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Characterization of [alpha]-adrenoceptor-mediated contractile responses in the isolated bovine tail artery and vein and an investigation of nerve-mediated contraction in the bovine tail artery in response to electrical field stimulation

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Characterization of α-adrenoceptor-mediated contractile responses in the isolated bovine tail artery and vein and an investigation of nerve-mediated contraction in the bovine tail artery in response to electrical field stimulation

by

Marina Val. Ioudina

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This is to certify that the Doctoral dissertation of

Marina Val. Ioudina

has met the dissertation requirements of Iowa State University

Signature was redacted for privacy.

Major Professor

Signature was redacted for privacy.

For the Major Program

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For the Graduate College

TABLE OF CONTENT

LIST OF ABBREVIATIONS AND DEFINITIONS

AC	adenylyl cylclase
ATP	adenosine triphosphate
cAMP	cyclic adenosine monophosphate
DAG	diacylglycerol
EC_{50}	effective concentration of agonist required to produce half-maximal response
pD_2	negative log of molar concentration of EC_{50}
G protein	guanidine nucleotide-binding regulatory protein
IP_3	inositol 1,4,5-triphosphate
K_A	dissociation constant for the agonist-receptor complex obtained from functional study
K_B	dissociation constant for antagonist-receptor complex obtained from functional study
pK_B	negative log of K_B
K_D	dissociation constant for the radioligand-receptor complex obtained from radioligand binding experiments in which saturation experiments were performed
K_i	dissociation constant for the radioligand-receptor complex obtained when inhibition of ligand binding was studied
pK_i	negative log of K_i
PA	phosphatidic acid
pA_2	the negative log of the antagonist concentration which requires that the agonist concentration be doubled to produce an equivalent response
PKC	protein kinase C
PLA_2	phospholipase A_2
PLC	phospholipase C
PIP_2	phospatidylinositol-4,5-biphosphate

ABSTRACT

The purpose of this study was to identify α-adrenoreceptor types and subtypes mediating contractile responses to adrenergic agonists in the bovine tail artery and vein and to investigate nerve-mediated contraction in the bovine tail artery in response to electrical field stimulation. Isolated tissue bath methods were used to study isometric contractile responses in vessel rings. Electrical field stimulation, under constant current conditions, was used to study vessel contraction as a result of neurotransmitter(s) release from sympathetic nerve terminals.

Evidence was obtained for the presence of functional α_{1A}- and α_{1D}-adrenoceptors in the tail artery. Whether functional α_{1B}-adrenoceptors are present is less certain but it is likely that α_{1B}-adrenoceptors do co-exist with other α_1-adrenoceptor subtypes. Of the α_1-adrenoceptor subtypes, the α_{1A}-adrenoceptor plays a dominant role in the contractile response to adrenergic agonists in the tail artery.

In the isolated endothelium denuded bovine tail vein both types of α-adrenoceptors (α_1 and α_2) are capable of mediating contractile responses to adrenergic agonists. α_1-Adrenoceptors play a significant role in adrenergic-mediated contractile responses in the bovine tail vein via activation of both α_{1A}- and α_{1B}-adrenoceptors, but not via α_{1D}-adrenoceptors. α_2-Adrenoceptor mediated contractions occurred via activation of α_{2C}-adrenoceptors. The possibility that other α_2-adrenoceptor subtypes (α_{2B} and/or α_{2D}) participate in adrenergic agonist-mediated contraction in the bovine tail vein cannot be excluded.

A purinergic agonist, α,β-meATP, produced contractile responses in the isolated endothelium intact bovine tail artery via activation of purinergic receptors (P_{2X}). The role of ATP as a sympathetic neurotransmitter in mediating contractile responses to sympathetic nerve stimulation in the bovine tail artery is not clear. Our findings suggest that there is a possibility of norepinephrine/purinergic co-transmission in the bovine tail artery. However, further experiments which incorporate the direct measurement of ATP release during field stimulation along with the use of more selective P_{2X} receptor antagonists will be required to resolve this issue.

CHAPTER I. GENERAL INTRODUCTION

Dissertation Organization

This dissertation is written in an alternative thesis format. It contains a general introduction, four research papers, a general discussion and a list of references that were cited in the general introduction and discussion sections. Chapter I, "General introduction", includes research objectives, background information and literature review. Chapter II, "Co-existence of α_1-adrenoceptor subtypes in the bovine tail artery", Chapter III, "Characterization of α_{1A} and α_{1B}-adrenoceptor subtypes in the bovine tail vein", Chapter IV, "The role of α_2-adrenoceptors in contractile responses in the bovine tail vein and artery" and Chapter V, "Evaluation of nerve-mediated contraction in the bovine tail artery in response to electrical field stimulation" have been submitted for publication to the *European Journal of Pharmacology*. Chapter VI, "General conclusion", includes a general discussion about the major findings for this research project.

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

Research Objectives

Vascular tone as a component of cardiovascular regulation plays an important role in the delivery of oxygen and nutrients to the tissues. The skeletal muscle vasculature has a great influence on systemic vascular resistance and this in turn has an effect on arterial blood pressure regulation. The regulation of vascular tone is a complex process and has at least three major elements: neuronal, hormonal and local tissue factors (e.g., autacoids) (Lefkowitz et al, 1996).

The sympathetic nervous system, as a part of the autonomic nervous system, plays a significant role in vascular tone regulation. Norepinephrine is a neurotransmitter which is released from the sympathetic nerve terminal in response to nerve stimulation and then binds

to postsynaptic adrenoceptors to produce the response. According to modern nomenclature there are two types of functional α-adrenoceptors (α_1 and α_2) and each type has three subtypes (α_{1A}, α_{1B}, α_{1D} and $\alpha_{2A/D}$, α_{2B}, α_{2C}) (Docherty, 1998). The purpose of this research was to identify α-adrenoceptor types and subtypes in the bovine tail artery and vein which are capable of playing a role in contractile responses to adrenergic agonists and also to evaluate the possibility of whether purinergic (ATP) co-transmission occurs in the isolated bovine tail artery when sympathetic nerves are stimulated. Evaluation of the role of purinergic receptors and identification of functional α-adrenoceptors was determined by studying contractile responses in the isolated bovine tail artery and vein. Evaluation of possible ATP co-transmission in the isolated bovine tail artery was studied using electrical field stimulation.

Background and Literature Review

This section provides background information related to the studies presented in this dissertation and includes information about: 1) α-adrenoceptors classification, structure and intracellular signaling pathways; 2) pharmacological characterization of α-adrenoceptor (α_1 and α_2) subtypes; 3) sympathetic nervous system co-transmitters (norepinephrine, ATP and neuropeptide Y); and 4) purinergic receptor mediated contractile responses in smooth muscle.

α-Adrenoceptors

Classification

Adrenoceptors mediate the action of the sympathetic nervous system and are found in many peripheral tissues and within the central nervous system (Bylund et al., 1994). Adrenoceptors are involved in the functioning of all organs and organ systems which are under the control of the sympathetic nervous system. Adrenoceptors are cell membrane receptors which have seven transmembrane domains and are coupled to guanine nucleotide-binding regulatory protein (G protein) (Docherty, 1998). There are two large categories of

adrenoceptors, α and β which were originally defined by Ahlquist (1948) based on the potency rank of a number of adrenergic agonists in producing a physiological response. The main excitatory receptor (except in the intestine) was designated as an α-adrenoceptor while the main inhibitory receptor (except in the heart) was named the β-adrenoceptor (Ahlquist, 1948). Later, in the 1970's two types of α-adrenoceptors, α_1- and α_2-adrenoceptors, were identified (Langer, 1974).

Initially, α_2-adrenoceptors were discovered as prejunctional autoreceptors on adrenergic neurons which function to modulate norepinephrine release (Langer, 1974). In general, α_1-adrenoceptors are characterized by having a higher affinity to prazosin than do α_2-adrenoceptors (Bylund et al., 1994; Docherty, 1998; Langer, 1999) while α_2-adrenoceptors are characterized by having higher affinity to the agonists, clonidine and UK 14,304, (5-bromo-6-[2-imidazolin-2-ylamino]-quinoxaline bitartrate) and to the antagonists rauwolscine and yohimbine (MacKinnon et al., 1994). The heterogenity of α_1-adrenoceptors and the existence of subgroups of α_1-adrenoceptors was proposed based on contractile study data about differences in tissue sensitivity to norepinephrine (Bevan et al., 1988; Bevan et al., 1986). However, identification of α-adrenoceptor subtypes became possible with the development of radioligand binding assays (Bylund, 1988). Both types of α-adrenoceptors (α_1 and α_2) were eventually subdivided into smaller groups, i.e. subtypes (Table 1).

Table 1. Modern nomenclature of α-adrenoceptors (Docherty, 1998; Langer, 1999).

	Type of α-adrenoceptors	
Characteristics	α_1	α_2
Cloned receptors (subtypes)	α_{1a} α_{1b} α_{1d}	α_{2A} α_{2B} α_{2C} α_{2D}
Native receptors (subtypes)	α_{1A} α_{1B} α_{1D}	$\alpha_{2A/D}$ α_{2B} α_{2C}

Three α_1-adrenoceptors have been cloned (α_{1a}, α_{1b}, α_{1d}) and three functional α_1-adrenoceptor subtypes (α_{1A}, α_{1B}, α_{1D}) have been identified (Bylund et al., 1994; Docherty, 1998; Langer, 1999). A different kind of α_1-adrenoceptor subtype, with a low affinity to prazosin has been identified in some tissues but has not been cloned (Langer, 1999). Another

classification scheme has divided α_1-adrenoceptors into two categories based on their affinity to prazosin: α_{1H}-adrenoceptors - those receptors with high affinity to prazosin (α_{1A}, α_{1B}, α_{1D}) and α_{1L}-adrenoceptors – those receptors with a low affinity to prazosin (Bylund et al., 1994; Docherty, 1998; Langer, 1999) (Fig. 1).

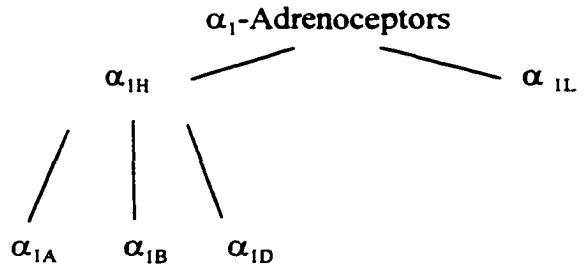

Figure 1. Subclassification of α_1-adrenoceptors (Langer, 1999).

Four subtypes of α_2-adrenoceptors have been cloned (α_{2A}, α_{2B}, α_{2C} and α_{2D}). Current classification recognizes three functional ($\alpha_{2A/D}$, α_{2B}, α_{2C}) subtypes (Bylund et al., 1994; Docherty, 1998). The α_{2A}- and α_{2D}-adrenoceptor subtypes are species homologues of the same receptor subtype ($\alpha_{2A/D}$-subtype) (Bylund et al., 1994). α_{2A}-Adrenoceptors are found in humans (Guimaraes et al., 1998), pigs (Trendelenburg et al., 1996) and rabbits (Trendelenburg et al., 1994), while the α_{2D}-adrenoceptor subtype is present in the rat (Guimaraes et al., 1998), mouse (Limberger et al., 1995), guinea pig (Guimaraes et al., 1998) and cattle (Bylund, et al., 1997).

Characterization of α-adrenoceptors

Several methods have been developed to characterize the presence of α-adrenoceptors in tissues: functional studies (Furchgott, 1972), radioligand binding assays (Buckner, et al., 1996), immunostaining assays (Hrometz, et al., 1999) and a variety of molecular biological techniques (Perez, et al., 1994). The following includes a brief review of the most commonly used methods in receptor characterization.

Functional study. In order to produce a response to a ligand (agonist), the ligand has to bind to the receptors and form the receptor-ligand (agonist) complex (Furchgott, 1966). This process can be described by an equation:

$$[\text{Ligand}] + [\text{Receptor}] \leftrightarrows [\text{Ligand} \times \text{Receptor}]$$

The response to an agonist is proportional to the number of occupied receptors (Furchgott, 1966). The ideal system for pharmacological characterization of the receptors is when the binding process is in thermodynamic equilibrium, i.e., the free-ligand and the receptor-bound ligand are in equilibrium (Furchgott, 1972). However, if the ligand mentioned above is norepinephrine there are some parameters that have to be controlled in order to achieve equilibrium conditions. The concentration of norepinephrine decreases over time from the receptor region due to the removal process which includes not only simple dilution of the norepinephrine away from the receptor but also transport (uptake) processes (Furchgott, 1966; Furchgott, 1972; Lefkowitz et al., 1996). Several uptake processes have been defined for norepinephrine (Furchgott, 1966; Lefkowitz et al., 1996). In order to inhibit these removal processes when α-adrenoceptors are characterized, it has been proposed that monoamine oxidase (MAO), catecholamine-*O*-methyltransferase, neuronal ($uptake_1$) and tissue uptake ($uptake_2$) be blocked with iproniazid, tropolone, cocaine and corticosterone, respectively (Furchgott, 1966; Furchgott and Garcia, 1968; Levin and Furchgott, 1970). Concentration-response relationships to an agonist are usually obtained by adding the agonist cumulatively to the tissue bath in half-log increments. Concentration-response relationships to an agonist are obtained twice, before and after incubation with one concentration of an antagonist. For each experiment a paired tissue not exposed to an antagonist is carried through the procedure to permit correction for time-dependent changes in responses to the agonist, as recommended by Furchgott (1972) and as used in our laboratory (Zhang and Dyer, 1990; Hill and Dyer, 1997).

There are some parameters which are commonly acquired in the characterization process. The receptor-agonist complex can be described by the effective concentration (EC_{50}, molar concentration) of agonist required to produce 50% of the maximal contraction ($-\log EC_{50} = pD_2$) and by determining the dissociation constant (K_A) for the receptor-agonist complex as described by Furchgott and Burzstyn (1967). In order to calculate the K_A value

for a given agonist, a concentration-response relationship is obtained before and after inactivation of a fraction of the receptors with dibenamine. Then a double reciprocal plot is constructed of equi-effective concentrations of an agonist before (1/[A]) and after (1/[A]') treatment with dibenamine (corrected for the time-dependent changes). The dissociation constant (K_A) can be calculated by using the equation: K_A = slope - 1/intersept (Ruffolo, 1982). Agonist affinity is a reciprocal of the K_A value ($1/K_A$) (Furchgott, 1972).

The dissociation constant (K_B) for the receptor-antagonist complex and a determination of an antagonist's potency (pA_2) can be obtained by methods described by Furchgott (1972) and as used in our laboratory (Zhang and Dyer, 1990). This method is based on a comparison of shifts of the agonist concentration-response curve at the EC_{50} in control and antagonist treated tissues. Briefly: agonist EC_{50} values are determined in the absence (A) and presence (A*) of each competitive antagonist concentration from agonist concentration-response curves. The concentration ratio, CR = A*/A (Furchgott, 1972), and the log (CR -1) are calculated after correction for the time-dependent changes in sensitivity to the agonist occurring during the course of the experiment. pA_2 values can be calculated, according to Arunlakshana and Schild (1959), by using the equation: log (CR-1) = log[B] - log K_B (Furchgott, 1972), where [B] is the concentration of the antagonist and K_B, the dissociation constant of the receptor-antagonist complex. The calculated log (CR-1) values are plotted versus the log [B] as a linear relationship and where the line intercepts the abscissa yields the pA_2 value (pA_2 = - log K_B).

Alternatively, the relative potency of antagonists can be determined by calculating pK_B values (pK_B = -logK_B) using a single concentration of an antagonist as described by Furchgott (1972). For this purpose EC_{50} values should be calculated from the concentration-response curves in the absence [A] and in the presence [A'] of each competitive antagonist concentration. The concentration ratio, CR = [A']/[A] (Furchgott, 1972), and the log (CR -1) should be calculated after correction for the time-dependent changes in sensitivity to the agonist. The K_B value can be calculated as: K_B = [B]/CR-1, where [B] is the concentration of the antagonist (Furchgott, 1972) and expressed as pK_B (-log K_B). However, this method

cannot provide information as to whether the antagonism was competitive as can the method discussed above by Arunlakshana and Schild (1959).

Functional studies have some limitations which are mainly related to the availability of selective agonists and/or antagonists. However, an advantage of this technique is that a physiologic response (e.g., tissue contraction) is used in the receptor characterization process.

Radioligand binding assay. Radioligand binding assay is a powerful tool used to determine the receptor type(s) on cell membranes. The principle of the radioligand binding assays is to measure the bound radioactivity which can be accomplished by three types of experiments: saturation, kinetic and/or inhibition (Bylund and Toews, 1993). The saturation method allows the determination of the receptor density [B_{max}] and the affinity of the receptors for the radioligand (dissociation constant, K_D). Dissociation rate constants can be estimated from kinetic data and the affinity (K_i) of the drug for the receptor can be estimated from inhibition experiments (Bylund and Toews, 1993). While radioligand binding studies were not performed for this dissertation, literature values of agonists or antagonists affinities obtained for cloned and native α-adrenoceptors by radioligand binding methods were used for comparison with data obtained from functional study experiments.

Other methods. Fluorescent ligand binding assay allows for the study of α-adrenoceptors receptors localization in live tissue (Piascik et al., 1997; Daly et al., 1998; Daniel et al., 1999). The use of fluorescent ligand binding assay in conjunction with confocal laser scanning microscopy allows the data to be quantified (Daly et al., 1998).

Identification of mRNA distribution of α-adrenoceptor types and subtypes in tissues can be used to determine tissue expression of receptors. (Piascik et al., 1994; Zhong and Minneman, 1999).

Molecular cloning techniques permit the study of receptor molecular structure (Graham et al., 1996; Schwinn and Price, 1999) and provide a scientific tool to study the mechanism of receptor function.

α_1-Adrenoceptors

This section is a general review of α_1-adrenoceptors and includes information about tissue distribution, molecular structure and intracellular signaling. Information about α_1-adrenoceptor subtypes and selective agonist and antagonists for these subtypes is also presented.

Tissue distribution. α_1-Adrenoceptors have been an extensively studied group of receptors. The presence of α_1-adrenoceptors has been reported in vascular tissues of different species, such as in: rat tail artery (Lachnit et al., 1996; Villalobos-Molina and Ibarra, 1996), rat hind limb (Zhu et al, 1997), rat mesenteric artery (Stassen et al, 1998; Piascik et al., 1997), rat aorta (Villalobos-Molina and Ibarra, 1996), rat carotid artery (Villalobos-Molina and Ibarra, 1996; Noble et al., 1997), rabbit ear artery (Fagura et al., 1997), pig splenic artery (Barbiery et al., 1998) and coronary artery (Yan et al., 1998), equine digital vein (Elliot, 1997), sheep uterine artery and umbilical vein (Hu and Dyer, 1997) and bovine tail artery (Hill and Dyer, 1997) as well as in non-vascular tissues in human (vas deferens, Furukawa et al., 1995; prostate, Teng et al., 1994; Chess-Williams et al., 1996; urethra, Taniguchi, et al., 1997); rabbit heart (Hattori and Kanno, 1997); pig female urethra (Alberts et al., 1999); rat liver (Stam et al., 1998), rat heart (Yu and Han, 1994) and vas deferens (Honner and Docherty, 1999).

