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Pharmacology of the ovine uterine artery and umbilical blood vessels: Serotonergic and α-adrenergic mechanisms

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Pharmacology of the ovine uterine artery and umbilical blood vessels: Serotonergic and α-adrenergic mechanisms

by

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ABBREVIATIONS

5-HT	5-hydroxytryptamine (serotonin)
cAMP	cyclic 3',5'-adenosine monophosphate
cGMP	cyclic 3',5'-guanosine monophosphate
CEC	chloroethylclonidine
$[Ca^{2+}]_e$	extracellular Ca^{2+}
$[Ca^{2+}]_i$	intracellular Ca^{2+}
CICR	Ca^{2+}-induced Ca^{2+} release
DAG	diacylglycerol
IICR	$Ins(1,4,5)P_3$-induced Ca^{2+} release
$Ins(1,4,5)P_3$	Inositol 1,4,5-trisphosphate
MLC	myosin light chain
MLCK	myosin light chain kinase
NE	norepinephrine
PDBu	phorbol 12,13-dibutyrate
PIP_2	phosphatidylinositol 4,5-bisphosphate
PKA	cAMP-dependent protein kinase
PKC	protein kinase C
aPKC	atypic portein kinase C
cPKC	conventional protein kinase C
nPKC	novel protein kinase C
PKG	cGMP-dependent protein kinase
PLC	phospholipase C
PC	phosphatidylcholine
PLD	phospholipase D
ROCs	receptor-operated Ca^{2+} channels
VOCs	voltage-operated Ca^{2+} Channels
WB 4101	2(N[2,6-dimethoxyphenoxyl])amino-methyl-1,4-benzodioxane

Epi	Epinephrine
NPY	Neuropeptide Y
EDRF	Endothelium-derived relaxing factor
CGRP	Calcitonin-gene related peptide
SP	Substance P
VIP	Vasoactive intestinal peptide
NO	Nitric oxide
SR	Sarcoplasmic reticulum

GENERAL INTRODUCTION

Statement of the Problem

During pregnancy, the blood flow through the uterine artery and umbilical vasculature is crucial for the survival and growth of the fetus. These two vascular systems are potential sites for the action of vasoconstrictors, such as norepinephrine (NE), 5-hydroxytryptamine (serotonin, 5-HT), endothelin-1 as well as others. Norepinephrine, 5-HT and endothelin-1 all have been found to be potent constrictors of uterine and umbilical vascular beds except for NE on the umbilical artery (Dyer, 1970; Ekstrom et al., 1991; Isla and Dyer, 1990; Mak et al., 1984; MacLean et al., 1992; Somlyo et al., 1965; Zhang and Dyer, 1990a, 1990b; 1991a). However, the mechanisms underlying the contractions induced by these agents are not fully investigated. Isolated tissues have been extensively used for investigating effects due to receptor-agonist interaction. Compared with studies in whole animals, the use of isolated tissue preparations greatly reduce problems associated with the distribution and metabolism of agonists. Isolated tissue preparations also eliminate many complications such as feedback effects. Our laboratory has been studying the constrictor mechanisms in the uterine and umbilical vessels. The use of isolated uterine and umbilical blood vessels permits us to study the mechanisms for vasoconstriction and vasodilation in depth.

5-Hydroxytryptamine$_2$ (serotonin$_2$, 5-HT$_2$) receptor-mediated hydrolysis of phosphoinositide has been documented in non-fetal vessels (Doyle et al., 1986; Roth et al., 1986). Previous study from our laboratory indicated the presence of 5-HT$_2$ receptors in the ovine umbilical artery (Zhang and Dyer, 1990b). In study one we examined whether

activation of 5-HT_2 receptors induces hydrolysis of phosphoinositide in the ovine umbilical artery.

In a physiological environment, it is possible for various vasoconstrictors to be present simultaneously in the vicinity of the vascular smooth muscle. The interaction between NE and 5-HT has been demonstrated in various vessels (Luscher and Vanhoutte, 1988; Meehan et al., 1986; Stupecky et al., 1986; van Nueten et al., 1981). The interaction between NE and 5-HT on the uterine vascular smooth muscle could play a role in regulating uterine blood flow during pregnancy. Study two was designed to investigate the interaction between NE and 5-HT in the ovine uterine artery.

Both the ovine uterine artery and umbilical vein contain α_1-adrenoceptors (Zhang and Dyer, 1990a; 1991a). Since the middle of 1980s, it has been clear that the α_1-adrenoceptor is heterogenous (Minneman and Esbenshade, 1994). In study three, we applied pharmacological tools to characterize the subtypes of α_1-adrenoceptors in the ovine uterine artery and umbilical vein.

Protein kinase C (PKC) has been suggested to have a role in agonist-induced contraction of smooth muscle (Rasmussen et al., 1987). Magness et al. (1991) reported the presence of PKC in the ovine uterine artery. In study four, the role of PKC in contractions induced by NE and endothelin-1 on the isolated ovine uterine artery was investigated.

Dissertation Organization

This dissertation is presented as a general introduction, a review of the literature, four papers prepared for publication, a general summary, a list of references cited in chapters other than papers, a general summary and acknowledgements.

This dissertation contains the experimental results obtained by the author during his graduate study under the supervision of his major professor, Dr. Donald C. Dyer.

REVIEW OF LITERATURE

1. Physiology and Pharmacology of the Uterine Vasculature

1.1. Anatomy of the Uterine Vasculature

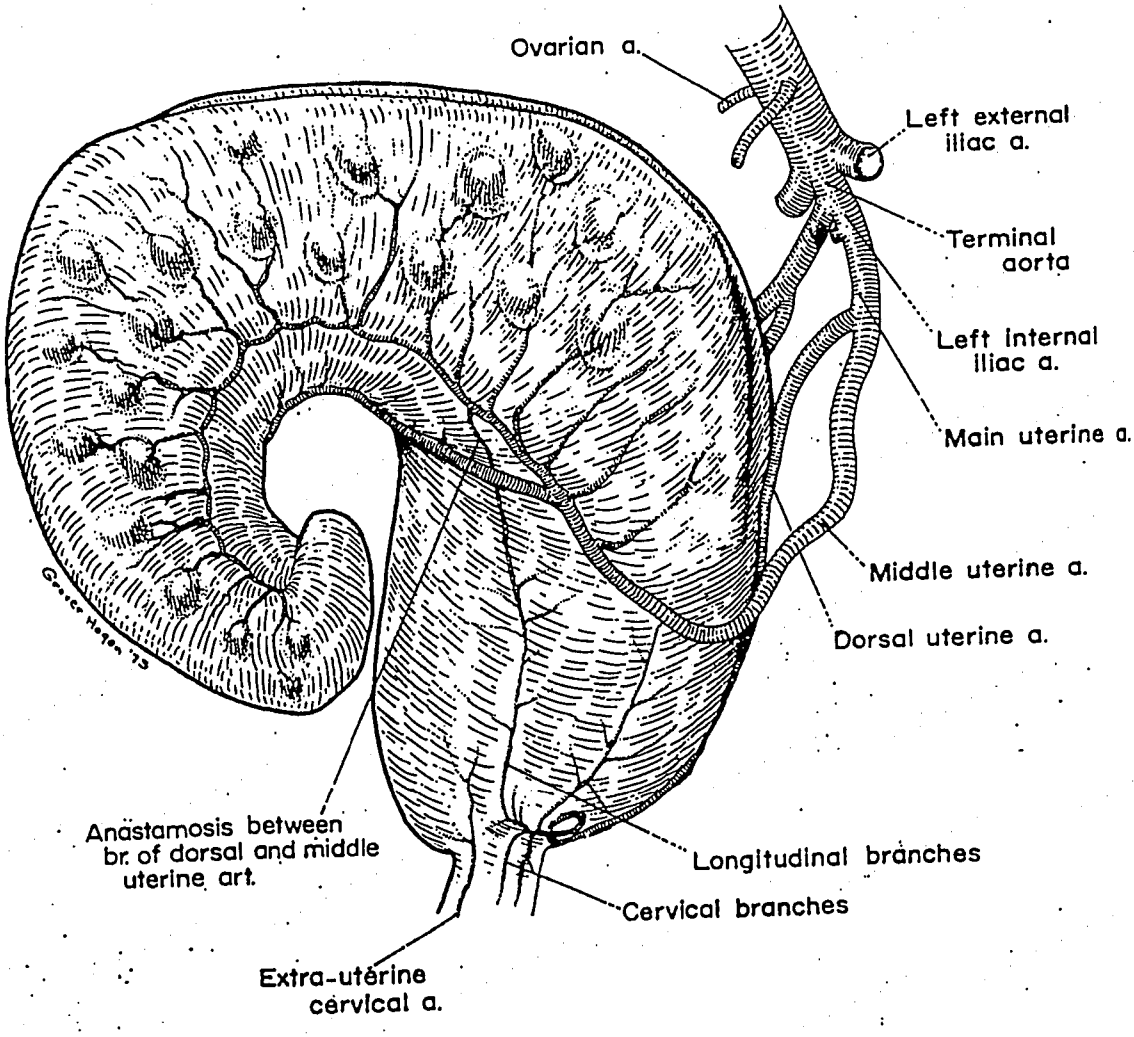
1.1.1. Anatomy of the uterine artery

In humans and in several laboratory animals (guinea pig, rat), the terminal part of the abdominal aorta divides into two iliac arteries; each iliac artery divides into an external and internal iliac artery. The uterine artery originates from the internal iliac artery. In the base of the broad ligament, the uterine artery progresses medially and divides into the cervicovaginal artery and the ascending uterine artery at the level of the supravaginal portion of the cervix. The ascending uterine artery provides a major branch to the upper portion of the cervix (Meschia, 1983).

In sheep, the caudal end of the abdominal aorta trifurcates into two external iliac arteries and a short common internal iliac artery. The common internal iliac artery is usually less than 1 cm in length before it divides into the left and right internal iliac arteries which are also referred to as the main uterine arteries. The main uterine artery divides into the larger middle uterine artery and smaller dorsal uterine artery. The middle uterine artery is comparable to the ascending uterine artery in the human and in sheep supplies the major portion of the uterine horn. The dorsal uterine artery supplies the lower uterine corpus and the cervix (Fuller et al., 1975) (fig. 1).

The arteries supplying the primate (including the human) uterus form a myometrial network (arcuate arteries) that encircles the organ. From this network arise the radial

Fig. 1. Vascular anatomy of the sheep uterine circulation (Fuller et al., 1975).


Ovarian a.
Left external iliac a.
Terminal aorta
Left internal iliac a.
Main uterine a.
Middle uterine a.
Dorsal uterine a.
Anastamosis between br. of dorsal and middle uterine art.
Longitudinal branches
Cervical branches
Extra-uterine cervical a.

arteries that are directed toward the uterine lumen. The radial arteries become spiral arteries as they pass into the uterine mucosa. Shortly after entering the endometrium the spiral arteries give off branches, the basal arteries, which ramify along the endometrial-myometrial border. Radial arteries supply the inner two-thirds of the uterus, while spiral arteries supply the endometrium, decidua, and placenta during pregnancy (Meschia, 1983).

In the ewe, the uterine artery branches within the broad ligament to supply blood to the uterine horn. Upon reaching the mesometrial border, these branches course dorsally and ventrally around the uterine horn (Ghoshal, 1975). These arteries are referred as circumferential arteries and are tortuous or coiled. They course between the outer and inner layer of uterine smooth muscle. One or two of these uterine arteries approach each cotyledon. The vessels divide into five or six trunks and pursue a tortuous course in the submucosa before dividing again and entering the cotyledon (Reynolds, 1963).

1.1.2. Innervation of the uterine vasculature

The uterine vascular bed is richly supplied with nerve fibers. The nature of the innervation of the uterine vessels and its role in the control of the uterine circulation probably varies from one animal species to another, and in the same species according to the hormonal status and gestational age. Earlier studies in Bell's laboratory revealed in detail the innervation of the uterine vasculature. In guinea pig, noradrenergic nerve fibers are distributed along the main uterine arteries as well as the secondary uterine arteries, while cholinergic nerve fibers were restricted to the main uterine arteries (Bell, 1968;

1971). Investigation of laboratory and domestic animals revealed that the main uterine arteries of dog and pig were supplied with cholinergic nerve fibers, while rat, cat, rabbit and cow were devoid of such innervation (Bell, 1971). The human uterine vein is not innervated with cholinergic nerves (Amenta et al., 1979).

Using histochemical, immunohistochemical and biochemical techniques, some recent studies have confirmed the innervation of uterine arteries by adrenergic nerve fibers in rat (Garfield, 1986; Papka and Traurig, 1988), cat (Alm et al., 1986), golden hamster (Sorger et al., 1983), human (Ekesbo et al., 1991; Zuspan et al., 1981), sheep (Massmann et al., 1992; Renegar and Rexroad, 1990), guinea pig (Fallgren et al., 1989; Morris et al., 1985) and pig (Taneike et al., 1994), and by cholinergic nerve fibers in guinea pig (Alm et al., 1988; Thorbet et al., 1977), human (Amenta et al., 1979; Ekesbo et al., 1991), sheep (Renegar and Rexroad, 1990) and mouse (Moscarini et al., 1982).

In addition, other neurotransmitters have been found to be present in uterine arteries of various species, i.e. neuropeptide Y (NPY) in human, rat and guinea pig (Morris et al., 1985; Papka and Traurig, 1988; Stjernquist et al., 1991); vasoactive intestinal peptide (VIP) in human, guinea pig, rat, cat, goat, pig and rabbit (Della et al., 1983; Ekesbo et al., 1991; Ottesen et al., 1981; Stjernquist et al., 1985); substance P (SP) in human and guinea pig (Fallgren et al., 1989; Heinrich et al., 1986); calcitonin gene-related peptide (CGRP) in guinea pig (Fallgren et al., 1989; Mione et al., 1988); dynorphin in guinea pig (Morris et al., 1985); somatostatin in human and guinea pig (Heinrich et al., 1986; Morris et al., 1985); and neurotensin in human (Heinrich et al., 1986).

In a most recent study, rat uterine blood vessels were

found to be innervated by 5-HT-like nerve fibers (Amenta et al., 1992). It was suggested that 5-HT released from vascular adrenergic nerve endings may contribute to the maintenance of local vascular tone (Kawasaki and Takasaki, 1984). However, the significance of this observation as to how 5-HT may affect/regulate the uterine artery is not clear. Also, Toda and coworkers (1994) found the human uterine artery to be innervated by vasodilator nerve fibers which use nitric oxide as a neurotransmitter.

The density of the uterine adrenergic nerve fibers during pregnancy has been found to be decreased in sheep (Sigger et al., 1986), human (Thorbert et al., 1979), dog (Ryan et al., 1974), cat (Alm et al. 1986), and guinea pig (Owman et al., 1980). The density of innervation of the uterine vasculature is also under the influence of pregnancy. The number of adrenergic nerve fibers in the uterine artery decreased during pregnancy in the guinea pig (Bell and Malcolm, 1978; Mione et al., 1990), sheep (Sigger et al., 1986) and dog (Ryan et al., 1974). This reduction could be mimicked by intrauterine progesterone treatment (Bell and Malcolm, 1978). The degeneration of adrenergic nerve fibers could result in the withdrawal of normal sympathetic vasoconstrictor tone as well as the development of hypersensitivity of the uterine vasculature to catecholamines.

1.2. Physiology of the Uterine Vasculature

1.2.1. Structural, biochemical and functional changes during pregnancy

The structure of the uterine artery changes markedly during pregnancy to accommodate the substantial rise in

uterine blood flow. Uterine artery diameter markedly increased during pregnancy in human (Annibale et al., 1990; Palmer et al., 1992), pig (Guenther et al., 1988) and sheep (Fuller et al., 1975). The increase in diameter of the uterine artery with pregnancy is not merely the result of passive dilation, since wall thickness is unchanged (Griendling et al., 1985). This finding suggests that there is active growth of the uterine arterial wall. Griendling et al. (1985) suggested that this arterial growth is accomplished by a 2.5-fold increase in smooth muscle mass. Biochemical changes in the uterine artery during pregnancy have also been observed. The collagen content of the porcine and ovine uterine artery declined progressively throughout pregnancy while elastin and RNA remained unchanged (Griendling et al., 1985; Guenther et al., 1988). Cytosol PKC of uterine artery declined with the advancement of gestation of pig whereas PKM (proteolytic product of PKC) progressively increased (Farley and Ford, 1992). In sheep, uterine arterial PKC activity was higher during the luteal phase than in follicular phase and decreased during pregnancy (Magness et al., 1991). Estrogen receptor level of rat uterine artery increased during pregnancy (Leiberman et al., 1993).

Contraction and relaxation of the uterine artery are also affected by pregnancy. The relaxant effect of sodium nitroprusside was attenuated in guinea pig uterine artery during pregnancy (Weiner et al., 1991). There was an increase in the relaxant response and efficacy to acetylcholine of the guinea pig uterine artery during pregnancy (Weiner et al., 1989). There was no change in potassium-induced contraction of guinea pig uterine artery during pregnancy (D'Angelo and Osol, 1993; Fallgren et al., 1988). However, potassium-induced vasoconstriction and $^{45}Ca^{2+}$

uptake were decreased in porcine uterine artery during pregnancy (Farley and Ford, 1992). Other alterations related to vasoactivity will be discussed in the coming sections.

Pregnancy is associated with dramatic increases in uteroplacental blood flow (Clapp, 1978; Guenther et al., 1988; Palmer et al., 1992; Rosenfeld, 1977; Thaler et al., 1990). In late pregnancy, the uterine arteries carry about 20% of the maternal cardiac output, with approximately 80% of the uterine blood flow going to the placenta. Such a large increase is thought to be the result of both an accelerated vessel growth and dilation of the vasculature. Although the blood vessels can still dilate in response to estrogen in late pregnancy, the magnitude of the response is decreased, suggesting that the vasculature is already almost maximally dilated.

1.3. Pharmacology of the Uterine Vasculature: Catecholamines and 5-Hydroxytryptamine

1.3.1. Catecholamines

Catecholamines, such as NE and epinephrine (Epi), are either released from sympathetic nerve fibers as neurotransmitter or secreted from the adrenal medulla as hormones. The uterine vasculature is sensitive to the vasoconstrictor action of both Epi and NE. The infusion of catecholamines into a pregnant animal reduces uteroplacental blood flow (Assali, 1981; Barton et al., 1974; Chestnut et al., 1986; Chez et al., 1978; Clark et al., 1990; Rosenfeld et al., 1976; Rosenfeld and West, 1977). The reduction of uteroplacental blood flow caused by Epi and NE can be completely inhibited by blocking α-adrenergic receptors with phenoxybenzamine (Egund and Carter, 1980; Greiss, 1972).

However, infusion of phenoxybenzamine alone had no effect on uterine blood flow (Chez, 1978).

α-Adrenoceptor agonist-induced uterine vasoconstriction is primarily mediated by α_1-adrenoceptors in the human (Stjernquist and Owman, 1990), sheep (Isla and Dyer, 1990), and guinea pig (Fallgren and Edvinsson, 1986). By using ligand binding techniques, α_1-adrenoceptors have been quantitively measured in the pig uterine artery (Farley et al., 1984). However, Ribeiro and Macedo (1986) argued that both α_1- and α_2-adrenoceptors are present in the human uterine artery. In addition, α_2-adrenoceptors are also present in the pig uterine artery (Guenther et al., 1988).

β-Adrenergic agonists are weak vasodilators in the uterine vascular bed of nonpregnant (Barton et al., 1974; Greiss and Gobble, 1967; Resnik et al., 1976) and pregnant ewes (Erkkola et al., 1981; Hasaart and de Haan, 1987). The vascular response to β-adrenergic agonists is reduced in pregnant animals (Erkkola et al., 1981). Isoproterenol had no relaxant effect on the $PGF_{2\alpha}$-contracted guinea pig uterine artery (Fallgre and Edvinsson, 1986). The human uterine artery was slightly relaxed by isoproterenol (Gough and Dyer, 1971). These results indicate that the uterine vasculature is under minor β-adrenergic control.

Pregnancy-induced refractoriness of the uterine artery to vasoconstrictors is controversial. During pregnancy, contractions to catecholamines were reduced in the guinea pig uterine artery when compared with arteries from nonpregnant status (Weiner et al., 1989; 1991). However, the sensitivity of the uterine artery to phenylephrine is increased in pregnant as compared to nonpregnant sheep (Annibale et al., 1989; D'Angelo and Osol, 1993; Osol and Cipolla, 1993). However, pregnancy did not change the sensitivity of the uterine arteries of the human (Suresh et

al., 1985) and rabbit (Moisey and Tulenko, 1983) to catecholamines.

Infusion of dopamine also reduced uterine blood flow in pregnant sheep (Fishburne et al., 1980; Santos et al., 1992). In vitro studies showed that dopamine was a weak vasoconstrictor of the isolated human uterine artery (Ekstrom et al., 1991;. Van Nimwegen and Dyer, 1974).

1.3.2. 5-hydroxytryptamine

5-Hydroxytryptamine is a naturally occurring substance that has a wide variety of effects. 5-Hydroxytryptamine causes vasoconstriction in both pregnant and nonpregnant sheep following direct administration into the uterine vasculature (Clark et al., 1980). A sub-vasoconstricting concentration of 5-HT potentiated the pressor response to angiotensin II in both the pregnant and nonpregnant guinea pig (Weiner et al. 1987). Serotonergic agonist-induced uterine vasoconstriction was mediated by 5-HT_2 receptors in the human (Ribeiro et al., 1991) and sheep (Zhang and Dyer, 1990). Activation of 5-HT_2 receptors increased Ca^{2+} influx in ovine uterine artery (Zhang and Dyer, 1991b). 5-Hydroxytryptamine-induced contraction of the guinea pig uterine artery was reduced during pregnancy (Weiner et al., 1992). The attenuation was endothelium dependent, however, neither nitric oxide nor prostacyclin was involved.

2. Physiology and Pharmacology of the Umbilical Vessels

2.1. Anatomy of the Umbilical Vessels

2.1.1. Umbilical vein

The oxygenated fetal blood is carried by numerous cotyledonary and intercotyledonary branches distributed on the fetal side of the placenta. These branches communicate with one another and finally converge to form the umbilical vein (Assali et al., 1968) (fig. 2).

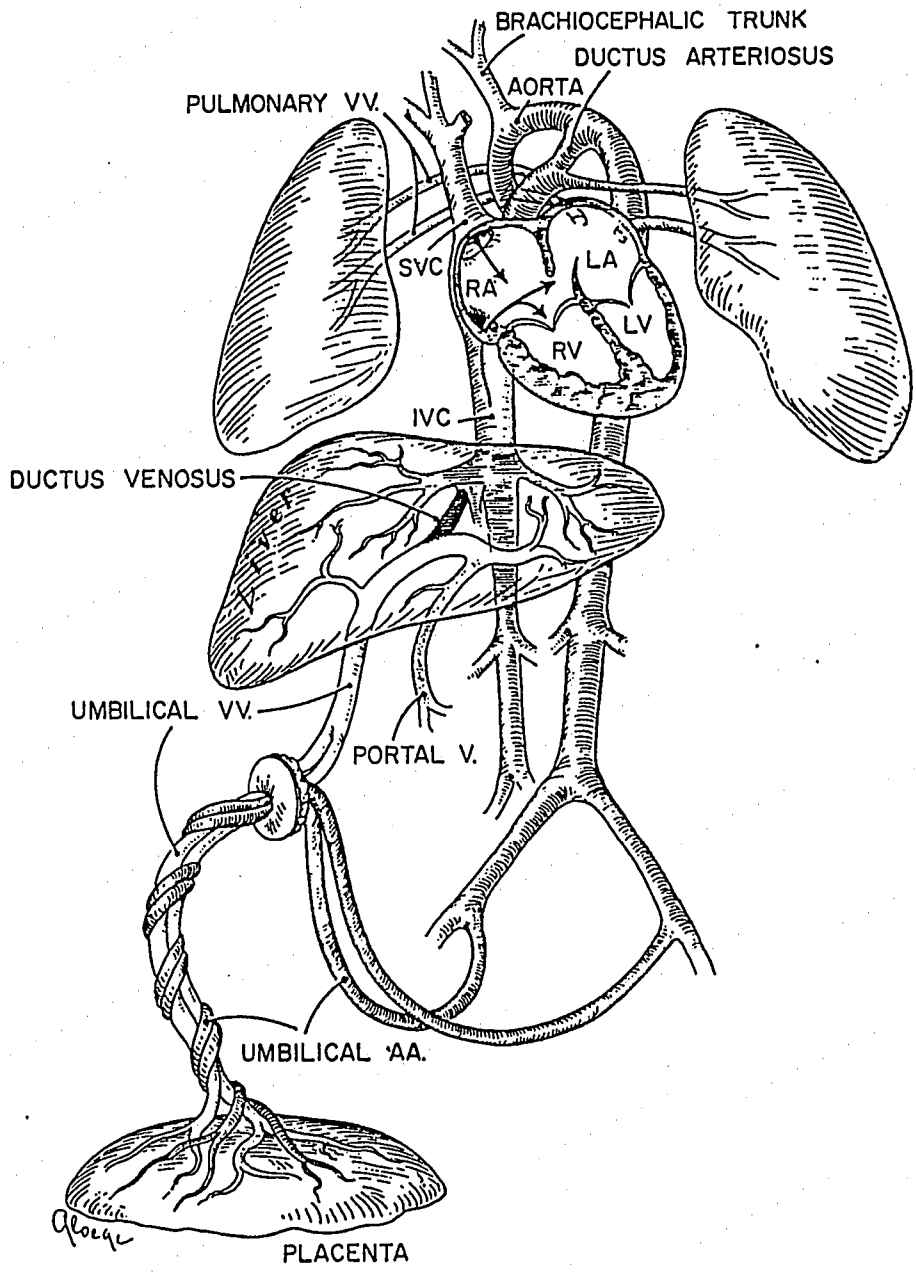
In man and primates, there is only one umbilical vein while in the sheep and goat there are two. In either case, the umbilical vein (or veins) courses along the umbilical arteries in the cord from its insertion in the placenta to the umbilical ring. Histologically, the vein possesses a muscular coat which is thinner than that of the umbilical arteries, but thicker than that of any other vein of the fetus. There is an attenuated elastic lamina (Sheppard and Bishop, 1973).

In humans, the single umbilical vein passes through the umbilical ring into the abdominal cavity of the fetus and runs along the abdominal wall enfolded by peritoneum. In the sheep, the two umbilical veins contained in the cord enter the abdominal cavity and fuse to form a single channel. This vessel runs about 2 to 3 cm before entering the liver.

2.1.2. Umbilical artery

The umbilical arteries may be considered as two large terminal branches of the descending aorta. Fetal blood, rich in carbon dioxide, enters the placenta via these arteries.

Fig. 2. Anatomy of the circulatory system in the fetal lamb (Assali et al., 1968).


BRACHIOCEPHALIC TRUNK
DUCTUS ARTERIOSUS
AORTA
PULMONARY VV.
SVC
RA
LA
LV
RV
IVC
DUCTUS VENOSUS
UMBILICAL VV.
PORTAL V.
UMBILICAL AA.
PLACENTA

Their cross-sectional diameter is smaller than that of the umbilical vein. The wall of the umbilical arteries are rather thick and very rich in muscular elements. However, unlike muscular arteries elsewhere, they lack elastic lamina (Shepard and Bishop, 1973).

2.1.3. Innervation of the umbilical vessels

The umbilical vessels are not innervated, at least in the human (Fox and Khong, 1990; Reilly and Russell, 1977; Spivack, 1943). Hence the umbilical vessels are mainly under humoral control. However, some studies indicate that the fetal end of the vessels is supplied with nerves (Baljet and Drukker, 1982; Kawano and Mori, 1990). Although not innervated, the umbilical vessels contain enzymes which metabolize catecholamines. Monoamine oxidase (MAO) was found to be present in the ovine umbilical blood vessels (Dyer and Weber, 1971). No subsequent studies have been done to subclassify the subtypes of MAO in the ovine umbilical blood vessels. Two subtypes of MAO, MAO-A and MAO-B as well as catechol *O*-methyl transferase (COMT) all have been found in the human umbilical vessels (Caramona, 1983; Precious and Lyles, 1988; Saarckoski, 1983). The human umbilical artery takes up norepinephrine possibly via an uptake$_2$ mechanism (Gulati and Sivaramakrishna, 1975).

2.2. Physiology of the Umbilical Vessels

The role of the endothelium in modulating the activities of the umbilical vessels is controversial. Some studies indicate that the human umbilical vessels have no relaxant response to those agents believed to release EDRF and removal of the endothelium does not alter vascular

responses to vasoconstrictors (Monuszko et al., 1990), While other studies find that histamine, A23187 and bradykinin but not acetylcholine and 5-HT (Chaudhuri et al., 1991; Van de Voorde et al., 1987) release EDRF in human umbilical artery and veins. A most recent study showed that acetylcholine relaxed isolated human umbilical artery via an endothelium-independent process (Xie and Triggle, 1994). Some investigators claimed that prostacyclin rather than nitric oxide (NO) is an endothelium-derived relaxing factor (EDRF) in the human umbilical artery (Klockenbusch et al., 1992), whereas others reported that human umbilical vein endothelial cells do release NO in response to α-thrombin, bradykinin and ionomycin (Tsukakora et al., 1993). Furthermore, infusion of NO synthetase inhibitor N^{ω}-nitro-L-Arginine decreased the ovine umbilical blood flow and increased umbilical-placental vascular resistance (Chang et al., 1992).

Responses of the umbilical artery to vasodilators are also different from that of the systemic vasculature (Renowden et al., 1992). The umbilical artery is less sensitive to vasodilators, such as sodium nitroprusside, forskolin, atrial natriuretic peptide and adenosine (Renowden et al., 1992; White, 1988). Forskolin did not affect the agonist (histamine and vasopressin)-induced increase in $[Ca^{2+}]_i$ in human umbilical vein smooth muscle cells (Sharma and Bhalla, 1989).

Studies using human umbilical artery and vein found that endothelial cells of these two vessels contain the neuropeptides VIP, SP, CGRP and vasopressin (Cai et al., 1993). However, their physiological roles are not yet clear. In addition, endothelin-1 is present in the human umbilical artery and vein (Hemsen et al., 1991; Salamonsen et al., 1992).

