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Characterization of a rat clonal [beta]-cell line, RINm5F, in a passage- and morphology-dependent manner

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Characterization of a rat clonal β-cell line, RINm5F, in a passage- and morphology-dependent manner

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LIST OF ABBREVIATIONS

ABC	Avidin-biotinylated peroxidase complex
AKAPs	A kinase Anchoring Proteins
ARs	Adrenergic receptors or Adrenoceptors
AMP	Adenosine monophosphate
ATP	Adenosine triphosphate
B_{max}	Maximum binding density
Ca^{2+}	Calcium ion
$[Ca^{2+}]_i$	Intracellular free Ca^{2+} concentrations
cAMP	Cyclic 3′ 5′ adenosine monophosphate
cGMP	Cyclic 3′ 5′ guanosine monophosphate
CHO	Chinese hamster ovary
CTX	Cholera toxin
DAB	Diaminobenzidine
DMEM	Dulbesso's modification of Eagles's minimal essential medium
EGF	Epidermal growth factor
ER	Endoplasmic reticulum
G-protein	GTP binding protein
^{125}I	Radioactive iodine, 125
IBMX	3-Isobutyl-1-methylxanthine
IC_{50}	Concentration to inhibit 50% of the maximal response
ICC	Immunocytochemistry
IGF	Insulin-like growth factor
IFNγ	Interferon-gamma
GAP	GTPase-activating protein,
GLUT	Glucose transporter
Grb-2	Growth factor receptor-bound protein 2
GTPγS	A nonhydrolyzable GTP analog,
HVA	High voltage activated
K^+	Potassium ion

LPA	Lysophosphatidic acid
MHC	Major histocompatibility complex
MLCK	Myosin light chain kinase
MTOC	Microtubule-organizing center
NGF	Nerve growth factor
NEDH	New England Deaconess Hospital
NIP	A novel insulin-like peptide
PAGE	Polyacrylamide gel electrophoresis
PDE	Phosphodiesterase
PDGF	Platelet-derived growth factor
PI	Phosphatidylinositol
PMA	Phorbol 12-myristate 13-acetate
Protein kinase A	Cyclic AMP-dependent protein kinase
PTX	Pertussis toxin
RIA	Radioimmunoassay
RINm5F	An insulin secreting clonal β-cell line
SDS	Sodium dodecyl sulfate
S.E.	Standard error
SH2 proteins	Src homology 2 domains
SRP	Signal recognition particle
VDCCs	Voltage-dependent calcium channels

CHAPTER I GENERAL INTRODUCTION

Dissertation Organization

This dissertation is written in an alternative thesis format, as permitted by the Graduate College. It includes a research objective, background and literature review, rationale and an experimental part of four manuscripts to be published, general discussion, general summary, list of references cited in the general introduction, rationale, and general discussion, and acknowledgements.

This dissertation contains the experimental results obtained by the author during his graduate study under the supervision of his major professor, Dr. Walter H. Hsu.

Research Objectives

Insulin secretion from pancreatic β-cells is essential for life and homeostasis of the concentration of glucose in the blood. Elevation of blood glucose causes insulin release through ATP-mediated cell depolarization, opening of voltage-dependent Ca^{2+} channels (VDCCs) and an increase in intracellular free Ca^{2+} concentration ($[Ca^{2+}]_i$). For the studies of the physiology and pharmacology of pancreatic β-cells, pancreatic tissue has been used. However, it is difficult to get pure β-cells from pancreatic islets of which β-cells comprise 60 - 75%. Therefore, clonal insulin-secreting cell lines have been established, and RINm5F cells are one of the cell lines which are established from the transplantable irradiation-induced NEDH rat insulinoma. The RINm5F cell line has facilitated numerous studies on β-cell function, including regulatory receptors, insulin gene expression, glucose transport and metabolism, ATP-sensitive K^+ channels, the regulation of $[Ca^{2+}]_i$ and insulin release. Despite the proven utility of RINm5F cells, insulin secretion is gradually decreased and the morphology change from a differentiated state (with neuron-like processes) to an undifferentiated state (with round shape) as the culture continues. The extent to which these changes are a genuine feature of the cells or merely a consequence of long-term maintenance is undetermined.

Insulin, with 51 amino acids, is made up of two chains of amino acids joined together by two disulfide linkages. The rat pancreas secretes equal amounts of two types of insulin, designated as rat insulin I and II; however, RINm5F cells have been found to express only rat insulin type I. In our preliminary study, undifferentiated RINm5F cells were demonstrated to produce a novel insulin-like immunoreactivity due to neither rat insulin I nor II, which has never been reported.

Epinephrine inhibits insulin release from pancreatic β-cells through activation of α_2-adrenergic receptors (ARs). When α_2-ARs are blocked, epinephrine increases insulin release by activating β_2-ARs. Despite the numerous studies on α_2-adrenergic mechanisms of insulin release in RINm5F cells, β-ARs/adenylyl cyclase-mediated insulin release has not been studied as much as α_2-AR-mediated insulin release. This may be attributable to the lack of information on the number of passages and the morphology (differentiated vs. undifferentiated state) of RINm5F cells which are appropriate for the study of α_2- or β-AR-mediated insulin release. Passage- and morphology-dependent changes of α- and β-ARs and adenylyl cyclase can be observed in these cells. The effect of epinephrine on insulin release was compared in order to characterize functional distribution of these receptors in RINm5F cells in the differentiated and undifferentiated state. In addition, the activities of adenylyl cyclase, phosphodiesterase (PDE), and cAMP-dependent protein kinase (protein kinase A) were characterized in RINm5F cells.

Ca^{2+} plays a central role in stimulus-secretion coupling in pancreatic β-cells and β-cell lines including RINm5F. The insulin secretagogue which triggers cell depolarization opens VDCCs leading to Ca^{2+} influx and insulin secretion. VDCCs have been classified into subclasses, i.e. T-, N-, L-, P-, and Q-type. RINm5F cells appear to have T-, L-, and Q-like-types mediating Ca^{2+} currents, but the presence of N-, P-, and Q-type VDCCs is still controversial. By utilizing two morphological features (differentiated and undifferentiated state), the presence of VDCCs that mediate Ca^{2+} currents, Ca^{2+} influx, and insulin release was characterized in RINm5F cells.

In summary, the purpose of this research was to characterize an insulin secreting clonal β-cell line RINm5F with regards to 1) a novel insulin-like immunoreactivity, 2) the α_1-, α_2-, and β-ARs, 3) adenylyl cyclase, PDE and protein kinase A, and 4) VDCCs in a passage- and morphology-dependent manner.

Background and Literature Review

The objective of this section is to provide concise background information for the study of 1) a novel insulin-like immunoreactivity, 2) the α_1-, α_2-, and β-ARs, 3) adenylyl cyclase, PDE and protein kinase A, and 4) VDCCs with a passage- and morphology-dependent manner in RINm5F cells.

The pancreatic islets

Anatomy

The pancreas is an exocrine and endocrine organ which is located in the upper right quadrant of the abdominal cavity in close association with the duodenum. The exocrine pancreas secretes pancreatic juice which consists of digestive enzymes required for the digestion of food. The endocrine glands of the pancreas, which are called islets of Langerhans, contain four types of endocrine cells. These cells are the A (or α) cells which secrete glucagon, the B (or β) cells which secrete insulin, the D (or δ) cells which secrete somatostatin, and F cells which secrete pancreatic polypeptide (Pelletier, 1977). These cells are not randomly distributed throughout the organ but are fairly well organized. The B cells are located in the center of islets and the A cells are located in the periphery of islets. The D cells are located between the A and B cells. The F cells are usually located near the A cells but are few in number and are not always found in the islets. The A cells comprise 20 - 30% of the islet cells, the B cells 60 - 75%, D cells 3 -10%, and F cells ‹ 2% (Karam, 1992). The A, B, D, and F cells make up 95 - 98% of islets volume (Larsson, 1980).

The islets of Langerhans are richly vascularized and both the islet cells and blood vessels are intimately associated with a variety of autonomic nerves. The origin of parasympathetic preganglionic neurons that distribute to the pancreas is in the brain stem, and that of the sympathetic system is in the spinal cord. Stimulation of parasympathetic nerves increases insulin secretion (Kaneto et al., 1967), whereas stimulation of the sympathetic nerves decreases insulin secretion (Miller, 1981).

Pathophysiology

Insulin and glucagon are the key hormones for maintaining glucose concentration in the blood (Hsu and Crump, 1989). Insulin decreases the concentration of blood glucose by facilitating the transport of glucose across the cell membrane. However, glucagon increases blood glucose concentration by increasing hepatic glycogenolysis and gluconeogenesis. In addition, insulin inhibits glucagon release, whereas glucagon stimulates insulin release. Somatostatin inhibits the release of insulin and glucagon. The role of pancreatic polypeptide, however, has not been clearly demonstrated in the regulation of insulin release.

Diminished rates of insulin secretion or tissue sensitivity to insulin cause diabetes mellitus classified as 1) insulin-dependent diabetes mellitus (IDDM) and 2) non-insulin-dependent diabetes mellitus (NIDDM). An estimated 8.3 million people in the USA are known to have diabetes, and 600,000 have the insulin-dependent type (Karam, 1992).

Insulin

Chemistry of insulin

Insulin with a M.W. of 56 KDa has two peptide chains (A chain with 21 amino acids and B chain with 30 amino acids) joined together by two disulfide bonds. The amino acid sequence of insulin is established (Sanger, 1960). The structures of human, porcine, and bovine insulins are shown in Fig. 1. Pancreatic β-cells synthesize insulin from a single-chain precursor, proinsulin, which consists of A and B subunits joined by the C-peptide (Orci et al., 1986). Proinsulin precursor is synthesized in the endoplasmic reticulum as preproinsulin, a precursor of proinsulin, which has at least 23 amino acids more at the N terminal than proinsulin (Chan et al., 1976). Conversion of proinsulin to insulin occurs by removing four amino acids and the C-peptide and is catalyzed by type I and type II carboxypeptidase H (Halban and Irminger, 1994).

Insulin exists as a monomer, a dimer and a hexamer but the biologically active form of insulin is a monomer. Hexameric zinc insulin is the storage form in the granules of β-cells. Three forms of insulin hexamers are present; T_6 (two zinc insulin hexamer), T_3R_3 (4 zinc insulin hexamer) and R_6 (a phenol-induced two zinc insulin hexamer with a high concentration of chloride ion). The T_6 hexamer has two zinc ions located 15.9 Å apart on the threefold symmetry axis, each in an octahedral array. The conversion

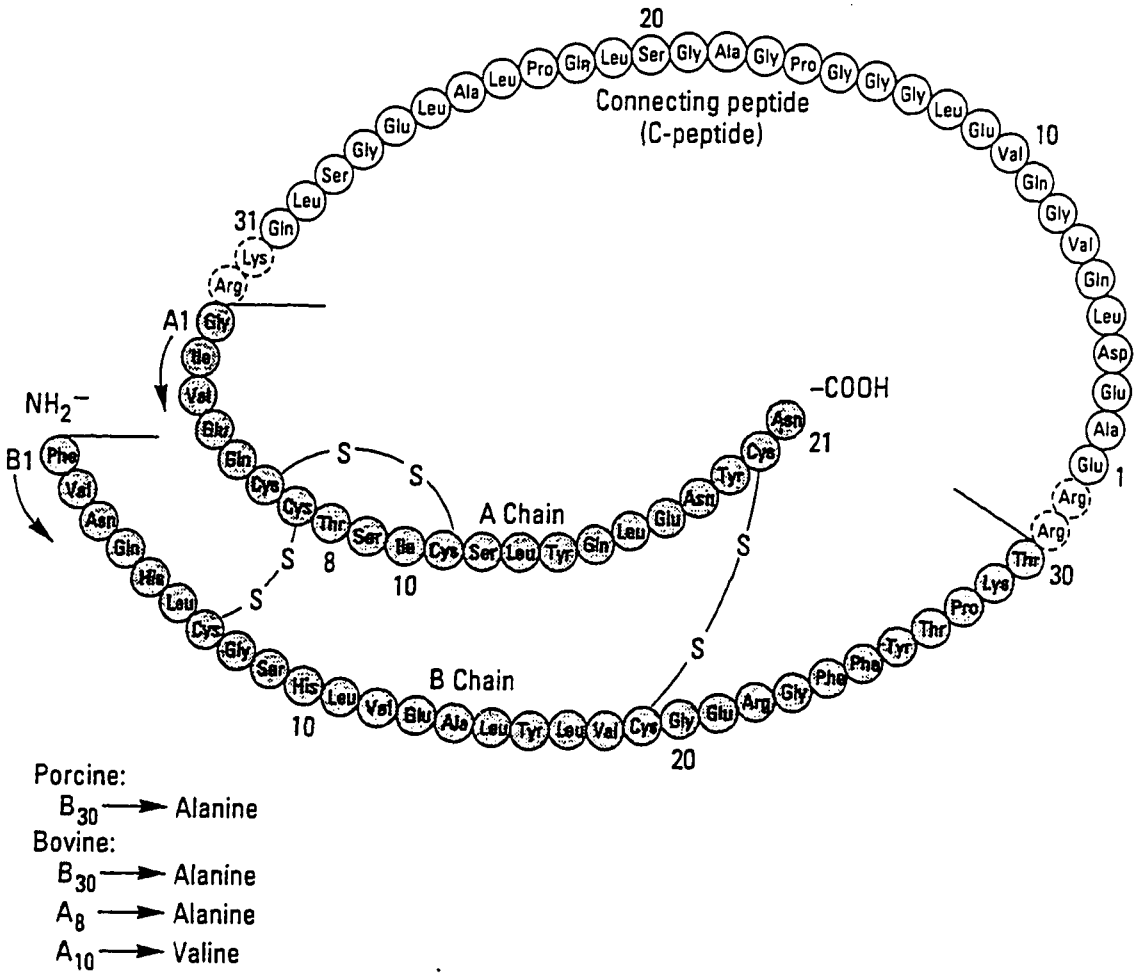

Fig. 1. The structure of human insulin. Insulin is shown as the shaded polypeptide A and B chains. Comparisons of human, porcine, and bovine C-peptides show considerable differences between species, with only about 40% homology. Species differences in the A and B chains are noted in the inset (from Karam, 1992).

from T_6 to R_6 occurs by changing from an extended B chain to a helix conformation and by positioning two zinc ions within the interior of the hexamer. The T_3R_3 hexamer is a stable intermediate between T_6 and R_6 conformational transition. The alternative conformations suggest that comparable conformational events may occur during biochemical signalling processes within the receptor-insulin complex. However, little is known about the relationship between the ligand-mediated conformational changes in the hexamer and the behavior of the insulin monomer *in vivo* (Brader and Dunn, 1991).

Genetic control of insulin

The expression of the insulin gene is regulated through both transcriptional and post-translational processes in pancreatic β-cells. Glucose rapidly increases the rate of transcription, an effect that may partly be mediated through cAMP (Nielsen et al., 1985; Welsh et al., 1986a), and stimulates translation (Itoh and Okamoto, 1980). Glucose-stimulated islets have greater amounts of insulin-specific mRNA in the membrane bound polysomes (Cordell et al., 1982). Post-transcriptional processes in insulin gene expression are enhanced by glucose which reduces degradation of insulin mRNA. In addition, translation of insulin is enhanced by glucose which increases initiation and elongation of signal recognition particle (SRP)-mediated transferral of cytosolic insulin mRNA to the rough endoplasmic reticulum (Welsh et al., 1986a). Leucine and 2-ketoisocaproate also play a stimulatory role in insulin gene expression (Welsh et al., 1986b). Glucocorticoids increase pancreatic islet mRNA content, but Ca^{2+} and a phorbol ester, an activator of protein kinase C, do not regulate insulin gene expression (Welsh et al., 1988).

Secretion of insulin

Insulin secretion is modulated by secretagogues in a regulated pathway, which allows for rapid and massive discharge of insulins stored in granules at the physiologically appropriate moments. The cytoskeletons, microtubules and microfilaments, in the β-cells link some of the granules to the cell membrane and provide internal pathways of controlled movement of the granules within the cells. The granules fuse with the membrane, rupture and liberate the content into the extracellular space (Lacy et al., 1968). Exocytosis from the constitutive pathway is a continuous

process limited only by the availability of product. The constitutive pathway is responsible for delivery of integral proteins to the cell surface (Halban and Irminger, 1994). In the regulated pathway, the secretory granules are formed in the *trans*-Golgi network (TGN) and coated with a protein called clathrin. The immature, clathrin-coated granules are where the conversion to proinsulin occurs. The maturation of secretory granules involves progressive acidification, proinsulin conversion and loss of the clathrin coat. The enzymes responsible for proinsulin conversion, type I and type II carboxypeptidase H and PAM (peptidyl α-amidating mono-oxidase), are present in secretory granules (Orci et al., 1985; Christie et al., 1991). However, rat proinsulin II and human proinsulin can be cleaved within the cells which contain only the constitutive pathway of secretion. Cleavage occurs at the C-A junction with tetrabasic sequence, Arg-X-(Lys/Arg)-Arg. This cleavage is likely due to the endogenous, Golgi-anchored, processing enzyme, furin or PACE4, which is present in the constitutive pathway of the cells (Groskreutz et al., 1994).

Species differences

Insulins of various mammals show a high degree of homology but species differences do exist. These differences involve amino acid residues 8, 9 and 10 of the A chain and amino acid residue 30 of the B chain (Fig. 1). The structure of porcine insulin is closest to human with the major difference being the presence of an alanine residue at the C-terminal of the human B chain. Bovine insulin differs from porcine insulin at A8 (Threonine → Alanine) and at A10 (Ileucine → Valine). Guinea pig insulin differs from porcine insulin in 10 residues of the B chain and 7 residues of the A chain. However, canine and fin whale insulins have a identical sequence with porcine insulin (Nicole and Smith, 1960; Smith, 1966). In addition to species differences, more than one insulin may exist in the same species. In rats, mice and fish, two different insulins have been isolated (Smith, 1966; Clark and Steiner, 1969; Markussen, 1971). The A chain of rat insulin has the same sequence and differs from porcine insulin at A4 glutamic acid. Rat insulin I B chain differs from porcine insulin at B3 (Asparagine → Lysine) and at B9 (Serine → Proline). Rat insulin II B chain differs from porcine insulin at B3 (Asparagine → Lysine) and at B29 (Lysine → Methionine) (Clark and Steiner, 1969).

Two different rat insulin cDNAs (mRNAs) have been isolated (Ullrich et al., 1977) and two nonallelic genes have been isolated and completely sequenced in the rat genome (Cordell et al., 1979). Insulin genes I and II are equally expressed and equal amounts of insulin I and II are secreted. However, insulin gene II is the ancestral gene in normal rat pancreatic β-cells (Bell et al., 1980). Equivalent amounts of insulin genes I and II are transcribed and mature mRNAs are present in RIN cells. However, rat preproinsulin I and II are 10-fold higher for insulin gene I than insulin gene II in RIN cells (Cordell et al., 1982). The presence of stable insulin gene II mRNAs in the cytoplasm which are not translated may be attributable to a cap-specific binding protein that differentially stimulates the translation of fully capped mRNAs compared with uncapped mRNAs (Sonenberg et al., 1980). RINm5F cells are suggested to have only rat insulin I mRNA (Fiedorek et al., 1990).

Mechanisms of insulin action

Effect of insulin on metabolism

Insulin controls blood glucose by stimulating glucose uptake and glycogen synthesis in muscle and adipocytes and by inhibiting gluconeogenesis and lipolysis in the liver and adipocytes. Furthermore, insulin modifies the expression or activity of a variety of enzymes and transport systems in nearly all cells (White and Kahn, 1994). Pyruvate dehydrogenase, a mitochondrial enzyme, is also dephosphorylated by insulin to oxidize pyruvate to fat reducing the formation of glucose from pyruvate (Reed et al., 1980). The activity of lipoprotein lipase is increased by insulin, which in turn makes fatty acids derived from circulating lipoproteins available to the cells (Steinberg, 1983). In protein metabolism, insulin increases amino acid uptake and protein synthesis and inhibits protein degradation (Draznin and Trowbridge, 1982). K^+ uptake is also promoted by insulin through stimulation of Na^+, K^+-ATPase (Resh et al., 1980).

Effect of insulin on glucose transport

Glucose is a major source of metabolic energy, and a transport system for glucose is present in all animal cells. The energy independent transport of glucose is mediated by the facilitative glucose transporter (GLUT) family that are present on the surface of the cells. The facilitative transport of glucose is saturable, stereoselective, and

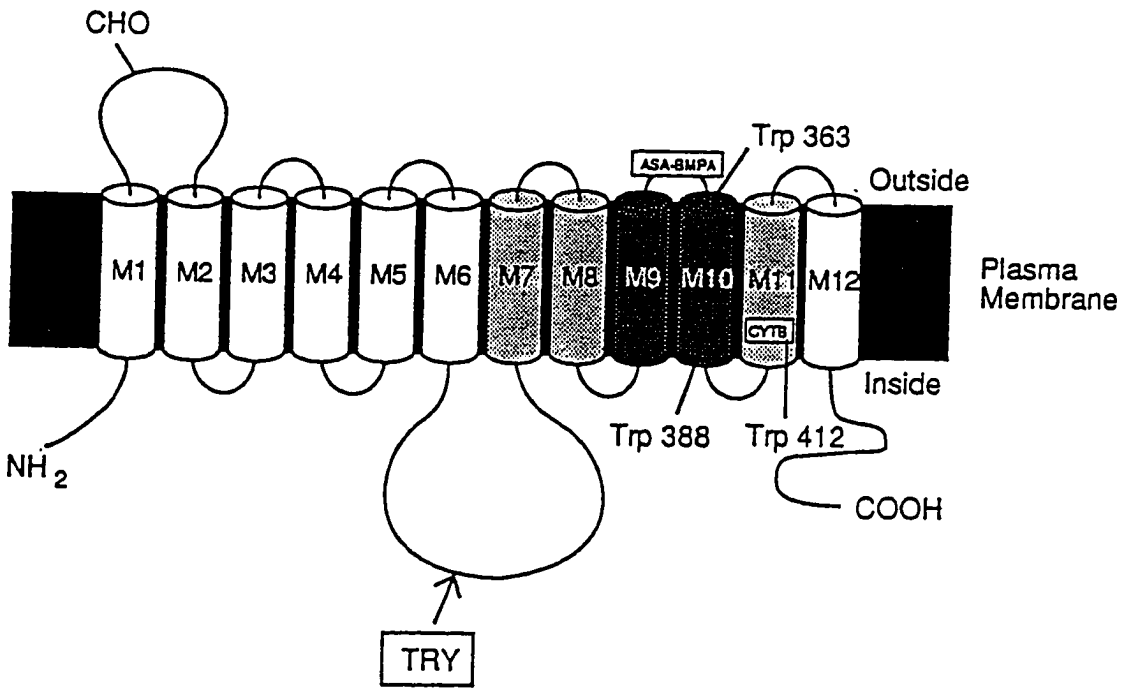

Fig. 2. Schematic representation of model showing two-dimensional membrane topology of the prototypical facilitative glucose transporter GLUT1. Putative membrane-spanning α-helices are labelled M1 - M12. M7, M8, and M11 have been putatively assigned as hydrophilic channels and M9 and M10 as the hydrophobic clefts (from Silverman, 1991).

bidirectional. The second family of glucose transporter (present in the lumen of the small intestine and nephron) is the intestinal Na^+/glucose cotransporter, SGLT1, which utilizes the electrochemical sodium gradient to transport glucose and galactose against their concentration gradient (Bell et al., 1993).

The rate of glucose transport is dramatically increased in muscle and fat when the circulating concentrations of insulin is high (Gould and Bell, 1990). GLUT1 (erythrocyte-type) is an integral membrane glycoprotein with a M.W. of 45 - 55 KDa and 12 transmembrane domains present in fetal tissues, brain, kidney, and colon (Fig. 2). With insulin challenge, the translocation of GLUT1 is not sufficient to account for the full stimulation of transport by insulin. GLUT2 (liver-type) is present in liver, pancreatic β-cells, small intestine and kidney but does not play a role in the increase of the rate of glucose uptake caused by insulin. The topological organization of GLUT2 is identical to that of GLUT1. GLUT2 in pancreatic β-cells plays a role in regulating insulin secretion and is important in maintaining blood glucose levels working in conjunction with glucokinase.

GLUT3 (brain-type) is present in brain, kidney and placenta but relatively low in adult muscle suggesting that this transporter is not the major one responsible for glucose uptake by muscle. The topological organization of GLUT3 is identical to that of GLUT1. GLUT4 (muscle-type) is found in skeletal muscle, heart and white and brown adipose tissues. Insulin causes a 20- to 30-fold increase in the rate of glucose transport through GLUT4 in adipocytes. The insulin-stimulated increase in glucose transport is due to the translocation of a latent pool of GLUT4 from intracellular locations to the plasma membrane. GLUT4 has 65%, 54% and 58% homology with GLUT1, 2, and 3, respectively.

GLUT5 (small intestine-type) is a fructose transporter expressed at highest levels in small intestine. However, it is unknown whether GLUT5 transports glucose or is a carrier for another sugar or other small polar molecule. GLUT5 has 42%, 40%, 39% and 42% homology with GLUT1, 2, 3 and 4, respectively. GLUT6 is a non-functional pseudogene and has been identified only in human. GLUT7 (microsomal-type) is a microsomal glucose transporter in liver. It comprises part of the glucose-6-phosphatase complex and mediates glucose release from the endoplasmic reticulum (ER).

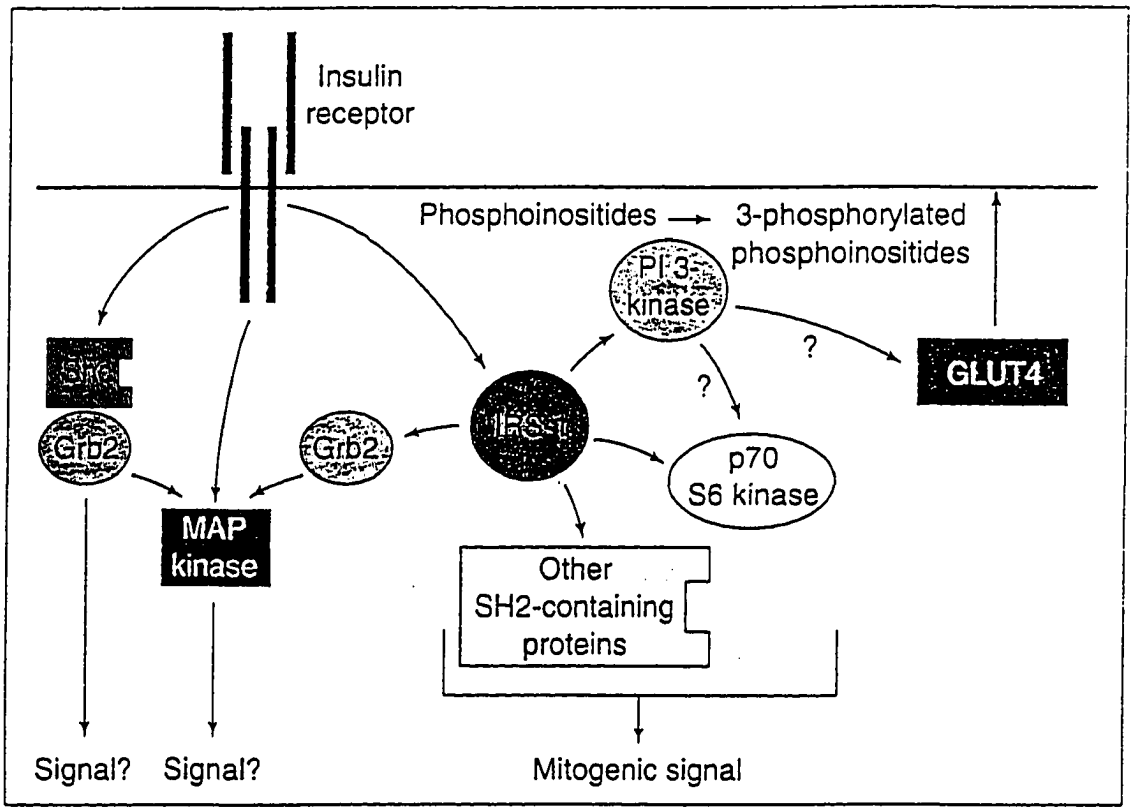

Fig. 3. Mechanisms of action through insulin receptor. IRS-1 is required for activation of PI 3'-kinase and p70 S6 kinase. The insulin receptor also mediates the phosphorylation of Shc on tyrosine and activation of small G-protein Ras and MAP kinase in the absence of IRS-1. The interaction of IRS-1 and Grb-2 can also play a role in MAP kinase signalling. PI 3'-kinase is suggested to be involved in movement of GLUT4-containing vesicles and activation of p70 S6 kinase (Myers et al., 1994).

Of the GLUT family, GLUT1 and 4 are responsible for increased glucose uptake through the stimulation of insulin, whereas other GLUTs do not need insulin to activate the glucose uptake (Gould and Bell, 1990; Silverman, 1991; Bell et al., 1993). GLUT1 regulates basal glucose uptake, however, GLUT4 is associated with an increased rate of glucose uptake in response to insulin stimulation. The effect of insulin on GLUT4 is acute, occurring with a half time of two or three minutes, and in the absence of new protein synthesis (Gould et al., 1994). Small G-proteins are believed to be involved in insulin-stimulated GLUT4 translocations from the observation that these G-proteins are colocalized with GLUT4 and GTPγS (a nonhydrolyzable GTP analog) and stimulate GLUT4 translocations (Etgen, Jr. et al., 1993).

Signal transduction through insulin receptor

The B25 phenylalanine of insulin plays a major role in directing the interaction of insulin with its receptor (Nakagawa and Tager, 1986). In addition, the A3 valine of insulin is also necessary for binding to the insulin receptor (Nanjo et al., 1986). Insulin action is mediated through the insulin receptor, a glycoprotein with intrinsic protein kinase activity, which has a kinase activity also found in receptors for EGF and PDGF (DeFronzo et al., 1992). The mechanism of insulin action is summarized in Fig. 3. Insulin receptors have two α subunits each with a M.W. of 125 KDa and two β subunits each a with M.W. of 90 KDa, joined together by two disulfide bonds. Activation of the receptor by insulin-induced dimerization stimulates autophosphorylation of tyrosine residues of the β-subunit (Ullrich et al., 1985; Myers et al., 1994). The activated receptor phosphorylates insulin receptor substrate-1 (IRS-1) with multiple tyrosine residues, which in turn binds to Src homology 2 domains (SH2 proteins) of phosphatidylinositol (PI) 3'-kinase, growth factor receptor-bound protein 2 (Grb-2), SH-PTP-2, and Nck (Backer et al., 1992; Skolnik et al., 1993). In addition to IRS-1, insulin stimulates tyrosine phosphorylation of Shc, a 52 KDa SH2-containing protein with a tyrosine phosphorylation site that associates with Grb-2. Regulation of Ras and the activation of MAP kinase can occur through at least two pathways: an IRS-1 dependent pathway and an Shc-dependent pathway (Myers et al., 1994).

In addition, insulin decreases intracellular cAMP concentrations by stimulating PDE and inhibiting adenylyl cyclase (Houslay et al., 1984) and lowers the affinity of protein

kinase A to cAMP without changing intracellular cAMP concentrations (Larner et al., 1982).

A clonal β-cell line, RINm5F

Establishment of RIN-m cell line

RINm5F cells were derived from a transplantable islet tumor induced by x-ray irradiation in an inbred NEDH (New England Deaconess Hospital) rat (Chick et al., 1977). The islet tumor was initiated and maintained by serially transplanting nine-times into NEDH rats (Gazdar et al., 1980). The tumor was again transplanted into athymic nude mice. The cell lines from the rat transplant were designated as RIN-r and from the nude mouse transplant as RIN-m. The RIN-m cell line was established after transplanting four times in nude mice. RIN-m cells was classified into 5 clones; designated as clone 1 to 5, RINm5F was one of the clones of RIN-m cells. The cells were further cultured in Dulbecco's modification of Eagles's minimal essential medium (DMEM) or RPMI-1640 medium supplemented with 10% fetal bovine serum. Population doubling times were 60 - 80 hr and varied with the culture medium and duration of culture. Growth rates were faster in DMEM than in RPMI-1640 medium and were also faster at later than at earlier passages.

Both epithelioid and fibroblastoid cells were present, but fibroblastoid cells of murine origin were completely eliminated by exposing the cells to cystine-free medium for 16 h. However, epithelioid cells were progressively grown in both media, with more rapid growth in the DMEM. RIN-m cells consisted of epithelioid cells without apparent fibroblastoid contamination (Gazdar et al., 1980), which was confirmed by isoenzyme analysis. The cells were free of contamination with mycoplasma and murine leukemia virus.

Characterization of the RIN-m cell line

Functional characterizations

The RINm5F cells secrete both insulin and somatostatin (Gazdar et al., 1980; Oie et al., 1983). The amount of insulin secreted is varied depending upon medium and duration of culture. There is an inverse relationship between proliferation and function. Maximal insulin secretion occurs by day 2 when cell growth is in a lag phase and a fall

in insulin secretion occurs when the cells rapidly proliferate post day 2 - 3 (Bartholomeusz et al., 1990). The changes in insulin production during culture may be due to changes in insulin mRNA stability (Muschel et al., 1986). Cells maintained in DMEM and RPMI-1640 media release relatively high levels of insulin (Gazdar et al., 1980). However, the level of insulin in DMEM is decreased and is not detectable after 190 days. In RPMI-1640 medium, insulin secretion decreases during the first 100 days but stabilizes at 150 - 250 μU/million cells per 24 hr. Moreover, the cells in RPMI-1640 medium keep secreting insulin for more than 400 days; although the insulin content of the cells is only 1% compared to pancreatic β-cells (Halban et al., 1983; Praz et al., 1983).

The level of somatostatin secreted did not widely fluctuate with culture time or type of medium and ranged from 3,150 - 8,700 pg/million cells per 24 hr. Therefore, variations in insulin secretion during culture may be influenced by the simultaneous secretion of somatostatin, an inhibitor of insulin secretion (Basabe et al., 1977).

Biochemical characterizations

RINm5F cells are not responsive to glucose but are responsive to glyceraldehyde-stimulated insulin release (Lenzen et al., 1987; Thomas et al., 1989). Glucose utilization is hyperbolic and reaches maximal rates at glucose concentrations of 3 - 5 mM (Halban et al., 1983). The ratios for glucose utilization and lactate/pyruvate output are much lower in RINm5F cells than in normal islet cells (Giroix et al., 1985).

The glucose insensitivity of the cells has been attributed to a lack of glucokinase and the presence of low K_m (hexokinase-like) glucose-phosphorylating activity (Halban et al., 1983; Lenzen et al., 1987; Vischer et al., 1987), and/or the reduced amounts of GLUT2 (Thorens et al., 1988). Although RINm5F cells do not have glucokinase, the cells are reported to have a glucokinase mRNA that is similar to the one in pancreatic islets (Fernandez-Mejia and Davidson, 1990). In addition, RINm5F cells have reduced amounts of GLUT2 compared to normal islets, and increased levels of GLUT1 (presumably as a result of the transformed phenotype). Since the K_m for glucose of GLUT1 is considerably lower than GLUT2, the rate of glucose entry into RINm5F cells is quite low compared to the cells expressing only GLUT2 (Thorens et al., 1988). In fact, the transfection of GLUT2 cDNA improves glucose transportation and glucose-

stimulated insulin secretion in RINm5F cells (Tiedge et al., 1993). However, contradictory findings have been reported on GLUT in RINm5F cells. The loss of glucose sensitivity is not due to the alteration of hexose transport because the cells have a passive glucose transport system with high capacity and low affinity (Trautmann and Wollheim, 1987).

The *in vivo* transplantation of cells markedly enhances the rate of glucose uptake (Hoenig et al., 1984; Flatt et al., 1988), however, such an effect is very rapidly lost in tissue culture (Flatt et al., 1988). In addition, basal insulin release and insulin release in response to pancreatic β-cell secretagogues (i.e. arginine, leucine, and KCl) are increased by *in vivo* transplantation but lost during subsequent cell culture (Flatt et al., 1988). This suggests that long-term maintenance in the physiological condition may enhance responsiveness of the cells by inducing glucokinase and other undetermined factors (Lenzen et al., 1987).

The cell lines resemble the APUD cells series in biochemical, functional, and morphological features (Pearse, 1980). RIN cells secrete polypeptide hormone, have high levels of L-dopa decarboxylase, aromatic L-amino acid decarboxylase, and exhibit fluorescence after being exposed to formaldehyde (Oie et al., 1983).

Immunological characterizations

RINm5F cells have been frequently employed as a model of islet cell growth and differentiation in that they are relatively undifferentiated compared to normal adult islet cells (Halban et al., 1988; Bartholomeusz et al., 1990; Polak et al., 1993). The differentiation of RINm5F cells has been characterized by using monoclonal antibodies (A2B5, 3G5, and R2D6) against gangliosides which are expressed on the plasma membrane. A2B5 and 3G5 are monoclonal antibodies which recognize gangliosides on many cells including β-cells. R2D6 is a monoclonal antibody to a ganglioside which is only expressed on the surface of pancreatic β-cells. Increased insulin gene expression accompanies increased expression of A2B5-reactive ganglioside, which is associated with β-cell differentiation (Halban et al., 1988; Bartholomeusz et al., 1990). A2B5-reactive ganglioside is expressed more in functional, more differentiated cells than in less differentiated RINm5F cells. In fact, fully differentiated adult islets are identified by reactivity with A2B5 (Eisenbarth et al., 1982). However, more 3G5 - but less

A2B5-reactive ganglioside is expressed in less differentiated cells than in more differentiated RINm5F cells. The expression of R2D6-reactive ganglioside is also expressed in RINm5F cells. Its expression is higher in cells at 1 - 2 days after subculture than those at 5 days after subculture, suggesting that cellular confluence or cell cycle may be a factor in differentiated surface antigen (Halban et al., 1988).

The differentiation of RINm5F cells has also been characterized by the expression of major histocompatibility complex (MHC) proteins. MHC is a glycoprotein expressed on the surface of nucleated cells of many tissues and classified into class I and class II MHC. It serves as a specific antigen of the cells which is responsible for immune rejection following allograft transplantation. More differentiated RINm5F cells exhibit reduced expression of class I MHC proteins suggesting that the expression of class I MHC proteins may be related to the state of islet cell differentiation (Bartholomeusz et al., 1990). RINm5F cells do not have detectable class II MHC proteins (Setum and Hegre, 1990). However, the expression of class I MHC is increased by reovirus infection (Campbell et al., 1988), and the expression of both class I and II MHCs is enhanced by interferon-gamma (IFNγ) (Varey et al., 1988).

Morphological changes of cells *in vitro*

Cells in culture usually undergo gross morphological changes. Cells attached to the substratum which is coated to the bottom of the culture flask grow neuron-like processes (differentiated) or completely flatten against the underlying surface. These cells become round (undifferentiated) during long-term culture (Willingham, 1976).

Stimulators of cellular processes

Cellular processes are either formed spontaneously or are induced by elevation of cAMP, phenylethyl alcohol, sodium butyrate, and nerve growth factor (NGF). cAMP appears to induce neuron-like processes in serum-free media (Nabika et al., 1988; Chaldakov et al., 1989) or in serum-contained media (Baorto et al., 1992). Increased intracellular cAMP concentration changes cell morphology in culture to look more like the normal parent cell, which is more spindly rather than round (Willingham, 1976). Dibutyryl cAMP (Bt_2cAMP), an analogue of cAMP, induces elongation of CHO (Chinese hamster ovary) cells (Hsie and Puck, 1971), Swiss 3T3 cells (Johnson and Pastan,

1972), cultured iris epithelial cells (Ortiz et al., 1973), cultured vascular smooth muscle cells (Nabika et al., 1988), primary cultured astrocytes (Baorto et al., 1992), NG 108-15 cells (Bergamaschi et al., 1992), NCB-20 cells (Mienville, 1992) and BHK21 cells (Edwards et al., 1993). N^6-substituted derivatives of adenosine can produce the processes without affecting cAMP levels. This raises the possibility that morphological changes may not be due to alteration of cAMP levels, but may act through the same effector mechanisms that cAMP regulates under normal conditions. However, protein kinase A is responsible for cAMP-dependent morphological transformation because microinjection of the catalytic subunit of protein kinase A triggers morphological changes from the differentiated to the undifferentiated state in thyroid epithelial cells (Roger et al., 1988).

Phenylethyl alcohol and sodium butyrate, a short-chain carboxylic acid, produce process elongation in CHO cells (Wright, 1973). The mechanisms of sodium butyrate-induced differentiation are reported to raise intracellular cAMP concentrations (Wright, 1973), to arrest proliferation at late G1 phase of cell cycle (Toscani, et al., 1988), and to decrease cellular content of polyamine (Sjoholm, 1993). NGF promotes neurite outgrowth and alters cellular morphology not through activating a protein kinase A but through activating tyrosine kinase in PC 12 cells (Greene and Tischler, 1976; Chijiwa et al., 1990) and in RINm5F cells (Polak et al., 1993). In addition, NGF and Bt_2cAMP synergistically stimulate neurite outgrowth in PC12 cells suggesting divergent pathways of action of NGF and cAMP in growth of cellular processes (Heidemann et al., 1985).

Inhibitors of cellular processes

Fetal bovine serum, platelet-derived growth factor (PDGF), epidermal growth factor (EGF), bombesin, insulin, and lysophosphatidic acid (LPA) have been shown to inhibit the formation of the cell processes which are formed by elongation of cytoplasm around cytoskeletal elements (Baorto et al., 1992). Reorganization of actin filaments forms stress fibers and in turn retracts processes (Ridley and Hall, 1992). PDGF, EGF, bombesin and insulin induce stress fiber formation in serum-starved Swiss 3T3 cells (Ridley et al., 1992; Ridley and Hall, 1992). LPA, bombesin and serum induce rapid stress fiber assembly (within 2 min), however, PDGF, EGF and insulin induce it more

slowly (5 to 10 min).

Serum decreases cAMP-induced process formation in BHK21, NIH-3T3 and CHO cells. However, factors present in serum which inhibit stellation are presently unknown (Edwards et al., 1993). It has been suggested that serum can be replaced by LPA to inhibit process formation (van Corven et al., 1992; Ridley and Hall, 1992). The effect of LPA on the inhibition of cAMP-induced process formation may suggest the reorganization of actin components of the cytoskeleton (Edwards et al., 1993). LPA in fibroblasts lowers cAMP accumulation (van Corven et al., 1989), but stimulates phosphoinositide hydrolysis which leads to a transient increase in $[Ca^{2+}]_i$ (van Corven et al., 1989). However, LPA-induced increase in $[Ca^{2+}]_i$ is not likely to activate stress fiber formation (Ridley and Hall, 1992).

There is a possibility that small GTP-binding proteins (G-proteins) are involved in the formation of stress fibers. The intracellular target for LPA has been reported to be rhoGAP, a GTPase-activating protein (Tsai et al., 1989). However, it is unclear whether LPA acts through a transmembrane receptor or an intracellular target. LPA, PDGF, and other growth factors presumably induce G-protein-dependent responses. They increase the level of GTP-bound rho and rac small G-proteins or inhibit rhoGAP which enhances GTP hydrolysis leading to actin filament accumulation in the plasma membrane of the cells (Ridley et al., 1992; Ridley and Hall, 1992). In fact, the rac and rho small G-proteins, which are approximately 60% identical, are essential components for organization of polymerized actin (Ridley et al., 1992).

The role of protein kinase C in the formation of stress fiber is still not clear. A phorbol ester, PMA (phorbol 12-myristate 13-acetate) which is an activator of protein kinase C, stimulates the accumulation of polymerized actin which leads to retract processes in serum starved Swiss 3T3 cells (Ridley et al., 1992). In addition, PMA prevents isoproterenol-induced process formation through activation of protein kinase C in vascular smooth muscle cells (Nabika et al., 1988). However, PMA causes cells to form long narrow processes in fibroblasts (Brown et al., 1989) and macrophages (Phaire-Washington et al., 1980).

Effect of cytoskeletal system on cellular processes

Effect of microtubules on cellular processes

Microtubules present in all eukaryotic cells are formed by polymerization of tubulin. The tubulin subunits are arranged in 13 parallel rows called protofilaments. Each protofilament is composed of repeating tubulin dimers linked head to tail with each dimer containing one α- and one β-tubulin molecule. Individual microtubules associated with intermediated filaments form a stable cytoskeleton and act as guides along which protein particles and organelles move up and down. In cultured cells, most microtubules radiate from the microtubule-organizing center (MTOC) and are consistently disassembling and reassembling into new ones (Amos and Baker, 1979; Mandelkow et al., 1986; Vale, 1987).

The morphological transformation of CHO cells *in vitro* by elevating intracellular cAMP concentration is related to the degree of organization, possibly increasing microtubular population (Ortiz et al., 1973). Agents that interfere with microtubular function, such as colchicine, prevent most of the morphological changes due to elevated cAMP levels (Hsie and Puck, 1971) and Bt_2cAMP treatment redistributes or increases microtubular structures (Willingham and Pastan, 1975).

Background information of actin

Actin microfilaments play a role in many cellular functions, including motility, chemotaxis, cell division, endocytosis and secretion (Ridley and Hall, 1992). Microfilaments, called F actin, are polymers of a globular protein subunit (a M.W. of 42 KDa) called globular actin, or G actin. Long bundles of filaments are termed stress fibers. Each actin subunit has a defined polarity, and the subunits polymerize head to tail. Most cellular actin is in microfilaments; although such filaments can grow and shrink. Actin filaments are much less likely to depolymerize than are microtubules. Under steady-state conditions the actin monomers are polymerized to microfilaments within cells.

A ras-related GTP-binding proteins (small G-proteins), rho and rac, stimulate the formation of stress fibers and focal adhesions in Swiss 3T3 cells. Stress fibers, which are contractile, are involved in the attachment of cultured cells to a substratum and in generation of the tension that produces the flattened shape of fibroblasts. Stress

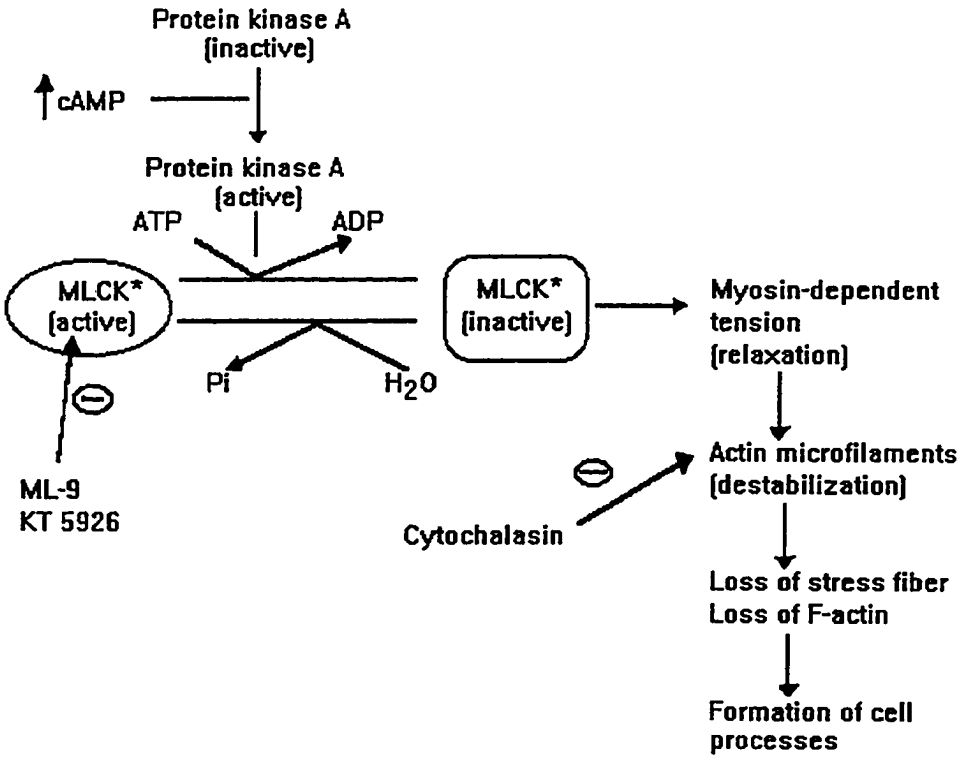

Fig. 4. Mechanisms of the cell process formation through increased intracellular cAMP concentration. ⊖: Inhibitory effect. *: Active MLCK binds Ca^{2+}/CaM strongly but inactive MLCK binds Ca^{2+}/CaM poorly. Ca^{2+}/CaM activates MLCK which in turn activates myosin. Activated myosin can bind to actin and actin microfilaments are stabilized. When actin microfilaments are stabilized, stress fibers are formed and the cell processes retract (summarized from the text).

fibers are located in the cytoplasm adjacent to the plasma membrane and contain myosin, α-actin, tropomyosin and myosin light chain kinase (MLCK) (Byers and Fujiwara, 1982; Pollard, 1986; Korn et al., 1987; Mitchison and Kirschner, 1988; Small 1988; Ridley and Hall, 1992).