Interestingly, in human tissues the highest concentration of mRNA for α_{1A}-adrenoceptors is found in the heart, for α_{1B}-adrenoceptors in spleen and for α_{1D}-adrenoceptors in urethra (Langer, 1998). The main physiological role of α_1-adrenoceptors in vascular tissues is in the control of blood pressure (Bylund et al., 1994). α_1-Adrenoceptors are also involved in growth of vascular smooth muscle and cardiac muscle (Zhong and Minneman, 1999).

Structure. α_1-Adrenoceptors are a heterogeneous group of single chain polypeptides with a molecular weight of about 65 - 80 kDa (Graham et al., 1996). The polypeptide chains of the rat cloned α_{1a}-, α_{1b}-, α_{1d}-adrenoceptors consist of 561, 515 and 466 amino acids,

respectively (Graham et al., 1996) (Table 2). Four different variants of human α_{1A}-adrenoceptors have been cloned: $\alpha_{1a\text{-}1}$ (wild type), $\alpha_{1a\text{-}2}$, $\alpha_{1a\text{-}3}$ and $\alpha_{1a\text{-}4}$ (Schwinn and Price, 1999). These variants have differences only in the carboxyl terminals but there are no differences in their pharmacology or in their intracellular second messenger systems (Schwinn and Price, 1999).

The helical membrane-spanning domains are composed of an extracellular pocket to bind the hydrophilic catecholamines and other adrenergic ligands (Graham et al., 1996; Chen et al., 1999). The ligand-binding pockets must be different among the α_1-adrenoceptor subtypes (Hwa et al., 1995).

Interestingly, α_{1B}-adrenoceptors can be desensitized. Homologous desensitization and phosphorylation of α_{1B}-adrenoceptors by agonists is the result of G-protein kinase-mediated phosphorylation at Ser^{404}, Ser^{408} and Ser^{410} sites, located on the carboxyl terminal of the receptor (Scheer et al., 1999; García-Sáinz et al., 2000).

Table 2. Characteristics of cloned α_1-adrenoceptors.

	α_1-Receptor Subtypes		
Characteristics	α_{1a}	α_{1b}	α_{1c}
Molecular weight (kDa)	80	80	≈65
Amino acids	466	515	561
Number of amino acids on amino	25	42	90
Glycosylation sites (N-terminus)	3	4	2
G-protein coupling	$G_{q/11/14}$	$G_{q/11/14/16}$, G_h	$G_{q/11}$
Receptor-coupled signaling	Ca^{2+} mobilization, PLC, PLA_2, PLD		

Characterization of the cloned α_1-adrenoceptors; PLC, phospholipase C; PLA_2, phospholipase A_2; PLD, phospholipase D, (modified from Graham et al., 1996).

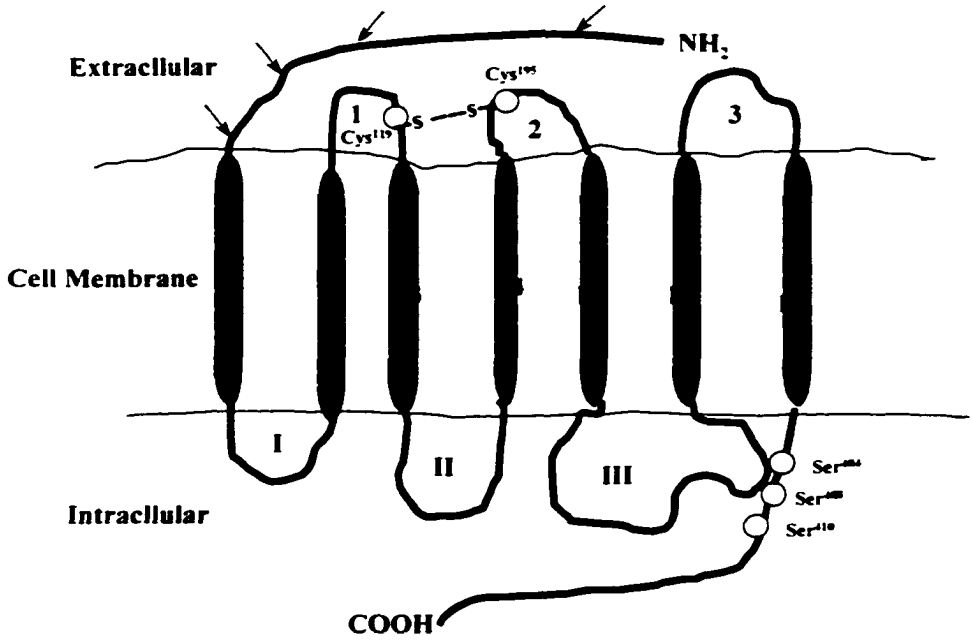

Figure 2. Model of α_1-adrenoceptors. α_1-Adrenoceptors have seven transmembrane domains (D1 - D7) connected by three extracellular (1 - 3) and three intracellular hydrophilic loops (I - III) (Graham et al., 1996). The extracellular amino termini contain 2 - 4 glycosylation sites (→) (Graham et al., 1996). In the α_{1B}-adrenoceptor subtype the essential disulfide bond between two amino acids Cys^{119} and Cys^{195} is inaccessible for solvents (Graham et al., 1996). Amino acids, Ser^{404}, Ser^{408} and Ser^{410}, located on the carboxyl terminal of the α_{1B}-adrenoceptors, are substrates for phosphorylation and desensitization of the receptor (Scheer et al., 1999; García-Sáinz et al., 2000).

α_1-Adrenoceptors belong to the G protein-coupled family of receptors. Each α_1-adrenoceptor has seven transmembrane domains of 20-28 hydrophobic amino acids connected by three extracellular and three intracellular hydrophilic loops (Graham et al., 1996) (Fig. 2).

The extracellular amino termini consists of a different number of amino acids depending on the α_1-adrenoceptor subtype and contains 2 - 4 glycosylation sites (Graham et al., 1996). The first and the second extracellular loops have a single cysteine which is

essential for folding. In the α_{1B}-adrenoceptor subtype the essential disulfide bond between Cys^{119} and Cys^{195} is inaccessible for solvents (Graham et al., 1996). The second and the third intracellular loops are involved in intracellular coupling to G-proteins. The third intracellular loop and the carboxyl terminal of α_1-adrenoceptors may be subtype-specific (Langer, 1998) and contain phosphorylation sites for protein kinase A (Graham et al., 1996) and be involved in receptor-mediated phosphoinositol signaling mechanisms (Graham et al., 1996) (Fig. 2).

The α_{1B}-adrenoceptor is also a substrate for heterologous desensitization via different mechanisms, such as: by phorbol esters (through activation of protein kinase C); via receptors coupled to G_q-proteins (such as endothelin receptors - ET_A), to G_i-proteins and to endogenous tyrosine kinase (growth factor receptors) (Scheer et al., 1999; García-Sáinz et al., 2000).

Signal transduction pathways. The main role of α_1-adrenoceptors on vascular smooth muscle cell membrane is in mediating contractile responses. The traditional pathway of the intracellular reactions is associated with pertussis toxin-insensitive G-proteins (G_q, G_{11}, G_{14}, G_{16} and G_h) coupled response. However, it has also been demonstrated that α_1-adrenoceptors can be coupled to pertussis toxin-sensitive G-proteins (G_i or G_o) (Graham et al., 1996). All α_1-adrenoceptors are coupled to G_q and G_{11} while α_{1A}- and α_{1B}-adrenoceptor subtypes are coupled to G_{14} and only α_{1B}-adrenoceptors are coupled to G_{16} and G_h (Table 2) (Graham et al., 1996). G-protein contains three subunits, which are designated as α, β and γ. When a ligand bind the α_1-adrenoceptors it causes changes in the conformation of the G-protein: the replacement of GDP by GTP, the dissociation of the α- from the $\beta\gamma$-subunit of the G-protein and formation of $G\alpha$-GTP, which in turn activates phospholipase C (PLC-β) (Marshall et al., 1999). Activation of PLC-β results in hydrolysis of phospatidylinositol-4,5-biphosphate (PIP_2) and formation of the cellular second messengers: inositol 1,4,5-triphosphate (IP_3) and diacylglycerol (DAG). IP_3 stimulates release of Ca^{2+} from sarcoplasmic reticulum and DAG activates protein kinase C (PKC) to produce cellular responses, (Hieble et al., 1995; Zhong and Minneman, 1999; Varma and Deng, 2000) which include activation of gene transcription (Graham et al., 1996).

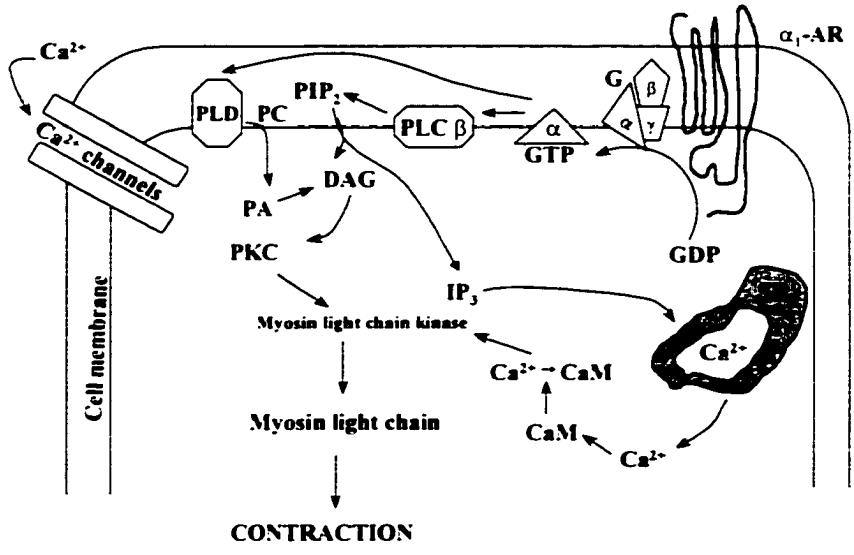

Figure 3. The pathway of intracellular reactions via activation of α_1-adrenoceptors. The activation of α_1-adrenoceptors (α_1-**AR**) causes changes in the conformations of the G-protein (**G**): the replacement of **GDP** by **GTP**, dissociation of the α- from the βγ-subunit of the G-protein and formation of Gα-GTP, which in turn activates phospholipase C (**PLC** β) (Marshall et al., 1999). Activation of PLC β results in hydrolysis of phospatidylinositol-4,5-biphosphate ($\mathbf{PIP_2}$) and formation of the cellular second messengers: inositol 1,4,5-triphosphate ($\mathbf{IP_3}$) and diacylglycerol (**DAG**). IP_3 stimulates release of $\mathbf{Ca^{2+}}$ from intracellular store (**SR** – sarcoplasmic reticulum) and diacylglycerol (**DAG**) activates protein kinase C (**PKC**) (Hieble et al., 1995; Zhong and Minneman, 1999; Varma and Deng, 2000). Intracellular Ca^{2+} concentration can be increased also via Ca^{2+} influx through voltage-dependent and independent channels (Zong and Minneman, 1999; Varma and Deng, 2000). Intracellular Ca^{2+} binds calmodulin and forms a Ca^{2+}-calmodulin complex which activates myosin light chain kinase. Activated myosin light chain kinase along with PKC initiates actin-myosin interaction by phosphorylation of myosin light chain, which in turn produces contraction of the smooth muscle cell (Voet and Voet, 1995). Stimulation of α_1-adrenoceptors can activate phospholipase D (**PLD**) (Zhong and Minneman, 1999; Varma and Deng, 2000) which can initiate hydrolysis of phosphatidylcholine (**PC**) to phosphatidic acid (**PA**) and this pathway leads to additional formation of DAG (Varma and Deng, 2000).

The cascade of intracellular reactions leads to an increase in intracellular Ca^{2+} concentration and formation of Ca^{2+}-calmodulin complex, which is essential to activate myosin light chain kinase. Activated myosin light chain kinase along with PKC initiates actin-myosin interaction by phosphorylation of myosin light chain which in turn produces contraction of the smooth muscle cell (Voet and Voet, 1995) (Fig. 3).

In addition to the traditional pathways of intracellular signaling several other mechanisms have been proposed for α_1-adrenoceptor-mediated responses. For example, intracellular Ca^{2+} can be increased not only via mobilization from intracellular stores but also via Ca^{2+} influx through voltage-dependent and independent channels (Zong and Minneman, 1999; Varma and Deng, 2000). Stimulation of α_1-adrenoceptors can activate phospholipase D (PLD) (Zhong and Minneman, 1999; Varma and Deng, 2000) and/or phospholipase A_2 (Graham et al., 1996; Ruan et al., 1998) (Fig. 3).

Activation of PLD can initiate hydrolysis of phosphatidylcholine to phosphatidic acid and this pathway leads to additional formation of DAG (Varma and Deng, 2000). Furthermore, activation of α_1-adrenoceptors can stimulate accumulation of cAMP via a potentiation of adenylyl cyclase through PKC activation and/or via inhibition of cAMP degradation (Graham et al., 1996).

Investigation of the role of α_1-adrenoceptors in cell growth and hypertrophy is a rapidly developing research area. It has been demonstrated that α_1-adrenoceptor agonists can activate mitogenic responses in vascular (Xin et al., 1997) and cardiac (Ramirez et al., 1997) muscle cells. Norepinephrine stimulates the growth of vascular smooth muscle cells isolated from rat aorta and this occurs via activation of either α_{1D}-adrenoceptors (Xin et al., 1997) or α_{1B}-adrenoceptors (Chen et al., 1995). The physiological significance and role of α_1-adrenoceptors in regulating smooth muscle growth and the mechanisms involved in its intracellular signaling needs further investigation (Zhong and Minneman, 1999).

α_1-Adrenoceptor subtypes

α_{1A}-Adrenoceptor. The α_{1A}-adrenoceptor is the most well characterized subtype among the α_1-adrenoceptors. The presence of functional α_{1A}-adrenoceptors has been reported in various vascular as well as in non-vascular smooth muscle. Activation of α_{1A}-adrenoceptors causes contraction of smooth muscle such as: rat tail artery (Lachnit et al., 1996; Villalobos-Molina and Ibarra, 1996), rat hind limb vasculature (Zhu et al. 1997), rat mesenteric artery (Stassen et al. 1998), rat aorta (Villalobos-Molina and Ibarra. 1996), rat vas deferens (Burt et al., 1998), porcine coronary artery (Yan et al., 1998), guinea pig nasal mucosa vasculature (Tanimitsu et al., 2000), female pig urethra (Alberts, et al., 1999), human urethra (Taniguchi et al., 1997), human prostate (Marshall et al., 1995) and human vas deferens (Furukawa et al., 1995).

The α_{1A}-adrenoceptor subtype has been characterized in experiments which used subtype selective agonists or antagonists. There are two commercially available adrenergic agonists with a high selectivity to the α_{1A}-adrenoceptors. A61603 ((±N-[5-(4,5-dihydro-1 H-imidazol-2yl)-2-hydroxy-5,6,7,8-tetrahydronaphthalen-1-yl] methanesulphonamide hydrobromide) and oxymetazoline. In radioligand binding experiments, A61603 has a high affinity (pK_i) for native α_{1A}-adrenoceptors in rat vas deferens, canine prostate and for cloned α_{1a}-adrenoceptors (Buckner, et al., 1996). A61603 caused a concentration dependent contraction of the isolated rat tail artery (pD_2 of 7.59) which was inhibited by prazosin and by the selective α_{1A}-adrenoceptor antagonist RS-17053 (N-[2-(2-cycloprorylmethoxyphenoxy)ethyl]-5-chloro-α,α,-dimethyl-1H-indole-3-ethanamine hydrochloride) (Lachnit et al., 1996), but not by idazoxan, an α_2-adrenoceptor antagonist, or by chloroethylclonidine, an irreversible α_{1B}- and α_{1D}-adrenoceptor antagonist (Lachnit et al., 1996). Oxymetazoline is reported to be an α_{1A}-adrenoceptor agonist and it produced contractions of the isolated female pig urethra ($pD_2 = 6.18$; Alberts et al., 1999) and rabbit iris dilator ($pD_2 = 6.77$, Nakamura et al., 1999). A61603 exhibits a higher affinity to α_{1A}-adrenoceptors when compared to that by norepinephrine and phenylephrine (Buckner et al., 1996). In radioligand binding experiments, oxymetazoline was found to have a higher

affinity (pK_i) for α_{1A}-adrenoceptors than for other α_1-adrenoceptor subtypes (Buckner et al., 1996; Perez et al., 1994). However, there are conflicting data in the literature about the specificity of oxymetazoline. In rat tail artery, oxymetazoline has been shown to act as a serotonin agonist (Lachnit et al., 1996) and it also antagonized contractile responses to A61603 (pK_i = 7.6) (Lachnit et al., 1996).

Two commercially available subtype selective antagonists have been commonly used to study α_{1A}-adrenoceptors namely: WB 4101 ((2-(2,6-dimethoxyphenoxyethyl) aminomethyl-1,4 benzodioxane) (Villalobos-Molina and Ibarra, 1996; Hu and Dyer, 1997; Marshall et all, 1995) and 5-methylurapidil (Fagura et al., 1997; Testa et al., 1995; Lachnit et al., 1996). WB 4101 has been used to characterize the α_{1A}-adrenoceptor subtype in the rat tail artery (pA_2 = 9.08) (Villalobos-Molina and Ibarra, 1996), in the isolated ovine uterine artery (pA_2 = 8.30) and ovine umbilical vein (pA_2 = 8.45) (Hu and Dyer, 1997), in the rat portal vein (pA_2 = 9.4) (Marshall et all, 1995) and in porcine coronary artery (pA_2 = 10.67). Another α_{1A}-adrenoceptor selective antagonist, 5-methylurapidil, was found to be a potent inhibitor of adrenergic-induced contraction in the rat aorta (pA_2 = 7.64 - 7.95, Testa et al., 1995; Fagura et al., 1997), in the rat tail artery (pA_2 = 9.0, Lachnit et al., 1996) and in the female pig urethra (pK_B = 8.59, Alberts, et al., 1999). There are some other antagonists which have been shown to have a greater selectivity to α_{1A}-adrenoceptor subtypes, such as: RS 17053 (N-[2-(2-cyclopropylmethoxyphenoxy)ethyl]-5-chloro-α,α-dimethyl-1H-indole-3-ethanamine hydrochloride), SNAP 5089 (2,6-dimethyl-4-(4-nitrophenyl)-1,4-dihydropyridine-3,5-dicarboxilic acid) and REC, 15/2739 (SB 216469, 8-3-[4-(2-methoxyphenyl)-1-benzopyran dihydrochloride). Contractile responses of the rat tail artery via activation of α_{1A}-adrenoceptor were inhibited by RS 17053 (pA_2 = 9.2), SNAP 5089 (pA_2 = 9.3) and REC, 15/2739 (pA_2 = 10.0) (Lachnit et al., 1996). However, Marshall and colleagues (1995) reported that the relative potency of RS 17053 was significantly lower for α_{1A}-adrenoceptors in the rat portal vein (pK_B = 7.1) and human prostate (pK_B = 7.1) than that to α_{1A}-adrenoceptors in rat epididimal vas deferens (pA_2 = 9.5) (Marshall et al., 1995). The differences in RS 17053's relative affinity for these α_{1A}-adrenoceptors are not clear and cannot be explained by species differences.