2.3. Pharmacology of the Umbilical Vessels: Catecholamines and 5-Hydroxytryptamine

2.3.1. Catecholamines

Infusion of α-adrenergic agonist into the ovine fetus did not significantly change the umbilical arterial blood flow (Adamson et al., 1989; Clark et al., 1990; Oakes et al., 1980; van Huisseling et al., 1991; Yoshimura et al., 1990), although umbilical vascular resistance increased (Adamson et al., 1989; Clark et al., 1990; Irion and Clark, 1990; Oakes et al., 1980). Paulick et al. (1991) found that NE and Epi only increased the umbilical venous pressure, but did not change the vascular resistance of the umbilical artery. In isolated human and sheep umbilical arteries, NE, Epi and phenylephrine were weak constrictors (Dyer, 1970; Mak et al., 1984; McGrath et al., 1985; Somlyo et al., 1965; Tuncer et al., 1985; Yashikawa and Chiba, 1991). It is concluded that there is no significant population of functional α_1-adrenoceptors in human umbilical artery (McGrath et al., 1985). α_2-Adrenergic agonists did not contract isolated human and ovine umbilical arteries (McGrath et al., 1985; Tuncer et al., 1985; Zhang and Dyer, 1991a). α_1-Adrenoceptors but not α_2-adrenoceptors were pharmacologically identified in ovine umbilical vein (Zhang and Dyer, 1991a).

α-Adrenoceptor antagonists did not change ovine umbilical blood flow (Chez et al., 1978; Edelstone et al., 1980; Oakes et al., 1980), confirming that the umbilical vessels are not under sympathetic control. The β-adrenoceptor agonist, isoproterenol also had no effect on ovine umbilical vascular resistance (Adamson et al., 1989; Berman et al., 1978) and produced a small increase in the

umbilical blood flow (Chez et al., 1978). Propranolol increased umbilical vascular resistance and decreased umbilical blood flow in sheep (Parer, 1983).

Dopamine did not change the ovine umbilical blood flow nor umbilical vascular resistance (Santos et al., 1992). In human umbilical artery, D_1 receptors have been identified (Ferrira de Almeida et al., 1993).

2.3.1. 5-Hydroxytryptamine

Infusion of 5-HT reduced umbilical blood flow and increased ovine umbilical vascular resistance (Berman et al., 1978). 5-Hydroxytryptamine is a strong vasoconstrictor of isolated human and ovine umbilical arteries (Altura et al., 1972; Dyer, 1970; MacLean et al., 1992; Somlyo et al., 1965; Yashikawa and Chiba, 1991) and veins (Altura et al., 1972; Dyer et al., 1972; Mak et al., 1984). However, in the perfused isolated human umbilical artery, 5-HT induced a biphasic response, vasodilation followed by a strong vasoconstriction (Bjoro and Stray-Pedersen, 1986; Haugen and Hovig, 1992). The dilatory response was endothelium-dependent (Haugen and Hovig, 1992). 5-Hydroxytryptamine$_2$ receptors have been identified with pharmacological tools in the ovine umbilical artery (Zhang and Dyer, 1990b) and vein (Zhang and Dyer, 1990c). 5-Hydroxytryptamine$_2$ receptors are also present in human umbilical artery and high oxygen tension revealed 5-HT$_1$-like receptors (MacLean et al., 1989; Templeton et al., 1991). 5-Hydroxytryptamine-induced vasoconstriction of the human umbilical artery is dependent on both extracellular and intracellular calcium (Dogan et al., 1991). However, in contrast to ovine uterine artery (Isla and Dyer, 1990), Ca^{2+} entry blockers were found more effective in inhibiting 5-HT- than K^+-induced contraction in

the isolated human umbilical artery (Medieros and Colixto, 1990).

3. Vasoconstriction and Signal Transduction Pathways in Vascular Smooth Muscle

3.1. Calcium and Vasoconstriction

It is well established that an increase in cytosolic free calcium initiates contraction of vascular smooth muscle (Stull et al., 1991). The rise of intracellular Ca^{2+} level leads to the binding of Ca^{2+} to calmodulin (CaM). The binding of Ca^{2+} to calmodulin then activates myosin light chain kinase (MLCK). Upon activation, MLCK catalyzes phosphorylation of serine 19 of the 20,000 dalton myosin light chain. When phosphorylated, myosin cross-bridges undergo rapid cyclic interactions with actin at the expense of ATP hydrolysis, which provides the energy for contraction (Kamm and Stull, 1985).

The Ca^{2+} required to activate the contractile reaction in smooth muscle comes either from the extracellular medium or from intracellular calcium stores (Bolton, 1979; Kuriyama et al., 1982). Extracellular calcium concentration is approximately 10,000 times greater than that inside the resting smooth muscle cell. Cytoplasmic Ca^{2+} ranges from approximately 100 nM in the resting state to about 600 to 800 nM in maximally contracting smooth muscle (Somlyo and Himpens, 1989; Somlyo and Somlyo, 1992).

Two different types of membrane channels are involved in the influx of extracellular calcium into the cell: voltage-operated Ca^{2+} channels (VOCs), which open when the

membrane is depolarized and receptor-operated Ca^{2+} channels (ROCs), which open when agonists react with their membrane receptors.

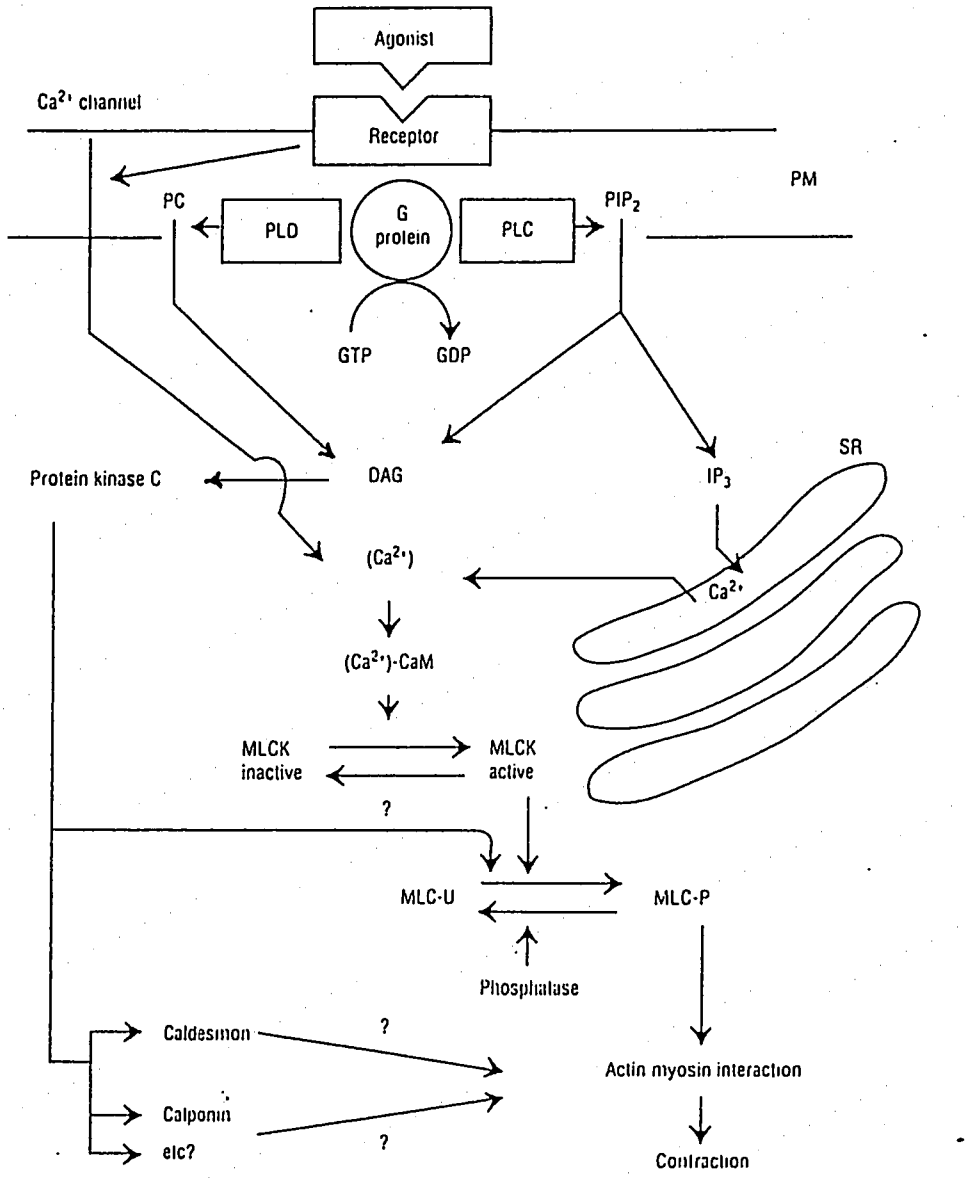
Voltage-operated Ca^{2+} channels in vascular smooth muscle can be classified into two major types according to electrophysiological studies: L-type and T-type. The high threshold current (L-type) is activated by a large depolarization and is relatively slowly inactivated (Bean et al., 1986; Benham et al., 1987; Ganiteuich and Isenberg, 1991; Loirand et al., 1986; Smirnov and Aaronson, 1992). This current is sensitive to 1,4-dihydropyridine. The L-type VOC has been cloned from rat aortic smooth muscle (Koch et al., 1990). A low-threshold (T-type) current that shows rapid inactivation has also been described by many of the above authors. The predominant Ca^{2+} current in vascular smooth muscle cells is carried through L-type Ca^{2+} channels (Bean, 1991). Because of their higher conductance and slower rate of inactivation, L-type VOC probably play a more important role in contraction of smooth muscle.

The observations that NE at certain concentrations can activate contractions of smooth muscle without causing a change in membrane potential or contract smooth muscle that are fully depolarized lead to the suggestion that ROCs exist in the plasma membranes of these cells (Bolton, 1979; van Breemen et al., 1979). Benham and Tsien (1987) showed that ATP directly activated ROCs in arterial smooth muscle cells. Receptor-operated Ca^{2+} channels are insensitive to depolarizing stimuli and to Ca^{2+} channel antagonists. In pig aortic microsomes, it was found that ROCs activated by NE are G-protein-dependent (Blayneys et al., 1992). The ROCs were inhibited by cGMP-dependent protein kinase (Blayney et al., 1991). Unlike VOCs, ROCs are still not fully characterized because of the lack of specific inhibitors.

In normal conditions, contraction in response to applied agonists often consists of two phases: an initial transient (phasic) component followed by a more sustained (tonic) component (Bolton, 1979; Kuriyama et al., 1982). In most vascular smooth muscle it appears that release of intracellular Ca^{2+} by inositol 1,4,5-trisphosphate ($Ins(1,4,5)P_3$) is required for the phasic component since contraction can be observed in Ca^{2+}-free-EGTA medium or in the presence of Ca^{2+} antagonists. Ca^{2+} entry from the extracellular fluid is required for the tonic component of contraction in most vascular smooth muscle. The role of the intracellular source of calcium in response to physiological agonists can be demonstrated by stimulation of vascular smooth muscle in Ca^{2+}-free medium (Steinsland et al., 1973). Further evidence came from the finding that $^{45}Ca^{2+}$ efflux increased when $^{45}Ca^{2+}$-loaded vascular smooth muscle was stimulated with agonists (van Breemen et al., 1986). Chelators of extracellular Ca^{2+} are widely used to inhibit the entry of extracellular Ca^{2+}. The contribution of stored intracellular Ca^{2+} has been determined by depleting releasible intracellular pools by repeated exposure to agonists or to caffeine and determining the residual contractile response. Studies have shown that a number of physiological agonists, such as NE, angiotensin II, vasopressin, histamine and prostaglandins, release Ca^{2+} from the same intracellular pool (Deth and Casteels, 1977; Deth and van Breemen, 1974). The importance of extracellular Ca^{2+} for agonist-induced contractions depends on the particular muscle and animal species being used (Minneman, 1988). The putative mechanisms for agonist-induced contraction is shown in fig. 3.

The sarcoplasmic reticulum (SR) is the physiological intracellular source and sink of activator Ca^{2+} (Bond et

Fig. 3. Mechanisms of agonist-induced vasoconstriction (Morgan et al., 1991). *CaM*, Calmodulin; *DAG*, diacylglycerol; IP_3, inositol 1,4,5-trisphosphate; *MLC-U*, myosin light chain unphosphorylated; *MLC-P*, myosin light chain phosphorlyted; *PC*, phosphatidylcholine; PIP_2, phosphatidylinositol 4,5-bisphosphate; *PLC*, phospholipase C; *PLD*, phospholipase D; *PM*, plasma membrane; *SR*, sarcoplasmic reticulum. Although DAG is shown in the cytosol, it actually remains in the plasma membrane (Berridge, 1987).


Agonist
Ca2+ channel
Receptor
PM
PC
PLD
G protein
PLC
PIP2
GTP
GDP
Protein kinase C
DAG
IP3
SR
(Ca2+)
Ca2+
(Ca2+)-CaM
MLCK inactive
MLCK active
?
MLC-U
MLC-P
Phosphatase
Caldesmon
?
Actin myosin interaction
Calponin
?
etc?
Contraction

al., 1984; Kowarski et al., 1985). The amount of calcium stored in the SR could be as high as 15 mM and is sufficient for the activation of maximal contraction (Bond et al., 1984; Iino, 1990). The signal for calcium release from SR is transmitted to the SR either by Ca^{2+} or $Ins(1,4,5)P_3$. The former process is called Ca^{2+}-induced Ca^{2+} release (CICR), and the latter $Ins(1,4,5)P_3$-induced Ca^{2+} release (IICR). Inositol 1,4,5-trisphosphate is the messenger product of the phosphoinositide cascade (Abdel-Latif, 1986; Berridge, 1987). These two different kinds of calcium release mechanisms were shown to be present in vascular smooth muscle (Endo et al., 1990; Hasimoto et al., 1986).

The major intracellular calcium release channels are the $Ins(1,4,5)P_3$ receptors located on the sarcoplasmic reticulum. Inositol 1,4,5-trisphosphate receptors have been purified from bovine aortic smooth muscle (Chadwick et al., 1990). A considerable amount of $Ins(1,4,5)P_3$ receptor mRNA was located in vascular smooth muscle (Furuichi et al., 1990). The purified $Ins(1,4,5)P_3$ receptor has been reconstituted into planar bilayer and shown to be an IICR channel (Mayerleitner et al., 1991). The $Ins(1,4,5)P_3$ receptor is inhibited by heparin, whereas this substance has no effect on the CICR (Ehrlich and Watras, 1988; Kobayashi et al., 1988). The CICR channel is often referred to as the ryanodine receptor, since it has a very high affinity for ryanodine (Lai et al., 1988). Ryanodine receptors are also present in the sarcoplasmic reticulum of vascular smooth muscle, however, the expression of these receptors is relatively low (Hakamata et al., 1992). The ryanodine receptor has been purified from vascular smooth muscle (Herrmann-Frank et al., 1991). This receptor is activated by Ca^{2+} and caffeine and inhibited by ruthenium red, substances

that have no effect on the Ins(1,4,5)P_3 receptor (Ehrlich and Watras, 1988). The increased $[Ca^{2+}]_i$ induced by Ins(1,4,5)P_3 also activates its own release through CICR (Blatter and Wier, 1992). Recently, cADP ribose, a nicotinamide adenine dinucleotide (NAD^+) metabolite, has been suggested to be a physiological agonist of the ryanodine receptors (Berridge, 1993; Galione, 1993). The physiological role of CICR in vasoconstriction is not fully understood.

3.2. Phosphoinositide Cascade and Vasoconstriction

In many cases, neurotransmitters, hormones, and growth factors bind to their corresponding receptors, triggering a series of events that lead to activation of phospholipase C (PLC) through G-proteins. The hydrolysis of phosphoinositide by PLC, which produces two second messengers, Ins(1,4,5)P_3 and diacylglycerol (DAG), is the major controller of subsequent events (Berridge, 1987; Rana and Hokin, 1990). Inositol 1,4,5-trisphosphate is released into the cytoplasm whereas DAG remains in the membrane. Inositol 1,4,5-trisphosphate has been shown to release calcium from nonmitochrondrial stores (Streb et al., 1983) while DAG activates PKC (Nishizuka, 1984). These actions trigger cellular responses, such as secretion, smooth muscle contraction and growth.

In animal cells there are three major myo-inositol-containing phospholipids: phosphatidylinositol (PI), which usually accounts for over 90% of the total inositol lipid, phosphatidylinositol 4-phosphate (PIP) and phosphatidylinositol 4,5-bisphosphate (PIP_2). The phosphorylated inositides (PI, PIP, and PIP_2) generally constitute 1-10% of the total inositol lipids. The amount of

PIP_2 that is available for hydrolysis is only sufficient to maintain Ins(1,4,5)P_3 production for a few minutes, however, this hormone-sensitive PIP_2 pool is rapidly replenished from part of the much larger PI pool by the action of PI and PIP kinases (Hokin, 1985).

3.2.1. Isoforms of phospholipase C

Three types of PLC have been identified in mammalian tissues by direct protein isolation, immunological characterization and molecular cloning studies: β, γ, and δ. Each type contains more than one subtype. There are three PLC-β (β_1, β_2 and β_3), two PLC-γ (γ_1 and γ_2) and three PLC-δ (δ_1, δ_2 and δ_3) subtypes. All three subtypes are single polypeptides and have similar catalytic properties. They hydrolyze three common phosphoinositides: PI, PIP and PIP_2 with the hydrolysis of PIP_2 being most sensitive. Taylor et al. (1992) have provided evidence that the PLC isozyme previously designated α is probably a proteolytic product of PLC-δ_1.

The G-proteins involved in signal transduction are heterotrimes composed of diverse α, β and γ subunits (Gilman, 1987). Currently, it is known that Gq and Gi subfamilies are the regulators coupled to the PLC pathway (Exton, 1994; Hepler and Gilman, 1992; Sternweis and Smrcka, 1992). To date, there are two discrete mechanisms by which PLC isozymes are activated by receptors. Phospholipase C-β_{1-3} are regulated both by Gα subunits of the Gq class (Gα q/11, Gα 16) and by G$\beta\gamma$ subunits. There is compelling evidence that the pertussis toxin-insensitive activation of PLC is mediated by the α subunit of Gq. All PLC-β isozymes are activated by Gαq, but PLC-β_1 and PLC-β_3 are more responsive. The pertussis toxin-sensitive activation of PLC is mediated

by the $\beta\gamma$ subunit of Gi, to which PLC-β_3 is most sensitive (Cockcraft and Thomas, 1992; Exton, 1994; Liscovitch and Cantley, 1994). Phospholipase C-γ isozymes are regulated by tyrosine kinases (Cockcraft and Thomas, 1992; Liscovitch and Cantley, 1994). The mechanism of PLC-δ activation has not been identified. It was suggested that the regulation of PLC-δ_1 is through changes in cytosolic Ca^{2+} (Exton, 1994).

3.2.2. Formation of Ins(1,4,5)P_3 and its metabolism

Inositol 1,4,5-trisphosphate is formed from the hydrolysis of the phosphodiester bond linking the phosphorylated inositol unit to the acetylated glycerol moiety by phospholipase C. At the same time, DAG is also generated by the action of PLC. The metabolism of Ins(1,4,5)P_3 is a more complex process and leads to increase in the levels of a variety of inositol phosphate isoforms. There are two routes for catabolism of Ins(1,4,5)P_3: 1) dephosphorylation and 2) phosphorylation followed by dephosphorylation. In the first route, 1) Ins(1,4,5)P_3 is converted to Ins(1,4)P_2 by inositol polyphosphate 5-phosphatase; 2) Ins(1,4)P_2 is hydrolyzed to Ins(4)P; and 3) Ins(4)P is dephosphorylated to inositol by inositol monophosphatase. In the second route, 1) Ins(1,4,5)P_3 is first phosphorylated to Ins(1,3,4,5)P_4; 2) Ins(1,3,4,5)P_4 is then dephosphorylated to Ins(1,3,4)P_3; 3) Ins(1,3,4)P_3 may then be converted to either Ins(1,3)P_2 or Ins(3,4)P_2 by inositol polyphosphate 4-phosphatase and inositol polyphosphate 1-phosphatase; 4) Ins(1,3)P_2 and Ins(3,4)P_2 are hydrolyzed to Ins(1)P and Ins(3)P by inositol polyphosphate 3-phosphatase and inositol polyphosphate 4-phosphatase, respectively; and 5) both Ins(1)P and Ins(3)P are dephosphorylated to inositol by inositol

monophosphatase.

3.2.3. Phosphoinositide hydrolysis in vascular smooth muscle

Investigation of the phosphoinositide pathway and its regulation in vascular smooth muscle has established the molecular basis of vasoconstriction. Membrane receptors, such as those for α_1-adrenergic agonists, endothelin-1 and 5-hydroxytryptamine, are coupled to phospholipase C via G-proteins.

Phospholipase C-δ_1 (Kato et al., 1992), PLC-α (Griendling et al., 1991) and PLC-γ1 (Inui et al., 1992) have been identified in vascular smooth muscle. The quantity of PLC isozymes has been investigated in rat aorta (Rhee et al., 1991). Phospholipase C-β_1 exists in low quantities in rat aorta (10 ng/mg protein), while PLC-γ_1 and PLC-δ_1 are relatively rich in the same tissue (120 ng/mg protein each). The existence of PLC-α is doubtful since a recent study indicated that PLC-α is actually a proteolytic fragment of PLC-δ1 (Taylor et al., 1992).

Receptor-mediated hydrolysis of phosphoinositide in vascular smooth muscle has been well documented, i.e., α_1-adrenoceptors for α-adrenergic agonists (Chiu et al., 1987; Fox et al., 1985; Rapoport, 1987), 5-HT_2 receptors for serotonergic agonists (Doyle et al., 1986; Roth et al., 1986), ET_A receptors for endothelin (Eguchi et al., 1992), AT_1 receptors for angiotensin II (Ko et al., 1992) and V_1 receptors for vasopressin (Plevin et al., 1992a). Usually, the receptor-mediated hydrolysis of phosphoinositide correlates well with the release of Ca^{2+} from intracellular stores (Chiu et al., 1987; Pijuan et al., 1993) or vasoconstriction (Gu et al., 1991; Pjiuan et al., 1993; Roth

et al., 1986), suggesting a link between these two phenomena.

3.2.4. Inositol 1,4,5-trisphosphate and vasoconstriction

The discovery that Ins(1,4,5)P_3, the product of PLC-mediated hydrolysis of PIP_2, functions as the second messenger for Ca^{2+} release from endoplasmic reticulum constitutes a major breakthrough in the quest to elucidate excitation-function coupling (Berridge, 1987). The concentration of Ins(1,4,5)P_3 in resting cells is about 0.1 μM and increases to 1-2 μM during agonist stimulation (Tarver et al., 1987). Inositol 1,4,5-trisphosphate has been shown to mobilize Ca^{2+} from the sarcoplasmic reticulum of vascular smooth muscle (Hashimoto et al., 1986; Somlyo et al., 1985; Suematsu et al., 1984; Yamamoto and van Breemen, 1985). Furthermore, light activation of caged Ins(1,4,5)P_3 showed that it rapidly released sarcoplasmic reticulum Ca^{2+} at submicromolar concentrations (Walker et al., 1987). In many of these cases the release of Ca^{2+} by Ins(1,4,5)P_3 was found to initiate contraction (Hashimoto et al., 1986; Somlyo et al., 1985). The role of Ins(1,4,5)P_3 in vasoconstriction was also discussed in the section 3.1..

3.2.5. Isoforms of PKC

Protein kinase C covers a family of serine- and threonine-specific protein kinases. Based on their structure and enzymatic properties, PKC isozymes have been classified into three groups: 1) the Ca^{2+}-dependent or conventional PKCs (cPKCs); 2) the Ca^{2+}-independent or novel PKCs (nPKCs); and 3) the atypic PKCs (aPKCs). The PKC isoforms α, β_1, β_2,

and γ define cPKC group, the isoforms δ, ϵ, η and θ belong to the nPKC group, and the isoforms ζ and λ are two members of the aPKC group (Liscovitch and Cantley, 1994; Nishizuka, 1992). The cPKC enzymes are activated by Ca^{2+}, phosphatidylserine, and DAG or phorbol esters. The nPKC enzymes do not require Ca^{2+} and are activated by phosphatidylserine and DAG or phorbol esters. The aPKC are dependent on phosphatidylserine but are not affected by DAG, phorbol esters, or Ca^{2+} (Nishizuka, 1992).

3.2.6. Sources of DAG

The triggers for the activation of the PKC signal transduction pathway, is the production of DAG. In response to agonists, the formation of DAG is usually biphasic: an early transient phase followed by a late sustained phase (Griendling et al., 1986; Sunako et al., 1989). The early phase parallels the increase in $Ins(1,4,5)P_3$. It is suggested that the early transient phase of formation of DAG results from the hydrolysis of PIP_2 and the late sustained phase is due to hydrolysis of phosphatidylcholine (PC) (Nishizuka, 1992). Diacyglycerol may be directly produced from PC by a PLC (Billah and Anthes, 1990; Exton, 1990; Löffelholz, 1989). Alternately, DAG may be produced indirectly via phospholipase D (PLD), yielding phosphatidic acid (Billah and Anthes, 1990; Exton, 1990; Löffelhalz, 1989; Thompson et al., 1991), which is then dephosphorylated by phosphatidic acid phosphohydrolase (Briendly, 1984). The activating effect of DAG on PKC can be mimicked by tumor-promoting phorbol esters *in vitro* (Castagna et al., 1982), which have become useful tools for studying the roles of PKC.

Agonist-induced activation of PLD or stimulation of

hydrolysis of PC in vascular smooth muscle have been well documented. These agonists include: 1) NE (Gu et al., 1992; Jones et al., 1993; Nally et al., 1992, Rapoport and Campbell, 1991); 2) endothelin (Liu et al., 1992); 3) angiotensin II (Lassegue et al. 1993); 4) vasopressin (Plevin et al., 1992b; Thibonnier et al., 1991; Welsh et al., 1990) and 5) ATP (Nally et al., 1992).

3.2.7. Protein kinase C and vasoconstriction

Studies of vascular smooth muscle revealed that phorbol esters initiate a slow but long-lasting contraction (Forder et al., 1985; Jinag and Morgan, 1987; Savineau et al., 1991). The calcium requirement of phorbol ester-induced vasoconstriction is controversial. An increase in force may occur without a change in $[Ca^{2+}]_i$ (Chatterjee and Tejada, 1986; Nishimura et al., 1990; Ruzycky and Morgan, 1989; Satoh et al., 1987) or be dependent on extracellular Ca^{2+} (Danthuluri and Deth, 1984; Gleason and Flaim, 1986; Sawamura et al., 1987). Phorbol esters have been shown to contract Ca^{2+}-depleted vascular smooth muscle (Itoh and Lederis, 1987).

Protein kinase C-α and -β isozymes of the cPKC group have been identified in vascular smooth muscle (Assender et al., 1994; Inoguchi et al., 1992; Singer et al., 1992) as well as in uterine smooth muscle (Karibe et al., 1991). Protein kinase C-ϵ and -ζ isozymes are also expressed in vascular smooth muscle (Dixon et al., 1994; Khalil et al., 1992). It was suggested that Ca^{2+}-independent vasoconstriction is associated with activation of PKC-ϵ (Khalil et al., 1992).

In contrast to the wealth of information accumulated on the activation of this signaling pathway and the

structures of the components within it, there is a dearth of information on physiological substrates for PKC and in particular those whose phosphorylation has a defined effect.

The mechanism of action of PKC on smooth muscle remains to be clarified. The contractile response could result from direct or indirect effects of PKC at one or more steps in the excitation-contraction coupling pathway. Phorbol esters could modulate VOCs in smooth muscle (Fish et al., 1988; Galizzi et al., 1987; Schuhmann and Groschner, 1994). Activation of PKC by phorbol ester has been shown to phosphorylate myosin light chain (Singer, 1990; Sato et al., 1992), MLCK (Nishikawa et al., 1985;) , desmin (Barany et al., 1992), caldesmon (Adam et al., 1989; Barany et al., 1992), and calponin (Winder and Walsh, 1990). It was suggested that the contractile effect initiated by PKC activators, such as phorbol esters, is not due to phosphorylation of myosin light chain by PKC (Inagaki et al., 1987). The physiological importance of phosphorylation of these proteins by PKC is not clear. Recently, it was reported that the activity of myosin light chain phosphatase was inhibited by PKC, and this inhibition is suggested to play an important role in Ca^{2+}-independent vasoconstriction (Itoh et al., 1993).

The effects of activation of PKC on agonist-induced vasoconstriction is complex. Both augmentation (Bendhack et al., 1988; Nally et al., 1992; Sheridan et al., 1991; Shima and Consigny, 1989) and attenuation (Danthuluri and Deth, 1984; Roth et al., 1986; Satoh et al., 1987; Shima and Blaustein, 1992; Villalobos-Molina et al., 1990) have been reported.

Activation of PKC by phorbol esters has been reported to have the following diverse actions on signal transduction pathways in vascular smooth muscle: 1) attenuation of

agonist-induced increase in intracellular Ca^{2+} (Aiyar et al., 1987; Berta et al., 1988; Brock et al., 1985; Caramelo et al., 1988; Satoh et al., 1987); 2) inhibition of agonist-induced phosphoinositide hydrolysis (Araki et al., 1989; Bazan et al., 1993; Chardonnens et al., 1990; Cotecchia et al., 1985; Go et al., 1988; Griendling et al., 1986; McMillan et al., 1986; Pfeilschifter et al., 1989; Reynolds et al., 1989; Roth et al., 1986; Vittet et al., 1986); 3) increased release of arachidonic acid (Reynolds et al., 1989); 4) decreased generation of cGMP (Hirata, 1988; Kawabe et al., 1992); and 5) enhanced formation of cAMP (Kubalak and Webb, 1993; Nabika et al., 1985; Phaneuf et al., 1988).

However, down-regulation of PKC was shown to cause the following: 1) increased agonist-induced phosphoinositide hydrolysis (Pfeilschifter et al., 1989); 2) enhanced cGMP accumulation (Kawabe et al., 1992); 3) inhibited activation of PLD by PKC (Konishi et al., 1991); and 4) enhanced agonist-induced Ca^{2+} mobilization (Pfeilschifter et al., 1989; Stassen et al., 1989).

It is generally agreed that myosin light chain phosphorylation plays an important role in initiating smooth muscle contraction (Kamm and Stull, 1985). However, it is also evident that myosin light chain phosphorylation is not necessary for maintaining isometric force in intact vascular smooth muscle, since intracellular levels of free Ca^{2+} decline toward basal level during the maintenance of tone (Morgan and Morgan, 1984). Therefore, additional regulatory mechanisms for maintaining contraction must be sensitive to lower concentration of intracellular Ca^{2+}.