Background information of myosin

Myosin proteins have a long rodlike tail and two globular heads at the one end. A myosin molecule has two identical heavy chains, each with a M.W. of 230 KDa and two pairs of light chains, each with a M.W. of 20 KDa. Type I myosin is present only in nonmuscle cells and contains the globular actin-binding region but lacks the fibrous tail. The fibrous tails are composed of 300 - 400 myosin dimers packed together to form a specific bipolar aggregate termed the thick filament. Type II myosin is present in muscle and in nonmuscle cells and contains a globular region that binds actin and a fibrous segment that allows it to aggregate into filaments (Tokunaga et al., 1987; Davis, 1988). Myosin has an ATPase activity. The S1 fragment retains all of the actin-stimulated ATPase activity of intact myosin. Binding of myosin S1 fragments to actin fibers reflects the directionality and helicity of the actin filaments (Tokunaga et al., 1987).

Effect of microfilaments on cellular processes

By elevating intracellular cAMP concentration, the subcellular distribution of microfilaments is changed (Fig. 4) (Willingham and Pastan, 1975). The process formation induced by increasing intracellular cAMP concentration can also be induced by cytochalasin, an agent that interferes with the function of microfilaments (Chaldakov et al., 1989). This suggests that actin microfilaments participate in the formation of the neuron-like processes. The cell processes induced by elevating cAMP are accompanied by depletion of actin microfilaments, particularly loss of stress fibers (Chaldakov et al., 1989; Baorto et al., 1992). Loss of F-actin can lead plasma membrane to form cellular processes (Edwards et al., 1993). In addition, the outgrowth of processes of BHK21 cells is accompanied by a high amount of F-actin at the tips of growing processes, indicating that the effect of elevated cAMP is selective for different subsets of microfilaments (Edwards et al., 1993). The relationship

between stellation and disappearance of sub-membrane actin emphasizes the importance of microtubule stabilization in neurite outgrowth (Heidemann et al., 1985). However, neurite outgrowth of PC12 cells happens in the absence of microtubules (Lamoureux et al., 1990).

The effect of cAMP on microfilaments may come from phosphorylation of MLCK through activating protein kinase A, which lowers the binding affinity of MLCK for the Ca^{2+}-calmodulin complex. As a result, myosin-dependent tension is relaxed and microfilaments are destabilized (Baorto et al., 1992). In addition, inhibitors of MLCK (ML-9 and KT 5926) and a calmodulin antagonist (W7) can induce the formation of cell processes by dephosphorylating a 19 KDa actin depolymerizing factor implicating MLCK as a control point in process initiation (Baorto et al., 1992).

Catecholamines

Chemistry

Catecholamines, sympathomimetic drugs, are derived from phenylethylamine by substituting positions 3 and 4 of the benzene ring with -OH groups. The structure of catecholamines and their biosynthesis pathways are illustrated in Fig. 4. Substitutions of phenylethylamine on the terminal amino group, of the benzene ring, and on the α or β carbons change affinity of the drugs for α- and β-ARs and influence the intrinsic activity to the receptors (Hoffman, 1992). For example, substitutions on the amino groups of phenylethylamine with alkyl or isopropyl groups increase β-AR activity; e.g., epinephrine and isoproterenol. The activities of catecholamines on α- and β-ARs are maximal when -OH groups are present at the 3 and 4 positions. The absence of one of these -OH group markedly decreases the potency of the drugs but makes them more resistant to catechol-O-methyltransferase (COMT) which methylates epinephrine or norepinephrine to metanephrine or normetanephrine, respectively. In addition, substitution on the α-carbon blocks oxidation by monoamine oxidase (MAO) and prolongs the action. Substitution of the OH group on the β carbon decreases lipid solubility but enhances agonistic activity both at α- and β-ARs. Catecholamines include epinephrine, norepinephrine, isoproterenol, and dopamine. Non-catecholamines include phenylephrine, methoxamine, ephedrine, amphetamine, and tyramine (Hoffman, 1992).

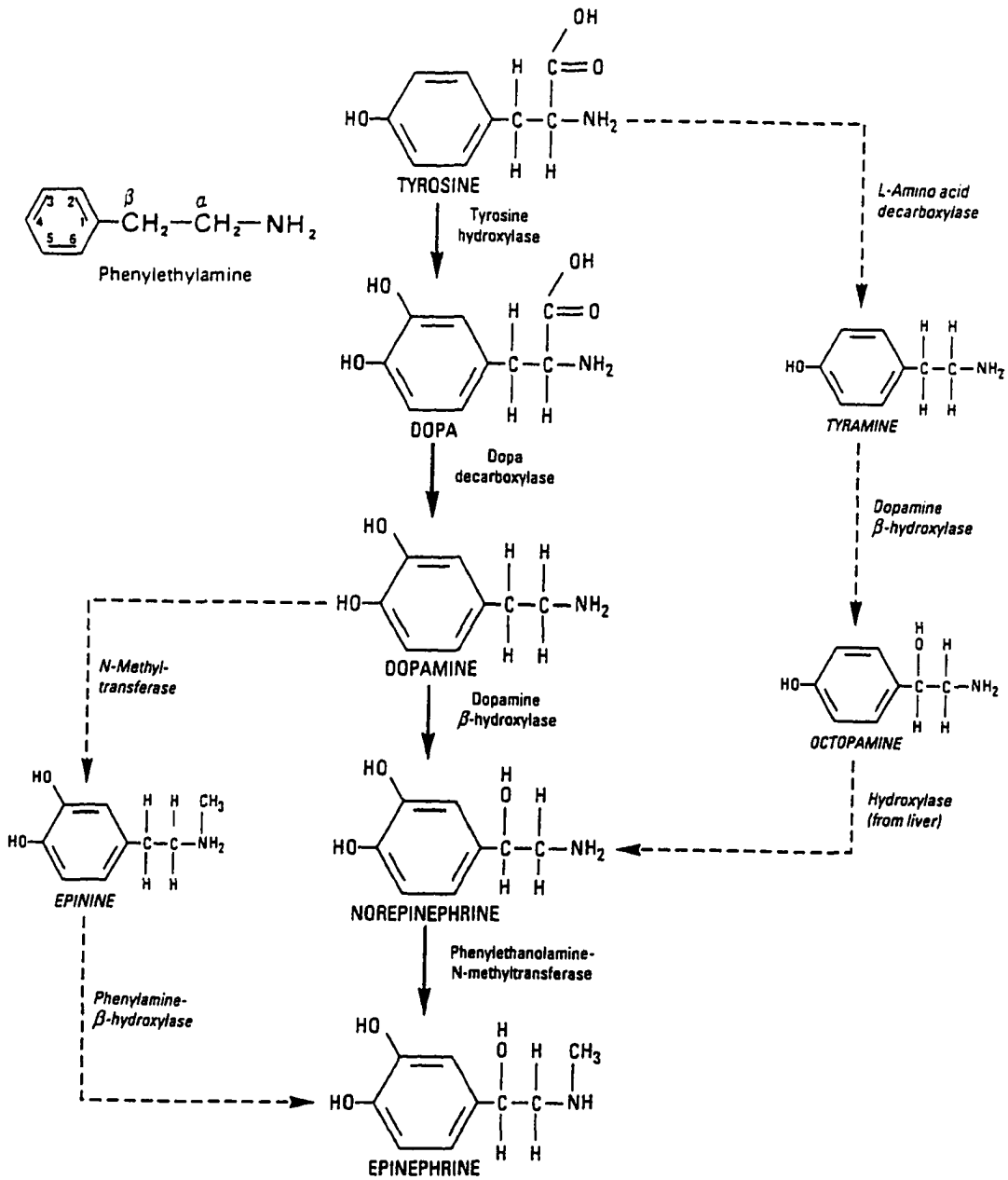

Fig. 5. Biosynthesis of catecholamines. The alternative pathways are shown by the dashed arrows. Phenylethylamine with the indication of α or β carbons is noted in the inset (from Katzung, 1992).

Synthesis of catecholamine

Catecholamines are synthesized from tyrosine by the hydroxylation of the phenolic ring, decarboxylation of the lateral ring, hydroxylation of the lateral ring, and N-methylation (Fig. 5). The major enzymes involved are tyrosine hydroxylase, L-aromatic amino acid decarboxylase, dopamine β-hydroxylase and phenylethanolamine-N-methyltransferase. Dopamine β-hydroxylase is found only in the granules but other enzymes are present in the cytosol of the chromaffin cells (Katzung, 1992).

Tyrosine, a precursor of catecholamine, is actively transported into the cells or derived from the hydroxylation of phenylalanine. Tyrosine is converted to DOPA (3,4-dihydroxy phenylalanine) by the tyrosine hydroxylase. The rate-limiting step in the biosynthesis of catecholamines is the hydroxylation of tyrosine. There are two forms of the tyrosine hydroxylase; a non-phosphorylated form which is only slightly active and a highly active phosphorylated form. The phosphorylation of tyrosine hydroxylase is a process of activation by protein kinase A and Ca^{2+}-activated protein kinase following stimulation of adrenergic nerves and the adrenal medulla (Yamauchi et al., 1981). In addition, the activity of tyrosine hydroxylase is inhibited by catechol compounds (competitive with the pterin receptor of tyrosine hydroxylase), α-methyltyrosine, and 3-iodotyrosine and its metabolites, all considered as feedback inhibition (Hanoune, 1990). DOPA is converted to dopamine by dopamine decarboxylase. Dopamine is transported to neuronal vesicles where the synthesis and the storage of norepinephrine take place. Hydroxylation of dopamine to norepinephrine is catalyzed by dopamine β-hydroxylase, in the presence of Ca^{2+}, ascorbic acid and molecular oxygen. Norepinephrine is stored in the granules of adrenergic nerve terminals (Ganong, 1991).

Release of catecholamine

Catecholamines are released from adrenergic fibers and the adrenal medulla by exocytosis. In the adrenal medulla, acetylcholine from the preganglionic fibers interact with nicotinic receptors to depolarize membrane potentials and to increase Ca^{2+} influx. Increase in $[Ca^{2+}]_i$ results in exocytosis of the granular content, including epinephrine, ATP, some neuroactive peptides and their precursors, chromogranins and dopamine-β-hydroxylase (Winkler et al., 1981). In adrenergic nerve terminals, Ca^{2+} also plays a

role in coupling the nerve impulse with norepinephrine (Burn and Gibbons, 1965).

Adrenergic receptors

Classification of adrenergic receptors

Catecholamines, epinephrine and norepinephrine, exert their effects on target tissue through direct binding with surface receptors, adrenergic receptors (ARs) (Fig. 6). Ahlquist (1948) proposed the two different subtypes (α and β) of ARs based on different rank of orders of potency with natural and synthetic agonists. This subclassification is also consistent when the sensitivity of ARs is tested with antagonists. Finally, this subclassification was proven by a partial agonist, dichloroisoproterenol, which was the first agent antagonizing β- but not α-AR-mediated responses (Powell and Slater, 1957).

α-ARs are further classified into subtypes, α_1- and α_2-ARs on an anatomical, functional, and pharmacological basis. Langer (1974) suggested that ARs which mediate effector organ responses through postjunctional α-AR be termed α_1-ARs and ARs which mediate neurotransmitter release through prejunctional α-AR be termed α_2-ARs. This anatomical classification, however, can not explain the phenomena that postjunctional events are mediated by agonists which are highly selective for α_2-AR. Berthelsen and Pettinger (1977) proposed a functional classification of α-ARs; ARs which mediate stimulatory responses be called α_1-ARs, and ARs which mediate inhibitory responses be called α_2-ARs. However, the vasoconstriction induced by norepinephrine is inhibited by both prazosin and yohimbine suggesting that postjunctional stimulation is mediated through both α_1 and α_2 (Drew and Whiting, 1979). Therefore, anatomical and functional characterizations can not reliably be used to classify α-ARs. Pharmacological classification is based on the relative potency of agonists and antagonists. α-ARs that are activated by methoxamine, cirazoline, or phenylephrine and are blocked by low concentration of prazosin, WB-4101, or corynanthine are classified as α_1-ARs. α-ARs that are activated by α-methylnorepinephrine, UK-14,304, B-HT 920, or B-HT 933 and are blocked by low concentrations of yohimbine, rauwolscine, or idazoxan is classified as α_2-ARs (Table 1) (Ruffolo, Jr., et al., 1991).

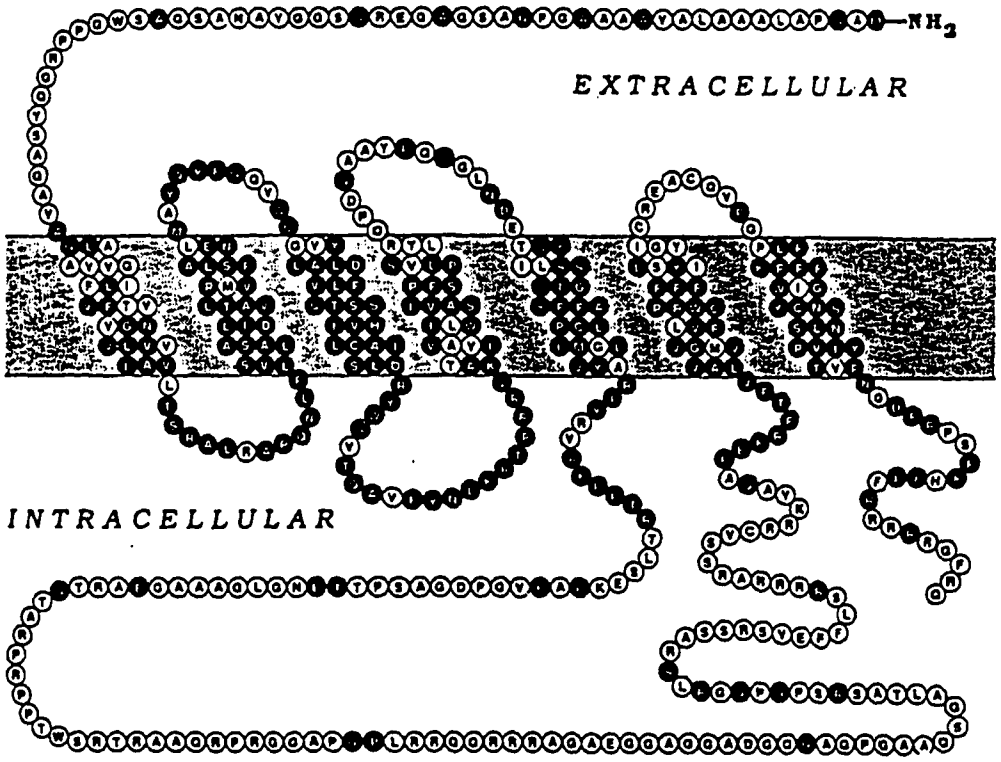

Fig. 6. Primary structure of the human α_2-AR. The amino acid sequence is represented by the one letter code. The arrangement of the receptor structure within the membrane is based on a model for rhodopsin and is thought to be general for other G-protein-coupled receptors (Ruffolo, Jr. et al., 1991).

Table 1. Relative selectivity of α-agonists and -antagonists

	agonists	antagonists
α_1 selective	phenylephrine, methoxamine, cirazoline, amidephrine, Sgd 101/75	prazosin, phenoxybenzamine, WB-4101, corynanthine
α_1 and α_2	epinephrine, norepinephrine	phentolamine, tolazoline
α_2 selective	clonidine, UK-14,304, B-HT 920, B-HT 933 α-methylnorepinephrine	yohimbine, rauwolscine, idazoxan

Modified from Ruffalo, R.R. Jr. et al: Structure and function of α-adrenoceptors. Pharmacol. Rev. 43: 475-505, 1991.

Table 2. Relative selectivity of β-agonists and -antagonists

	agonists	antagonists
β_1 selective	norepinephrine, dobutamine, prenalterol, xamoterol, denopamine	metoprolol, acebutolol, practolol, alprenolol, atenolol, esmolol, betaxolol, bisoprolol, CGP 20712A
β_1 and β_2	isoproterenol, epinephrine	propranolol, timolol, nadolol, pindolol, CGP 12177
β_2 selective	fenoterol, albuterol, terbutaline, metaproterenol, salbutamol, salmeterol	butoxamine, ICI 118551
β_3 selective	BRL 37344, CGP 12177*, ICI 198157, CL 316243	SR 59230A

* Partial agonistic activity.

Modified from Bylund, D.B. et al.: IV. International union of pharmacology nomenclature of adrenoceptors. Pharmacol. Rev. 46: 121-136, 1994.

β-ARs have been subdivided into β_1-, β_2- and β_3-ARs on the basis of the relative selectivity of effects of both agonists and antagonists (Table 1) (Bylund et al., 1994). Heterogeneity of β-ARs will be reviewed in page 28 in detail.

Heterogeneity of α-adrenergic receptors

α_1-Adrenergic receptors

α_1-ARs are subclassified as α_{1A}-, α_{1B}-, and α_{1D}-ARs based on functional, radioligand binding, and molecular biology technique (Table 3) (Hieble et al., 1995). Subclassification between α_{1A}- and α_{1B}-ARs is based on the receptor dissociation constants for 5-methylurapidil or (+)-niguldipine and on sensitivity to irreversible inactivation by chloroethylclonidine. 5-Methylurapidil and (+)-niguldipine are at least 100-fold more selective for the α_{1A}-ARs than α_{1B}-ARs. In addition, indoramin and its analog, SNAP-1069, have approximately 10-fold selectivity for α_{1A}-ARs compared with the other two α_1-AR subtypes (Forray et al., 1994). Chloroethylclonidine selectively alkylates the α_{1B}-ARs. There is no currently no competitive antagonist for α_{1B}-ARs, but spiperone is reported to have a 10-fold higher affinity for α_{1B}- ARs than α_{1A}-ARs (Michel et al., 1989). α_{1D}-ARs have affinity for 5-methylurapidil that is intermediate between α_{1A}- and α_{1B}-ARs. In addition, α_{1D}-ARs have 50- to 100-fold selectivity for antagonists, BMY 7378 and SK&F 105854, and are alkylated by chloroethylclonidine (Hieble et al., 1995).

α_1-ARs are also subclassified as α_{1a}-, α_{1b}- and α_{1d}-ARs by using recombinant technique. α_{1a}-AR is a seven transmembrane spanning, G-protein-coupled receptor and pharmacologically represents α_{1A}-AR (Lomasney et al., 1991; Hieble et al., 1995). α_{1b}-AR is a G-protein-coupled receptor and pharmacologically represents α_{1B}-AR (Ramarao et al., 1992). The chromosomal locations of α_{1A}-, α_{1B}-, and α_{1D}-ARs are C8, C5, and C20 in human, respectively.

α_2-Adrenergic receptors

α_2-ARs are subclassified into 4 subtypes which can be blocked by yohimbine and rauwolscine with different affinities (Table 4). α_{2A}-ARs have a low affinity for prazosin but higher affinity for oxymetazoline and BRL 44408 than α_{2B}-ARs. The rank order of potency for α_{2A}-ARs is RX 821002 > yohimbine = rauwolscine $\approx$ oxymetazoline >

Table 3. Pharmacological properties of the α_1-adrenergic receptor subtypes

Subtypes	Clone	Human chromosomal location	Chloroethylclonidine selective selectivity	antagonist
α_{1A}	α_{1a}	C8	±	SNAP-5089 (+) niguldipine 5-methylurapidil indoramin
α_{1B}	α_{1b}	C5	+ + + +	?
α_{1D}	α_{1d}	C20	+ + +	BMY 7378 SK&F 105854

Modified from Hieble, J.P. et al.: International union of pharmacology.
X. Recommendation for nomenclature of α_1-adrenoceptors: consensus update.
Pharmacol. Rev. 47: 267-270, 1995

Table 4. Pharmacological properties of the α_2-adrenergic receptor subtypes

Subtypes	Clone	Rank order of potency
α_{2A}	α_2C10	RX 821002 > yohimbine = rauwolscine ≈ oxymetazoline > phentolamine ≈ idazoxan > prazosin ≈ chlorpromazine ≈ ARC 239
α_{2B}	α_2C2	rauwolscine > yohimbine > ARC 239 ≈ prazosin ≈ idazoxan ≈ phentolamine > chlorpromazine ≈ oxymetazoline
α_{2C}	α_2C4	rauwolscine > yohimbine > phentolamine ≈ prazosin ≈ ARC 239 ≈ oxymetazoline > chlorpromazine
α_{2D}	?	RX 821002 > oxymetazoline ≈ phentolamine ≈ idazoxan > yohimbine = rauwolscine > prazosin ≈ chlorpromazine ≈ ARC 239

Modified from Remaury, A. and Paris, H. : The insulin-secreting cell line, RINm5F, expresses an alpha-2D adrenoceptor and nonadrenergic idazoxan-binding sites. J. Pharmacol. Exp. Ther. 260: 417-425, 1990.

phentolamine ≈ idazoxan >> prazosin ≈ chlorpromazine ≈ ARC 239. α_{2B}-ARs have high affinity for prazosin and selective affinity for ARC 239, spiroxatrine, and imiloxan (Young et al., 1989). The rank order of potency for α_{2B}-ARs is rauwolscine > yohimbine > ARC 239 ≈ prazosin ≈ idazoxan ≈ phentolamine > chlorpromazine ≈ oxymetazoline. The α_{2C}-ARs are similar to the α_{2B}-ARs regarding the relatively high affinity for prazosin, ARC 239, and spiroxatrine, but it has higher affinity for rauwolscine. The rank order of potency for α_{2C}-ARs is rauwolscine > yohimbine > phentolamine ≈ prazosin ≈ ARC 239 ≈ oxymetazoline > chlorpromazine. α_{2C}-ARs are present in opossum kidney tissue and Y 79 cells. α_{2D}-ARs, present in bovine and rat tissues, have a low affinity for prazosin, ARC 239 and spiroxatrine, and have a lower affinity for rauwolscine and yohimbine than α_{2C}-ARs. The rank order of potency for α_{2D}-ARs is RX 821002 > oxymetazoline ≈ phentolamine ≈ idazoxan > yohimbine = rauwolscine > prazosin ≈ chlorpromazine ≈ ARC 239. α_{2D}-ARs are the rat and bovine homologs of the human α_{2A}-ARs (Bylund and Ray-Prenger, 1989; Remaury and Paris, 1992; Bylund et al., 1994).

α_2-ARs are also subclassified as α_2C10, α_2C2, and α_2C4 in human by using recombinant technique. The α_2C10 and α_2C2 clones pharmacologically represent α_{2A}-AR and α_{2B}-AR, respectively. The α_2C4 clone has pharmacological characteristics of the α_{2C}-AR. The clone which represents α_{2D}-ARs has not been clearly demonstrated, however, rat RG20 clone is suggested to have characteristics of α_{2D}-ARs (Lomasney et al., 1990; Bylund et al., 1992; Bylund et al., 1994). Cloned α_2-ARs in the rat are designated as pA_2d, RG10, cA_2-47 and RG 20. However, mice have a high degree of homology with human α_2C10, α_2C2, and α_2C4 (Bylund et al., 1994).

Heterogeneity of β-adrenergic receptors

β-ARs are subclassified into three subtypes; β_1, β_2 and β_3 (Table 2). The three subtypes of β-ARs are activated by norepinephrine and epinephrine with differential affinities (Bylund et al., 1994). Norepinephrine and epinephrine are equally potent for β_1-AR, but epinephrine has 100-fold more selectivity for β_2-AR than for β_1-AR. In contrast, norepinephrine has higher affinity for β_3-AR than epinephrine. The synthetic catecholamine, isoproterenol, is a potent agonist with equal selectivity for all β-ARs. Selective agonists (denopamine, Ro 363 and xamoterol) for β_1-ARs have low selectivity

and efficacy, however, selective agonists (terbutaline, salbutamol, salmeterol and zinterol) for β_2-ARs have high selectivity. Selective agonists for β_3-ARs are BRL 37344, ICI 198157 and CL 316243, however, CGP 12177, a β_1/β_2-AR antagonist, is a partial agonist for β_3-AR.

Highly selective antagonists for β_1-ARs include metoprolol, practolol, atenolol, betaxolol and CGP 20712A. Highly selective antagonists for β_2-ARs are butoxamine and ICI 118551 (Dooley et al., 1986; O'Donnell and Wanstall, 1980; Emorine et al., 1992). In addition, propranolol is a potent antagonist for β_1- and β_2-ARs, but much less selective for β_3-ARs (Bylund et al., 1994). SR 59230A is the selective antagonist for β_3-ARs (Manara et al., 1995).

All of the β-ARs subclassified in pharmacological profile have been cloned and expressed. The pharmacologic characteristics of the recombinant receptors correspond well with those of three receptor subtypes, although there are some differences in β_3-ARs. Although the clone is derived from a human genomic library, BRL 37344, a β_3-AR agonist, corresponds more closely to those of the rat adipocyte than those of human adipocyte . This may reflect species differences in the β_3-AR density (Zaagsma and Nahorski, 1990).

Adrenergic receptors in pancreatic β-cell

Insulin secretion is modulated by the sympathetic and parasympathetic nervous systems, although glucose and other substrates are the principal regulators of insulin secretion. The parasympathetic preganglionic neurons are originated from the brain stem, and the sympathetic system is from the spinal cord. Stimulation of parasympathetic nerves increases insulin secretion (Kaneto et al., 1967), whereas stimulation of the sympathetic nerves decreases insulin secretion (Miller, 1981). Both α_2- and β-ARs have been demonstrated on pancreatic islets (Ahren, et al., 1984), but the presence of α_1-ARs is still controversial. An α_1-AR agonist, amidephrine mesylate, fails to affect glucose-stimulated insulin release in rat pancreatic β-cells (Malaisse and Moratinos, 1986), but an α_1-AR agonist, phenylephrine, causes a slight but significant increases of insulin release in RINm5F cells (Ullrich and Wollheim, 1985). In porcine pancreatic islet, however, an α_1-AR agonist, phenylephrine, inhibits insulin release, and the inhibitory effect of epinephrine is abolished by an α_1-AR antagonist, prazosin, but

not inhibited by an α_2-AR antagonist, idazoxan (Gregersen et al., 1991). In pancreatic β-cells, there are separate imidazoline binding sites, which are not α_2-ARs. New selective α_2-antagonists of imidazoline family, phentolamine and idazoxan, can stimulate insulin secretion by binding to these sites.

Glucose-induced insulin secretion is inhibited by α_2-agonists and stimulated by β-agonists. β-ARs are β_2-subtype and α_2-ARs are α_{2D}-subtype in rats (Remaury and Paris, 1992). α_2-ARs have also been suggested as α_{2A}-subtype in rat pancreas (Niddam et al., 1990), however, this observation was made without considering the presence of α_{2D}-subtype. In fact, α_{2D}-subtype is suggested as a rat homologue of α_{2A}-subtype.

Voltage-dependent Ca^{2+} channels (VDCCs)

VDCCs in general

VDCCs are classified as L (long-lasting), T (transient or tiny), N (neither L nor T) and P (Purkinje cells) according to their activation and inactivation kinetics, conductance, ion specificities, and sensitivity to drugs and toxins (Table 5). In addition, the Q-type channel has been characterized in the brain (Spedding and Paoletti, 1992; Hofmann et al., 1994).

T-type VDCC

The T-type (low voltage-activated or slow-deactivating) channel is activated at membrane potentials of around -50 mV and exhibits a voltage-dependent inactivation, which may be important for pacemaker activity in several tissues. The T-type channels are present in the sinoatrial node, the atrioventricular node, smooth muscle cells, and neurons where they are responsible for burst firing (White et al., 1989). There are no specific blockers for T-type channels, however, Ni^{2+}, ethosuximide, tetrandine and felodipine show some selectivity for blocking T-type channels.

L-type VDCC

The L-type (high-voltage activated or fast-deactivating) channel is activated at membrane potentials of around -40 mV and reaches its maximum at -10 to 0 mV, and the inactivation of the calcium current is Ca^{2+}-dependent (Pollo et al., 1993). The L-type channel is characterized by its sensitivity to dihydropyridines (DHP), being

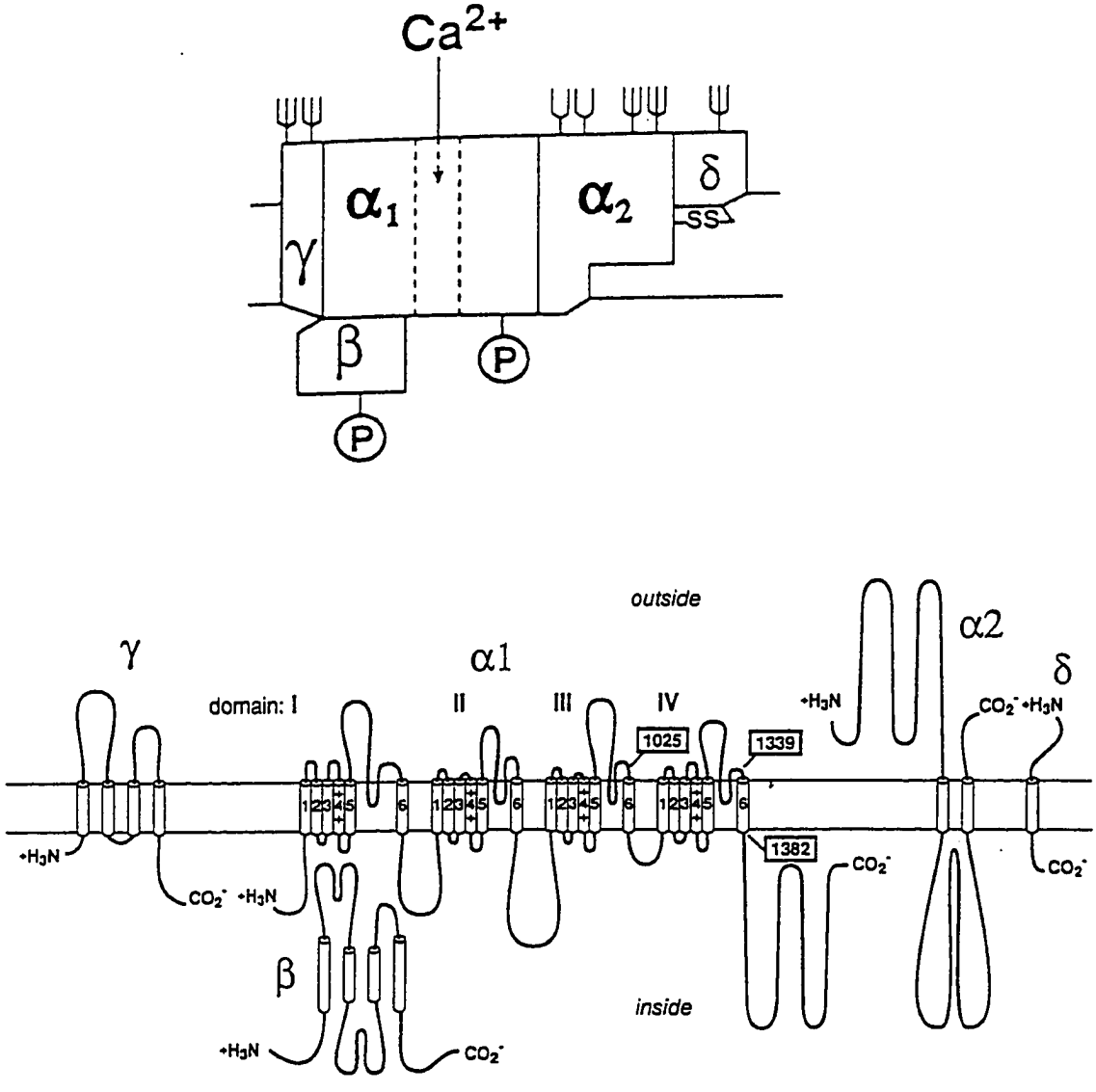

Fig. 7. Structural model of L-type Ca^{2+} channel. Upper panel: subunit structure of an L-type Ca^{2+} channel. P, phosphorylation site. Lower panel: proposed folding patterns of the polypeptide components of an L-type Ca^{2+} channel as predicted from the amino acid sequences deduced from cDNA cloning and sequencing (from Catterall and Striessnig, 1992).

inhibited by the DHP antagonist, nimodipine, and activated by a DHP agonist, Bay K 8644, (Smith et al., 1993). DHPs have a high affinity for the inactivated state of the channel and low affinity for other states (activated and resting). 40 mM KCl shifts the equilibrium to the inactivated state in approximately 70% of cardiac muscle cells and increases the affinity of DHPs to produce pharmacological effects (Bean, 1984). Changes in membrane potentials are critical in the potency of DHPs, therefore, a wide range of holding potentials, stimulation frequencies, charge carriers, etc. should be performed in electrophysiological studies (Catterall and Striessnig, 1992; Spedding and Paoletti, 1992).

L-type channels consist of five subunits; α_1, α_2, β, γ and δ. The α_1 subunit, with a M.W. of 165 KDa, contains sites for phosphorylation and binding sites for L-type channel agonists and antagonists. The subunit has four repeating units (I to IV), each containing six membrane-spanning domains (S1 to S6), of which the S4 is a voltage sensor (Fig. 7). The subunit has 55% homology with sodium channels, therefore, selective drugs have affinity for both sodium channels and L-type channels. The subunit forms an ion-selective pore and binding sites for DHPs, phenylalkylamines and benzodiazepines. The DHP-binding site is localized close to the SS1 - SS2 region of repeat III and to a sequence following the IVS6 (Hofmann et al., 1994). The α_2-subunit does not participate in channel formation and serves as sites for drug binding. The β subunit increases the rate of activation and inactivation of the channel and modifies DHP binding to the α_1 subunit. The structure of γ subunit has been determined but the function is still unknown. The δ subunit is linked to the α_2-subunit by sulfhydryl bonds. There appears to be 4 isoforms of the L-type channel; distinguished as L1 to L4 (Spedding and Paoletti, 1992).

N-, P-, and Q-type VDCCs

The N-type channels, which are coupled to neurotransmitter release, are sensitive to ω-conotoxin GVIA but not sensitive to L-type channel blockers. Some N-type channels are predominantly voltage activated, but others may be modulated by receptor-linked second messenger systems such as G-proteins (Kongsamut et al., 1989). The P-type channels, present in cerebellar Purkinje cells and squid giant axons, are sensitive to ω-agatoxin IVA but not sensitive to either DHPs or ω-conotoxin GVIA

Table 5. Classes of voltage-dependent calcium channels

	Channel types				
	L	N	T	P	Q
Conductance	25 pS	12-20 pS	8 pS	10-12 pS	
Activation	High voltage	High voltage	Low voltage	Moderate high voltage	High voltage
Inactivation	Slow	Moderate	Transient	Very Slow	Fast
Location/ function	Widespread, muscle, nerve	Neuronal transmitter release	Widespread, pacemaker	Neuronal, purkinje	Neuronal
Blockers	DHP, calciseptine, phenylalkylamines	ω-CTX GVIA	Flunarizine?	ω-Aga IVA	ω-Aga IVA ω-CTX MVIIC

Abbreviations: CTX; conotoxin, Aga: agatoxin, DHP; dihydropyridine.

Modified from Spedding, M. and Paoletti, R.: III. Classification of calcium channels and the sites of action of drugs modifying channel function. Pharmacol. Rev. 44: 363-376, 1992. And also summarized from Olivia, B.M. et al.: Calcium channel diversity and neurotransmitter release: The ω-conotoxins and ω-agatoxins. Ann. Rev. Biochem. 63: 823-867, 1994.

(Regan et al., 1991). These channels form a larger portion of Ca^{2+} channels in the brain and are responsible for neurotransmitter release. The Q-type channels, present in the brain, are blocked by a higher concentration of ω-agatoxin IVA than the P-type channels, and are also blocked by ω-conotoxin MVIIC (Hofmann et al., 1994).

VDCCs in pancreatic β-cells

VDCCs are characterized as T- and L-types in pancreatic β-cells and in β-cell lines including RINm5F (Wollheim and Pozzan, 1984; Ashcroft et al., 1990). In addition, the presence of N-, P-, and Q-types has been suggested (Magnelli et al., 1995). The α_1 subunit of L-type VDCCs has been cloned in human pancreatic β-cells and shares 65%, 64%, and 41% homology with the α_1 subunit of rabbit heart, skeletal muscle and brain, respectively (Seino et al., 1992). The properties of L- and T-type channels in pancreatic β-cells are similar to those of skeletal and cardiac muscle and neurons. The L-type channels control voltage-dependent insulin release, but the significance of T-type channels remains to be determined. With the patch-clamp technique, the additional presence of DHP-resistant high voltage activated (HVA) channels is suggested in RINm5F cells (Pollo et al., 1993; Magnelli et al., 1995). The L-type channels contribute to approximately 50% of the HVA current, the N-type channels to 15%, Q-like-channels (kinetically similar to the P- and N-type channels) to 30% and the R-type channels to the remainder of the HVA currents. KCl-induced increases in $[Ca^{2+}]_i$ and insulin release are completely inhibited by DHP antagonists in rat islet cells and in RINm5F cells, but are not attenuated by ω-conotoxin GVIA (Komatsu et al., 1989). Therefore, irrespective of the presence of other types of VDCCs or hormone-regulated Ca^{2+} channels, only the L-type channels appear to be involved in the mechanism of depolarization-induced insulin release. However, contradictory finding has been suggested. D-glyceraldehyde and alanine-induced Ca^{2+}-dependent insulin release is attenuated by ω-conotoxin GVIA in RINm5F cells (Sher et al., 1992). This raises a possibility that depolarization-induced increases in $[Ca^{2+}]_i$ have differential signal transduction pathways from D-glyceraldehyde and alanine-induced increases in $[Ca^{2+}]_i$.

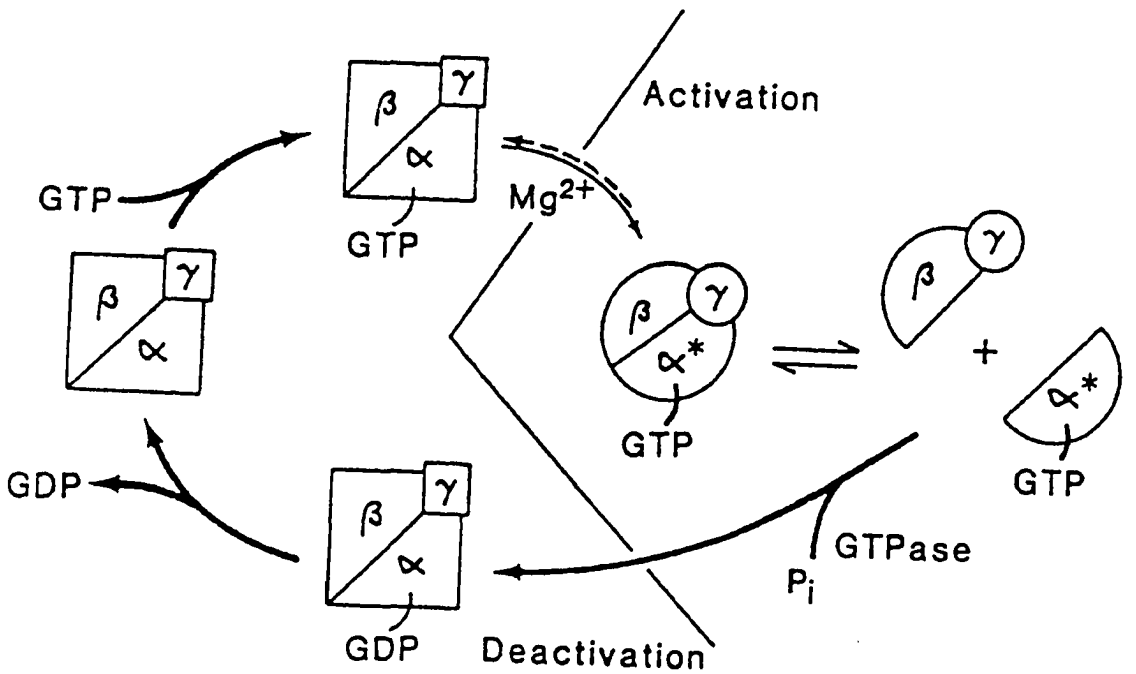

Fig. 8. Regulatory cycle of a G-protein. Squares and semisquares represent inactive conformations of the G-protein as they relate to modulation of effector functions. Circles and semicircles represent activated forms of the G-protein. Activation is both GTP and Mg^{2+} dependent and is stabilized by subunit dissociation to give an G_{α}*-GTP complex and the $G_{\beta\gamma}$ dimer. Hydrolysis of GTP by the G_{α} subunit deactivates it, increases its affinity for $G_{\beta\gamma}$, and leads to reassociation to give an inactive holo-G-protein with GDP bound to it (from Birnbaumer, 1990)

GTP-binding proteins (G-proteins)

G-proteins in signal transduction

G-proteins consist of three subunits named α, β and γ in order of decreasing molecular size. The α-subunits have a single, high affinity binding site for GTP or GDP. The β- and γ-subunits are tightly associated and function as a unit. The GDP-bound form of the α-subunit is an inactive state and is bound tightly to the $\beta\gamma$ subunit, which stabilizes the GDP-bound form of the α-subunit. When GTP binds to an α-subunit, the α-subunit is dissociated from the $\beta\gamma$ subunit and functions as a regulator of effector proteins (Fig. 8). In addition, the α-subunit has an intrinsic GTPase activity and hydrolyses the terminal phosphate of GTP to GDP and a free phosphate. Bacterial toxins covalently modify specific residues of the α-subunit. Cholera toxin (CTX), an 87 KDa protein with one A and five B subunits, covalently modifies the α-subunit of CTX-sensitive-G-proteins (G_s and G_{olf}) amplifying hormonal mechanisms. The A_1 subunit of CTX catalyses the transfer of an ADP-ribose unit from NAD^+ to a specific arginine side chain. This ADP-ribosylation of CTX-sensitive-G-proteins block their capacity to hydrolyze bound GTP to GDP. Pertussis toxin (PTX) ADP-ribosylates a specific cysteine residue of the α-subunit and prevents receptor-mediated activation of G-proteins. The ADP-ribosylation of PTX-sensitive G-proteins (G_i, G_o and G_t) blocks their capability to bind to GTP by releasing GDP. Consequently, both CTX and PTX play a role in increasing levels of cAMP depending on cell type. G-proteins are bound to the plasma membrane by anchoring γ-subunits of G-proteins to a lipid molecule called an isoprenoid or by anchoring α-subunits to a lipid called myristic acid (Hepler and Gillman, 1992). Therefore, lipid modifications can change the affinity of G-proteins to the membrane and the affinity of the α-subunit for the $\beta\gamma$ subunit (Birnbaumer, 1990; Hepler and Gillman, 1992).

Four G-protein families exist based on amino acid sequence relationships; G_s, G_i, G_q and G_{12} (Hepler and Gilman, 1992). The α-subunit of the G_s family (G_s and G_{olf}) stimulates adenylyl cyclase and enhances the rate of cAMP synthesis. The $G_{s\alpha}$-proteins stimulate DHP-sensitive VDCCs (L-type) in skeletal muscle (Mattera et al., 1989) and inhibit Na^+ channels in cardiac muscle (Schubert et al., 1989). The α subunit of the G_i family (G_i, G_o, G_t, G_g and G_z) inhibits adenylyl cyclase or Ca^{2+} channels. The $\beta\gamma$ subunits from G_i may inhibit adenylyl cyclase indirectly by interaction with $G_{s\alpha}$ (Gilman,

1984). The CTX- and PTX-insensitive G_q family (G_q, G_{11}, G_{14}, G_{15} and G_{16}) activates phospholipase C which catalyses hydrolysis of phosphoinositol 4,5-bisphosphate (PIP_2) to form inositol 1,4,5-trisphosphate (IP_3) and diacylglycerol (DAG). The G_{12} family (G_{12} and G_{13}) is present ubiquitously in tissues but its role is still unclear.

G-proteins in pancreatic β-cells

G-proteins link many hormone receptors to adenylyl cyclase, ion channels, phospholipase C, and exocytotic apparatus in pancreatic β-cells. β-ARs are coupled to the G_s-proteins to stimulate adenylyl cyclase and increase cAMP and insulin release (Sharp, 1979). Two molecular forms of $G_{s\alpha}$-proteins are present in HIT cells (derived from hamster islets with Simian virus-40 infection): with one form at 45 KDa and the other form at 52 KDa (Walseth et al., 1989). The 45 KDa form is more efficacious than the 52 KDa form as an activator of cAMP. Epinephrine inhibits insulin release through α_2-ARs, which are coupled to G_i-proteins. PTX pretreatment (initially termed as islet activating protein) prevents the inhibitory effect of epinephrine by lowering cAMP (Katada and Ui, 1979; Hsu et al., 1991a). Five PTX substrates are found as three forms of G_i (G_{i1}, G_{i2} and G_{i3}) and two forms of G_o (G_{o1} and G_{o2}) in RINm5F cells (Gillison and Sharp, 1994). In addition, three forms G_i-proteins and two forms of G_o-proteins are found in HIT cells (Robertson et al., 1991). Two molecular forms of $G_{i\alpha}$-proteins are present in RINm5F cells: with one form at 39 KDa and the other form at 42 KDa (Gillison and Sharp, 1994).

The effects of prostaglandin E_2, somatostatin and galanin depend on the activation of a G_i-protein (Robertson et al., 1987; Hsu et al., 1991b; Gillison and Sharp, 1994). All inhibitory effects of somatostatin are mediated by a PTX-sensitive mechanism, but the inhibitory effect of epinephrine is mediated through both PTX-sensitive and -insensitive mechanisms (Roberson et al., 1991). The PTX-sensitive G-proteins on the effect of epinephrine are known as G_i-proteins which inhibit adenylyl cyclase and proposed as G_{ei}-proteins which exert their effects by directly coupling to exocytotic apparatus (Ullrich and Wollheim, 1988). The PTX-insensitive G-proteins which mediate the inhibitory effect of epinephrine are not clear. However, the PTX-insensitive mechanism of epinephrine is suggested as G_z-proteins (Freissmuth et al., 1989; Robertson et al., 1991).