α_{1B}-Adrenoceptors. The role of the α_{1B}-adrenoceptor subtype in contractile responses of vascular and non-vascular smooth muscle has been recently studied. Activation of α_{1B}-adrenoceptors causes contractile responses in smooth muscle via phosphatidylinositol hydrolysis and Ca^{2+} mobilization (Zhong and Minneman, 1999). The presence of α_{1B}-adrenoceptors has been reported in the rat spleen (Burt et al., 1995; Noble et al., 1997), rat heart (Yu and Han, 1994), human prostate (Teng et al., 1994) and in rat mesenteric artery (Piascik et al., 1997).

Table 3. Antagonist potency (pA_2) for α_1-adrenoceptors reported from functional studies.

Antagonists	α_{1A}	α_{1B}	α_{1D}
Prazosin	8.78[a] 8.46*[a] 9.04[b] 9.02[c]	10.02[a]	9.35[a]
WB 4101	9.69[a] 8.87*[a] 9.48[b] 9.08[c]	9.39[a]	9.36[a] 9.17[c]
5-Methylurapidil	8.39[a] 8.76*[a] 8.10[b] 9.0[c] 8.19[c]	7.17[a]	7.83[a] 7.72[c] 7.95[d]
ARC 239	8.47[a] 8.98*[a]	8.86[a]	9.46[a]
Phentolamine	8.01[a] 7.53*[a]	7.47[a]	7.72[a]
BMY 7378	6.66[b] 6.09[c] 6.3[e]	---	9.08[d] 8.72[c]

a - Buckner et al., 1996: α_{1A}- rat vas deferens, α_{1A}- canine prostate*, α_{1D}- rat spleen and α_{1B} - rat aorta;

b - Zhu et al., 1997: α_{1A}- in perfused rat hind limb;

c - Villalobos -Molina and Ibarra, 1996: α_{1A}- α_{1D}- rat tail artery;

d - Fagura et al., 1997; α_{1D}- rat aorta;

e - Lachnit et al., 1996: α_{1A}- rat caudal artery.

Chloroethylclonidine, an irreversible antagonist, has been used to discriminate α_{1B}-adrenoceptors (Testa et al., 1995; Hattori and Kanno, 1997; Perez et al., 1994; O'Rourke et al., 1995) from the α_{1A}-adrenoceptor subtype based on its higher alkylation rate for α_{1B}-adrenoceptors (Xiao and Jeffries, 1998).

Table 4. Radioligand affinities (pK_i) for several agonists and antagonists in cloned α_1-adrenoceptors.

Agonists	α_{1a}	α_{1b}	α_{1d}
Norepinephrine	5.66[b] 6.38[b*]	6.54[b]	7.69[b]
Epinephrine	6.62 [b] 6.64[b*]	6.91[b]	7.45[b]
Cirazoline	7.02 [b] 7.00[b*]	6.70[b]	7.32[b]
Phenylephrine	6.03 [b] 5.76[b*]	5.90[b]	6.79[b]
Oxymetazoline	7.88 [b] 7.86[b*]	6.69[b]	6.47[b]
A61603	7.52 [b] 7.61[b*]	5.68[b]	5.87[b]
Methoxamine	5.26 [b] 5.54[b*]	4.35[b]	5.24[b]
Antagonists			
Prazosin	9.34[a]	9.16[a]	9.69[a]
WB 4101	9.28[a]	7.29[a]	8.42[a]
RS-17053	8.59[a]	7.06[a]	7.40[a]
5-Methylurapidil	8.24[a]	6.40[a]	6.76[a]
BMY 7378	6.11[a]	6.40[a]	8.29[a]
L-765,314	6.30[c]	8.27[c]	7.30[c]

a - Zhu et al., 1997; with ^{125}I-BE2254, in HEK cells;

b - Buckner et al., 1996; with [^{3}H]prazosin; α_{1a}- bovine cloned, α_{1a}*- human cloned, α_{1b}- hamster cloned and α_{1d} - rat cloned;

c – Patane et al., 1998; with [^{125}]HEAT for the cloned receptors.

However, chloroethylclonidine does not distinguish between α_{1B}- and α_{1D}-adrenoceptor subtypes (O'Rourke et al., 1997; Xiao and Jeffries, 1998). Complicating the issue is that α_{1B}- and α_{1D}-adrenoceptors have a similar affinity to most adrenergic antagonists (Piascik et al., 1997) and this creates a problem in receptor subtype identification when functional methods are used and when tissue contains both α_{1B}- and α_{1D}-adrenoceptor

subtypes. In addition, the interactions of chloroethylclonidine with α-adrenoceptors needs more study.

The synthesis of novel α_{1B}-adrenoceptor subtype selective antagonists, cyclazosin ([4-(4-amino-6,7-dimethoxyquinazolin-2 yl)-*cis*-octahydroquinoxalin-1-yl]furan-2-yl-methanone) (Giardina et al., 1996) and L-765,314 (4-amino-2-[4-[1-(benzyloxycarbonyl)-2(S)-[[(1,1-dimethylethyl)amino] carbonyl]-piperazinyl]-6,7-dimethoxyquinazoline), which is related to prazosin, has been recently reported (Patane, et al., 1998).

Cyclazosin exhibited a higher affinity (pK_i = 9.68), as determined by radioligand binding assays, for the α_{1B}-adrenoceptor in rat liver (Stam et al., 1998) but failed to discriminate α_{1B}-adrenoceptors in functional studies. Cyclazosin's relative potency (pK_B) against phenylephrine-induced contraction was 7.96 for α_{1B}-adrenoceptors in rat spleen while the potency (pA_2) for $\alpha_{1A/L}$-adrenoceptors in rat small mesenteric artery and α_{1D}-adrenoceptors (pK_B) in rat aorta were 7.78 and 6.86, respectively (Stam et al., 1998). A newly described α_{1B}-adrenoceptor antagonist, L-765,314, was found to have a ten-fold greater affinity for α_{1B}- over the α_{1D}-adrenoceptors and more than a 100-fold greater affinity for the α_{1B}- over the α_{1A}-adrenoceptor (Patane, et al., 1998).

α_{1D}-Adrenoceptors. When α_{1D}-adrenoceptors were cloned they were mistakenly reported to be like the α_{1A}-adrenoceptor. With the discovery of the selective competitive α_{1D}-adrenoceptor antagonist, BMY 7378 (8-(2-(4-(2-methoxyphenyl)-1-piperazinyl)ethyl)-8-azaspiro(4,5)decane-7,9-dione dihydrochloride) it became possible to pharmacologically distinguish the α_{1D}-adrenoceptor subtype (Goetz et al., 1995; Piascik et al., 1995). The presence of α_{1D}-adrenoceptors has been studied in a number of rat vascular beds including rat aorta (Piascik et al., 1995; Buckner et al., 1996; Villalobos-Molina and Ibarra, 1996), rat iliac artery (Piascik et al., 1995), rat carotid and mesenteric artery (Villalobos-Molina and Ibarra, 1996). There are no selective agonists for α_{1D}-adrenoceptors. BMY 7378 exhibits a high affinity for the α_{1D}-adrenoceptor subtype in the rat aorta and yielded pA_2 values of 8.72 (Villalobos-Molina and Ibarra, 1996) and 9.08 (Fagura et al., 1997).

Affinities and relative potencies of adrenergic agonists and antagonists for different α_1-adrenoceptor subtypes obtained from functional and radioligand binding experiments are presented in the tables 3 and 4.

α_2-Adrenoceptors

This section is a literature review of α_2-adrenoceptors and includes general information about tissue distribution, molecular structure, intracellular signaling pathways, α_2-adrenoceptor subtypes identified primarily in vascular smooth muscle and information about affinities of selective agonists and antagonists. In general, α_2-adrenoceptors are characterized as having a high affinity to the agonists, clonidine and UK 14,304 and to the antagonists rauwolscine and yohimbine and by having a low affinity to the α_1-adrenoceptor antagonist prazosin (see MacKinnon et al., 1994 for review).

Tissue distribution. The presence of α_2-adrenoceptors has been reported in many species and different tissues such as human right atrium (Rump et al., 1995), rabbit spleen (Michel et al., 1990), rabbit iris (Fuder and Selbach, 1993), opposum kidney (Blaxall et al., 1991), rat kidney (Michel et al., 1990), rat cortex (Ho et al., 1998), rat atrium (Ho et al., 1998), mouse brain (Limberger, et al., 1995), porcine uterus (Kitazawa et al., 2000), bovine ocular tissue (Bylund et al., 1997) and guinea pig kidney (Uhlén et al., 1994). α_2-Adrenoceptors have also been found in a variety of vascular beds such as the equine digital vein (Elliot, 1997), dog mesenteric artery and vein (Daniel et al., 1995; Paiva et al., 1999), dog saphenous vein (Hicks et al., 1991; MacLennan et al., 1997), rat femoral vein (Paiva et al., 1999), human saphenous vein (Smith et al., 1992; Roberts et al., 1992; Gavin et al., 1997) and human femoral vein (Glusa and Markwardt, 1983). There are both prejunctional and postjunctional α_2-adrenoceptors. Prejunctional α_2-adrenoceptors are located on the membrane of the sympathetic nerve terminal and mediate an inhibition of norepinephrine release through a negative feedback in response to a build-up of the norepinephrine concentration in the postganglionic sympathetic neuro-effector junction (Docherty 1998, for review). Postjunctional α_2-adrenoceptors are also located on the membrane of the effector

cell (Docherty 1998, for review). Stimulation of α_2-adrenoceptors on vascular smooth muscle mediates contraction (Elliot, 1997; Daniel et al., 1995; Paiva et al., 1999; Hicks et al., 1991; MacLennan et al., 1997; Smith et al., 1992; Roberts et al., 1992; Gavin et al., 1997; Glusa and Markwardt, 1983).

Structure. α_2-Adrenoceptors are glycoproteins which have seven transmembrane domains connected by three extracellular and three intracellular hydrophilic loops and two terminals: an extracellular amino terminal with sites for glycosylation and an intracellular carboxyl terminal. The helical structure of the fifth transmembrane domain has been demonstrated experimentally (Marjamäki et al., 1998).

Three pharmacological subtypes (α_{2A}, α_{2B} and α_{2C}) of human α_2-adrenoceptors are encoded by three genes (α_2C10, α_2C2 and α_2C4, respectively) (MacKinnon et al., 1994; Schaak et al., 1997). The gene (rg20), found in rat tissue, for the α_{2D}-adrenoceptor and the human gene, α_2C10 for the human α_{2A}-adrenoceptor, encode a similar protein (MacKinnon et al., 1994).

It is now accepted that the α_{2D}-adrenoceptor is a species homolog of the human α_{2A}-adrenoceptor subtype (O'Rourke et al., 1994; MacKinnon, 1994; Schaak et al., 1997; Bylund et al., 1997) and this is due to a difference in one amino acid located within the fifth transmembrane domain. Thus, the α_{2A}-adrenoceptor subtype contains Ser^{201} while the α_{2D}-adrenoceptor subtype contains Cys^{201} (MacKinnon, 1994). Variation in the amino acid (serine versus cysteine) in this position determines the pharmacological (subtype) differences in the ligand binding properties of these homologues receptors (Cockroft, et al., 2000).

The second and third intracellular loops of the α_2-receptor are responsible for binding a specific G-protein (Small et al., 2000; Näsman et al., 1997; Eason and Liggett, 1996) which initiates the cascade of intracellular reactions.

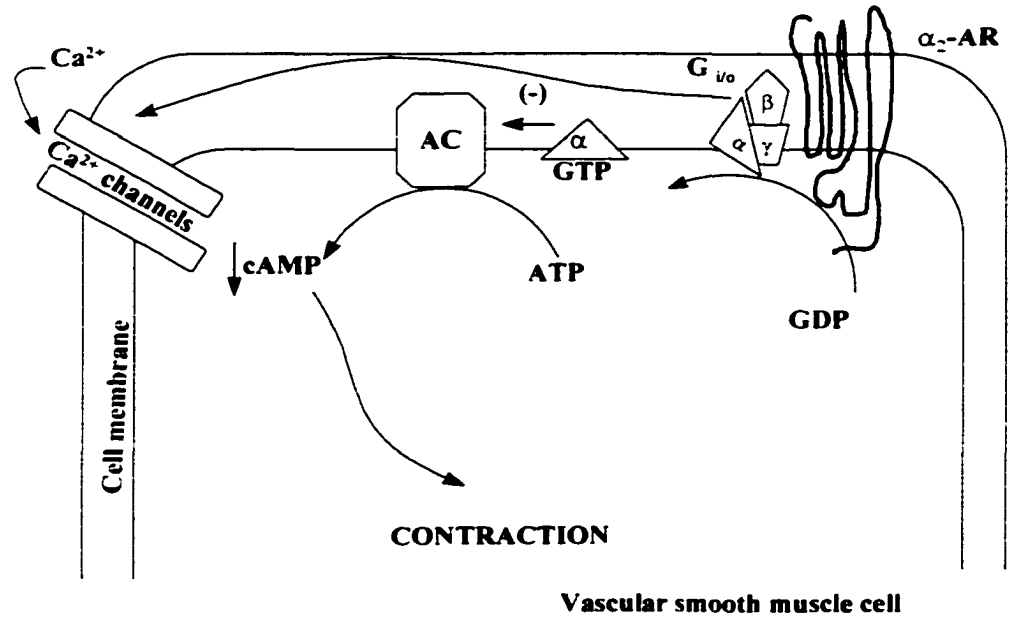

Figure 4. Intracellular reactions initiated by via activation of α_2-adrenoceptors. α_2-Adrenoceptors (α_2-**AR**) are coupled to a pertussis toxin sensitive G_i or G_o protein ($\mathbf{G_{i/o}}$) (Docherty, 1998). Activation of α_2-adrenoceptors is associated with inhibition of adenylyl cyclase (**AC**) which leads to inhibition of cAMP synthesis and which results in contraction of vascular smooth muscle. α_2-Adrenoceptors coupled to pertussis toxin-sensitive G protein (G_i) mediate contraction of the human subcutaneous artery mostly via an influx of extracellular Ca^{2+} through voltage-gated calcium channels (Parkinson and Hughes, 1995).

Signal transduction pathways. α_2-Adrenoceptors are coupled to a pertussis toxin sensitive G_i or G_o protein (Docherty, 1998) and can also be coupled to G_s (Eason and Liggett, 1996). The general pathway (Fig. 4) of intracellular signaling subsequent to the activation of α_2-adrenoceptors is associated with an inhibition of adenylyl cyclase which leads to inhibition of cAMP synthesis and which results in contraction of vascular smooth muscle. However, this is not the only intracellular signaling pathway known for α_2-adrenoceptor-mediated responses (Docherty, 1998).

In porcine palmer lateral vein, activation of α_2-adrenoceptors caused a contractile response via a reduction of previously elevated (by stimulation of adenylyl cyclase) level of cAMP but not in the tissue with a normal level of cAMP (Wright et al., 1995).

Postjunctional α_2-adrenoceptors

The presence of functional postjunctional α_2-adrenoceptors has been reported in several vascular beds, such as: equine digital vein (Elliot, 1997), dog mesenteric artery (Daniel et al., 1995), dog saphenous vein (Hicks et al., 1991; MacLennan et al., 1997) and human saphenous vein (Roberts et al., 1992). α_2-Adrenoceptor subtype identification, by using functional experiments, i.e. vasoconstriction, has some potential difficulties and these are related to the lack of potent subtype selective agonists and antagonists. In order to identify the subtypes of a receptor in a tissue it is often useful to correlate the data obtained from functional experiments with data obtained from radioligand binding experiments for the known (native or cloned) α_2-adrenoceptor subtype (MacLennan et al., 1997).

α_{2A}-Adrenoceptors. The α_{2A}-adrenoceptor subtype has been identified in isolated dog saphenous vein (MacLennan et al., 1997) and dog mesenteric vein (Paiva et al., 1999). The affinities (pK_A) of the following α_{2A}-adrenoceptor subtype selective agonists were: A-54741 (5,6-dihydroxy-1,2,3,4-tetrahydro-1-naphthyl-imidazoline) (8.03), oxymetazoline (7.67), guanfacine (6.79) and guanabenz (7.02) (MacLennan et al., 1997) and were higher than that of norepinephrine (5.74) in the isolated dog saphenous vein (MacLennan et al., 1997). Contractile responses to norepinephrine were inhibited by a number of antagonists, and their affinity (pK_B) was calculated as follows: prazosin (5.19), spiroxatrine (6.59), tolazoline (6.21), WB 4101 (7.42), rauwolscine (8.66) and idazoxan (7.11). Agonist pK_A values and antagonist pK_B values were significantly correlated (correlation coefficient, r = 0.82) with the affinities obtained from radioligand binding assays for the cloned human α_{2A}-adrenoceptor (MacLennan et al., 1997) expressed in Chinese hamster lung cells.

The pA_2 values of antagonists against the α_2-adrenoceptor selective agonist, UK 14,304 (5-bromo-6-[imidazolin-2-ylamino]-quinoxaline tartrate) (Paiva et al., 1999) were

significantly correlated with pK_i values from radioligand binding assays obtained on the cloned human α_{2A}-adrenoceptor (MacLennan et al., 1997).