Studies have shown that force can be developed in the complete absence of increases in myosin light chain phosphorylation (Chatterjee and Tajeda, 1986; Singer and Baker, 1987), that the crossbridge cycling rate can be

regulated independent of changes in myosin light chain phosphorylation (Moreland and Moreland, 1987). Some evidence suggests that PKC can, apparently, directly initiate the development of force supported by a specific population of crossbridges characterized by unphosphorylated myosin light chain and low cycling rates (Fulginiti et al., 1994). Rasmussen (1987) proposed that PKC is responsible for the maintenance of tonic force. Later, it was hypothesized that these two regulatory systems act in parallel in smooth muscle to regulate contraction (Moreland et al., 1991; Morgan et al., 1988). One of these is the Ca^{2+}-dependent myosin phosphorylation-dephosphorylation system responsible for the rapid development of force and the second is a hypothesized Ca^{2+}-dependent system responsible for slow development of force as well as the maintenance of previously developed force. This second system has a higher Ca^{2+} sensitivity than that for myosin light chain phosphorylation (i.e. functioning at a lower $[Ca]_i$) and may be activated by PKC. The total force maintained by smooth muscle is the result of these two regulatory systems acting in concert.

The sensitivity of the regulatory/contractile apparatus to Ca^{2+} can be increased by agonists (Kitazawa et al., 1989; Nishimura et al., 1990). The amount of force developed at a given $[Ca^{2+}]_i$ is significantly greater during excitation by an agonist than during depolarization with high K^+ (Bradley and Morgan, 1987; Himpens et al., 1990; Himpens and Casteels, 1987; Rembold and Murphy, 1988). The Ca^{2+}-sensitizing action of agonists is mediated by G-proteins, as it can be mimicked by GTPγS and inhibited by GDBβS (Kitazawa et al., 1989; Nishimura et al., 1988). Some studies have showed that phorbol esters increased the Ca^{2+} sensitivity of the contractile apparatus (Brozovich et al.

1990; Nishimura et al., 1990).

4. Heterogeneity of α_1-Adrenoceptors

4.1. Adrenoceptor Subtypes

Catecholamines such as NE and Epi, are produced in and released from nerve terminals and the adrenal chromaffin tissue to produce a wide range of effects by activating adrenoceptors. Adrenoceptors were initially subclassified into α and β subtypes on the basis of the rank orders of potency of a series of structurally related catecholamines (Ahlquist, 1948). Later both β- and α-adrenoceptors were subdivided into β_1- and β_2-adrenoceptors (Lands et al., 1967) and α_1- and α_2-adrenoceptors (Langer, 1974). The β-adrenoceptors were divided into two classes, β_1- and β_2-, based on the relative potencies of isoproterenol, Epi and NE (Lands et al, 1967). β_1-Adrenoceptors demonstrate approximately equal affinity for Epi and NE, whereas β_2-adrenoceptors recognize Epi with higher affinity than NE. β_1-Adrenoceptors were found predominantly in heart, while β_2-adrenoceptors were found predominantly in smooth muscle. Langer (1974) proposed that α_1-adrenoceptors were postsynaptic α-adrenoceptors that mediate effector organ response, while α_2-adrenoceptors were presynaptic α-adrenoceptors that regulated neurotransmitter release. However, the discovery of postsynaptic α_2-adrenoceptors in vascular smooth muscle and platelets (Timmermans and van Zwieten, 1981) made it necessary to refine the subclassification of α-adrenoceptor subtypes. At present, the designations of α_1- and α_2-adrenoceptors are based exclusively on the selectivities of agonists and antagonists

for their respective subtypes (Starke, 1981).

Adrenoceptors, both α and β, appear to belong to a large superfamily of membrane receptors that transmit information into the interior of cells through coupling to G-proteins (Gilman, 1987; Lefkowitz and Caron, 1988). However, the signaling mechanisms of the α- and β-adrenoceptor classes are distinct. β-Adrenoceptors are linked to the activation of adenylate cyclase resulting in the generation of the second messenger cAMP. This is true for all β-adrenoceptor types (Bylund, 1992; Lefkowitz et al., 1983). Agonist binding to α_1-adrenoceptors leads to the activation of PLC and alteration in the intracellular calcium concentration (Minneman, 1988). α_2-Adrenoceptors appear to be linked to adenylate cyclase but in an inhibitory fashion (Jakobs et al., 1976). α_2-Adrenoceptors also mediate Ca^{2+} influx through a pertussis toxin-sensitive G protein (Ruffalo et al., 1991).

α_1-Adrenoceptors play a dominant role in the control of blood pressure, nasal congestion, prostate function, and other processes (Minneman, 1988). As such, the number of α_1-adrenoceptors and the drug specificities of each subtypes are of particular therapeutic interest. Therefore, the foregoing brief review will focused on α_1-adrenoceptor subtypes.

4.2. α_1-Adrenoceptor Heterogeneity

α_1-Adrenoceptors are generally defined as receptors which are potently blocked by the competitive antagonists phentolamine and prazosin, and irreversibly blocked by the alkylating agent phenoxybenzamine, and selectively stimulated by the agonists phenylephrine and methoxamine.

Increasing evidence suggests that α_1-adrenoceptors are

not homogenous. In the late 1980s, the development of more selective drugs and the use of molecular biology techniques has provided confirmation of the presence of multiple subtypes (Bylund, 1992). The first definitive classification of α_1-adrenoceptor subtypes, established by use of radioligand binding, was proposed by Morrow and Creese (1986) based on the ability of certain antagonists such as WB4101 and phentolamine to discriminate between high and low affinity binding sites in rat cerebral cortex. Morrow and Creese (1986) designated α_1-adrenoceptors with subnanomolar affinity for WB4101 as α_{1A} and the α_1-adrenoceptors with lower affinity as α_{1B}. Further characterization with the alkylating agent chloroethylclonidine (CEC) was carried by Johnson and Minneman (1987). They demonstrated that CEC could inactivate only approximately 50% of the α_1-adrenoceptors in the rat cerebral cortex and could not inactivate any α_1-adrenoceptors in rat hippocampus despite the fact that all of the α_1-adrenoceptors in both brain regions were sensitive to alkylation by phenoxybenzamine. Thus it appeared that CEC could discriminate between α_1-adrenoceptor subtypes and that these subtypes had a differential distribution within different regions of the brain. Further studies showed that CEC could inactivate nearly all of the α_1-adrenoceptors in rat liver and spleen but very few of the α_1-adrenoceptors in rat vas deferens (Han et al., 1987). Those α_1-adrenoceptors with low affinity sites for WB4101, which are sensitive to CEC , were designated α_{1B}, and those receptors with high affinity for WB 4101 were called α_{1A} (Minneman, 1988). These findings supported the proposed subdivision of α_1-adrenoceptors by Morrow and Creese (1986).

Substantial evidence suggests that α_{1A}-adrenoceptors have a 20- to 100-fold higher affinity than do the α_{1B}-

adrenoceptors for the competitive antagonists 5-methyl-urapidil (Gross et al., 1989; Hanft et al., 1989), (+)-niguldipine (Boer et al., 1989), WB4101 (Morrow and Creese, 1986) and benoxathian (Han et al., 1987b). α_{1B}-Adrenoceptors are highly sensitive to inactivation by the site-directed alkylating agent CEC. Chloroethylclonidine binds equally well to both α_{1A}- and α_{1B}-adrenoceptors, but it does not inactivate the α_{1A} subtype (Han et al., 1987a; Johnson and Minneman, 1987).

In 1986, there was another attempt to classify α_1-adrenoceptors. Prazosin is generally accepted as a potent and highly selective antagonist of α-adrenoceptors. When the receptor dissociation constants for prazosin against a variety of α_1-adrenoceptor-mediated responses are examined, there is a nearly a 100-fold range in the observed antagonist affinity (Flavahan and Vanhoutte, 1986). Based on prazosin's variable antagonism of functional response, Flavahan and Vanhoutte (1986) proposed α_{1L} (low affinity for prazosin) and α_{1H} (high affinity for prazosin) subtypes. Later, Muramatsu and coworkers (1990a) further extended this subdivision to three subtypes: α_{1L}, α_{1H} and α_{1N} (non-α_{1L} or α_{1H}), based on their antagonist affinity and susceptibility to CEC in functional studies. However, this proposal was mainly based on the data obtained from blood vessels and has not been confirmed with binding studies.

4.3. Molecular Biology of α_1-Adrenoceptor Subtypes

Recently, molecular cloning techniques have identified significant heterogeneity within the α_1-adrenoceptor family. Three α_1-adrenoceptor cDNA clones have been isolated, but their relationship to the pharmacologically defined α_{1A}- and α_{1B}-adrenoceptor subtypes remains controversial.

The first α_1-adrenoceptor cDNA clone was isolated from a hamster smooth muscle DDT_1-MF_2 cell library (Cotecchia et al., 1988). Northern analysis showed the mRNA for this clone had a tissue distribution similar to that predicted for the α_{1B}-adrenoceptors. This clone encoded a protein of 515 amino acid residues.

An additional cDNA that encoded a 466-residue polypeptide was isolated from a bovine brain library (Schwinn et al., 1990). Expression of this cDNA resulted in a novel subtype with a unique drug specificity that had a relatively high affinity for α_{1A}-selective drugs but was sensitive to inactivation by CEC. The α_1-adrenoceptor encoded by this clone was designated α_{1C} (Schwinn et al., 1990). Initially, mRNA for α_{1C}-adrenoceptor was shown to have an extremely restricted distribution and was found in detectable quantities in only human hippocampus and rabbit liver (Schwinn et al., 1990). However, a most recent study indicated that α_{1C}-adrenoceptor mRNA predominates in many human tissues, such as liver, heart, cerebellum, and cerebral cortex (Price et al., 1994).

Two other cDNA clones, with sequences differing by only two codons, were isolated independently from rat brain libraries (Lomasney et al., 1991; Perez et al., 1991) and have been designated as α_{1A}- and α_{1D}-adrenoceptor subtypes, respectively. Stringent pharmacological analysis (Perez et al., 1991; Schwinn and Lomasney, 1992) suggested that these two clones were identical and that both express proteins with primarily α_{1B}-subtype pharmacology. This clone has thus been suggested to be designated as the $\alpha_{1A/D}$ subtype (Schwinn and Lomasney, 1992)

However, questions have been raised regarding whether the cloned $\alpha_{1A/D}$-adrenoceptor actually represents the true pharmacologically defined α_{1A} subtype or, a fourth α_{1D}-

adrenoceptor subtype (Perez et al., 1992). Until this issue is resolved, it has been suggested that the cloned α_{1A}-adrenoceptor be referred to as the $\alpha_{1A/D}$ subtype (Schwinn and Lomasney, 1992).

4.4. Signal Transduction Mechanisms

In most cells, the primary functional consequence of α_1-adrenoceptor activation is an increase in intracellular Ca^{2+}. This increase appears to result from the release of Ca^{2+} from intracellular stores and/or the influx of extracellular Ca^{2+} into the cells. Minneman (1988) initially proposed on the basis of functional studies that α_{1A}-adrenoceptors were linked to the influx of extracellular Ca^{2+} and α_{1B}-adrenoceptors were coupled to PLC and release of intracellular Ca^{2+}. However, the signaling mechanisms for α_1-adrenoceptor subtypes are more complex than they were originally proposed. Investigators now generally agree that activation of α_{1B}-adrenoceptors increases the formation of DAG and Ins(1,4,5)P_3 and that Ins(1,4,5)P_3 mobilize Ca^{2+} from intracellular stores. The signaling mechanism for activation by the α_{1A} subtype, however, is less clear. Despite the evidence for influx of extracellular Ca^{2+} through voltage-operated channels (Han et al., 1987b; Han et al., 1990a; Minneman, 1988; Suzuki et al., 1990a; Tsujimoto et al. 1989), α_{1A}-adrenoceptor also increase inositol phosphate formation (Cohen and Almazan, 1993; Esbenshade et al., 1993; Han et al., 1990b; Michel et al., 1993; Wilson and Minneman, 1990). In addition, α_{1B}-adrenoceptors can activate Ca^{2+} influx (Esbenshade et al., 1994; Han et al., 1992; Klijn et al., 1991). Furthermore, all three α_1-adrenoceptor subtypes have been shown to activate PLC via $G\alpha_q$ and $G\alpha_{11}$ (Wu et al., 1992). α_1-Adrenoceptors have also been shown to activate

phospholipase A_2 (Perez et al., 1993) and D (Gu et al., 1992; Llahi and Fain, 1992). The above evidence indicates that α_1-adrenoceptor subtypes may be coupled to multiple signaling mechanisms.

4.5. α_1-Adrenoceptor Subtypes in Vascular Smooth Muscle

Extensive studies have been done on vascular smooth muscle to characterize α_1-adrenoceptor subtypes. Table 1 shows an incomplete summary of α_1-adrenoceptor subtypes in vascular smooth muscle from functional studies. The observations in table 1 suggest that the distribution of the subtypes of α_1-adrenoceptors is species- and tissue-dependent. However, current studies with pharmacological tools also cause confusion. For example, in rat aorta, some authors claimed that only the α_{1B} subtype is present (Han et al., 1990a), while other investigators found the presence of both α_{1A} and α_{1B} subtypes (Piascik et al., 1991). Furthermore, α_{1A} and $\alpha_{1atypic}$ (non-α_{1B}) subtypes have also been found in this vessel (Oriowo and Ruffolo, 1992).

Activation of α_1-adrenoceptors has been reported to increase the influx of extracellular calcium and release of calcium from intracellular stores (Awad et al., 1983; Deth and Lynch, 1981). The rapid phasic contraction caused by norepinephrine are mainly caused by Ca^{2+} release from internal stores, whereas slow tonic contractions requires Ca^{2+} influx from the extracellular space (Deth and van Breemen, 1974, Karaki et al., 1979). Suzuki et al. (1990a) observed that α_{1A}-adrenoceptors caused a tonic response which was predominantly dependent on the influx of extracellular Ca^{2+}, whereas α_{1B}-adrenoceptors stimulated PI hydrolysis/intracellular Ca^{2+} mobilization and caused a phasic response in rabbit aorta. This finding was in

Table 1. Subtypes of α_1-Adrenoceptor in Vascular Smooth Muscle

Tissue	Subtype(s)	Reference
Rat aorta	α_{1A} + $\alpha_{1atypic}$	Aboud et al., 1993
Rat aorta	α_{1A} + $\alpha_{1atypic}$	Oriowo and Ruffolo, 1992
Rat aorta	α_{1B}	Han et al., 1990a
Rat aorta	α_{1B}	Oriowo and Bevan, 1990
Rat aorta	α_{1A} + α_{1B}	Piascik et al. 1991
Rat renal artery	α_{1A}	Han et al., 1990a
Rat mesenteric artery	α_{1A} + α_{1B}	Han et al., 1990a
Rat portal vein	α_{1A} + α_{1B}	Han et al., 1990a
Rat portal vein	α_{1A}	Schwietert et al., 1991a
Rat vena cava	α_{1B}	Sayet et al., 1993
Rabbit aorta	α_{1A} + α_{1B}	Suzuki et al., 1990a
Rabbit aorta	α_{1A} + α_{1B}	Takayanagi et al., 1991
Rabbit aorta	α_{1A}	Torres-Marquez et al., 1991
Rabbit aorta	α_{1A} + $\alpha_{1atypic}$	Oriowo and Ruffolo, 1992
Rabbit iliac artery	α_{1A} + α_{1B}	Takayanagi et al., 1991
Canine aorta	α_{1B}	Hoo et al., 1994
Canine aorta	α_{1A} + $\alpha_{1atypic}$	Oriowo and Ruffolo, 1992
Canine lingual artery	α_{1A}	Skrbic and Chiba, 1992
Guinea pig aorta	$\alpha_{1atypic}$	Oriowo and Ruffolo, 1992
Monkey lingual artery	α_{1A} + α_{1B}	Skrbic and Chiba, 1992

agreement with the scheme for subclassifying α_1-adrenoceptors proposed by Minneman (1988). However, several other lines of evidence questioned the scheme: 1) α_{1B}-Adrenoceptors in rat vena cava was found to be linked to Ca^{2+} influx (Sayet et al., 1993); 2) Rat portal vein mainly contains the α_{1A}-adrenoceptor subtype and both the phasic and tonic responses are mediated by the α_{1A} subtype (Schwietert et al., 1991b); Using myocytes from rat portal vein, it has been found that activation of α_{1A}-adrenoceptors induces the formation of Ins (1,4,5)P_3 and mobilizes calcium from intracellular stores (Lepretre et al., 1994); 3) WB 4101 (a selective α_{1A}-adrenoceptor antagonist), 5-methyl-urapidil (a selective α_{1A}-adrenoceptor antagonist) and CEC (an α_{1B}-adrenoceptor antagonist) all inhibited both phasic and tonic contraction induced by sympathetic nerve stimulation in the perfused rat tail artery (Sulpizio and Hieble, 1991); 4) 5-methyl-urapidil (a selective α_{1A} antagonist) and CEC could not discriminate α_1-adrenoceptor-mediated Ca^{2+} entry from the extracellular fluid and Ca^{2+} release from intracellular stores (Schwietert et al., 1992)

It was suggested that α_{1A}-adrenoceptors play a role in the tonic maintenance of arterial blood pressure and that α_{1B}-adrenoceptors participate in the response to exogenously administered agonists, since CEC treatment had no significant effect on resting systemic arterial blood pressure (Piascik et al., 1990). In support of this was the finding that CEC had only minor effects on α_1-adrenoceptor-mediated increase in diastolic blood pressure in the pithed rat (Schwietert et al., 1992).

5. 5-Hydroxytryptamine Receptors in Vascular Smooth Muscle

5.1. 5-Hydroxytryptamine Receptor Subtypes

It is well known that the biogenic amine 5-hydroxytryptamine (5-HT, serotonin) causes numerous physiological and pharmacological effects in various organs and tissues. Thus, it has been suggested for a long time that 5-HT might act on multiple 5-HT receptors. The first pharmacological evidence for the existence of 5-HT receptor subtypes came from Gaddum and Picarelli in 1957. Their subdivision into M and D receptor subtypes was based on findings in the isolated guinea pig ileum where 5-HT induced contractions by two different mechanisms. In one instance, a direct contractile action on the smooth muscle by 5-HT was antagonized by phenoxybenzamine (Dibenzyline) and the sites which mediated this action were designated as D receptors. A second mechanism involved an indirect action via the release of acetylcholine and this was antagonized by Morphine. The sites responsible for this action were called M receptors (Gaddum and Picarelli, 1957).

The development of radioligand binding and molecular biology techniques has brought a major breakthrough in receptor classification. Based on radioligand binding studies, Bradley et al. (1986) proposed that 5-HT receptors could be divided into three subtypes: 5-HT_1, 5-HT_2 and 5-HT_3. Now it is apparent that 5-HT receptors can be classified into seven subtypes (Hoyer et al., 1994). They comprise the 5-HT_1, 5-HT_2 and 5-HT_3 subtypes, as well as the uncloned 5-HT_4 receptor. The 5-HT_5, 5-HT_6 and 5-HT_7 receptor genes were recently cloned, but the receptors have yet to be fully characterized (Hoyer et al., 1994).

Based on transductional characteristics, it is clear

that receptors for 5-HT fall into two super families: a ligand-gated ion channel super-family (the 5-HT_3 subtype) and a G-protein coupled super-family (the remaining families).

The 5-HT_1 receptor family includes 5-HT_{1A}, 5-HT_{1B}, 5-HT_{1D}, 5-HT_{1E} and 5-HT_{1F} receptors. The 5-HT_{1A}, 5-HT_{1B}, 5-HT_{1D}, 5-HT_{1E} and 5-HT_{1F} receptors have been cloned and shown to share a high degree of homology and to have intronless genes in the coding sequence region. They also share a common transduction system in being negatively coupled to adenylate cyclase, presumably via a common or similar G-protein link. 5-Hydroxytryptamine$_1$ receptors have a high affinity for 5-hydroxytryptamine and 5-carboxamidotryptamine (5-CT) and mediate effects that are antagonized by methiothepin and methysergide, but not by antagonists at 5-HT_2 (ketanserin, spiperone), 5-HT_3 (MDL 72222, tropisetron) or 5-HT_4 (GR 113808, SC 53606) receptors (Brode, 1990; Hoyer et al., 1994).

5-Hydroxytryptamine$_{2A}$, 5-HT_{2B} and 5-HT_{2C} are members of the 5-HT_2 receptor family. All three 5-HT_2 receptors are G-protein-linked and mediate their effects through activation of phospholipase C. In addition, all three subtypes have close homology. 5-Hydroxytryptamine$_2$ receptors have a low affinity for 5-HT. Activation of 5-HT_2 receptors mediates effects that are susceptible to antagonism by ketanserin, but are resistant to antagonism by 5-HT_3 receptor antagonists (MDL 72222, tropisetron) and 5-HT_4 receptor antagonists (GR 113808, SC 53606) (Brode, 1990; Hoyer et al., 1994).

5-Hydroxytryptamine$_3$ receptor activation triggers a rapid depolarization because of a transient inward current response contingent on the opening of cation selective channels (Peters et al., 1992). 5-HT_3 receptors are characterized by their high affinity for cocaine derivatives (ICS 205-930 and MDL 72222) (Brode, 1990; Hoyer et al., 1994).

5-Hydroxytryptamine$_4$ receptors are positively linked to

adenylate cyclase (Dumis et al., 1988). GR 113808 and SB 204070 are the most potent and selective 5-HT_4 receptor antagonist described to date (Ford and Clarke, 1993).

5.2. 5-Hydroxytryptamine Receptors in Vascular Smooth Muscle

The effects of 5-HT on vascular smooth muscle are complex. 5-Hydroxytryptamine can cause either vasoconstriction or vasodilation, depending on the conditions. This complexity is due to the fact that 5-HT can act on different cell types of one tissue (e.g. on smooth muscle or on endothelial cells of the blood vessel wall) or on different subtypes of 5-HT receptors (e.g., 5-HT_1, 5-HT_2 or 5-HT_4 receptors in vascular smooth muscle). To date, three families of 5-HT receptors (5-HT_1, 5-HT_2 and 5-HT_4 subtypes) have been functionally demonstrated to be involved in the vascular responses to 5-HT (Martin, 1994; Saxena and Villalon, 1990).

5-HT_1-like receptors are a group of receptors that have pharmacological characteristics that are similar to 5-HT_1 receptors, but which have not been positively equated with any of the 5-HT_1-binding site subtypes. 5-HT_1-like receptors appear to mediate a number of functional responses which include vasoconstriction and vasorelaxation.

In the majority of blood vessels examined to date, vasoconstrictor 5-HT_1-like receptors co-exist to varying degrees with 5-HT_2 receptors (Docherty and Hyland, 1986; Maclennan and Martin, 1992; van Nueten et al., 1985). However, the distribution and functional importance of this 5-HT receptor type may well be underestimated. For example, in physiological O_2 tension only 5-HT_2 receptors were present in the human umbilical artery, whereas high O_2 tension revealed a vasoconstrictor 5-HT_1-like receptors (MacLennan et al., 1989). In dog saphenous vein, the vasoconstrictor 5-HT_1-like

receptor, with a pharmacological similarity to 5-HT_{1D}, appears to inhibit adenylate cyclase (Sumner and Humphrey, 1990). How this transduction pathway leading to vasoconstriction by 5-HT_1-like receptors needs more study.

A number of reports have now described 5-HT relaxation of tonically constricted, isolated blood vessels in which endothelium was denuded (Feniuk et al., 1983; Martin et al., 1987; Sumner et al., 1989). Available evidence indicates that the 5-HT_1 receptor mediating vasorelaxation is positively coupled to cAMP formation (Sumner et al., 1989; Trevethick et al., 1986).

The 5-HT_1-like receptors which mediate vasoconstriction display a close pharmacological similarity to the 5-HT_{1D} subtypes (Kaumann et al., 1993). However, the 5-HT_1-like receptors which mediate vasorelaxation display a resemblance to the 5-HT_7 receptors (Bard et al., 1993). However, in both cases identity between the functional receptor and the respective gene product remains to be established.

Activation of 5-HT_1-like receptors has been demonstrated to elicit vasorelaxation indirectly, by stimulating the release of an endothelium-derived relaxing factor (EDRF) (Bodelson et al., 1993; Glusa, 1992; Leff et al., 1987; Sumner and Humphrey, 1988). It has been claimed that endothelial 5-HT_1-like receptors are of the 5-HT_{1D} subtype (Schoeffter and Hoyer, 1990).

5-Hydroxytryptamine$_2$ receptors are widely distributed in peripheral tissues (Bradley et al., 1986). 5-HT_{2A} receptors mediate the powerful constrictor actions of 5-HT in a wide range of arteries and veins, especially conduit vessels (Apperley et al., 1976; Cohen et al., 1981; Feniuk et al., 1983; Leff and Martin, 1986). In vascular smooth muscle, activation of 5-HT_2 receptors results in the production of Ins(1,4,5)P_3 and DAG (Cory et al., 1986; Roth et al., 1984;

1986). In addition, 5-HT_2 receptors also mediate Ca^{2+} influx in vascular smooth muscle (Zhang and Dyer, 1991c).

The existence of 5-HT_4 receptors in vascular smooth muscle was reported in sheep pulmonary vein by Cocks and Arnold (1992). These authors found that 5-HT potently relaxed this vessel when it was precontracted with endothelin-1. A similar result was observed in the same tissue by Zhang and Dyer (unpublished observation). Activation of 5-HT_4 receptors in bovine pulmonary artery smooth muscle cells increased the accumulation of cAMP, which was mediated by Gs (Becker et al., 1992).

In addition to its direct vasoconstriction, 5-HT also amplifies responses to other vasoconstrictors (Chang and Owman, 1989; de la Lande, 1992; Meehan et al., 1986; Szabo et al., 1991; van Nueten et al., 1982; Yang et al., 1992). The amplifying effects appear to be mediated via either 5-HT_1-like receptors (Chang and Owman, 1989; de la Lande, 1992) or 5-HT_2 receptors (Meehan et al., 1986; Szabo et al., 1991; van Nueten et al., 1982).

5-HYDROXYTRYPTAMINE INDUCES PHOSPHOINOSITIDE HYDROLYSIS IN THE OVINE UMBILICAL ARTERY

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Abstract

5-Hydroxytryptamine (5-HT) is a potent constrictor of the ovine umbilical artery and contracts the vessel via activation of 5-HT_2 receptors. The present studies were designed to investigate whether 5-HT induces the hydrolysis of phosphoinositide in the ovine umbilical artery. Segments of the ovine umbilical artery were preincubated with [^{3}H]inositol prior to determining the time-course and concentration-response relationship to 5-HT. Inositol

monophosphate ($InsP_1$), inositol bisphosphate ($InsP_2$) and inositol trisphosphate ($InsP_3$) were isolated by anion-exchange chromatography and the radioactivity was counted. Generation of inositol phosphates in response to 5-HT was rapid (5-10 sec), although the increases in the accumulation were not significant. The accumulation quickly returned to unstimulated basal levels followed by secondary significant rises. 5-HT (10^{-6}-10^{-4} M) in a concentration-dependent manner stimulated the hydrolysis of phosphoinositide during a 30 min exposure to the agonist. The 5-HT-induced accumulation of inositol phosphates was inhibited by ketanserin, a selective 5-HT_2 receptor antagonist, suggesting the involvement of 5-HT_2 receptors.

Phosphoinositide; 5-hydroxytryptamine; Umbilical artery

1. Introduction

It is well known that agonist-induced vasoconstriction is accompanied by activation of phospholipase C (Abdel-Latif, 1986). The hydrolysis of phosphatidylinositol 4,5-bisphosphate (PIP_2) generates inositol 1,4,5-trisphosphate ($Ins(1,4,5)P_3$ and diacylglycerol (DAG). Inositol 1,4,5-trisphosphate has been shown to release Ca^{2+} from intracellular stores in smooth muscle thereby initiating contraction (Somlyo et al., 1985; Suematsu et al., 1984). Activation of PKC may have a role in maintaining the sustained phase of contraction (Rasmussen et al., 1987).

Increased formation of inositol phosphates induced by 5-hydroxytryptamine (5-HT) has been shown in various vascular smooth muscle (Berta et al., 1986; Doyle et al.,

1986; Nakaki et al., 1985). 5-Hydroxytryptamine$_2$ receptor antagonists inhibit the 5-HT-induced increase in inositol phosphates suggesting that activation of 5-HT_2 receptors are involved in phosphoinositide turnover (Cohen and Wittenauer, 1987; Roth et al., 1986).

5-hydroxytryptamine is a potent constrictor of umbilical artery (Dyer, 1970; Maclean et al., 1992). The contraction induced by 5-HT was found to be mediated by 5-HT_2 receptors (Zhang and Dyer, 1990). In the present study we have investigated the effect of 5-HT on phosphoinositide hydrolysis in the ovine umbilical artery.

2. Materials and Methods

2.1. *Materials*

Myo-[^{3}H]inositol (16 Ci/mmol) was obtained from New England Nuclear (Boston, MA). Dowex AG 1X8 (formate form) and disposable columns (10 ml) were from Bio-Rad Laboratories (Richmond, CA). 5-Hydroxytryptamine creatinine was purchased from Sigma Chemical (St. Louis, MO). Ketanserin tartrate was from Janssen (Beerse, Belgium).

2.2. *Tissue preparation.*

Mixed breed sheep within 2 weeks of term (145 days) were euthanized with an i.v. injection of pentobarbital sodium. Umbilical cords were quickly removed and placed in an oxygenated modified Krebs' solution of the following composition (in mM): NaCl, 115.21; KCl, 4.70; $CaCl_2$, 1.80; $MgSO_4$, 1.16; KH_2PO_4, 1.18; $NaHCO_3$, 22.14 and dextrose, 7.88. Disodium EDTA (0.03 mM) was added to suppress the oxidation

of amines. The arteries were carefully cleaned free from connective tissue and cut into 0.4-0.5 cm wide ring segments.

2.3. *Inositol Phosphates Determination.*

Production of inositol phosphates was measured by modification of the procedure previously described by Roth et al. (1986). Briefly, the segments were preincubated in Krebs' solution at 37 °C for 30 min under a 95% O_2-5% CO_2 atmosphere and then transferred to vials containing 20 μCi/ml of myo-[^{3}H]inositol. The incubation was continued for 4 hrs. After washing 3 times with warm oxygenated Krebs' solution to remove free myo-[^{3}H]inositol, LiCl (final concentration 10 mM) was then added to the incubation solution. Following 15 min of incubation, the following protocols were used : 1) 10^{-4} M 5-HT was applied (for time-course study) and the tissues were incubated for various times; 2) 5-HT was added at various concentrations for the concentration-response study; 3) ketanserin (10^{-7} M) was added 30 min before 10^{-4} M 5-HT was applied. At the end of the incubation, the reaction was terminated by immersing the ring segments in liquid N_2. The tissues were then transferred to 2 ml ice-cold methanol:chloroform:HCl (200:100:1) for approximately 3 min and then were rapidly blotted dry, weighed and homogenized in the same methanol:chloroform:HCl mixture. Chloroform (1 ml) and water (1.1 ml) were added to the homogenate. After standing on ice for 4 hrs, the homogenate were centrifuged to separate the phases. The upper phase was then added to 1 ml of Dowex AG 1X8 (formate form) resin packed in a disposable column (Bio-Rad). The columns were then washed sequentially with 15 ml of water to elute free myo-[^{3}H]inositol, 15 ml of 0.2 mM

ammonium formate-0.1 M formic acid to elute inositol monophosphate ($InsP_1$), 15 ml of 0.6 M ammonium formate-0.1 M formic acid to elute inositol bisphosphate ($InsP_2$) and finally 15 ml of 1.0 M ammonium formate-0.1 M formic acid to elute inositol trisphosphate ($InsP_3$). One ml of each fraction was then transferred to liquid scintillation vials and 15 ml scintillation cocktail was added and radioactivity was determined by a liquid scintillation spectrophotometry.