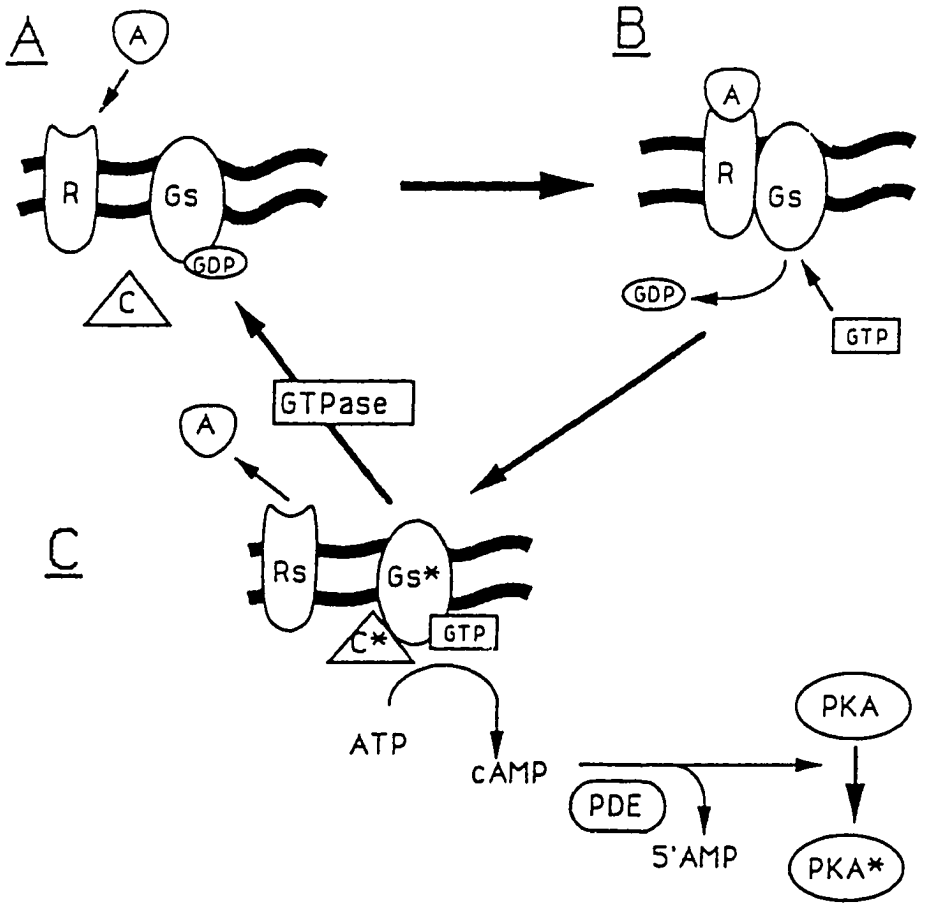

Fig. 9. Molecular mechanism of β-AR activation of adenylyl cyclase and protein kinase A. Panel A shows stimulatory components of the complex in the inactive state. (A) represents β-agonist, (G_s) the stimulatory G-protein and (C) the catalytic component. Panel B shows activation of G_s by an agonist-bound receptor and GTP and the formation of the transient high-affinity complex. Panel C shows activated G_s* which in turn activates C*. G_s* is subsequently deactivated by GTPase activity inherent within the G-protein. Increased cAMP concentration stimulates protein kinase A activity. cAMP is degraded to AMP by PDE (from Maier et al., 1989)

Adenylyl cyclase

Chemistry of adenylyl cyclase

The formation of cAMP from ATP is catalyzed by adenylyl cyclase, an enzyme associated with the plasma membrane in mammalian cells. The six mammalian isoenzymes of adenylyl cyclase (AC I to AC VI) differ in tissue distribution and mode of regulation (Tang and Gilman, 1992). Eight adenylyl cyclases have been cloned; six of these (types I - VI) are from mammalian cells and two others (types VII and VIII) are products of Drosophila and Dictyostellium. Adenylyl cyclase is thought to be either a dimer or tetramer with a M.W. of 120 KDa. There are at least three classes of membrane-bound adenylyl cyclases. The most common motif in higher eukaryotes has one amino-terminal domain (N) and two cytoplasmic domains (C1 and C2), connected by two hydrophobic transmembrane domains (M1 and M2). M1 and M2 have a six transmembrane helix. The hydrophobic domains may serve as a site for activation by forskolin, an activator of adenylyl cyclase. Among the eight adenylyl cyclases, C1a and C2a domains (portions of C1 and C2 domains, respectively) have 50% - 92% homology among mammalian enzymes. The catalytic activity of adenylyl cyclases requires both C1a and C2a domains.

Physiology of adenylyl cyclase

Adenylyl cyclase is regulated by hormones and neurotransmitters, acting through both receptors (generally with seven transmembrane domains) and G-proteins. Adenylyl cyclase is stimulated by GTP-bound $G_{s\alpha}$-proteins and inactivated by hydrolysis of GTP from $G_{s\alpha}$-protein (Fig. 9). Inhibition of adenylyl cyclase is mediated by the α subunit of G_i protein. The $\beta\gamma$ subunits also inhibit type I adenylyl cyclase by binding to stimulated α subunits of G_s-proteins, thereby blocking their access to adenylyl cyclase. Therefore, the inhibition by $\beta\gamma$ subunits occurs only when G_s is present. By engaging the stimulatory α subunit, the inhibitory $\beta\gamma$ subunits prevent $G_{s\alpha}$-proteins from interacting with adenylyl cyclase. However, the $\beta\gamma$ subunits greatly potentiate the stimulatory effect of $G_{s\alpha}$-protein on types II and IV. The concentration of the $\beta\gamma$ subunits required to regulate the activity of adenylyl cyclase is greater than that of the α subunit of G_s protein. Thus, the G_s-protein may not be the source of the $\beta\gamma$ subunits for such regulation. Rather, the necessary concentrations of the $\beta\gamma$ subunits may be

contributed by G-proteins such as G_i and G_o, which are more abundant than G_s (Hepler and Gillman, 1992). Although all the isoenzymes are stimulated by the GTP-bound α-subunits of G_s-proteins, they differentially respond to other regulators such as Ca^{2+} and calmodulin (CaM). Types I and III adenylyl cyclase are stimulated by Ca^{2+}/CaM, while types II and IV are not sensitive to Ca^{2+}/CaM (Lustig et al., 1993).

Adenylyl cyclase in pancreatic β-cells

Adenylyl cyclase in pancreatic islets is regulated by Ca^{2+}-CaM (Thams et al., 1982). Intracellular Ca^{2+} may affect adenylyl cyclase to control cellular cAMP levels by 1) stimulating adenylyl cyclase via CaM, 2) directly inhibiting adenylyl cyclase, 3) stimulating cAMP-dependent PDE via CaM and 4) stimulating CaM-dependent kinase or protein kinase C-mediated effects on adenylyl cyclase (Caldwell et al., 1992). In RINm5F cells, Ca^{2+} stimulates adenylyl cyclase via a CaM-dependent mechanism. Ca^{2+} inhibits adenylyl cyclase activity in the absence of CaM but stimulates adenylate cyclase in the presence of CaM. Maximal stimulation is achieved by 1 μM $[Ca^{2+}]_i$ in the presence of 1 μM CaM (Caldwell et al., 1992). Inhibitors of Ca^{2+}-calmodulin kinase II, KN-62 and KN-93, inhibit forskolin-stimulated insulin release, suggesting the possible involvement of Ca^{2+}-calmodulin kinase II in the control of insulin release after adenylyl cyclase is activated (Niki et al., 1993). The differences in the response of Ca^{2+}/CaM-dependent adenylyl cyclase between cell types may reflect the relative distribution of the CaM-dependent and CaM-independent forms of adenylyl cyclase. However, in pancreatic islets, subtypes of adenylyl cyclase have not been demonstrated.

Adenylyl cyclase in pancreatic islets is also regulated by Na^+ . β-AR- and forskoin-stimulated activation of adenylyl cyclase is enhanced by Na^+ suggesting that Na^+ can play a role in regulating G_s-proteins/adenylyl cyclase system (Watson et al., 1989).

<u>cAMP-dependent protein kinase (protein kinase A)</u>

Chemistry of protein kinase A

cAMP primarily activates protein kinase A to control diverse phenomena such as metabolism, gene transcription, and memory (Krebs and Bevo, 1979). Protein kinase A is present in all mammalian cells at a cellular level of 0.2 - 2 μM. In the absence of cAMP, protein kinase A is an inactive tetramer composed of two regulatory subunits

and two catalytic subunits. Two isoforms of regulatory subunits (I and II with M.W. of 43 and 45 KDa, respectively) are products of different genes. Because regulatory subunits I and II isoforms have a complex with the same catalytic subunit isoforms, the release and activation of catalytic subunit from either holoenzyme would target the same substrates (Doskeland et al., 1993). The regulatory subunits are homodimers formed through interactions of monomers near the amino termini, although heterodimers also occur (Jahnsen et al., 1986).

Three isoforms of the catalytic subunit (α, β and γ with a M.W. of 40 KDa) are the products of separate genes. In the absence of cAMP, the regulatory homodimer is combined with any of the catalytic subunit isoenzymes with a concentration of 0.2 μM. The catalytic activity of the catalytic subunit is latent because of the inhibition by the inhibitory domain of regulatory subunits. When two cAMP molecules bind per regulatory subunit, the affinity of regulatory subunit for catalytic subunit decreases 10,000 - 100,000-fold. Tetrameric protein kinase A dissociates into dimeric regulatory subunits and two monomers of catalytic subunits, and inhibition of catalytic subunit by regulatory subunit is concomitantly released (Francis and Corbin, 1994). After the catalytic subunit is activated, it catalyzes the transfer of the γ-phosphate of ATP to protein or peptide substrates containing the -RRXSX- substrate consensus amino acid sequence (Yeaman et al., 1977).

The regulatory subunits of protein kinase A are classified into type I and type II based on the order of holoenzyme elutes in anion exchange resins (Corbin et al., 1975) and their amino acid sequence (Takio et al., 1984). In addition, type I and type II holoenzymes can functionally be distinguished on the basis of their potential for autophosphorylation and interaction with MgATP. The type II holoenzyme contains an autophosphorylation site on the regulatory subunit, whereas the type I holoenzyme is not autophosphorylated but has a high affinity binding site for MgATP (Hoffmann et al., 1975). The isoenzymes are equally activated because MgATP stabilizes type I protein kinase A, and autophosphorylation stabilize type II protein kinase A.

Physiology of protein kinase A

Protein kinase A, primarily the type II isoform, is associated with the cytoskeleton, Golgi complex, and microtubule organizing centers (Bregman et al., 1989; Bregman et al., 1991). In neurons, more than 70% of protein kinase A is localized to the postsynaptic densities and dendritic cytoskeletal elements. Proteins that interact with regulatory subunit II to anchor the catalytic subunits or regulatory subunits to subcellular compartments have been called A kinase Anchoring Proteins (AKAPs). AKAPs vary in size, amino acid sequence and relative affinities for either regulatory subunits IIα or IIβ (Meinkoth et al., 1990). AKAPs may specifically localize protein kinase A to regions/substrates for which rapid regulatory control is important.

Regulation of cellular processes by protein kinase A occurs over a narrow range of activities, because two- to three-fold increases in cAMP produce maximum physiological responses in most tissues. One mechanism for limiting cAMP elevation is through negative feedback control to regulate its own steady-state activity. The activated protein kinase A phosphorylates and activates a PDE, which accelerates the rate of cAMP degradation (Gettys et al., 1987). This PDE is designated as low K_m PDE, cGMP-inhibited PDE or type III PDE.

Protein kinase A in pancreatic β-cells

Two isoforms of protein kinase A are present in pancreatic β-cells (Sugden et al., 1979) and several islet proteins are phosphorylated in the presence of cAMP (Harrison et al., 1984). Although protein kinase A phosphorylates some substrates, the nature of the substrates and their role in cAMP-mediated responses remains unclear. In HIT cells, protein kinase A increases Ca^{2+} influx through voltage-dependent Ca^{2+} channels by phosphorylating Ca^{2+} channels or closely associated proteins (Rajan et al., 1989). However, in pancreatic β-cells and RINm5F cells, forskolin potentiates glucose-induced insulin release without changing $[Ca^{2+}]_i$ (Wollheim et al., 1984; Rorsman and Abrahamsson, 1985). Furthermore, forskolin stimulates insulin release while $[Ca^{2+}]_i$ is held constant (Tamagawa et al., 1985). Thus, the potentiating effect of cAMP on glucose-stimulated insulin secretion may not be mediated by VDCCs in the pancreatic β-cells (Hill et al., 1987). In addition, protein kinase A closes ATP-sensitive K^+ channels (Holz et al., 1993) and/or directly interacts with the secretory machinery

(Ammala et al., 1993). In HIT cells, changes in cAMP content and the subsequent effect on $[Ca^{2+}]_i$ appears to be dependent on glucose, suggesting that an unidentified glucose-generated signal is required for the action of cAMP (Rajan et al., 1989).

There are contradictory observations regarding cAMP/protein kinase A-mediated insulin release. An increase in cAMP concentration can potentiate, but does not initiate secretion from the pancreatic β-cells. This suggests that an elevation in intracellular cAMP concentration is an insufficient stimulus for the initiation of insulin secretion and cAMP plays a potentiating role in the secretory processes (Persaud et al., 1990). In addition, cAMP can act through mechanisms other than the activation of protein kinase A. The regulatory subunit of protein kinase A containing bound cAMP can function independently to interact with the catalytic subunit of protein kinase A. An unidentified cAMP-binding protein may mediate cAMP effects without interacting with protein kinase A (Weber et al., 1987). Furthermore, cAMP may act by binding to cGMP-dependent protein kinase or ion channels (Ludwig et al., 1990; Jiang et al., 1992; Holz, 4th. et al., 1995).

CHAPTER II RATIONALE

Since a rat clonal insulin-secreting cell line RINm5F was established from rat insulinoma, RINm5F cells have facilitated numerous studies on β-cell functions such as receptors, the regulation of $[Ca^{2+}]_i$ and insulin release. However, their utilization has been limited because insulin secretion gradually is decreased and the morphology is changed from differentiated to undifferentiated state as the culture ages. Our preliminary studies showed that the effect of epinephrine on insulin release was inhibitory in differentiated cells and stimulatory in undifferentiated cells. The effect of KCl and Bay K 8644 increased $[Ca^{2+}]_i$ and insulin release more in differentiated cells than in undifferentiated cells. Although insulin secretion was greater in differentiated cells than in undifferentiated cells, a novel insulin-like immunoreactivity was demonstrated only in undifferentiated cells. Therefore, we were interested in characterizing RINm5F cells in a passage- and morphology-dependent manner to determine whether these changes are a genuine feature of the cells or merely a consequence of long-term maintenance.

In rat pancreatic β-cells and RINm5F cells, information has accumulated on the heterogeneity and structure of insulin. Rat pancreas secretes 2 types of insulin (rat insulins I and II), but RINm5F cells express only rat insulin type I. In chapter III, we first demonstrated a novel insulin-like immunoreactivity in undifferentiated RINm5F cells which was attributable to neither rat insulin I nor II by using radioimmunoassay (RIA), immunocytochemistry (ICC) and immunoblot assay.

Specific tools for the investigation of insulin have been RIA, ICC, and immunoblot assay. The measurement of insulin is relatively simple, precise and rapid when measured by RIA. Insulin measurement by RIA techniques is based on specific binding of an anti-insulin antibody to insulin (Parker, 1981). RIA techniques require insulin labelled with ^{125}I and depend on the ability of the unlabeled insulin to inhibit binding of the radioactive insulin by antibody. To maximize sensitivity, the assay is carried out in the presence of only enough antibody to achieve substantial radioactive insulin binding. In this study, the dilutions of antibodies selected gave approximately 30% binding of radioactive insulin when unlabeled insulin is absent. The process is a simple competition in which insulin occupies a portion of the antibody-combining sites

reducing the free antibodies available to radioactive insulin. Fixed concentrations of antibody and labeled ^{125}I-insulin are incubated in the absence and in the presence of the unknown amounts of insulin. Once adequate binding of labeled ^{125}I-insulin has been established, free and antibody-bound ^{125}I-insulin are separated and measured by a γ-counter. The unknown concentration of insulin is determined by comparing the decrease of ^{125}I-insulin binding with a standard curve obtained by adding known amounts of insulin to the assay system.

Localization of insulin within cells can be determined by ICC. After the cells are permeabilized by Triton-X 100, the cells are preincubated with normal goat serum to minimize nonspecific staining, and subsequently incubated with a primary antiserum vs. insulin. After removal of the primary antiserum, the cells are treated with biotinylated secondary antibodies and an avidin-biotinylated peroxidase complex (ABC). Peroxidase staining is obtained by incubating the cells in diaminobenzidine (DAB) and H_2O_2. In addition to ICC, sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and immunoblot are sensitive and capable of a high degree of resolution for insulin. A SDS-PAGE system separates proteins in a M.W. range from 1 to 100 KDa. The use of tricine as a trailing ion allows a resolution of insulin at lower acrylamide concentrations than in glycine-SDS-PAGE systems (Schagger and Jagow, 1987). Samples are electrophoresed on an SDS-PAGE and transferred to a nitrocellulose paper for reaction with a subsequently added antiserum vs. insulin. The insulin-antibody vs. insulin on the sheet is incubated with biotinylated secondary antibodies and ABC. Peroxidase generates a colored product after incubating the sheets in DAB.

Epinephrine potentially modulates insulin release through ARs, with α_2-ARs inhibiting and α_1- and β-ARs stimulating insulin release. The presence of α_1, α_2 and β_2-ARs has been demonstrated in pancreatic β-cells and β-cell lines including RINm5F cells. In pancreatic β-cells and RINm5F cells, the α_2-actions on the effect of epinephrine in insulin release have been relatively well demonstrated; however, α_1- and β_2-actions have not. RINm5F cells are useful in classifying and characterizing ARs and signal transduction mechanisms through their receptors. Furthermore, alterations of functional distributions of ARs can be studied when the morphology of the cells is changed from differentiated to undifferentiated state as the culture ages. The efficiency of the stimulus through ARs is influenced by the density of the receptors and

the nature of the signal transduction mechanisms which translate external signals into the cell responses. Therefore, specific AR-agonists and -antagonists can demonstrate the functional involvement of ARs on the cells, and specific enzyme activators and inhibitors can provide information on the contribution of signal transduction mechanisms through the receptors. In chapter IV, we characterized a passage- and morphology-dependent change of ARs with regard to their relative contributions to cAMP production and insulin release. In addition, in chapter V, we further demonstrated the possible contribution of G-protein, adenylyl cyclase, PDE and protein kinase A for the action mediated by ARs. These studies identify the distinct distribution of ARs between differentiated and undifferentiated cells and provide information for further utilization of RINm5F cells in characterizing the function of ARs and adenylyl cyclase in the cells.

An increase in $[Ca^{2+}]_i$ induces insulin release in pancreatic β-cells. Stimulus-secretion coupling in pancreatic β-cells, therefore, refers primarily to the membrane and intracellular events that mediate an increase in $[Ca^{2+}]_i$ sufficient to modulate insulin release in the cells. In RINm5F cells, the contribution of VDCCs to increases in $[Ca^{2+}]_i$ and insulin release has been studied; however, the relative distribution of VDCC subtypes is still controversial. In chapter VI, we employed RINm5F cells with two morphological features to further characterize the subtypes of VDCCs with regards to their voltage dependency, kinetics, and pharmacology. This study provides information to better understand the functional roles of VDCCs in electrophysiological and chemical signallings. The functional role of VDCCs is important as they are frequent targets for modulation by hormone, neurotransmitter, and drugs.

A fluorescent probe, Fura-2, allows reliable quantitification of intracellular free Ca^{2+} concentration from a resting level of approximately 100 nM to a maximal level of several micromolar. Fura-2 is a Ca^{2+}-chelating dye that changes its fluorescent properties after binding to free Ca^{2+}. Fura-2 has an excitation wavelength between 340 and 380 nm, and the emission of the two wavelengths is monitored at 510 nm. An increase in $[Ca^{2+}]_i$ results in an increase in the ratio of 340/380 nm. An advantage of Fura-2 is that some variabilities from nonuniform dye concentrations, probe leakage, dye bleaching and cell thickness do not affect the ratio measurements. In addition, the ratio measurement has time resolution to the millisecond, which allows for recording of

$[Ca^{2+}]_i$ in real time.

Ca^{2+} conductance through its channels is directly proportional to the activity of the channel. The whole-cell patch-clamp technique makes it possible to study Ca^{2+} channels. The patch-clamp technique provides an electrical isolation of a small membrane patch from the rest of the cell membrane. The patch pipette is pressed against the cell membrane and a slight negative pressure forms a seal with high resistance on the order of 10^9 ohm (Gigaohm), a so called gigaohm seal. After the gigaohm seal is established, the membrane is permeabilized by amphotericin B to eliminate "run-down" (diminution of Ca^{2+} currents without apparent reason) and the dialysis of cytoplasmic constituents with the pipette solution leading to the loss of regulatory second messengers. When stimulated by voltage or ligands, the Ca^{2+} channels in the plasma membrane open and the charged ions move through the channel due to an electrical gradient. The movement of the charged ions is called current, which passes through the wire, is amplified, and then recorded by the amplifier.

The methodology and techniques described above can be used to characterize 1) a novel insulin-like immunoreactivity, 2) the effect of epinephrine on cAMP production and insulin release, 3) the effect of Gs-protein, adenylyl cyclase, PDE, and protein kinase A, and 4) a characterization of VDCCs in differentiated and undifferentiated RINm5F cells.

CHAPTER III CHARACTERIZATION OF A NOVEL INSULIN-LIKE IMMUNOREACTIVITY IN A RAT CLONAL BETA-CELL LINE, RINM5F IN A PASSAGE- AND MORPHOLOGY-DEPENDENT MANNER

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Richard L. Engen, Cheryl Clark, Ter-Hsin Chen and Walter H. Hsu

ABSTRACT

A rat clonal β-cell line, RINm5F, is derived from a radiation-induced insulinoma and secretes insulin. We have utilized radioimmunoassay, immunocytochemistry and immunoblotting by using antisera vs. rat, porcine and bovine insulins to characterize a novel insulin-like immunoreactivity in undifferentiated RINm5F cells. Antisera vs. rat and porcine insulins recognized all rat, porcine and bovine insulins. However, the antiserum vs. bovine insulin did not recognize rat insulin but did recognize porcine and bovine insulins.

The RINm5F cells exhibited one of two morphological features; the differentiated state with long neuron-like processes and the undifferentiated state without the processes. As the morphologies of the cells were changed from differentiated to undifferentiated cells, the amount of secreted insulin decreased. By applying antisera vs. rat and porcine insulins in RIA, the amount of secreted insulin from RINm5F cells was gradually decreased; however, it was gradually increased by antiserum vs. bovine insulin. Transmission electron microscopy revealed that more dense core secretory vesicles were contained in differentiated cells than in undifferentiated cells. Examination by immunocytochemistry and immunoblot demonstrated that antisera vs. rat and porcine insulins reacted with insulin in both differentiated and undifferentiated cells. However, antiserum vs. bovine insulin reacted with insulin in undifferentiated cells, but not with insulin in differentiated cells. In immunoblotting, insulin from cell homogenate was found as a single band at a M.W. of 6 KDa. Antiserum vs. porcine insulin reacted with insulin from both differentiated and undifferentiated cell

homogenate. However, antiserum vs. bovine insulin reacted with insulin from undifferentiated cell-homogenate, but not with insulin from differentiated cell-homogenate. These results suggested that a novel insulin-like peptide (NIP) is present in undifferentiated RINm5F cells, which is not attributable to rat insulin I nor II. In addition, NIP secreted from undifferentiated cells was neither rat insulin I and II nor insulin like growth factor (IGF)-I and -II.

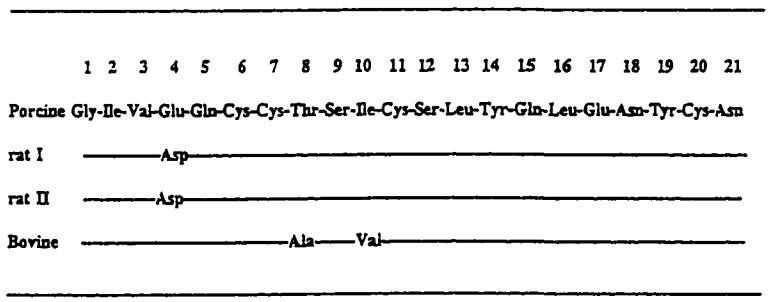
INTRODUCTION

Insulin, with a 5.6 KDa and 51 amino acids, is composed of two amino acid chains joined together by two disulfide linkages (Miaza and Wilkin, 1989). The two chains are compactly arranged, with the A chain positioned above the central helical portion of the B chain. Pancreatic β-cells synthesize insulin from a single-chain precursor called proinsulin. On conversion of proinsulin to insulin, four basic amino acids and the remaining connector or C-peptide are removed by proteolysis. Preproinsulin has been identified as the precursor of proinsulin. Preproinsulin is synthesized and cleaved to proinsulin in the endoplasmic reticulum, and is therefore not a product that is accumulated in the β-cell (Chan et al., 1976).

In contrast to the majority of mammals, not one but two different insulins are present in the pancreas of rats (Smith, 1964; Clark and Steiner, 1969) and mice (Balant et al., 1971). In the rat, these insulins are nonallelic and coded by two distinct genes, with the corresponding mRNAs showing about 93% homology (Ullrich et al., 1977; Villa-Komaroff et al., 1978). The primary translation products, rat preproinsulin I and II, differ by 3 amino acid substitutions in the signal peptide sequence, 2 in the C-peptide region and 2 in the B-chain (Cordell et al., 1979; Villa-Komaroff et al., 1978). Many species variations of insulin are known and the major sites of structural difference among porcine, bovine, and rat insulins are in positions 8, 9, and 10 of the A chain (Rathjen et al., 1986). The structural differences of bovine, porcine and rat (I and II) insulins are shown in figure 1.

The rat insulinoma cell line RINm5F is derived from a radiation-induced insulinoma transplanted for further growth in nude mice. The expression of the two nonallelic genes in rat insulinoma cells has been found to be 10-fold higher for rat insulin I than

A Chain



B Chain

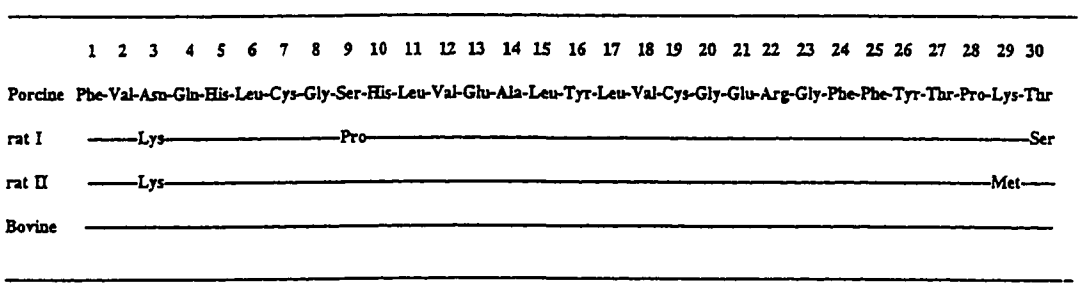


Fig. 1. Comparison of the amino acid sequences of insulins (Rathjen et al, 1986) used in this study.

rat insulin II (Cordell et al., 1982). It also has been demonstrated that only the rat insulin type I gene is expressed despite the presence of the rat insulin II gene in rat insulinoma cells (Fiedorek Jr. et al., 1990). These differences in insulin production rates could be the result of differences in transcription of either of the two nonallelic genes present in rats (Lomedico et al., 1979) or subsequent steps involving processing and stability of either of the two gene products (Fiedorek Jr. et al., 1990).

In a preliminary study, we observed that the amount of secreted insulin from RINm5F cells was decreased as the morphologies of the cells were changed from neuron-like processed (differentiated) cells to non-processed (undifferentiated) cells. Furthermore, antiserum vs. rat insulin could recognize insulin secreted by both cell types but antiserum vs. bovine insulin recognized insulin secreted only by undifferentiated cells. These findings suggested that a novel insulin-like immunoreactivity was present in undifferentiated cells and prompted us to further characterize the novel insulin-like immunoreactivity in undifferentiated RINm5F cells. We hypothesized that undifferentiated RINm5F cells secrete a novel insulin-like peptide (NIP) that is different from the insulin secreted by differentiated RINm5F cells. Therefore, this study was performed to characterize the novel insulin-like immunoreactivity in undifferentiated RINm5F cells.

MATERIALS AND METHODS

Materials

The following materials were used: RPMI-1640, fetal bovine serum, IGF-I, IGF-II, and poly-L-lysine (all from Sigma Chemical,St. Louis, MO), 24-well culture plate (Corning Glass Works, Corning, NY), rat insulins I and II, porcine insulin and bovine insulin (Eli Lilly, Indianapolis, IN), antiserum vs. rat insulin (Linco Research Inc., St Louis, MO), antiserum vs. porcine insulin (a gift from Dr. Joseph Dunbar, Wayne State University, Detroit, MI), antiserum vs. bovine insulin (a gift from Dr. Allen Trenkle, Iowa State University, Ames, IA), secondary biotinylated anti-guinea pig IgG antibody and avidin-biotin complex kit (all from Vector, Burlingham, CA), Tricine (Fisher, Pittsburgh, PA), Tricine-gel (Jule Inc., New Haven, CT), nitrocellulose paper (Bio-Rad, Hercules, CA), and a low molecular marker (Novex, San Diego, CA).

Cell culture

RINm5F cells were obtained from Dr. S.B. Pek (University of Michigan Medical Center, Ann Arbor, MI). The cells were maintained in RPMI-1640 medium with 10% fetal bovine serum as described previously (Chen et al., 1994). The cells were subcultured every 7 days and the medium was changed at day 3 and day 5 after subculture. All experiments were performed by use of cells from passages 28-40. No antibiotics were used.

Insulin secretion

Static incubations were performed to study insulin release as previously described (Hsu et al., 1991). Briefly, RINm5F cells were plated into 24-well Corning plates and grown for 5 days. For experiment, every other passage from 28 to 40 was used. For passages from 28 to 32, the cells were plated at 0.2 million cells/well and for passages after 34, the cells were plated at 0.1 million cells/well. Growth medium was removed and the monolayer cells were washed with modified Krebs-Ringer bicarbonate buffer (KRB) containing (in mM): 136 NaCl, 4.8 KCl, 2.5 $CaCl_2$, 1.2 KH_2PO_4, 1.2 $MgSO_4$, 5 $NaHCO_3$, 10 HEPES, 1.67 glucose and 0.1% bovine serum albumin at pH 7.4. The cells were preincubated for 15 min, and incubated for 15 min at 37 °C. After experiments, the cells were detached by using trypsin-EDTA and the cell numbers were counted in each well. Insulin in the media was measured by radioimmunoassay (RIA) as previously described (Hsu et al., 1991).

Immunocytochemistry

The cells were seeded into Petri dishes with histological cover slides that were previously coated with poly-L-lysine and then cultured for 5 days. The cells were then fixed with 4% paraformaldehyde and stained for insulin by an avidin-biotin complex method described previously (Hill et al., 1987). Briefly, the cells were treated with 0.3% H_2O_2 to block endogenous peroxidase activity and permeabilized with 0.4% Triton-X 100. The cells were incubated with antiserum vs. rat or bovine insulin for 24 h at room temperature. The secondary biotinylated anti-guinea pig IgG antibody (0.02%) was applied for 2 h, followed by an incubation with 1% avidin-biotin-horseradish-peroxidase complex. Immunologically bound peroxidase was visualized

with 0.4 mg/ml diaminobenzidine and 3% H_2O_2. The cells were counterstained with Neutral Red and dehydrated with ethanol series from 70% to 100%.

Electron microscopy

The cells were grown on poly-L-lysine coated coverslides for 5 days and fixed in phosphate-buffered 5% glutaraldehyde. The cell were processed as previously described (Dellmann and Stahl, 1984). Briefly, the cells were postfixed in 1% OsO_4 plus 1.5% potassium ferricyanide in veronal acetate buffer for 1 h, stained *en bloc* for 2 h in uranyl acetate in the same buffer, dehydrated and embedded in an epon-araldite mixture for transmission electron microscopy. Following orientation in semi-thin sections, thin sections were examined at 50 KV with a Hitachi HU 12 A electron microscope.

Immunoblotting

RINm5F cells in suspension were homogenized with a tissue homogenizer and the cell debris was pelleted by 30 min of centrifugation at 10,000 x g. Insulin was separated by tricine-SDS-gel electrophoresis as previously described (Schagger H., and von Jagow, G., 1987). Briefly, a tricine buffer system with stacking and spacing gels and a separating gel of 16.5% polyacrylamide was applied. The running time was 2 h. The proteins separated were transferred to nitrocellulose paper. For immunoblotting, the nitrocellulose paper was blocked by incubation in 2% nonfat dried milk in Tris-buffered saline (50 mM Tris, 150 mM NaCl, pH 7.4) for 1 h as described previously (Fiedorek Jr. and Parkinson, 1992). The antiserum vs. porcine or bovine insulin were applied for 24 h followed by applying biotinylated anti-guinea pig secondary antibody for 1 h. The avidin-biotin-horseradish-peroxidase complex was applied and immunologically bound peroxidase was visualized with diaminobenzidine and H_2O_2.

Statistical analysis

All values were expressed as the mean ± S.E. Results were analyzed by using analysis of variance (ANOVA). The least significance test was used to test for differences between means of end points for which the ANOVA indicated a significant ($P < 0.05$) *F* ratio.

RESULTS

Changes in morphology of RINm5F cells

RINm5F cells exhibited one of two morphological features; the differentiated state with neuron-like processes and the undifferentiated state without the processes (Fig. 2). Transmission electron microscopy revealed the presence of dense core secretory granules typical for vesicles in unstimulated RINm5F cells (Fig. 3). RINm5F cells contained scattered dense-core secretory granules which exhibited variable morphologies. Some cells exhibited the characteristic clear halo of islet β-cell granules and others displayed a more immature, halo-devoid appearance. The dense core secretory granules were heterogeneously dispersed predominantly along plasma membranes and cellular processes. The dense core secretory vesicles were more numerous in the differentiated cells than undifferentiated cells. In differentiated cells, the endoplasmic reticulum (ER) was composed of elongated cisternae and occurred in stacks. In contrast, in undifferentiated cells the ER was composed of short or elongated cisternae and dispersed in a poorly organized way (Fig. 3). More than 80% of the cells between passages 28 and 32 had neuron-like processes. However, the cells gradually lost processes to become non-processed cells with more than 99% of the cells after passage 35 not exhibiting processes (Fig. 4). We defined a passage which had $>90\%$ of neuron-like processes as differentiated cells, and a passage which had $<1\%$ of the processes as undifferentiated cells. Therefore, the passages 28 - 30 were differentiated cells and the passages $\geq$ 35 were undifferentiated cells (Fig. 4).

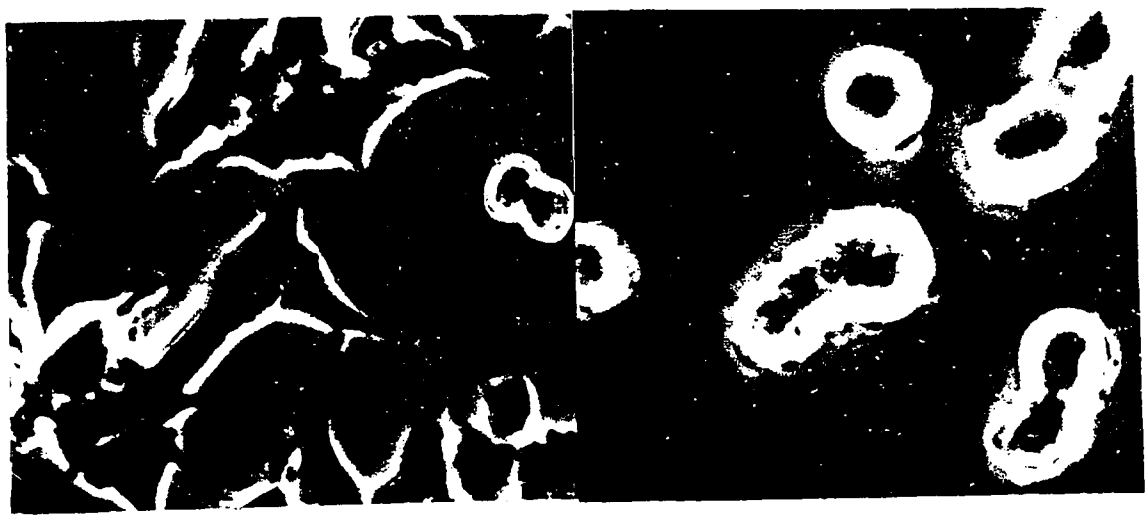
Specificities of antisera vs. rat-, porcine- and bovine-insulins on rat, porcine and bovine insulins and insulin-like growth factors

Experiments were performed by incubating antiserum vs. rat, porcine or bovine insulin with ^{125}I-porcine insulin and increasing concentrations of rat, porcine or bovine insulin from 31.25 to 8000 pg/ml. The binding affinities of antiserum vs. rat, porcine or bovine insulin were calculated from the concentration of the insulins required to inhibit the binding of ^{125}I-porcine insulin by 50% (IC_{50}). IC_{50}'s of rat, porcine, and bovine insulins to antiserum vs. rat insulin were 362, 387 and 241 pg/ml, respectively.

Fig. 2. Phase-contrast photographs of differentiated (A) and undifferentiated (B) RINm5F cells. Magnification 400 X. These pictures were taken 48 h after subculture. (C) Change of the percentage of cells with processes with passage-dependent manner in RINm5F cells. One representative result out of 5 observations. The cells were counted 48 h after subculture.

A Differentiated cells

B Undifferentiated cells



C

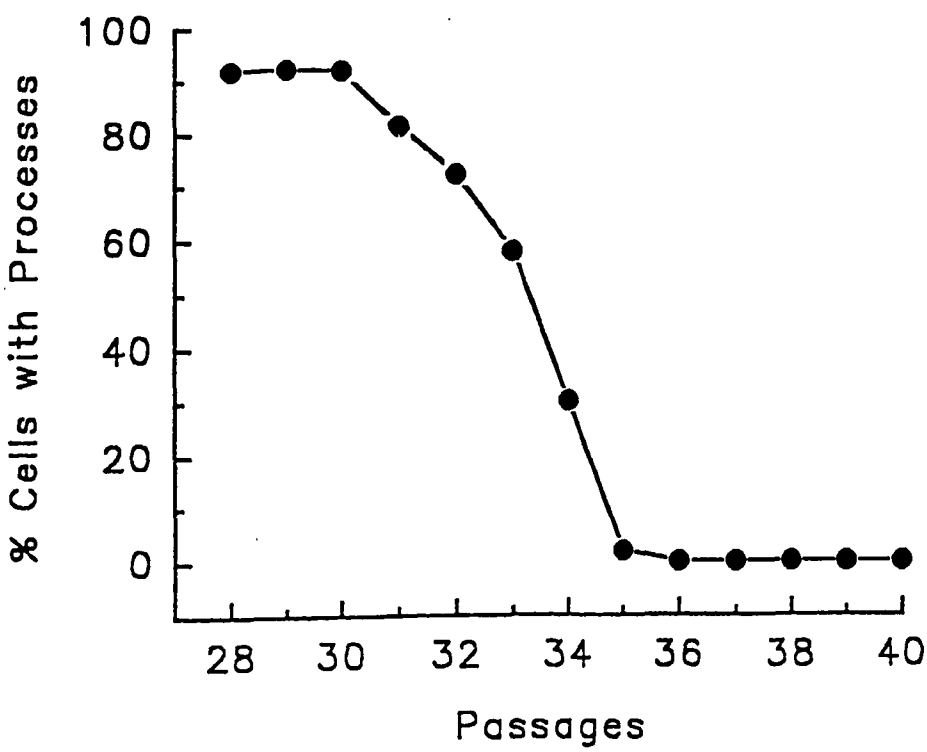


Fig. 3. Ultrastructures of differentiated (left panel) and undifferentiated (right panel) RINm5F cells. A and B are representatives of most fields from differentiated cells. C and D are representatives from undifferentiated cells. Dense core secretory vesicles (⇑) and endoplasmic reticulum (↑) are more apparent in differentiated cells than in undifferentiated cells. The secretory granules are found along with plasma membrane (⌓), mitochondria (▲), endoplasmic reticulum (↑) and Golgi apparatus (★). Magnifications; A and B 3,750 X, C and D 15,000 X.

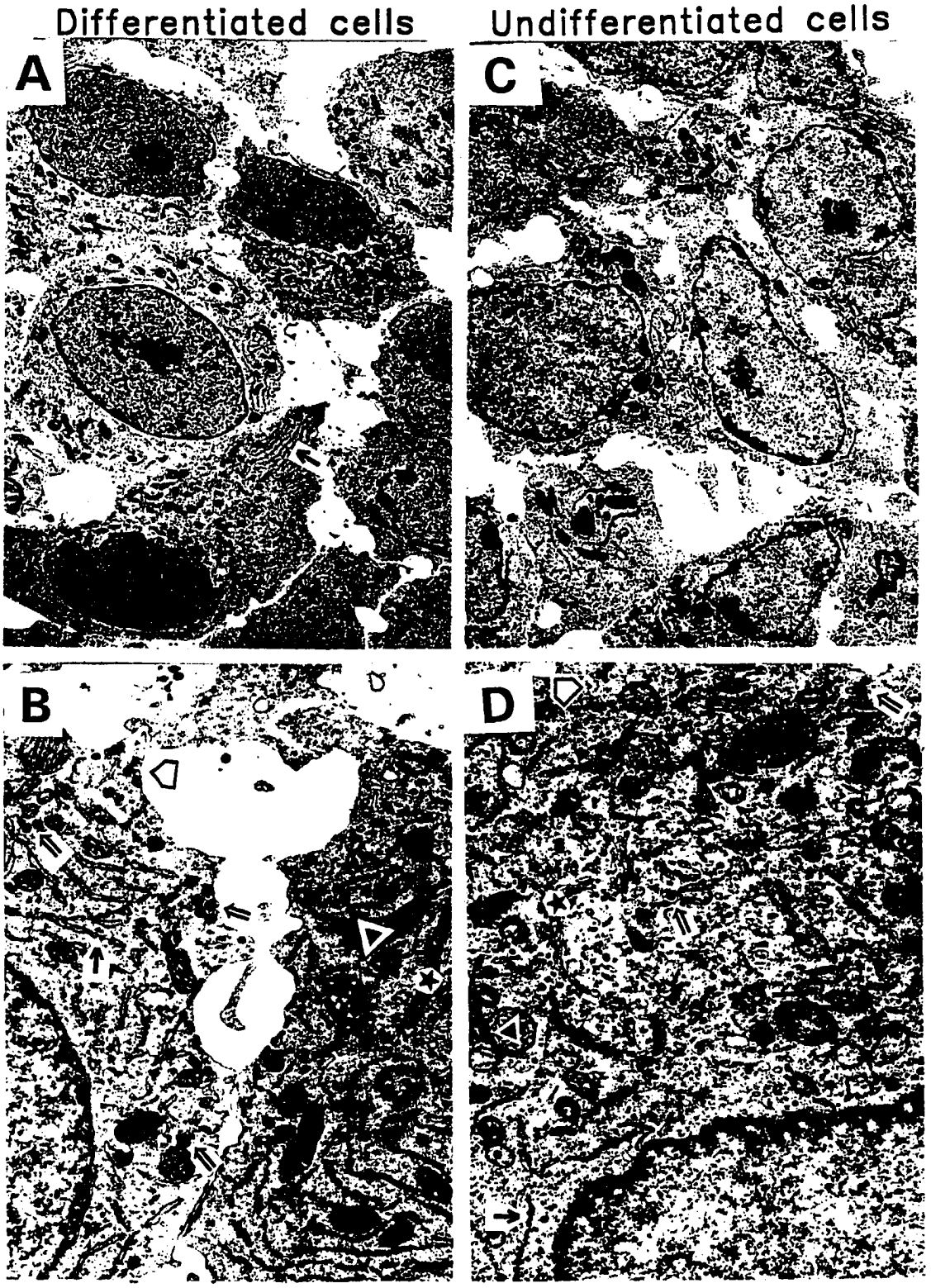
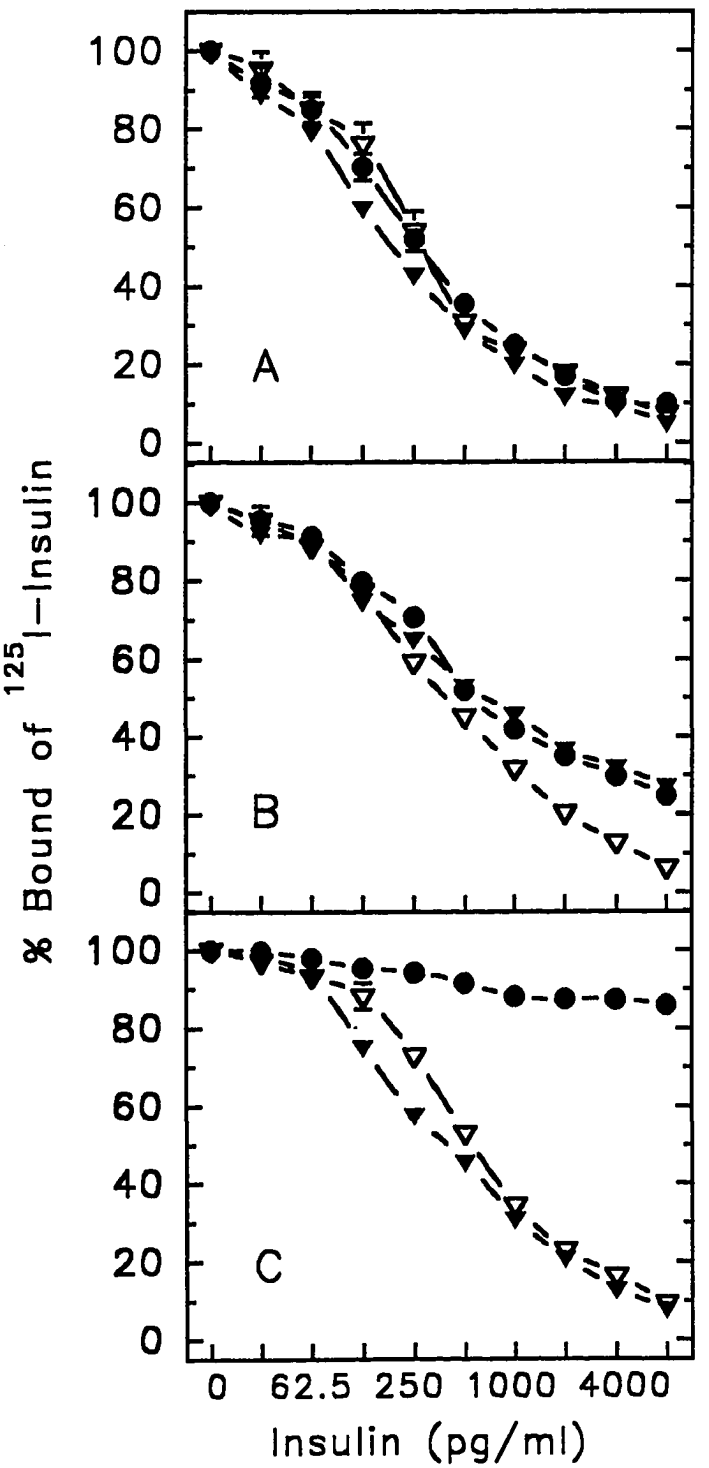

Differentiated cells
Undifferentiated cells
A
C
B
D

Fig. 4. Specificities of antisera vs. rat (A), porcine (B) and bovine (C) insulins. Insulin labelled with ^{125}I was incubated in the presence of rat (●), porcine (▽) or bovine (▼) insulin. Once adequate binding of labelled ^{125}I-insulin was established, free and antibody-bound ^{125}I-insulin was separated and measured by a γ-counter. Mean ± S.E. (n = 4) are shown.