α_{2B}-Adrenoceptors. The presence of functional α_{2B}-adrenoceptors in human saphenous vein were found by Smith and co-workers (1992). They obtained pA_2 values for the following antagonists: yohimbine (7.37), HV 723 ((α-ethyl-3,4,5-trimethoxy-α-(3((2-(2-methoxyphenoxy)ethyl)-amino)-propyl)-benzene acetonitrile fumarate) (7.52), ARC 239 ((2-(2,4-(o-methoxyphenyl)-piperazin-1-yl)-ethyl)-4,4-dimethyl-1,3-(2H,4H)-isoquuinolindine chloride) (7.05), prazosin (6.44) and BRL 44408 ((2-((4,5-dihydro-1H-imidazol-2-yl)-methyl)-2,3-dihydro-1-methyl-1H-isoindole) (5.72) and these pA_2 values were correlated ($r = 0.71$) with the antagonist's affinity at α_{2B}-ligand binding sites in rat kidney cells. In contrast, the rank of potencies for the α_{2A}-ligand binding site of human platelets was different from those in the human saphenous vein and their affinities were not strongly correlated.

α_{2C}-Adrenoceptors. The presence of functional α_{2C}-adrenoceptors has been reported in human saphenous vein (Gavin et al., 1997). The affinities (pK_B) for prazosin (6.62) and BRL 44408 (5.72) were lower than those for ARC 239 (7.19) and yohimbine (7.23). The affinities for the antagonists mentioned above were correlated with the affinities (pK_i) for the human cloned α_{2C}-adrenoceptor expressed on Sf9 cells, but not with the α_{2B}-ligand binding site in rat kidney cells or with the α_{2A}-ligand binding site in human platelets (Gavin et al., 1997).

α_{2D}-Adrenoceptors. The α_{2D}-adrenoceptor is a species homologue of the α_{2A}-subtype ($\alpha_{2A/D}$-subtype) (Bylund et al., 1994) but pharmacological differences between α_{2A}- and α_{2D}-adrenoceptor subtypes (Bohmann et al., 1994; Guimaraes et al., 1998; Ali, et al., 1998) have been reported. α_{2D}-Adrenoceptors are present in the rat (Guimaraes et al., 1998), mouse (Limberger et al., 1995), guinea pig (Guimaraes et al., 1998) and cattle (Bylund et al., 1997).

Prejunctional α_2-adrenoceptors

Prejunctional (i.e., presynaptic) α_2-adrenoceptors inhibit norepinephrine release from the sympathetic nerve terminal (Langer, 1974).

The presence of functional prejunctional α_2-adrenoceptors can be demonstrated in experiments by measuring [^{3}H]norepinephrine overflow (Guimaraes et al., 1998; Molderings and Gothert, 1995; Rump et al., 1995) or by measuring the contractile force developed in smooth muscle (Ali et al., 1998) in response to electrical stimulation of sympathetic nerve terminals in the presence and absence of selective inhibitors.

α_{2A}-Adrenoceptors. Prejunctional α_{2A}-adrenoceptors have been found in the rabbit pulmonary artery and human saphenous vein by measuring overflow of [^{3}H]norepinephrine (Molderings and Gothert, 1995) . In their experiment the α_2-adrenoceptor antagonist, rauwolscine, but not the α_1-adrenoceptor antagonist, prazosin, was very potent and facilitated the electrically stimulated release of [^{3}H]norepinephrine in both vessels. The potency of the antagonists in increasing [^{3}H]norepinephrine release was highly correlated with the antagonist's affinities (pK_i) for the α_{2A}-binding sites in human platelets.

In the isolated human gastric and ileocolic arteries, the α_2-adrenoceptor antagonists rauwolscine, RX 821002 (2-[2-methoxy-1,4-benzodioxan-2-yl]-2-imidazoline) and yohimibine, were more potent than prazosin in increasing [^{3}H]norepinephrine overflow (Guimaraes et al., 1998). They found a high correlation between the potencies (EC_{30}) of the eight α_2-antagonists, rauwolscine, RX 821002, yohimibine, prazosin, phentolamine, idazoxan, WB 4101 and spiroxatrine in human gastric and ileocolic arteries and the affinities (pK_i) of the same antagonists for the cloned human α_{2A}-subtype expressed in Chinese hamster lung cells.

α_{2B}-Adrenoceptors. Interestingly, that there are no reports, to my knowledge, about the presence of prejunctional α_{2B}-adrenoceptors.

Table 5. Radioligand affinities (pK_i or pK_D) for several agonists and antagonists for cloned α_2-adrenoceptors cited from radioligand binding studies.

Agonists	α_{2A}	α_{2B}	α_{2C}	α_{2D}
Oxymetazoline	7.30[a] 7.88[b] 8.11[g]	5.70[a] 6.13[b] 6.60[g]	6.67[a] 7.14[f]	8.6[e]
Norepinephrine	5.56[b] 6.37[g]	5.80[b] 6.68[g]		
Clonidine	7.71[a] 7.23 [b]	6.83[a] 7.45[b]	6.81[a]	
Guanfacine	7.75[a] 7.51 [b]	5.73[a] 6.01[b]	6.08[a]	
Guanabenz	7.91[a] 7.88 [b]	6.34[a] 6.70[b]	6.80[a]	
UK 14.304	6.76[b] 7.46[g]	6.53 [b] 6.61[g]	6.67[a]	
Antagonists				
RX 821002	9.87[a] 9.62[b]	8.10[a] 8.89 [b]	8.96[a]	8.8[e]
Idazoxan	8.69[a] 8.79[b] 7.12[g]	6.84[a] 7.76 [b] 7.29[g]	7.71[a] 8.62[f]	8.2[d] 8.0[e]
WB 4101	7.43[a] 7.26[b] 7.08[c] 8.17[g]	6.77[a] 7.80 [b] 6.94[c]	8.12[a] 8.80[f]	6.58[c] 7.3[d] 8.0[e]
Yohimbine	7.39[a] 7.53[b] 8.04[c]	6.67[a] 8.14[b] 7.93[c]	8.25[a] 9.40[f]	7.34[c]
Prazosin	5.94[a] 6.42[b] 5.62[c] 5.33[g]	6.89[a] 7.76[b] 7.12[c] 7.22[g]	6.89[a] 7.44[f]	6.24[c] 6.1[d] 6.3[e]
ARC 239	6.41[a] 6.47[b] 5.45[c]	7.28 [a] 8.11 [b] 7.06[c]	7.07[a] 7.32[f]	5.54[c] 6.7[d] 6.6[e]
Phentolamine	7.61[c] 7.78[g]	7.24[c] 7.97[g]		7.31[c] 8.5[d] 8.0[e]
SKF104078	6.56[b] 7.00[g]	6.83 [b] 6.96[g]		7.4[d] 7.4[e] 6.6[e]
Rauwolscine	7.56[a] 7.69[b] 8.06[g]	6.66 [a] 8.88 [b]	8.12[a] 10.10[f]	8.0[d] 8.3[e]

a - pK_D values published by Uhlen et al., 1995, with[^{3}H]-MK: α_{2A} and α_{2C} in guinea pig cerebral cortex, α_{2B} in guinea pig kidney;

b – pK_D values published by Renouard et al., 1994, with [^{3}H]RX821002: α_{2A} in rat cerebral cortex cells and α_{2B} in neonatal rat lung cells.

c – pK_i values published by Smith et al., 1995, with [^{3}H]yohimbine: α_{2A} in human platelet, α_{2B} in rat kidney and α_{2D} in rat submandibular gland;

d - pK_D values published by Limberger et al., 1995, with [^{3}H]norepinephrine: α_{2D} in mouse brain cortex;

e - pK_D values published by Wahl et al., 1996. [^{3}H]norepinephrine: α_{2D} in mouse heart atria;

f - pK_i values published by Blaxall et al., 1991, [^{3}H]rauwolscine: α_{2C} in opossum kidney cell;

g- pK_i values published by Michel et al., 1990, [^{3}H]rauwolscine: α_{2A} in rabbit spleen and α_{2B} in rat kidney.

α_{2C}-Adrenoceptors. Prejunctional α_{2C}-adrenoceptors have been found in the human right atrium (Rump et al., 1995). Their presence was based upon: 1) the similar rank of antagonist affinities (rauwolscine > WB 4101 > phentolamine > prazosin) in response to the non-selective α-adrenoceptor agonist, norepinephrine, and the selective α_2-adrenoceptor agonist, UK 14,304. 2) A high correlation ($r = 0.909$) of antagonist potency (EC_{30}) with the affinity (pK_D) of those antagonists was obtained from radioligand binding experiments at α_{2C}-binding sites of opossum kidney cells.

α_{2D}-Adrenoceptors. Prejunctional α_{2D}-adrenoceptors were found in the rat kidney (Bohmann et al., 1994) and in the rat heart (Smith et al., 1995) and these resembled the α_{2D}-adrenoceptor binding site in rat submandibular gland.

Non-subtype selective α_2-adrenoceptor agonists and antagonists

Two non-subtype selective α_2-adrenoceptor agonists have been used to study α_2-adrenoceptors (Table 5). The non-subtype selective α_2-adrenoceptor agonist, UK 14,304 (5-bromo-6-[2-imidazolin-2-ylamino]-quinoxaline bitartrate) is a potent vasoconstrictor in the isolated equine digital vein (Elliot, 1997) and in the isolated porcine thoracic aorta (pD_2 = 6.24) and marginal ear vein ($pD_2 = 7.51$, Wright et al., 1995). UK 14,304 has a higher affinity for all cloned α_2-adrenoceptor subtypes (pK_i values: 6.3 - 6.7) than does norepinephrine (pK_i values: 4.9 - 5.8) (Renouard et al., 1994; Uhlén et al., 1992). Clonidine also has been used as a non-subtype selective α_2-adrenoceptor agonist. The direct application of clonidine *in vivo* caused concentration (10 nM - 1mM) dependent contractions in canine pial artery and vein (Ishiyama et al., 1998) via postjunctional receptors. Activation of prejunctional α_2-adrenoceptors by clonidine has been demonstrated to produce concentration dependent inhibition of electrically-evoked contractions in rat vas deferens ($pD_2 = 9.29$), rat tail artery ($pD_2 = 7.98$) and guinea-pig ileum ($pD_2 = 7.28$, Ali et al., 1998). These responses to clonidine were almost as potent as those to UK 14,304 and were inhibited by phentolamine and rauwolscine (Ali et al., 1998).

There are no available α_2-adrenoceptor subtype selective antagonists because all of them are able to bind all α_2-adrenoceptor subtypes. However, some α_2-adrenoceptor antagonists have a slight selectivity to one of the α_2-adrenoceptor subtypes and this can be used for subtype identification.

For example, prazosin can discriminate α_{2B}- and α_{2C}-adrenoceptor subtypes from $\alpha_{2A/D}$-adrenoceptors because prazosin has a higher affinity (pK_i) for α_{2B}- and/or α_{2C}-adrenoceptor subtypes (Uhlén et al., 1994, MacLenann et al., 1997) and a lower affinity (pK_i) for $\alpha_{2A/D}$-adrenoceptors (Trendelenburg et al., 1994, Bohmann et al., 1994, Limberger et al., 1995, Wahl et al., 1996).

However, prazosin is unable to discriminate between α_{2B}- and α_{2C}-adrenoceptor subtypes. For this purpose the antagonist potencies of ARC 239 and rauwolscine can be compared. Even though ARC 239 and rauwolscine exhibit affinity for all α_2-adrenoceptor subtypes, ARC 239 has a slightly greater selectivity for α_{2B}-adrenoceptors (Blaxall et al., 1991, Uhlén et al., 1994, Renouard et al., 1994) while rauwolscine is more selective for α_{2C}-adrenoceptors (Blaxall et al., 1991, Uhlén et al., 1994). In order to discriminate between α_{2B}- and α_{2C}-adrenoceptor subtypes, the relative potencies (Blaxall et al., 1991, Fuder and Selbach, 1993) of ARC 239 and rauwolscine for α_{2B}- and α_{2C}-adrenoceptors can be compared.

An antagonist's potency to α_2-adrenoceptors, in functional studies, is a very useful piece of information in characterizing the receptor subtype. The following literature values of antagonist potency (pA_2), determined from functional studies for α_2-adrenoceptors, has been reported: yohimbine (8.22) in equine digital vein (Elliot, 1997) and in human saphenous vein (7.23 - 7.40) (Connaughton and Docherty, 1990; Smith et al., 1992; Gavin et al., 1997); for rauwolscine (6.09) in porcine thoracic aorta (Wright et al., 1995) and in dog saphenous vein (6.0) (Low et al, 1999); for RX 812002 (2-[2-methoxy-1,4-benzodioxan-2-yl]-2-imidazoline) in equine digital vein (9.04) (Elliot, 1997); and for RS-15385-197 ((8aR,12aS,13aS)-5,8,8,a,9,10,11,12,12a,13,13a,decahydro-3-methoxy-12-(methylsulphonyl)6H-isoquino[2,1-g][1,6]-naphthyridine) in dog saphenous vein (10.0) (Brown et al., 1993).

Sympathetic neurotransmitters

The hypothesis that the sympathetic nervous system regulates organ functions via chemical neurotransmission where adrenaline-like substance might be released from sympathetic nerve was expressed almost a century ago (Elliott, 1905). The fact that norepinephrine is the primarily neurotransmitter released from the sympathetic nerve terminal which in turn produces the effect was identified by Euler (1946). It is now commonly accepted that regulation of vascular tone by sympathetic nerves depends on neurotransmitter(s) release from the nerve terminal and also by the presence of functional receptors. Substances such as neuropeptide Y (NPY) (Lundberg et al. 1982) and adenosine 5'-triphosphate (ATP) (Lagercrantz and Stjarne, 1974; Burnstock, 1976) can be stored and released from the adrenergic nerve terminal along with norepinephrine and are thought to be involved in producing the physiological response. The ATP co-transmission process has been demonstrated in dog mesenteric artery (Muramatsu, 1986), rabbit ear artery (O'Connor et al., 1990), rabbit aorta (Sedaa et al., 1990), canine splenic artery (Ren et al., 1996), guinea-pig and rat vas deferens (Fedan et al., 1981), guinea-pig taenia coli and rat duodenum (Windscheif et al., 1995). Investigation of possible ATP co-transmission in the bovine tail artery was a part of the experimental work for this dissertation.

Neurotransmitter(s) release or physiological responses mediated by neurotransmitter(s) can be studied in vitro by using transmural electrical field stimulation of sympathetic nerve terminals (Burnstock, 1976; Levitt and Westfall, 1982; Machaly et al., 1988; Evans and Kennedy, 1994; Lundberg, 1996). Depolarization of the sympathetic nerve ending by an electrical field causes Ca^{2+} influx through voltage-dependent Ca^{2+} channels, an event essential for neurotransmitter release (Lefkowitz et al., 1996). Depolarization of the nerve terminal can be abolished by tetrodotoxin, a voltage-dependent Na^{+}-channel blocker (Bao and Stjärne,1993; Lundberg, 1996). Tetrodotoxin (1 μM) or guanethidine, an inhibitor of norepiphrine release, have been commonly used to determine whether the response produced by field stimulation occurred via neurotransmitter release or was due to the direct

stimulation of smooth muscle by field stimulation (Ren and Burnstock, 1997; Bao and Stjärne, 1993).

The use of electrical procedures which incorporate a constant current between a pair of electrodes in the tissue bath has been proposed as the best method to use to stimulate the nerve and not the smooth muscle (Duckles and Silverman, 1980). A frequency-response relationship is usually obtained to field stimulation in such experiments (Duckles and Silverman, 1980; Ren and Burnstock, 1997).

Norepinephrine as a neurotransmitter

Norepinephrine is synthesized in the sympathetic neuron from the amino acid tyrosine, stored in vesicles and ultimately released from the sympathetic nerve in response to neuron stimulation (Lefkowitz et al., 1996). The effect of norepinephrine occurs when it diffuses across the sympathetic cleft and binds to the adrenergic receptors on the membrane of the effector cell. Once released, the concentration of norepinephrine in the sympathetic cleft decreases because of several removal processes. These processes lead to a termination of norepinephrine's action and include simple dilution of the norepinephrine concentration in the receptor region and by active transport ($uptake_1$) of norepinephrine back into the neuron and by diffusing into the effector tissues ($uptake_2$) (Furchgott, 1966; Lefkowitz et al., 1996). Neuronal uptake ($uptake_1$) of norepinephrine can be blocked by cocaine while non-neuronal uptake ($uptake_2$) can be blocked with corticosterone. Norepinephrine can be deaminated by the intracellular neuronal enzyme monoamine oxidase (MAO) and be degraded within non-neuronal tissue by both catechol-O-methyltransferase (COMT) and MAO (Furchgott, 1966). Since these four processes are constantly removing norepinephrine from the receptors site, equilibrium conditions never occur for norepinephrine and receptors. That is why Furchgott (1966) recommended that removal processes be blocked when considering the characterization of receptors using agonists and antagonists.

As a neurotransmitter, norepinephrine can inhibit its own release through the activation of prejunctional α_2-adrenoceptors (Langer, 1974; Guimaraes et al., 1998; Molderings and Gothert, 1995; Rump et al., 1995).

ATP as a neurotransmitter in sympathetic transmission

ATP as a neurotransmitter is released from the sympathetic neuron terminal (Lagercrantz and Stjarne, 1974; Burnstock, 1976; Levitt and Westfall, 1982; Sedaa et al., 1990) and through activation of purinergic receptors causes physiologic responses (Ralevic and Burnstock, 1998). Evaluation of whether ATP is a neurotransmitter can be studied by measuring [^{3}H]purine outflow from [^{3}H]ATP-labeled sympathetic nerve terminals (Levitt and Westfall, 1982; Sedaa et al., 1990) and/or by monitoring contractile responses (Windscheif et al., 1994; Ziyal et al. 1997) to field stimulation. ATP co-transmission has been reported in several vascular beds (Muramatsu, 1986; O'Connor et al., 1990; Sedaa et al., 1990; Ren et al., 1996) but information concerning whether ATP co-transmission occurs in skeletal muscle vasculature is limited (Sajag et al., 1990; Burnstock and Warland, 1987; Dzielak, 1983).

Some difficulties exist in determining whether ATP is involved in a particular co-transmission process. The major problem is related to the chemical properties of ATP as a neurotransmitter. Thus, neuronally released ATP is a very unstable compound and is rapidly, within 100 milliseconds (Bao and Stjärne, 1993), enzymatically inactivated by adenosine 5'-triphosphatase (ecto-ATPase) to adenosine (Gordon, 1986). Adenosine can cause cellular responses on its own via activation of adenosine receptors and thereby mask the role of ATP (Ralevic and Burnstock, 1998). The second major problem is related to the possibility that multiple purinergic receptors can be present in the same tissue.