2.4. *Statistics.*

Results are expressed as means ± S. E.; n, the number of observations refers to the animals used. Student's t test was used and a p value of <0.05 was taken as significant.

3. Results

3.1. *Time-course of 5-HT-stimulated formation of inositol phosphates*

The effect of duration of stimulation on the accumulation of individual inositol phosphates in response to 5-HT in the ovine umbilical artery was investigated. As shown in fig. 1, following stimulation with 10^{-4} M 5-HT, there were small transient but not significant increases in the formation of $InsP_1$ and $InsP_2$ (20.3% and 7.5%, respectively), then the accumulation of these two inositol phosphates declined towards unstimulated basal levels followed by a secondary increase after 5 min which were significantly higher than the unstimulated basal values at 30 min (107% and 98% for $InsP_1$ and $InsP_2$, respectively). The accumulation of $InsP_3$ was biphasic. 5-Hydroxytryptamine

stimulated a slow rise of accumulation of $InsP_3$ which reached a peak of approximately 35% at 60 to 90 sec. $InsP_3$ then declined towards unstimulated basal level at 2 min and then continued to rise during the 5-30 min period.

3.2. *Concentration-response of 5-HT-stimulated formation of inositol phosphates*

Fig. 2 illustrates the concentration-response curve for 5-HT-induced formation of inositol phosphates in the ovine umbilical artery. Stimulation of the umbilical artery with 5-HT for 30 min increased the accumulation of $InsP_1$ and $InsP_2$ at 5-HT concentrations of 10^{-6}-2 X 10^{-4} M. The significant accumulation of $InsP_3$ was observed only at the concentrations of 10^{-5}, 10^{-4} M and 2 X 10^{-4} M.

3.3. Effect of Ketanserin on 5-HT-induced formation of inositol phosphates

As shown in fig. 3, 10^{-4} M 5-HT produces significant increases in the accumulation of $InsP_1$, $InsP_2$ and $InsP_3$. Pretreatment of tissues with ketanserin (10^{-7} M), a $5\text{-}HT_2$ antagonist, significantly blocked the increase of inositol phosphates, indicating involvement of $5\text{-}HT_2$ receptors.

4. Discussion

The hydrolysis of phosphoinositide is an essential step in signal transducing mechanism for vasoconstrictyors. Extensive studies of 5-HT-induced phosphoinositide turnover in blood vessels have been made in aorta (Cohen and

Fig. 1. Time-course for 5-HT-induced accumulation of inositol phosphates in the ovine umbilical artery. The basal accumulation at time 0 for $InsP_1$, $InsP_2$ and $InsP_3$ were 144.4±13.3, 89.0±11.7 and 22.5±6.8 cpm/mg wet tissue, respectively. Each point represents the mean±S.E. of 5-6 animals. * Significantly different from corresponding basal accumulation, $P<0.05$.

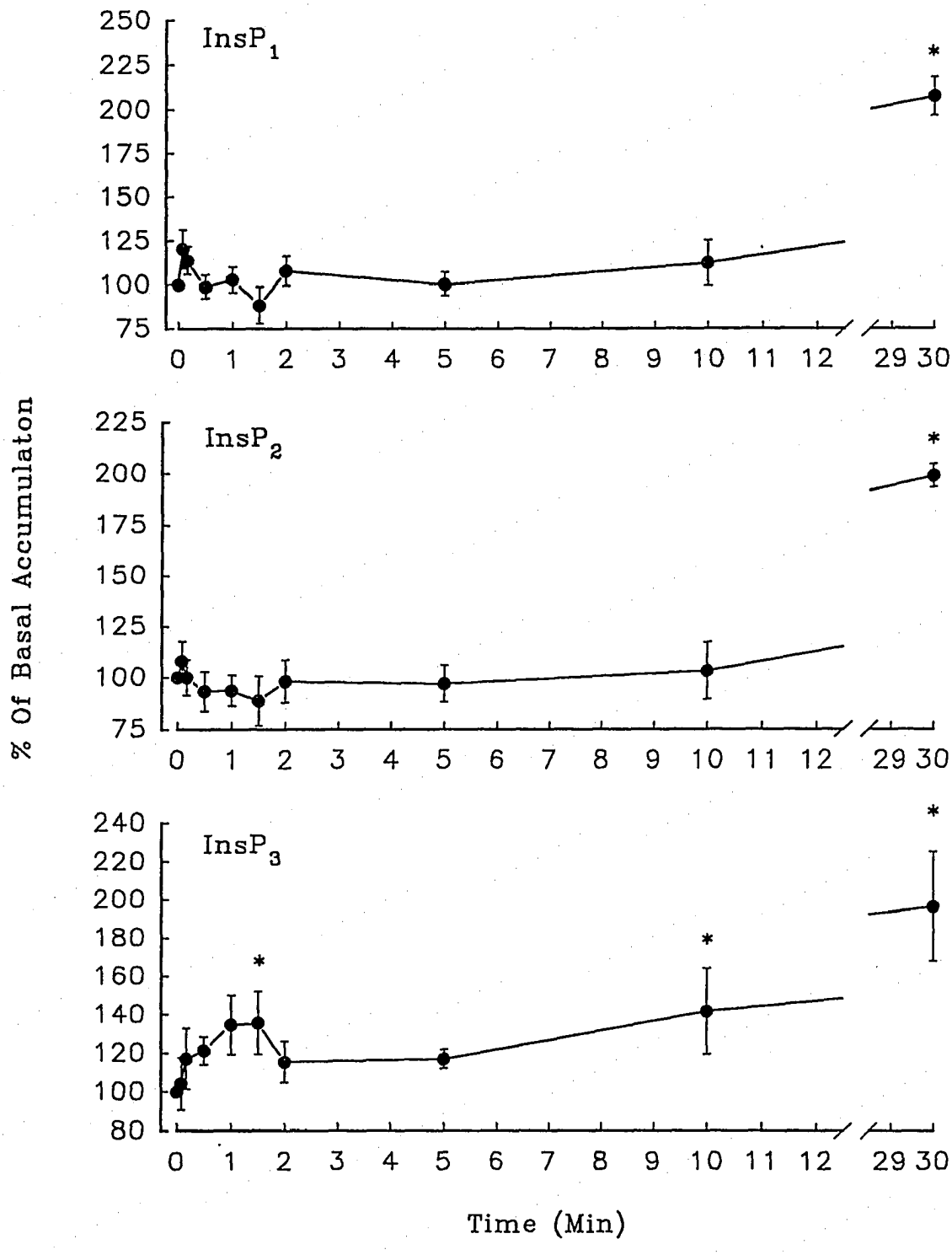

InsP1
InsP2
InsP3
% Of Basal Accumulaton
Time (Min)
250
225
200
175
150
125
100
75
240
220
200
180
160
140
120
100
80
0 1 2 3 4 5 6 7 8 9 10 11 12 29 30
*

Fig. 2. Concentration-response relationship for 5-HT-induced accumulation of inositol phosphates in the ovine umbilical artery. The basal accumulation (tissue incubated without 5-HT for 30 min) for $InsP_1$, $InsP_2$ and $InsP_3$ were 127.8±17.8, 63.7±7.8 and 38.5±9.4 cpm/mg wet tissue, respectively. Each points represents the mean±S.E. of 5-6 animals.
* Significantly different from corresponding basal accumulation, $P<0.05$.

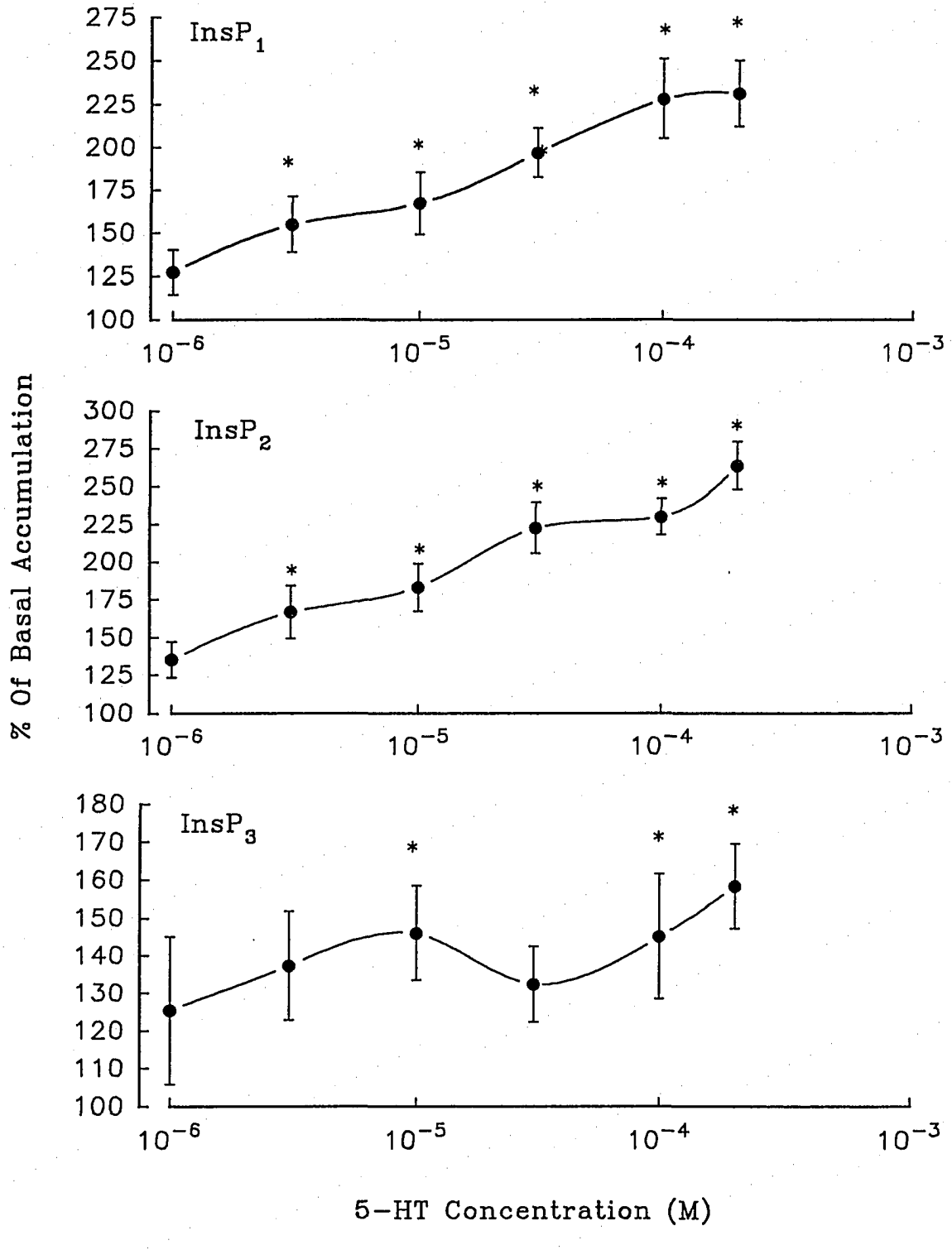
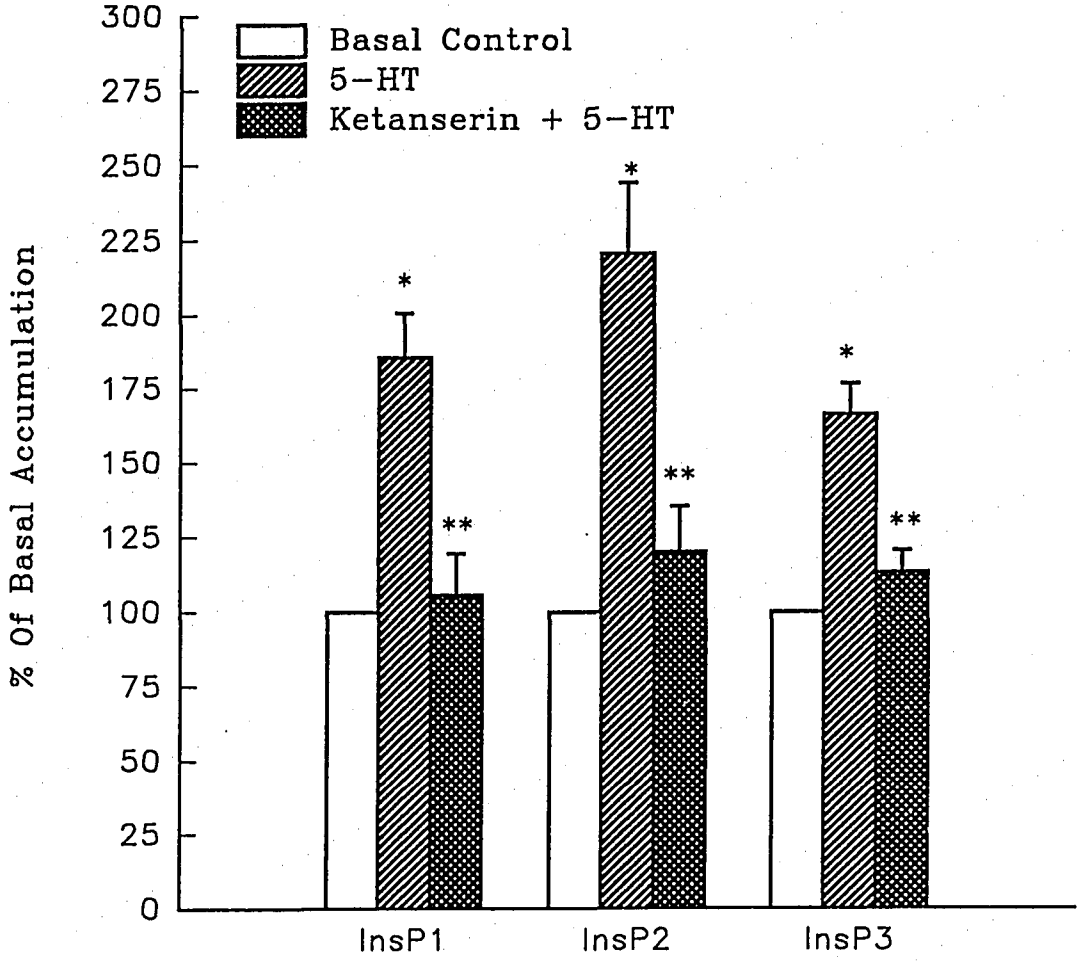

InsP1
InsP2
InsP3
% Of Basal Accumulation
5-HT Concentration (M)
10^-6
10^-5
10^-4
10^-3

Fig. 3. Effects of ketanserin (10^{-7} M) on the accumulation of inositol phosphates in response to 5-HT (10^{-4} M) in the ovine umbilical artery. The basal accumulation (tissue incubated without 5-HT for 30 min) for $InsP_1$, $InsP_2$ and $InsP_3$ were 117.6±12.6, 60.4±5.0 and 37.5±8.1 cpm/mg wet tissue, respectively. Each point represents the mean±S.E. of 5 animals. * Significantly different from corresponding basal accumulation, $P<0.05$. ** Significantly different from 5-HT-stimulated accumulation, $P<0.05$.


Basal Control
5-HT
Ketanserin + 5-HT
% Of Basal Accumulation
300
275
250
225
200
175
150
125
100
75
50
25
0
*
**
*
**
*
**
InsP1
InsP2
InsP3

Wittenauer, 1987; Nakaki et al., 1985; Roth et al., 1986) and smooth muscle cell lines derived from the aorta (Doyle et al., 1986; Pauwels et al., 1989). To our knowledge, there is no study of phosphoinositide hydrolysis conducted on the umbilical artery.

5-Hydroxytryptamine contracts vascular smooth muscle via interaction with 5-HT_2 and 5-HT_1-like receptors (Saxena, 1989). In the ovine umbilical artery, 5-HT produces vasoconstriction via 5-HT_2 receptors (Zhang and Dyer, 1990). Interaction with 5-HT_2 receptors has been shown to increase phosphoinositide hydrolysis in vascular smooth muscle (Roth et al., 1986; Doyle et al., 1986; Cohen and Wittenauer, 1987) and there is a high correlation between 5-HT-induced contraction and phosphoinositide hydrolysis (Roth et al., 1986). Our results suggest that as in the aorta (Roth et al. 1986), 5-HT stimulates the hydrolysis of phosphoinositide via 5-HT_2 receptors in the umbilical artery.

The breakdown of PIP_2 is a rapid process, which is usually observed within seconds (Rana and Hokin, 1990). Generally, time-course studies have showed that agonists increase the accumulation of $InsP_3$ by 10-20% within 15 sec (Abdel-Latif, 1986). Our data showed an increase of 17% of $InsP_3$ at 10 sec, although this increase was not significant. Our finding is consistent with the conclusion by Abdel-Latif (1986). There are two alternative routes for the metabolism of Ins(1,4,5)P_3. In one pathway it is sequentially dephosphorylated to free inositol, whereas in the other pathway it is transformed into Ins(1,3,4,5)P_4 before being dephosphorylated. The half-life of Ins(1,4,5)P_3 is ~4 sec and Ins(1,3,4)P_3 has a slower turnover than Ins(1,4,5)P_3 (Berridge, 1987; Rana and Hokin, 1990). In addition, lithium has been shown to selectively enhance accumulation of

Ins(1,3,4)P_3 but not that of Ins(1,4,5)P_3 (Rana and Hokin, 1990). Therefore, the continuous increase in $InsP_3$ after 5-10 sec in our study may reflect the accumulation of Ins(1,3,4)P_3, since isoforms of $InsP_3$ could not be separated with the method used in our study. The accumulation of $InsP_3$ in response to 3 X 10^{-5} M was less than 10^{-5} M 5-HT. A similar phenomenon was also observed by Labelle and Murray (1990) for norepinephrine-induced formation of $InsP_3$ in rat tail artery. Such an event could result from the rapid degradation of $InsP_3$. The difficulty of measuring $InsP_3$ in vascular smooth muscle have been noted by some laboratories (Fox et al., 1985; McMillan et al., 1986).

The accumulation of inositol phosphates in response to 5-HT in the ovine umbilical artery are concentration-dependent, which is similar to that found by Roth et al. (1986) in the rat aorta. Similarly, the accumulation of inositol phosphates was time-dependent, which is in agreement with the findings in cultured vascular smooth muscle cells (Leysen and Pauwels, 1990).

In summary, we have demonstrated that 5-HT induced an increase in inositol phosphates in the ovine umbilical artery. The accumulation of inositol phosphates induced by 5-HT is time-dependent and concentration-dependent and is mediated by 5-HT_2 receptors.

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AMPLIFICATION BY NOREPINEPHRINE OF 5-HYDROXYTRYPTAMINE-INDUCED VASOCONSTRICTION IN THE ISOLATED OVINE UTERINE ARTERY

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Abstract

The interaction between norepinephrine and 5-hydroxytryptamine was investigated in ovine uterine artery. The contractions in response to 5-hydroxytryptamine (5×10^{-8} M) were amplified by pretreatment with subthreshold (10^{-8} M) and threshold (3×10^{-8} M) concentrations of norepinephrine. The amplification by subthreshold concentration of norepinephrine of contractions induced by 5-hydroxytryptamine was abolished by prazosin (10^{-8} M), an α_1-adrenoceptor antagonist, but not by yohimbine (10^{-7} M), an

α_2-adrenoceptor antagonist. The amplifying effects were also markedly inhibited by nifedipine (10^{-6} M), a L-type voltage-operated calcium channel blocker and by H-7 (10^{-5} M), the putative protein kinase C (PKC) inhibitor. Our results suggest that norepinephrine amplifies 5-hydroxytryptamine-induced contractions of ovine uterine artery through α_1-adrenoceptors, and an increase in Ca^{2+} influx and activation of PKC.

Uterine artery; 5-hydroxytryptamine; norepinephrine; amplification

1. Introduction

Both norepinephrine (NE) and 5-hydroxytryptamine (5-HT) are potent vasoconstrictors of the uterine artery (Dyer and Gough, 1971; Ekstrom et al., 1991). The uterine artery is innervated by the sympathetic nervous system and NE is the principal neurotransmitter (Ekesbo et al., 1991). In response to NE, vasoconstriction occurs as a result of activation of α_1-adrenoceptors in the ovine uterine artery (Isla and Dyer, 1990). The plasma concentration of 5-HT is extremely low and almost all of the 5-HT in the blood is contained in the platelets. However, when the platelets aggregate, they will release 5-HT into the blood leading to a high local concentration. Platelets constitute the main source of 5-HT which is potentially available to vascular smooth muscle. 5-Hydroxytryptamine-immunofluorescent nerves have been found in uterine vasculature (Amenta et al., 1992), and it is suggested that these nerves are actually adrenergic nerves in which 5-HT is taken up and stored and that 5-HT could be released as a "false transmitter"

(Jackowski et al., 1989). Kawasaki and Takasaki (1984) suggested that 5-HT released from vascular adrenergic nerve endings may contribute to the maintenance of local vascular tone. The 5-HT-induced contraction of the ovine uterine artery is mediated by 5-HT_2 receptors (Zhang and Dyer, 1990). It is possible, in certain situations, that NE and 5-HT may interact with each other on the uterine vasculature.

In various species, 5-HT has been documented to enhance α_1-adrenoceptor-mediated vasoconstriction (van Nueten et al., 1981; Meehan et al., 1988; Luscher and Vanhoutte, 1988; Xiao and Rand, 1989). In addition, 5-HT also potentiated the contractile responses to acetylcholine (Asano and Hidaka, 1980), histamine (van Nueten et al., 1982), angiotensin II (van Nueten et al., 1982), prostaglandin $F_{2\alpha}$, vasopressin (Xiao and Rand, 1989), and endothelin (Yang et al., 1992).

However, reports on the amplification by NE of vasoconstriction induced by other agonists are relatively few. Norepinephrine was found to amplify contractile responses to acetylcholine (Asano and Hidaka, 1980), 5-HT (Stupecky et al., 1986) and angiotensin II (Prins et al., 1992).

During pregnancy, blood flow through the uterine (caruncular) arteries is the main source to the fetus for oxygen and nutrients. Decreased uterine blood flow in response to vasoactive substances could compromise fetal-maternal exchange. Therefore, interaction between NE and 5-HT on the uterine vasculature during pregnancy may be of importance.

The purpose of the present study was to investigate: 1) whether NE amplifies the 5-HT-induced vasoconstriction on the isolated ovine uterine artery; and 2) the underlying mechanism(s) for the possible amplification.

2. Material and Methods

2.1. *Tissue Preparation*

Adult pregnant mixed breed sheep near term were euthanized with an i.v. injection of phentobarbital. Uterine arteries were carefully removed without stretching and placed in a modified Krebs solution of the following composition (mM): NaCl, 115.21; KCl, 4.70; $CaCl_2$, 1.80; $MgSO_4$, 1.16; KH_2PO_4, 1.18; $NaHCO_3$, 22.14; and dextrose, 7.88. EDTA (0.03 mM) was added to suppress oxidation of amines. Uterine arteries were cleaned of extraneous fat and connective tissue and cut into 3-4 mm rings. The rings were mounted between two stainless wires in 10 ml organ baths containing Krebs solution maintained at 37°C. The Krebs solution was aerated with 95% O_2-5%CO_2. One wire was attached to a fixed support while the second wire was connected to Grass FT 0.3 transducers and contractions were recorded by a Grass polygraph (model 7). The arterial rings were equilibrated under 2 g tension over 60-90 min. The tissues were then exposed to 10^{-6} M 5-HT for 10 min. Subsequently, the rings were washed with fresh Krebs solution twice and allowed to relax to base line. After equilibration for another 30 min, the experimental protocol began.

2.2. *Experimental Protocol*

The rings were repeatedly exposed to 5 X 10^{-8} M 5-HT until a reproducible response was obtained. The interval between each addition of 5-HT was 30 min. Then a subthreshold concentration of NE (10^{-8} M) was added into the

bath for 5 min and followed by the addition of 5 X 10^{-8} M 5-HT. In some experiments, a threshold concentration of NE (3 X 10^{-8} M) was used followed by 5 X 10^{-8} M 5-HT.

In the experiments in which prazosin (10^{-8} M) and yohimbine (10^{-7} M) were used, they were added to the bath 25 min before the addition of the subthreshold concentration of NE (10^{-8} M).

Nifedipine (10^{-6} M) and H-7 (10^{-5} M) were preincubated with the tissues for 25 min before addition of subthreshold concentration of NE (10^{-8} M) or vehicle. Nifedipine experiments were carried out in a darkened room.

2.3. *Drugs*

The following drugs were used: NE bitartrate, 5-HT creatinine sulfate, 1-(5-isoquinolinesulphonyl)-2-metylpiperazine (H-7) (Sigma Chemical Co., St. Louis, MO), prazosin HCl (Pfizer Inc., Brooklyn, NY), yohimbine HCl (Merck, Rahway, NJ), and nifedipine HCl (Ciba Pharmaceuticals, Summit, NJ).

2.4. *Statistics*

Results are expressed as mean ± S. E., n, referred to the number of animals used. Student's t test was used for statistic analysis and $P<0.05$ was taken as significant.

Results

In the isolated ovine uterine artery, both subthreshold and threshold concentrations of NE significantly potentiated 5-HT (5 X 10^{-8} M)-induced

vasoconstriction (Fig. 1). Pretreatment with subthreshold concentrations of NE (10^{-8} M) resulted a 2.7 fold increase in the contractile response initiated by 5-HT (5 X 10^{-8} M). Moreover, a threshold concentration of NE (3 X 10^{-8} M), which by itself evoked only a small increase in tension, amplified almost 4.5 fold the 5-HT-induced contraction. The response to the threshold concentration of norepinephrine was subtracted from the combined response.

The α-adrenoceptor antagonists, prazosin and yohimbine were used to investigate the subtype of α-adrenoceptor involved in the potentiating effect. Amplification of 5-HT by a subthreshold concentration NE (10^{-8} M) in the ovine uterine artery was completely blocked by the α_1-adrenoceptor antagonist, prazosin (10^{-8} M) (Fig. 2), suggesting the involvement of α_1-adrenoceptors. The α_2-adrenoceptor antagonist, yohimbine (10^{-7}) had no effect on the amplification response (Fig. 3), indicating no role for α_2 adrenoceptors in the potentiation of 5-HT-induced contraction by subthreshold concentration of NE. Neither prazosin (10^{-8} M) nor yohimbine (10^{-7} M) significantly altered responses to 5-HT (5 X 10^{-8} M) in the ovine uterine artery (data not shown).

Nifedipine (10^{-6} M), a calcium entry blocker, significantly attenuated (68.1%) the response to 5-HT (5 X 10^{-8} M) in the ovine uterine artery (fig. 4). In the presence of nifedipine, a subthreshold concentration of NE had no potentiating effect on the 5-HT-induced vasoconstriction.

The protein kinase C (PKC) inhibitor, H-7 (10^{-5} M), markedly decreased (45.6%) the 5-HT-induced contractile response in the ovine uterine artery. The amplification of 5-HT-induced vasoconstriction by a subthreshold concentration NE was also blocked by H-7 (fig 5).

Fig. 1. Effect of norepinephrine on contraction induced by 5-hydroxytryptamine (5-HT, 5 X 10^{-8} M) on isolated rings of ovine uterine artery. $NE_{(S)}$: subthreshold concentration of norepinephrine ($NE_{(S)}$, 1 X 10^{-8} M); $NE_{(T)}$: threshold concentration of norepinephrine ($NE_{(T)}$, 3 X 10^{-8} M). The force produced by threshold concentration of norepinephrine was subtracted from the combined force. The values are mean ± S.E. of 5-7 animals. * indicates that values are signficantly different form the control response to 5-hydroxytryptamine ($P < 0.05$).

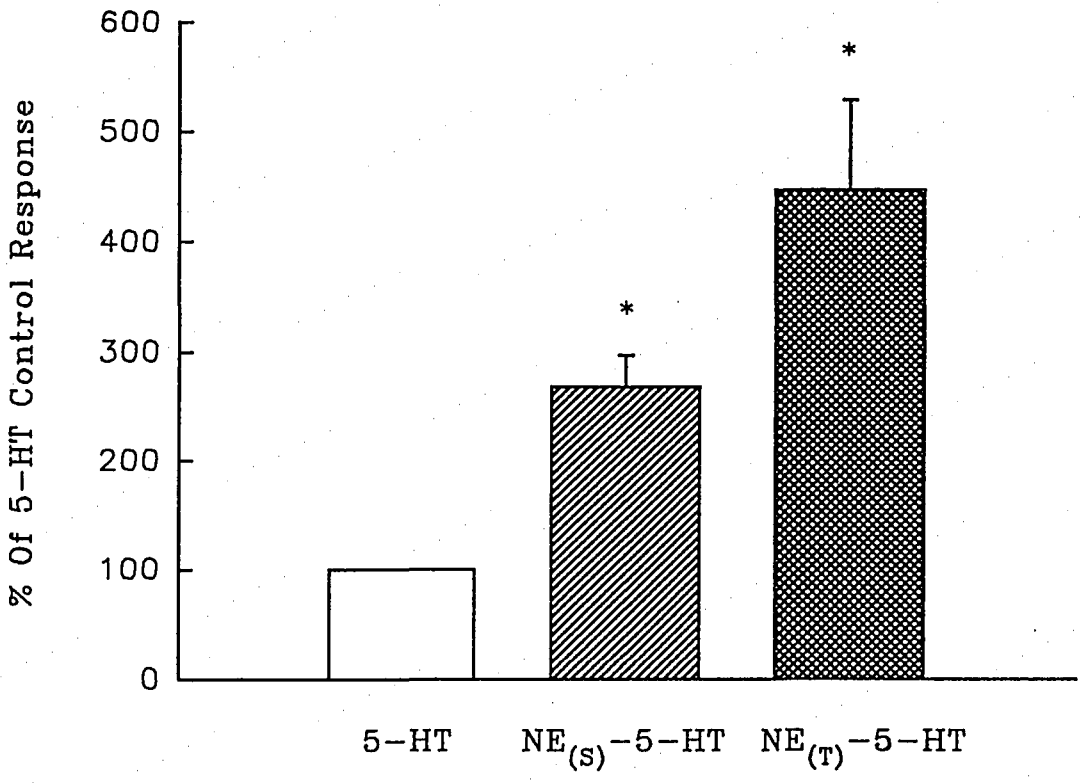

% Of 5-HT Control Response
600
500
400
300
200
100
0
*
*
5-HT
NE(S)-5-HT
NE(T)-5-HT

Fig. 2. Effect of prazosin (Praz, 10^{-8} M) on contractions induced by 5-hydroxytryptamine (5-HT, 5 X 10^{-8} M) in the presence of a subthreshold concentration of norepinephrine ($NE_{(S)}$, 10^{-8} M) on ringss of ovine uterine artery. The values are mean ± S. E. of 5 animals. * Indicates that values are significantly different from the control response to 5-hydroxytryptamine, $P < 0.05$. ** Indicates that values are significantly different from the response to 5-hydroxytryptamine in the presence of $NE_{(S)}$ ($P < 0.05$).