100
80
60
40
20
0
A
B
C
% Bound of ^{125}I–Insulin
0
62.5
250
1000
4000
Insulin (pg/ml)

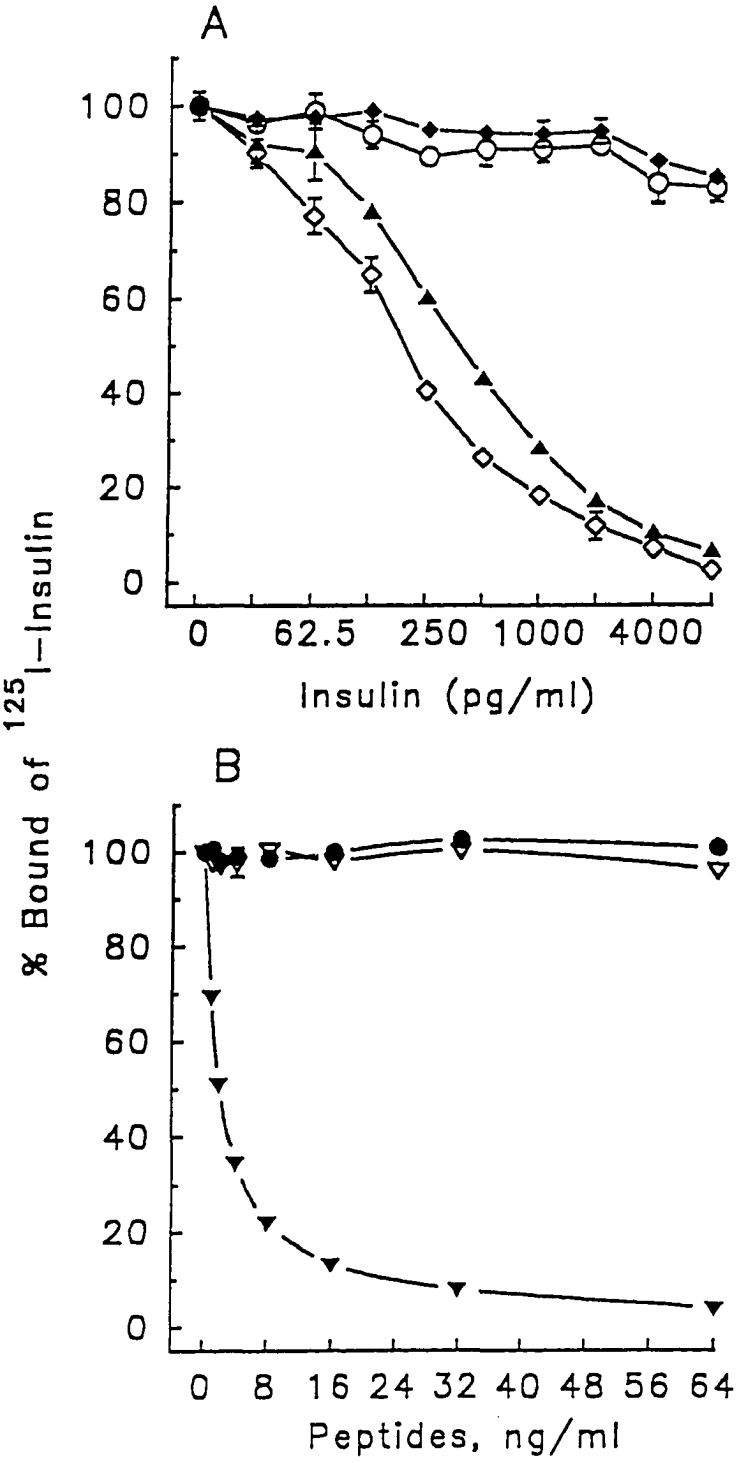
The IC_{50} values of rat, porcine, and bovine insulins to antiserum vs. porcine insulin were 1907, 474 and 982 pg/ml, respectively. The IC_{50} values of porcine and bovine insulins to antiserum vs. bovine insulin were 688 and 545 pg/ml, respectively. However, The IC_{50} values of rat insulin to antiserum vs.bovine insulin could not be determined because rat insulin only inhibited the binding of ^{125}I-porcine insulin by 14% at a maximal concentration of rat insulin at 8000 pg/ml (Fig. 5). Furthermore, even a high concentration of 64 ng/ml, rat insulin inhibited the binding of ^{125}I-porcine insulin to antiserum vs. bovine insulin only by 20%. Rat insulins I and II (8000 pg/ml) inhibited the binding of ^{125}I-porcine insulin to antiserum vs. bovine insulin by only 15% (Fig. 5A). Since IGFs have considerable homology with insulin and proinsulin, the specificity of antiserum vs. bovine insulin on IGF-I and -II was determined. The antiserum was incubated with ^{125}I-porcine insulin and increasing concentrations of IGF -I and -II from 2 to 64 ng/ml and even a single high concentration of 512 ng/ml. IGF -I and -II at 2 to 64 ng/ml and 512 ng/ml did not significantly inhibit the binding of 125I-porcine insulin to antiserum vs.bovine insulin (Fig. 5B).

Changes in insulin release and a novel insulin-like immunoreactivity in RINm5F cells

The cells secreted insulin in all passages studied (28 - 40). When antisera vs. rat or porcine insulins were used to recognize the insulin, the amount of basal release of insulin was gradually decreased as morphologies of the cells were changed from differentiated to undifferentiated cells. However, when antiserum vs. bovine insulin was used, the amount of insulin secreted was gradually increased from nearly undetectable concentrations, as the morphology of the cells changed from the differentiated state to undifferentiated state (Fig. 6).

To further characterize a novel insulin-like peptide (NIP) secreted from undifferentiated cells, competitive inhibition of the peptide for the binding of ^{125}I-porcine insulin to antiserum vs. insulin was compared with the inhibition of insulin for the binding of ^{125}I-porcine insulin. The experiment was performed by incubating antiserum vs. rat, porcine or bovine insulin with ^{125}I-porcine insulin and NIP from undifferentiated cell homogenate. NIP (31.25 - 8000 pg/ml) dose-dependently inhibited

Fig. 5. (A) Specificities of antisera vs. rat (▲ and ◇) and bovine (◆ and ○) insulins on rat insulin I (◆ and ▲) and II (○ and ◇). ^{125}I-insulin was incubated with rat insulin I and II. (B) Specificity of antiserum vs. bovine insulin on IGF-I (●) and IGF-II (▽) and bovine insulin (▼). ^{125}I-insulin was incubated in the presence of IGF-I, IGF-II, and bovine insulin. Mean ± S.E. (n = 4) are shown.


A
100
80
60
40
20
0
0
62.5
250
1000
4000
Insulin (pg/ml)
% Bound of ^{125}I-Insulin
B
100
80
60
40
20
0
0
8
16
24
32
40
48
56
64
Peptides, ng/ml

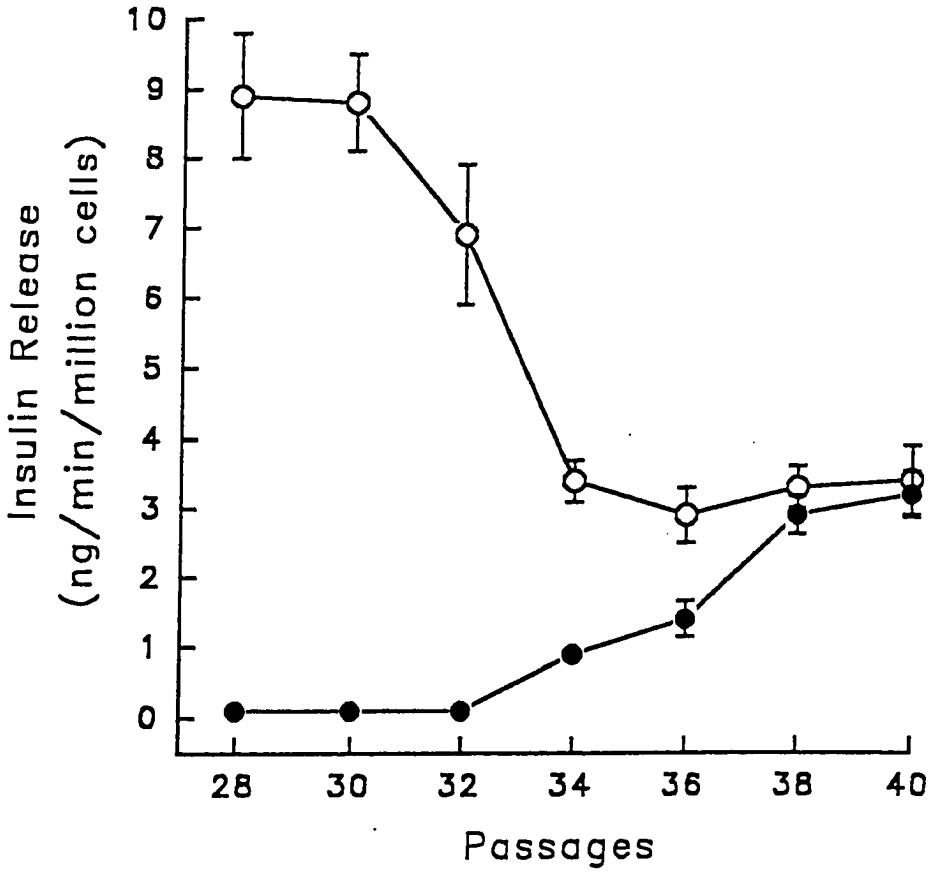
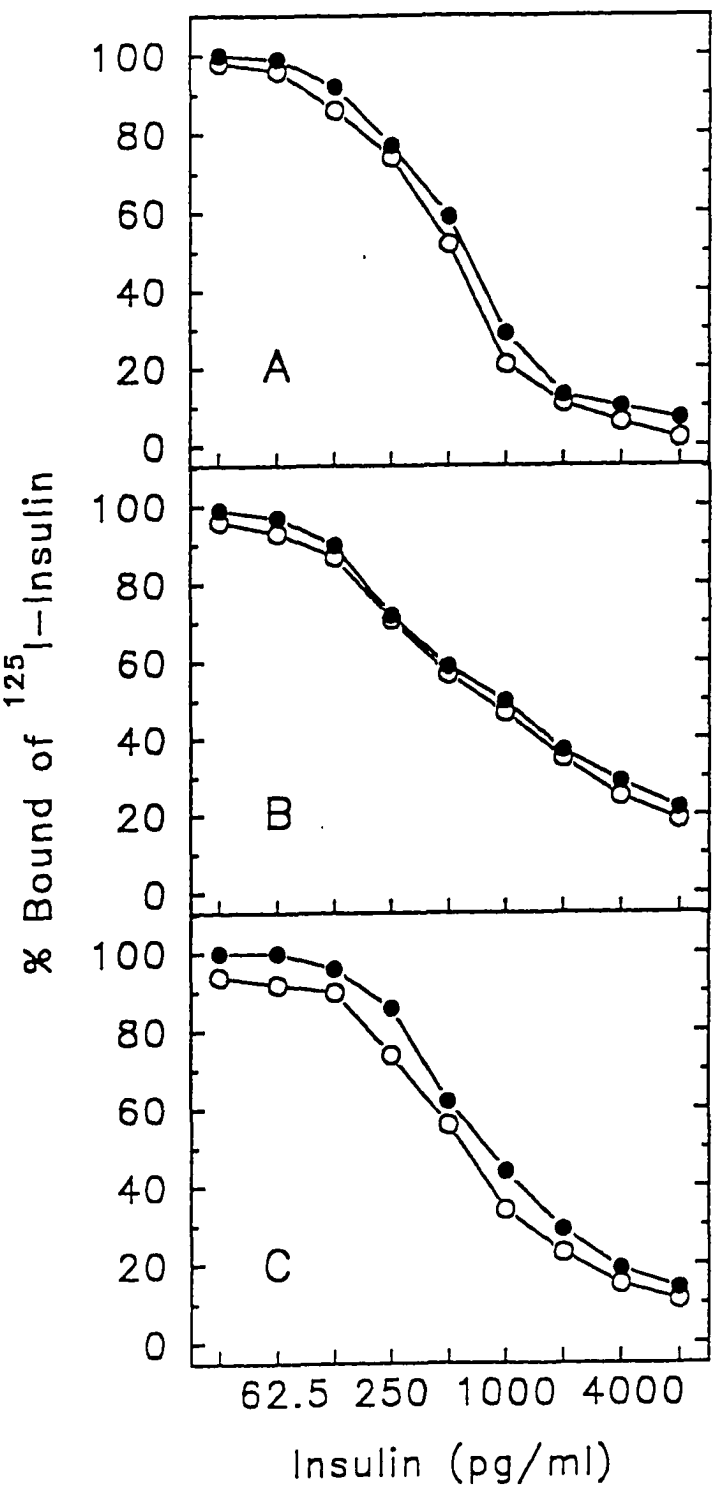


Fig. 6. Passage dependency of basal insulin secretion in RINm5F cells. The basal insulin was measured by antiserum vs. rat insulin (O) and by antiserum vs. bovine insulin (●). After KRB buffer was removed, the cells were detached by using trypsin-EDTA and the cell numbers were counted in each well. Mean ± S.E. (n = 16) are shown.

Fig. 7. Dose-dependent inhibition by a novel insulin-like peptide (NIP) from undifferentiated cell-homogenate for the binding of ^{125}I-insulin. Rat insulin (A and B) and porcine insulin (C) were used as a standard with antisera vs. rat (A), porcine (B), and bovine insulin (C). ^{125}I-insulin was incubated with NIP (●) and insulin (○). Once adequate binding of labelled ^{125}I-insulin was established, free and antibody-bound ^{125}I-insulin was separated and measured. Mean (n = 4) is shown.


100
80
60
40
20
0
A
B
C
% Bound of ^{125}I–Insulin
62.5 250 1000 4000
Insulin (pg/ml)

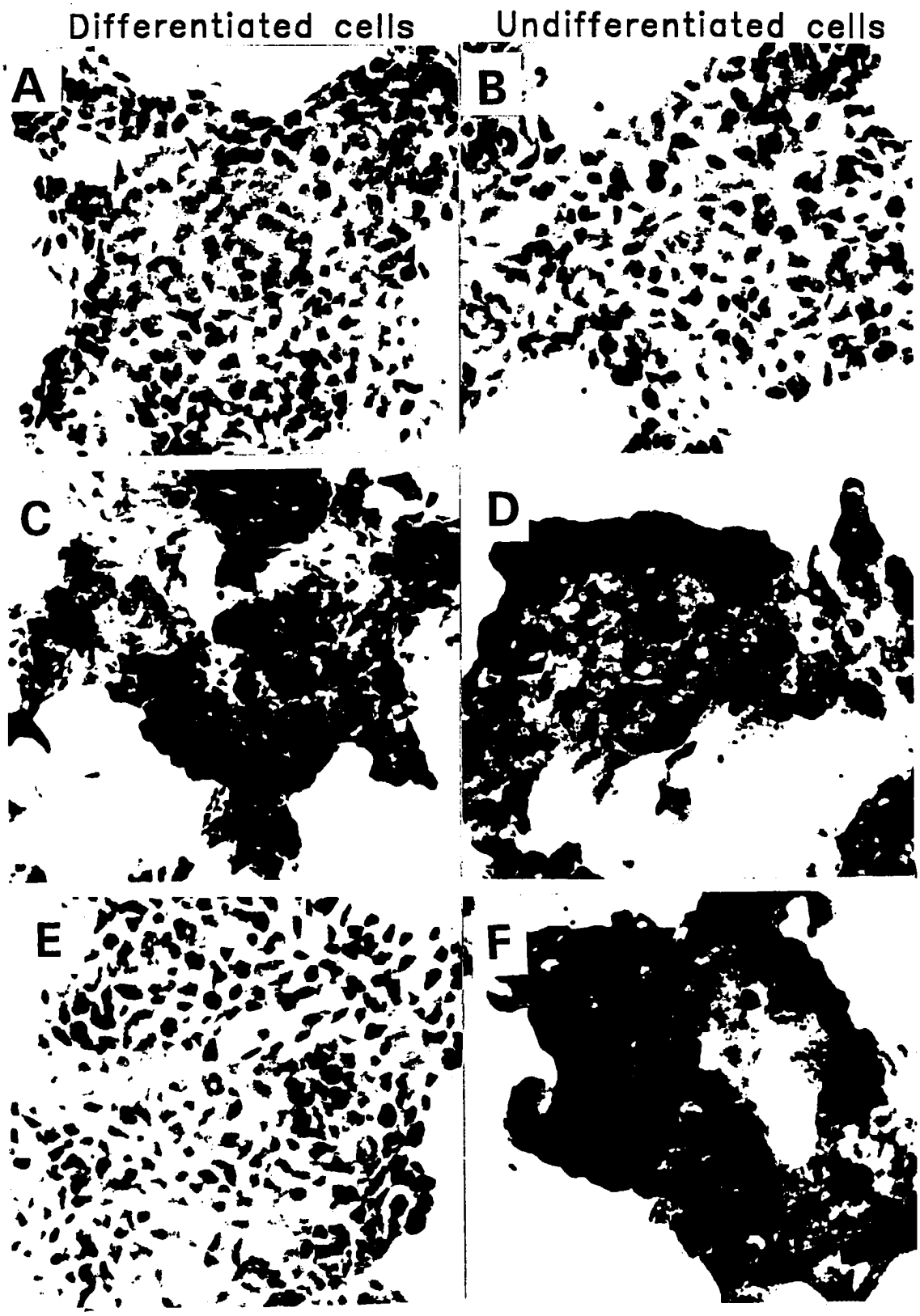
the binding of ^{125}I-porcine insulin to antisera vs. rat, porcine, and bovine insulins (Fig. 7). In addition, the pattern of dose-dependent inhibition by NIP paralleled the pattern of the inhibition by rat and porcine insulins. This suggested that NIP contained in undifferentiated RINm5F cells was most likely insulin.

To determine whether all of NIP secreted by undifferentiated cells was immunoreactive to antiserum vs. bovine insulin, two standard systems were applied by using porcine insulin with antiserum vs. porcine insulin, and by using porcine insulin with antiserum vs. bovine insulin. The amount of NIP recognized by a standard of porcine insulin with antiserum vs. porcine insulin was 2.6 ± 0.1 and 3.0 ± 0.2 ng/min/million cells in passages 38 and 40, respectively. The amount of NIP recognized by a standard of porcine insulin with antiserum vs. bovine insulin were 2.9 ± 0.2 and 3.3 ± 0.1 ng/min/million cells in passages 38 and 40, respectively. Between these standard systems, the amount of NIP was not significantly different between two standard systems in passages 38 and 40. This suggested that all of the NIP secreted in undifferentiated cells was immunoreactive to antiserum vs. bovine insulin.

Localization of insulin using immunocytochemistry and immunoblot analysis in RINm5F cells

Immunoreactive insulin was localized in RINm5F cells using immunocytochemistry. Insulins, which were not treated with antisera vs. rat and bovine insulins, were not immunostained in either differentiated or undifferentiated cells (Figs. 8A and 8B). In addition, insulins, which were not treated with biotinylated anti-guinea pig antibody, were not immunostained in either differentiated or undifferentiated cells (data not shown). Both differentiated and undifferentiated cells were immunostained for insulin with antiserum vs. rat insulin (Figs. 8C and 8D). However, with antiserum vs. bovine insulin, insulin was immunostained in undifferentiated cells (Fig. 8F) but not in differentiated cells (Fig. 8E). To further characterize a novel insulin-like immunoreactivity, immunoblotting was applied (Fig. 9). Antiserum vs. porcine insulin reacted with rat insulin I (*lane 2*), however, antiserum vs. bovine insulin did not (*lane 5*). In addition, antiserum vs. porcine insulin recognized insulin from both cell homogenates (*lanes 3 and 4*), however, antiserum vs. bovine insulin recognized insulin

Fig. 8. Localizations of insulin by immunocytochemistry in differentiated (left panel) and undifferentiated (right panel) RINm5F cells. As a negative control (A and B), antiserum vs. insulin or biotinylated anti-guinea pig antibody was omitted. Antiserum vs. rat insulin (C and D) or antiserum vs. bovine insulin (E and F) were treated to stain insulin. The cells were counterstained with Neutral Red. These pictures are representatives of most fields from differentiated and undifferentiated RINm5F cells. Magnification 128 X.


Differentiated cells
Undifferentiated cells
A
B
C
D
E
F

only from the undifferentiated cell homogenate (*lanes 6 and 7*). Insulin from cell homogenates were not immunostained when they were not treated with either antiserum vs. insulin or biotinylated anti-guinea pig antibody (data not shown). In immunoblotting, insulins from both cell homogenates and rat insulin I had same mobility and a single band. M.W of insulin from both cell homogenates and rat insulin I were near 6 KDa (Fig. 10).

DISCUSSION

We have characterized a novel insulin-like immunoreactivity in undifferentiated RINm5F cells, which does not represent either rat insulins I or II. As the morphology of the cells changed from the differentiated state to undifferentiated state, the amount of secreted insulin was decreased; however, the immunoreactivities of antiserum vs. bovine insulin were increased from nearly undetectable concentrations. Studies from immunocytochemistry and immunoblot further confirmed that insulins from the undifferentiated cells were immunoreactive with all antisera vs. rat, porcine and bovine insulins, although insulins from the differentiated cells were immunoreactive with both antisera vs. rat and porcine insulins but not with antiserum vs. bovine insulin.

Previous investigations have demonstrated the presence of rat insulins I and II in pancreatic β-cells and insulinoma derived cell lines (Smith, 1966; Clark and Steiner, 1969; Cordell et al., 1982; Gross et al., 1988; Fiedorek Jr. et al., 1990). The expression of two insulin genes I and II in a transplantable β-cell tumor has been found to be 10-fold higher for rat insulin I than rat insulin II, while in normal pancreatic islets there are approximately equal amounts of each insulin (Cordell et al., 1982). In contrast, it has also been demonstrated that RINm5F cells contain only rat insulin I mRNA and selective expression of the insulin I gene is a relatively general characteristic of all RIN cells (Fiedorek Jr. et al., 1990). However, a novel insulin-like immunoreactivity in RINm5F cells, which was not due to rat insulin I and II, has not been previously demonstrated.

Electron microscopic study revealed that more insulin secretory vesicles were contained in differentiated than undifferentiated cells. These findings are consistent with a report that sodium butyrate, an inducer of cellular differentiation, induces

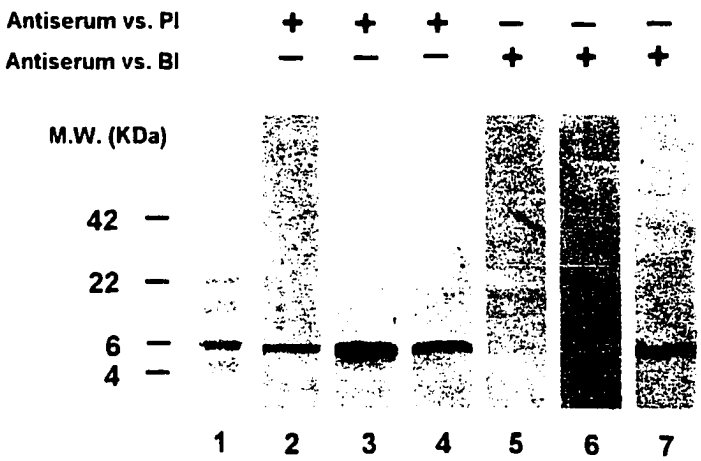


Fig. 9. Immunoblot of rat insulin I and cell homogenates from differentiated and undifferentiated RINm5F cells. Lane 1, a M.W. marker; lanes 2 and 5, rat insulin I; lanes 3 and 6, cell-homogenate from differentiated RINm5F cells, and lanes 4 and 7, cell-homogenate from undifferentiated RINm5F cells. Lanes 2, 3, and 4 were reacted with antiserum vs. porcine insulin (PI) and lanes 5, 6, and 7 with antiserum vs. bovine insulin (BI). The position of a M.W. marker is given on the left.

changes in the morphology of RIN cells to differentiated features and increases insulin mRNA 10 fold over control levels (Philippe et al., 1987; Bartholomeusz et al., 1990). Thus, there is a possibility that decreased insulin release is related to the morphological differentiation or is the result of the long-term culture *in vitro*.

It is likely that antigenic determinants of the insulin secreted in undifferentiated cells are changed because the amino acid residue(s) are changed. Antigenic specificity to a species-specific insulin is dependent on the presence of a suitable antigenic determinant on the molecule even though insulins of mammals show a high degree of homology (Bullesbach and Schwabe, 1994). Thus, anti-insulin antibody formation is initiated through recognition of the conformation of specific loops by changes in the amino acid sequence (Sasaki et al., 1988). The interaction between A- and B-chains is necessary for spatial configuration of the molecule and for the integrity of most antigenic determinants of insulin (Arquilla and Bromer, 1967). The antibody specificity of antiserum vs. bovine insulin was a major finding of this study. Porcine and bovine insulins differ by only two amino acids; however, antibody specificities between antisera vs. porcine and bovine insulins were distinct. The only differences between porcine and bovine insulins are amino acids in A8 and A10. A8 is alanine and A10 is valine in bovine insulin, while threonine and isoleucine are located in these positions in porcine insulin as shown in Figure 1. In addition, the amino acids in A8 and A10 in rat insulins I and II are identical to those in porcine insulin. Therefore, it is most likely that the amino acids in A8 and A10 contribute the specificity of antisera vs. rat and bovine insulins. This is supported by the findings that A8 - 10 may play an important role in insulin antigenicity and receptor binding ability because of differences in primary structure and a high degree of mobility (Sasaki et al., 1988; Bullesbach and Schwabe, 1994). Therefore, the changes in antigenic determinant of the insulin secreted by undifferentiated RINm5F cells might contribute to the novel insulin-like immunoreactivity. This can be confirmed by identifying the amino acid sequence of NIP by using molecular cloning technique in the future.

The implication of our present findings remains to be established. However, RINm5F cells do not share surface features in common with native β-cells (Bartholomeusz et al., 1990) and are relatively undifferentiated in comparison with normal adult islet cells (Gazdar et al., 1980). In addition, RINm5F cells have frequently

been employed as a model of islet cell growth and an early stage in pancreatic β-cell differentiation (Philippe et al., 1987; Bartholomeusz et al., 1990; polak et al., 1993). Therefore, we speculate that the third gene which produce the novel insulin-like immunoreactivity may serve as an ancestral gene, although insulin gene II has been known as an ancestral gene in normal pancreatic β-cells (Bell et al., 1980). This should be further tested by molecular cloning techniques.

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CHAPTER IV ALTERATIONS OF ADRENERGIC RECEPTORS IN RESPONSE TO EPINEPHRINE ON INSULIN RELEASE FROM RINM5F CELLS IN A PASSAGE- AND MORPHOLOGY-DEPENDENT MANNER

A paper to be submitted to Journal of
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Bum-sup Lee, Dong-Ho Shin, Ter-Hsin Chen, Chi Yang and Walter H. Hsu

ABSTRACT

The α_1-, α_2- and β-adrenergic receptors (ARs) are present in pancreatic β-cells. In the present study, the functional distributions of these receptors were investigated in RINm5F cells, a cloned β-cell line, in conjunction with the changes of morphologic features from differentiated to undifferentiated states. Thus, the cells in passages between 28 and 30 were differentiated and the cells after passage 35 were undifferentiated. Epinephrine (1 - 10 μM) significantly decreased cAMP production and insulin release in differentiated cells; however, epinephrine (10 nM - 10 μM) dose-dependently increased cAMP production and insulin release in undifferentiated cells. In addition, epinephrine (1 μM) decreased forskolin-induced intracellular cAMP concentration in differentiated cells but not in undifferentiated cells. In differentiated cells, epinephrine (1 μM) decreased KCl (30 mM)-induced insulin release but RX 821002 (0.1 μM), an α_2-AR antagonist, abolished the epinephrine-induced inhibition. Prazosin (0.1 μM), an α_1-AR antagonist, and propranolol (1 μM), a β-AR antagonist, failed to alter the effect of epinephrine. In undifferentiated cells, however, epinephrine (1 μM) increased insulin release and enhanced KCl (30 mM)-induced insulin release. RX 821002 further potentiated the effect of epinephrine on KCl's action. Propranolol (1 μM) abolished the effect of epinephrine on KCl's action but prazosin (0.1 μM) did not. In addition, when α_2-ARs were blocked, cirazoline (10 μM) and methoxamine (100 μM), α_1-AR agonists, did not increase insulin release in either differentiated or undifferentiated cells. Medetomidine (0.001-1 μM), a specific α_2-AR agonist, inhibited

KCl (30 mM)-induced insulin release in a dose-dependent manner in differentiated cells, but did not in undifferentiated cells. Isoproterenol, a β-AR agonist (1 μM), failed to increase intracellular cAMP concentration and insulin release in differentiated cells but increased them in undifferentiated cells. Taken together, these results suggested that in RINm5F cells, 1) the differentiated states are functionally equipped with more α_2-, but fewer β-adrenergic receptors, than the undifferentiated states, and 2) α_1-adrenergic receptors do not participate in insulin release in either differentiated or undifferentiated RINm5F cells.

INTRODUCTION

The islets of Langerhans are richly vascularized and both the islet cells and blood vessels are intimately associated with autonomic nerves. In general, stimulation of parasympathetic nerves increases insulin secretion (Kaneto et al., 1967), whereas stimulation of the sympathetic nerve decreases insulin release (Miller, 1981). Norepinephrine and epinephrine inhibit insulin release by activating α_2-ARs of the β-cells. When α_2-ARs are blocked, norepinephrine and epinephrine increase insulin release by activating β_2-adrenergic receptors (Miller, 1981). Activation of α_2-ARs decreases insulin release by inhibiting adenylyl cyclase activity (Thomsen and Neubig, 1989). However, activation of β_2-adrenergic receptors stimulates insulin release by activating adenylyl cyclase activity (Ross and Gilman, 1980). A clonal β-cell line, RINm5F cells, have α_1-, α_2-and β-ARs and have been widely used as a model to characterize the cellular mechanisms of these receptors in pancreatic β-cells (Ullrich and Wollheim, 1985; Ullrich and Wollheim, 1989; Aicardi et al., 1991; Chan et al., 1991; Suzuki et al., 1992).

RINm5F cells change their morphology from a differentiated state (with a few neuron-like processes) to an undifferentiated state (without the processes) during long-term culture (Polak et al., 1993). In a preliminary study, we found that when RINm5F cells changed from the differentiated to the undifferentiated state, the effect of epinephrine on insulin release was changed from inhibitory to stimulatory. The morphological changes of RINm5F cells after long-term culture have been noticed in many laboratories (Polak et al., 1993), but the functional changes in conjunction with

morphological changes have not been reported.

In the present study, we characterized the α_1-, α_2-, and β-ARs mediating insulin release in differentiated and undifferentiated cells. We performed the experiment in passages 28 - 40 because these passages presented a marked contrast between differentiated and undifferentiated cells. Our results suggested that differentiated RINm5F cells possess more α_2-adrenergic receptors than the undifferentiated cells but undifferentiated cells possess more functional β-adrenergic receptors than differentiated cells. In addition, α_1-adrenergic receptors did not participate in insulin release in either differentiated or undifferentiated RINm5F cells.

MATERIALS AND METHODS

Materials

The following materials were used: RPMI-1640, fetal bovine serum, epinephrine, isoproterenol, propranolol and forskolin (all from Sigma Chemical,St. Louis, MO), 24-well culture plate (Corning Glass Works, Corning, NY), medetomidine HCl (Farmos, Turku, Finland), Prazosin HCl (Pfizer Inc., Groton, CT), RX 821002 (Research Biochemicals Inc., Natick, MA), antiserum vs. rat insulin (Linco Research Inc., St Louis, MO).

Cell culture

RINm5F cells were obtained from Dr. S.B. Pek (University of Michigan Medical Center, Ann Arbor, MI). The cells were maintained in RPMI-1640 medium with 10% fetal bovine serum as described previously (Chen et al., 1994). The cells were subcultured every 7 days and the medium was changed at day 3 and day 5 after subculture. All experiments were performed by use of cells from passages 28-40. No antibiotics were used.

Insulin release

Static incubations were performed to study insulin release in response to test agents as previously described (Hsu et al., 1991b). Cells from later passages grew more rapidly; consequently, the cells were plated onto 24-well plates at 2×10^5 cells/well for passages 28-32 and at 7×10^4 cells/well for passages 34-40 and grown for 5 days. The growth medium was then removed, and the cells were washed with Krebs-Ringer buffer (KRB) containing (in mM): 136 NaCl, 4.8 KCl, 2.5 $CaCl_2$, 1.2 KH_2PO_4, 1.2 $MgSO_4$, 5 $NaHCO_3$, 10 HEPES, 1.67 glucose and 0.1% bovine serum albumin, pH 7.4. The cells were then preincubated for a 15-min basal period at 37°C, followed by 15-min incubation in KRB with test agents. When needed, the antagonist was given 5 min before the administration of the agonist to ensure blockade of the receptors before the addition of the agonist. Insulin in the media was measured by radioimmunoassay (RIA) as previously described (Hsu et al., 1991).

Statistical analysis

All values were expressed as the mean ± S.E. Analysis of variance (ANOVA) was used to determine the treatment or dose effect. The least significance test was used to test for differences between means of end points for which the ANOVA indicated a significant ($P<0.05$) *F* ratio.

RESULTS

Effects of epinephrine on intracellular cAMP concentration and insulin release in differentiated and undifferentiated RINm5F cells

In the differentiated state, RINm5F cells had a few neuron-like processes. However, in the undifferentiated state, the cells did not exhibit morphological features of neuron-like processes. More than 90% of the cells between passages 28 and 30 were differentiated. However, the cells gradually lost processes to become undifferentiated cells and > 99% of the cells after passage 35 were undifferentiated (Fig. 1. in chapter III).

To demonstrate the effects of epinephrine on insulin release, epinephrine was tested on every other passage between 28 and 40. Since cAMP has been implicated as a second messenger mediating α_2-adrenergic effects, intracellular cAMP

concentrations were also measured in parallel to insulin release. In passages 28 and 30, epinephrine (1 μM) significantly inhibited basal insulin release by 12% and intracellular cAMP concentration by 14 - 17%. However, in passages after 35, epinephrine (1 μM) significantly increased basal insulin release by 28 - 39% and intracellular cAMP concentration by 20 - 37% (Fig. 1). Dose-dependent effects of epinephrine were studied between differentiated and undifferentiated cells. Epinephrine (1 - 10 μM) significantly decreased basal cAMP production and insulin release, but epinephrine (10 nM - 10 μM) increased basal cAMP production and insulin release in a dose-dependent manner (Fig. 2).

Since the effect of an inhibitor on intracellular cAMP is best demonstrated in stimulated cells, forskolin, an adenylate cyclase activator, was used to increase intracellular cAMP concentration. The intracellular cAMP concentration after the administration of forskolin was set at 100%, and changes were expressed as a percentage of the forskolin control value. Forskolin (5 μM) markedly increased intracellular cAMP concentration by 85.7-fold in differentiated cells and forskolin (0.5 μM) increased it by 98.5-fold in undifferentiated cells (Fig. 3A). Epinephrine (1 μM) significantly inhibited forskolin (5 μM)-stimulated intracellular cAMP concentration by 86% in differentiated cells but did not inhibit forskolin (0.5 μM)-stimulated intracellular cAMP concentration in undifferentiated cells (Fig. 3A). With a blockade of α_2-ARs by RX 821002 (0.1 μM), the inhibitory effect of epinephrine on cAMP in differentiated cells was abolished (data not shown). Furthermore, when the effect of epinephrine on the forskolin-stimulated intracellular cAMP production was tested on every other passage between 28 and 40, epinephrine (1 μM) significantly inhibited the forskolin (10 μM)-stimulated intracellular cAMP concentration by 68 - 86% in differentiated cells but did not in undifferentiated cells (Fig. 3B).

Effects of prazosin, propranolol and RX 821002 on KCl-stimulated insulin release in differentiated and undifferentiated RINm5F cells

The functional receptors which mediated the inhibitory and stimulatory effects of epinephrine on insulin release were further characterized by applying KCl with a number of AR antagonists in both differentiated and undifferentiated RINm5F cells. In differentiated cells, epinephrine (1 μM) significantly inhibited KCl (30 mM)-induced

Fig. 1. Passage-dependent responses of RINm5F cells to epinephrine (1 μM) on cAMP production (A) and insulin release (B). After each experiment, the cells were detached by using trypsin-EDTA and the cell numbers were counted in each well. The basal insulin release was 6.9 - 8.9 ng/min/million cells in differentiated cells and 2.9 - 3.4 ng/min/million cells in undifferentiated cells. Mean ± S.E. (n=16) are shown. *$P<0.05$, compared with basal control group.

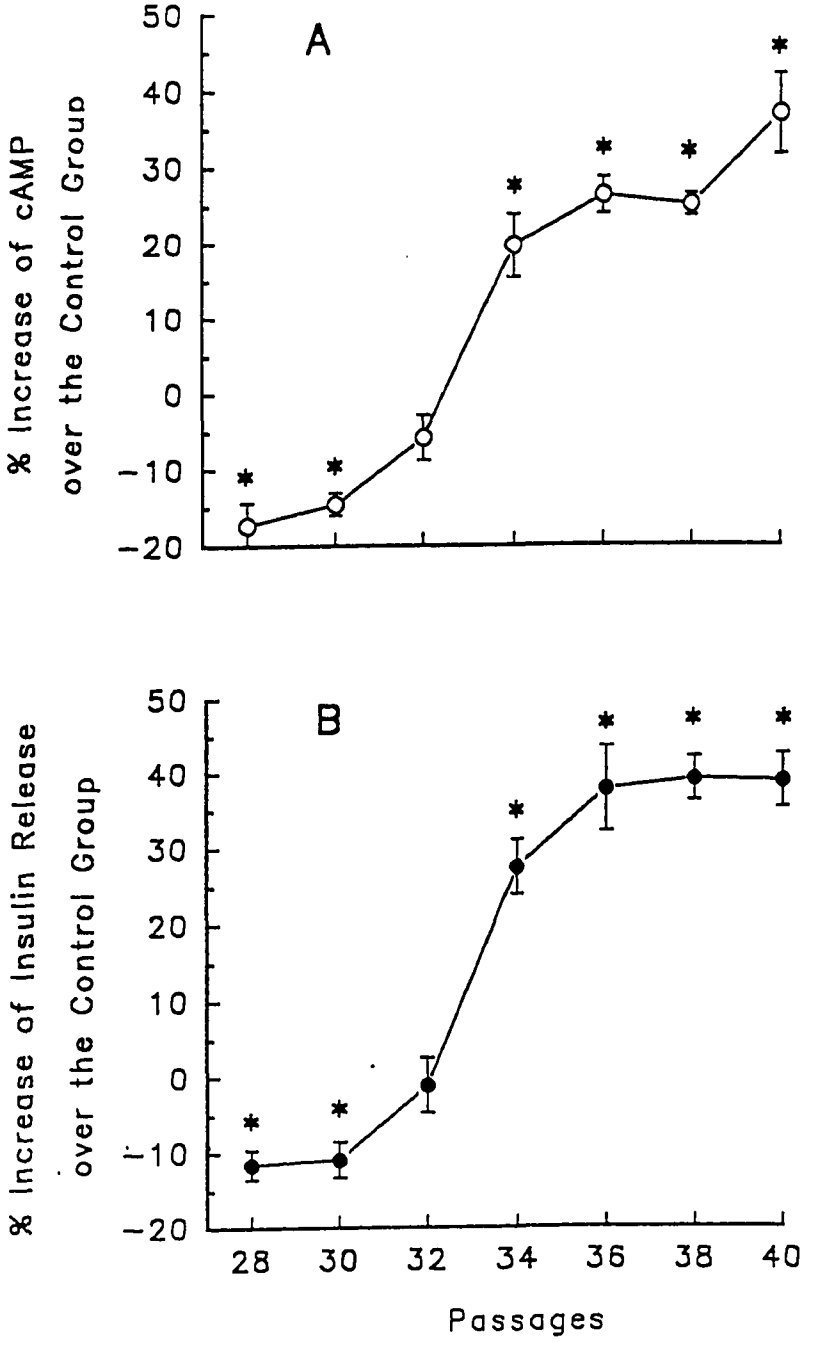

A
% Increase of cAMP over the Control Group
50
40
30
20
10
0
-10
-20
B
% Increase of Insulin Release over the Control Group
28
30
32
34
36
38
40
Passages

Fig. 2. Dose-dependent responses of differentiated (◯) and undifferentiated (●) RINm5F cells to epinephrine (1 nM - 10 μM) on cAMP production (A) and insulin release (B). Mean ± S.E. (n=4) are shown. *$P<0.05$, compared with basal control group.

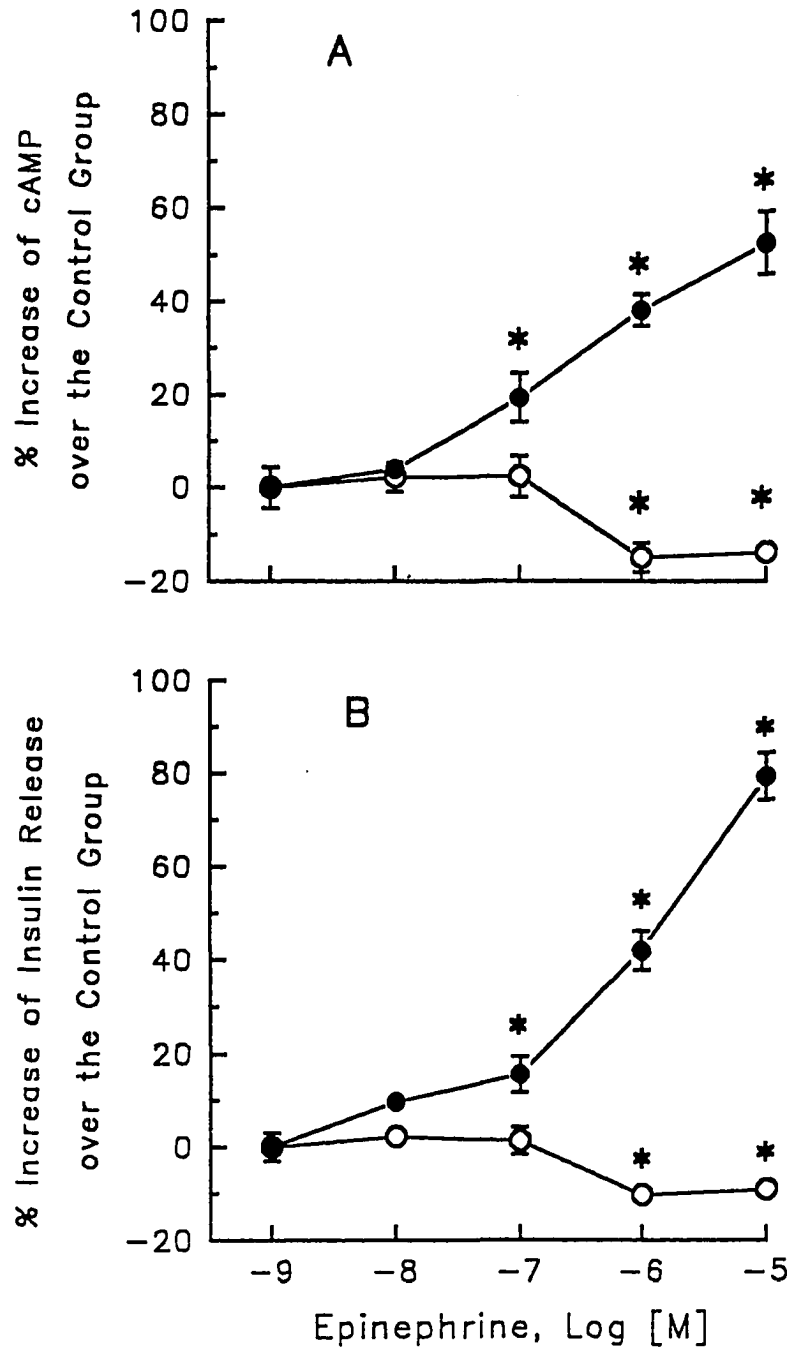
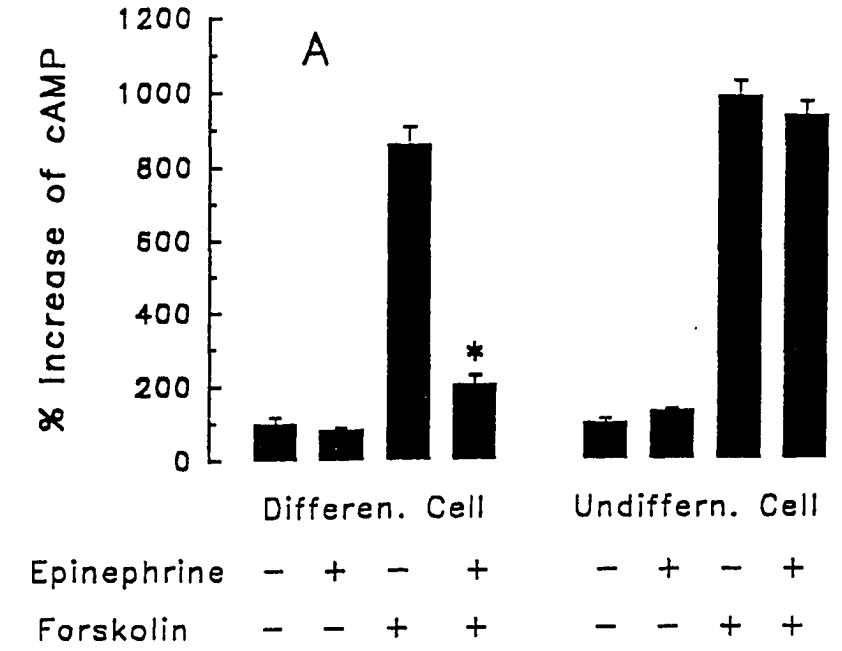
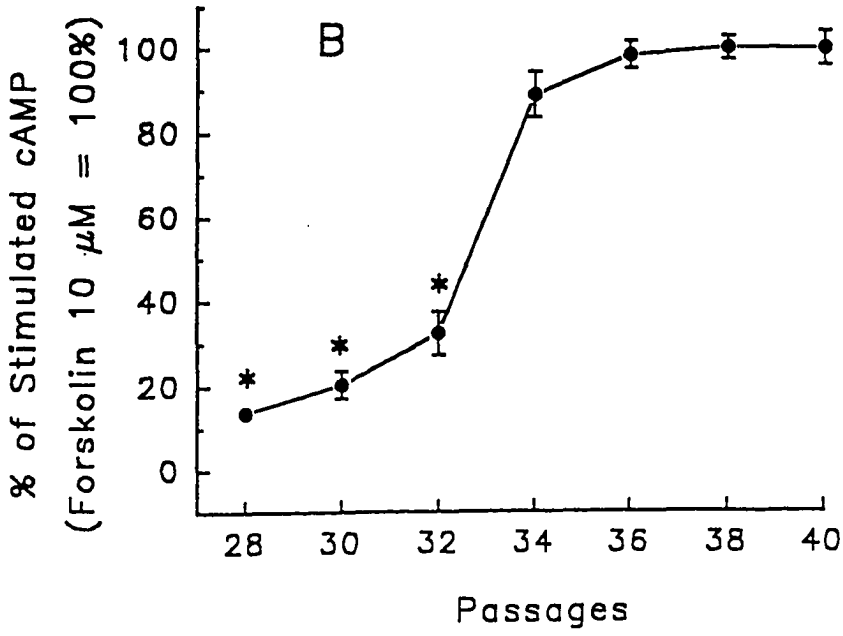

A
B
% Increase of cAMP over the Control Group
% Increase of Insulin Release over the Control Group
100
80
60
40
20
0
−20
−9
−8
−7
−6
−5
Epinephrine, Log [M]
*

Fig. 3. (A) Effect of epinephrine (1 μM) on forskolin-induced cAMP production in differentiated and undifferentiated RINm5F cells. Various concentrations of forskolin were applied to differentiated and undifferentiated cells to equally increase intracellular cAMP concentration, which were 5 μM and 0.5 μM, respectively. Mean ± S.E. (n = 4) are shown. *$P < 0.05$, compared with forskolin-stimulated group. (B) The passage-dependent effect of epinephrine (1 μM) on forskolin (10 μM)-induced cAMP production. Mean ± S.E. (n = 12) are shown. *$P < 0.05$, compared with basal control group.


A
% Increase of cAMP
1200
1000
800
600
400
200
0
*
Differen. Cell
Undiffern. Cell
Epinephrine − + − + − + − +
Forskolin − − + + − − + +


B
% of Stimulated cAMP
(Forskolin 10 μM = 100%)
100
80
60
40
20
0
*
*
*
28
30
32
34
36
38
40
Passages

insulin release by 86% (Fig. 4A). An α_2-AR antagonist, RX 821002 (0.1 μM), abolished the epinephrine-induced inhibition. However, Prazosin (0.1 μM), an α_1-AR antagonist, and propranolol (1 μM), a β-AR antagonist, failed to alter the effect of epinephrine (1 μM) (Fig. 4A). In undifferentiated cells, epinephrine (1 μM) increased insulin release by 40% and enhanced KCl (30 mM)-stimulated insulin release (Fig. 4B). In the presence of α_2-AR blockade by RX 821002 (0.1 μM), epinephrine further potentiated KCl (30 mM)-induced insulin release by 51%. Propranolol (1 μM) abolished the stimulatory effect of epinephrine on KCl's action but prazosin (0.1 μM) did not (Fig. 4B). With a blockade of α_2-AR by RX 821002 (0.1 μM) and α_1 -AR by prazosin (0.1 μM), epinephrine (1 μM) potentiated KCl-induced insulin release by 47%, but with a blockade of α_2-AR by RX 821002 (0.1 μM) and β-AR by propranolol (1 μM), epinephrine failed to change it.