Different approaches have been used in order to eliminate the potential problems in evaluating the role of ATP as a neurotransmitter. For example, contractile responses to field stimulation can be conducted on intact (Ziyal et al., 1997) or endothelium denuded blood vessels (Sedaa, et al., 1990; Evans and Kennedy, 1994). The use of endothelium denuded isolated vessels has been proposed as a way to eliminate the effect of the endothelium on vascular smooth muscle contraction (Furchgott and Zawadski, 1980). An ecto-ATPase inhibitor, ARL 67156 (6-N,N-diethyl-D-β,γ-dibromomethyleneATP, Westfall et al., 1996), has been used to slow down the rapid breakdown of the released ATP within the synaptic cleft. To eliminate the possible effect of adenosine, a non-selective P1 receptor inhibitor, 8-sulphophenyltheophylline (O'Connor et al., 1990; Crack et al., 1994), has been included in

the experimental design. In some preparations cyclooxygenase inhibitors (e.g. indomethacin) have been used to inhibit prostaglandin synthesis (Crack et al., 1994).

Neuropeptide Y (NPY) as a neurotransmitter in sympathetic transmission

It has been demonstrated that neuropeptide Y might be a sympathetic co-transmitter released from the sympathetic nerve terminal (Lundberg et al. 1982) and which might modulate contractile responses to sympathetic nerve stimulation by inhibiting neurotransmitter (norepinephrine and/or ATP) release from the nerve terminal (Lundberg, 1996). Neuropeptide Y is a peptide consisting of 36 amino acids and can be synthesized within the sympathetic nerve and stored in vesicles. Neuropeptide Y can cause vasoconstriction through activation of its own receptors (Lundberg, 1996). Three types of neuropeptide Y receptors have been identified (Y_1, Y_2 and Y_3) and all of them are linked to increases in intracellular Ca^{2+} concentration and stimulation of cAMP synthesis (Lundberg, 1996). Activation of the Y_1 receptors causes vasoconstriction, presumably by acting postsynaptically, while the Y_2 receptors act presynaptically to modulate neurotransmitters release. The Y_3 receptor mediates inhibition of secretion in bovine chromaffin cells by inhibiting adenylyl cyclase (Lundberg, 1996).

Although neuropeptide Y may function in the bovine tail artery as a co-transmitter, this possibility was not investigated in this research project.

Purinergic receptors

A part of this dissertation concerned the role of purinergic receptors in contractile responses in the bovine tail artery and was necessitated by the results obtained when the sympathetic nerves in the tail artery were stimulated. The following review covers the classification of purinergic receptors, their structures, intracellular signaling mechanisms and possible involvement in regulation of vascular tone and a discussion of agonists and antagonists.

Current classification of purinergic receptors divides them into two categories: P1 (adenosine receptors) and P2 (ATP receptors) (Ralevic and Burnstock, 1998; Fredholm et al.,

1997). Purinergic receptors are cell-surface membrane-bound receptors and have been found in various tissues including the heart and vasculature (Ralevic and Burnstock, 1998).

Adenosine receptors. Adenosine receptors (P1) are G-protein coupled receptors (Table 6). Adenosine receptors have been found in the following tissues: heart, brain, kidney and blood vessels. Adenosine receptors are divided into four subtypes, A_1, A_{2A}, A_{2B} and A_3, based on molecular structure, tissue distribution and pharmacological characteristics. A_2-receptors are the most common subtype of adenosine receptors and are found in vascular tissues where they mediate relaxation of the blood vessels.

Table 6. Classification, general intracellular signaling mechanisms and effects in blood vessels of adenosine receptors (modified from Ralevic and Burnstock, 1998).

Receptors	**A_1**	**A_{2A}**	**A_{2B}**	**A_3**
G-protein-coupling	$G_{i/o}$	G_s	G_s	G_i G_q
Intracellular effects	↓cAMP ↑IP_3	↑cAMP	↑cAMP	↓cAMP ↑IP_3, Ca^{2+}
Predominant effect in the blood vessels	Direct effect is rare Relaxation via prejunctional inhibition of sympathetic neurotransmitter release	Relaxation	Relaxation	Contraction or Relaxation*

* - The physiological role of A_3-receptors is not completely understood. Activation of A_3-receptors on mast cells causes degranulation of the mast cells and the release of vasoactive substances (i.e., histamine) which in turn causes the vascular response (Ralevic and Burnstock, 1998).

In general, the activation of A_1 causes inhibition of cAMP level through $G_{i/o}$ protein-coupled response, while activation of A_2 receptors causes stimulation of cAMP synthesis though stimulation of adenylyl cyclase via a G_s protein-coupled mechanism. However, A_{2B} can be coupled to G_q and G_i proteins. A_3-receptors are predominantly G_i coupled and their

mRNA has been found in many tissues, including: lung, brain, heart, spleen, uterus, bladder, kidney and liver. However, the physiological role of A_3-receptors is not completely understood (Ralevic and Burnstock, 1998).

ATP receptors. ATP receptors (P2) are subdivided into two classes: P2X and P2Y, where P2X receptors are ATP-gated ion channels (Na^+, K^+ and Ca^{2+}) and P2Y receptors are G-protein coupled receptors (Table 7). In addition, seven subtypes of P2X and five subtypes of P2Y receptors have been cloned (Ralevic and Burnstock, 1998; Kennedy and Leff, 1995; Fredholm et al., 1997). ATP receptors have been found in many tissues, especially in the central nervous system and in vascular tissues. Activation of P2X receptors causes contraction of vascular (Ren and Burnstock, 1997; Windschief et al., 1994; Ziyal et al., 1997) and non-vascular (Westfall et al., 1996; Tuluc et al., 1998; Najbar-Kaszkiel and Rand, 1996) smooth muscle while activation of P2Y receptors on endothelial cells causes relaxation of vascular smooth muscle via the endothelium-dependent mechanisms (Malmsjö et al., 1999; Thapaliya et al., 1999). General characteristics of ATP receptors are given in Table 7 (Ralevic and Burnstock, 1998; Kennedy and Leff, 1995).

P2X receptors. P2X receptors are proteins and their structure has been predicted based on cloned P2X receptors. P2X receptors consist of 379 - 472 amino acids, which form an ion channel with two hydrophobic transmembrane domains and one hydrophilic extracellular loop (Fig. 5). The extracellular loop has 10 conserved (for all P2X receptor subtypes) cysteine and 14 glycine residues and there are 2 to 6 potential glycosylation sites (Ralevic and Burnstock, 1998).

Activated P2X receptors mediate the influx of Na^+, K^+ and Ca^{2+} and this produces cellular responses due to an increase in intracellular Ca^{2+} concentration and cell depolarization (Ralevic and Burnstock, 1998). Some P2X receptor subtypes, but not all, can be desensitized by ATP (Ralevic and Burnstock, 1998; Fredholm et al., 1997). The mechanism of desensitization is not completely understood. Different portions of P2X receptors have been suggested to be involved in the desensitization process, i.e. hydrophobic domains, carboxyl ($P2X_2$) or amino terminal ($P2X_3$) (Ralevic and Burnstock, 1998).

Table 7. Characterization of purinergic receptors.

	P2X	P2Y
Receptor type	ATP-gated ion channel	G-protein coupled: $G_{q\,11}$, G_i
Number of amino acids	379 - 472	308 - 377
Intracellular enzymes involved	not applicable	PLC, AC, PLD, PKC, MAPK
Effectors	$Ca^{2+} > Na^+ > K^+$	$\uparrow IP_3$, $\uparrow$DAG, $\uparrow Ca^{2}$ $\downarrow$cAMP
Subtypes	$P2X_1$, $P2X_2$, $P2X_3$, $P2X_4$, $P2X_5$, $P2X_6$, $P2X_7$	$P2Y_1$, $P2Y_2$, $P2Y_4$, $P2Y_6$, $P2Y_{11}$
Effect on vascular tone	Contraction	Relaxation* or Contraction

Phospholipase C (PLC); phospholipase D (PLD); proteinkinase C (PKC), mitoge-activated protein kinase (MAPK), adenylyl cyclase (AC), diacylglycerol (DAG) (modified from Ralevic and Burnstock, 1998). * - Activation of P2Y receptors on the endothelial cell causes endothelium-dependent relaxation of vascular smooth muscle.

Based on its ability to be desensitized, P2X receptors are divided into three categories: rapidly (within 100 -300 msec) desensitizing, slowly desensitizing and non-desensitizing (Ralevic and Burnstock, 1998).

In addition, non-desensitizing P2X receptors might be either α,β-meATP-sensitive or α,β-meATP-insensitive. Desensitization of P2X receptors can be used as a method to inactivate these receptors in order to study their functional role (Ren and Burnstock, 1997). In vascular smooth muscle both categories of α,β-meATP-sensitive, desensitizing and non-desensitizing receptors, have been found (Ralevic and Burnstock, 1998).

All seven subtypes of P2X receptors ($P2X_1$, $P2X_2$, $P2X_3$, $P2X_4$, $P2X_5$, $P2X_6$, $P2X_7$) have been cloned (Fredholm, et al., 1997; Ralevic and Burnstock, 1998). All subtypes are

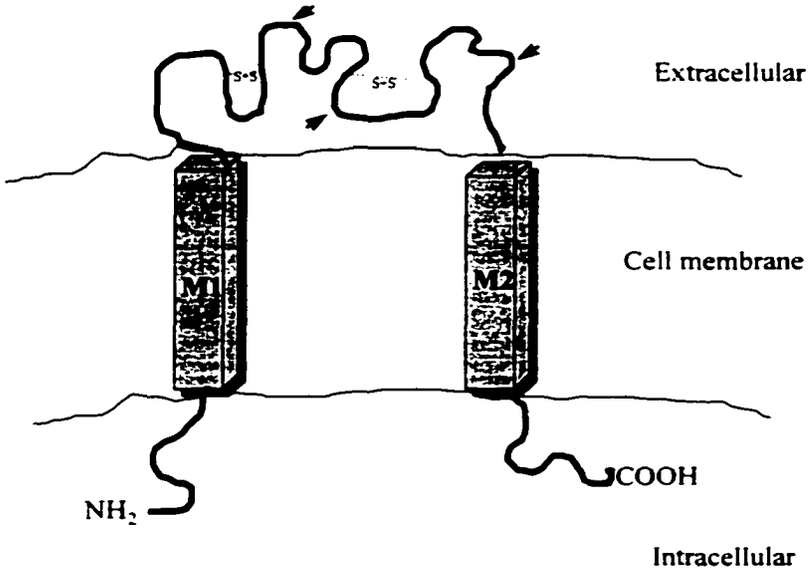

Figure 5. Diagram of a proposed $P2X_2$ receptor structure. The diagram shows that both the N- and C-terminals are intracellular. There are two transmembrane hydrophobic spanning segments (M1 and M2) and one extracellular hydrophilic loop with two disulfide-bond (-s-s-) and three glycosylation sites (→) (modified from Ralevic and Burnstock, 1998).

widely spread in spinal cord and peripheral neurons. $P2X_1$ is one of the most significant subtypes present in vascular smooth muscle (Ralevic and Burnstock, 1998).

P2Y receptors. P2Y receptors belong to a large group of G-protein coupled receptors. Their primary pathway of intracellular responses is associated with $G_{q/11}$-protein coupled activation of phospholipase C which leads to the formation of IP_3 and increases in intracellular Ca^{2+} concentration. However, some P2Y receptor subtypes can be negatively coupled to adenylyl cylclase through G_i protein (Ralevic and Burnstock, 1998). In addition, intracellular signaling mechanisms of P2Y receptors have been described which link them to multiple intracellular effectors such as, phospholipases (C, D, A_2), diacylglycerol, protein-kinase C and voltage-dependent Ca^{2+} channels (Ralevic and Burnstock, 1998). P2Y receptors have been found in various tissues and $P2Y_1$ is the primary subtype presented in blood vessels. P2Y receptors have been found on endothelial and vascular smooth muscle cells. Activation of P2Y receptors mediates vasodilatation via Ca^{2+}-dependent synthesis of nitric

oxide and/or synthesis of endothelium-derived relaxing or hyperpolarizing factors. P2Y receptors can also play a role in vascular smooth muscle proliferation via activation of mitogenic-activated protein kinase (MAPK) (Ralevic and Burnstock, 1998). P2Y receptors can be desensitized through phosphorylation of intracellular regions of the receptor but the mechanisms of desensitization are not completely understood (Ralevic and Burnstock, 1998).

Purinergic agonists and antagonists

There are no selective agonists for P2X or P2Y receptors and most agonists can couple to both receptor types. All purinergic receptors can be activated by ATP, which is not a stable compound. Several synthetic ATP analogs are commercially available to study the effects of purines on biological processes. 2MeSATP (2-methylthio ATP), a synthetic agonist, can stimulate both P2X and P2Y receptors (Malmsjö et al., 1999) and exhibits a higher potency than ATP for some P2X receptor subtypes (Liu et al., 1999; Ralevic and Burnstock, 1998). α,β-meATP and β,γ-meATP have been used to study P2X receptors (Kennedy and Leff, 1995; Ralevic and Burnstock, 1998). α,β-MeATP causes contraction of vascular (Ren and Burnstock, 1997; Windschief et al., 1994; Ziyal et al., 1997) and non-vascular (Westfall et al., 1996) smooth muscle via activation of P2X receptors and is more potent than 2meSATP in rat perfused mesenteric arteries (Windschief et al., 1994), in rabbit ear artery (O'Connor et al., 1990) and in rat urinary bladder (Bo and Burnstock, 1990). ATP was less potent than α,β-meATP and 2meSATP in rat mesenteric arteries (Windschief, et al., 1994) and in rabbit ear artery (O'Connor et al., 1990) while in the rat urinary bladder the rank of potencies was different: α,β-meATP > ATP> 2-MeSATP (Bo and Burnstock, 1990).

There are no subtype selective P2X and P2Y receptor antagonists. Suramin and PPADS (pyridoxalphosphatte-6-azophenyl-2',4'-disulphonic acid) are the most commonly used antagonists to study P2X and P2Y receptors (Ohara, et al., 1998; Bao and Stjärne, 1993; McLaren et al., 1994; Windscheif et al., 1995).

Suramin is a slow-equilibrating antagonist (Leff et al., 1990) and exhibited competitive antagonism to α,β-meATP-induced contraction (Leff et al., 1990; Ziayl et al., 1997). Suramin inhibited contractile responses to α,β-meATP via P2X receptors in rabbit ear

artery (pK_B = 4.79; Leff et al., 1990), in rabbit saphenous artery (pA_2 = 4.79; Ziayl et al., 1997) and inhibited relaxant responses via P2Y receptors in rabbit aorta (Ziayl et al., 1997). Suramin did not affect contraction to KCl (Leff et al., 1990; Bao and Stjärne, 1993) or to norepinephrine in guinea pig small cutaneous artery (Morris, 1999) and in rat tail artery (Bao and Stjärne, 1993) but inhibited contractile responses to phenylephrine and histamine in rabbit ear artery (Leff et al., 1990). Suramin's effect on contractile responses to phenylephrine and histamine contraction is not completely understood. Even though suramin is a competitive purinergic antagonist, it is also a G protein inhibitor whereby it inhibits the activation of the G_α subunit (Freissmuth et al., 1996; Hohenegger et al., 1997).

NF023 (3'-urea of 8-(benzamido)naphthalene-1,3,5-trisulphonic acid), another purinergic antagonist (Ralevic and Burnstock, 1998), exhibited a higher potency (pA_2 = 5.69) than suramin in inhibition of α,β-meATP-induced contraction but it had no effect on relaxant responses to α,β-meATP in the rabbit aorta (Ziayl et al., 1997).

PPADS is not a selective purinergic antagonist (Ziganshin et al., 1993; Windschief et al., 1994; McLaren et al., 1994) and has been shown to inhibit contractile responses to α,β-meATP via P2X receptors (Ziganshin et al., 1993) and via P2Y receptors in guinea-pig taenia coli and rat duodenum (Windschief et al., 1995). Some other purinergic antagonists such as reactive blue 2, reactive red (Ralevic and Burnstock, 1998; Burnstock and Warland, 1987), diadenosine pentaphosphate (Ap_5A) (Van der Giet et al., 1999) and its product, diinosine pentaphosphate (Ip_5I) (King, et al., 1999) have been used to study purinergic receptors.

CHAPTER II. CO-EXISTENCE OF α_1-ADRENOCEPTOR SUBTYPES IN THE BOVINE TAIL ARTERY.

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ABSTRACT

The purpose of this study was to identify the α_1-adrenoreceptor subtypes present in the bovine tail artery which mediate contractions to adrenergic agonists. α_1-Adrenergic agonists caused concentration-dependent contraction of the isolated bovine tail artery. A61603, an α_{1A}-selective agonist, was more potent (pD_2 = 7.7 ± 0.15; n=9) in causing contraction of the bovine tail artery compared to that of norepinephrine (6.0 ± 0.07, n=6) and phenylephrine (5.9 ± 0.13, n=8). The calculated dissociation constant (K_A) of A61603 was 0.149 ± 0.04 μM (n= 6). Antagonists, ARC 239, BMY 7378, WB 4101 and 5-methylurapidil, caused a parallel shift to the right of the concentration-response curve to A61603 with pA_2 values of 9.29, 6.62, 9.27 and 8.86, respectively. Prazosin, BMY 7378 and WB 4101 shifted the concentration-response curve to phenylephrine to the right with pA_2 values of 9.47, 7.17 and 9.73, respectively. WB 4101, a selective α_{1A}-adrenoceptor antagonist, was equally potent against phenylephrine and A61603. The affinities (pA_2 value) obtained for 5-methylurapidil, WB 4101, BMY 7378 and prazosin against α_1-adrenoceptor agonists were significantly correlated with pK_i values (Zhu et al., 1997) for the cloned α_{1a}-adrenoceptor (r=0.99, $P < 0.05$), but not with the α_{1b}-adrenoceptor (r=0.78, n.s.) or the cloned α_{1d}-adrenoceptor (r=0.43, n.s.). BMY 7378, a selective α_{1D}-adrenoceptor antagonist, was significantly more potent against the non-subtype selective agonist phenylephrine than to A61603. Chloroethylclonidine (50 μM for 10 min) did not affect contractile responses (at EC_{30} and maximum) to A61603, but caused a significant ($P<0.05$) inhibition of contractile responses to phenylephrine. In conclusion, it appears that all three subtypes (α_{1A}-, α_{1B}- and

α_{1D}) of α_1-adrenoceptors may play a role in adrenergic mediated contraction in the bovine tail artery. However, α_{1A}-adrenoceptors play a dominant role in the contractile response to adrenergic agonists in this tissue while the other two α-adrenoceptor subtypes (α_{1B}- and α_{1D}-) very likely co-exist in the bovine tail artery but have less of an impact on contractile responses.

Keywords: α_1-adrenoceptor subtypes, vascular smooth muscle, bovine, median caudal artery.