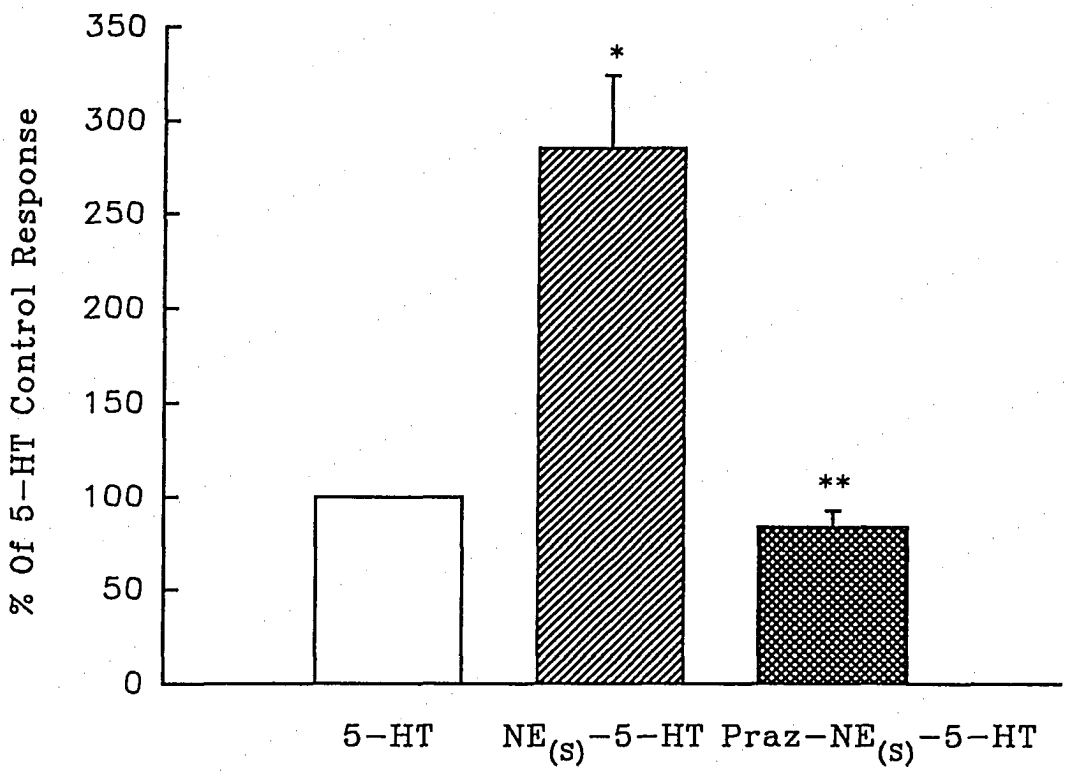

% Of 5-HT Control Response
350
300
250
200
150
100
50
0
*
**
5-HT
NE(S)-5-HT
Praz-NE(S)-5-HT

Fig. 3. Effect of yohimbine (Yoh, 10^{-7} M) on contraction induced by 5-hydroxytryptamine (5-HT, 5 X 10^{-8} M) in the presence of a subthreshold concentration of norepinephrine ($NE_{(S)}$, 10^{-8} M) in rings of ovine uterine artery. The values are mean ± S. E. of 6 animals. * indicates that values are significantly different from the control response to 5-hydroxytryptamine ($P < 0.05$).

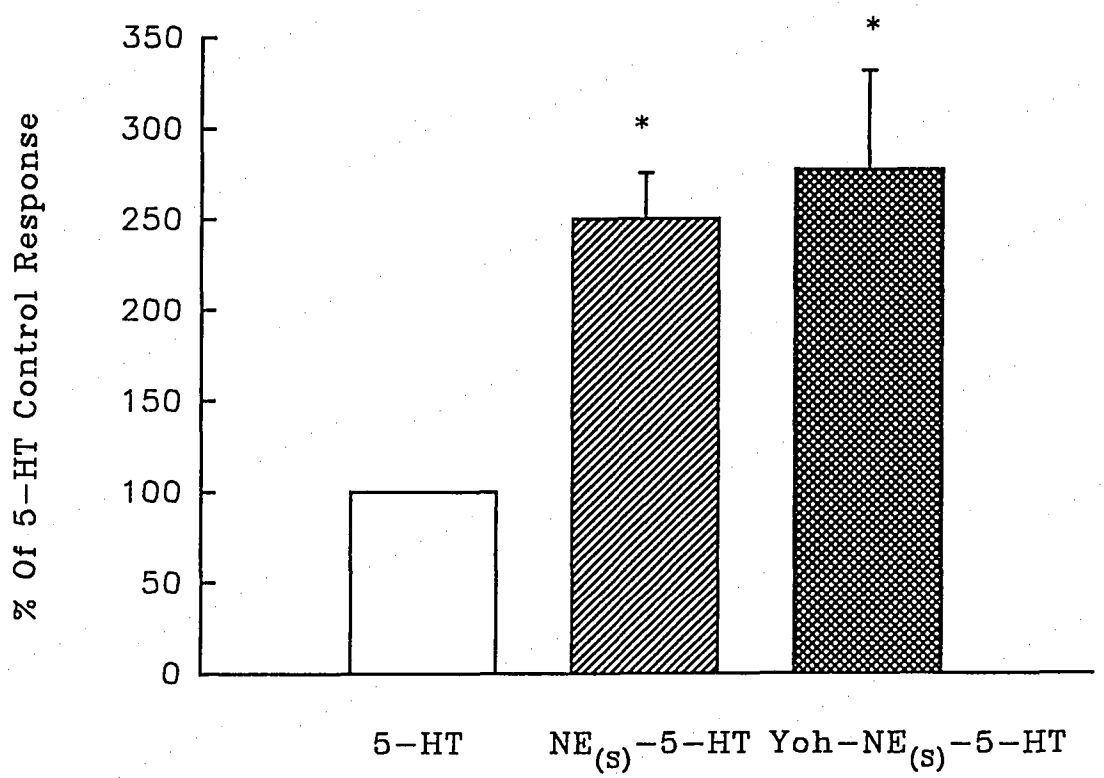

% Of 5-HT Control Response
350
300
250
200
150
100
50
0
*
*
5-HT
NE(s)-5-HT
Yoh-NE(s)-5-HT

Fig. 4. Effect of nifedipine (Nif, 10^{-6} M) on contractions induced by 5-hydroxytryptamine (5-HT, 5 X 10^{-8} M) in the presence or absence of a subthreshold concentration of norepinephrine ($NE_{(S)}$, 10^{-8} M) in rings of ovine uterine artery. The values are mean ± S. E. of 5-7 animals. * indicates that the values are significantly different from the control response to 5-hydroxytryptamine, $P < 0.05$. ** indicates that values are significantly different from the response to 5-hydroxytryptamine in the presence of $NE_{(S)}$ ($P < 0.05$).

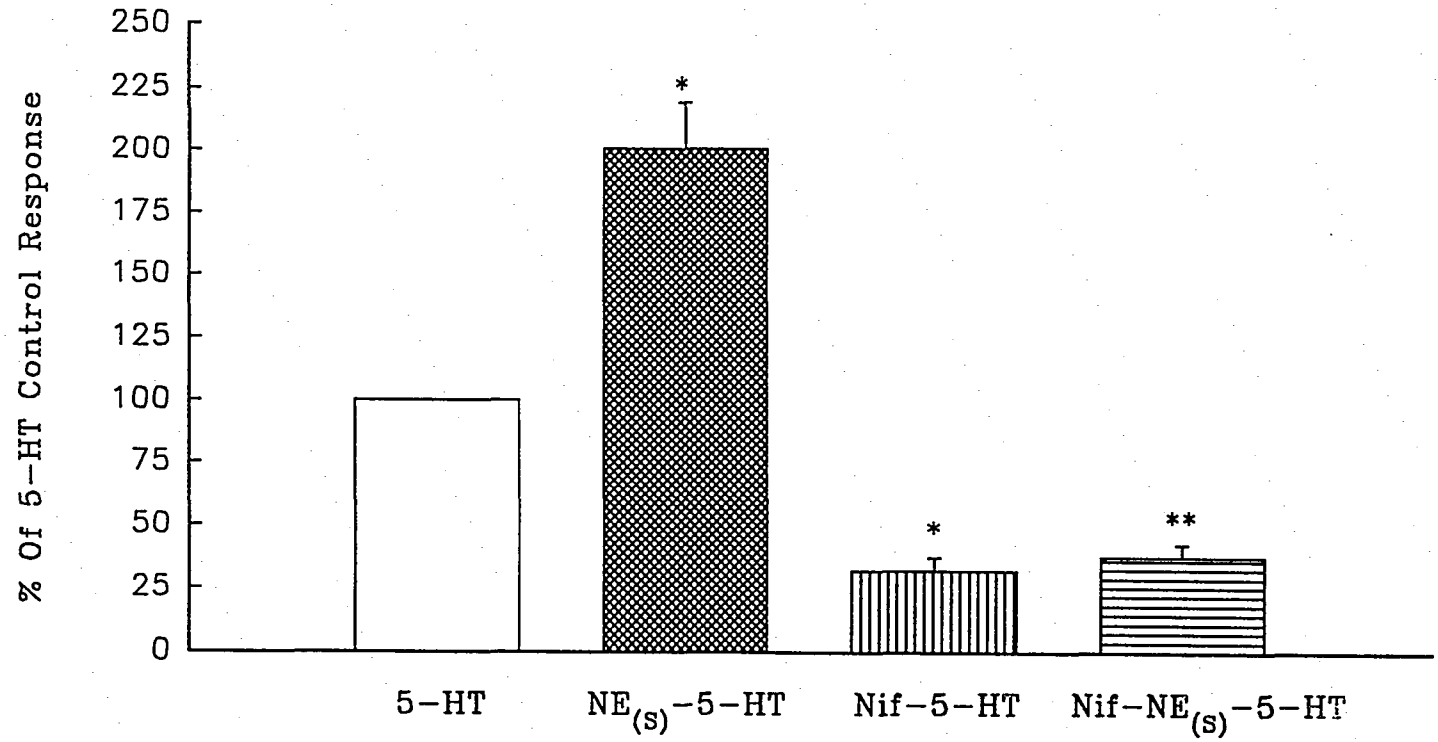
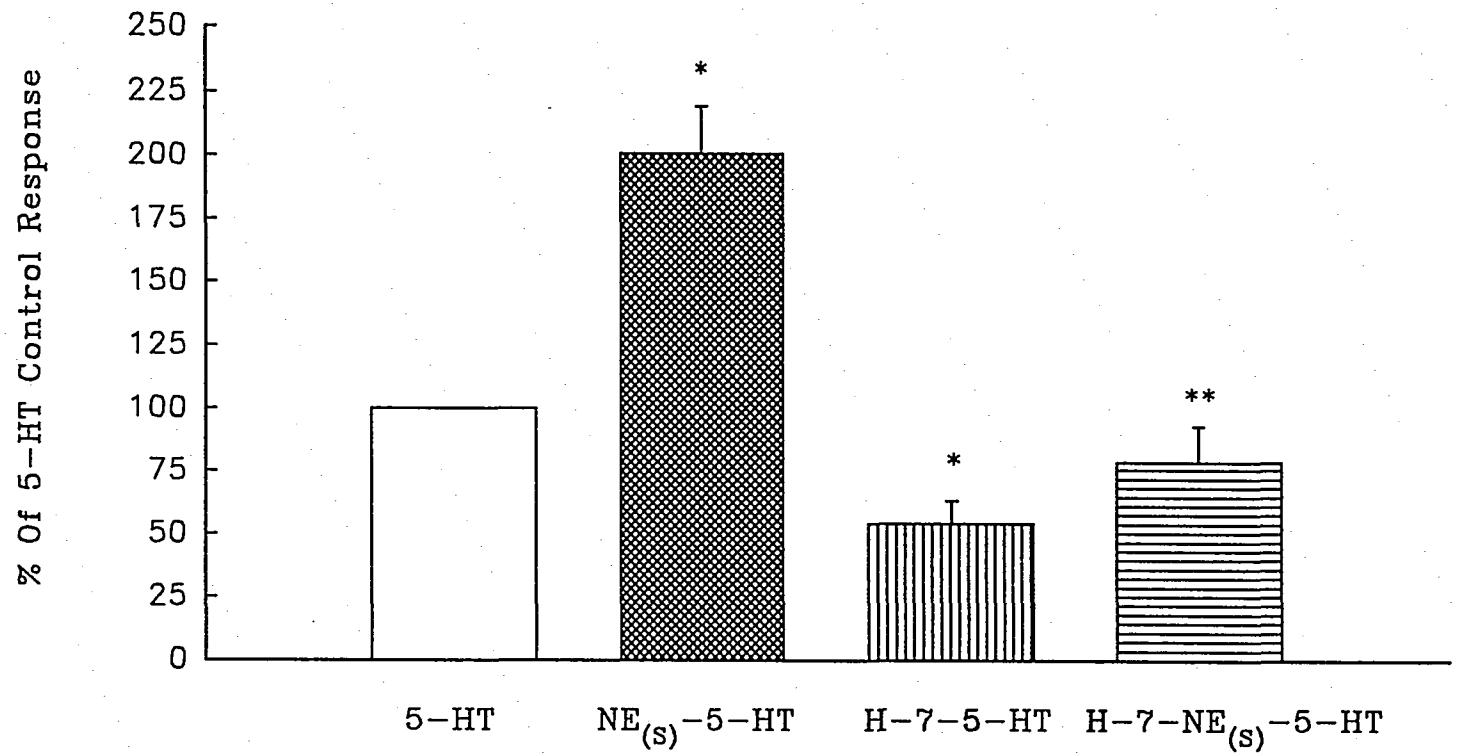

% Of 5-HT Control Response
250
225
200
175
150
125
100
75
50
25
0
*
*
**
5-HT
NE(s)-5-HT
Nif-5-HT
Nif-NE(s)-5-HT

Fig. 5. Effect of H-7 (10^{-5} M) on contractions induced by 5-hydroxytryptamine (5-HT, 5 X 10^{-8} M) in the presence or absence of a subthreshold concentration of norepinephrine ($NE_{(S)}$, 10^{-8} M) in rings of ovine uterine artery. The values are mean ± S. E. of 5-7 animals. * indicates that values are significantly from the control response to 5-hyddroxytryptamine, $P < 0.05$. ** indicates that values are significantly form the response to 5-hydroxytryptamine in the presence of $NE_{(S)}$ ($P < 0.05$).


% Of 5-HT Control Response
250
225
200
175
150
125
100
75
50
25
0
*
*
**
5-HT
NE(S)-5-HT
H-7-5-HT
H-7-NE(S)-5-HT

Comment

In the physiological environment, vascular smooth muscle is exposed to a variety of agonists. Hence the response of vascular smooth muscle to an agonist may be modified by the presence of other agonists. The present study demonstrated that both subthreshold and threshold concentrations of NE amplified 5-HT-induced contraction of ovine uterine artery.

The amplification of 5-HT-induced vasoconstriction by a subthreshold concentration of NE requires the activation of α_1- but not α_2-adrenoceptors, since the amplification response was completely blocked by prazosin, a selective α_1-adrenoceptor antagonist, but not by yohimbine, a selective α_2-adrenoceptor antagonist. These findings are consistent with previous work from this laboratory which found that only α_1- but not α_2-adrenoceptors mediated contraction of the ovine uterine artery (Isla and Dyer, 1990).

Three potential events have been suggested for agonist-induced contraction of smooth muscle: 1) Ins $(1,3,5)P_3$ induced Ca^{2+} release from the sarcoplasmic reticulum; 2) influx of extracellular Ca^{2+}; and 3) modulation of the Ca^{2+}-sensitivity of the contractile regulatory apparatus (Somlyo et al., 1991). Any of these three events could be influenced by the presence of either antagonists or other agonists.

The contractile response to 5-HT of the uterine artery was significantly attenuated by H-7, a putative PKC inhibitor. This observation is compatible with the findings that PKC is present in the ovine uterine artery (Magness et al, 1992) and that phorbol 12,13-dibutyrate contracted the uterine arteries of the guinea pig and sheep (Fallgren et

al., 1989; Hu and Dyer, 1994). The present finding that the contraction to 5-HT was markedly inhibited by nifedipine, a calcium channel blocker, is compatible with observations previously reported from our laboratory (Isla and Dyer, 1990; Zhang and Dyer, 1991).

In the presence of nifedipine or H-7, the amplification of 5-HT-induced contraction by NE was significantly attenuated or completely blocked, indicating the involvement of both voltage-operated calcium channels (VOCs) and PKC. Activation of either α_1-adrenoceptors or 5-HT_2 receptors will increase Ca^{2+} influx (Cauvin et al., 1982; Chiu et al., 1986; Zhang and Dyer, 1991) and stimulate phosphoinositide hydrolysis (Chiu et al., 1987; Roth et al., 1986) in vascular smooth muscle. These observations suggest that activation of α_1-adrenoceptors and 5-HT_2-receptors act on the same signalling pathways to produce contraction.

Subthreshold concentrations of NE might increase the sensitivity of the VOCs to 5-HT and hence increase the availability of Ca^{2+} to the contractile apparatus. There are at least two potential processes by which subthreshold concentrations of NE might amplify 5-HT-induced contraction: 1) partially depolarize the plasma membrane of the vascular smooth muscle, and 2) increase the opening probability of VOCs (Bean, 1989; Nelson, 1990). Either of these two processes would enhance the coupling between VOCs and 5-HT receptors and the availability of Ca^{2+} to the contractile apparatus and thereby amplify the contractions induced by 5-HT. Our results are similar to findings in rabbit femoral artery in which the amplification of angiotensin II-induced vasoconstriction by NE was blocked by nifedipine (Prins et al., 1992), although a threshold concentration of NE was used in their experiment. Furthermore, Bay K8644, a Ca^{2+} channel activator, at concentrations which did not elicit a

contraction, potentiated NE-induced contraction in the rabbit basilar artery (Laher et al., 1989).

Subthreshold concentrations of NE could also enhance the 5-HT-stimulated activity of PKC. As indicated earlier, both α_1-adrenoceptors and 5-HT_2 receptors are coupled to the phosphoinositide cascade. It is possible that G-protein coupled phospholipase C could be partially activated by NE at subthreshold concentration and consequently potentiate 5-HT stimulated formation of Ins(1,4,5)P_3 and diacylglycerol. It has been reported that angiotensin II and endothelin-1 potentiate NE-induced contraction by activating PKC in the rabbit facial artery and aorta (Henrion et al., 1992a; Henrion and Laher, 1993). In addition, the activation of PKC by phorbol ester also amplifies phenylephrine-induced vasoconstriction in the guinea pig aorta (Molderings and Schumann, 1989).

α_1-Adrenoceptor agonists, such as NE and phenylephrine, have been shown to increase the calcium sensitivity of the contractile apparatus in vascular smooth muscle (Kitazawa et al., 1989; Nishimura et al., 1990). The increase in the Ca^{2+} sensitivity of the contractile apparatus is mediated via a G protein and secondary to an increase in myosin light chain phosphorylation and inhibition of myosin light chain phosphatase (Kitazawa et al., 1989). The amplification response could be due to the activation of PKC (Drenth et al., 1989; Nishimura et al., 1990; Ruzycky and Morgan, 1989), since phorbol esters mimic the amplifying effects. Supporting the amplification effect may be an inhibition of myosin light chain phosphatase activity, since phorbol 12,13-dibutyrate inhibited myosin light chain phosphatase activity in the rat aorta (Itoh et al., 1993).

In the rabbit femoral artery, amplification by

angiotensin II of NE-induced contraction was accompanied by an increased Ca^{2+} influx (Purdy and Weber, 1988). However, Henrion et al. (1992a, 1992b) found only PKC but not Ca^{2+} was involved in the amplification by angiotensin II of NE-induced contractions in the rabbit facial artery and aorta. Our results indicated that both Ca^{2+} influx and activation of PKC participated in the amplifying effect by NE of 5-HT-induced contraction of the ovine uterine artery. While our study can not be directly compared to the two above reports, it is possible that the amplification response may vary with species and/or vascular beds.

In summary, we have demonstrated that NE amplifies 5-HT-induced contraction of ovine uterine artery via activation of α_1-adrenoceptors. The amplification involves participation of both VOCs and PKC.

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PHARMACOLOGICAL CHARACTERIZATION OF THE SUBTYPES OF α_1-ADRENOCEPTORS IN THE OVINE UTERINE ARTERY AND UMBILICAL VEIN

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Abstract

The subtypes of α_1-adrenoceptors in the ovine uterine artery and umbilical vein were investigated. The irreversible α_{1B}-adrenoceptor antagonist, chlorethylclonidine (CEC) (5 X 10^{-5} M) inhibited norepinephrine-induced contraction in the ovine uterine artery without depressing the maximal response. Chlorethylconidine significantly reduced the maximal response in the ovine umbilical vein. The calcium channel blocker, nifedipine (10^{-6} M), inhibited norepinephrine-

induced contractions on both blood vessels. The competitive α_{1A}-adrenoceptor antagonist, WB4101, inhibited the norepinephrine-induced contractile response in ovine uterine artery and umbilical vein with pA_2 values of 8.30 and 8.45, respectively. Combined use of CEC with either WB 4101 or nifedipine produced an additive inhibition of norepinephrine-induced contractions on both blood vessels. Prazosin, WB 4101 and CEC all inhibited norepinephrine-induced contraction due to release of calcium from intracellular stores. Our results suggest there is heterogeneity of α_1-adrenoceptors in the ovine uterine artery and umbilical vein and that α_{1A}-adrenoceptors may also be involved in the release of intracellular stored calcium.

α_1-Adrenoceptors; subtypes; uterine artery; umbilical vein

1. Introduction

α_1-Adrenoceptors are not homogeneous and have been subclassified into two subtypes: α_{1A} and α_{1B} based on both binding and functional studies (Bylund et al., 1994; Minneman, 1988). WB4101 and chlorethylclonidine (CEC) have frequently been used as pharmacological tools to assist in the subclassification of α_1-adrenoceptors. Those receptors which are CEC-insensitive and have a high affinity for WB4101 are designated as α_{1A}-adrenoceptors, while those which are CEC-sensitive and have a low affinity for WB4101 are classified as α_{1B}-adrenoceptors. Molecular cloning has confirmed the existence of three subtypes of α_1-adrenoceptors (Catecchia et al., 1988; Lomasney et al.,

1991; Perez et al., 1991; Schwinn et al., 1990). $\alpha_{1A/D}$, α_{1B} and α_{1C} Subtypes are all expressed in vascular smooth muscle (Lomasney et al., 1991; Piascik et al., 1994; Ping and Faber, 1993; Price, 1994).

Blood flow in the uterine artery and umbilical vein are crucial for the growth of the fetus. The uterine artery transports blood rich in O_2 and nutrients to the placenta where O_2 and nutrients are taken up by the fetal blood and CO_2 is discharged into the maternal circulation. The umbilical vein transports the oxygenated blood from the placenta to the fetus. Although the uterine artery is innervated by adrenergic nerve fibers, degeneration occurs during pregnancy (Bell and Malcolm, 1978; Sigger et al., 1986). On the other hand, umbilical vessels are not innervated (Fox and Khong, 1990; Spivack, 1943). Hence, the uterine artery during pregnancy and the umbilical vein are somewhat similar in that they are under little or no influence from the sympathetic nervous system. However, in response to α_1-adrenoceptor agonists, both vessels contract via activation of α_1-adrenoceptors (Isla and Dyer, 1990; Zhang and Dyer, 1991).

The objective of the present study was to investigate and characterize the subtypes of α_1-adrenoceptors in the ovine uterine artery and umbilical vein.

2. Materials and Methods

2.1. *Tissue Preparation.*

Adult pregnant mixed breed sheep near term were euthanized with an injection of pentobarbital sodium. Uterine arteries and umbilical cord were carefully removed

without stretching and placed in a modified Krebs' solution of the following composition (mM): NaCl, 115; KCl, 4.70; $CaCl_2$, 1.80; $MgSO_4$, 1.16; KH_2PO_4, 1.18; $NaHCO_3$, 22.14; and dextrose, 7.88. EDTA (0.03 mM) was added to suppress oxidation of amines. The uterine artery and umbilical vein were cleaned of connective tissues and cut into 3-4 mm ring segments. The ring segments were mounted between two wires in 10 ml organ baths containing Krebs' solution maintained at 37°C. The Krebs' solution was aerated with a mixture of 95% O_2-5% CO_2. One wire was attached to a fixed support while the second wire was connected to Grass FT 0.3 transducers and contractions were recorded by a Grass polygraph (model 7) or a Beckman polygraph (model R611). The segments were equilibrated under 2 g tension over 60-90 min with regular replacement of the bath fluid at 20 min intervals. The ring segments were then primed with 10^{-5} M norepinephrine. After complete washout of the agonist, desimipramine (10^{-7} M), corticosterone acetate (10^{-5} M) and propranolol (10^{-5} M) were included in the Krebs' solution to block neuronal $uptake_1$, extraneuronal $uptake_2$ and β-adrenoceptors, respectively. These agents were in contact with the tissues for 30 min before a protocol began and throughout the protocol. Concentration-response curves were generated by cumulative addition of the agonist in approximately one-half log increments, and the response to each concentration of agonist was allowed to stabilize before the next addition.

2.2. *Determination of pA_2 values for WB 4101.*

Control concentration-response relationship for norepinephrine were constructed in the absence of WB 4101. Following complete washout of the norepinephrine and return

to baseline, one of three concentrations (10^{-8}, 3 X 10^{-8} and 10^{-7} M) of WB 4101 were added to the organ baths and allowed to equilibrate with the tissues for 30 min before obtaining a second concentration-response relationship to the agonist in the presence of the antagonist. PA_2 (=-log K_B) values were determined as described by Arunlakshana and Schild (1959). EC_{50} values for the agonist in the absence (EC_{50}) and presence of the antagonist (EC_{50}') were used to calculate the concentration ratio (CR) (CR = EC_{50}'/EC_{50}) and a plot of log (CR-1) against -log [antagonist] was made to obtain the pA_2 value (X -intercept) and slope of the regression line.

2.3. *Effects of CEC on norepinephrine-induced contractions.*

For studies in which the irreversible α_{1B}-adrenoceptor-alkylating agent, CEC, was used, a control concentration-response relationship was first constructed for norepinephrine. After complete washout and return to baseline, the tissues were then exposed to CEC (5 X 10^{-5} M) for 30 min, followed by complete washout of the antagonist for 60 min at 15 min intervals. Subsequently, a second concentration-response relationship was obtained for norepinephrine.

2.4. *Effects of nifedipine on norepinephrine-induced contractions.*

For studies in which the calcium channel blocker, nifedipine was used, tissues were incubated with nifedipine (10^{-6} M) for 30 min in a darkened room following complete washout of the agonist after obtaining the first concentration-response relationship. After incubation with

nifedipine, a second concentration-response relationship was obtained.

2.5. *Effects of combined use of CEC with prazosin, WB 4101 or nifedipine*

In this protocol, a control concentration-response relationship to norepinephrine was obtained, followed by washout of the agonist from the bath. Subsequently, tissues were exposed to CEC (5×10^{-5} M, 30 min), followed by washout of the antagonist for 60 min at 15 min-interval. The tissues were then incubated in the presence or absence of prazosin (10^{-8} M), WB 4101 (10^{-8} M) or nifedipine (10^{-6} M) for 30 min before obtaining a second concentration-response relationship. The dissociation constants (K_B) for prazosin and WB 4101 were calculated using the equation (Furchgott, 1972): $K_B = [B]/(CR-1)$, where [B] is the concentration of the antagonist, and CR is the concentration ratio described in section 2.2.. Nifedipine studies were conducted in a darkened room.

2.6. *Correction for changes in tissue sensitivity*

In all the above studies, one tissue was run in parallel with the experimental tissues, but received no antagonists and was used to correct for time-dependent changes in agonist sensitivity during the course of the experiment (Furchgott, 1972).

2.7. *Effects of α_1-adrenoceptor antagonists on contractions to norepinephrine in the absence of external calcium*

Tissue were challenged with norepinephrine (10^{-5} M) repeatedly in normal Krebs' solution (1.8 mM Ca^{2+}) until a reproducible response was obtained. The interval between each addition of norepinephrine was 45 min. After complete washout of the agonist and equilibration in normal Krebs' solution for 60 min, the Krebs' solution was replaced with a Ca^{2+}-free Krebs' solution containing EGTA (1 mM). The tissues were washed with Ca^{2+}-free Krebs' solution 3 times for a period of 10 min. α_1-Antagonists (prazosin, 10^{-8} M or WB 4101 10^{-8} M) were then added to the bath for 20 min. The tissues were then exposed to norepinephrine (10^{-5} M). In a separate tissue, after complete washout of norepinephrine (10^{-5} M) in normal Krebs' solution, the tissues were incubated with CEC (5 X 10^{-5} M) for 30 min. The tissue was then washed over 30 min with normal Krebs' solution to remove CEC. The tissue was then washed with Ca^{2+}-free Krebs' solution containing EGTA (1 mM) 3 times and then incubated in the Ca^{2+}-free Krebs' solution continued for another 20 min. The tissue was then exposed to norepinephrine (10^{-5} M). The contractile response to norepinephrine for each tissue in the normal Krebs' solution was taken as 100%.

2.8. *Drugs.*

(-)Norepinephrine bitartrate, desimipramine hydrochloride, propranolol hydrochloride and corticosterone acetate were purchased from Sigma Chemical Co. (St. Louis,

MO). WB4101 and CEC were obtained form Research Biochemical Inc. (Natick, MA). Nifedipine and prazosin hydrochloride were gifts from Ciba Pharmaceuticals (Summit, NJ) and Pfizer Inc. (Brooklyn, NY), respectively.

2.9. *Statistics.*

Results are given as mean ± S.E.. Differences between means were tested for significance using the Student's t-test for paired or unpaired data. A P values of less than 0.05 was taken as significant.