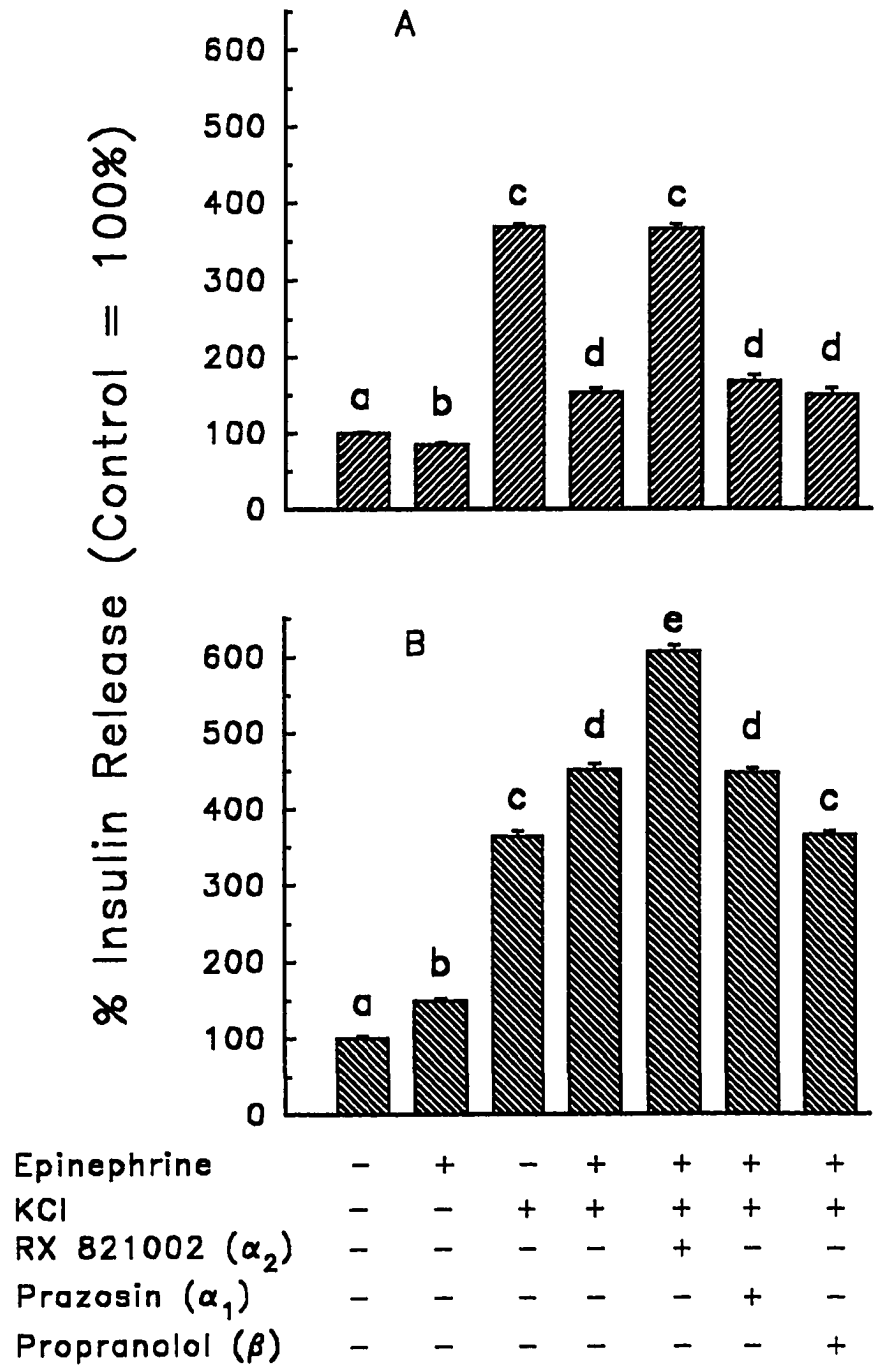
Effects of cirazoline, methoxamine and medetomidine on insulin release in differentiated and undifferentiated RINm5F cells

Since we demonstrated the insignificant participation of α_1-AR in insulin release by using its antagonist, prazosin, other α_1-AR agonists were tested to support these findings. Cirazoline (10 μM) and methoxamine (100 μM), α_1-AR agonists, in the presence of α_2-AR antagonist, RX 821002 (0.1 μM), did not significantly increase insulin release in either differentiated or undifferentiated RINm5F cells. Medetomidine (0.001 - 1 μM), a potent and specific α_2-AR agonist (Virtanen et al., 1988), inhibited KCl (30 mM)-stimulated insulin release in a dose-dependent manner (IC_{50} of 48.5 $\pm$ 0.9 nM) in differentiated cells but not in undifferentiated cells (Fig. 5). In this experiment, insulin release after the administration of KCl was set at 100%, and all changes are expressed as a percentage of the KCl-stimulated value.

Effect of isoproterenol on intracellular cAMP concentration and insulin release in differentiated and undifferentiated RINm5F cells

Since the significant participation of β-ARs was demonstrated by the antagonism study, the effect of its agonist, isoproterenol, was further tested on every other passage between 28 and 40 to determine if there was a passage-dependent effect.

Fig. 4. Effect of epinephrine (1 μM) on KCl (30 mM)-induced insulin release and effects of adrenergic receptor antagonists (0.1 μM prazosin, 1 μM propranolol and 0.1 μM RX 821002) on epinephrine-induced insulin release in differentiated cells (A), and undifferentiated RINm5F cells (B). The antagonists were given 5 min before epinephrine. Mean ± S.E. (n=8) are shown. The different letters in the same panel indicate a significant difference ($P<0.05$) between the treatment groups.


A
B
% Insulin Release (Control = 100%)
600
500
400
300
200
100
0
a
b
c
d
e
Epinephrine – + – + + + +
KCl – – + + + + +
RX 821002 (α_2) – – – – + – –
Prazosin (α_1) – – – – – + –
Propranolol (β) – – – – – – +

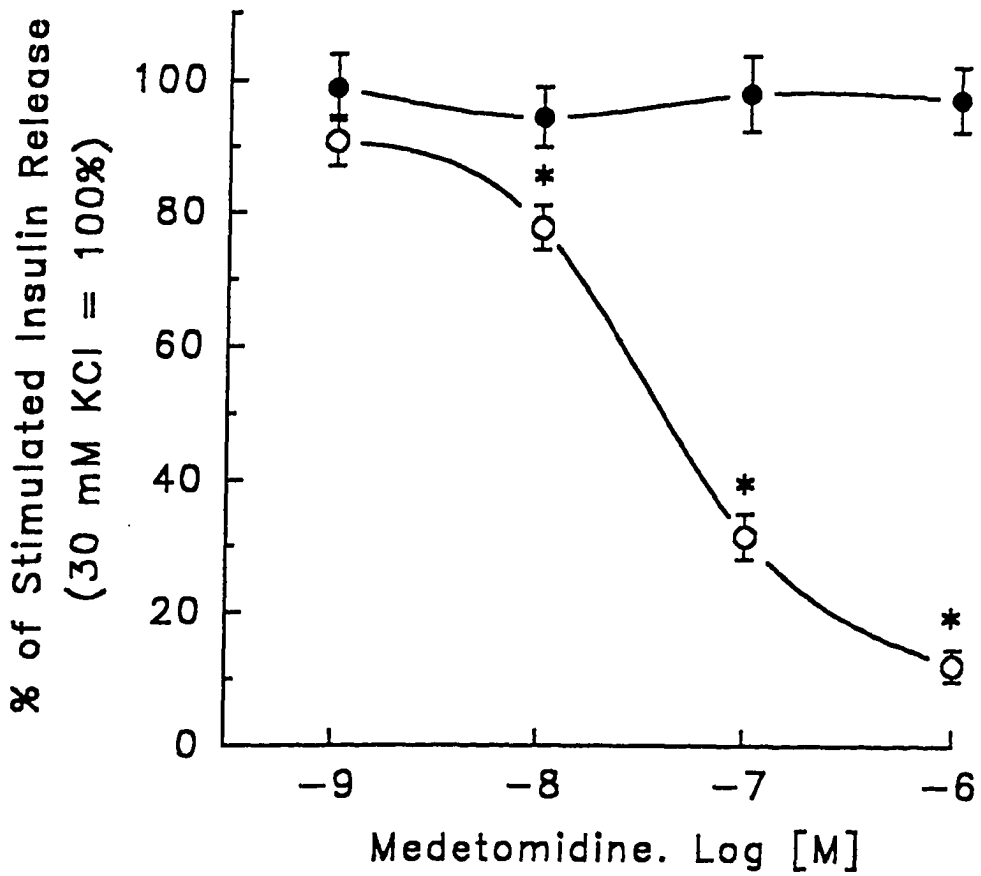


Fig. 5. Effect of medetomidine on KCl (30 mM)-induced insulin release in differentiated cells (○) and undifferentiated RINm5F cells (●). Mean ± S.E. (n=8) are shown. *$P<0.05$, compared with basal control group.

Fig. 6. Passage-dependent responses of RINm5F cells to isoproterenol (1 μM) on cAMP production (A) and insulin release (B). Mean ± S.E. (n = 16) are shown. *$P < 0.05$, compared with basal control group.

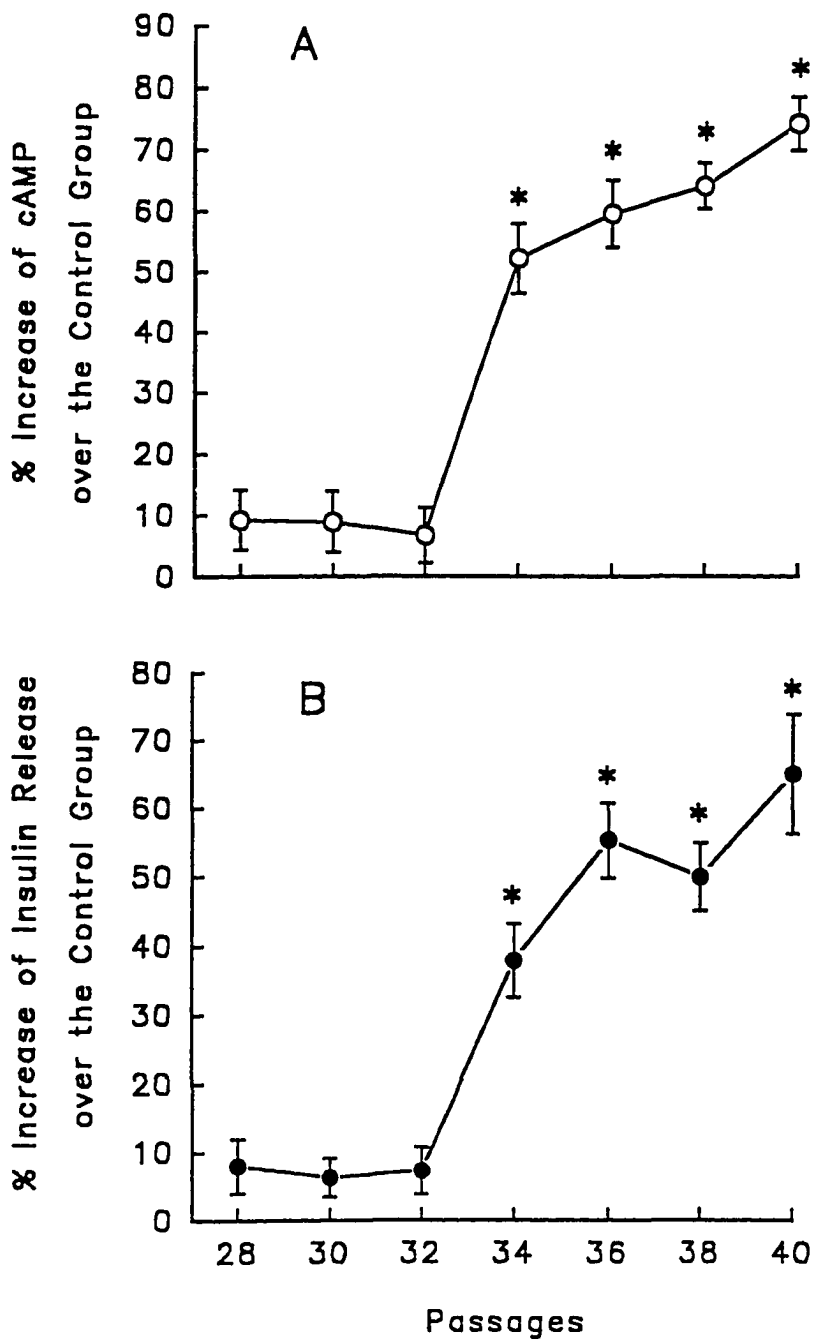
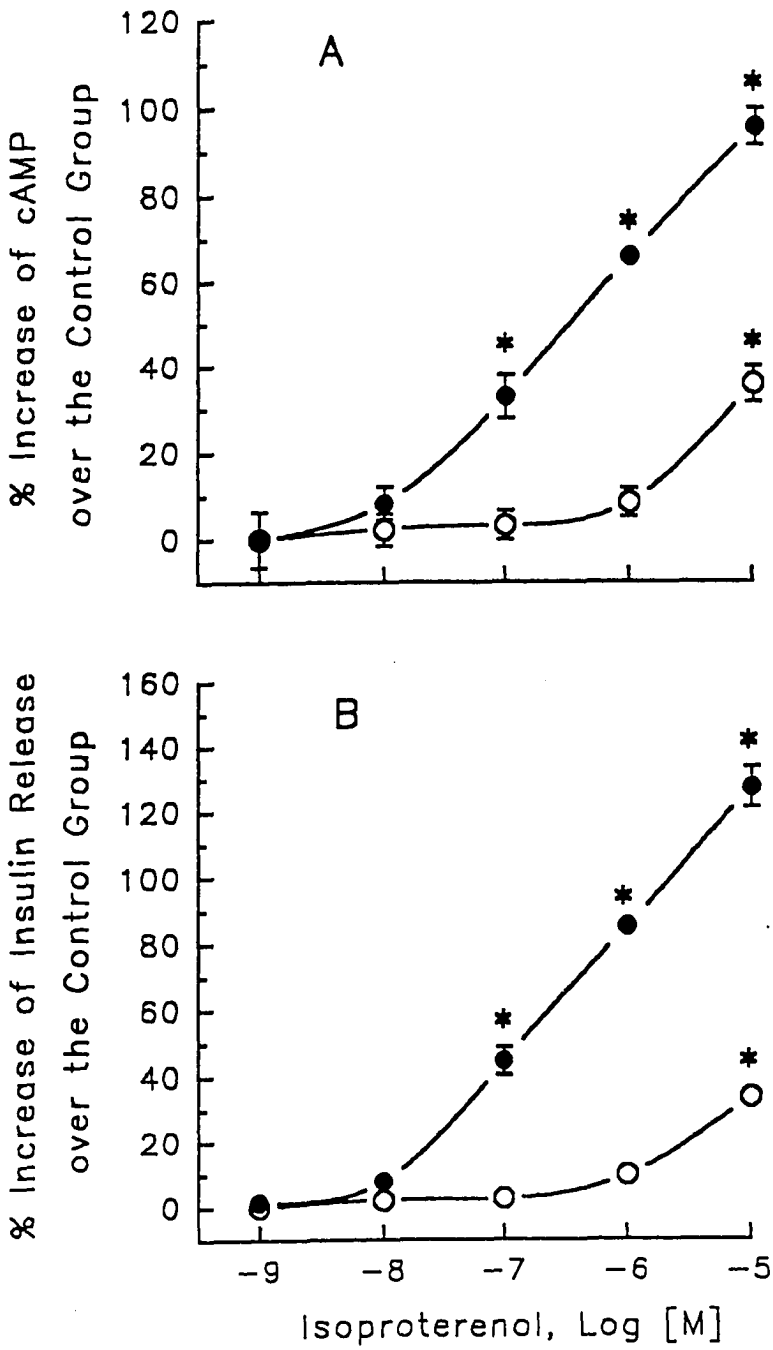

A
% Increase of cAMP over the Control Group
90
80
70
60
50
40
30
20
10
0
B
% Increase of Insulin Release over the Control Group
80
70
60
50
40
30
20
10
0
28
30
32
34
36
38
40
Passages

Fig. 7. Dose-dependent changes of differentiated (◯) and undifferentiated (●) RINm5F cells to isoproterenol (1 nM - 10 μM) on cAMP production (A) and insulin release (B). Mean ± S.E. (n=8) are shown. *$P<0.05$, compared with basal control group.


A
% Increase of cAMP over the Control Group
120
100
80
60
40
20
0
B
% Increase of Insulin Release over the Control Group
160
140
120
100
80
60
40
20
0
−9
−8
−7
−6
−5
Isoproterenol, Log [M]

Isoproterenol (1 μM) failed to change insulin release in differentiated cells but increased it by 55-65% in undifferentiated cells (Fig. 6A). In addition, isoproterenol failed to change intracellular cAMP concentration in differentiated cells but increased it by 52-74% in undifferentiated cells (Fig. 6B). Dose-dependent increases of intracellular cAMP and insulin release by isoproterenol (1 nM-10 μM) were higher in undifferentiated cells than in differentiated cells (Fig. 7).

DISCUSSION

This study was undertaken to characterize the functional ARs which mediate the inhibitory and stimulatory effects of epinephrine on insulin release in differentiated and undifferentiated RINm5F cells. In differentiated cells, α_2-ARs mediated the inhibitory effect of epinephrine on KCl- and forskolin-induced insulin release and cAMP production. In undifferentiated cells, β-ARs mediated the stimulatory effect of epinephrine or isoproterenol on insulin release and cAMP production. However, α_1-ARs did not mediate the effect of epinephrine on insulin release in either differentiated cells or undifferentiated RINm5F cells.

Our findings in the differentiated cells were consistent with the observation that epinephrine inhibits basal cAMP concentration and insulin release by activating α_2-ARs in RINm5F cells (Ullrich and Wollheim, 1988). In addition, forskolin-stimulated cAMP production is lowered by epinephrine and clonidine, an α_2-AR agonist, in rat pancreatic islets and RINm5F cells (Garcia-Morales et al., 1984; Ullrich and Wollheim, 1988). In a clonal β-cell line HIT-T15 (derived from hamster islets with Simian virus-40 infection), isoproterenol causes more dramatic increase in intracellular cAMP concentration in the later passages, although HIT-T15 cells are morphologically undifferentiated in all the passages studied (Zhang et al., 1989). Thus, it has to be further tested whether passage numbers or morphology plays a role in the effect of epinephrine on insulin release in RINm5F cells.

There are inconsistencies in the literatures regarding the α_1-adrenergic effects on insulin release. An α_1-AR agonist, amidephrine mesylate (1 μM), failed to affect glucose-stimulated insulin release in rat pancreatic β-cells (Malaisse and Moratinos, 1986), but an α_1-AR agonist, phenylephrine (100 μM), caused a slight increase of

insulin release from RINm5F cells (Ullrich and Wollheim, 1985). In porcine pancreatic islets, an α_1-AR agonist, phenylephrine (0.1 μM), inhibited insulin release, and the inhibitory effect of epinephrine was abolished by an α_1-AR antagonist, prazosin, but not by an α_2-AR antagonist, idazoxan (Gregersen et al., 1991). The reason for these discrepancies is unclear.

The functional distribution of ARs was distinct between differentiated and undifferentiated RINm5F cells. Functional studies are based on the receptor occupation theory which states that the occupation of a receptor by a drug leads to a stimulus and a subsequent response (Kenakin, 1984). In our studies, insulin release and cAMP production were used to test the potency of epinephrine on α- and β-ARs and to distinguish the subtypes of ARs. The lack of agonistic activity of epinephrine through α_1 and β-ARs may be a result of the low concentration of these ARs in differentiated RINm5F cells. Similarly, the lack of epinephrine's action through α_1- and α_2-ARs in undifferentiated RINm5F cells may also be a result of low density of these ARs. Our speculation on the density of ARs which mediate the dual inhibitory and stimulatory effects of epinephrine in RINm5F cells should be further tested by radioligand binding studies.

Alterations in affinity of ARs to G-proteins and in the quantities of G-proteins may be secondary to differences in receptor numbers and subtypes. Epinephrine inhibits adenylyl cyclase and insulin release through α_2-ARs coupled to PTX-sensitive G_i-proteins (Katada and Ui, 1979; Hsu et al., 1991a), but stimulates them through β-ARs coupled to CTX-sensitive G_s-proteins (Sharp, 1979). Five PTX substrates are found as three forms of G_i (G_{i1}, G_{i2} and G_{i3}) and two forms of G_o (G_{o1} and G_{o2}) in RINm5F cells (Gillison and Sharp, 1994). Two molecular forms of $G_{i\alpha}$-proteins are present in RINm5F cells: one form with 39 KDa and the other form with 42 KDa (Gillison and Sharp, 1994). Two molecular forms of $G_{s\alpha}$-proteins are present in HIT cells: one form with 45 KDa and the other form with 52 KDa. The 45 KDa form is more efficacious than the 52 KDa form as an activator of cAMP (Walseth et al., 1989). In addition, greater quantities of the 45 KDa forms than 52 KDa forms of $G_{s\alpha}$-proteins are present with increasing passages in HIT-15 cells (Walseth et al., 1989) and this contributes to a more marked increase in isoproterenol-induced cAMP production in the later passages of HIT-T15 cells (Zhang et al., 1989). In RINm5F cells, it remains to be determined

whether changes in G_i- or G_s-proteins are responsible for the epinephrine-effect on insulin release, which is inhibitory in differentiated cells and stimulatory in undifferentiated cells.

Greater β- than α_2-adrenergic activity in undifferentiated cells and greater α_2- than β-adrenergic activity in differentiated cells can be associated with the development of fetal pancreatic β-cells. We speculate that dedifferentiation during oncogenesis resembles the morphological and functional changes of RINm5F cells towards the early stage of embryonic β-cells. In fact, RINm5F cells are less differentiated than their native counterparts in several key respects and represent the fetal β-cells (Gazdar et al., 1980; Halban et al., 1983; Polak et al., 1993). The undifferentiated RINm5F cells might represent more primitive fetal β-cells than the differentiated cells. Comparison between differentiated and undifferentiated RINm5F cells may provide information on the alteration of adrenergic receptors in the embryonic development of pancreatic β-cells. In some cases, immature tissues contain higher receptor concentrations that subsequently decline with development. This pattern of fetal receptor overexpression is found for α_2- and β-ARs in the liver (McMillian et al., 1983), for β-ARs in the thymus (Rossi, 1987), and for α_2-ARs in the heart (Lin et al., 1992). The β-receptor overexpression in fetal rat is a widespread phenomenon and receptor subtype selectivities (β_2 in liver, β_1 in heart, predominantly β_2 in whole fetus) have been confirmed (Slotkin and Seidler, 1994).

The tissue distribution of β-ARs during fetal life is more clearly defined than their functional significance. The activation of β_2-ARs elicits a program of growth and differentiation in thyroid cell line FTRL (Hen et al., 1989), human lung adenocarcinoma cell line (Schuller and Cole, 1989), mouse T lymphoma TL 2-9 (Buscail et al., 1990), and human submandibular cell line A253 (Marmary et al., 1989). β_2-ARs are also involved in regulating the growth and differentiation of tumor cells such as rat hepatoma (Miyamoto, et al., 1985), rat mammary tumor (Marchetti et al., 1989), and in neoplastic human thyroid tissues (Ling et al., 1992). In addition, undifferentiated keratinocytes *in vitro* expresses more β_2-adrenergic receptors than differentiated ones (Schallreuter et al., 1993).

Fetal tissues can be exposed to catecholamines via two sources. First, norepinephrine and epinephrine are released by the fetal adrenal medulla into the

circulation, but these effects are more prominent toward the end of gestation and during parturition (Lagercrantz and Bistoletti, 1973; Jones et al., 1988). Second, a variety of different cell types can express adrenergic phenotype and the ability to synthesize catecholamines transiently during development (Cochard et al., 1978; Jonakait et al., 1979). The cellular processes in developing pancreatic β-cells might alter the sensitivity or selectivity of target cells to catecholamines to increase or decrease insulin release depending on whether β- or α-ARs are activated (Polak et al., 1993). Greater functional involvement of β-ARs in undifferentiated cells than in differentiated cells might represent the development of early fetal pancreatic β-cells and contribute to the growth and differentiation of fetal β-cells.

In conclusion, our present findings suggested that differentiated RINm5F cells possess more functional α_2-, but fewer β-ARs, than the undifferentiated cells. In addition, α_1-ARs apparently do not participate in insulin release in either differentiated or undifferentiated RINm5F cells. The implication of our present findings remains to be established. However, since RINm5F cells functionally express α_2- and β-ARs in differentiated and undifferentiated states, respectively, the cells can be used as a model for cellular mechanisms of these receptors in pancreatic β-cells. Our present findings might also be useful in studying the early development of pancreatic β-cells in conjunction with distribution of ARs.

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CHAPTER V ELEVATED ACTIVITY OF ADENYLYL CYCLASE IN UNDIFFERENTIATED RINM5F CELLS IN A PASSAGE- AND MORPHOLOGY-DEPENDENT MANNER

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ABSTRACT

We have demonstrated that undifferentiated RINm5F cells have greater activity of β-ARs than differentiated cells (in Chapter IV). In the present study, we further characterized a cAMP-dependent signalling pathway which may contribute to the elevated activity of β-ARs in undifferentiated cells. Activities of G_s-protein, adenylyl cyclase, phosphodiesterase (PDE) and cAMP-dependent protein kinase (protein kinase A) were characterized in a clonal β-cell line, RINm5F. The cells were equipped with two morphological features; the cells with neuron-like processes (differentiated cells) in passages between 28 and 30 and the cells without processes (undifferentiated cells) in passages after 35. Cholera toxin (CTX, 1 μg/ml), an activator of G_s-proteins, increased intracellular cAMP concentration in both differentiated and undifferentiated cells without significant differences between these cells. CTX also increased insulin release in both cell types but was 2.6-fold more potent in undifferentiated cells than in differentiated cells. The effect of forskolin (10 μM) on cAMP production and insulin release was 2- and 6-fold more potent, respectively, in undifferentiated cells than in differentiated cells. The phosphodiesterase inhibitor, IBMX (100 μM), significantly increased intracellular concentration of cAMP in differentiated and undifferentiated cells without significant differences between these cells. The stimulatory effect of IBMX on insulin release, however, was 6-fold greater in undifferentiated cells than in differentiated cells.

Isoproterenol, a β-adrenergic agonist, significantly potentiated forskolin-induced cAMP production and insulin release in undifferentiated cells, but not in differentiated cells. CTX (1 μg/ml) significantly potentiated forskolin-induced cAMP production and insulin release in undifferentiated cells, but not in differentiated cells. In addition, forskolin potentiated KCl-induced insulin release in undifferentiated cells, but not in differentiated cells. There were no significant differences in intracellular cAMP concentrations or $[Ca^{2+}]_i$ between the forskolin alone group and forskolin + KCl group. The activity of protein kinase A was not significantly different between differentiated and undifferentiated cells. Taken together, these results suggested that the activity of adenylyl cyclase is higher in undifferentiated cells than in differentiated cells, and this may be partly responsible for the elevated activity of β-ARs in undifferentiated cells. The activities of G_s-protein, PDE and protein kinase A were similar in differentiated and undifferentiated RINm5F cells. However, the G_s-protein- and protein kinase A-dependent exocytotic apparatus may be more functional in undifferentiated cells than in differentiated cells, which may partly contribute to the elevated activity of β-ARs in undifferentiated cells.

INTRODUCTION

GTP binding proteins (G-proteins) consist of three subunits named α, β and γ in order of decreasing molecular size. The α-subunits have a single, high affinity binding site for GTP or GDP. The β- and γ-subunits are tightly associated and function as a unit. G-proteins link many hormone receptors to adenylyl cyclase, ion channels, phospholipase C, and exocytotic apparatus in pancreatic β-cells. β-ARs are coupled to the G_s-proteins to stimulate adenylyl cyclase and increase cAMP and insulin release (Sharp, 1979). Two molecular forms of $G_{s\alpha}$-proteins are present in HIT cells: one form with 45 KDa and the other form with 52 KDa (Walseth et al., 1989). Cholera toxin (CTX), an 87 KDa protein with one A and five B subunits, covalently modifies the α-subunit of CTX-sensitive-G-proteins (G_s and G_{olf}) amplifying hormonal mechanisms. The ADP-ribosylation of CTX-sensitive-G-proteins block their capacity to hydrolyse bound GTP to GDP, consequently activating adenylyl cyclase and increasing intracellular concentration of cAMP (Birnbaumer, 1990).

Both forskolin, an adenylyl cyclase activator, and IBMX (3-isobutyl-1-methylxanthine), a phosphodiesterase (PDE) inhibitor, increase insulin release through elevating intracellular concentration of cAMP in pancreatic β-cells and clonal β-cell lines including RINm5F (Wiedenkeller and Sharp, 1981; Hermansen, 1985; Suzuki, et al., 1992; Chen and Hsu, 1994). cAMP activates primarily protein kinase A, to control diverse phenomena such as metabolism, gene transcription, and memory (Krebs and Bevo, 1979). In pancreatic β-cells, protein kinase A increases Ca^{2+} influx through voltage-dependent Ca^{2+} channels (Rajan et al., 1989), closes ATP-sensitive K^+ channels (Holz et al., 1993) and/or directly interacts with the secretory machinery (Ammala et al., 1993). However, cAMP may act through mechanisms other than activation of protein kinase A. An unidentified cAMP-binding protein may mediate cAMP effects without interacting with protein kinase A (Weber et al., 1987). In addition, cAMP may act by binding to the cGMP-dependent protein kinase or ion channels (Jiang et al., 1992; Holz, IV. et al., 1995).

The clonal insulin-secreting cell line RINm5F is derived from NEDH rat insulinoma with X-ray irradiation (Gazdar et al., 1980). Numerous studies of the physiology and pathophysiology of pancreatic β-cells have been facilitated by RINm5F cells which have a virtually pure population of β-cells unlike pancreatic islets which have various types of endocrine cells. However, insulin secretion and the response to secretagogues of the cell line have been observed to vary with passage number (passage is defined as a subculture of cells from one culture vessel to another in 7 days). In addition, RINm5F cells change their morphology from the differentiated to undifferentiated state after long-term culture.

We have demonstrated that undifferentiated cells are functionally equipped with more β-ARs than differentiated RINm5F cells. However, differentiated cells are functionally equipped with more α_2-ARs than undifferentiated RINm5F cells. Therefore, we further characterized cAMP-dependent signal transduction pathways responsible for the action mediated by α_2- and β-ARs. In the present study, we evaluated the activities of G_s-proteins, adenylyl cyclase, PDE and protein kinase A in a passage- and morphology-dependent manner in RINm5F cells. To perform these studies, we used the cells from passage 28 to 40 because the cells in the passages before 30 were differentiated and gradually changed to become undifferentiated state after passage

35.

MATERIALS AND METHODS

Materials

The following materials were used: RPMI-1640, fetal bovine serum, HEPES, BSA (fraction V), isoproterenol, IBMX, leupeptin, and forskolin (all from Sigma Chemical,St. Louis, MO), 24-well culture plate (Corning Glass Works, Corning, NY), colorimetric protein kinase A assay kit (Pierce, Rockford, IL), and cholera toxin (ICN Biochemicals, Aurora, OH).

Cell culture

RINm5F cells were obtained from Dr. S.B. Pek (University of Michigan Medical Center, Ann Arbor, MI). The cells were maintained in RPMI-1640 medium with 10% fetal bovine serum as described previously (Chen and Hsu, 1994). The cells were subcultured every 7 days and the medium was changed at day 3 and day 5 after subculture. All experiments were performed by use of cells from passages 28-40. No antibiotics were used.

Insulin release

Static incubations were performed to study insulin release in response to test agents as previously described (Hsu et al., 1991). Cells from later passages grew more rapidly; consequently, the cells were plated onto 24-well plates at 2×10^5 cells/well for passages 28 - 32 and at 7×10^4 cells/well for passages 34 - 40 and grown for 5 days. The medium was changed at the day before the experiment. The growth medium was then removed, and the cell monolayer was washed with Krebs-Ringer buffer (KRB) containing (in mM): 136 NaCl, 4.8 KCl, 2.5 $CaCl_2$, 1.2 KH_2PO_4, 1.2 $MgSO_4$, 5 $NaHCO_3$, 10 HEPES, 1.67 glucose and 0.1% bovine serum albumin, pH 7.4. The cells were then preincubated for a 15-min basal period at 37°C, followed by 15-min incubation in KRB with test agents. Insulin in the media was measured by radioimmunoassay (RIA) as previously described (Hsu et al., 1991).

cAMP measurement

Intracellular cAMP concentrations were measured after 15 min of static incubations with test agents. Cells were treated with cholera toxin (CTX) to study ADP ribosylation of the G_s-proteins. After CTX holoenzyme was activated at 30 °C for 20 min in 5 mM dithiothreitol and 20 mM Tris at pH 7.4 as described previously (Gillison and Sharp, 1994), cells were treated with CTX (1 μg/ml) for 5 h. After the experiment, cells were scraped from the plates in 0.01 N HCl and incubated in a water bath at 75 °C for 20 min to heat-inactivate PDE. After centrifugation, the cell extracts were neutralized by 0.01 N NaOH and resuspended in the assay buffer. cAMP concentrations were measured by RIA as previously described (Chen and Hsu, 1994).

$[Ca^{2+}]_i$ measurement

The cells were loaded with 2 μM fura-2 acetoxymethylester (AM) in KRB for 30 min at 37 °C. The loaded cells were washed with KRB and kept at room temperature. The cells were resuspended in a concentration of 10^6 cells/ml and 1.5 ml of aliquot was used for measuring $[Ca^{2+}]_i$. The 340/380 nm fluorescence ratios were monitored in a SLM-8000 fluorescence spectrophotometer (SLM, Urbana, IL). The $[Ca^{2+}]_i$ was calibrated after cell lysis as described previously (Hsu et al., 1991).

Protein kinase A assay

Cells were grown for 5 days, harvested and washed with ice-cold homogenization buffer containing 5 mM K_2HPO_4, 5 mM KH_2PO_4, 1 mM EDTA, 0.1% Triton-X 100, 0.1 mM dithiothreitol, 10 μg/ml leupeptin, and 50 mM NaF at pH 6.8 as previously described (Flockhart and Corbin, 1984). The homogenate was centrifuged at 20,000 x g for 30 minutes at 4 °C. After centrifugation, the soluble fraction was retained for further treatment and assay, and the particulate fraction was discarded. The activity of protein kinase A was determined by a colorimetric protein kinase A assay (Pierce, Rockford, IL).

The soluble fraction and pre-mixed reaction mixture including reaction buffer, protein kinase A substrate, and activator solution were incubated for 45 minutes at 30 °C. After incubation, each sample was transferred directly onto the affinity membrane. Phosphopeptide binding buffer was applied to the affinity membrane and centrifuged at

3,450 x g for 1 minute. Phosphopeptide elution buffer was applied and the samples were centrifuged. Enzyme protein in the eluted fractions was measured by Spectronic spectrophotometer (Milton Roy, Rochester, NY) at 570 nm.

Statistical analysis

All values were expressed as the mean ± S.E. Data were analyzed with Student's unpaired *t* test (two-tail) or one way analysis of variance (ANOVA). The least significance test was used to test for differences between means of end points for which the ANOVA indicated a significant ($P<0.05$) *F* ratio.

RESULTS

Effects of CTX, forskolin, and IBMX on cAMP production and insulin release in RINm5F cells

More than 90% of the cells had neuron-like processes (differentiated) in passages between 28 and 30 . However, the cells gradually lost processes to become round cells (undifferentiated) and > 99% of the cells were undifferentiated in passages after 35 (Fig. 1 in chapter III). CTX (1 μg/ml) significantly increased intracellular cAMP concentration in both differentiated and undifferentiated cells. The increases of intracellular cAMP by CTX were 73.5 ± 6.6% in differentiated cells and 80.5 ± 4.5% in undifferentiated cells (Fig. 1A). Basal cAMP concentrations were 961 - 1112 *f*mol/million cells without significant differences among all passages studied (28 - 40). However, insulin release in response to CTX was greater in undifferentiated cells (105.4 ± 2.1%) than in differentiated cells (39.9 ± 1.0 %) (Fig. 1B).

Forskolin (10 μM), an activator of adenylyl cyclase, significantly increased intracellular cAMP concentration by 150 - 186 fold in differentiated cells and by 311 - 370 fold in undifferentiated cells (Fig. 2A). Forskolin (10 μM) significantly increased insulin release by 15 - 32% in differentiated cells and by 104 - 130% in

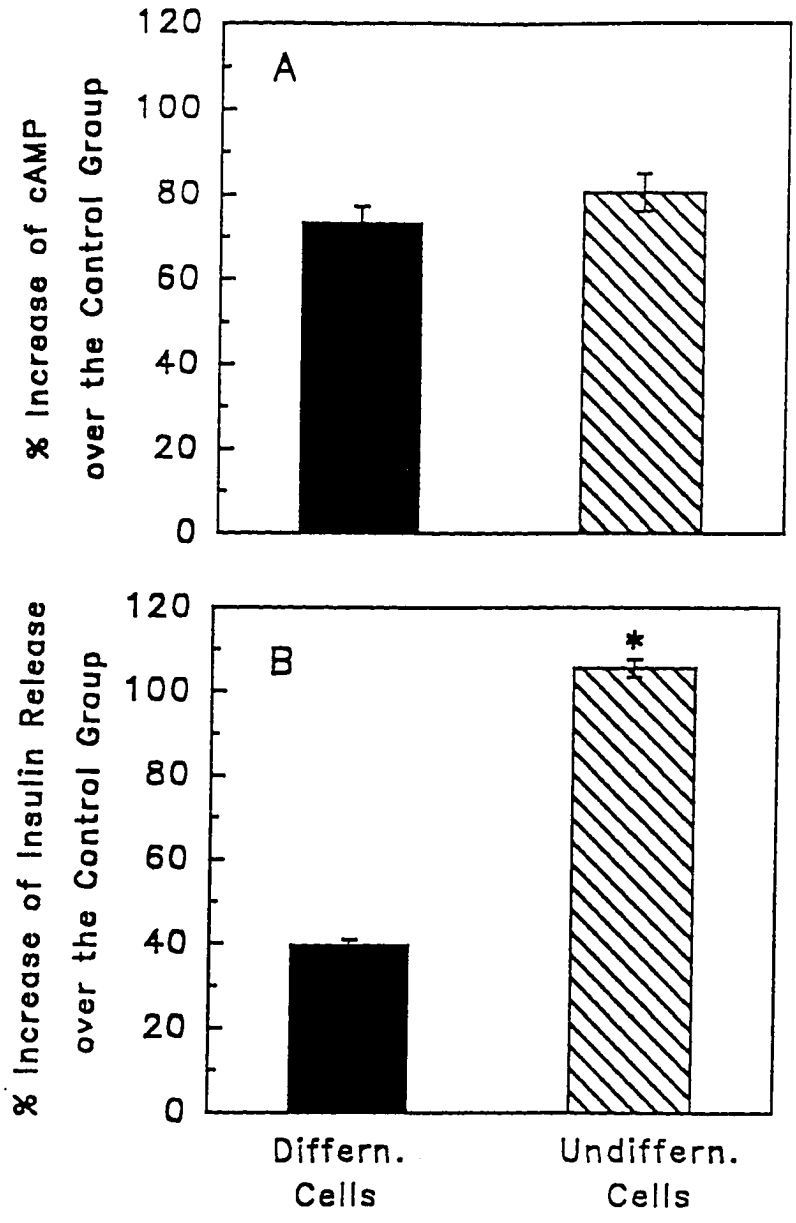


Fig. 1. Effect of CTX on cAMP production (A) and insulin release (B) in RINm5F cells. Mean ± S.E. (n = 4) for cAMP production and insulin release are shown. *$P < 0.05$, compared with differentiated cells.

Fig. 2. Passage-dependent effect of forskolin (10 μM) on cAMP production (A) and insulin release (B) in RINm5F cells. Passage-dependent effect of IBMX (100 μM) on cAMP production (C) and insulin release (D) in RINm5F cells. Mean ± S.E. (n = 16) are shown for both intracellular cAMP concentration and insulin release.. Intracellular cAMP concentrations and insulin release by forskolin (10 μM) and IBMX (100 μM) were significantly increased in all the passages studied (28 - 40) at $P<0.05$, compared with basal control group. The intracellular concentrations of cAMP in control group were 961 - 1112 *f*mol/million cells in differentiated and undifferentiated cells, without significant differences at $P<0.05$. The insulin release of control group were 6.9 - 8.9 ng/min/million cells in differentiated and 2.9 - 3.4 ng/min/million cells in undifferentiated cells.

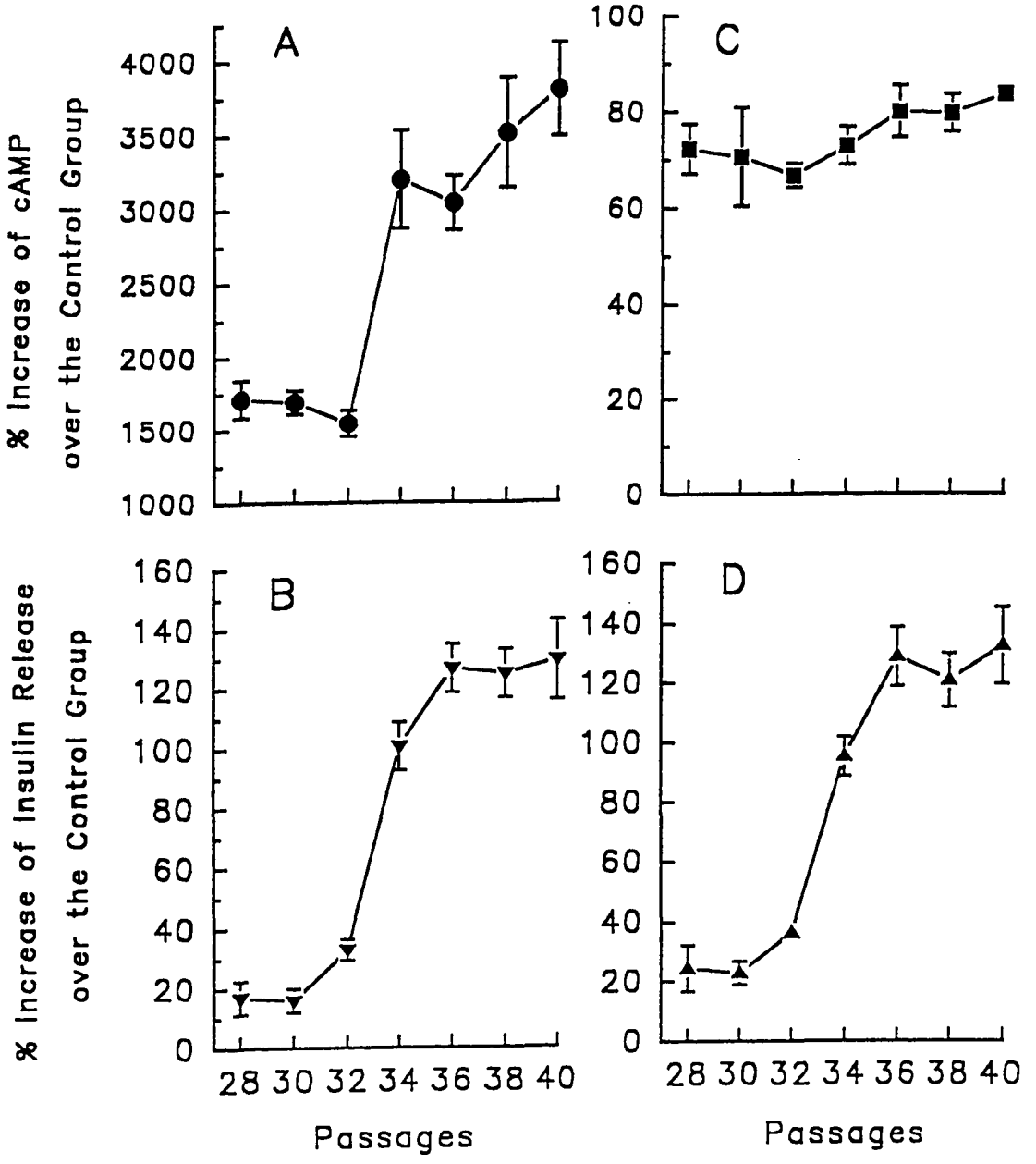
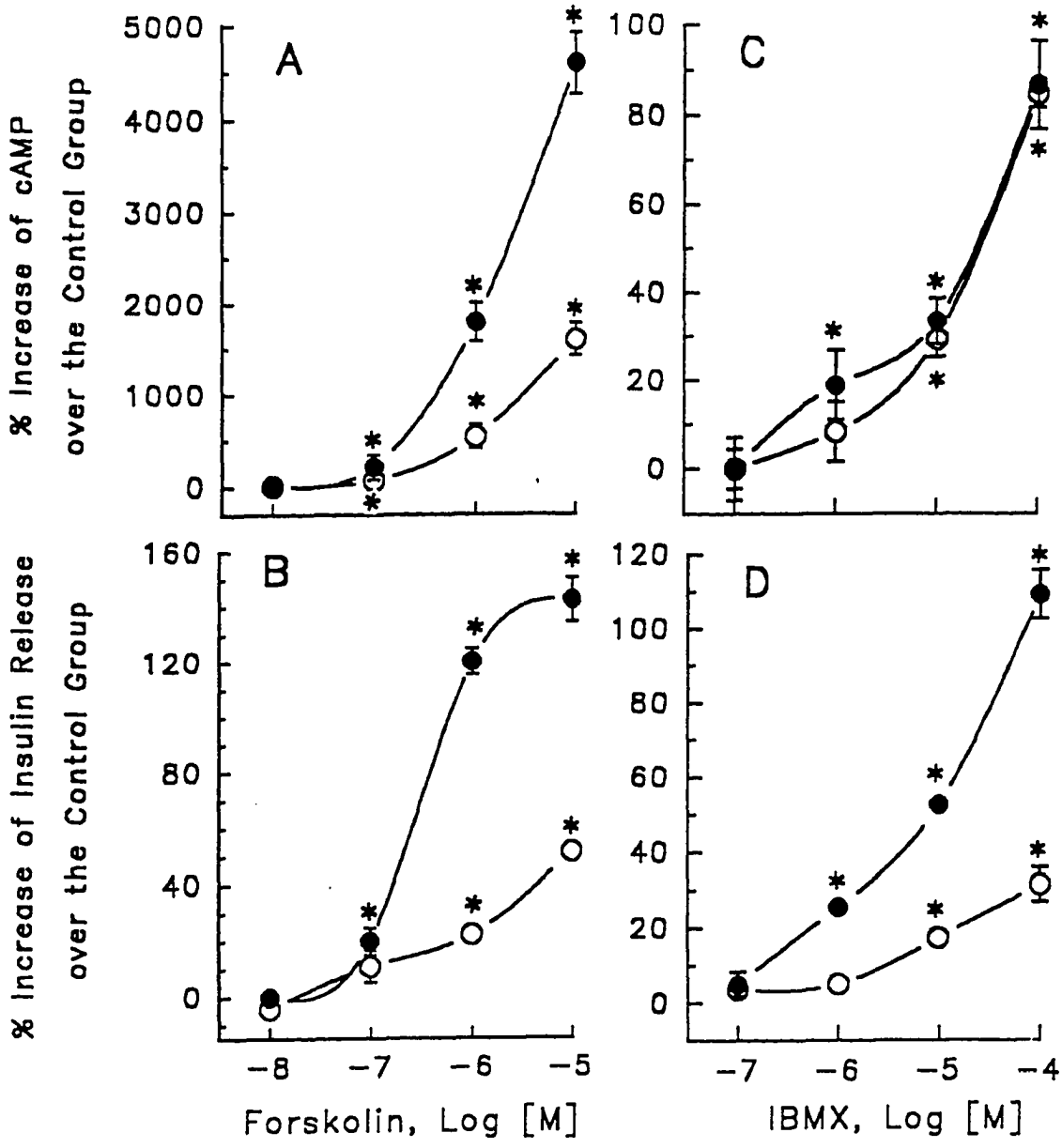

A
B
C
D
% Increase of cAMP over the Control Group
% Increase of Insulin Release over the Control Group
4000
3500
3000
2500
2000
1500
1000
100
80
60
40
20
0
160
140
120
100
80
60
40
20
0
28 30 32 34 36 38 40
Passages
Passages

Fig. 3. Dose-dependent increase of forskolin on cAMP production (A) and insulin release (B) in differentiated (○)and undifferentiated (●) RINm5F cells. Dose-dependent response of IBMX on cAMP production (C) and insulin release (D) in differentiated (○)and undifferentiated (●) RINm5F cells. Mean ± S.E. for insulin release (n=8) and for intracellular cAMP concentration (n=4) are shown. *$P<0.05$, compared with basal control group.