INTRODUCTION

Modern classification divides α-adrenoceptors into two types: α_1 and α_2. α_1-Adrenoceptors have three native subtypes: α_{1A}, α_{1B} and α_{1D} (Docherty, 1998). Three recombinant α_1-adrenoceptor subtypes have been cloned and current classification determines them as α_{1a}, α_{1b} and α_{1d} (Hieble et al., 1995; Docherty, 1998). Three native ($\alpha_{2A\,2D}$-, α_{2B}-, α_{2C}) and four cloned (α_{2A}-, α_{2B}-, α_{2C}- and α_{2D}) subtypes of α_2-adrenoceptors have been identified (Docherty, 1998) by modern classification.

The presence of functional α_1-adrenoceptors has been reported in vascular tissues (Lachnit et al., 1996; Villalobos-Molina and Ibarra, 1996; Zhu et al, 1997; Stassen et al, 1998; Hu and Dyer, 1997; Hill and Dyer, 1997; Fagura et al., 1997) as well as in non-vascular (Furukawa et al., 1995; Yu and Han, 1994; Teng et al., 1994; Alberts et al., 1999; Marshall et al., 1995; Hattori and Kanno, 1997) tissues. α_1-Adrenoceptors have been extensively studied in a variety of species such as rat (Lachnit et al., 1996; Villalobos-Molina and Ibarra, 1996; Zhu et al, 1997; Stassen et al, 1998), rabbit (Fagura et al.,1997, Hattory and Kanno, 1997), cattle (Hill and Dyer, 1997), sheep (Hu and Dyer, 1997), human (Teng et al., 1994; Furukawa et al., 1995; Marshall et al., 1995) and pig (Alberts et al., 1999).

The α_{1A}- subtype is the most well defined adrenoceptor subtype and has been studied with selective α_{1A}-adrenoceptor agonists and/or antagonists. There are at least two commercially available agonists with high selectivity to the α_{1A}-adrenoceptor subtype: A61603 ((±N-[5-(4,5-dihydro-1 H-imidazol-2yl)-2-hydroxy-5,6,7,8-tetrahydronaphthalen-1-yl] methanesulphonamide hydrobromide) and oxymetazoline (Buckner et al., 1996). WB

4101 ((2-(2,6-dimethoxyphenoxyethyl) aminomethyl-1,4 benzodioxane) and 5-methylurapidil are two subtype selective α_{1A}-adrenoceptor antagonists, which discriminate for the α_{1A}-adrenoceptor (Marshall et all, 1995; Zhu et al., 1997; Furukawa et al., 1995; Fagura et al., 1997).

Limited information is available about the α_{1B}-adrenoceptor subtype, which has also been found in both vascular and non-vascular smooth muscle. The presence of α_{1B}-adrenoceptors has been reported in the rat aorta (Han et al., 1990; Testa et al., 1995), rat heart (Yu and Han,1994), rat mesenteric artery (Piascik et al., 1997), rabbit heart (Hattori and Kanno, 1997) and human prostate (Teng et al., 1994). Unfortunately, there are no selective competitive antagonists for the α_{1B}-adrenoceptor. Chloroethylclonidine has been employed to distinguish between α_{1A}-adrenoceptors and α_{1B}-adrenoceptors (Teng et al., 1994; Testa et al., 1995; Hattori and Kanno, 1997). Chloroethylclonidine alkylates the α_{1B}-adrenoceptor subtype and serves as an irreversible antagonist (O'Rourke et al., 1995). However, the interactions of chloroethylclonidine and α-adrenoceptors have not been fully studied. Thus, it has been demonstrated that chloroethylclonidine can cause contraction of the dog saphenous vein (Nunes and Guimarães, 1993) and the rabbit ear artery (Fagura et al., 1997). O'Rourke and co-workers (1997) demonstrated using radioligand binding methods that chloroethylclonidine significantly inhibited the maximum binding of [^{3}H]prazosin to α_{1B}-adrenoceptors in rat spleen membranes to 21% and the binding of [^{3}H]yohimbine to α_{2D}-adrenoceptors in rat submandibulary gland membranes to 35%. In addition, they reported that chloroethylclonidine (100 μM) irreversibly bound α_{2D}-adrenoceptors in rat aorta and produced contraction only in combination with norepinephrine, acting as "a silent irreversible agonist" (O'Rourke et al., 1997).

The presence of α_{1D}-adrenoceptors has been studied in a number of rat vascular beds including rat aorta (Fagura et al., 1997; Buckner et al., 1996; Villalobos-Molina and Ibarra, 1996), rat renal artery (Villalobos-Molina et al., 1997), rat carotid and mesenteric artery (Villalobos-Molina and Ibarra, 1996). BMY 7378 ((8-(2-(4-(2-methoxyphenyl)-1-piperazinyl)ethyl)-8-azaspiro(4,5)decane-7,9-dione dihydrochloride)) is reported to be

selective for the cloned α_{1d}-adrenoceptor subtype (Goetz et al.,1995) and has been used to study α_{1D}-adrenoceptors (Fagura et al., 1997; Villalobos-Molina et al., 1997).

Adrenergic induced contractile responses in the isolated bovine tail artery were investigated by Hill and Dyer (1997). They reported that norepinephrine and phenylephrine caused contractions of the isolated bovine tail artery which were significantly inhibited by prazosin, an α_1-adrenoceptor antagonist. Whereas, the selective α_2-adrenoceptor agonist, B-HT 920 (5,6,7,8-tetrahydro-6-(2-propenyl)-4H-thiazolo[4,5-d]azepin-2-amine dihydrochloride), failed to cause contraction. They concluded that adrenergic agonists caused contraction of isolated bovine tail artery primarily through α_1-adrenoceptors and not through α_2-adrenoceptors. No attempt was made to characterize the subtype(s) of the α_1-adrenoceptors present in the bovine tail artery.

The purpose of this study was to identify the α_1-adrenoreceptor subtypes present in the bovine tail artery which mediate contractions to adrenergic agonists.

MATERIALS AND METHODS

Tissue preparation

Bovine tails (steers) were obtained from a local abattoir. The tissue was transported to the laboratory on ice where the median caudal artery was dissected from the tail. Tissue preparation protocol was similar to that described by Hill and Dyer (1997). Dissected artery was 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; dextrose, 7.88; and 0.03 sodium EDTA. Endothelium was removed by the careful rotation of a blunt wooden stick inside the lumen of the vessels. Vessels were cut into rings 4-5 mm long. Each ring was placed in a 10 ml tissue bath filled with Krebs' solution at 38.5^0 C and continually aerated by bubbling O_2 : CO_2 (95:5) from the bottom of the bath. The tissue rings were suspended in the bath by two stainless steel wires passing through the lumen of each ring. One wire was stationary and secured near the bottom of the tissue bath while the second wire was connected to the force transducer. Up to 8 rings were studied per experimental day.

Isometric contraction was continually monitored by using a MacLab system connected to a computer. The artery rings were initially stretched to a tension of 15 g and then allowed to relax over a 60 min period. Before beginning the experiments baseline tension was established by adjusting the tension to 2 g. During the equilibration period all tissues were incubated with 0.36 mM iproniazid in order to inhibit monoamine oxidase (MAO). Endothelium removal was confirmed by absence of a relaxation response to 1μM acetylcholine in rings contracted by 45 mM KCl. The artery rings were then washed with fresh Krebs' solution every 10 minutes for 30 minutes until the basal tension of 2 g was re-established. Artery rings were treated with 10 μM tropolone, 1 μM cocaine and 10 μM corticosterone for 15 minutes, prior to obtaining concentration-response relationships to an α-adrenoceptor agonist, in order to block catecholamine-*O*-methyltransferase, neuronal ($uptake_1$) and tissue ($uptake_2$) uptake, respectively (Furchgott and Garcia, 1968; Levin and Furchgott, 1970; Hill and Dyer, 1997). All concentrations of agonists, norepinephrine (10 nM - 0.1 mM), phenylephrine (10 nM - 0.1 mM) and A61603 (1 nM - 0.1 mM), were added to the bath cumulatively in half-log increments. In each experiment with an antagonist, concentration-response relationships to an agonist were obtained twice, before and 1 hour after incubation with one concentration of an antagonist. The maximal contraction to an agonist obtained during the first concentration-response relationship was set as the 100% response for that tissue. The effective concentrations of an agonist required to produce 30% and 50% (when mentioned) of the maximal contraction (EC_{30} and EC_{50}, respectively) obtained from the control and the antagonist treated tissues were determined. The negative log molar concentration of agonist inducing 50% of the maximal response (-log EC_{50}) was expressed as pD_2. Shifts in agonist EC_{30} values by antagonists were used to calculate antagonist potencies (Schild, 1949). For every experiment a tissue not exposed to an antagonist was carried through the procedure to permit corrections for time-dependent changes in the response of the tissues to the agonist and not due to the antagonist, as recommended by Furchgott (1972) and as used in our laboratory (Zhang and Dyer, 1990; Hill and Dyer, 1997).

Analysis of responses to agonists and antagonists

The dissociation constant (K_A) of the α_{1A}-selective agonist, A61603, was determined for the artery using the procedure described by Furchgott and Burzstyn (1967) and as used in our laboratory (Hill and Dyer, 1997). Briefly, the tissues were prepared as described above and a concentration response relationship to A61603 (1 nM - 30 μM) was obtained. In order to inactivate a fraction of the α_{1A}-adrenoceptors, tissues were incubated with dibenamine (0.3 μM), an irreversible α-adrenoceptor antagonist, for 20 min followed by washing the tissues 4-5 times over 30 min. Responses to A61603 before (A) and after (A') treatment with dibenamine were plotted after the corrections were made to account for time-dependent changes in the response of the tissue to A61603. A double reciprocal plot of equi-effecive concentrations of A61603 yielded a slope of 1/q and an abscissa intercept of (1-q)/q K_A. The dissociation constant (K_A), where q is the fraction of inhibited receptors, was calculated by using the equation: 1/[A] = 1-q/q[A'] + 1/q (K_A), or K_A = slope - 1/intercept (Furchgott and Burzstyn, 1967).

The antagonist potencies for each adrenoceptor antagonist, pA_2, was obtained as described by Furchgott (1972) and as used in our laboratory (Zhang and Dyer, 1990). Artery rings were prepared as described above. Antagonists, WB 4101 (1-30 nM) and BMY 7378 (0.3-30 μM) were tested against A61603 and phenylephrine; 5-methylurapidil (10 nM - 0.1 μM) and ARC 239 (10 nM - 0.1 μM) were tested against A61603; prazosin (1-30 nM) was tested against phenylephrine and norepinephrine. Antagonist potencies (pA_2) were calculated based on shifts of the agonist concentration-response curves at the EC_{30}. Briefly: EC_{30} was calculated in the absence (A) and presence (A*) of each competitive antagonist concentration from the concentration-response curves. The concentration ratio, CR = A*/A (Furchgott, 1972), and the log (CR -1) was calculated after correction for the time-dependent changes in sensitivity to the agonist. The K_B value is inversely related to affinity and can be calculated as: K_B = [B]/CR-1, where [B] is the concentration of the antagonist. The pA_2 values were calculated according to Arunlakshana and Schild (1959) by using the equation: log (CR-1) = log[B] - log K_B (Furchgott, 1972), where [B] is the concentration of the antagonist and K_B, the dissociation constant of the receptor-antagonist complex, is inversely

related to affinity ($pA_2 = - \log K_B$). The calculated log (CR-1) values were plotted versus the log [B] as a linear relationship and where the line intercepts the abscissa yields the pA_2 value.

Chloroethylclonidine was used to study α_{1B}-adrenoceptors (Villalobos- Molina et al., 1997; Zhu et al., 1997; Marshall et al., 1995). Tissues were prepared as described above. After the first concentration-response relationship to phenylephrine, artery rings were incubated with 50 μM of chloroethylclonidine for 10, 20 or 30 min or with 100 μM of chloroethylclonidine for 30 min. Then tissues were rinsed thoroughly with fresh Krebs' solution for 30 min. A second concentration-response relationship to phenylephrine or a concentration-response relationship to A61603 was obtained. The EC_{30} and maximal responses in the absence and presence of chloroethylclondine treatment were determined for each agonist. The response to A61603 was plotted as a percentage of the original response to phenylephrine.

Drugs

The following drugs were used: (−)-norepinephrine bitartrate, cocaine hydrocholride, corticosterone 21-acetate, ipronizid (phosphate salt), acetylcholine chloride, phenylephrine hydrochloride, idazoxan (Sigma Chemical, St. Louis, MO), A61603 ((N-[5-(4,5-dihydro-1H-imidazol-2yl)-2-hydroxy-5,6,7,8-tetrahydronaphthalen-1-yl]methanesulfonamide hydrobromide), ARC 239 ((2-[2,4-(methoxyphenyl)piperazin-1-yl]-ethyl)-4,4-dimethyl-1,3-(2H,4H)-isoquinolindine dihydrochloride) (Tocris, Ballwin, MO), chloroethylclonidine, WB 4101 ((2-(2,6-dimethoxyphenoxyethyl) aminomethyl-1,4 benzodioxane) hydrochloride), 5-methylurapidil, BMY 7378 (8-[2-[4-(2-methoxyphenyl)-1-piperazinyl]ethyl]-8-azaspiro[4,5]decane-7,9-dione dihydrochloride) (Research Biochemical International, Natick, MA), prazosin (Pfizer, Brooklyn, NY), tropolone (Aldrich, Milwaukee), dibenamine HCl (Smith, Kline and French, Philadelphia, PA). Prazosin was initially prepared in lactic acid and then diluted in isotonic saline solution. Norepinephrine and phenylephrine solutions were prepared in isotonic saline which was acidified with hydrochloric acid. Dibenamine and corticosterone were dissolved in ethanol. All other drugs were dissolved in isotonic saline.

Statistical Analysis

The results are presented as mean ± S.E.M. and *n* is the number of animals used for each experiment. EC_{50} values for agonists, were calculated from their concentration-response relationships by using the GraphPad Prism computer program (GraphPad Software Inc, San Diego, CA). EC_{30} values of agonists, were calculated by using linear function between two of the closest points which overlap the 30% response. Student's unpaired t-test was performed to define the differences between two group means. A *P* value of 0.05 or less was considered significant.

RESULTS

α_1-Adrenergic agonists caused concentration-dependent contractions of the isolated bovine tail artery. The potency of norepinephrine, expressed as pD_2, was 6.0 ± 0.07 (n=6) and was not different from the potency of phenylephrine 5.9 ± 0.13 (n=8). A61603, an α_{1A}-selective agonist, was more potent (pD_2 = 7.7 ± 0.15; n=9) in causing contraction of the bovine tail artery. The calculated dissociation constant (K_A) of A61603 was 0.149 ± 0.04 μM (n= 6).

The potency of antagonists, prazosin, BMY 7378, WB 4101 and 5-methylurapidil, in blocking α-adrenoceptor agonists is presented in Table 1. All antagonists shifted the concentration-response curves to these α-adrenoceptor agonists to the right (Fig. 1 and 2) and exhibited the characteristics of competitive antagonism since the slopes of the Schild plots were not different from unity. BMY 7378, an α_{1D}-adrenoceptor antagonist, was more potent against phenylephrine (pA_2 = 7.17) than A61603 (pA_2 = 6.62).

Chloroethylclonidine caused inhibition of contractile responses to α_1-adrenoceptor agonists (Fig. 3), and was more potent against contractile responses to phenylephrine than those to A61603. Thus, incubation with 50 μM chloroethylclonidine for 10 min did not affect contractile response (at EC_{30} and maximum) to A61603, but caused significant ($P<0.05$) inhibition of contractile responses to phenylephrine. However, incubation with 50 μM of chloroethylclonidine for 20 min (data not shown) or 30 min or with 100 μM of

chloroethylclonidine for 30 min caused significant inhibition of contractile responses when determined at the EC_{30} and the maximal contraction to both agonists.

DISCUSSION

Our results support data published by Hill and Dyer (1997) that adrenergic agonists cause contraction of the bovine tail artery through activation of α_1-adrenoceptors. We extended their study and evaluated the involvement of α_1-adrenoceptor subtypes in the vasoconstriction response. We used endothelium denuded bovine tail artery rings to avoid possible interference by the endothelium on the contractile response. The α_{1A}-adrenoceptor subtype has been identified and extensively studied in vascular smooth muscle (Lachnit et al., 1996; Villalobos-Molina and Ibarra, 1996; Stassen et al., 1998). We used A61603, an α_{1A}-adrenoceptor selective agonist (Buckner et al., 1996), to investigate the possible role of the α_{1A}-adrenoceptor subtype in contractile responses in bovine tail artery. A61603 caused concentration dependent contractions and was more potent than the non-selective α_1-adrenoceptor agonists, norepinephrine and phenylephrine, based on a comparison of EC_{50} values. These findings are consistent with those of Buckner and co-workers (1996). In radioligand binding experiments, using bovine cloned α_{1a}-adrenoceptor, they found the following affinities (pK_i): A61603 (7.52), norepinephrine (6.36) and phenylephrine (6.03). The affinity of A61603 for hamster clonal α_{1b}-adrenoceptors (5.68) and rat clonal α_{1d}-adrenoceptors (5.87) was less than those of norepinephrine and phenylephrine. Data from our study is consistent with theirs since we found that the potency of A61603, expressed as a K_A value, was higher (K_A = 0.149 μM) than that of norepinephrine (K_A = 3.11 μM; Hill and Dyer, 1997).

The affinities of four α_1-adrenoceptor antagonists (5-methylurapidil, WB 4101, BMY 7378 and ARC 239) were determined against A61603. 5-Methylurapidil (Table 1), an α_{1A}-adrenoceptor selective antagonist, inhibited A61603 induced contraction with a higher potency (pA_2 = 8.86 ± 0.05) than that of BMY 7378 (pA_2 = 6.62 ± 0.15), an α_{1D}-adrenoceptor selective antagonist. The affinities (pA_2 value) obtained for 5-methylurapidil, WB 4101,

BMY 7378, ARC 239 and prazosin against α_1-adrenoceptor agonists were significantly correlated (Fig. 4) with pK_i values (Zhu et al., 1997) for the cloned α_{1a}-adrenoceptor ($r = 0.99$, $P < 0.05$), but not with the α_{1b}-adrenoceptor ($r=0.78$, $P > 0.05$) or the cloned α_{1d}-adrenoceptor ($r=0.43$, $P > 0.05$). Our data suggest that the α_{1A}-adrenoceptor subtype plays a significant role in contractile responses to adrenergic agonists in the bovine tail artery.