3. Results

3.1. *Norepinephrine sensitivity pD_2 (- $logEC_{50}$) of the uterine artery and umbilical vein*

Norepinephrine produced concentration-dependent contractions of both the ovine uterine artery and umbilical vein (fig. 1). The pD_2 values for norepinephrine were 6.54 ± 0.08 and 6.31 ± 0.07 in the uterine artery and umbilical vein, respectively. There was no difference between the uterine artery and umbilical vein (Table 1).

3.2. *Effects of CEC on norepinephrine contractions*

Chloroethylclonidine (5×10^{-5} M), the selective, irreversible α_{1B}-adrenoceptor antagonist, displaced the concentration-response curve for norepinephrine in the uterine artery to the right but did not reduce the maximal response (fig. 2A, table 2). However, in the umbilical

Fig. 1. Contractile responses induced by norepinephrine in the ovine uterine artery and umbilical vein. Each points represents mean ± S.E. of 5-7 animals.

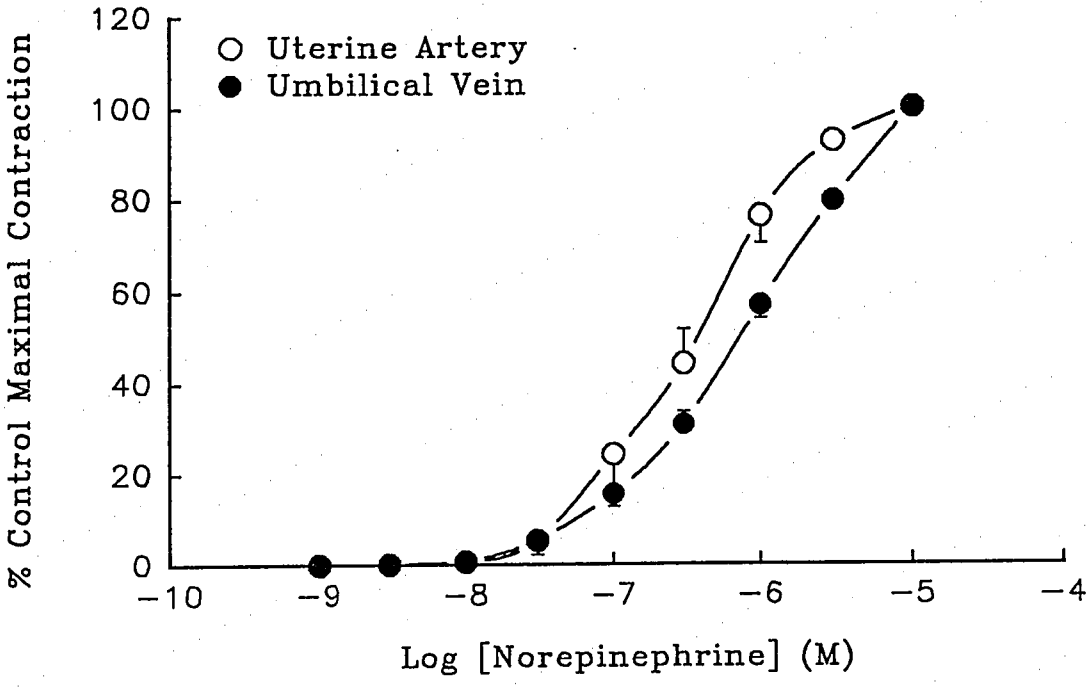

% Control Maximal Contraction
120
100
80
60
40
20
0
Uterine Artery
Umbilical Vein
−10
−9
−8
−7
−6
−5
−4
Log [Norepinephrine] (M)

Table 1

pD_2 values and maximal contractions for norepinephrine in the uterine artery and umbilical vein. Mean ± S.E.

	pD_2	Maximal Contraction (g)
Uterine Artery	6.5 ± 0.1 (5)	31.7 ± 3.3 (5)
Umbilical Vein	6.3 ± 0.1 (7)	11.8 ± 1.7[a] (7)

[a] $P<0.05$, comparing to uterine artery.

Fig. 2. Concentration-response curves for norepinephrine in the absence and presence of CEC (5×10^{-5} M, 30 min) in the ovine uterine artery and umbilical vein. Each point represents mean ± S.E. of 5-7 animals.

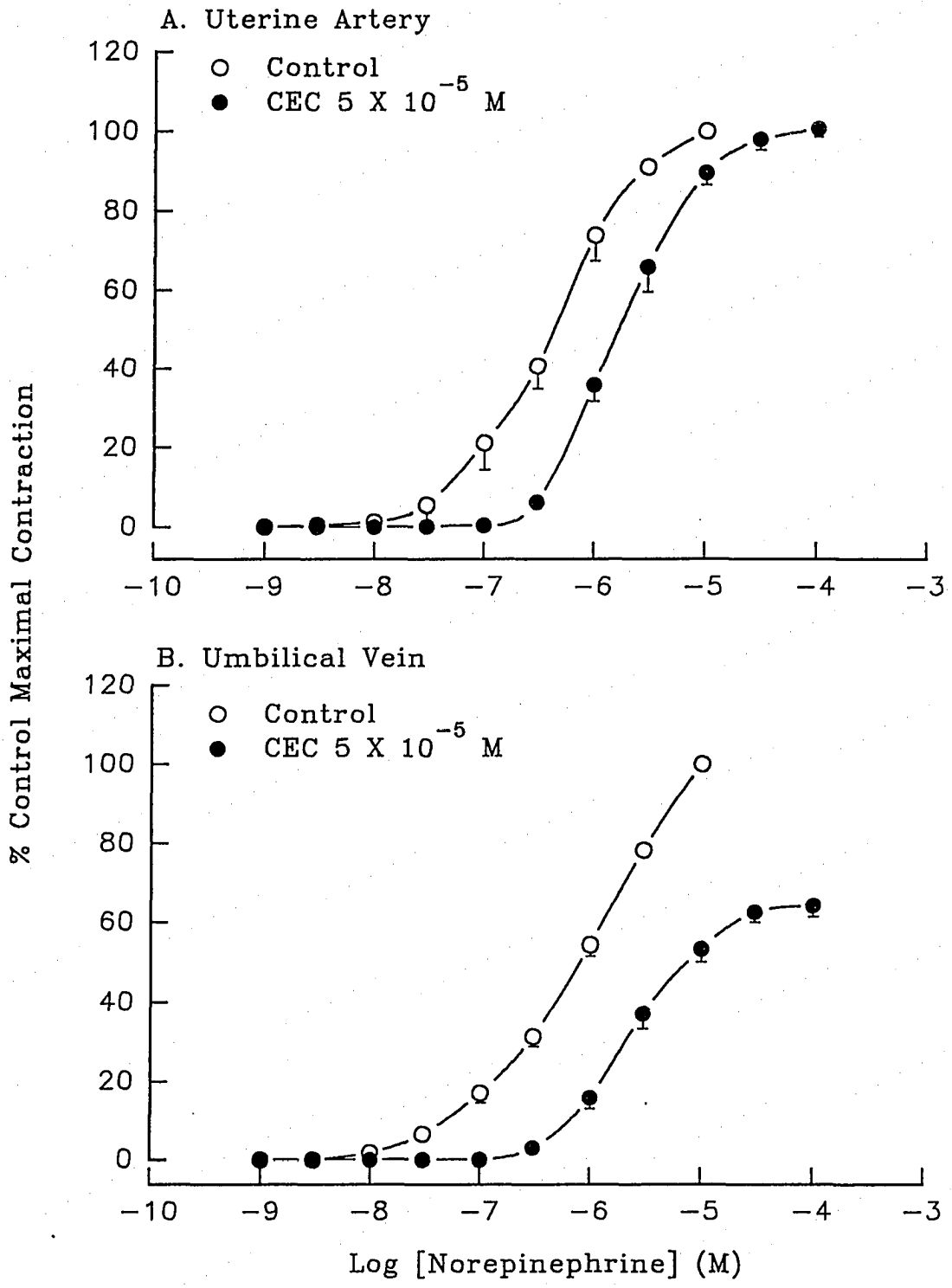

A. Uterine Artery
Control
CEC 5 X 10^-5 M
120
100
80
60
40
20
0
−10
−9
−8
−7
−6
−5
−4
−3
B. Umbilical Vein
Control
CEC 5 X 10^-5 M
% Control Maximal Contraction
Log [Norepinephrine] (M)

Table 2

Effects of CEC on norepinephrine-induced vasoconstriction in the uterine artery and umbilical vein. Mean ± S.E.

	Uterine Artery		Umbilical Vein	
	pD_2	Maximal Contraction (g)	pD_2	Maximal Contraction (g)
Control	6.6±0.1 (5)	30.5±3.6 (5)	6.3±0.1 (7)	13.8±2.7 (7)
CEC	5.8±0.1[a] (5)	30.6±3.6 (5)	4.8±0.1[a] (7)	8.8±1.7[a] (7)

[a] $P<0.05$, comparing to control

vein, CEC pretreatment significantly depressed the maximal contraction (63.8% of control) and shifted the concentration-response curve to the right (fig. 2B, table 2). Chloroethylclonidine did not contract either the uterine artery or umbilical vein.

3.3. *Effects of nifedipine on norepinephrine contractions*

Blockade of calcium channels by nifedipine (10^{-6} M) in the uterine artery shifted the concentration-response curve to norepinephrine to the right without significantly decreasing the maximal response (fig 3A). In the umbilical vein, nifedipine displaced the concentration-reponse curve to norepinephrine to the right and significantly depressed the maximal contraction to norepinephrine (fig. 3B, table 3).

3.4. *Competitive antagonism by WB4101*

In the uterine artery, the selective α_{1A}-adrenoceptor antagonist, WB4101 (10^{-8}, 3 X 10^{-8} and 10^{-7} M), shifted the concentration-response curves for norepinephrine to the right in both the uterine artery and umbilical vein (fig. 4). A Schild plot for WB4101 against norepinephrine yield a straight line with a slope of unity for both vessels (-1.08 for both the uterine artery and umbilical vein) (fig. 5). The pA_2 values for WB4101 against norepinephrine were 8.30 and 8.46 in the uterine artery and umbilical vein, respectively.

Fig. 3. Concentration-response curves for norepinephrine in the absence and presence of nifedipine (10^{-6} M, 30 min) in the ovine uterine artery and umbilical vein. Each point represents mean ± S.E. of 5-7 animals.

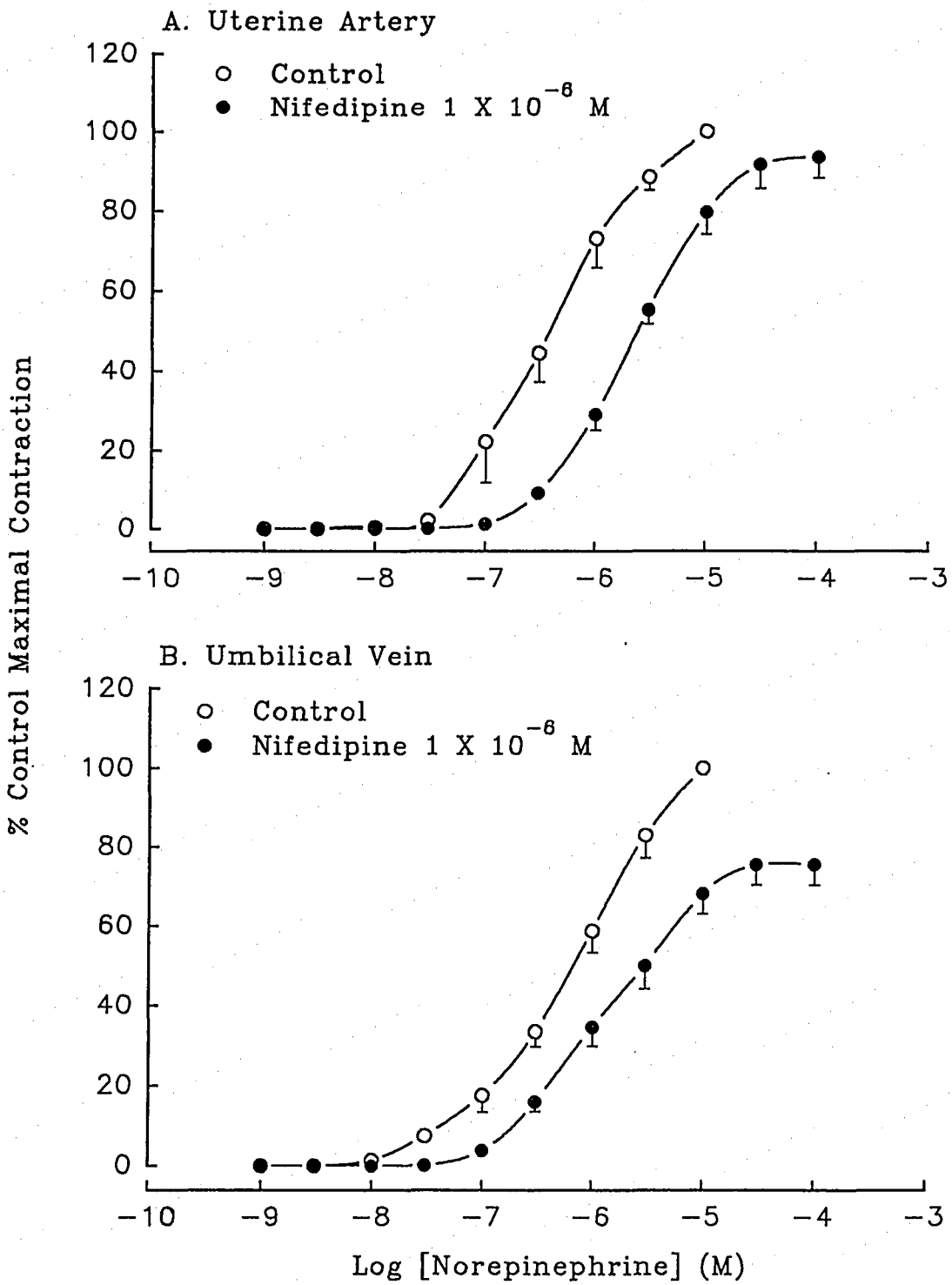

A. Uterine Artery
Control
Nifedipine 1 X 10⁻⁶ M
B. Umbilical Vein
Control
Nifedipine 1 X 10⁻⁶ M
% Control Maximal Contraction
Log [Norepinephrine] (M)
120
100
80
60
40
20
0
−10
−9
−8
−7
−6
−5
−4
−3

Table 3

Effects of nifedipine on norepinephrine-induced vasoconstriction in the uterine artery and umbilical vein. Mean ± S.E.

	Uterine Artery		Umbilical Vein	
	pD_2	Maximal Contraction (g)	pD_2	Maximal Contraction (g)
Control	6.5±0.1 (5)	28.0±2.0 (5)	6.4±0.1 (7)	12.8±2.9 (7)
Nifedipine	5.7±0.1[a] (5)	26.0±1.5 (5)	5.4±0.1[a] (7)	9.7±2.4[a] (7)

[a] $P<0.05$, comparing to control

Fig. 4. Concentration-response curves for norepinephrine in the absence and presence of WB 4101 (10^{-8} - 10^{-7} M, 30 min) in the ovine uterine artery and umbilical vein. Each point represents mean ± S.E. of 5-7 animals.

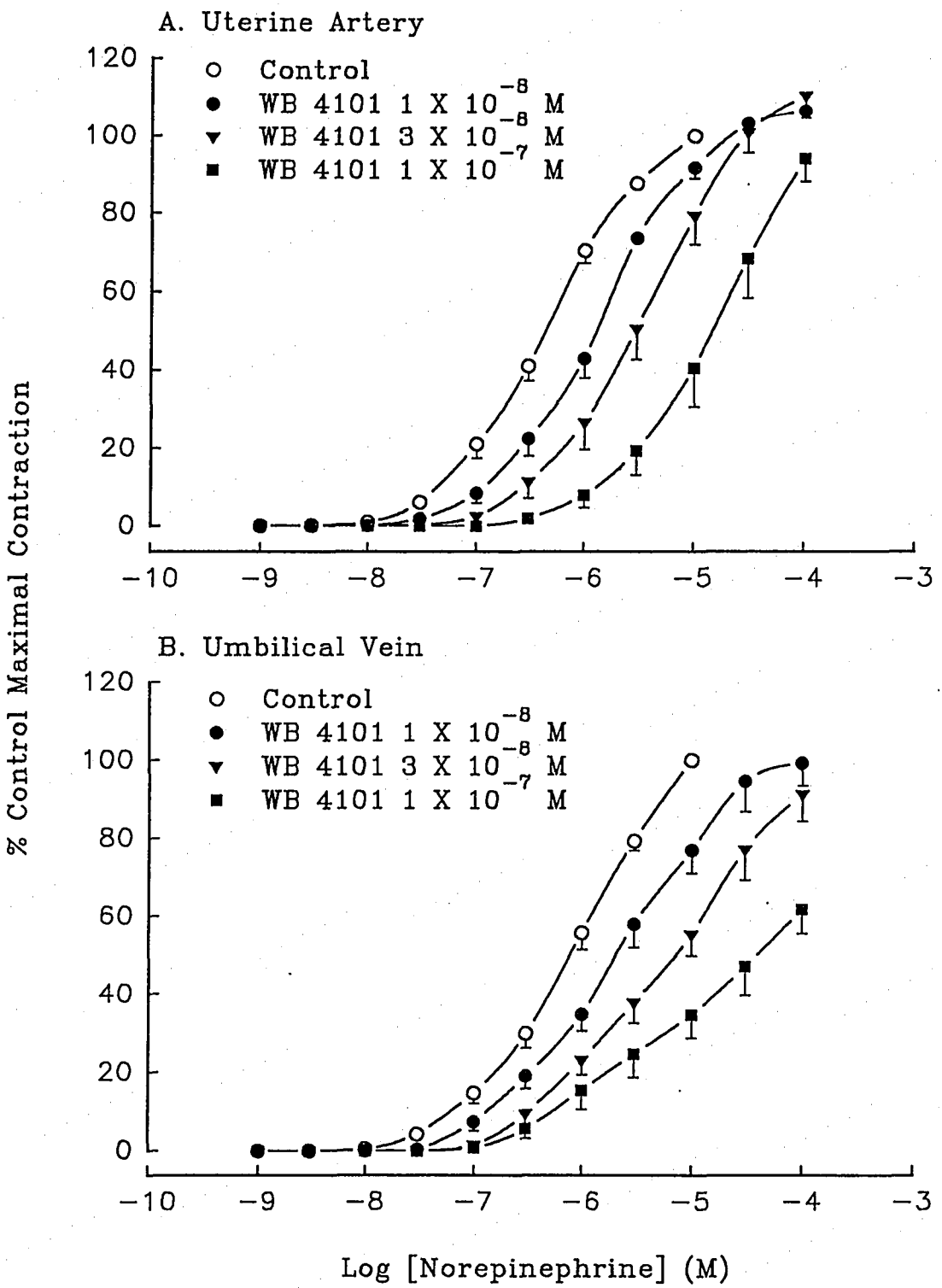
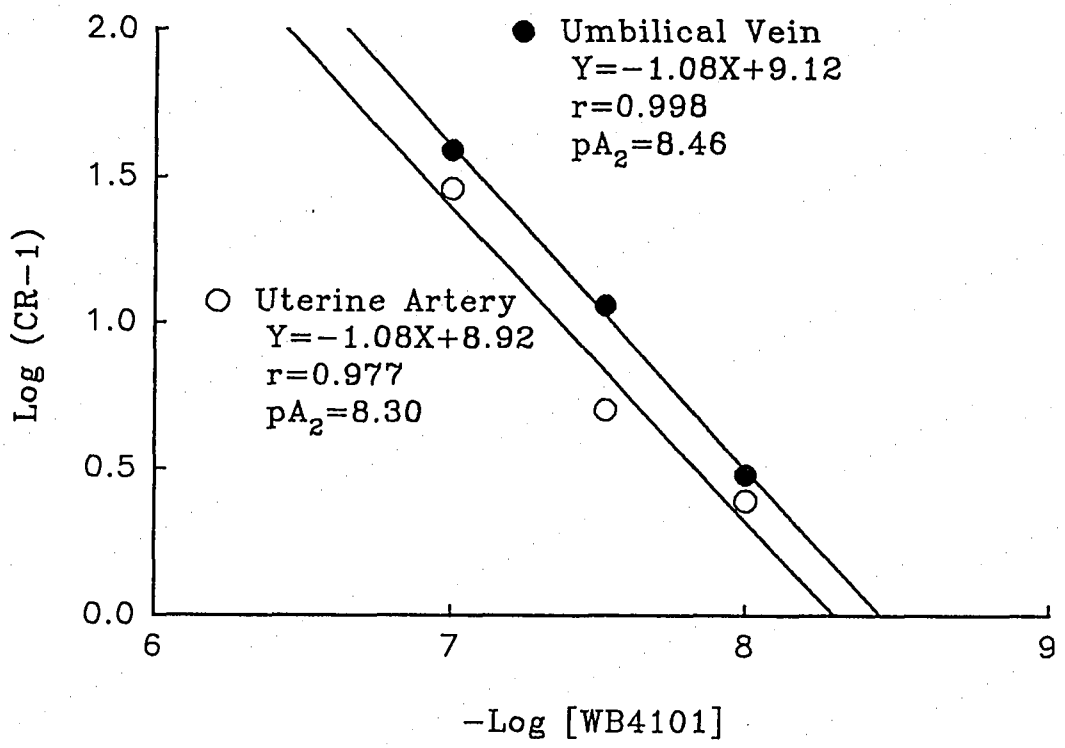

A. Uterine Artery
Control
WB 4101 1 X 10⁻⁸ M
WB 4101 3 X 10⁻⁸ M
WB 4101 1 X 10⁻⁷ M
120
100
80
60
40
20
0
−10
−9
−8
−7
−6
−5
−4
−3
B. Umbilical Vein
Control
WB 4101 1 X 10⁻⁸ M
WB 4101 3 X 10⁻⁸ M
WB 4101 1 X 10⁻⁷ M
% Control Maximal Contraction
Log [Norepinephrine] (M)

Fig. 5. A Schild plot for the antagonism between WB 4101 and norepinephrine in the ovine uterine artery and umbilical vein. Each point represents the average of 5-7 animals. r: correlation coefficient.


2.0
1.5
1.0
0.5
0.0
6
7
8
9
Log (CR-1)
−Log [WB4101]
● Umbilical Vein
Y=−1.08X+9.12
r=0.998
pA2=8.46
○ Uterine Artery
Y=−1.08X+8.92
r=0.977
pA2=8.30

3.5. *Effects of combining CEC with prazosin, WB 4101 or nifedipine*

Similar to the studies reported in section 3.2., CEC shifted the concentration-response curve for norepinephrine to the right in both the uterine artery and umbilical vein. However, the maximal responses to norepinephrine was depressed in the umbilical vein but not in the uterine artery.

Prazosin (10^{-8} M) alone displaced the concentration-response curve for norepinephrine to the right in both ovine uterine artery and umbilical vein. The -log K_B values for prazosin against norepinephrine were 8.8 and 9.4 in the uterine artery and umbilical vein, respectively. Pretreatment with CEC (5 X 10^{-5} M) followed by prazosin further shifted the concentration-response curve for norepinephrine to the right in both vessels (fig. 6). The -log K_B values for prazosin did essentially not change after CEC pretreatment, i.e. 8.8 and 9.4 in the uterine artery and umbilical vein, respectively (table 4).

WB 4101 at the concentration of 10^{-8} M shifted the concentration-response curve for norepinephrine to the right in both the uterine artery and umbilical vein, which is similar to the data reported in section 3.4.. Pretreatment of the tissues with CEC (5 X 10^{-5} M) followed by WB 4101 further shifted the concentration-response curve to norepinephrine to the right in both the uterine artery and umbilical vein (fig. 7). The -log K_B values were 8.2 and 8.5 in the ovine uterine artery and umbilical vein, respectively (table 4), which are almost the same as those obtained using the Schild plot method. After pretreatment with CEC, the -log K_B values were almost the same as those before CEC treatment, i.e. 8.2 and 8.6 in the ovine uterine artery and

Fig. 6. Effects of prazosin (10^{-8} M) alone or with CEC (5×10^{-5} M, 30 min) pretreatment on norepinephrine-induced contractions of the ovine uterine artery and umbilical vein. Each point represents mean ± S.E. of 5 animals.

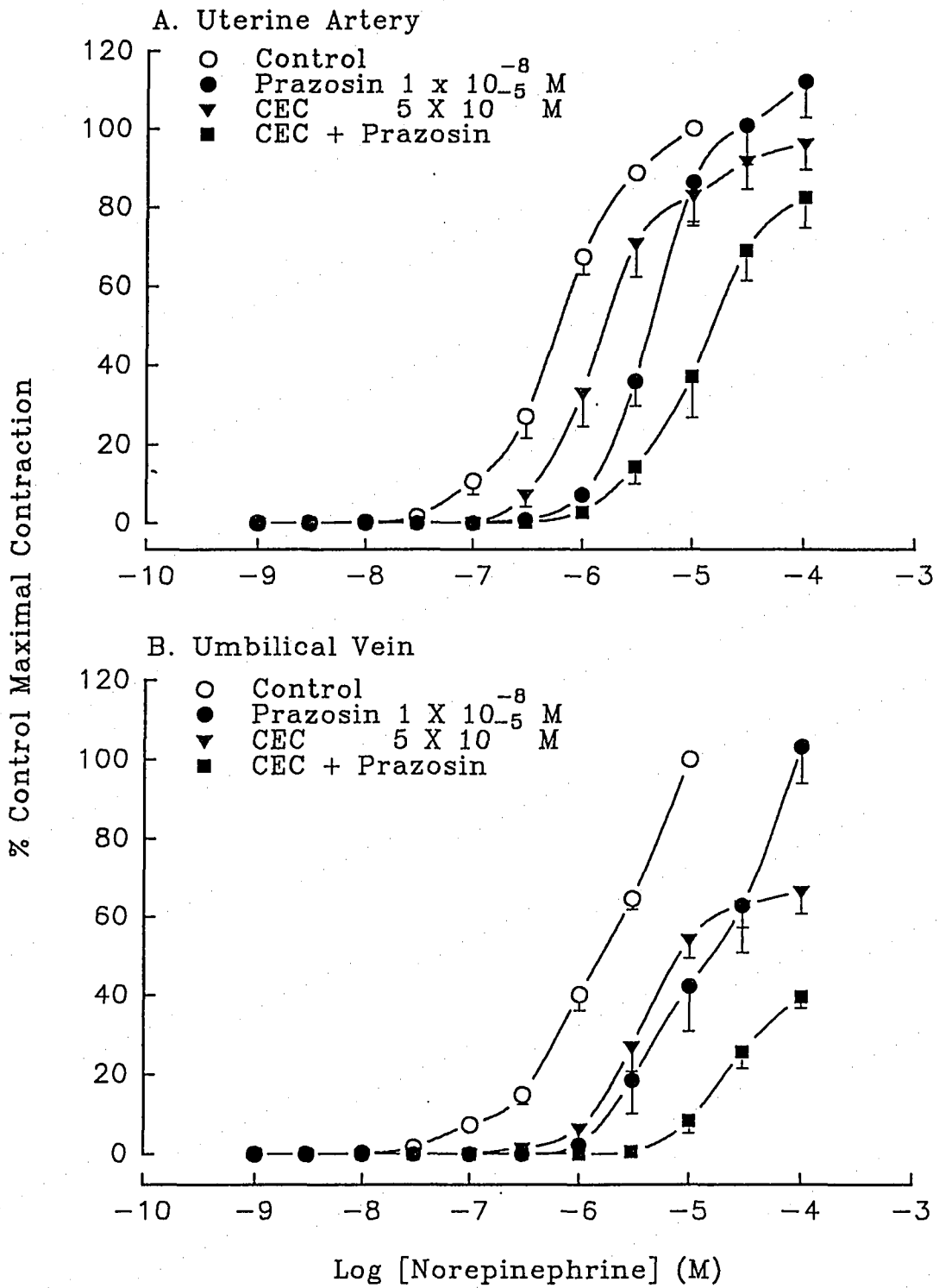
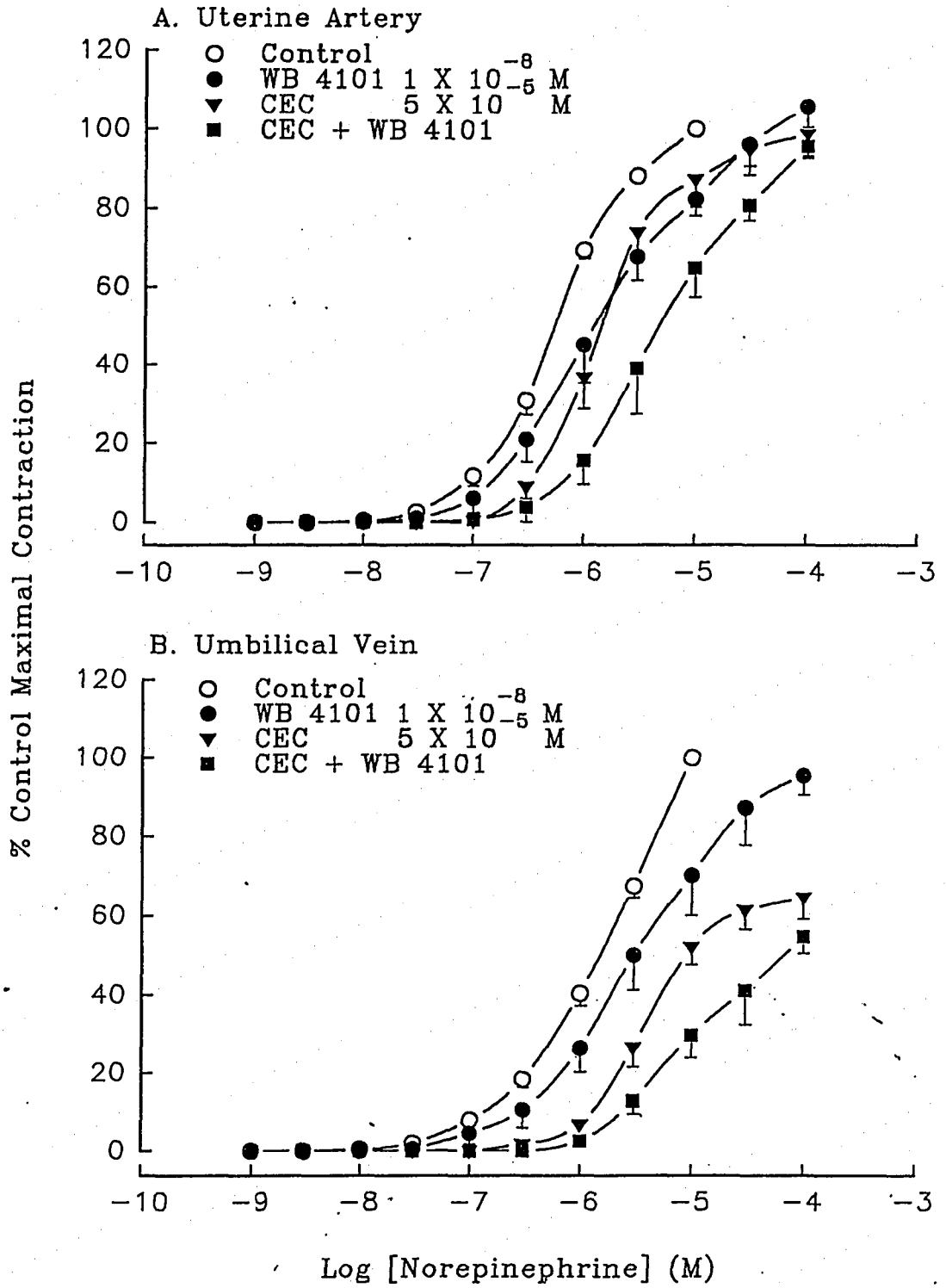

A. Uterine Artery
Control
Prazosin 1 x 10-8 M
CEC 5 X 10-5 M
CEC + Prazosin
B. Umbilical Vein
Control
Prazosin 1 X 10-8 M
CEC 5 X 10-5 M
CEC + Prazosin
% Control Maximal Contraction
Log [Norepinephrine] (M)

Table 4 . Efffects of CEC pretreatment on antagoinst affinity of α_1-adrenoceptors in the uterine artery and umbilical vein. n=5-6.