% Increase of cAMP over the Control Group
% Increase of Insulin Release over the Control Group
A
B
C
D
5000
4000
3000
2000
1000
0
160
120
80
40
100
60
20
−8
−7
−6
−5
−4
Forskolin, Log [M]
IBMX, Log [M]

undifferentiated cells (Fig. 2B). IBMX (100 μM) significantly increased intracellular cAMP concentration without significant differences between differentiated and undifferentiated cells, in which the increases were 63 - 86% (Fig. 2C). Furthermore, IBMX (0.1 mM) significantly increased insulin release by 11 - 48% from differentiated cells and by 110 - 132% from undifferentiated cells (Fig. 2D).

Forskolin (0.01 - 10 μM) increased intracellular cAMP concentration and insulin release in a dose-dependent manner in both differentiated and undifferentiated cells (Figs. 3A and 3B). However, increases in intracellular cAMP concentration and insulin release were higher in undifferentiated cells than differentiated cells. In addition, dose-dependent increases of insulin release by IBMX (0.1 - 100 μM) was higher in undifferentiated cells than in differentiated cells. However, the IBMX-induced cAMP increase was not significantly different between differentiated cells and undifferentiated cells (Figs. 3C and 3D).

Effects of isoproterenol and CTX on forskolin-induced cAMP production and insulin release in RINm5F cells

To study the effect of isoproterenol on forskolin-induced cAMP production and insulin release, various concentrations of isoproterenol and forskolin were applied to determine the concentrations of isoproterenol and forskolin that cause equal intracellular cAMP concentrations in differentiated and undifferentiated cells. Isoproterenol at 10 μM in differentiated cells and 0.1 μM in undifferentiated cells increased intracellular cAMP concentration by 30.4 ± 3.1% and 32.5 ± 2.6%, respectively. In addition, forskolin at 1 μM in differentiated cells and 0.3 μM in undifferentiated cells increased intracellular cAMP concentration by 509 ± 32% and 485 ± 29%, respectively. In differentiated cells, isoproterenol (10 μM) did not significantly potentiate forskolin (1 μM)-induced cAMP production or insulin release (Fig. 4). However, in undifferentiated cells, isoproterenol (0.1 μM) potentiated forskolin (0.3 μM)-induced cAMP production and insulin release by 355% and 135%, respectively.

Fig. 4. Effect of isoproterenol on forskolin-stimulated cAMP production (A) and insulin release (B) in RINm5F cells. Closed bar; isoproterenol, open bar; forskolin, and hatched bar, isoproterenol + forskolin. Isoproterenol at 10 μM in differentiated cells and 0.1 μM in undifferentiated cells increased intracellular cAMP concentration equally in differentiated and undifferentiated cells. In addition, to equally increase intracellular cAMP concentration, forskolin at 1 μM and 0.3 μM was applied in differentiated and undifferentiated cells, respectively. Mean ± S.E. (n=4) for intracellular cAMP concentration and (n=8) insulin release are shown. $^*P<0.05$, compared with the combined value from each forskolin and isoproterenol value.

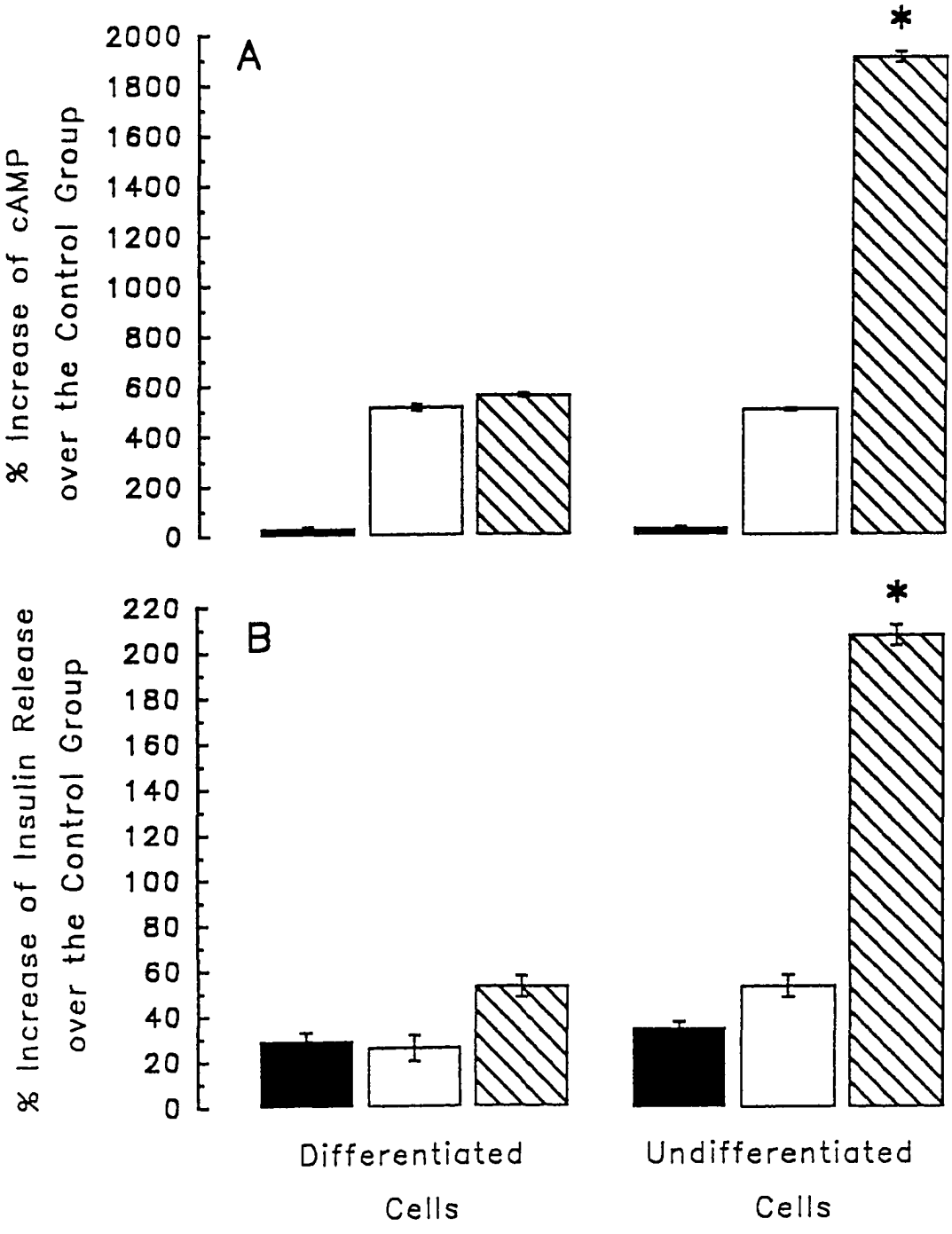
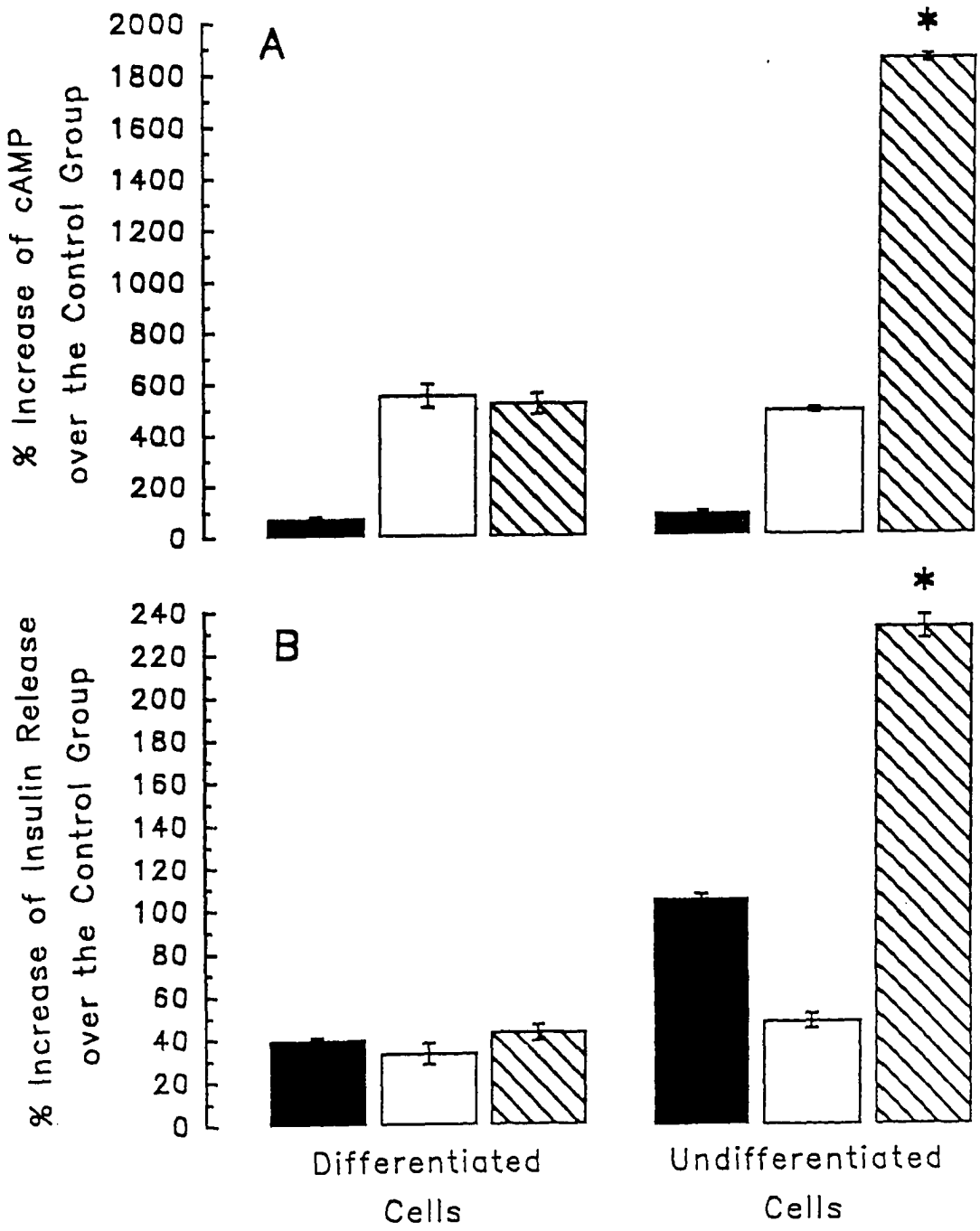

A
% Increase of cAMP over the Control Group
2000
1800
1600
1400
1200
1000
800
600
400
200
0
*
B
% Increase of Insulin Release over the Control Group
220
200
180
160
140
120
100
80
60
40
20
0
*
Differentiated Cells
Undifferentiated Cells

Fig. 5. Effect of CTX on forskolin-stimulated cAMP production (A) and insulin release (B) in RINm5F cells. Closed bar; CTX, open bar; forskolin, and hatched bar, CTX and forskolin. CTX (1 μg/ml) was used to ADP-ribosylate G_s-proteins in both differentiated and undifferentiated cells. However, forskolin at 1 μM in differentiated cells and 0.3 μM in undifferentiated cells increased intracellular cAMP concentration equally in differentiated and undifferentiated cells. Mean ± S.E. (n = 4) for intracellular cAMP concentration and insulin release are shown. $^*P < 0.05$, compared with the combined value from each forskolin and isoproterenol value.


A
% Increase of cAMP over the Control Group
2000
1800
1600
1400
1200
1000
800
600
400
200
0
*
B
% Increase of Insulin Release over the Control Group
240
220
200
180
160
140
120
100
80
60
40
20
0
*
Differentiated Cells
Undifferentiated Cells

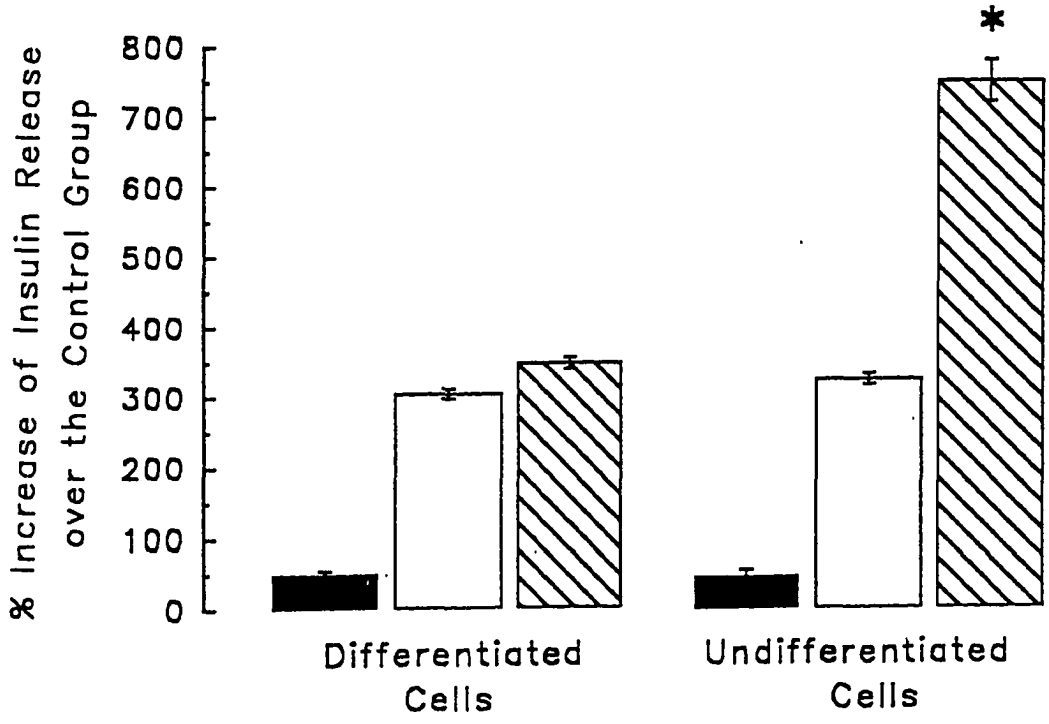


Fig. 6. Effect of forskolin on KCl (30 mM)-stimulated insulin release in RINm5F cells. Closed bar; forskolin, open bar; KCl, and hatched bar, forskolin and KCl. Forskolin at 10 μM and 0.3 μM was applied to equally increase insulin release in differentiated and undifferentiated cells, respectively. Mean ± S.E. (n = 8) for insulin release are shown. *$P < 0.05$, compared with the combined value from each forskolin and KCl value.

To study the effect of ADP ribosylation of G_s-protein by CTX on forskolin-induced cAMP production and insulin release, forskolin at 1 μM in differentiated cells and 0.3 μM in undifferentiated cells was used to obtain similar increase in intracellular cAMP concentration in differentiated cells (549 ± 46%) and in undifferentiated cells (488 ± 10%). In undifferentiated cells, CTX (1 μg/ml) significantly potentiated forskolin-induced cAMP production and insulin release by 326% and 152% (n=4), respectively. In differentiated cells, CTX, however, did not potentiate forskolin-induced cAMP production and insulin release (Fig. 5).

Effect of forskolin on KCl-stimulated cAMP production and insulin release in RINm5F cells

To study the effect of forskolin on KCl-induced insulin release, concentrations of forskolin were applied to equally increase insulin release, as described above. Forskolin at 10 μM in differentiated cells and 0.3 μM in undifferentiated cells equally increased insulin release by 50.3 ± 3.6% and 47.2 ± 5.5%, respectively. In undifferentiated cells, forskolin (0.3 μM) potentiated KCl (30 mM)-induced insulin release by 102% but in differentiated cells forskolin (10 μM) failed to do so (Fig. 6). In undifferentiated cells there were no significant differences in intracellular cAMP concentrations between the forskolin alone group and the forskolin + KCl group (557 ± 43% and 479 ± 38%, respectively;(n=4)). In addition, there was no significant differences in the increase of $[Ca^{2+}]_i$ between KCl alone group and forskolin + KCl group (data not shown).

Activity of protein kinase A in RINm5F cells

To study the activity of protein kinase A in differentiated and undifferentiated cells, 100 μM cAMP was added to 10 μl soluble fractions of cell homogenate from 1 million cells. Activity of protein kinase A was not significantly different between differentiated cells and undifferentiated cells. The concentrations of protein kinase A were 191 ± 9 U/mg protein in differentiated cells (n=4) and 181 ± 4 U/mg protein in undifferentiated cells (n=4).

DISCUSSION

This study was undertaken to characterize cAMP-dependent signal transduction pathways mediating the action of β-ARs in RINm5F cells. Our findings demonstrated that activity of adenylyl cyclase is higher in undifferentiated cells than in differentiated RINm5F cells. However, the activities of G_s-proteins, PDE and protein kinase A remain unchanged between differentiated and undifferentiated RINm5F cells. Our results further suggested that elevated activity of adenylyl cyclase and sensitivity of G_s-protein- and protein kinase A-dependent exocytosis may be responsible for the higher activities of β-ARs in undifferentiated cells than in differentiated cells.

CTX, which causes ADP-ribosylation of G_s-proteins, equally increased intracellular cAMP concentrations suggesting the presence of equal activity of G_s-proteins in differentiated and undifferentiated cells. In HIT-T15 cells (derived from hamster islets with Simian virus-40 infection), increased potency of isoproterenol and forskolin in the later passages is responsible for greater quantities of 45 KDa subunit of $G_{\alpha s}$-protein between 45 and 52 KDa subunits (Zhang et al., 1989; Walseth et al., 1989). In addition, CTX stimulated more cAMP production, suggesting that these are greater quantities of $G_{\alpha s}$-proteins in the later passages than in earlier passages (Walseth et al., 1989). Whether the quantity and/or subunit of G_s-proteins contribute to the activity of G_s-proteins in RINm5F cells requires further study.

CTX increased more insulin release in undifferentiated cells than in differentiated cells. CTX activates G_s-proteins and increases intracellular cAMP concentration. Increased intracellular cAMP activates protein kinase A which may increase insulin release partly through elevating $[Ca^{2+}]_i$ via opening of VDCCs, and partly through some unknown mechanisms. Our results showed that the activity of protein kinase A was not significantly different and forskolin (10 μM) caused an insignificant increase in $[Ca^{2+}]_i$ compared to its effect on insulin release (unpublished observations) in both cell types. Therefore, G_s-proteins may be directly coupled to the exocytotic apparatus, if so, are more involved in undifferentiated cells than in differentiated cells. In fact, the presence of G_{es}-proteins, which may be directly involved in the activation of exocytosis, has been suggested in RINm5F cells (Ullrich and Wollheim, 1988).

Forskolin stimulated more cAMP production and insulin release in undifferentiated cells, which indicates that the activity of adenylyl cyclase is greater than in differentiated cells. The activity of adenylyl cyclase in pancreatic islets is regulated by Ca^{2+}-calmodulin (Thams et al., 1982). In the absence of calmodulin, Ca^{2+} inhibits adenylyl cyclase activity but in the presence of calmodulin, Ca^{2+} stimulates adenylyl cyclase in RINm5F cells (Caldwell et al., 1992). In addition, β-AR- and forskolin-stimulated activation of adenylyl cyclase is enhanced by Na^+ suggesting that Na^+ can play a role in regulating the G_s-proteins/adenylyl cyclase system (Watson et al., 1989). In our findings, the effect of forskolin on cAMP production and insulin release was further potentiated by isoproterenol and CTX in undifferentiated cells, but not in differentiated cells suggesting that G_s-proteins may be directly associated with the activity of adenylyl cyclase. In fact, the affinity of forskolin is higher for adenylyl cyclase (types II, V and VI, but not type I) which is associated with the α-subunit of G_s-proteins than for adenylyl cyclase alone (Alousi et al., 1991; Sutkowski et al., 1994). Therefore, our findings suggested that greater association of $G_{\alpha s}$-protein with adenylyl cyclase may contribute to an increased activity of adenylyl cyclase on cAMP production in undifferentiated than differentiated cells, although equal activity of G_s-proteins is present in both cell types.

Intracellular cAMP concentration is determined not only by the rate of cAMP production through the activation of adenylyl cyclase but also by the rate of hydrolysis through the activation of PDE. IBMX, a PDE inhibitor, primarily increases intracellular cAMP concentration (Wiedenkeller and Sharp, 1981), although IBMX may have other cAMP-independent mechanisms such as a promoter for release of Ca^{2+} stores (Shemesh et al., 1984) and an inhibitor of the sustained calcium current in RINm5F cells (Simasko and Yan, 1993). According to the current guidelines for PDE nomenclature (Beavo and Reifsnyder, 1990), cAMP is hydrolyzed to 5'-AMP by at least four classes of PDE isozymes: (1) a Ca^{2+}-calmodulin-dependent PDE (PDE I), (2) a cGMP-stimulated PDE (PDE II), (3) a cGMP-inhibited PDE (PDE III), and (4) a cAMP-specific PDE (PDE IV). Since these studies were performed with IBMX which is a nonselective PDE inhibitor (Akita et al., 1994), the possible changes of PDE isotypes were not evaluated and remain to be further characterized.

Increased intracellular cAMP concentrations primarily activate protein kinase A and stimulate insulin release in pancreatic β-cells and clonal β-cell lines including RINm5F (Wiedenkeller and Sharp, 1981; Hermansen, 1985; Suzuki, et al., 1992; Chen and Hsu, 1994). cAMP-dependent phosphorylation may be responsible for insulin release by increasing Ca^{2+} entry into the β-cells (Eddlestone et al., 1985) or by releasing Ca^{2+} from intracellular stores (Mandarino et al., 1980; Hahn et al., 1979). In our findings, total (activated and inactivated) activity of protein kinase A was not significantly different between differentiated and undifferentiated RINm5F cells. However, insulin release containing equal amounts of cAMP was greater in undifferentiated cells than in differentiated cells. The greater insulin release in undifferentiated cells may be due not only to more sensitivity of protein kinase A to cAMP but also by more binding of cAMP to a target such as a channel other than activation of protein kinase A (Weber et al., 1987; Jiang et al., 1992; Holz, 4th. et al., 1995). Further studies are required to test these hypotheses.

The precise relationship of cAMP to ion channels in pancreatic β-cells is still controversial. Ca^{2+}-activated nonselective cation channels are activated by cAMP to generate I_{cAMP} which is an inward Na^+ current induced by cAMP. I_{cAMP} results in membrane depolarization and raises $[Ca^{2+}]_i$ by activation of VDCCs. I_{cAMP} may also increase $[Na^+]_i$ which slows Na^+/Ca^{2+} exchange which, in turn, increases $[Ca^{2+}]_i$. An increase in $[Ca^{2+}]_i$ promotes Ca^{2+}-induced Ca^{2+} release (CICR) from intracellular Ca^{2+} stores (Sturgess et al., 1986; Reale et al., 1994; Holz, 4th. et al., 1995). In addition, cAMP increases $[Ca^{2+}]_i$ not only through VDCCs, but also through an uncharacterized voltage-independent process (Holz, 4th. et al., 1995). However, forskolin potentiated KCl-induced insulin release in RINm5F cells without significantly changing $[Ca^{2+}]_i$ (our unpublished observations). Based on these findings, modulation of exocytosis by protein kinases A, at a distal step to the elevation of Ca^{2+}, may also be much greater in undifferentiated cells than in differentiated cells. Activation of protein kinase A or inhibition of protein phosphatases dramatically potentiates exocytosis of insulin without an enhancement of Ca^{2+} influx in pancreatic β-cells. This suggests that protein kinase A is of greater quantitative importance than Ca^{2+} (Ammala et al., 1993; Gillis and Misler, 1993; Ammala et al., 1994).

RINm5F cells may be valuable tools for further studies on the relationship between changes in insulin release and β-ARs/cAMP metabolism during the differentiation of fetal pancreatic β-cells. In fact, RINm5F cells resemble incompletely differentiated fetal pancreatic β-cells (Gazdar et al., 1980; Halban et al., 1983; Polak et al., 1993). By comparing the morphologic and physiologic changes which occur when the cells change from differentiated to undifferentiated state during oncogenesis, RINm5F cells may provide useful information on the embryonic development of pancreatic β-cells. Since the morphologically undifferentiated RINm5F cells might represent more poorly differentiated fetal β-cells than the differentiated cells. The β-adrenoceptor/adenylyl cyclase system is well characterized in embryonic development (Maier et al., 1989). In rats, basal and forskolin-stimulated activities of adenylyl cyclase show a developmental spike by gestational day 18 (Slotkin et al., 1994) and are greater in the fetus and neonate than in the adult (Katz et al., 1985; Tobise et al., 1994). Stimulation of adenylyl cyclase by GTP also decreases during development suggesting that developmental alterations of the nucleotide regulatory component and/or its interaction with the catalytic component occur (Katz et al., 1985). Altered plasma membrane fluidity has been reported during development (Kutchai et al., 1976), and fluidity of the membrane in turn modulates coupling of G-proteins to adenylyl cyclase (Salesse et al., 1982). Developmental changes of membrane lipid content may also exert a regulatory influence on the enzyme (Kalish et al., 1977). In addition, in the ventricle, a decrease in the content of the type VI, but not type V, isoform of adenylyl cyclase correlates with a decrease in catalytic activity with development and aging (Tobise et al., 1994). For insulin secretion, aged rats (2 years old) do not show a response to forskolin in a cAMP- dependent pathway, whereas, young rats (12 weeks old) do (Aizawa et al., 1994). Similarly, IBMX stimulates cAMP production and insulin release in 4 - 6 day old rats but does not stimulate insulin release in 12 day or 3 month old rats (although cAMP concentration is significantly enhanced) (Ziegler et al., 1982). In rabbit ventricular cells, the PDE isoenzyme changes from PDE IV to PDE III to increase calcium currents during the postnatal period (Akita et al., 1994). Activation of adenylyl cyclase stimulates differentiation and growth, gene expression, the response to *in utero* stress and the adaptation of organs to birth (Slotkin et al., 1987; Odom et al., 1987; Jones and Ritchie, 1983; Curtis and Zalin, 1985; McDonald et al., 1986). Therefore,

we speculate that the activity of adenylyl cyclase is higher early in embryonic and fetal pancreatic β-cells in order to contribute to cell migration and differentiation, and RINm5F cells may be a useful model to study development in this tissue.

In conclusion, our present findings suggested that the activity of adenylyl cyclase is greater in undifferentiated cells than in differentiated RINm5F cells and this may be due, in part, to a greater association of $G_{\alpha s}$-proteins with adenylyl cyclase. The activities of G_s-proteins, PDE and protein kinase A remain unchanged between differentiated and undifferentiated RINm5F cells. However, it is possible that G_s-protein- and protein kinase A-dependent exocytosis of insulin is more functional in undifferentiated cells than in differentiated cells.

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CHAPTER VI CHARACTERIZATION OF VOLTAGE-DEPENDENT CALCIUM CHANNELS IN RINM5F CELLS IN A PASSAGE- AND MORPHOLOGY-DEPENDENT MANNER

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ABSTRACT

Changes in intracellular Ca^{2+} concentrations have a major role in the regulation of insulin release from pancreatic β-cells. In the present study, voltage-dependent calcium channels (VDCCs) were characterized in a passage-and morphology-dependent manner in a clonal β-cell line RINm5F. Passages from 28 to 40 were employed to contrast two morphological features: the differentiated cells with neuron-like processes and the undifferentiated cells without the processes. Passages $\leq$ 30 were differentiated cells and passages $\geq$ 35 were undifferentiated cells. The effect of KCl (15 mM) on $[Ca^{2+}]_i$ and insulin release was 3-fold higher and Bay K 8644 (1 μM) was 6-fold higher in differentiated cells than in undifferentiated cells. Furthermore, the dose-dependent increases in $[Ca^{2+}]_i$ and insulin release by the L-type Ca^{2+} channel agonist, Bay K 8644 (1 nM - 1 μM), were higher in differentiated cells than in undifferentiated cells. However, dose-dependent increases in $[Ca^{2+}]_i$ and insulin release induced by low concentrations of KCl (7.5 - 30 mM) were greater in differentiated cells than in undifferentiated cells but the results were reversed in the presence of higher concentrations of KCl (30 - 45 mM). In addition, the inhibitory effect of nimodipine on KCl and Bay K 8644 was studied by equally increasing $[Ca^{2+}]_i$ and insulin release in differentiated and undifferentiated cells. Thus, 12 mM and 15 mM KCl was used in differentiated and undifferentiated cells, respectively, and 0.1 μM and 1 μM Bay K 8644 was used in differentiated and undifferentiated cells, respectively. Dose-dependent inhibitions in KCl- and Bay K 8644-induced $[Ca^{2+}]_i$ and insulin release by nimodipine (1 nM - 1 μM) were greater in differentiated cells than in undifferentiated

cells.

In whole-cell patch-clamp recordings, with a holding potential of -80 mV, inward Ca^{2+} currents were first observed during a depolarization to -50 mV in both differentiated cells and undifferentiated cells. However, the maximal Ca^{2+} current was higher in undifferentiated cells than in differentiated cells. Bay K 8644 (1 μM) increased Ca^{2+} currents by 74 ± 13% in differentiated cells and 20 ± 7 % in undifferentiated cells. Nimodipine (1 μM) caused an inhibition of control Ca^{2+} currents by 48 ± 17% in differentiated cells and failed to inhibit them in undifferentiated cells. In addition, nimodipine (1 μM) inhibited Bay K 8644-induced Ca^{2+} current by 83 ± 8 % in the differentiated cells, but failed to inhibit them in undifferentiated cells.

Since nimodipine did not totally inhibit control Ca^{2+} currents and Bay K 8644-induced increases in $[Ca^{2+}]_i$ and insulin release in undifferentiated cells, it was further studied for two possibilities: 1) the functional involvement of other VDCCs such as N-, P- and Q-type and 2) the voltage-dependence of L-type Ca^{2+} channels. An N-type channel blocker, ω-conotoxin GVIA (1 μM), and a P- and Q-type channel blocker, ω-agatoxin IVA (300 nM), did not inhibit basal Ca^{2+} currents nor did they inhibit KCl- and Bay K 8644-induced increases in $[Ca^{2+}]_i$ and insulin release in both differentiated and undifferentiated cells. The voltage-dependence of L-type channels was studied by using nimodipine, whose binding could be enhanced by prolonged membrane depolarization. KCl (30 mM)-induced increases in $[Ca^{2+}]_i$ and insulin release was dose-dependently inhibited by nimodipine (0.1 nM - 1 μM) in both differentiated and undifferentiated cells. The potency of nimodipine on $[Ca^{2+}]_i$ and insulin release by 30 mM KCl was 17- to 22-fold higher than by 15 mM KCl in undifferentiated cells. However, in differentiated cells, there were no significant differences in the potency of nimodipine on $[Ca^{2+}]_i$ and insulin release induced by 12 and 30 mM KCl. In addition, in undifferentiated cells, the potency of nimodipine in inhibiting control Ca^{2+} currents was compared by holding membrane potentials at -80 mV and 0 mV in the whole-cell patch-clamp recordings. Nimodipine (1 μM) failed to inhibit control Ca^{2+} currents at the holding potentials of -80 mV, but inhibited them by 70.3 ± 7.7% at the holding potentials of 0 mV. The current-voltage relationship shifted to more negative potentials in differentiated cells than in undifferentiated cells. The voltage at which one-half of Ca^{2+} currents was activated was -24.3 ± 3.6 mV in differentiated cells and -10.6 ±

2.5 mV in undifferentiated cells. The prepulse potential at which one-half of the maximal Ca^{2+} currents was inactivated shifted from -21.4 ± 3.9 mV in differentiated cells to 1.8 ± 4.3 mV in undifferentiated cells. Taken together, these results suggested that differentiated RINm5F cells are functionally equipped with more L-type Ca^{2+} channels than the undifferentiated cells, and the voltage-dependence of L-type Ca^{2+} channels was different between differentiated and undifferentiated cells. However, N-, P- and Q-type VDCCs were not detected in differentiated and undifferentiated RINm5F cells.

INTRODUCTION

Intracellular Ca^{2+} serves as a major signal for insulin release (Smith et al, 1993). Ca^{2+} entry through voltage-dependent Ca^{2+} channels (VDCCs) is a major pathway for raising the intracellular Ca^{2+} concentration in most of living cells including β-cells (Ashcroft et al., 1990). As Ca^{2+} entry in β-cells is primarily controlled by VDCCs, the identification and characterization of these channels are of importance for understanding β-cell stimulus-secretion coupling. VDCCs are primarily categorized as T (transient)-, N (neither L nor T)-, L (long-lasting)- and P (Purkinje)-types. In addition, the Q-type channel has been characterized in the brain (Hofmann et al., 1994). The T-type (low voltage-activated or slow-deactivating) channel is activated at membrane potentials of around -50 mV and exhibits a voltage-dependent inactivation. The L-type (high-voltage activated or fast-deactivating) channel of β-cells is activated at membrane potentials of around -40 mV, and the inactivation of calcium current is Ca^{2+}-dependent (Pollo et al., 1993). The L-type channel is distinguished by its sensitivity to dihydropyridines (DHP), being inhibited by DHP antagonists such as nimodipine and activated by DHP agonists such as Bay K 8644 (Smith et al., 1993). The N- and P-type channels are blocked by ω-conotoxin GVIA and ω-agatoxin IVA, respectively. The Q-type channel is blocked by higher concentrations (300 nM) of ω-agatoxin IVA than the P-type channel (30 nM). In addition, the Q-type channel is blocked by ω-conotoxin MVIIC (Hofmann et al., 1994).

There is growing evidence that the pancreatic β-cell possesses a heterogeneity of

Ca^{2+} channels and that the expression of the different channel types may vary among species (Ashcroft et al., 1994). In mouse β-cells, the L-type Ca^{2+} channel accounts for most of the Ca^{2+} influx (Smith et al., 1993), and both L- and T-type channels have been identified in rat and human β-cells (Ashcroft et al., 1990) and RINm5F cells (Velasco, 1987). Recently, the presence of multiple types of high voltage-activated (HVA) Ca^{2+} channels has been suggested in pancreatic β-cells and RINm5F cells (Aicardi et al., 1991; Sher et al., 1992; Pollo et al., 1993; Magnelli et al., 1995). DHPs inhibit the HVA current by 55%, ω-conotoxin GVIA by 10-20%, ω-agatoxin IVA by 24%, and ω-conotoxin MVIIC by 45% in RINm5F cells suggesting that N-, P- and Q-type VDCCs in addition to L-type may be present (Aicardi et al., 1991; Sher et al., 1992; Pollo et al., 1993; Magnelli et al., 1995).

In a preliminary study, we observed that RINm5F cells had neuron-like long processes (differentiated cells) and lost them to become round cells (undifferentiated cells) after a long-term (> 8 weeks) culture . As the cells changed their morphologies, the functional responses to Bay K 8644 and KCl for insulin release and intracellular calcium ($[Ca^{2+}]_i$) were distinct between differentiated and undifferentiated cells. These findings led us to further characterize the role of VDCCs on increases in $[Ca^{2+}]_i$ and insulin release by applying a number of calcium channel agonists and antagonists in differentiated and undifferentiated RINm5F cells. The present findings indicated that L-type Ca^{2+} channels are functionally more predominant in differentiated cells than in undifferentiated cells, whereas, the voltage-dependency of L-type Ca^{2+} channels was different between differentiated cells and undifferentiated RINm5F cells . However, N-, P- and Q-type calcium channels were not evident in either differentiated or undifferentiated RINm5F cells.

MATERIALS AND METHODS

Materials

The following materials were used: RPMI-1640, fetal bovine serum, and ω-conotoxin GVIA (Sigma Chemical,St. Louis, MO), 24-well culture plate (Corning Glass Works, Corning, NY), Bay K 8644 and nimodipine (Research Biochemicals International, Natic, MA), CGP 28392 (Ciba-Geigy Limited, Basel, Switzerland), and ω-agatoxin IVA

(Pfizer Inc., Groton, CT).

Cell culture

RINm5F cells were obtained from Dr. S. B. Pek (University of Michigan Medical Center, Ann Arbor, MI). The cells were maintained in RPMI-1640 medium with 10% fetal bovine serum as described previously (Chen et al., 1994). The cells were subcultured every 7 days and the medium was changed at day 3 and day 5 after subculture. All experiments were performed by use of cells from passages 28-40. No antibiotics were used.

$[Ca^{2+}]_i$ measurement

Intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) was monitored as previously described (Hsu et al., 1991a). Briefly, the cells were loaded with 2 μM fura-2 acetoxymethylester (AM) in KRB for 30 min at 37 °C. The loaded cells were washed with KRB and kept at room temperature. The cells were resuspended in a concentration of 10^6 cells/ml and 1.5 ml of aliquot was used for measuring $[Ca^{2+}]_i$. The 340/380 nm fluorescence ratios were monitored in a SLM-8000 fluorescence spectrophotometer (SLM, Urbana, IL). The $[Ca^{2+}]_i$ was calibrated after cell lysis as described previously (Hsu et al., 1991a).

Ca^{2+} current recordings

The perforated patch clamp was performed as described previously (Horn and Marty, 1988) using an Axopatch-1D clamp amplifier (Axon Instruments, Foster City, CA). Patch pipettes (3-5 MΩ) were made on a micropipette puller (Narishige Instruments, Tokyo) by using disposable glass pipettes (VWR Scientific, West Chester, PA), and were polished on a custom-made microforge. A high-resistant seal between the edge of the electrode and the membrane was obtained by applying slight suction to the pipette after gently pushing it on to the surface of the cell. The liquid junction potentials were nullified with an offset circuit before the high-resistance seal formed. Ca^{2+} currents were evoked by depolarizing pulses from a holding potential of -80 mV to a potential between -60 to +50 mV at 10 mV increments. Data were collected and analyzed using an IBM-PC computer with an analogue-digital interface board and

pClamp software 5.5. (Axon Instruments, Foster city, CA). The patch pipette was filled with a solution containing 120 mM glutamic acid, 130 mM CsOH, 250 μg/ml amphotericin B and 10 mM Hepes (pH 7.2). The bath solution contained in mM: 140 tetraethylammonium chloride, 5 4-aminopyridine, 2 $CaCl_2$, 1.67 glucose, 5.4 KCl and 10 Hepes (pH 7.4). Agents were applied to the bath solution directly and all experiments were performed at room temperature.

Insulin release

Static incubations were performed to study insulin release in response to test agents as previously described (Hsu et al., 1991b). Cells from later passages grew more rapidly; consequently, the cells were plated onto 24-well plates at 2 x 10^5 cells/well for passages 28-32 and at 7 x 10^4 cells/well for passages 34-40 and grown for 5 days. The growth medium was then removed, and the cells were washed with Krebs-Ringer buffer (KRB) containing (in mM): 136 NaCl, 4.8 KCl, 2.5 $CaCl_2$, 1.2 KH_2PO_4, 1.2 $MgSO_4$, 5 $NaHCO_3$, 10 HEPES, 1.67 glucose and 0.1% bovine serum albumin, pH 7.4. The cells were then preincubated for a 15-min basal period at 37°C, followed by a 15-min incubation in KRB with test agents. When needed, the antagonist was given 5 min before the administration of the agonist to ensure blockade of the receptors before the addition of the agonist. Insulin in the media was measured by radioimmunoassay (RIA) as previously described (Hsu et al., 1991b).

Statistical analysis

All values were expressed as mean ± S.E. Analysis of variance (ANOVA) was used to determine the treatment or dose effect. The least significance test was used to test for differences between means of end points for which the ANOVA indicated a significant ($P<0.05$) *F* ratio.

RESULTS

Effects of KCl and Bay K 8644 on $[Ca^{2+}]_i$ and insulin release in differentiated and undifferentiated RINm5F cells

In the differentiated state, RINm5F cells had neuron-like processes; however, in the

undifferentiated state, the cells did not possess neuron-like processes. More than 90% of the cells were differentiated in passages ≤ 30. However, the cells gradually lost processes to become undifferentiated cells and > 99% of the cells were undifferentiated after passage 35 (Fig. 1 in Chapter III).

KCl (15 mM) and Bay K 8644 (1 μM) caused a rapid transient increase in $[Ca^{2+}]_i$ which was followed by a slowly decaying plateau in both differentiated and undifferentiated cells, but increases were greater in differentiated cells than in undifferentiated cells (Fig. 1). KCl was 3-fold more potent and Bay K 8644 6-fold more potent in producing a $[Ca^{2+}]_i$ increase in differentiated cells than in undifferentiated cells (Fig. 2). Similarly, KCl (15 mM) and Bay K 8644 (1 μM) induced insulin release in both differentiated and undifferentiated cells but KCl was 3-fold more potent and Bay K 8644 6-fold more potent on insulin release in differentiated cells than in undifferentiated cells (Fig. 2). Bay K 8644 (1 nM - 1 μM) dose-dependently increased $[Ca^{2+}]_i$ and insulin release in both cell types, but increases were greater in differentiated cells than in undifferentiated cells (Fig. 3). KCl (7.5 - 45 mM) increased $[Ca^{2+}]_i$ and insulin release in a dose-dependent manner in both differentiated and undifferentiated cells, but patterns of the increases were distinct (Fig. 4). At lower concentrations of KCl (10 - 30 mM), the dose-dependent increases were greater in differentiated cells while at higher concentrations of KCl (30 - 45 mM) the increases were greater in undifferentiated cells. These findings suggested that differentiated cells are functionally equipped with more L-type channels than undifferentiated cells. In addition, the voltage-dependence of the HVA-Ca^{2+} channels may be different between differentiated and undifferentiated cells because KCl, which depolarizes membrane potentials and opens VDCCs, increased $[Ca^{2+}]_i$ and insulin release in a discrete manner between differentiated and undifferentiated cells (Fig. 4).

Effects of depolarization and Bay K 8644 on Ca^{2+} currents in RINm5F cells

In the whole-cell patch-clamp recordings, after blocking K^+ currents by adding a high concentration of Cs^+ (30 mM) to the pipette solution, and tetraethylammonium (TEA) and 4-aminopyridine (4-AP) to the bath solution to block K^+ channels, inward Ca^{2+} currents were elicited by depolarizing from -60 to +50 mV from a holding

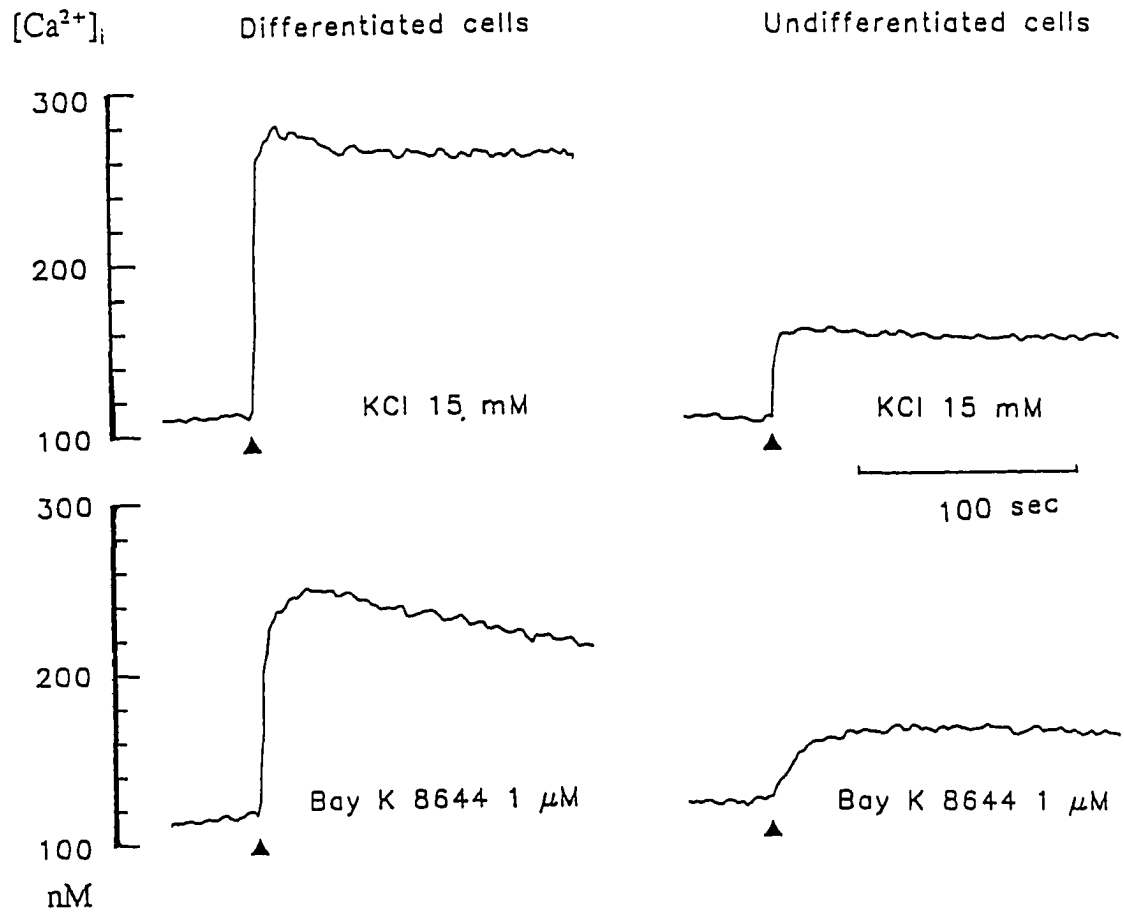


Fig. 1. Effects of KCl (15 mM) and Bay K 8644 (1 μM) on $[Ca^{2+}]_i$ in differentiated (left panel) and undifferentiated (right panel) RINm5F cells. Arrowheads show the times when the agents were added. One representitive result out of 8 observations.

Fig. 2. (A and C) Passage-dependent effects of KCl (15 mM) on $[Ca^{2+}]_i$ (A) and insulin release (C) in RINm5F cells. Percentage of increase of $[Ca^{2+}]_i$ was calculated from the changes between basal and peak concentration. Basal concentration of $[Ca^{2+}]_i$ was 105 - 134 nM in passages between 28 and 40. (B and D) Passage-dependent effect of Bay K 8644 (1 μM) on $[Ca^{2+}]_i$ (B) and insulin release (D) in RINm5F cells. Mean ± S.E. ($n = 8$) for $[Ca^{2+}]_i$ and ($n = 16$) for insulin release are shown.