Additional support for the dominance of the α_{1A}-adrenoceptor in adrenergic mediated contractions of the bovine tail artery comes from the finding that the affinity of BMY 7378 ($pA_2 = 7.17 \pm 0.05$) against phenylephrine was significantly higher than against A61603 ($pA_2 = 6.62 \pm 0.15$). BMY 7378, a selective α_{1D}-adrenoceptor antagonist has higher affinity to α_{1D}- than to α_{1A}-adrenoceptors (Goetz et al., 1995; Zhu et al., 1997) and has been commonly used to discriminate the α_{1D}-adrenoceptor subtype (Fagura et al., 1997; Villalobos-Molina et al., 1997; Zhu et al., 1997). Greater antagonism by BMY 7378 against phenylephrine, a non-subtype selective α_1-adrenoceptor agonist when compared to that against A61603, a selective α_{1A}-agonist, can be taken as evidence for the presence of functional α_{1D}-adrenoceptors in the bovine tail artery.

We used chloroethylclonidine to study the presence of α_{1B}-adrenoceptors by evaluating the differences in alkylation rate of α_1-adrenoceptors subtypes. Our method was based on previous experiments by Xiao and Jeffries (1997) concerning the kinetics of alkylation of cloned α_1-adrenoceptors. Xiao and Jeffries (1997) reported that the lowest alkylation rate was for the α_{1a}-adrenoceptors when compared to α_{1b}- or α_{1d}-adrenoceptors. We found that treatment with a low concentration of chloroethylclonidine (50 μM) for 10 min significantly inhibited contractile responses to phenylephrine, but had no affect on the concentration-response relationship to A61603. Increasing the concentration of chloroethylclonidine (to 100 μM) or increasing the incubation time (to 20 or 30 min) significantly inhibited contractions to both, phenylephrine and A61603. These findings suggest that alkylation of α_1-adrenoceptors with 50 μM (10 min) of chloroethylclonidine caused inactivation of other than α_{1A}-adrenoceptor subtypes. Unfortunately, alkylation of α_1-adrenoceptors with chloroethylclonidine is unable to discriminate between α_{1B}- and α_{1D}-adrenoceptor subtypes and therefore our data cannot exclude the possibility of the presence of

both functional α_{1B}- and α_{1D}-adrenoceptors. Defining the presence of functional α_{1B}-adrenoceptors in the bovine tail artery awaits the disclosure of more specific agonist/antagonists for α_{1B}-adrenoceptors.

In summary, it appears that all three subtypes (α_{1A}-, α_{1B}- and α_{1D}) of α_1-adrenoceptors may play a role in adrenergic mediated contraction in the bovine tail artery. α_{1A}-Adrenoceptors play a dominant role in contractile responses to adrenergic agonists in this tissue. This conclusion is based on the high potency of the α_{1A}-adrenoceptor agonist A61603 in bovine tail artery and a significant correlation of antagonist affinities (pA_2 values) with literature radioligand binding affinities (pK_i values) for the cloned α_{1a}-adrenoceptor. The other two α-adrenoceptor subtypes (α_{1B}- and α_{1D}-) very likely co-exist in the bovine tail artery but have less of an impact on contractile responses to adrenergic agonists than the α_{1A}-adrenoceptors.

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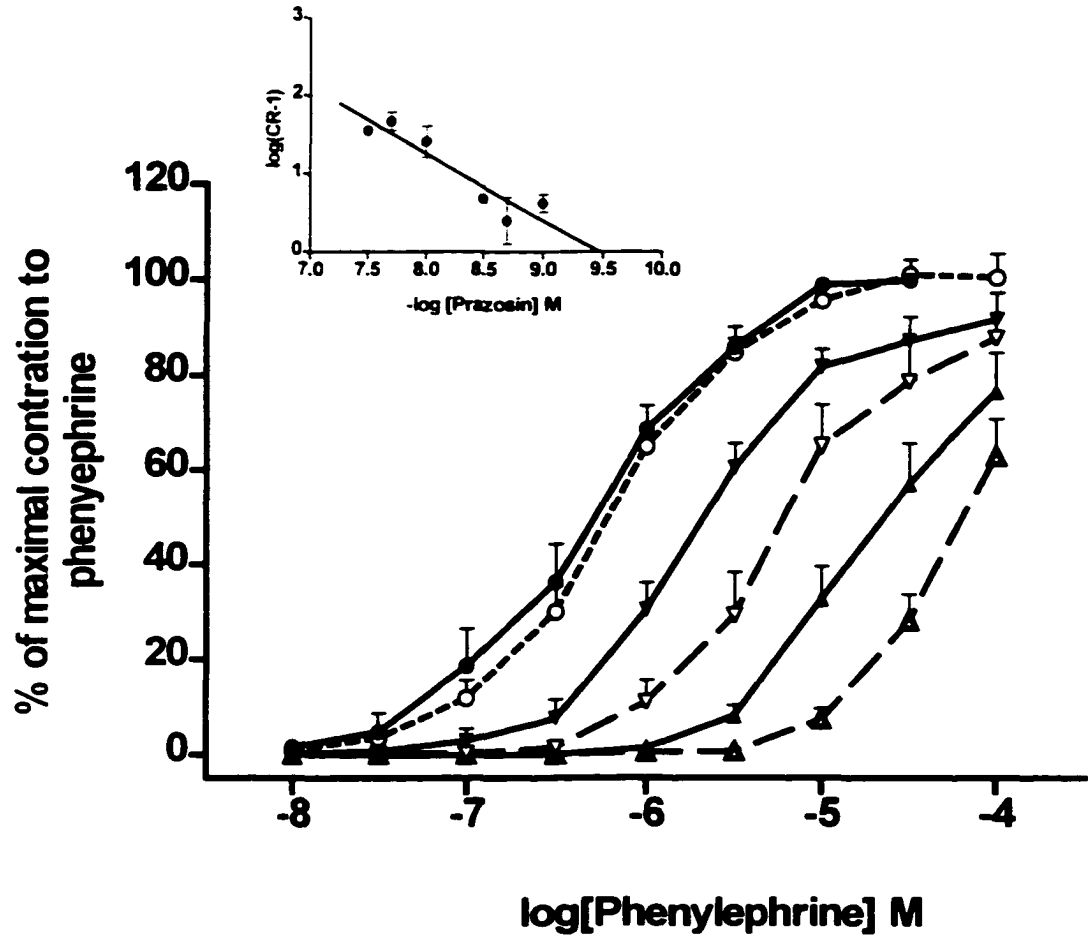
Figure 1. Concentration-response relationships to phenylephrine in endothelium denuded bovine tail artery rings in the absence of antagonists (control (●) and time-matched (○) control tissue) and in the presence of adrenergic antagonists: **(A)** prazosin (▼) 1nM, (▽) 3 nM, (▲) 10 nM, (Δ) 30 nM, n=6; **(B)** WB 4101 (▼) 1nM, (▲) 10 nM, (Δ) 30 nM, n=5 and **(C)** BMY 7378 (□) 0.3 μM, (◆) 1 μM, (◇) 30 μM, n=5. Each point on the concentration-response curve represents the mean ± S.E.M. Schild plots were constructed for each antagonist.

A


% of maximal contration to phenyephrine
120
100
80
60
40
20
0
-8
-7
-6
-5
-4
log[Phenylephrine] M
log(CR-1)
3
2
1
0
7.0
7.5
8.0
8.5
9.0
9.5
10.0
-log [Prazosin] M

B

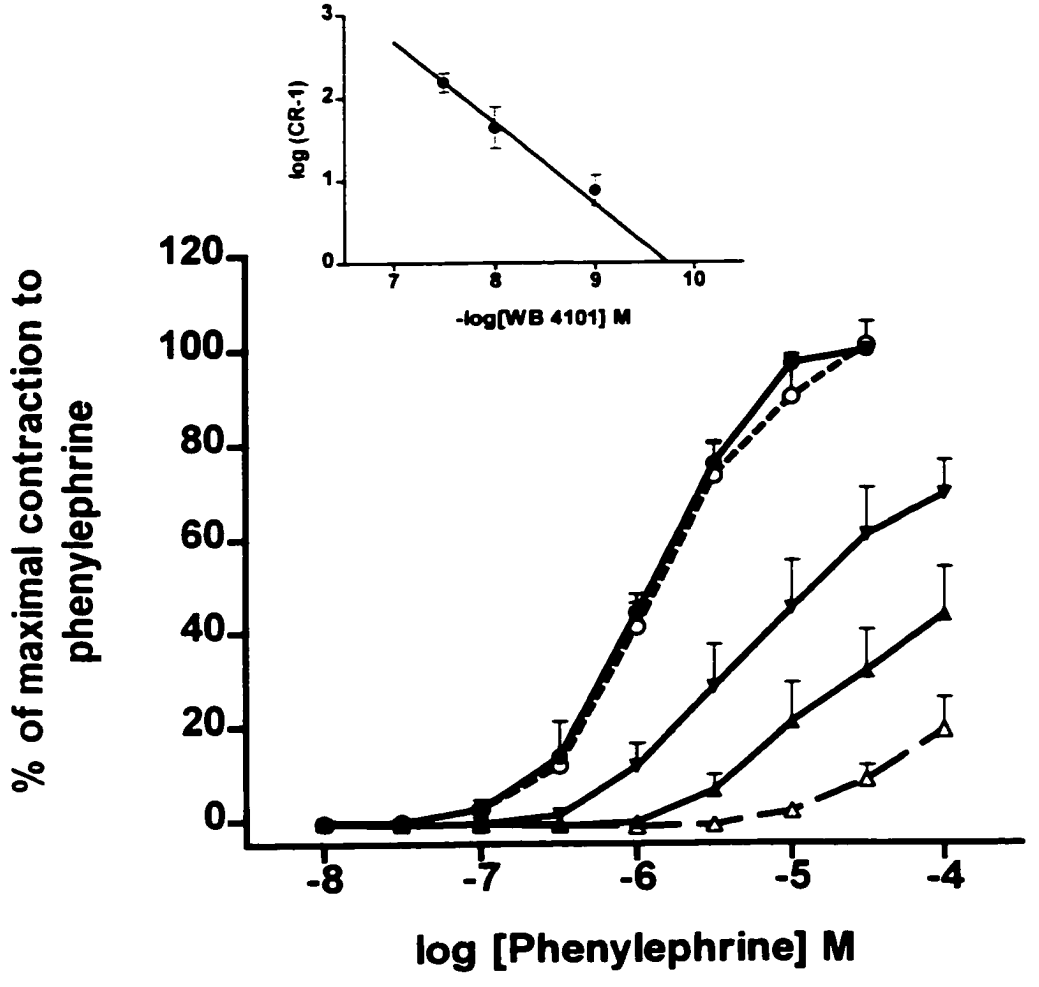


Figure 1. (continued)

C

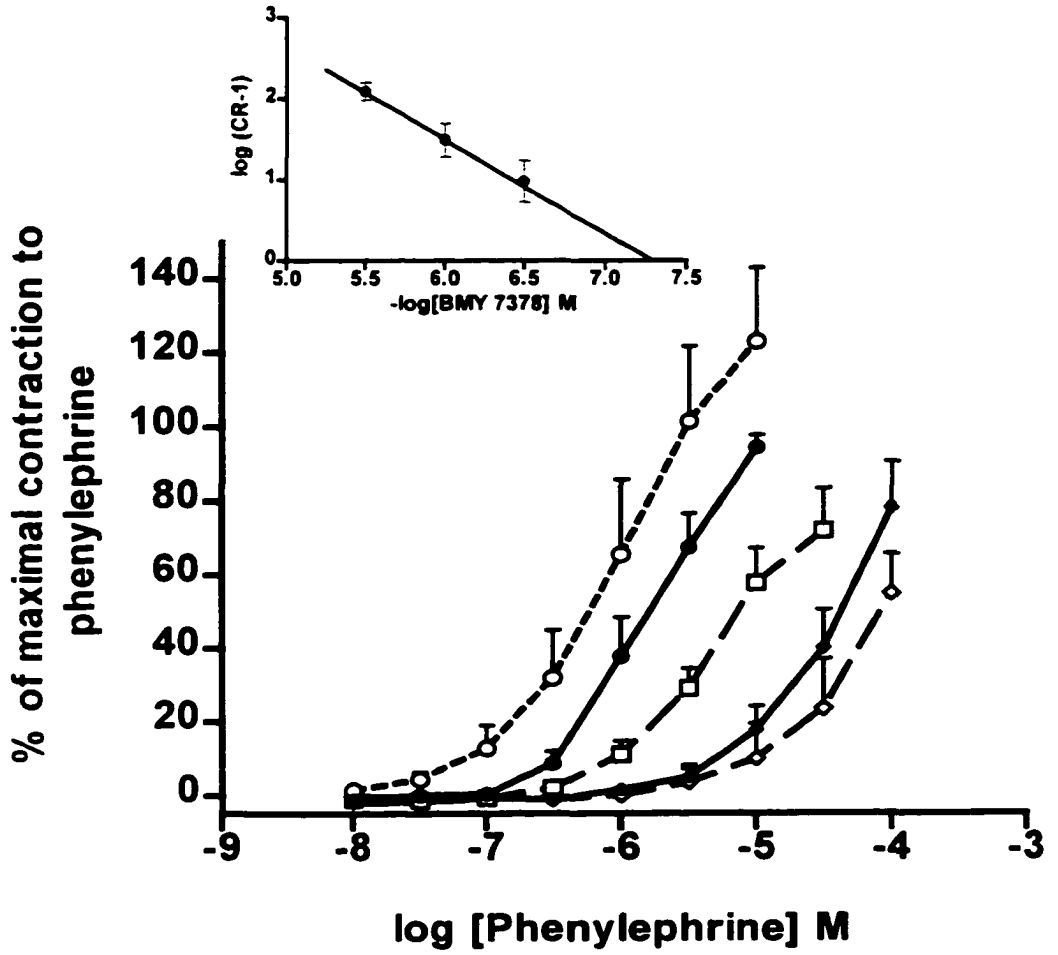
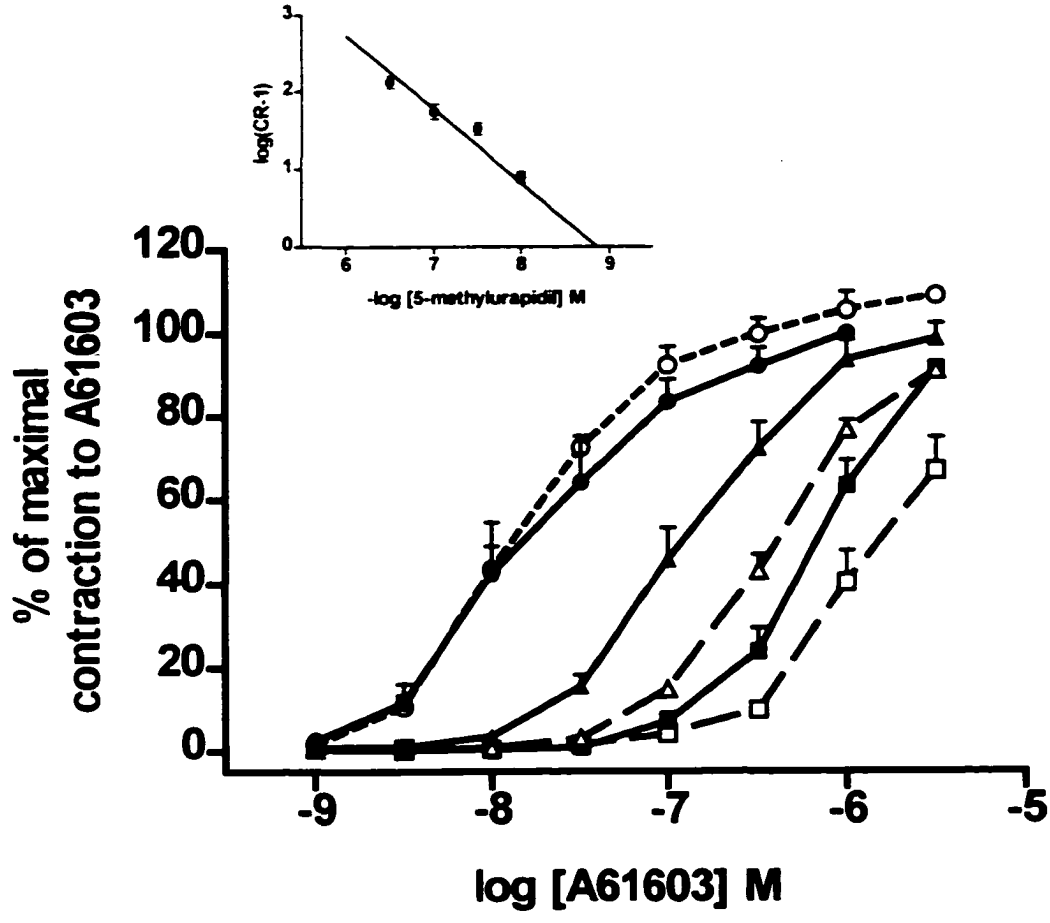


Figure 1. (continued)

Figure 2. Concentration-response relationships to A61603 in endothelium denuded bovine tail artery rings in the absence of antagonists (control (●) and time-matched (○) control tissue) and in the presence of adrenergic antagonists: **(A)** 5-methylurapidil (▲) 10 nM, (Δ) 30 nM, (■) 0.1μM, (□) 0.3 μM; n=4; **(B)** WB 4101 (▼) 1nM, (▽) 3 nM, (▲) 10 nM, (Δ) 30 nM, n=4; **(C)** BMY 7378 (□) 0.3 μM, (◆) 1 μM, (◇) 30 μM, n=5 and **(D)** ARC 239 (▲) 10 nM, (Δ) 30 nM, (■) 0.1μM, n=5. Each point on the concentration-response curve represents the mean ± S.E.M. Schild plots were constructed for each antagonist.