Antagonist	- Log K_B			
	Uterine Artery		Umbilical Vein	
	Before CEC	After CEC	Before CEC	After CEC
Prazosin	8.8	8.8	9.4	9.4
WB 4101	8.2	8.2	8.5	8.6

Fig. 7. Effects of WB 4101 (10^{-8} M) alone or with CEC (5×10^{-5} M, 30 min) pretreatment on norepinephrine-induced contractions of the ovine uterine artery and umbilical vein. Each point represents mean ± S.E. of 6 animals.


A. Uterine Artery
Control
WB 4101 1 X 10-8 M
CEC 5 X 10-5 M
CEC + WB 4101
B. Umbilical Vein
Control
WB 4101 1 X 10-8 M
CEC 5 X 10-5 M
CEC + WB 4101
% Control Maximal Contraction
Log [Norepinephrine] (M)

umbilical vein, respectively (table 4). The inhibition was additive for the combined use of CEC and WB 4101.

Nifedipine (10^{-6} M), a L-type calcium channel blocker which was suggested to be selective for the α_{1A}-adrenoceptors, caused a rightward shift of the concentration-response curve for norepinephrine in both the uterine artery and umbilical vein. The pD_2 values for norepinephrine in the presence of nifedipine were 5.7 ± 0.1 and 5.5 ± 0.1 for the uterine artery and umbilical vein, respectively, which were almost the same as those reports in section 3.3. After pretreatment with CEC, nifedipine further shifted the concentration-response curves to norepinephrine to the right in both the uterine artery and umbilical vein (fig. 8). The pD_2 values for norepinephrine in the presence of nifedipine after CEC pretreatment were 4.9 ± 0.1 and 4.2 ± 0.2 for the uterine artery and umbilical vein, respectively, which are significantly different form those without CEC pretreatment ($p < 0.05$). The inhibition of responses to norepinephrine by CEC and nifedipine was additive in both the uterine artery and umbilical vein.

3.6. *Effects of α_1-antagonists on contractions to norepinephrine in the absence of external calcium*

In normal Krebs' solution, norepinephrine (10^{-5} M) produced a contraction in both the uterine artery and umbilical vein which was composed of two components: a phasic contraction followed by a tonic contraction. In Ca^{2+}-free Krebs' solution, norepinephrine (10^{-5} M) produced a transient contraction in both blood vessels. The contractions in response to norepinephrine (10^{-5} M) in Ca^{2+}-free medium were 39.7 ± 3.0 % and 18.1 ± 3.2 % in the uterine artery and umbilical vein, respectively, of that in

Fig. 8. Effects of nifedipine (10^{-6} M) alone or CEC (5 X 10^{-5} M, 30 min) pretreatment on norepinephrine-induced contractions of the ovine uterine artery and umbilical vein. Each point represents mean ± S.E. of 6 animals.

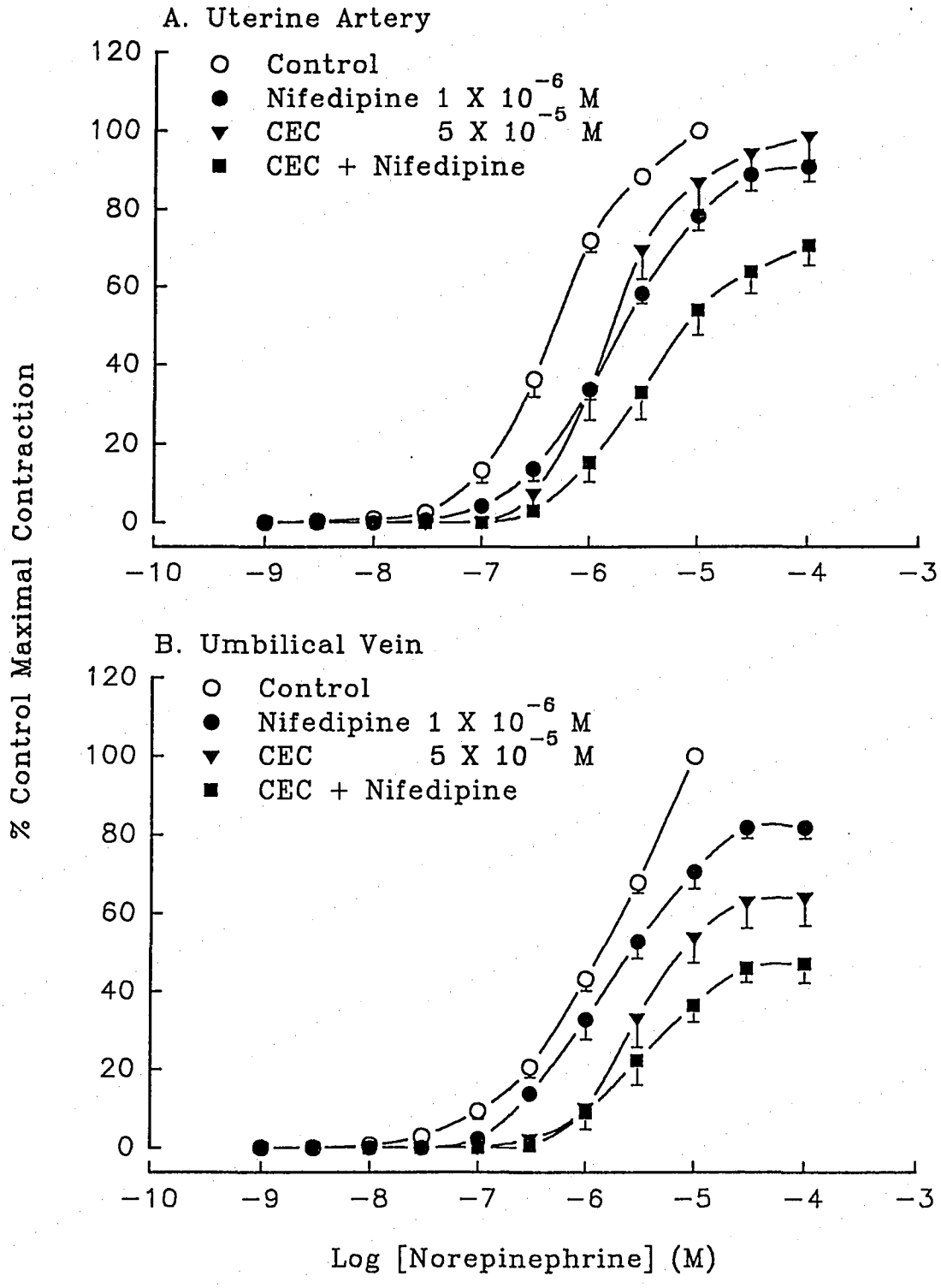
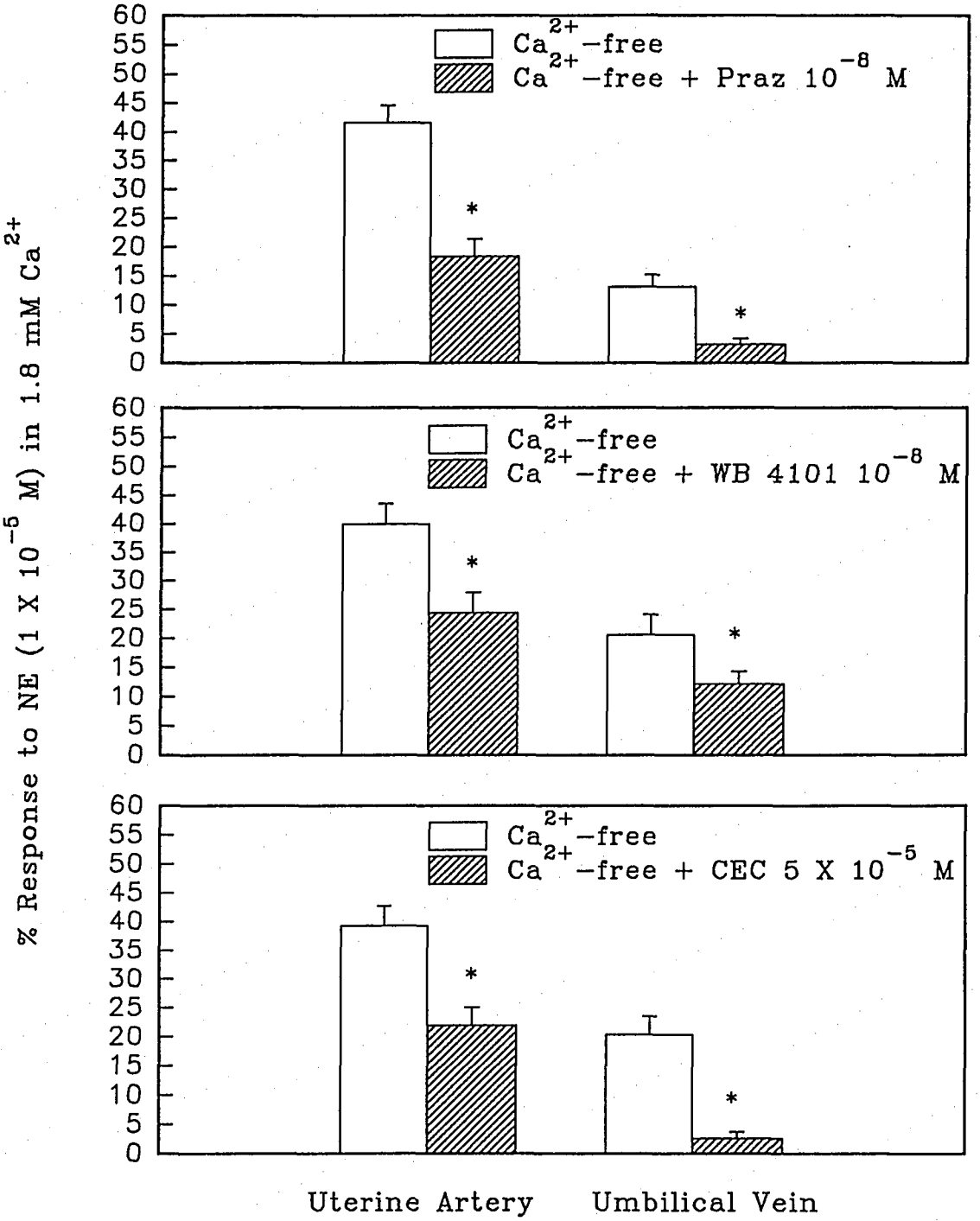

A. Uterine Artery
Control
Nifedipine 1 X 10^-6 M
CEC 5 X 10^-5 M
CEC + Nifedipine
120
100
80
60
40
20
0
−10
−9
−8
−7
−6
−5
−4
−3
B. Umbilical Vein
Control
Nifedipine 1 X 10^-6 M
CEC 5 X 10^-5 M
CEC + Nifedipine
% Control Maximal Contraction
Log [Norepinephrine] (M)

Fig. 9. Effects of α_1-adrenoceptor antagonists (prazosin (Praz), WB 4101 and CEC) on contractions induced by norepinephrine (NE) in the absence of external calcium in the ovine uterine artery and umbilical vein. The values are mean ± S.E. of 5-9 animals. * Indicates that values are significantly different form control values in the absence of external calcium, $P < 0.05$.

Response to NE (1 X 10^{-5} M) in the absence of external Ca^{2+}



normal Krebs' solution (fig. 9). Prazosin (10^{-8} M), a selective α_1-adrenoceptor antagonist, WB 4101 (10^{-8} M), a selective α_{1A} antagonist, and CEC (5 X 10^{-5} M), an irreversible α_{1B} antagonist, all significantly inhibited norepinephrine (10^{-5} M) contractions in both the uterine artery and umbilical vein in the absence of external calcium. The magnitude of the inhibition was similar for all three antagonists in the uterine artery. However, in the umbilical vein, prazosin and CEC were more potent in inhibiting contractions to norepinephrine.

4. Discussion

Vasoconstriction elicited by alpha-adrenoceptor agonists in the ovine uterine artery and umbilical vein were shown to be mediated via by α_1-adrenoceptors (Isla and Dyer, 1990; Zhang and Dyer, 1991). Using pharmacological tools we have investigated the possible heterogeneity of α_1-adrenoceptors in these two vessels.

Prazosin, a selective α_1-adrenoceptor antagonist, competitively inhibited contractile responses induced by norepinephrine in both the uterine artery and umbilical vein, the K_B values for prazosin were similar to those observations in previous studies from our laboratory (Isla and Dyer, 1990; Zhang and Dyer, 1991). These observations suggested the presence of α_1-adrenoceptors in both tissues.

Chlorethylclonidine (CEC) is an irreversible alkylating derivative of clonidine and possesses different effectiveness in blocking α_1-adrenoceptors in rat tissues (Han et al., 1987a; Johonson and Minneman, 1987). It was proposed that those α_1-adrenoceptors which are sensitive to CEC alkylation are of the α_{1B} subtype, while those α_1-

adrenoceptors which are insensitive to CEC alkylation are of the α_{1A}-subtype (Minneman, 1988). In the present study, CEC shifted the concentration-response curves for norepinephrine to the right in both the uterine artery and umbilical vein. In the uterine artery, CEC shifted the concentration-response curve for norepinephrine to the right without depressing the maximal contraction. Similar observations have been made in other vascular smooth muscle (Aboud et al., 1993; Oriowo et al., 1992; Oriowo and Ruffolo, 1992; Tian et al., 1990). It is not clear why CEC depresses the maximal response in some vessels but not in others. It is unlikely that receptor reserve has a role in this phenomenon, since previous studies showed that there was no substantial α_1-adrenoceptor reserve in the uterine artery (Isla and Dyer, 1990). During pregnancy, the contractility of the uterine artery in response to norepinephrine increased in sheep (Annibale et al., 1989) and guinea pig (Fallgren et al., 1988). However, there was no change in the amount of α_1-adrenoceptors in pig uterine artery during pregnancy (Guenther et al., 1988). It may be possible that norepinephrine has high receptor coupling efficacy in the uterine artery during pregnancy. Bevan et al. (1989) proposed that the affinity of receptor for either agonists or antagonist could be modified by the environment of the receptor. The uterine artery is under extensive influence of sex hormones during pregnancy. It is possible that the microenvironment and the structure of α_{1B}-adrenoceptors, as well as intracellular signaling pathways coupled to α_1-adrenoceptors in the uterine artery have been modified during pregnancy. Our results may also imply that even without the participation of α_{1B}-adrenoceptors, activation of α_{1A}-adrenoceptors alone by norepinephrine could still produce the maximal contractile response in the uterine

artery. However, activation of both α_{1A}- and α_{1B}-adrenoceptors is required to obtain the maximal contractile response in the umbilical vein. Recently, it has been reported that α_{1D}-adrenoceptor is expressed in vascular smooth muscle (Piascik et al., 1994) and that this subtype is partially inactivated by CEC (Perez et al., 1991). It is possible that both α_{1B}- and α_{1D}-adrenoceptor may be present in the uterine artery. Our results suggest that there is heterogeneity of α_1-adrenoceptors in both the uterine artery and umbilical vein.

Radioligand binding and functional studies using a competitive α_{1A}-adrenoceptor antagonist, WB4101, demonstrated two separate population of α_1-adrenoceptors in rat brain tissues (Han et al., 1987a, 1987b; Morrow and Creese, 1986). The α_{1A} subtype is designated as having high affinity for WB4101 and α_{1B} subtype with low affinity for WB4101. In the present study, WB4101 shifted the concentration-response curve for norepinephrine to the right in a parallel manner. The Schild plot yielded a straight line whose slope was not significantly different from unity in both the uterine artery and umbilical vein, suggesting competitive antagonism (Kenakin, 1982). The pA_2 values for WB4101 in the uterine artery and umbilical vein were 8.30 and 8.45, respectively, which are comparable to those values (8.07-9.49) reported in other blood vessels (Aboud et al., 1993; Han et al., 1990a; Muramatsu et al., 1990; Oriowo and Ruffalo, 1992; Takayanagi et al., 1991). These results indicate the existence of α_{1A}-adrenoceptors in both the uterine artery and umbilical vein.

In the presence of nifedipine, a L-type calcium channel blocker, the concentration-response curves to norepinephrine were shifted to the right in both the uterine artery and umbilical vein. α_{1A}-Adrenoceptor-mediated

contraction is thought to be coupled to dihydropyridine-sensitive calcium channels (Minneman, 1988). Our results suggest a possibility that Ca^{2+} influx is involved in α_{1A}-adrenoceptor-mediated contractions in both blood vessels.

The findings of contractile responses to norepinephrine after CEC pretreatment in both the uterine artery and umbilical vein further suggests the possibility of α_1-adrenoceptor heterogeneity in both blood vessels. After pretreatment with CEC, contractions to norepinephrine were further inhibited by prazosin in both the uterine artery and umbilical vein. This indicates that the response which is resistant to CEC inactivation is still sensitive to antagonism by prazosin. Furthermore, we found that CEC-resistant contractions to norepinephrine were also sensitive to antagonism by WB 4101 and nifedipine. The potencies of prazosin and WB 4101 were essentially unchanged following CEC pretreatment. These findings further suggest that contractions to norepinephrine which are CEC-resistant are mediated by α_{1A}-adrenoceptor and that Ca^{2+} influx participates in α_{1A}-adrenoceptor-mediated contractile responses in both the uterine artery and umbilical vein, and provides addition evidence that both α_{1A}- and α_{1B}-adrenoceptors co-exist in both the uterine artery and umbilical vein. Our results differ from studies reported by others in which neither prazosin nor WB 4101 antagonized the residual contractile responses to norepinephrine in the rat and dog aorta after pretreatment with CEC (Oriowo and Bevan, 1990; Oriowo and Ruffolo, 1992). Oriowo and Bevan (1990) suggested that the CEC-resistant contraction to norepinephrine was mediated by a non-adrenoceptor. This is not the case in the uterine artery and umbilical vein, since combined use of CEC with prazosin or WB 4101 produced an additive inhibition of contractile responses to

norepinephrine. In addition, Oriowo and Ruffolo (1992) observed that CEC-resistant contractions to norepinephrine were sensitive to antagonism by prazosin and WB 4101 in rabbit aorta. These observations suggest that the ability of prazosin or WB 4101 to antagonize CEC-resistant contractions to norepinephrine is tissue-dependent.

As proposed by Minneman (1988), α_{1A}-adrenoceptor-mediated responses are associated with calcium influx from extracellular fluid and are sensitive to antagonism by WB4101 and nifedipine. α_{1B}-Adrenoceptor-mediated responses are thought to be linked to phospholipase C and mobilization of intracellular calcium and are sensitive to alkylation by CEC. Contractions induced by agonists in Ca^{2+}-free medium are thought to be the result of release of intracellular stored calcium by $Ins(1,4,5)P_3$ (Bohr, 1988). Inhibition of contractions to norepinephrine by CEC in Ca^{2+}-free medium in both the uterine artery and umbilical vein confirmed the suggestion that the α_{1B}-adrenoceptor is coupled to the release of intracellular calcium (Han et al., 1987b; Minneman, 1988). However, inhibition of contractions to norepinephrine by a low concentration of WB 4101 in Ca^{2+}-free medium in both vessels may suggest that the α_{1A}-adrenoceptor is also capable of releasing intracellular calcium. This finding is comparable with observations by Lepretre et al. (1994). These investigators demonstrated that activation of α_{1A}-adrenoceptors stimulates phosphoinositide hydrolysis and mobilizes intracellular calcium in the rat portal vein.

Both the α_{1A}- and α_{1B}-adrenoceptor subtype may not be coupled to only a single transduction pathway. In fact, besides the coupling mechanisms of α_{1A}- and α_{1B}-adrenoceptors originally proposed by Minneman (1988), α_{1A}-adrenoceptor subtype has recently been found to stimulate the hydrolysis

of phosphoinositide (Han et al., 1990b; Michel et al., 1993), while α_{1B}-adrenoceptor subtype are reported to stimulate Ca^{2+} influx (Han et al., 1992).

Based on their observations, Yamaguchi and Kopin (1980) proposed that the α_1-adrenoceptors are located at the neuroeffector junctions (junctional receptors), while α_2-adrenoceptors are located at extrajunctional sites (extrajunctional receptors). This proposal was extended by Langer and Shepperson (1982a,b), who suggested that α_1-adrenoceptors are located in the vicinity of uptake$_1$ sites. Piascik and coworkers (1990a, b) further suggested that α_{1A}-adrenoceptors are located junctionally while α_{1B}-adrenoceptors are located extrajunctionally. Since the umbilical vessels are not innervated (Fox and Khong, 1990; Spivack, 1943), the previous finding (Zhang and Dyer, 1991) and present observations in the umbilical vein suggest the possibility of an extrajunctional location for both α_{1A} and α_{1B}-adrenoceptors. It is generally accepted that the physiological role of postjunctional α_1-adrenoceptors appears to be responsible for maintaining resting vascular tone, while extrajunctional α_2-adrenoceptors may respond to circulating epinephrine (Langer and Shepperson, 1982b; Ruffolo, 1991). Recently, Piascik et al., (1990a, b) proposed that α_{1A}-adrenoceptors appear to play a role in the maintenance of vascular tone, while α_{1B}-adrenoceptors respond to circulating catecholamines. Our findings suggest that both α_{1A}- and α_{1B}-adrenoceptors in the umbilical vein could respond to circulating catecholamines and participate in the regulation of resting tone, since there are no apparent α_2-adrenoceptors in the umbilical vein (Zhang and Dyer, 1991).

In summary, our study found evidence for the

heterogeneity of α_1-adrenoceptors in both the ovine uterine artery and umbilical vein. In addition, our findings also suggest that the α_{1A}-adrenoceptor may be linked to the release of intracellular calcium.

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EFFECTS OF STAUROSPORINE, A PROTEIN KINASE C INHIBITOR, ON CONTRACTIONS INDUCED BY NOREPINEPHRINE, ENDOTHELIN-1, KCL AND PHORBOL 12,13-DIBUTYRATE ON THE ISOLATED OVINE UTERINE ARTERY

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Abstract

The role of protein kinase C (PKC) in vasoconstriction of the ovine uterine artery was investigated using the putative PKC inhibitor, staurosporine. Norepinephrine, endothelin-1, KCl and phorbol 12,13-dibutyrate (PDBu) all produced concentration-dependent contractions. Endothelin was a more potent vasoconstrictor than norepinephrine in the

ovine uterine artery. PDBu induced-contractions were inhibited by staurosporine in a concentration-dependent manner, and were almost completely blocked by 10^{-7} M staurosporine. Staurosporine had no inhibitory effect on contractions induced by KCl, suggesting that staurosporine is a relative specific PKC inhibitor in the ovine uterine artery. The contractile response induced by norepinephrine and endothelin-1 were inhibited by staurosporine, however, the inhibition was incomplete. These results suggest that activation of PKC is involved in agonist-induced contractions of the ovine uterine artery and that mechanisms other than PKC may contribute to the contraction process.

Key words: protein kinase C, phorbol 12,13-dibutyrate, norepinephrine, endothelin-1, uterine artery

1. Introduction

Activation of protein kinase C (PKC) results in diverse biological responses (Nishizuka, 1984). Diacylglycerol (DAG) is the physiological activator of PKC in cells. At present, there are two known sources of formation of DAG: 1) hydrolysis of phosphoinositide by phospholipase C; and 2) hydrolysis of phosphatidylcholine by phospholipase C and D (Billah and Anthes, 1990; Exton, 1990). Hydrolysis of phosphoinositide contributes to the early transient accumulation of DAG and the hydrolysis of phosphatidylcholine results in the late sustained accumulation of DAG (Nishizuka, 1992). Both norepinephrine and endothelin have been shown to induce the hydrolysis of

phosphoinositide (Labelle and Murray, 1990; Resink, 1990) and phosphatidylcholine (Gu et al., 1992; Liu et al., 1992) in vascular smooth muscle.The activating effect of DAG on PKC could be mimicked by tumor-promoting phorbol ester (Castagna et al., 1982). Activation of PKC by phorbol esters induced a slowly developing and sustained vasoconstriction (Forder et al., 1985; Gleason and Flaim, 1986; Khalil and van Breemen, 1988), suggesting that PKC may play a role in vasoconstriction (Rasmussen et al., 1987).

The uterine artery during pregnancy transports blood to placenta to supply the fetus with oxygen and nutrients. Both norepinephrine and endothelin are potent vasoconstrictors of the uterine artery (Dyer and Guogh, 1970; Edstrom et al., 1991). It has been reported that PKC activities in the sheep (Magness et al., 1991) and pig (Farley and Ford, 1992) uterine arteries decreased during pregnancy. However, the role of PKC in agonist-induced contractions of the uterine artery has been rarely studied.

In the present study we have used staurosporine , a putative PKC inhibitor to examine the possibility that activation of PKC contributes to contractions induced by norepinephrine, endothelin-1, KCl or phorbol 12,13-dibutyrate (PDBu).

2. Materials and Methods

2.1. Tissue Preparation

Adult pregnant mixed breed sheep near term were euthanized with an injection of pentobarbital. Uterine arteries were carefully removed without stretching and placed in a modified Krebs' solution of the following

composition (mM): NaCl, 115; KCl, 4.70; Cal_2, 1.80; $MgSO_4$, 1.16; KH_2PO_4, 1.18; $NaHCO_3$, 22.14; dextrose, 7.88 and EDTA, 0.03. The uterine artery was cleaned free of connective tissue and cut into 3-4 mm ring segments. The ring segments were mounted between two wires in 10 ml organ baths containing Krebs' solution maintained at 37°C. The Krebs' solution was aerated with a mixture of 95% O_2-5% CO_2. One wire was attached to a fixed support while the second wire was connected to Grass FT 0.3 transducers and contractions were recorded by a Grass polygraph (model 7). The segments were equilibrated under 2g tension over 60-90 min with regular replacement of Krebs' solution at 20 min intervals. Subsequently, all tissues were exposed to 90 mM KCl. All subsequent contractile responses in each ring segment were expressed as a percentage of the contractile response to KCl in that vessel.

2.2. *Effects of staurosporine on contractions induced by norepinephrine and KCl*

Two sequential concentration-response curves were generated by the cumulative addition of the contractile agent (norepinephrine or KCl). Staurosporine (10^{-8}-10^{-7} M) was equilibrated with the tissues for 30 min before obtaining the second concentration-response relationship to norepinephrine or KCl. In all studies with norepinephrine and KCl, one ring segment received no staurosporine and was run in parallel with the experimental tissues. This tissue was used to correct for time-dependent changes in agonist sensitivity (time control). Response to norepinephrine was obtained in the presence of desimipramine (10^{-7} M), corticosterone acetate (10^{-5} M) and propranolol (10^{-6} M) to block neuronal $uptake_1$, extraneuronal $uptake_2$ and β-

adrenoceptors, respectively. Response to KCl was obtained in the presence of phentolamine (10^{-7} M) in order to block the effects of any neurally released norepinephrine. These agents were in contact with tissues for 30 min before a protocol began and throughout the protocol.

2.3. *Effects of staurosporine on contractions induced by endothelin-1 and phorbol 12,13-dibutyrate (PDBu)*

Concentration-response curves to endothelin-1 and PDBu were generated in the presence or absence of staurosporine (10^{-8}-10^{-7} M) on paired preparations. Staurosporine was added to the baths 30 min before obtaining a concentration-response relationship to endothelin-1 and PDBu.

2.3. *Drugs*

(-) Norepinephrine hydrochloride, phorbol 12,13-dibutyrate, endothelin-1, staurosporine and bovine serum albumin were purchased from Sigma Chemical Co. (St. Louis, MO). Norepinephrine was dissolved in saline (0.9% NaCl) and endothelin-1 was dissolved in saline containing 0.5% bovine serum albumin in order to prevent nonspecific binding to glassware (Kasuya et al., 1989). Initial stock solutions of PDBu and staurosporine were in dimethylsulfoxide (DMSO) and subsequent dilutions were made with saline. The final concentration of DMSO in the bath medium was less than 0.1% and had no effect on the contractility of the ovine uterine artery to KCl.

2.4. *Statistics*

The results are expressed as mean ± S.E.. The data were analyzed by Student's t-test. A P value less than 0.05 was taken as significant.

3. Results

3.1. *Comparison of contractions induced by norepinephrine, KCl, endothelin-1 and PDBu*

In the isolated ovine uterine artery, norepinephrine induced a rapid contraction, whereas endothelin-1 and PDBu produced slow contractions. Norepinephrine, endothelin-1 and PDBu all caused concentration-dependent contractions in the ovine uterine artery (fig. 1). The pD_2 (-log EC_{50}) values are 6.66 ± 0.16, 7.45 ± 0.13 and 5.33 ± 0.19 (n = 5-6) for norepinephrine, endothelin-1 and PDBu, respectively. The potencies of these agents were in the order of endothelin-1 > norepinephrine > PDBu and were significantly different from each other ($P < 0.05$).

3.2. *Effect of staurosporine on contraction induced by KCl*

KCl induced concentration-dependent contractions in the ovine uterine artery. KCl-induced contractions were not affected by any concentration of staurosporine tested (10^{-8}, 3 X 10^{-8} and 10^{-7} M) (fig 2).

Fig. 1. Contractile responses induced by norepinephrine, endothelin-1 and phorbol 12,13-dibutyrate in the isolated ovine uterine artery. Each point represents mean ± S.E. of 5-6 animals.