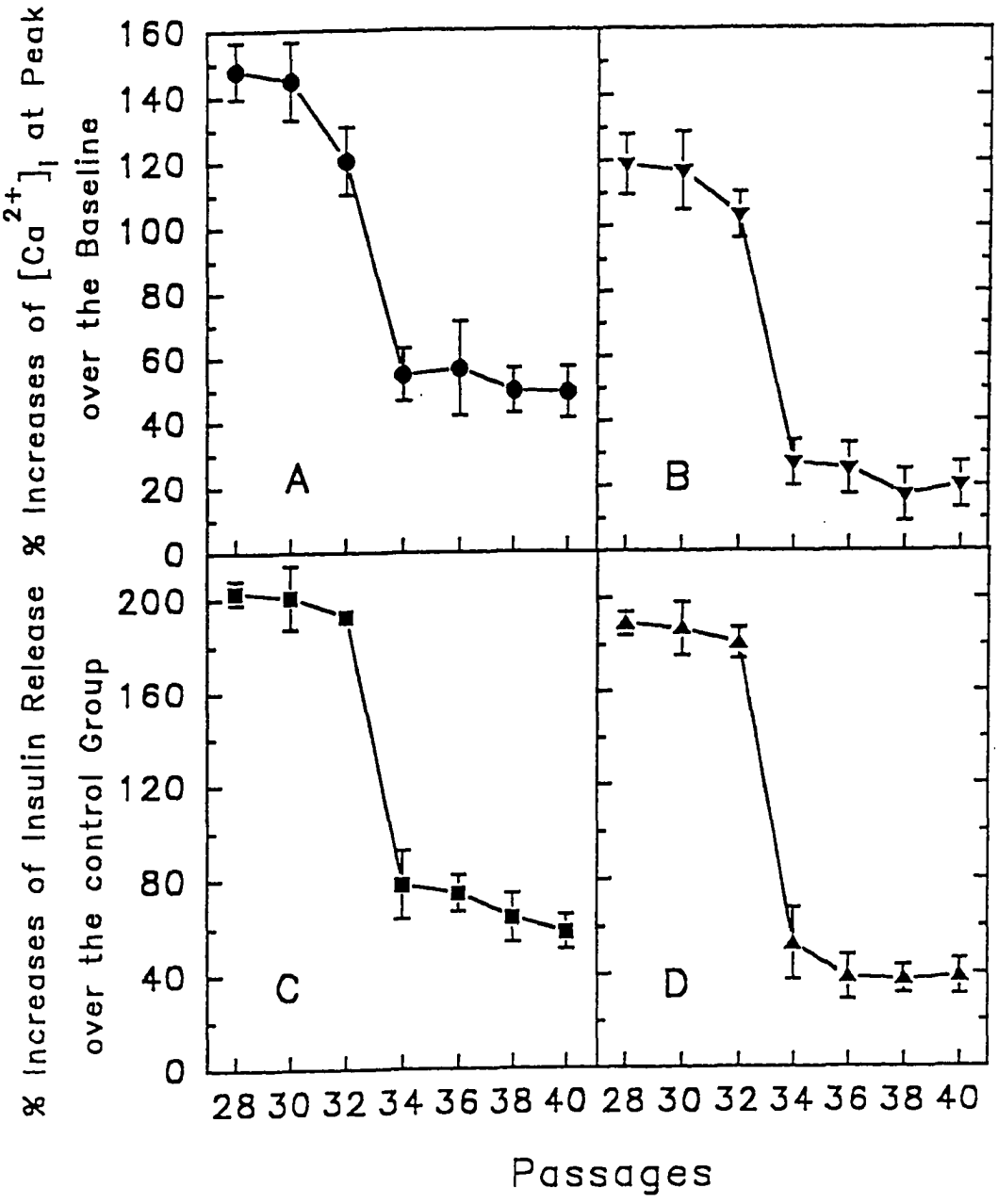
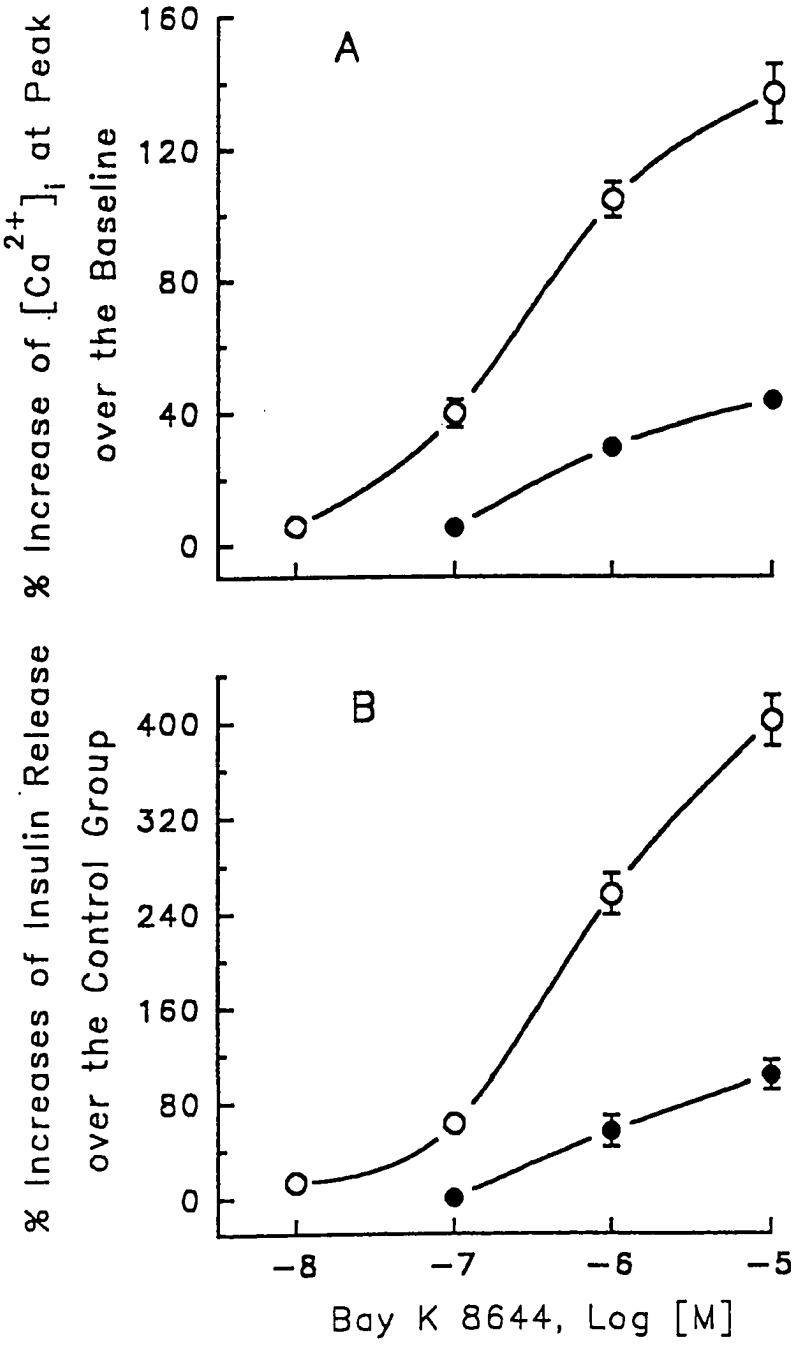

% Increases of Insulin Release over the control Group
% Increases of $[Ca^{2+}]_i$ at Peak over the Baseline
160
140
120
100
80
60
40
20
0
200
160
120
80
40
0
A
B
C
D
28 30 32 34 36 38 40
28 30 32 34 36 38 40
Passages

Fig. 3. Dose-dependent effect of Bay K 8644 on $[Ca^{2+}]_i$ (A) and insulin release (B) in differentiated (○) and undifferentiated (●) RINm5F cells. Percentage of increase of $[Ca^{2+}]_i$ was calculated from the changes between basal and peak concentration. Mean ± S.E. for $[Ca^{2+}]_i$ (n=4) and for insulin release (n=8) are shown.


A
% Increase of $[Ca^{2+}]_i$ at Peak over the Baseline
160
120
80
40
0
B
% Increases of Insulin Release over the Control Group
400
320
240
160
80
0
−8
−7
−6
−5
Bay K 8644, Log [M]

potential of -80 mV (Fig. 5) in the presence of 2 mM Ca^{2+} in the bath solution. Under control conditions, inward Ca^{2+} currents were first observed during a depolarization to -50 mV and reached maximum at membrane potentials of about 0 mV in the differentiated cells and 20 mV in the undifferentiated cells (Fig. 5). The Ca^{2+} currents were typically biphasic, consisting of a sustained and an inactivating component. The maximal amplitude was 103 ± 22 pA (n = 10) in the differentiated cells and 386 ± 90 pA (n = 12) in the undifferentiated cells. The activation of both currents was fast while inactivation developed slowly and was largely incomplete after 300 ms depolarizing pulses. Bay K 8644 (1 μM) increased the peak amplitude by 74 ± 13% (n = 6) in the differentiated cells and 22 ± 5% (n = 8) in the undifferentiated cells. The Ca^{2+} currents recovered after the washout.

Effects of DHP antagonists on the KCl- and Bay K 8644-stimulated $[Ca^{2+}]_i$ and insulin release in RINm5F cells

To further evaluate the activity of L-type Ca^{2+} channels, the inhibitory effects of two DHP antagonists, nimodipine and nifedipine, on $[Ca^{2+}]_i$ and insulin release were studied in differentiated and undifferentiated RINm5F cells. In this experiment, $[Ca^{2+}]_i$ and insulin release were equally elevated by 12 and 15 mM KCl in differentiated and undifferentiated cells, respectively, and 0.1 and 1 μM Bay K 8644 in differentiated and undifferentiated cells, respectively. Nimodipine (1 nM - 1 μM) dose-dependently inhibited KCl- and Bay K 8644-induced $[Ca^{2+}]_i$ and insulin release in both differentiated and undifferentiated cells (Fig. 6). IC_{50} of nimodipine on the KCl-induced $[Ca^{2+}]_i$ were 5 ± 1 nM (n = 4) in differentiated cells and 41 ± 4 nM (n = 4) in undifferentiated cells. IC_{50} of nimodipine on the KCl-induced insulin release were 3 ± 3 nM (n = 8) in differentiated cells and 30 ± 3 nM (n = 8) in undifferentiated cells. IC_{50} of nimodipine on the Bay K 8644 (0.1 μM)-induced $[Ca^{2+}]_i$ and insulin release in the differentiated cells were 13 ± 4 nM (n = 4) and 5 ± 2 nM (n = 8), respectively. However, IC_{50} of nimodipine on the Bay K 8644 (1 μM)-induced $[Ca^{2+}]_i$ and insulin release in undifferentiated cells was not estimated because of poor inhibitory effect of nimodipine in the cells. Therefore, by comparing IC_{50} values of nimodipine on KCl-induced $[Ca^{2+}]_i$ and insulin release, nimodipine was 8- to 10-fold more potent in differentiated cells than in undifferentiated cells.

Fig. 4. Dose-dependent effect of KCl on $[Ca^{2+}]_i$ (A) and insulin release (B) in differentiated (O) and undifferentiated (●) RINm5F cells. Percentage of increase of $[Ca^{2+}]_i$ was calculated from the changes between basal and peak concentration. Mean ± S.E. for $[Ca^{2+}]_i$ (n = 4) and for insulin release (n = 8) are shown.

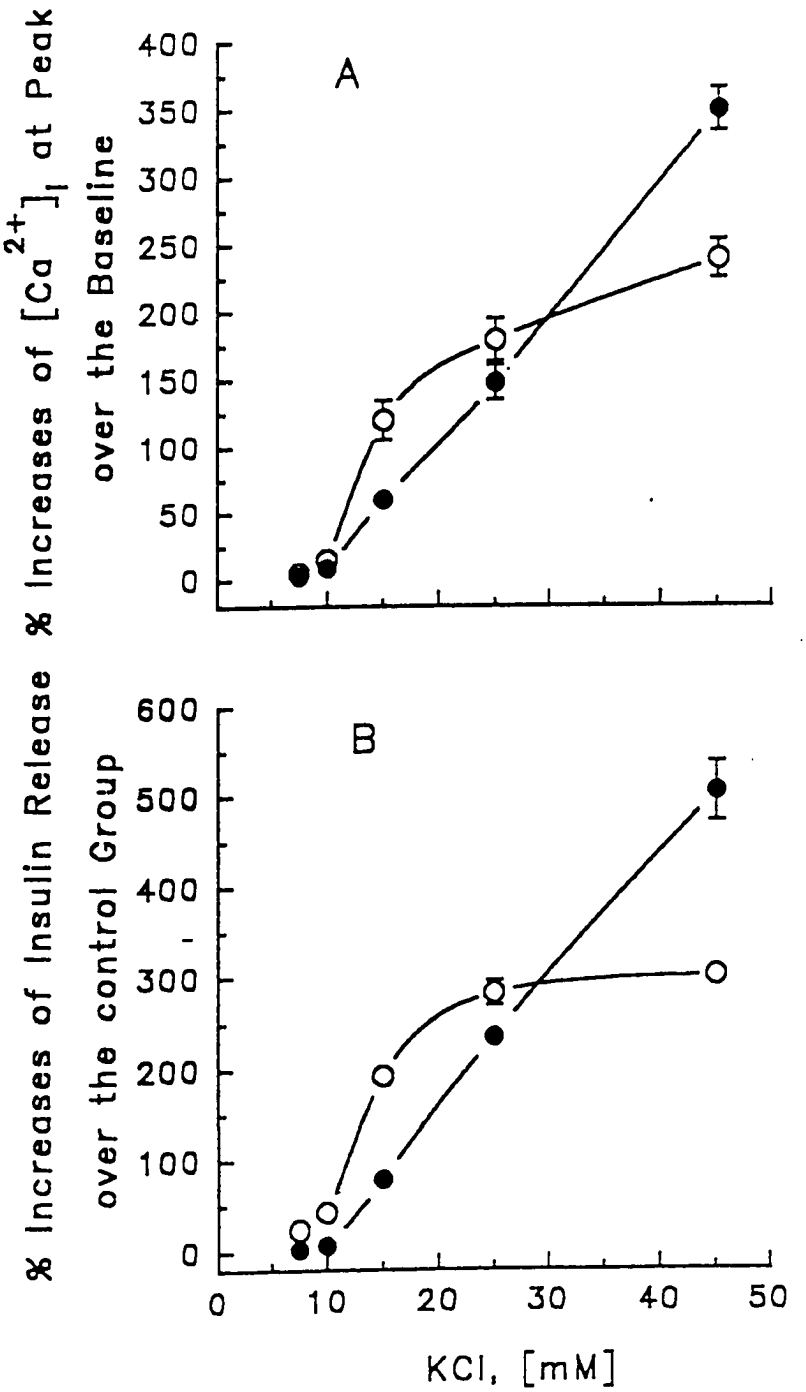
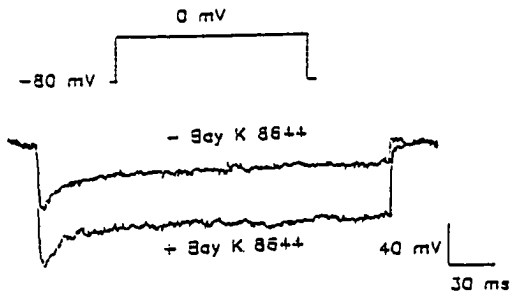

% Increases of Insulin Release over the control Group
% Increases of $[Ca^{2+}]_i$ at Peak over the Baseline
A
B
400
350
300
250
200
150
100
50
0
600
500
400
300
200
100
0
0
10
20
30
40
50
KCl, [mM]

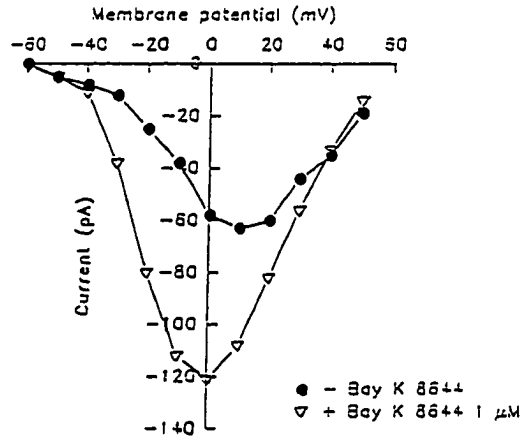
Fig. 5. Effect of Bay K 8644 (1 μM) on evoking calcium current in differentiated (A) and undifferentiated (B) RINm5F cells. (Aa) Superimposed current traces obtained in the absence (O) and presence of Bay K 8644 (●) in differentiated cells. The holding potential and test potential were -80 mV and +0 mV, respectively. (Ab) The voltage-current relationship. (Ba) Superimposed current traces obtained in the absence (O) and presence of Bay K 8644 (●) in undifferentiated cells. The holding potential and test potential were -80 mV and +20 mV, respectively. (Bb) The voltage-current relationship. (C) Average increase of Ca^{2+} currents by Bay K 8644. Mean ± S.E. for Ca^{2+} currents in differentiated cells (n=6) and in undifferentiated cells (n=8) are shown.

A

a

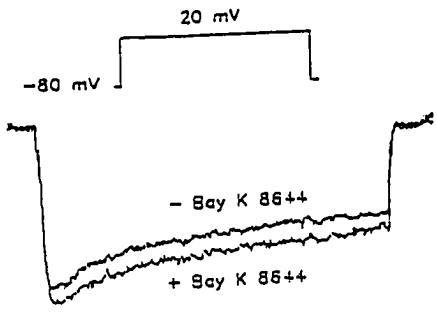

0 mV
-80 mV
- Bay K 8644
+ Bay K 8644
40 mV
30 ms

b

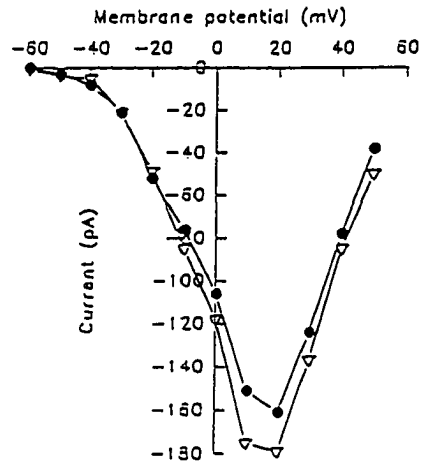

Membrane potential (mV)
-60 -40 -20 0 20 40 60
-20
-40
-60
-80
-100
-120
-140
Current (pA)
- Bay K 8644
+ Bay K 8644 1 μM

B

a


20 mV
-80 mV
- Bay K 8644
+ Bay K 8644

b


Membrane potential (mV)
-60 -40 -20 0 20 40 60
-20
-40
-60
-80
-100
-120
-140
-160
-180
Current (pA)

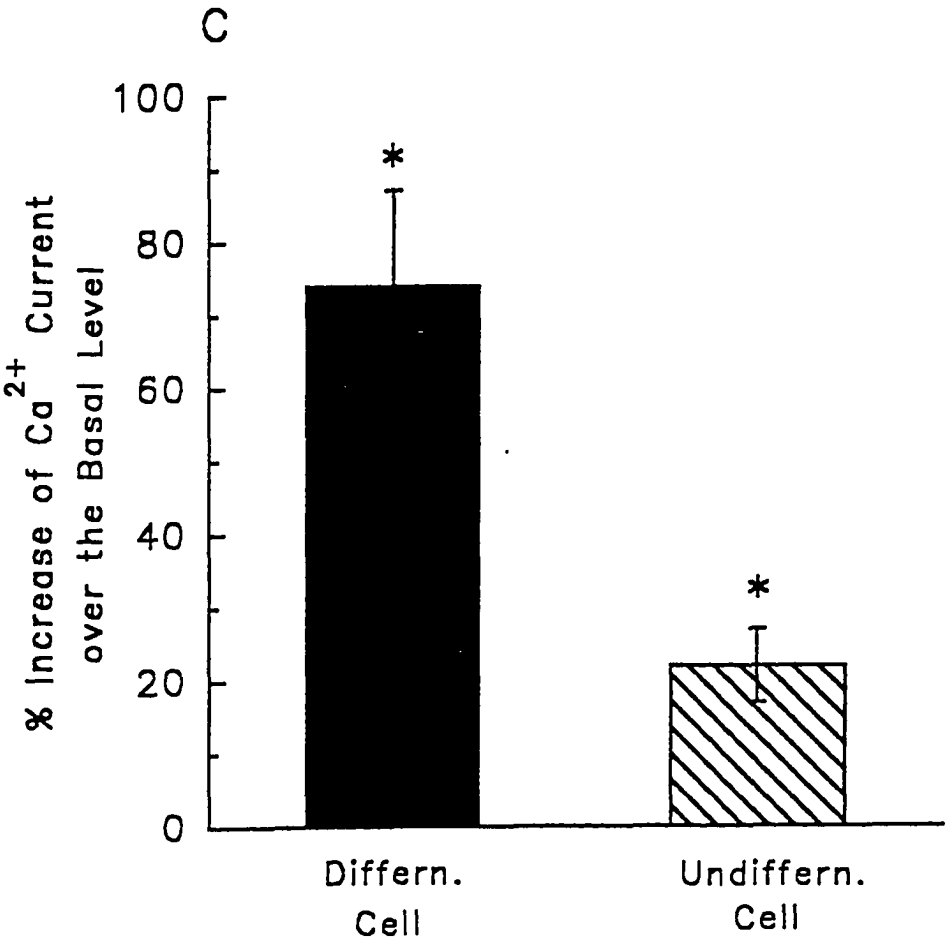
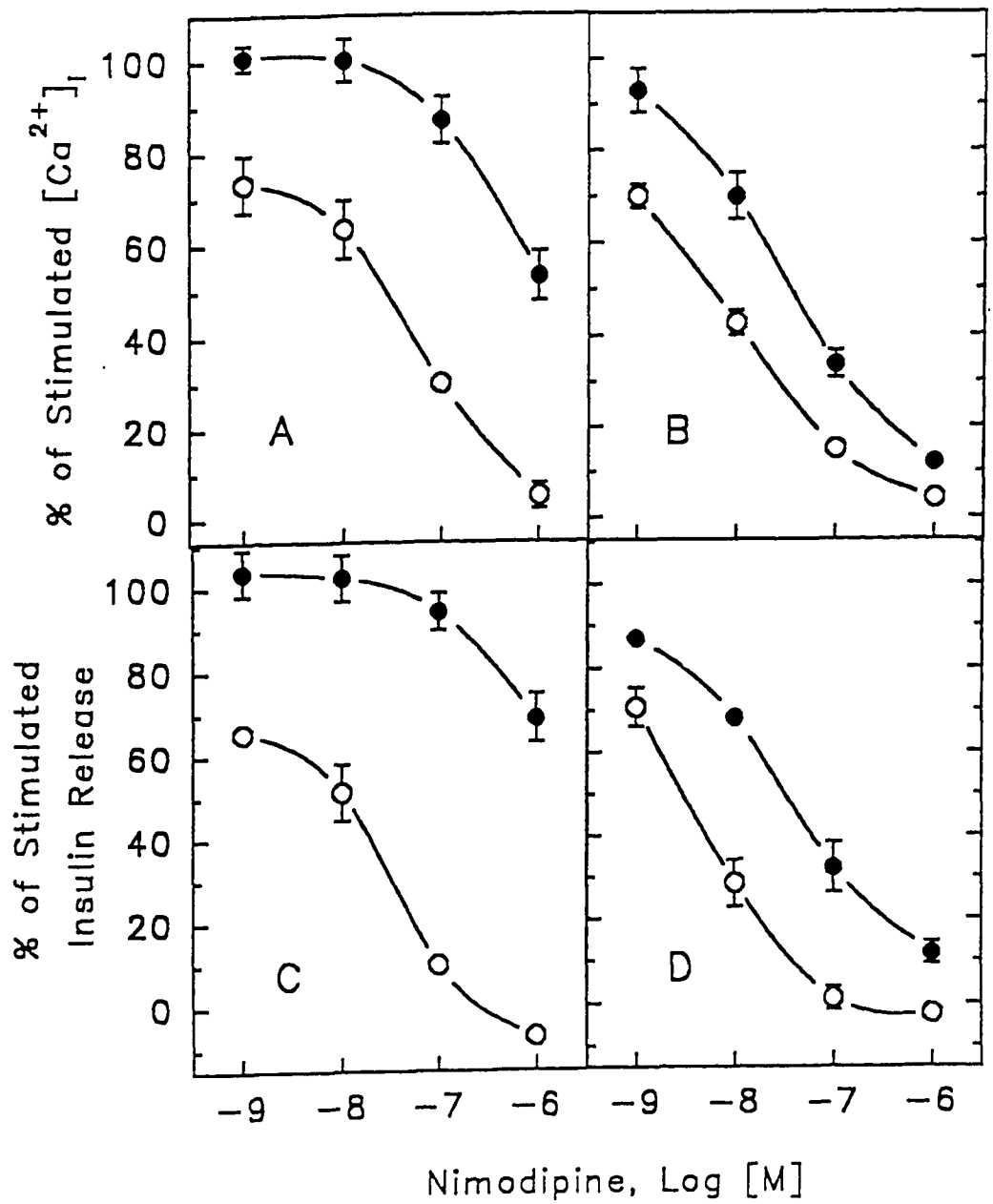

C
100
80
60
40
20
0
% Increase of Ca^{2+} Current over the Basal Level
*
*
Differn. Cell
Undiffern. Cell

Fig. 6. (A and C) Effect of nimodipine (1 nM - 1 μM) on Bay K 8644-induced $[Ca^{2+}]_i$ (A) and insulin release (C) in differentiated (O) and undifferentiated (●) RINm5F cells. (B and D) The effect of nimodipine (1 nM - 1 μM) on KCl -induced $[Ca^{2+}]_i$ (B) and insulin release (D) in differentiated (O) and undifferentiated (●) RINm5F cells. 12 and 15 mM KCl was applied to equally increase $[Ca^{2+}]_i$ and insulin release in differenatiated and undifferentiated cells, respectively. In addition, to equally increase $[Ca^{2+}]_i$ and insulin release, 0.1 and 1 μM Bay K 8644 was applied in differentiated and undifferentiated cells, respectively. Mean ± S.E. for $[Ca^{2+}]_i$ (n=4) and for insulin release (n=8) are shown.


% of Stimulated $[Ca^{2+}]_i$
% of Stimulated Insulin Release
100
80
60
40
20
0
A
B
C
D
−9
−8
−7
−6
Nimodipine, Log [M]

Effect of nimodipine on depolarization- and Bay K 8644-induced Ca^{2+} currents in RINm5F cells

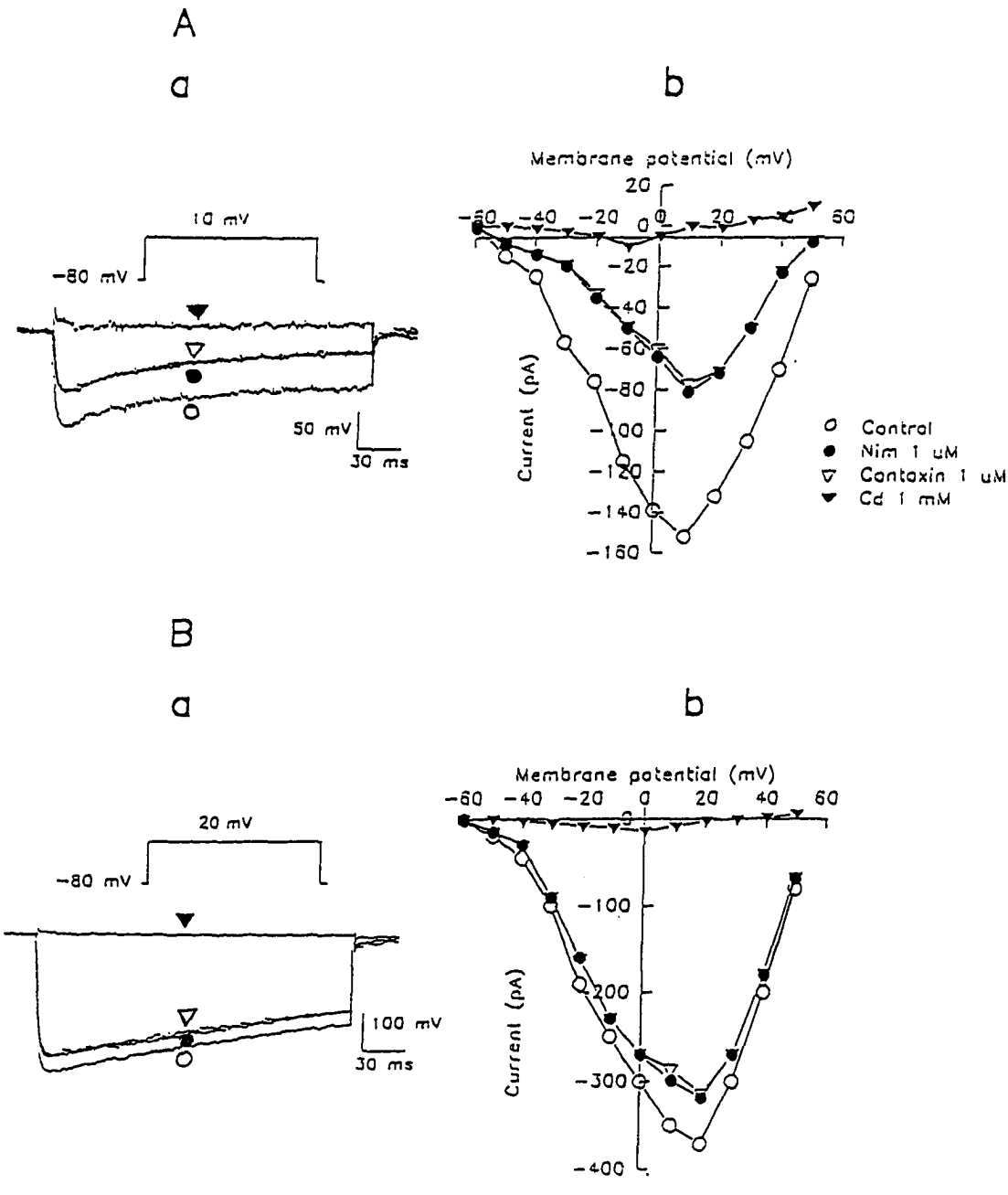
Ca^{2+} currents were activated at about -50 mV in both differentiated and undifferentiated cells reaching maximal amplitude at 0 mV in the differentiated cells and 20 mV in the undifferentiated cells. The activation of these currents was rapid while inactivation developed slowly and was largely incomplete after 300 ms of depolarizing pulses. The maximal Ca^{2+} current was 123 ± 25 pA (n=5) in the differentiated cells and 446 ± 111 pA (n=5) in the undifferentiated cells. The addition of nimodipine (1 μM) produced a reduction of maximal control Ca^{2+} currents by 47.6 ± 17% (n=5) in differentiated cells and 8 ± 3% (insignificant changes at $P<0.05$; n=5) in undifferentiated cells (Fig. 7). The Ca^{2+} currents recovered after the washout.

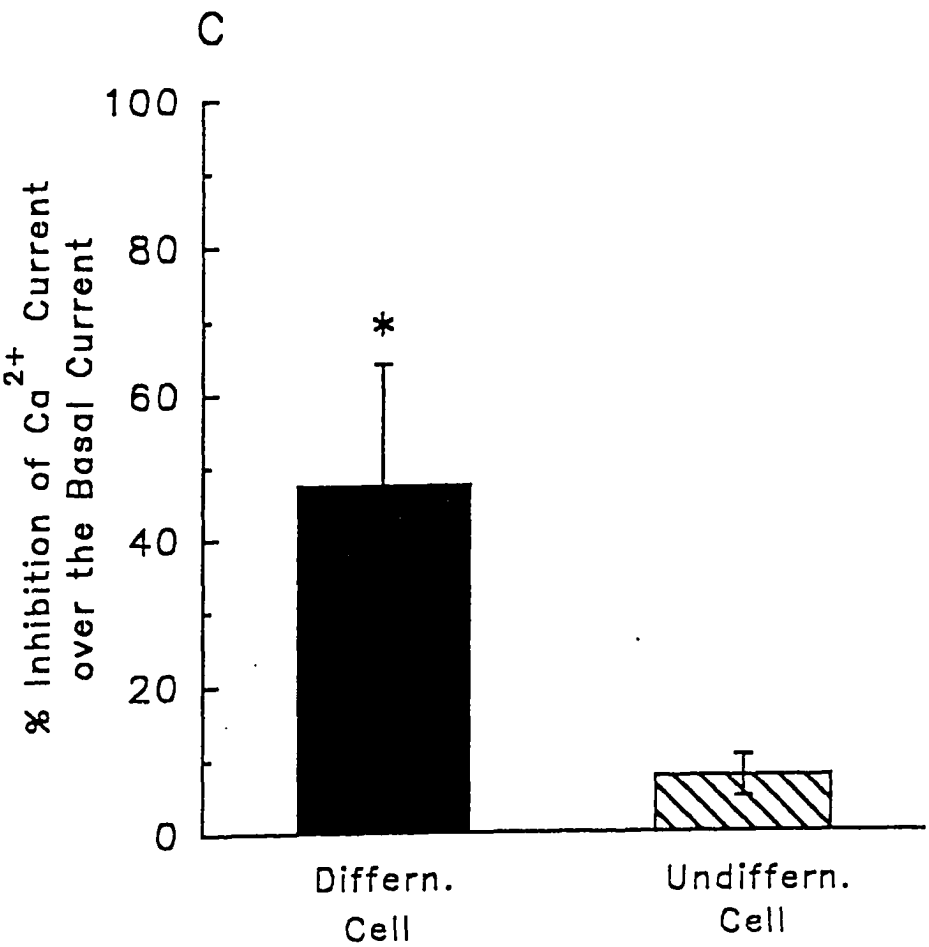
To further evaluate the relative contribution of nimodipine and Bay K 8644-sensitive Ca^{2+} channels in both differentiated and undifferentiated cells, the effect of nimodipine on Bay K 8644-activated Ca^{2+} currents was studied. The maximal amplitude was 128 ± 18 pA (n=5) in the differentiated cells and 422 ± 85 pA (n=5) in the undifferentiated cells. Bay K 8644 (1 μM)-induced Ca^{2+} currents were inhibited by nimodipine (1 μM) by 83 ± 8% (n=5) in differentiated cells, but not in undifferentiated cells (at $P<0.05$ compared with controls; n=5) (Fig. 8). Ca^{2+} currents also recovered after the washout.

Effects of ω-conotoxin GVIA and ω-agatoxin IVA on Ca^{2+} currents, $[Ca^{2+}]_i$ and insulin release in RINm5F cells

To test the possibility that other Ca^{2+} channels besides the L-type may be involved in RINm5F cells, ω-conotoxin GVIA and ω-agatoxin IVA were applied to both differentiated and undifferentiated cells. ω-Conotoxin GVIA (1 μM), an N-type channel blocker, failed to change KCl (30 mM)-induced insulin release in differentiated and undifferentiated cells, which were 103 ± 3% and 102 ± 1%, respectively (n=4). ω-Agatoxin IVA (300 nM), a P- and Q-type calcium channel blocker, did not significantly inhibit KCl (30 mM)-induced insulin release in differentiated and undifferentiated cells, which were 99 ± 4% and 90 ± 5%, respectively (n=8). ω-Agatoxin IVA (300 nM) failed to change KCl (15 and 30 mM)-induced increase in $[Ca^{2+}]_i$ in differentiated and undifferentiated cells. In addition, ω-conotoxin GVIA (1 μM) and ω-agatoxin IVA (300

Fig. 7. Effects of nimodipine (Nim, 1 μM) and ω-conotoxin (1 μM) on Ca^{2+} currents in differentiated cells (A) and undifferentiated (B) RINm5F cells. (Aa) Superimposed current traces obtained in the absence (○) and presence of nimodipine (●), ω-conotoxin GVIA (▽), and Cd^{2+} (▾) in differentiated cells. The holding potential and test potential were -80 mV and +10 mV, respectively. (Ab) The voltage-current relationship. (Ba) Superimposed current traces obtained in the absence (○) and presence of nimodipine (●), ω-conotoxin GVIA (▽), and Cd^{2+} (▾) in undifferentiated cells. The holding potential and test potential were -80 mV and +20 mV, respectively. (Bb) The voltage-current relationship. (C) Average inhibition of Ca^{2+} currents by nimodipine. Mean ± S.E. (n=5) for Ca^{2+} currents in differentiated cells and in undifferentiated cells are shown.


A
a
b
10 mV
−80 mV
50 mV
30 ms
Membrane potential (mV)
20
−60 −40 −20 0 20 60
−20
−40
−60
−80
−100
−120
−140
−160
Current (pA)
Control
Nim 1 uM
Contoxin 1 uM
Cd 1 mM
B
a
b
20 mV
−80 mV
100 mV
30 ms
Membrane potential (mV)
−60 −40 −20 0 20 40 60
−100
−200
−300
−400
Current (pA)


C
100
80
60
40
20
0
% Inhibition of Ca^{2+} Current over the Basal Current
*
Differn. Cell
Undiffern. Cell

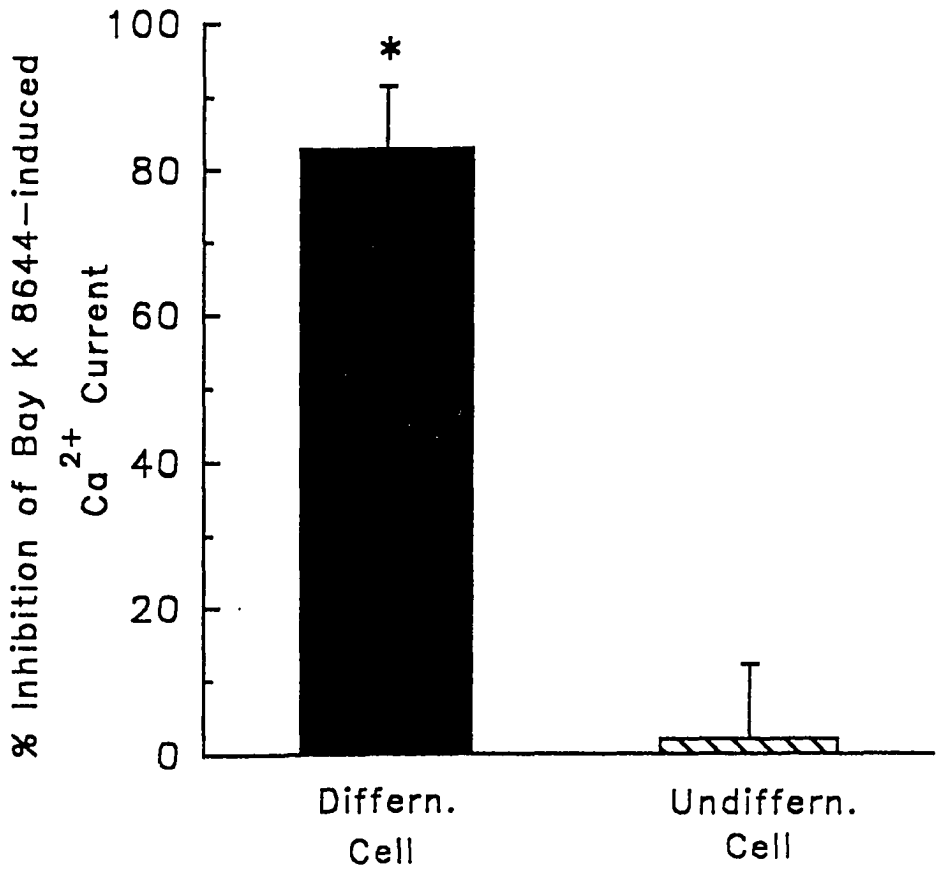


Fig. 8. Effect of nimodipine (1 μM) on Bay K 8644-induced Ca^{2+} currents in differentiated (solid bar) and undifferentiated (hatched bar) RINm5F cells. Mean ± S.E. (n = 5) are shown.

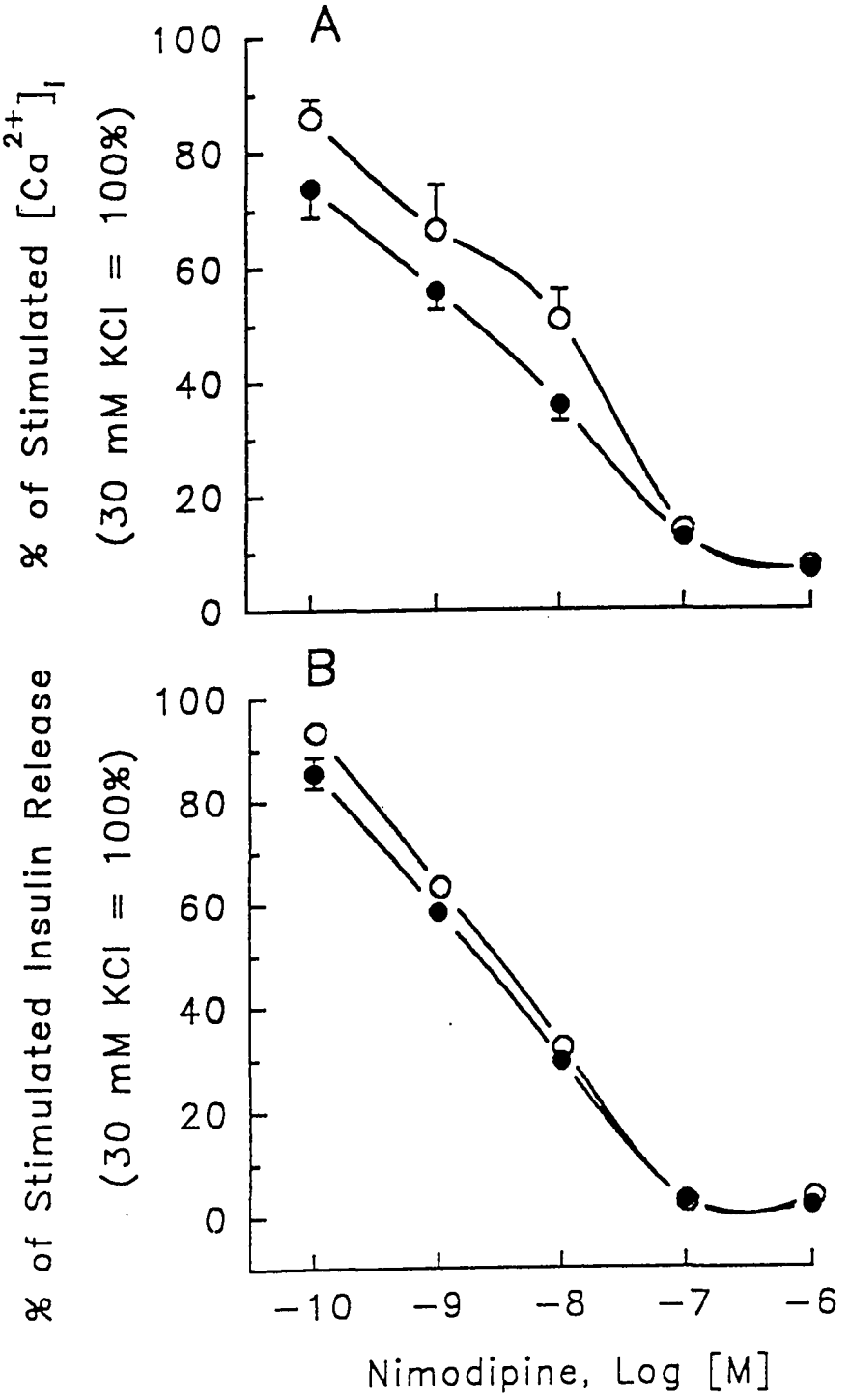
nM) failed to inhibit the control Ca^{2+} currents and KCl (15 and 30 mM)-induced increase in $[Ca^{2+}]_i$ in both differentiated cells and undifferentiated cells.

Voltage-dependence of L-type channels on Ca^{2+} currents, $[Ca^{2+}]_i$ and insulin release in RINm5F cells

To test the possibility that the affinity of nimodipine might be lowered in undifferentiated cells and it can be enhanced by prolonged depolarization (Catterall and Striessnig, 1992; Hille, 1992), 30 mM KCl was applied to equally elevate $[Ca^{2+}]_i$ and insulin release (Fig. 4) in differentiated and undifferentiated RINm5F cells. Nimodipine (0.1 nM - 1 μM) dose-dependently inhibited KCl (30 mM)-induced $[Ca^{2+}]_i$ and insulin release in both differentiated and undifferentiated cells (Fig. 9). IC_{50} of nimodipine on KCl-induced $[Ca^{2+}]_i$ were 6.0 ± 2.2 nM (n=4) in differentiated cells and 1.8 ± 0.6 nM (n=4) in undifferentiated cells. In addition, IC_{50} of nimodipine on KCl-induced insulin release was 2.5 ± 0.1 nM (n=8) in differentiated cells and 1.8 ± 0.1 nM (n=8) in undifferentiated cells. The potency of nimodipine on $[Ca^{2+}]_i$ and insulin release induced by 30 mM KCl was 17- to 22-fold higher than by 15 mM KCl in undifferentiated RINm5F cells. However, in differentiated RINm5F cells, there were no significant differences in the potency of nimodipine on $[Ca^{2+}]_i$ and insulin release induced by 12 and 30 mM KCl. Therefore, these findings suggested that the greater inhibitory effect of nimodipine resulted from a further decrease in membrane potentials in undifferentiated cells, but not in differentiated cells. The voltage-dependence of RINm5F cells may be different between differentiated and undifferentiated cells, since the change of membrane potentials produced by 15 mM to 30 mM KCl affected the potency of nimodipine in undifferentiated cells, but not in differentiated cells,

We further studied the voltage-dependence of RINm5F cells, particularly in undifferentiated cells. In the whole-cell patch-clamp recordings, the potency of nimodipine in inhibiting control Ca^{2+} currents was further compared by applying two different membrane holding potentials (-80 mV and 0 mV) in undifferentiated cells, because the resting membrane potentials of RINm5F cells were -80 mV and 50% of the Ca^{2+} channels was inactivated at membrane potentials of 0 mV. With a holding potential of -80 mV, inward Ca^{2+} currents were first observed during a depolarization

Fig. 9. Effect of nimodipine (0.1 nM - 1 μM) on KCl (30 mM)-induced $[Ca^{2+}]_i$ (A) and insulin release (B) in differentiated (O) and undifferentiated (●) RINm5F cells. 30 mM KCl was applied to equally elevate $[Ca^{2+}]_i$ and insulin release. Mean ± S.E. for $[Ca^{2+}]_i$ (n = 4) and for insulin release (n = 8) are shown.

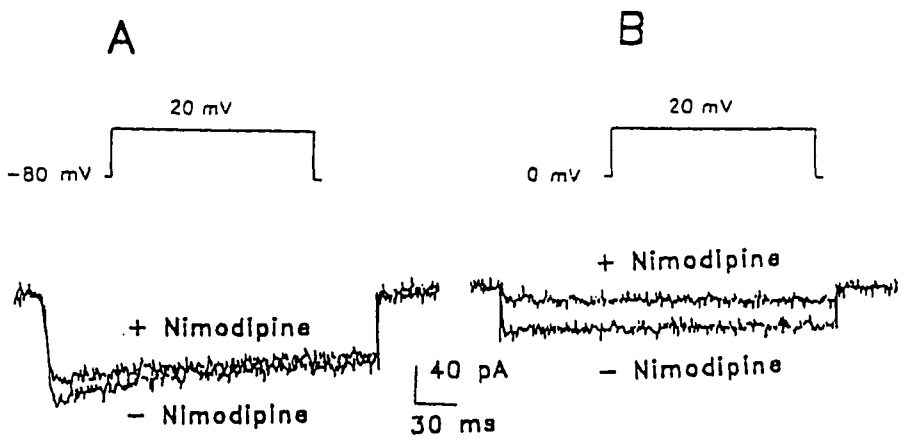

A
B
100
80
60
40
20
0
% of Stimulated $[Ca^{2+}]_i$
(30 mM KCl = 100%)
% of Stimulated Insulin Release
(30 mM KCl = 100%)
−10
−9
−8
−7
−6
Nimodipine, Log [M]

to -50 mV, reaching maximum at membrane potentials of 20 mV. With a holding potential of 0 mV, inward Ca^{2+} currents were first observed during a depolarization to +5 mV, reaching maximum at membrane potentials of 20 mV. In both holding potentials, the Ca^{2+} currents were also typically biphasic, consisting of a sustained and an inactivating component. The maximal Ca^{2+} current was approximately 121 ± 24 pA (n=5) in the holding potential of -80 mV and 39 ± 9 pA (n=4) in the holding potential of 0 mV (Fig. 10a). The activation of both currents was fast while inactivation developed slowly and was largely incomplete after 300 ms depolarizing pulses in both holding potentials. Nimodipine (1 μM) failed to inhibit control Ca^{2+} currents at the holding potentials of -80 mV (n=5), but inhibited them by 70.3 ± 7.7% (n=4) at the holding potentials of 0 mV in undifferentiated cells (Fig. 10b). The Ca^{2+} currents recovered after the washout.

Voltage dependence of activation and steady-state inactivation of Ca^{2+} currents in RINm5F cells

Analysis of voltage-dependent activation and inactivation is one approach to differentiate L-type Ca^{2+} currents. The activation (I_{step}/I_{max}) was estimated by normalizing the currents evoked by test potentials to the maximal current. The average data were fitted to the Boltzmann equation: $I_{step}/I_{max} = 1/[1 + \exp\{(V_{1/2} - V)/k\}]$ where $V_{1/2}$ is the half-activation potential and k is the slope factor, a measure of the steepness of the voltage dependence of activations. By fitting to the Boltzmann equation, $V_{1/2}$ for activation of Ca^{2+} currents was -24.3 ± 3.6 mV in differentiated cells (n=4) and -10.6 ± 2.5 mV in undifferentiated cells (n=5) (Fig. 11a). The steady-state inactivation was characterized using the two-pulse protocol consisting of a long prepulse (5 s) and a 300 ms test pulse where the peak current is produced. The average values were fitted well by the following function: $I_{step}/I_{max} = 1/[1 + \exp\{(V - V_{1/2})/k\}]$ where V is the prepulse, $V_{1/2}$ is the potential required for half inactivation of the currents, and k is the slope factor. The inactivation was measured by the ratio of I_{step}/I_{max} in which I_{max} is the maximum current amplitude evoked during the test pulse after the most hyperpolarizing prepulse and I_{step} is the currents evoked by test pulse. $V_{1/2}$ for inactivation of Ca^{2+} currents was -21.4 ± 3.9 mV in differentiated cells (n=4) and 1.8 ± 4.3 mV in undifferentiated cells (n=4) (Fig. 11b). These findings suggested that the current-

Fig. 10. Effect of nimodipine (1 μM) on control Ca^{2+} currents with holding potentials at -80 mV and 0 mV in undifferentiated RINm5F cells. Superimposed traces (A and B) of currents elicited by 300 ms depolarizing pulses (amplitude indicated on each pair from holding poteintials of -80 mV (A) and 0 mV (B) for Ca^{2+} currents). Note in B the change in holding potential altered the currents significantly. (C) Average changes of Ca^{2+} currents by nimodipine. Mean ± S.E. ((n=5) for the holding potentials at -80 mV; (n=4) for the holding potentials at -10 mV) are shown .


A
B
20 mV
-80 mV
20 mV
0 mV
+ Nimodipine
+ Nimodipine
40 pA
- Nimodipine
- Nimodipine
30 ms

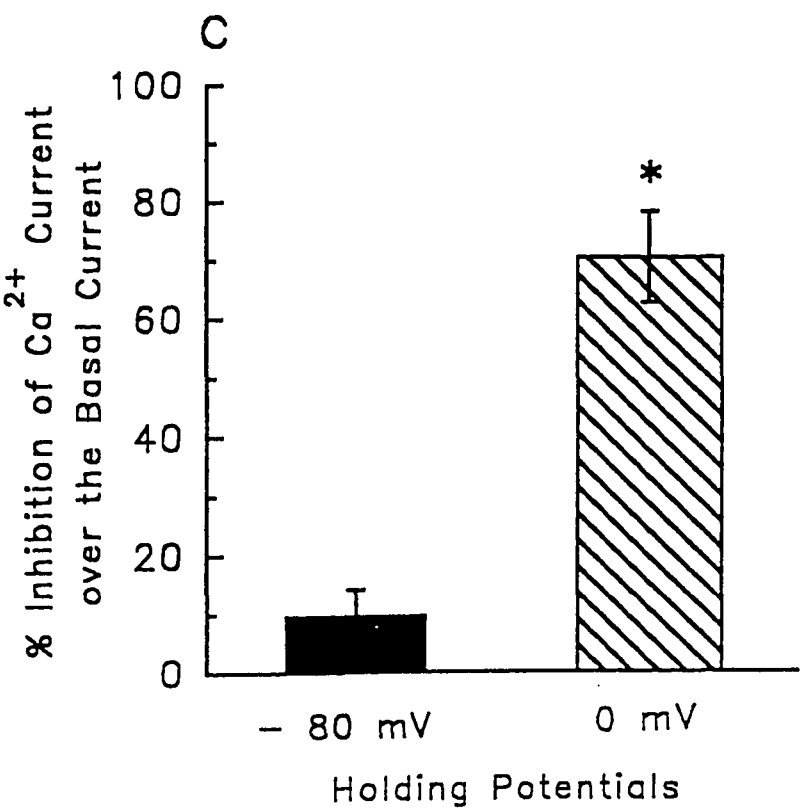
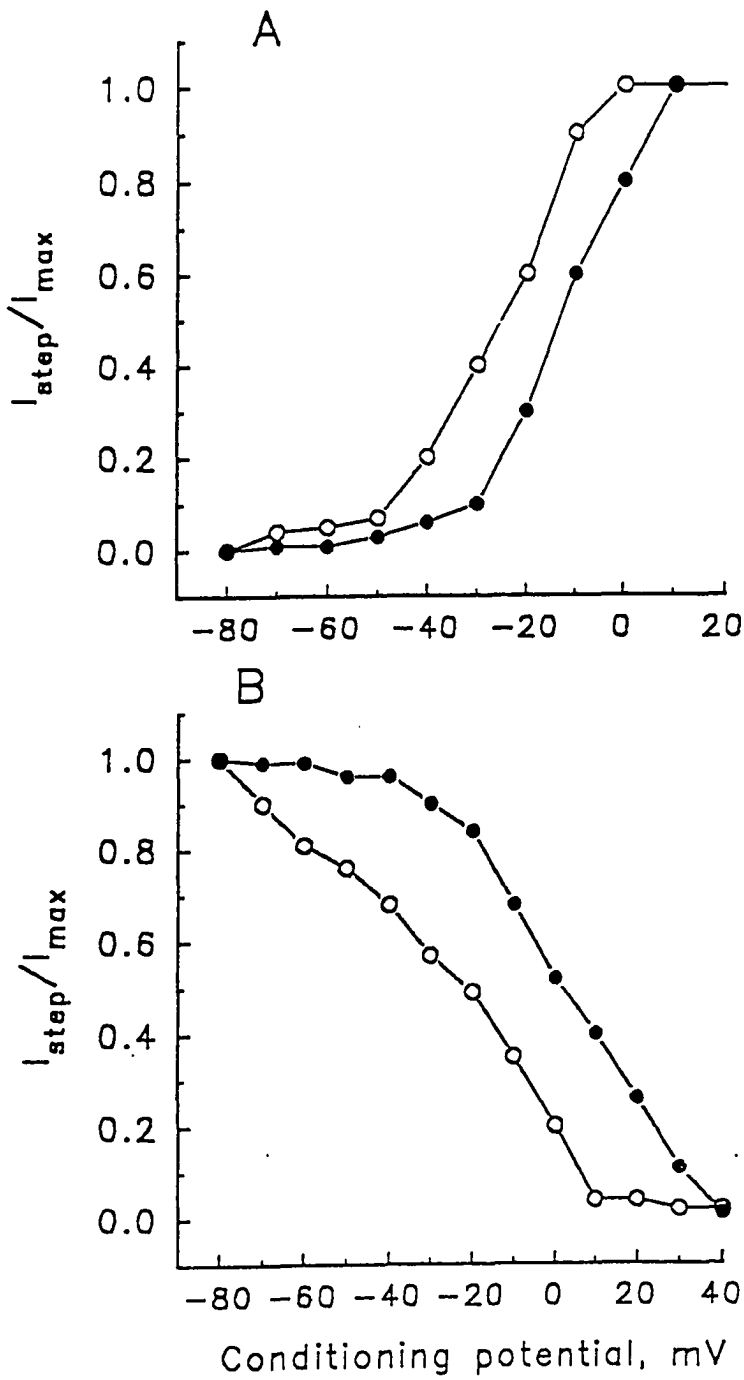

C
% Inhibition of Ca2+ Current over the Basal Current
100
80
60
40
20
0
*
- 80 mV
0 mV
Holding Potentials

Fig. 11. (A) Voltage dependence of activation of L-type Ca^{2+} currents in differentiated (○) and undifferentiated (●) RINm5F cells. The activation of Ca^{2+} currents was measured at a variety of test potentials (I_{step}) and was normalized to the maximum current (I_{max}). Data points are means from differentiated cells (n = 4) and undifferentiated cells (n = 5). (B) Steady-state inactivation of L-type Ca^{2+} currents in differentiated (○) and undifferentiated (●) RINm5F cells. Data points are means from differentiated cells (n = 4) and undifferentiated cells (n = 5).


A
B
1.0
0.8
0.6
0.4
0.2
0.0
I_{step}/I_{max}
−80 −60 −40 −20 0 20
−80 −60 −40 −20 0 20 40
Conditioning potential, mV

voltage relationship of activation and inactivation of L-type channels shifted to more negative membrane potentials in differentiated cells than in undifferentiated cells.

DISCUSSION

Although VDCCs are known to play a crucial role in stimulus-secretion coupling in pancreatic β cells, little is known about their molecular properties and pharmacology. This is of interest in light of increasing evidence of the coexpression of multiple subtypes of VDCCs in pancreatic β-cells and RINm5F cells (Aicardi et al., 1991; Pollo et al., 1993; Magnelli et al., 1995). In the present study, we performed a long-term culture of RINm5F cells which led to morphologic changes possibly linked to the concomitant changes in the VDCC physiology. Our results demonstrated that predominantly L-type Ca^{2+} channels are functionally involved in both the differentiated cells and undifferentiated cells. However, differentiated cells are functionally equipped with more L-type channels than undifferentiated RINm5F cells. In addition, voltage dependence of activation and steady-state inactivation of Ca^{2+} currents were shifted to more negative membrane potentials in undifferentiated cells than in differentiated RINm5F cells. The possible involvements of N-, P- and Q-type Ca^{2+} channels were excluded from both differentiated and undifferentiated RINm5F cells.

Differentiated cells showed greater response to depolarization- and Bay K 8644-induced increase in Ca^{2+} currents and $[Ca^{2+}]_i$ than undifferentiated cells. In addition, voltage dependence of activation and steady-state inactivation of Ca^{2+} currents in differentiated cells were shifted to more negative potentials than those in undifferentiated RINm5F cells. Since the α_1 subunit of L-type channel contains the binding sites for DHPs and is the pore-forming subunit, increases in Ca^{2+} currents and $[Ca^{2+}]_i$ are directly associated with the α_1 subunit (Singer et al., 1991). In *Xenopus* oocytes, Bay K 8644 induces Ca^{2+} currents with the expression of α_1 subunit alone, but does not with the expression of γ subunit or the coexpression of both α_2/δ and β subunits. However, Bay K 8644 enhances Ca^{2+} currents with the coexpression of α_2/δ or β subunit with α_1 subunit, and further potentiates Ca^{2+} currents with the coexpression of α_2/δ and β subunit with α_1 subunit (Singer et al., 1991). In addition, activation and inactivation kinetics of VDCCs are slow when only α_1 subunit of Ca^{2+}

channels is expressed. With the coexpression of α_1 and β subunits of L- or T-type Ca^{2+} channels, however, voltage dependence of activation and steady-state inactivation of Ca^{2+} currents are normal and are shifted to more negative membrane potentials than with the expression of α_1 subunit alone (Lacerda et al., 1991; Singer et al., 1991; Soong et al., 1993). Therefore, our findings suggested that smaller number of L-type channels or fewer α_1 and β subunits are expressed in undifferentiated cells than in differentiated cells. However, further studies are required to confirm this hypothesis.

Although Bay K 8644 and nimodipine are DHP agonist and antagonist, respectively, their effects on L-type Ca^{2+} channels were not consistent between differentiated and undifferentiated RINm5F cells. Nimodipine at the highest dose studied (1 μM) abolished Bay K 8644-induced Ca^{2+} currents, $[Ca^{2+}]_i$ and insulin release in differentiated cells but only partially inhibited those in undifferentiated cells. These findings raised the possibility that the binding sites for Bay K 8644 and nimodipine are different. In fact, it has been suggested that there are two DHP binding sites in L-type Ca^{2+} channels: one is close to the SS1-SS2 region of repeat III (Striessnig et al, 1991; Nakayama et al., 1991) and the other is close to a sequence following IVS6 segment of the α_1 subunits of L-type Ca^{2+} channels (Regulla et al., 1991). Another possibility is that the affinity of nimodipine might be lowered in undifferentiated cells. The affinity of a DHP antagonist is enhanced by prolonged depolarization (Catterall and Striessnig, 1992; Hille, 1992) and binds far more tightly to the inactivated state than to the resting state of the Ca^{2+} channel (Bean, 1984). Thus, the properties of the DHP receptor depend on the gating state of the Ca^{2+} channels (Hill, 1992). We demonstrated that nimodipine was a more potent inhibitor of Ca^{2+} currents and $[Ca^{2+}]_i$ increases and insulin release when membrane potentials were decreased or membrane holding potentials were increased. These findings suggested that the gating state of DHP-sensitive Ca^{2+} channels are different and this contributes to the different effect of nimodipine between differentiated and undifferentiated RINm5F cells.

RINm5F cells have been suggested to possess at least three HVA Ca^{2+} channels; an L-type channel that contributes to about 50% of the total current, an N-type (10-20%) and other non-L-, non-N-type channels contributing to the slowly inactivating current ($\approx$35%) (Pollo et al., 1993). In addition, HVA Ba^{2+} currents in the whole-cell patch clamp of RINm5F cells are partially blocked by both ω-agatoxin IVA and ω-

conotoxin MVIIC (Magnelli et al., 1995), in that 30 nM and 300 nM ω-agatoxin IVA block P- and Q-type channels, respectively, and micromolar concentrations of ω-conotoxin MVIIC blocks Q-type channels (Mintz et al., 1992; Wheeler et al., 1994). Therefore, the presence of "Q-like" channels, kinetically more similar to the P- and N-type channels than to the Q-type, have been suggested in RINm5F cells (Magnelli et al., 1995). However, in our findings, N-, P- and Q-type Ca^{2+} channels were not involved in either differentiated or undifferentiated RINm5F cells. Therefore, it is likely that in differentiated and undifferentiated RINm5F cells, only L-type Ca^{2+} channels are involved in the increase in Ca^{2+} currents, $[Ca^{2+}]_i$ and KCl-mediated insulin release. This is in accordance with the observations that KCl-induced $^{45}Ca^{2+}$ net uptake into RINm5F cells are completely inhibited by (+)-PN 200-110, a DHP Ca^{2+} channel blocker (Quar et al., 1988). Depolarization-induced insulin release from rat pancreatic β-cells (Komatsu et al., 1989) and RINm5F cells (Roenfeldt et al., 1992) is not attenuated by ω-conotoxin GVIA. The complete inhibition of KCl-induced insulin release by nimodipine (0.1 μM) in our study and by PN 200-110 (0.1 μM) from Roenfeldt et al. (1992) suggests that in RINm5F cells, irrespective of the presence of other types of VDCCs or hormone regulated Ca^{2+} channels, only the L-type channel is involved in the mechanism of depolarization-induced insulin release (Roenfeldt et al., 1992).

The increased activity of L-type Ca^{2+} channels in differentiated RINm5F cells may be directly associated with differentiation of the normal pancreatic β-cell. Decreased activity of L-type Ca^{2+} channels may occur when the cells undergo undifferentiation during oncogenesis. In fact, RINm5F cells resemble the incompletely differentiated fetal β-cells in that they contain and secrete minimal amounts of insulin and respond only weakly to glucose. They also secrete small amount of glucagon and somatostatin (Gazdar et al., 1980; Halban et al., 1983). The morphologically undifferentiated RINm5F cells may represent more poorly differentiated fetal β-cells than the differentiated cells. In this aspect, the comparison between differentiated and undifferentiated RINm5F cells may provide information on the embryonic development of VDCCs in pancreatic β-cells. In rat skeletal muscle, the levels of α_1 subunits of L-type VDCCs are quite low during the first 10 days after birth, then rise dramatically and, by day 20, approach those found in adult muscle (Morton and Froehner, 1989). VDCC α_1 mRNA is not detectable in the myoblast form of C2C12 cells while its

expression is induced in differentiated myotubules (Varadi et al., 1989). It has been suggested that the VDCC α_1 subunit is also expressed in pancreatic β-cells and RINm5F cells (Seino et al., 1992). In addition, the B_{max} value for [^{3}H]-nitrendipine, a DHP antagonist, increases with age between fetal and neonatal ages in rat ventricular muscles (Kojima et al., 1990). Altogether, poorly undifferentiated fetal pancreatic β-cells may have a lower activity of L-type channels than differentiated β-cells, but by progressive cell differentiation, pancreatic β-cells might have a higher activity of L-type channels than undifferentiated β-cells.

In neuroblastoma x glioma hybrid NG 108-15 cells and neuroblastoma x Chinese hamster brain NCB-20 cells, the differentiated cells show a marked response to depolarization- and Bay K 8644-induced increases in Ca^{2+} currents and $[Ca^{2+}]_i$ but the undifferentiated cells do not (Mienville, 1992; Jin et al., 1994). Sodium butyrate (NaB), an inducer of cell differentiation, decreases cell growth and increases insulin release and the expression of ganglioside which represents β-cell differentiation (Bartholomeusz et al., 1990). In addition, NaB decreases the cellular content of polyamine (Sjoholm, 1993) which also can be depleted by the ornithine decarboxylase inhibitor, difluoromethylornithine (DFMO), in RINm5F cells (Sjoholm et al., 1993). In DMFO-treated RINm5F cells, $[Ca^{2+}]_i$ and insulin release are markedly enhanced after KCl (25 mM)-induced depolarization. Furthermore, the behavior of the stimulus-secretion coupling of polyamine-depleted RINm5F cells changes towards that of native β-cells (Sjoholm et al., 1993). Nerve growth factor (NGF) is a good candidate for comparing morphologic changes to the changes in the VDCC physiology because NGF has been reported to induce differentiation in RINm5F cells (Polak et al., 1993). However, we could not induce differentiation from the undifferentiated state by using NGF in RINm5F cells. The relationship between morphology and VDCC physiology in RINm5F cells should be further studied.

In conclusion, our present findings suggested that for increases in $[Ca^{2+}]_i$ and insulin release, the activity of L-type Ca^{2+} channels is predominant in both differentiated cells and undifferentiated cells and there is no involvement of N-, P- or Q-type Ca^{2+} channels. However, differentiated cells are functionally equipped with more L-type channels than undifferentiated RINm5F cells. In addition, voltage dependence of activation and steady-state inactivation of Ca^{2+} currents in differentiated cells were

shifted to more negative membrane potentials than in undifferentiated cells than in differentiated RINm5F cells. Therefore, we suggest that differentiated RINm5F cells have higher density of L-type channels and/or more functional α_1 and β subunits than undifferentiated cells. The density of L-type channels which mediate the effects of L-type channel agonist and antagonist in RINm5F cells should be further evaluated by a radioligand binding studies.

The physiological implication of our present findings remains to be established. However, as discussed above, we speculate that both cell migration and differentiation are influenced by changes in $[Ca^{2+}]_i$ through L-type channels and, as a consequence, the modulation of $[Ca^{2+}]_i$ is important in β-cell differentiation (Moran, 1991). Neurite outgrowth and cell adhesive function are dependent upon Ca^{2+} (Lankford and Letourneau, 1989) and maintenance of the differentiated state also requires regulation at the L-type Ca^{2+} channels (Moran, 1991). It is conceivable that, as the cells migrate and differentiate, they encounter stimuli that may promote the clustering of L-type channels. In this context, VDCCs are believed to provide more Ca^{2+} to the specific cell region undergoing the cytomorphological change (i.e. the growth of cellular processes) (Silver et al., 1990).

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CHAPTER VII GENERAL DISCUSSION

Detailed discussion of the results obtained in this dissertation may be found in the Discussion section of each chapter. This chapter will outline the major conclusions derived from the presented data and discuss possible physiological implications and mechanisms underlying our experimental findings.

A novel insulin-like immunoreactivity in RINm5F cells

Based on the specificity of antiserum vs. bovine insulin, we suggest that undifferentiated RINm5F cells have a novel insulin-like peptide (NIP), which is most likely insulin. Changes in the antigenic determinant of the insulin secreted by undifferentiated RINm5F cells may contribute to the specificity of antiserum vs. bovine insulin producing a novel insulin-like immunoreactivity. The amino acid sequence of NIP may be very similar to porcine and bovine insulins, but not to rat insulin, especially at residues A8 and A10 which may act as antigenic determinants in insulin (Sasaki et al., 1988; Bullesbach and Schwabe, 1994). However, we speculate that amino acids A8 and A10 may not significantly contribute to the antigenic determinant between bovine and porcine insulins. Although these insulins only exhibit differences at the A8 and A10 residues, antisera vs. bovine and porcine insulin cross-react to recognize either insulin. The possible antigenic determinants of rat insulin are amino acids at A4, B3, B9, B29 and B30 based on these residues being the only differences between rat and bovine insulin. Rat insulin differs from bovine and porcine insulin at A4 (Glu → Asp), B3 (Asn → Lys), B9 (Ser → Pro), B29 (Lys → Met) and B30 (Thr → Ser). However, the changed amino acid residues of NIP are most likely at A8 and A10. The significance of the changes at A4, B3, B9, B29 and B30 can be ruled out, because antiserum vs. porcine insulin recognizes rat insulin even though amino acid residues of porcine insulin are different from rat insulin at these residues. Therefore, we speculate that the amino acid sequence of NIP should be similar to that of bovine insulin and major changes occur at A8 and A10. Antibody specificities between antisera vs. porcine and bovine insulins may be attributable to these two residues; and may also

contribute to the specificity of antiserum vs. bovine insulin to rat insulin. Furthermore, it is possible that antibody vs. bovine insulin is heteroclitic. The antibody vs. bovine insulin with low affinity to rat insulin fits loosely to the epitope of rat insulin I and II. This increases the possibility of its being heteroclitic, and permits greater binding to cross-reacting epitopes of NIP (Mirza and Wilkin, 1989).

In addition, the changed affinity of insulin to antibody vs. bovine insulin may contribute to the novel insulin-like immunoreactivity. Since the gaps and holes that exist between the antibody and the antigen may be filled by water molecules, hydrogen bonding and other charge-charge interactions may strengthen the binding (Amit et al., 1986).

To further characterize the insulin-like immunoreactivity, it is necessary to examine the relationship between morphological changes and the novel insulin-like immunoreactivity. It has been shown that a differentiating inducer, sodium butyrate, induces RINm5F cells to synthesize more insulin without demonstrating changes of morphology. However, sodium butyrate induces morphological changes in another system (Wright, 1973). Nerve growth factor (NGF), another differentiating inducer, changes morphology from the undifferentiated to differentiated state in RINm5F cells (Polak et al., 1993). Therefore, the relationship between the morphology and a novel insulin-like immunoreactivity requires further study, possibly using differentiating inducers such as sodium butyrate or NGF. To study the relationship between the morphology and function, we have employed NGF in serum-free condition to induce morphological changes from the undifferentiated state to the differentiated state. However, NGF did not significantly change the morphology from the undifferentiated state to differentiated state (unpublished observations). Therefore, other agent(s) except NGF should be used to test the relationship between the morphology and the physiology.

It would be important to consider the long-term effect of a culture system *in vitro* without supplying physiological factors which might contribute to the unusual immunoreactivity of insulin. *In vivo* transplantation of cells enhances insulin secretion and responses to secretagogues, but improved responses are lost during subsequent subculture (Flatt et al., 1988). This suggests that long-term maintenance of the cells *in vitro* causes changes in morphology and induces a novel insulin-like immunoreactivity.

Transplantation of cells to rats may demonstrate the effect of long-term culture.

Physiological implications of NIP remains to be determined. However, RINm5F cells resemble incompletely differentiated fetal β-cells (Gazdar et al., 1980; Halban et al., 1983). When the cells morphologically change from the differentiate state to the undifferentiated state during oncogenesis, the undifferentiated cells may represent more poorly differentiated fetal β-cells than the differentiated cells. NIP may provide information concerning the development of insulin gene expression. We speculate that during differentiation of fetal pancreatic β-cells, the genes of insulin I and II are differentially expressed. The gene of insulin II is considered to be the ancestral gene in normal pancreatic β-cells (Bell et al., 1980), however, the third insulin gene may serve as an ancestral gene.

In summary, undifferentiated RINm5F cells produce NIP which is neither rat insulin I nor II and is immunoreactive to antisera vs. rat, porcine and bovine insulin.. We speculate that NIP may be developed and utilized as an universal insulin which does not produce a significant immune reaction when it is injected into either humans or animals.

Adrenergic receptors and their signal transduction mechanisms in RINm5F cells

The functional distribution of ARs was distinct between differentiated and undifferentiated RINm5F cells. Functional studies are based on the receptor occupation theory which states that the occupation of a receptor by a drug leads to a stimulus and a subsequent response (Kenakin, 1984). Insulin release and cAMP production were used to test the potency of epinephrine on α- and β-ARs and to distinguish the subtypes of ARs. The lack of agonistic activity of epinephrine through α_1 and β-ARs may be a result of the low concentration of these ARs in differentiated RINm5F cells. In addition, the lack of epinephrine's action through α_1- and α_2-ARs may also be a result of low density of these ARs in undifferentiated RINm5F cells. Our speculation on the density of ARs which mediate the dual inhibitory and stimulatory effects of epinephrine in RINm5F cells should be further tested by using a radioligand binding study.

Differences in the relative potency of epinephrine and isoproterenol may result from differences in their relative affinity for receptors and /or their relative efficacy. The potency of the agonists may be affected by a number of factors such as the number of receptors and the second messenger system. Therefore, we speculate that signal transduction after plasma membrane receptor binding may play a significant role in the effect of epinephrine on insulin release. Heterotrimeric G-proteins play an essential role in linking cell-surface receptors to effector proteins at the plasma membrane, and in transferring the external signals to internal signals. G_i- or G_s-proteins, which are coupled to the ARs, may contribute to epinephrine's action. It is possible that higher quantities of G_i-proteins may contribute to the significant functional activities of α_2-ARs in differentiated cells, and higher quantities of G_s-proteins in the mediation of β-ARs in undifferentiated RINm5F cells. In fact, greater quantities of the 45 KDa subunit of $G_{\alpha s}$-protein are responsible for increased activities of β-ARs in HIT-T15 cells (Zhang et al., 1989; Walseth et al., 1989). However, our findings demonstrated that the activity of G_s-proteins is not significantly different between differentiated and undifferentiated cells. Therefore, we speculate that the quantity of G_s-proteins is not different between these two types of RINm5F cells.

Our results suggested that the activity of adenylyl cyclase is higher in undifferentiated cells than in differentiated cells, although the activity of G_s-proteins is not different between these cells. Therefore, we speculate that higher association of G_s-proteins with adenylyl cyclase in undifferentiated cells than in differentiated cells may contribute to the increased activities of adenylyl cyclase and agonistic effect of epinephrine. These differences may be responsible for the elevated potency of epinephrine and isoproterenol through β-ARs and elevated potency of forskolin in undifferentiated cells when compared to differentiated cells.

Intracellular cAMP concentration is determined not only by the rate of cAMP production by adenylyl cyclase, but also the rate of hydrolysis by PDE. Activity of PDE remained unchanged between differentiated cells and undifferentiated cells because increase in intracellular cAMP by IBMX was not significantly different between these cells. In addition, the increased activity of protein kinase A may be responsible for the increased activity of β-ARs in undifferentiated cells, although cAMP can act through mechanisms other than activation of protein kinase A (Weber et al., 1987; Ludwig et

al., 1990; Jiang et al., 1992). However, in our study, the total (activated and inactivated) activity of protein kinase A was not different between differentiated and undifferentiated RINm5F cells. These findings suggested that mechanisms mediated by cAMP other than activation of protein kinase A may be more important in β-AR-mediated insulin release in undifferentiated cells. The R subunit of protein kinase A containing bound cAMP can function independently to interact with the C subunit of protein kinase A. An unidentified cAMP-binding protein may mediate cAMP effects without interacting with protein kinase A (Weber et al., 1987). Furthermore, cAMP may act by binding to cGMP-dependent protein kinase or ion channels (Ludwig et al., 1990; Jiang et al., 1992; Holz, 4th. et al., 1995).

These findings may have physiological implications with differentiation of fetal pancreatic β-cells because RINm5F cells represent the early stage of embryonic β-cells (Gazdar et al., 1980; Halban et al., 1983). During oncogenesis, the morphologically undifferentiated RINm5F cells might represent more poorly differentiated fetal β-cells than the differentiated cells. We speculate that β-ARs are predominant in the early development of pancreatic β-cells, decline with further development as α_2-ARs become the dominant AR's type. In fact, the β-AR overexpression in the fetal rat is a widespread phenomenon and receptor subtype selectivities (predominantly β_2 in the whole fetus) are already in place (Slotkin et al., 1994). Fetal tissues can be exposed to catecholamines released by the fetal adrenal medulla and a variety of different cell types which synthesize catecholamines (Jonakait et al., 1979; Jones et al., 1988). Therefore, we speculate that β_2-ARs may elicit a program of growth and differentiation, a response to *in utero* stress and the adaptation of pancreatic β-cells to birth as in other cells and cell lines (Slotkin et al., 1987; Hen et al., 1989; Schuller and Cole, 1989; Schallreuter et al., 1993).

The β-AR/adenylyl cyclase system has been characterized in the study of embryonic development (Maier et al., 1989). Activities of adenylyl cyclase and interaction between adenylyl cyclase and G-proteins are greater in the fetus and neonate than in the adult (Katz et al., 1985; Tobise et al., 1994). We speculate that changes in the cytoskeletal system and membrane fluidity between differentiated cells and undifferentiated cells may contribute to the coupling of G-proteins to adenylyl cyclase, in which G-proteins are more closely associated with adenylyl cyclase in

undifferentiated cells than in differentiated cells. Therefore, the cellular processes in developing pancreatic β-cells may function to increase the sensitivity or target cell selectivity to catecholamines leading to increases or decreases in insulin release depending on whether β- or α-ARs are activated (Polak et al., 1993). However, we do not rule out the possibility that different isoforms of adenylyl cyclase are correlated with changes of activity of the enzyme.

In summary, we suggest that the higher density of α_2-ARs and lower activities of adenylyl cyclase may contribute to the higher functional activity of α_2-ARs and lower activity of β-ARs in differentiated cells than in undifferentiated cells. In addition, the higher concentration of β-ARs, higher association of G_s-proteins with adenylyl cyclase and higher activities of adenylyl cyclase may contribute to the higher functional activity of β-ARs and lower activity of α_2-ARs in undifferentiated cells than in differentiated cells.

Voltage-dependent Ca^{2+} channels in RINm5F cells

Based on pharmacological properties, we suggest that both differentiated and undifferentiated RINm5F cells have predominantly L-type Ca^{2+} channels, which have been identified in pancreatic β-cells and insulin-secreting cell lines including RINm5F. The L-type Ca^{2+} currents in RINm5F cells in the whole-cell patch-clamp recordings are activated at relatively high membrane potential. Activation of Ca^{2+} currents are fast with long duration and slow inactivation. In addition to being voltage-dependent, the L-type currents are regulated by Ca^{2+}. An increase in $[Ca^{2+}]_i$ induced by opening of the channel down-regulates the activity of the channel. The down-regulation may be due to the decrease in the electrochemical driving force for Ca^{2+} influx. In addition, the binding of Ca^{2+} with the inside of the channel (S6 of α_1 subunit) or with extracellular and cytoplasmic domains may contribute to the Ca^{2+}-induced down-regulation of the channel (Zhang et al., 1994). Ca^{2+}-activated K^+ channels and non-selective cation channels are also activated by an increase in $[Ca^{2+}]_i$ and result in the repolarization of membrane potentials which close the L-type channels (Cole and Sanders, 1989).

RINm5F cells have a large conductance, long-lasting opening and high density of L-type Ca^{2+} channels, which are responsible for the upstroke of the action potential and Ca^{2+} influx with membrane depolarization. In this study, we found no involvement of N-, P- and Q-type channels in either differentiated or undifferentiated cells, although the presence of these channels has been suggested in differentiated RINm5F cells (Magnelli et al., 1995). In addition, we were not able to identify T-type channels in RINm5F cells, although the T-type channels are present in rat pancreatic β-cells. T-type currents may play an important physiological role in the near resting membrane potential to regulate excitability, as a pacemaker, due to their low threshold. Therefore, it is possible that T-type channels may be demonstrated when more RINm5F cells are studied using whole-cell patch-clamp recordings. To test this possibility, the percentage of cells containing T-type channels can be studied in differentiated and undifferentiated cells by using the properties of voltage-dependency and kinetics of T-type channels. However, T-type channel blockers can not be used to characterize the channels due to the lack of specific T-type channel blockers.

The functional distributions of L-type channels were distinct between differentiated and undifferentiated RINm5F cells. The low agonistic activity of Bay K 8644 in undifferentiated cells may be a result of the low density of L-type channels. Our speculation on the density of L-type channels which mediate the effects of L-type channel agonist and antagonist in RINm5F cells should be further tested by radioligand binding studies. In addition, differences in relative potency of nimodipine may result from differences in relative affinity for receptors. The affinity of DHP antagonist is enhanced by prolonged depolarization (Catterall and Striessnig, 1992) and binds far more tightly to the inactivated state than to the resting state of the Ca^{2+} channels (Bean, 1984). Thus, the properties of the DHP receptor depend on the gating state of the Ca^{2+} channels. In our findings, when membrane holding potentials were increased, nimodipine was more potent to inhibit Ca^{2+} currents and increase $[Ca^{2+}]_i$ and insulin release. These findings suggest that the gating state of L-type channels is different between differentiated and undifferentiated RINm5F cells. In addition, the non-inactivating component of the Ca^{2+}-independent K currents are inhibited by DHP antagonists, nicardipine and nimodipine, but not by DHP agonist, Bay K 8644 (Valmier et al., 1991; Hume, 1985). This observation suggests that in RINm5F cells, nimodipine

may inhibit Ca^{2+}-independent K currents, which depolarize membrane potentials and, if so, contribute to the inhibitory effect of nimodipine on Bay K 8644-induced $[Ca^{2+}]_i$ and insulin release. However, this hypothesis remains to be tested.

Undifferentiated RINm5F cells can be utilized as a model for the binding site of DHP agonists and antagonists. This model is based on the findings that nimodipine totally abolishes Bay K 8644-induced Ca^{2+} currents, increases $[Ca^{2+}]_i$ and insulin release in differentiated cells, but not in undifferentiated cells. However, we do not rule out the possibility that the affinity of nimodipine is lower in undifferentiated cells than in differentiated cells. Two distinct binding sites for DHPs may exist; one for agonists such as Bay K 8644 and another for antagonists such as nimodipine. The DHP binding site in L-type Ca^{2+} channels is localized close to the SS1-SS2 region of repeat III (Striessnig et al, 1991; Nakayama et al., 1991) and to a sequence following the IVS6 segment of the α_1 subunits of L-type Ca^{2+} channels (Regulla et al., 1991). The region responsible for the binding of either the agonist or antagonist has not been determined.

The higher activity of L-type Ca^{2+} channels in differentiated RINm5F cells may be directly associated with differentiation of normal pancreatic β-cells, because RINm5F cells resemble incompletely differentiated fetal β-cells (Gazdar et al., 1980; Halban et al., 1983). Therefore, the morphologically undifferentiated RINm5F cells might represent more poorly differentiated fetal β-cells than the differentiated cells. In rat skeletal muscle, the levels of α_1 subunits of L-type VDCCs are quite low during the first 10 days after birth, then rise dramatically and, by day 20, approach those found in adult muscle (Morton and Froehner, 1989). VDCC α_1 mRNA is not detectable in the myoblast form of C2C12 cells while its expression is induced in differentiated myotubules (Varadi et al., 1989). In addition, the B_{max} value for [^{3}H]-nitrendipine, a DHP antagonist, increases with age between fetal and neonatal ages in rat ventricular muscles (Kojima et al., 1990). Therefore, we speculate that the differences in changes of $[Ca^{2+}]_i$ mediated through L-type channels may be attributed to the levels of α_1 subunits of L-type VDCCs between the differentiated and undifferentiated cells. It is conceivable that the levels of α_1 subunit of L-type channels are low in undifferentiated cells but high in differentiated RINm5F cells. Therefore, the levels of α_1 subunits of L-type channels are quite low during early development of pancreatic β-cells, then rise

dramatically and approach those found in adult pancreatic β-cells. The developmental changes of L-type channels have been demonstrated in rat skeletal muscle and ventricular muscles (Morton and Froehner, 1989; Kojima et al., 1990). In addition, the developmental changes of L-type channels have been implicated in some cell lines (neuroblastoma x glioma hybrid NG 108-15 cells and neuroblastoma x Chinese hamster brain NCB-20 cells), which have two morphological features as RINm5F cells do (Mienville, 1992; Jin et al., 1994).

In summary, differentiated cells demonstrate higher activity of L-type channels than undifferentiated RINm5F cells, and the voltage-dependence of activation and inactivation of these channels differs between differentiated and undifferentiated cells. N-, P- and Q-type VDCCs may not be present in differentiated and undifferentiated RINm5F cells. We speculate that poorly undifferentiated fetal pancreatic β-cells may have lower activity of L-type channels than differentiated β-cells , but with cell differentiation, pancreatic β-cells might have higher activity of L-type channel than undifferentiated β-cells.

CHAPTER VIII GENERAL SUMMARY

A rat clonal β-cell line, RINm5F, is originated from rat pancreatic islets following X-ray irradiation. The cells exhibited two morphological features: the differentiated state with long neuron-like processes and the undifferentiated state without the processes. A novel insulin-like immunoreactivity, adrenergic receptors (ARs), second messenger systems through ARs and voltage-dependent Ca^{2+} channels (VDCCs) were characterized in a passage- and morphology dependent manner.

As morphology of the cells changed from differentiated to undifferentiated cells, the amount of secreted insulin decreased. By applying antisera vs. rat and porcine insulins in RIA, the amount of secreted insulin from RINm5F cells gradually decreased; however, with antiserum vs. bovine insulin, it gradually increased from a barely detectable amount. Transmission electron microscopy revealed that dense core secretory vesicles were more numerous in differentiated cells than in undifferentiated cells. Immunocytochemistry and immunoblot analysis showed that antisera vs. rat and porcine insulins reacted with insulins from both cell types. There was no reaction of insulins with antisera vs. bovine insulin in differentiated cells, however, there was a reaction in undifferentiated cells. These results suggested that a novel insulin-like immunoreactivity was present only in undifferentiated cells. In immunoblotting, insulin from homogenized cells was found as a single band with a M.W. of 6 KDa. All insulins secreted from undifferentiated cells were neither rat insulin I and II nor insulin like growth factor (IGF)-I and -II. These results suggested that a novel insulin-like immunoreactivity is present in undifferentiated RINm5F cells, which is different from rat insulin I and II.

Epinephrine (1 μM) significantly decreased basal intracellular cAMP concentration and insulin release in differentiated cells, but significantly increased intracellular cAMP concentration and insulin release in undifferentiated cells. In addition, epinephrine (1 μM) decreased forskolin-induced intracellular cAMP concentration in differentiated cells but not in undifferentiated cells. In differentiated cells, epinephrine (1 μM) decreased KCl (30 mM)-induced insulin release but RX 821002 (0.1 μM), an α_2-AR antagonist, abolished the epinephrine-induced inhibition. Prazosin (0.1 μM), an α_1-AR antagonist, and propranolol (1 μM), a β-AR antagonist, failed to alter the effect of epinephrine. In

undifferentiated cells, however, epinephrine (1 μM) increased insulin release and enhanced KCl (30 mM)-induced insulin release. RX 821002 further potentiated the effect of epinephrine on KCl's action. Propranolol (1 μM) abolished the effect of epinephrine on KCl's action but prazosin (0.1 μM) did not. In addition, under α_2-AR blockade, the α_1-AR agonists cirazoline (10 μM) and methoxamine (100 μM) did not increase insulin release in either differentiated or undifferentiated cells. Medetomidine (0.001 - 1 μM), a potent and specific α_2-AR agonist, inhibited KCl (30 mM)-induced insulin release in a dose-dependent manner in differentiated cells, but not in undifferentiated cells. Isoproterenol, a β-AR agonist (1 μM), failed to increase intracellular cAMP concentration and insulin release in differentiated cells but increased them in undifferentiated cells. These results suggested that in RINm5F cells, 1) the differentiated states are functionally equipped with more α_2-, but fewer β-ARs, than the undifferentiated states, and 2) α_1-ARs do not participate in insulin release in either differentiated or undifferentiated RINm5F cells.

To further study the higher activity of β-ARs, which may arise from higher activity of second messenger systems, we characterized the activities of G_s-protein, adenylyl cyclase, phosphodiesterase (PDE) and cAMP-dependent protein kinase (protein kinase A). Cholera toxin (CTX, 1 μg/ml), an activator of G_s-proteins, increased intracellular cAMP concentration in both differentiated and undifferentiated cells without significant differences between these cells. CTX also increased insulin release in these cells, however, CTX was 2.6-fold more potent in undifferentiated cells than in differentiated cells. The effect of forskolin (10 μM) on cAMP production and insulin release was 2- and 6-fold more potent, respectively, and the effect of IBMX (100 μM) on insulin release was 6-fold more potent in undifferentiated cells than in differentiated cells. IBMX, however, significantly increased the intracellular concentration of cAMP equally in differentiated and undifferentiated cells. Isoproterenol potentiated forskolin-induced cAMP production and insulin release in undifferentiated cells, but not in differentiated cells. CTX potentiated forskolin-induced cAMP production and insulin release in undifferentiated cells, but not in differentiated cells. Furthermore, forskolin potentiated KCl-induced insulin release in undifferentiated cells, but not in differentiated cells, without significant differences of intracellular cAMP concentrations and $[Ca2+]_i$ between the forskolin alone group and the forskolin + KCl group. However, activity of

protein kinase A was not significantly different between differentiated and undifferentiated cells. These results suggested that activity of adenylyl cyclase is higher in undifferentiated cells than in differentiated cells, and the activities of $G_{\alpha s}$-proteins, PDE and protein kinase A remain unchanged between differentiated and undifferentiated RINm5F cells. Furthermore, greater association of $G_{\alpha s}$-protein with adenylyl cyclase may contribute to the increased activity of adenylyl cyclase in undifferentiated RINm5F cells.

VDCCs were characterized in a passage- and morphology-dependent manner. The effect of KCl (15 mM) on $[Ca^{2+}]_i$ and insulin release was 3-fold higher and Bay K 8644 (1 μM) was 6-fold higher in differentiated cells than in undifferentiated cells. Furthermore, the dose-dependent increases in $[Ca^{2+}]_i$ and insulin release by the L-type Ca^{2+} channel agonist, Bay K 8644 (1 nM - 1 μM), was greater in differentiated cells than in undifferentiated cells. However, at lower concentrations of KCl (10 - 30 mM), the dose-dependent increases were greater in differentiated cells while at higher concentrations of KCl (30 - 45 mM) the increases were greater in undifferentiated cells. In addition, the inhibitory effect of nimodipine on KCl and Bay K 8644 were studied by equally increasing $[Ca^{2+}]_i$ and insulin release between differentiated and undifferentiated cells. Thus, 12 mM and 15 mM KCl was used in differentiated and undifferentiated cells, respectively, and 0.1 μM and 1 μM Bay K 8644 was used in differentiated and undifferentiated cells, respectively. Dose-dependent inhibitions in KCl- and Bay K 8644-induced $[Ca^{2+}]_i$ and insulin release by nimodipine (1 nM - 1 μM) were greater in differentiated cells than in undifferentiated cells.

In whole-cell patch-clamp recordings, with a holding potential of -80 mV, inward Ca^{2+} currents were first observed during a depolarization to -50 mV in both differentiated cells and undifferentiated cells. However, the maximal Ca^{2+} current was greater in undifferentiated cells than in differentiated cells. Bay K 8644 (1 μM) increased Ca^{2+} currents by 74 $\pm$ 13% in differentiated cells and 20 $\pm$ 7 % in undifferentiated cells. Nimodipine (1 μM) caused an inhibition of control Ca^{2+} currents by 48 $\pm$ 17% in differentiated cells and failed to inhibit them in undifferentiated cells. In addition, nimodipine (1 μM) inhibited Bay K 8644-induced Ca^{2+} current by 83 $\pm$ 8 % in the differentiated cells, but failed to inhibit them in undifferentiated cells.

Since nimodipine did not totally inhibit control Ca^{2+} currents and Bay K 8644-

induced increases in $[Ca^{2+}]_i$ and insulin release in undifferentiated cells, it was further studied for two possibilities: 1) the functional involvement of other VDCCs such as N-, P- and Q-type and 2) the voltage-dependency of L-type Ca^{2+} channels. An N-type channel blocker, ω-conotoxin GVIA (1 μM), and a P- and Q-type channel blocker, ω-agatoxin IVA (300 nM), did not inhibit basal Ca^{2+} currents nor did they inhibit KCl- and Bay K 8644-induced increases in $[Ca^{2+}]_i$ and insulin release in either differentiated or undifferentiated cells. The voltage-dependence of L-type channels was studied by using nimodipine, whose binding could be enhanced by prolonged membrane depolarization. KCl (30 mM)-induced increases in $[Ca^{2+}]_i$ and insulin release was dose-dependently inhibited by nimodipine (0.1 nM - 1 μM) in both differentiated and undifferentiated cells. The potency of nimodipine on $[Ca^{2+}]_i$ and insulin release by 30 mM KCl was 17- to 22-fold higher than that by 15 mM KCl in undifferentiated cells. However, in differentiated cells, there were no significant differences in the potency of nimodipine on $[Ca^{2+}]_i$ and insulin release induced by 12 and 30 mM KCl. In addition, in undifferentiated cells, the potency of nimodipine in inhibiting control Ca^{2+} currents was compared by holding membrane potentials at -80 mV and 0 mV in the whole-cell patch-clamp recordings. Nimodipine (1 μM) failed to inhibit control Ca^{2+} currents at the holding potentials of -80 mV, but inhibited them by 70.3 $\pm$ 7.7% at the holding potentials of 0 mV. The current-voltage relationship shifted to more negative potentials in differentiated cells than in undifferentiated cells. The voltage at which one-half of Ca^{2+} currents was activated ($V_{1/2}$) was -24.3 $\pm$ 3.6 mV in differentiated cells and -10.6 $\pm$ 2.5 mV in undifferentiated cells. The prepulse potential at which one-half of the maximal Ca^{2+} currents was inactivated ($V_{1/2}$) shifted from -21.4 $\pm$ 3.9 mV in differentiated cells to 1.8 $\pm$ 4.3 mV in undifferentiated cells. These results suggested that differentiated RINm5F cells are functionally equipped with more L-type Ca^{2+} channels than the undifferentiated cells, and the voltage-dependence of L-type Ca^{2+} channels was different between differentiated and undifferentiated cells. However, N-, P- and Q-type VDCCs may not be present in differentiated and undifferentiated RINm5F cells.

In summary, we suggest that a novel insulin-like immunoreactivity is present in undifferentiated RINm5F cells, which is not due to rat insulin I nor II. In addition, the differentiated cells are functionally equipped with more α_2-, but fewer β-ARs, than the

undifferentiated cells. The increased activity of β-ARs in undifferentiated cells is partly due to higher association of $G_{\alpha s}$-protein with adenylyl cyclase and increased activity of adenylyl cyclase. We also suggest that RINm5F cells have predominantly L-type channels with no N-, P- and Q-type channels. However, differentiated cells are functionally equipped with more L-type Ca^{2+} channels than the undifferentiated cells and the voltage-dependency of L-type Ca^{2+} channels was different between differentiated and undifferentiated cells.

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