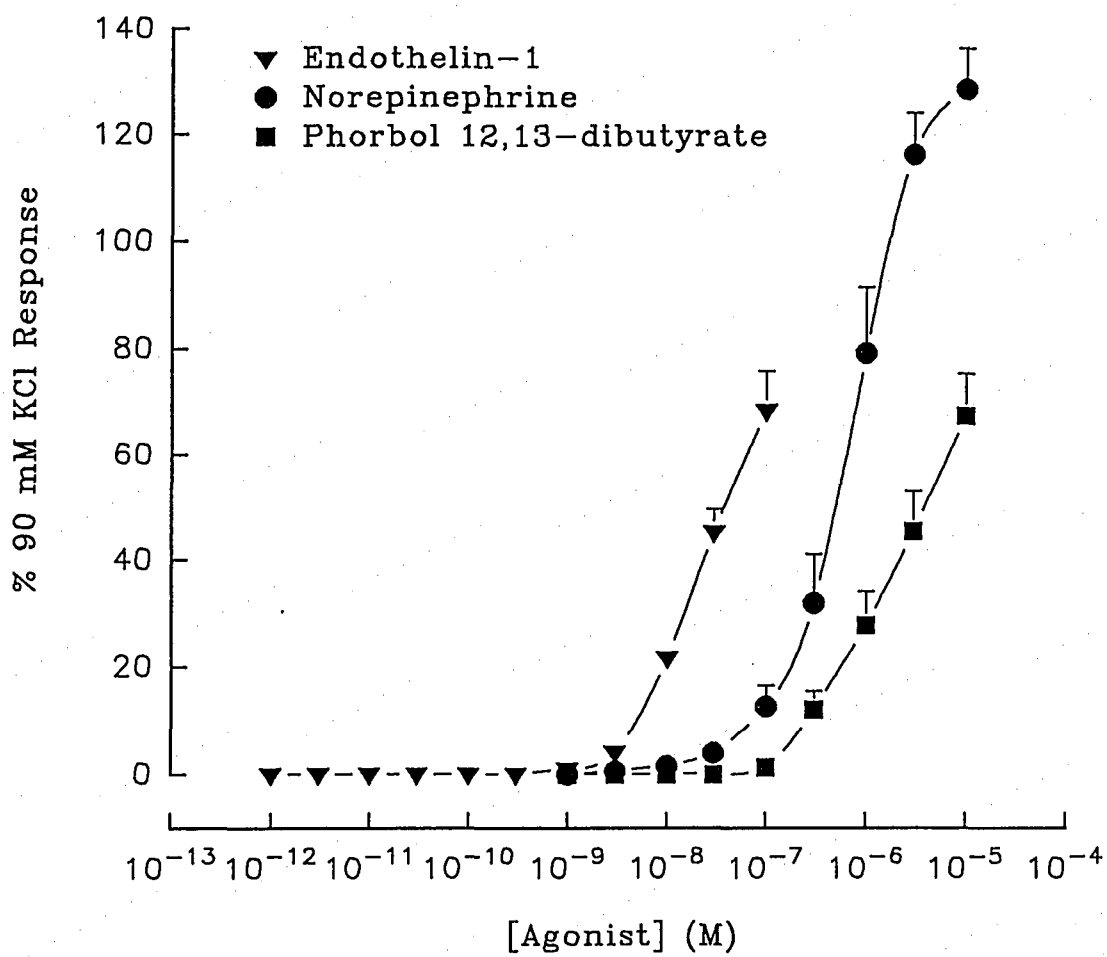
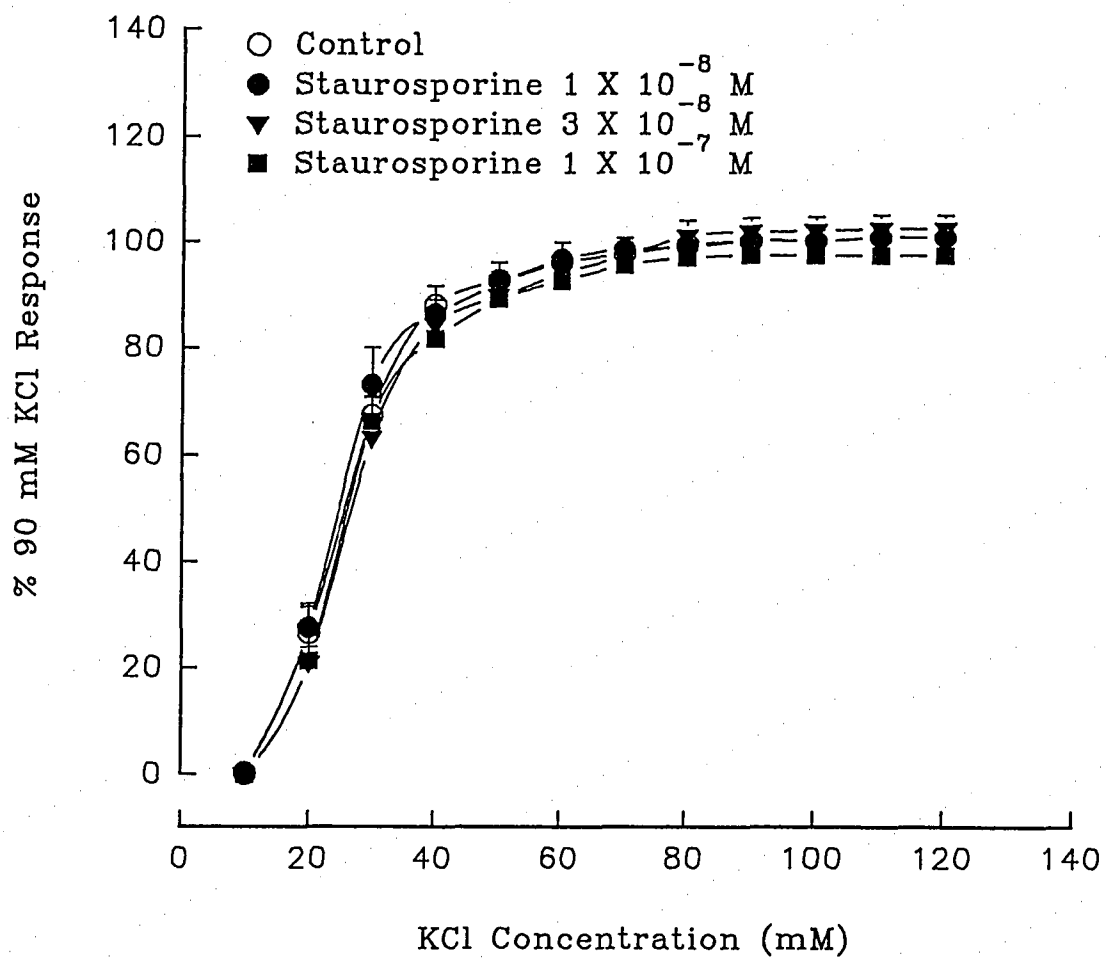

Endothelin-1
Norepinephrine
Phorbol 12,13-dibutyrate
% 90 mM KCl Response
140
120
100
80
60
40
20
0
10^{-13} 10^{-12} 10^{-11} 10^{-10} 10^{-9} 10^{-8} 10^{-7} 10^{-6} 10^{-5} 10^{-4}
[Agonist] (M)

Fig. 2. Effects of staurosporine (10^{-8} - 10^{-7} M) on contraction induced by KCl in the ovine uterine artery. Each point represents mean ± S.E. of 6-7 animals.


Control
Staurosporine 1 X 10 -8 M
Staurosporine 3 X 10 -8 M
Staurosporine 1 X 10 -7 M
% 90 mM KCl Response
KCl Concentration (mM)
0
20
40
60
80
100
120
140

3.3. *Effects of staurosporine on contraction induced by PDBu*

Staurosporine inhibited PDBu-induced contraction in a concentration-dependent manner in the ovine uterine artery (fig. 3). At the concentration of 10^{-7} M, staurosporine inhibited the contraction to PDBu (10^{-5} M) by 80.2 ± 5.2%.

3.4. *Effect of staurosporine on contraction induced by norepinephrine*

The concentration-response curves for norepinephrine in the ovine uterine artery were shifted to the right by staurosporine (10^{-8}-10^{-7} M) (fig. 4). Staurosporine at 10^{-8} M had no apparent effect on the norepinephrine (10^{-5} M)-induced contraction, whereas higher concentrations of staurosporine (3 X 10^{-8} and 10^{-7} M) significantly depressed the contractile responses to norepinephrine (10^{-5} M) (22.2 ± 4.6% and 26.9 ± 5.0%, respectively).

3.5. *Effect of staurosporine on contraction induced by endothelin-1*

Similarly, staurosporine also inhibited contractions induced by endothelin-1 in a concentration-dependent manner in the ovine uterine artery (fig. 5). The contractile responses to endothelin-1 (10^{-7} M) was significantly attenuated by 3 X 10^{-8} M and 10^{-7} M staurosporine (28.1 ± 3.3% and 37.8 ± 4.9%, respectively).

Fig. 3. Effects of staurosporine (10^{-8} - 10^{-7} M) on contraction induced by phorbol 12,13-dibutyrate (PDBu) in the isolated ovine uterine artery. Each point represents mean ± S.E. of 5-6 animals.

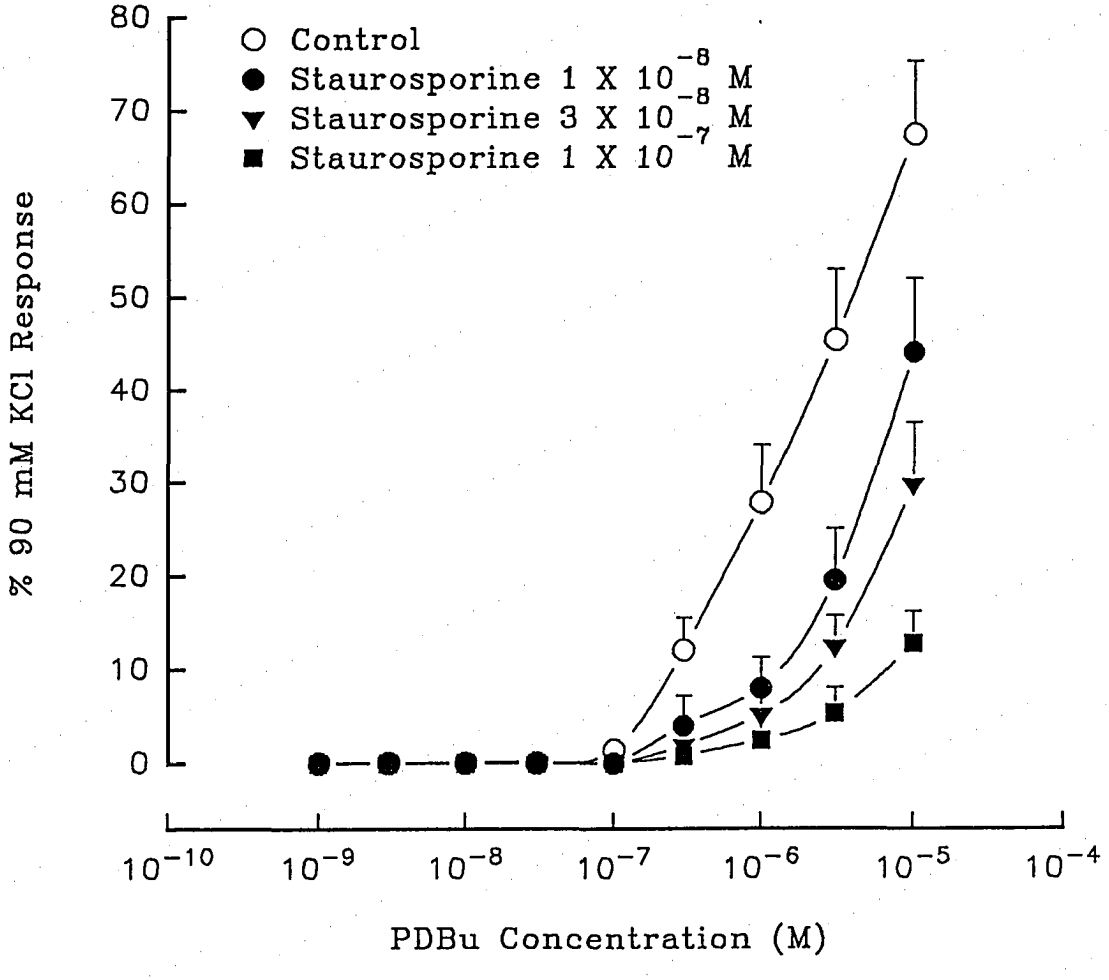

Control
Staurosporine 1 X 10^-8 M
Staurosporine 3 X 10^-8 M
Staurosporine 1 X 10^-7 M
% 90 mM KCl Response
80
70
60
50
40
30
20
10
0
10^-10
10^-9
10^-8
10^-7
10^-6
10^-5
10^-4
PDBu Concentration (M)

Fig. 4. Effects of staurosprine (10^{-8} - 10^{-7} M) on contraction induced by norepinephrine in the isolated ovine uterine artery. Each point represents mean ± S.E. of 5-6 animals.

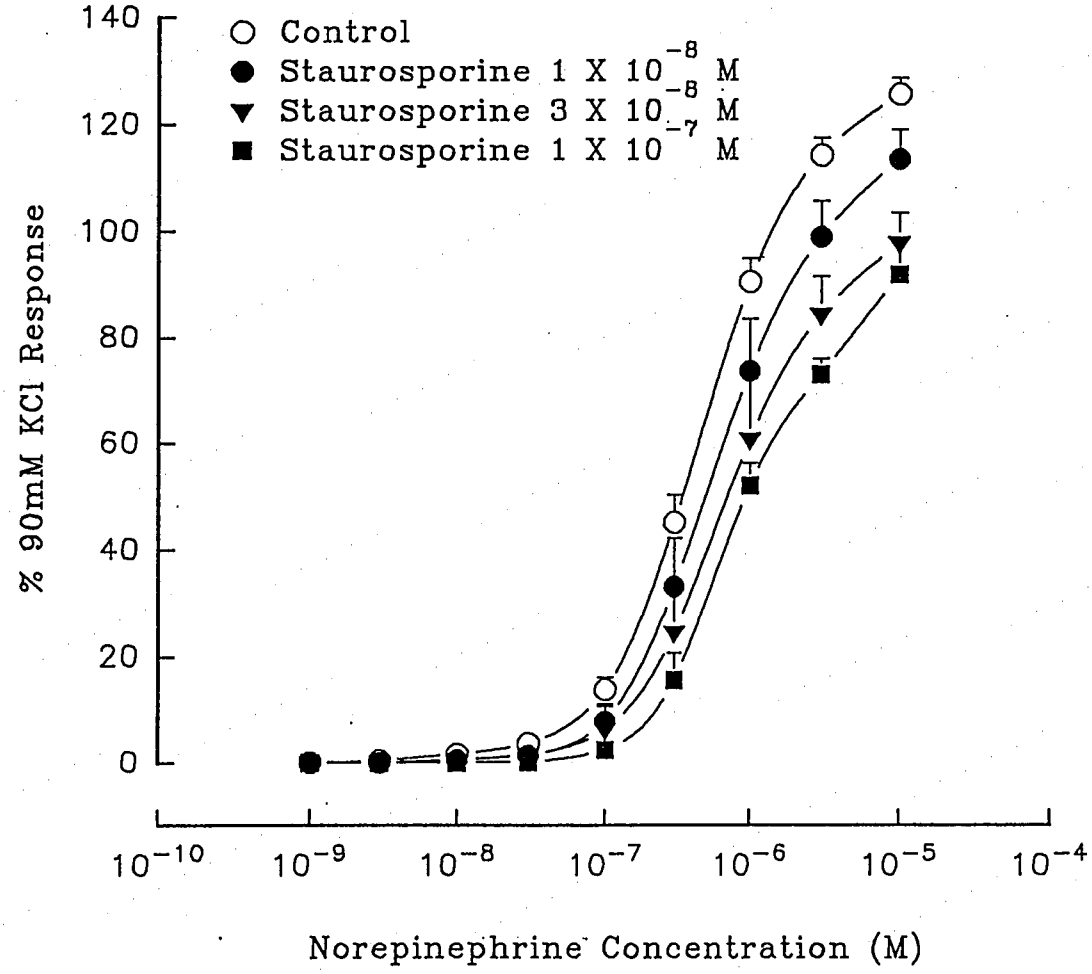
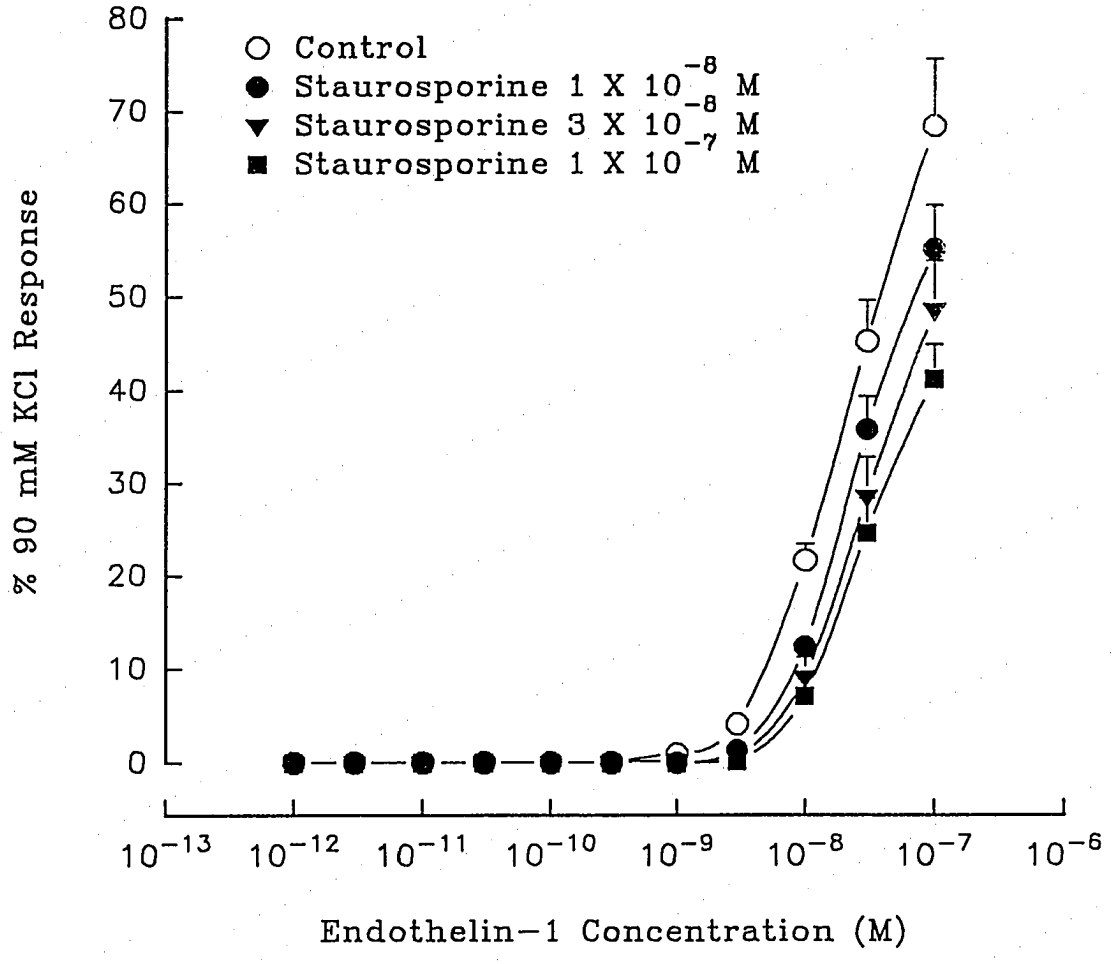

Control
Staurosporine 1 X 10⁻⁸ M
Staurosporine 3 X 10⁻⁸ M
Staurosporine 1 X 10⁻⁷ M
% 90mM KCl Response
140
120
100
80
60
40
20
0
10⁻¹⁰
10⁻⁹
10⁻⁸
10⁻⁷
10⁻⁶
10⁻⁵
10⁻⁴
Norepinephrine Concentration (M)

Fig. 5. Effects of staurosporine (10^{-8} - 10^{-7} M) on contraction induced by endothelin-1 in the isolated ovine uterine artery. Each point represents mean ± S.E. of 5-6 animals.


Control
Staurosporine 1 X 10⁻⁸ M
Staurosporine 3 X 10⁻⁸ M
Staurosporine 1 X 10⁻⁷ M
% 90 mM KCl Response
80
70
60
50
40
30
20
10
0
10⁻¹³ 10⁻¹² 10⁻¹¹ 10⁻¹⁰ 10⁻⁹ 10⁻⁸ 10⁻⁷ 10⁻⁶
Endothelin-1 Concentration (M)

4. Discussion

Receptor-mediated vasoconstriction is accompanied with an increased formation of Ins(1,4,5)P_3 and DAG from phosphatidylinositol 4,5-bisphosphate hydrolysis (Abedel-Latif, 1986). In addition, the breakdown of phosphatidylcholine also contributes to the accumulation of DAG in vascular smooth muscle (Billah and Anthes, 1990; Exton, 1990;Nishizuka, 1992). It is widely accepted that release of calcium from intracellular stores by Ins(1,4,5)P_3 is implicated in receptor-mediated vasoconstriction. However, there is not a general agreement on the role of PKC in vasoconstriction. While some investigators claimed that calcium is the determinant factor in vasoconstriction (Rembold and Murphy, 1988), other investigators argued that PKC is responsible for maintaining contraction (Rasmussen, 1987).

KCl contracts vascular smooth muscle via depolarization of the membrane which in turn stimulates Ca^{2+} influx through voltage-operated calcium channels (Bolton, 1979). There is no involvement of PKC in KCl-induced contraction of smooth muscle (Haller et al., 1990). Staurosporine is a microbial product with an inhibitory effect on protein kinase C by inhibiting the catalytic domain (Tamoki and Nakano, 1990). The specificity of the action of staurosporine was examined with KCl. The failure of staurosporine to inhibit KCl-induced contraction suggest that staurosporine is not a nonspecific PKC inhibitor in the ovine uterine artery. In the rat aorta, staurosporine inhibited KCl-induced contraction and was suggested to be a nonselective PKC inhibitor (Shimamoto et al., 1993). Rüegg and Burgess (1989) suggested that staurosporine act on myosin light chain kinase instead of protein kinase C and

thereby prevent vasoconstriction. However, our results do not support this suggestion, since KCl-induced contraction of vascular smooth muscle is solely dependent on myosin light chain kinase. The discrepancy could result from tissue selectivity to staurosporine.

PDBu, the widely used phorbol ester in studying PKC, produced a slow developing and sustained contraction in the ovine uterine artery. Similar results have been reported in the guinea pig uterine artery (Fallgren et al., 1989) as well as in other vascular beds (Chatterjee and Tejada, 1986; Forder et al., 1985; Gleason and Flaim, 1986). In the present study, we found that staurosporine at 10^{-7} M, which had no inhibitory effect on KCl-induced contraction in the ovine uterine artery, produced an almost complete inhibition of PDBu-induced contraction. PKC is the PDBu receptor protein (Kikkawa et al., 1983). Furthermore, Magness et al. (1992) reported the presence of protein kinase C in the ovine uterine artery. Taking these observations together, we concluded that PDBu-induced contractions are mediated by activation of PKC in the ovine uterine artery. In another study, we found that PDBu-induced contractions of the uterine artery were attenuated by 10^{-6} M nifedipine (Hu and Dyer, unpublished data). This phenomenon indicates that Ca^{2+} influx is involved in contraction induced by PDBu in the ovine uterine artery, which could result from modulation of voltage-operated calcium channels by PKC (Fish et al., 1988).

Norepinephrine contracts ovine uterine artery through activation of α_1-adrenoceptors (Isla and Dyer, 1990). Recently, two subtypes of α_1-adrenoceptors, α_{1A} and α_{1B}, have been pharmacologically identified in this vessel (Hu and Dyer, 1994). The hydrolysis of phosphoinositide mediated by α_{1A}- and α_{1B}-adrenoceptors (Han et al., 1987; Lepretre et

al., 1994) and the hydrolysis of phosphatidylcholine mediated by α_1-adrenoceptors (Gu et al., 1992) has been observed in vascular smooth muscle. These could constitute the sources of DAG for activation of protein kinase C. The inhibition of norepinephrine-induced contraction by staurosporine indicates that activation of PKC plays a role in norepinephrine-induced vasoconstriction of the ovine uterine artery. The inhibition of norepinephrine-induced contractions by staurosphrine has been demonstrated in other vascular smooth muscle (Abebe and MacLeod, 1990; Merkel et al., 1991). However, the partial inhibition of norepinephrine-induced contractions by 10^{-7} M staurosporine, which almost abolished the contraction induced by PDBu, suggests that other mechanism besides PKC contribute to the norepinephrine-induced contractile responses. Khalil and van Breemen (1988) suggested Ca^{2+} influx is more important than activation of protein kinase C in phenylephrine-induced contractions. We also found that norepinephrine-induced contraction of the ovine uterine artery was inhibited by nifedipine, a blocker of voltage-operated calcium channel (Hu and Dyer, 1994). In addition, the Ca^{2+}-sensitivity of the contractile apparatus may be increased by norepinephrine (Nishimura et al., 1990).

Endothelin-1 is a potent vasoconstrictor (Yanagisawa et al., 1988). An *in vivo* study revealed that endothelin-1 was more potent than norepinephrine or phenylephrine as a vasoconstrictor of the ovine uterine artery (Yang and Clark, 1992). Our present *in vitro* observation confirmed the *in vivo* finding. The mechanisms for endothelin-induced contraction are not yet clear. Like norepinephrine, endothelin-1 also stimulates the hydrolysis of phosphoinositide and phosphatidylcholine to produce Ins(1,4,5)P_3 and DAG in vascular smooth muscle (Liu et al.,

1992; Marden et al., 1989; Resnik et al., 1988). The activity of protein kinase C was increased by endothelin in vascular smooth muscle (Griendling et al., 1989; Haller et al., 1990; Lee et al., 1989). It has been suggested that the activation of PKC may contribute to endothelin-1-induced contractions (Ohlstein et al., 1989; Danthuluri and Brock, 1990). Data from our studies confirme the involvement of PKC in endothelin-1-induced contraction of the ovine uterine artery. However, the partial inhibition of endothelin-1-induced contraction by 10^{-7} M staurosporine, which almost completely inhibited the response to PDBu, suggest that mechanisms other than PKC may also be involved. Goto et al. (1989) reported the activation of voltage-operated calcium channels by endothelin in vascular smooth muscle. Endothelin-1 was also found to increase the Ca^{2+}-sensitivity of the contractile apparatus in vascular smooth muscle (Nishimura et al., 1992). These mechanisms could have a role in endothelin-1-induced contraction of the ovine uterine artery.

Although there is a dramatic decrease in PKC activities in the ovine uterine artery in late pregnancy (Magness; 1991), our studies demonstrated that PKC still plays a role in agonist-induced contractions of the ovine uterine artery.

In summary, our studies indicate that staurosporine is a specific PKC inhibitor in the ovine uterine artery since it inhibited contractions to PDBu but not KCl. The inhibition of contractions to norepinephrine and endothelin-1 suggest the involvement of PKC in the contractions to these agents. However, other mechanisms may also contribute to contractions induced by norepinephrine and endothelin-1.

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GENERAL SUMMARY

Uteroplacental blood flow is one of the most important rate-limiting factors for normal fetal growth and development. Uterine and umbilical vessels are under extensive influence of many factors, which could have an impact on fetal well-being. The vasoconstrictor effects of catecholamines (Dyer, 1970, Ekstrom et al., 1991; Fallgren and Edvisson, 1986; Isla and Dyer, 1990; Mak et al., 1984; Somlyo et al., 1965; Stjernquest and Owman, 1990; Tuncer et al., 1985; Zhang and Dyer, 1990a, 1991) and 5-HT (MacLean et al., 1989; Ribeiro et al., 1991; Somlyo et al., 1965; Templeton et al., 1991; Zhang and Dyer, 1990b, 1990c) have been documented in the uterine artery and umbilical vessels. The receptor subtypes mediated contractions induced by catecholamines and 5-HT in sheep are α_1-adrenoceptors in the uterine artery as well as umbilical vein (Isla and Dyer, 1990; Zhang and Dyer, 1991) and 5-HT_2 receptors in the uterine and umbilical arteries (Zhang and Dyer, 1990a, 1990b), receptively. The α_1-adrenergic and serotonergic mechanisms have been further investigated in the present studies.

Receptor-mediated vasoconstriction is accompanied by phosphoinositide hydrolysis (Abdel-Latif, 1986). It has been reported that there is a good correlation between these two events (Gu et al., 1991; Rapoport, 1987; Roth et al., 1986). In the ovine umbilical artery, we demonstrated that 5-HT induces phosphoinositide hydrolysis via 5-HT_2 receptors, which is time- and concnetration-dependent. Our findings may imply that receptor-mediated hydrolysis of phosphoinositide

could be involved in contractions induced by 5-hydroxytryptamine in the ovine umbilical artery.

Usually, there exist several agonists in the vicinity of the vascular smooth muscle. It is possible for these agonists to interact with each other. The amplification of one agonist-induced contraction by the other agonist has been reported in various vascular beds (Luscher and Vanhoutte, 1988; Prins et al., 1992; van Nueten et al., 1981; Xiao et al., 1989; Yang et al., 1992). We observed that subthreshold concentration of NE via α_1-adrenoceptors amplifies contractions induced by 5-HT in the ovine uterine artery. Ca^{2+} influx through L-type VOCs and activation of PKC are involved in the amplifying effects.

In the middle 1980s, it was found that α_1-adrenoceptors were not homoglous (Minneman, 1988). Previous studies from our laboratory have shown the existence of α_1-adrenoceptors in both the ovine uterine artery and umbilical vein (Isla and Dyer, 1990, Zhang and Dyer, 1991). We further characterized the subtypes of α_1-adrenoceptor using pharmacological tools and found the heterogeneity of α_1-adrenoceptors in both blood vessels. In addition, we also observed that α_{1A}-adrenoceptor might release intracellular Ca^{2+}, which disagrees with the signaling mechanism for α_{1A}-adrenoceptor suggested by Minneman (1988).

Receptor-mediated hydrolysis of phosphoinositide generates two second messengers: Ins(1,4,5)P_3 and DAG (Berridge, 1987). Inositol 1,4,5-trisphosphate releases Ca^{2+} from intracellular stores (Streb et al., 1983), while DAG activates PKC (Nishizuka, 1984). Activation of PKC has been suggested to play a role in agonist-induced contractions

(Rasmussen, 1987). Using the putative PKC inhibitor, staurosporine, we demonstrated that activation of PKC is involved in contractions induced by NE and endothelin-1 in ovine uterine artery, however, other mechanisms may also participate. Staurosporine is a relative selective PKC inhibitor in the ovine uterine artery, since the KCl-induced contractile response was not affected by staurosporine.

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