A


log(CR-1)
-log [5-methylurapidil] M
% of maximal contraction to A61603
log [A61603] M

B

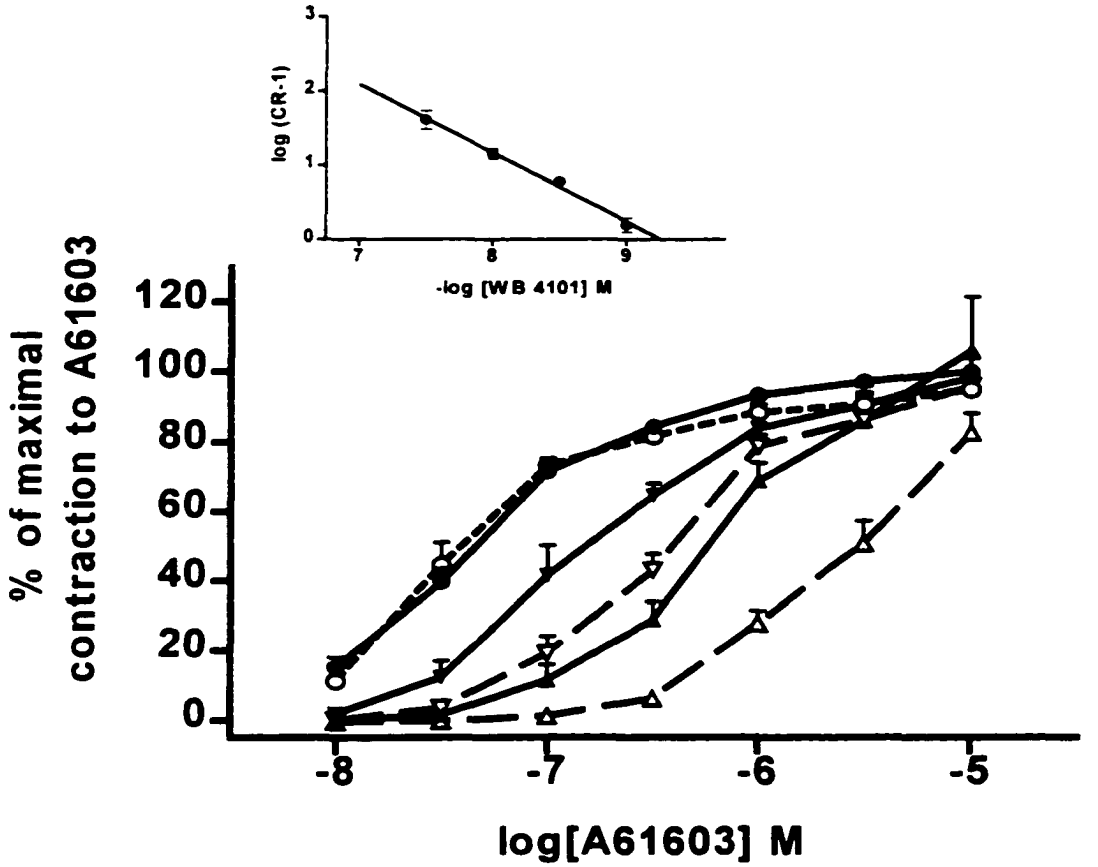


Figure 2. (continued)

C

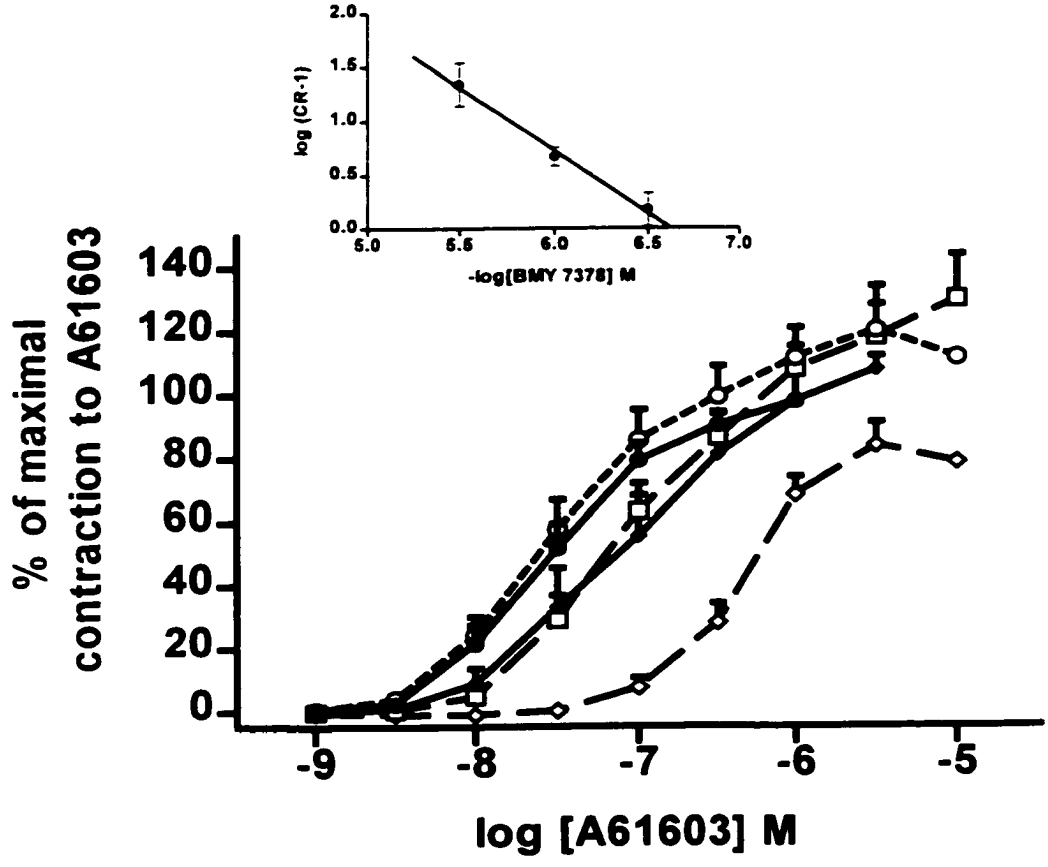


Figure 2. (continued)

D

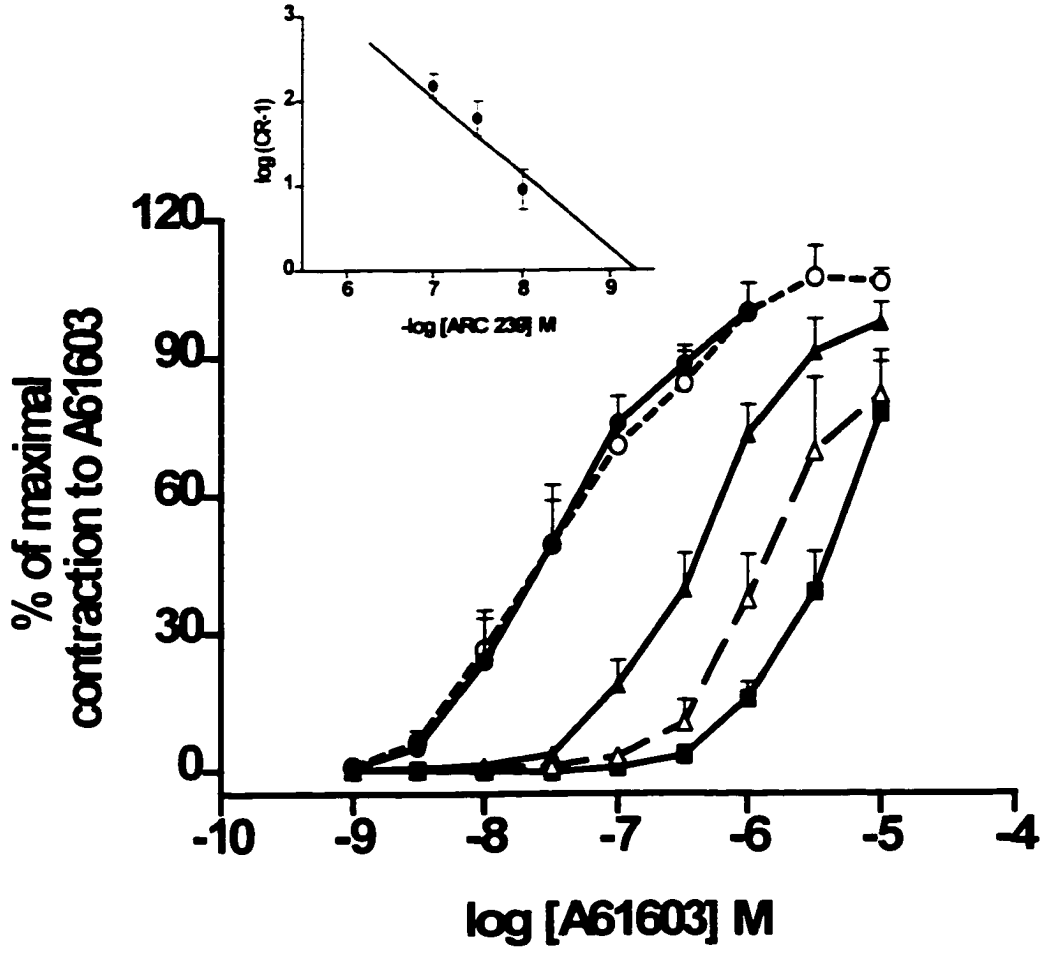


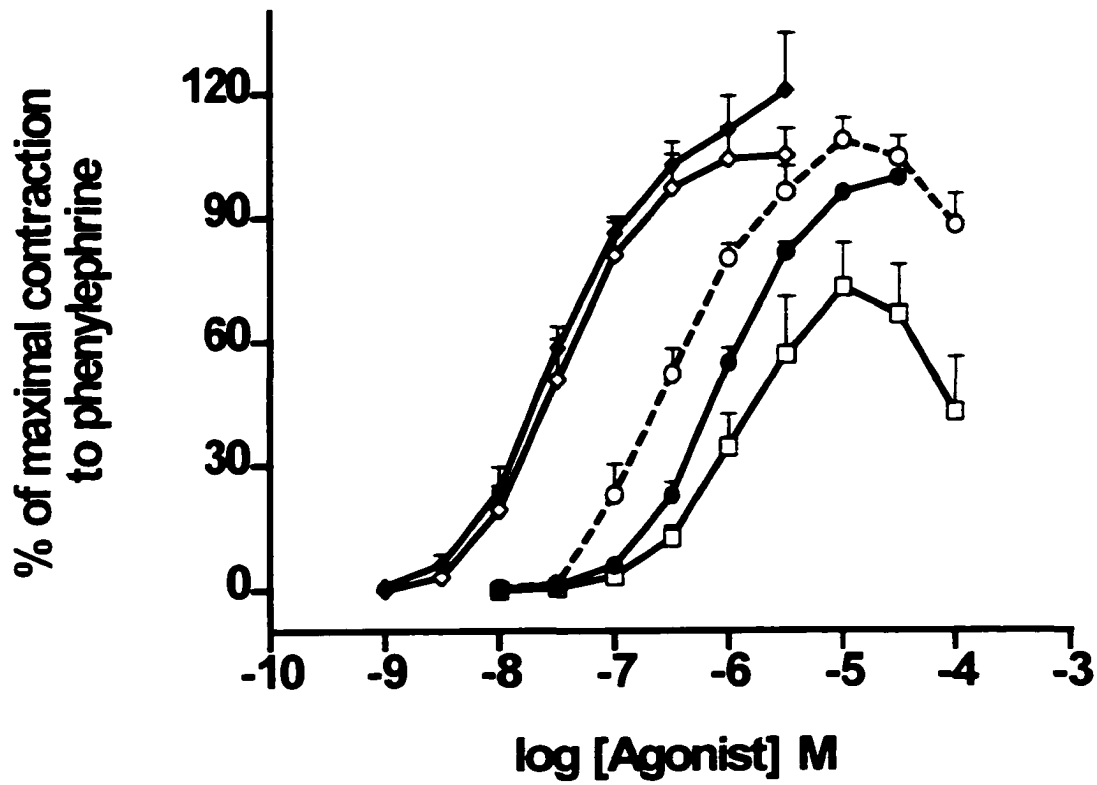
Figure 2. (continued)

Figure 3. Concentration-response relationships in isolated bovine tail artery rings to phenylephrine and A61603 alone and in experiments with chloroethylclonidine. **(A)** The effect of incubation with 50 μM of chloroethylclonidine for 10 min and **(B)** the effect of incubation with 50 μM and 100 μM of chloroethylclonidine for 30 min.

(**A**). Explanation of symbols: (●) the first concentration-response relationship to phenylephrine (control tissue), (○) the second concentration-response relationship to phenylephrine for the untreated tissue (time-matched control), (□) concentration-response relationship to phenylephrine after exposure to chloroethylclonidine (50 μM) for 10 min. (◆) concentration-response relationship to A61603 in untreated (control) tissue and (◇) concentration-response relationship to A61603 in chloroethylclonidine (50 μM, 10 min) treated tissues. Each point on the concentration-response curve represents the mean ± S.E.M. n=4.

(**B**). Explanation of symbols: concentration-response relationship to phenylephrine in (●) control tissue, (○) time-matched control, and after exposure to (▲) 50 μM and (Δ) 100 μM chloroethylclonidine for 30 min. Concentration-response relationship to A61603 in (◆) untreated (control) tissue and in tissues treated with (▼) 50 μM and (▽) 100 μM chloroethylclonidine for 30 min. Each point on the concentration-response curve represent the mean ± S.E.M. n=4.

A


% of maximal contraction to phenylephrine
120
90
60
30
0
-10
-9
-8
-7
-6
-5
-4
-3
log [Agonist] M

B

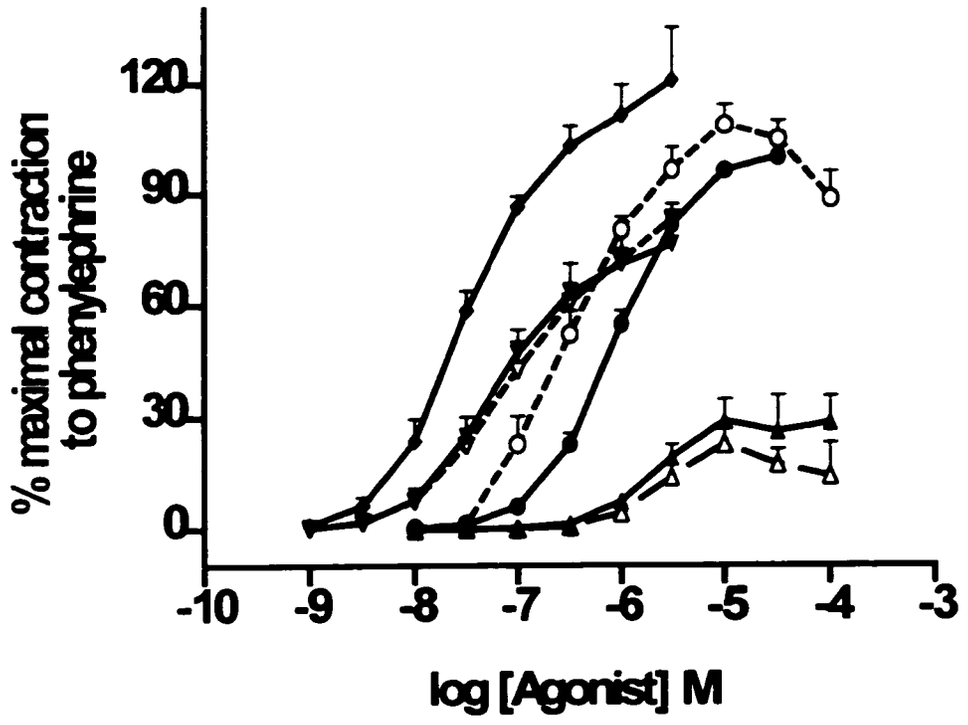


Figure 3. (continued)

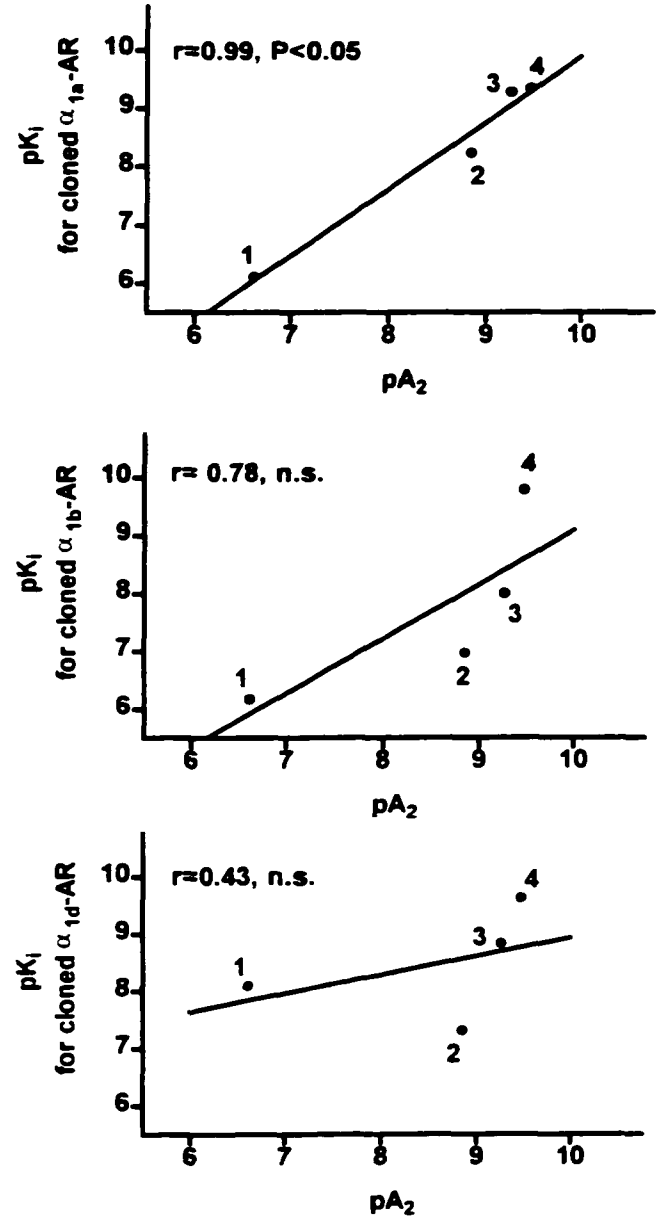


Figure 4. Correlation between the potencies (pA_2) of α_1-adrenoceptor antagonists (**1** - BMY 7378, **2** - 5-methylurapidil, **3** - WB 4101, **4** - prazosin) against contractile responses induced by A61603 in the isolated endothelium denuded bovine tail artery and bindings affinities (pK_i) reported for cloned α_{1a}, α_{1b}, and α_{1d} – adrenoceptors by Zhu and co-workers (1997). There was a significant correlation for α_{1a}-adrenoceptors, n.s means not significant.

Table 1. pA_2 values for α_1-adrenoceptor antagonists in the bovine tail artery.

Agonist	Antagonist	pA_2	Slope	*n*
Phenylephrine	Prazosin	9.47 ± 0.21	-0.89 ± 0.09	6
Phenylephrine	BMY 7378 *	7.17 ± 0.05	-1.21 ± 0.13	5
Phenylephrine	WB 4101	9.73 ± 0.18	-1.04 ± 0.05	5
A61603	ARC 239	9.29 ± 0.26	-1.05 ± 0.09	5
A61603	BMY 7378	6.62 ± 0.15	-1.16 ± 0.14	5
A61603	WB 4101	9.27 ± 0.09	-0.93 ± 0.06	4
A61603	5-methylurapidil	8.86 ± 0.05	-0.98 ± 0.05	4

Data are expressed as mean ± SEM of the number (*n*) of animal used. Slopes are not different from unity. * pA_2 values are significantly different from that found for A61603, $P < 0.05$.

CHAPTER III. CHARACTERIZATION OF α_{1A}- AND α_{1B}-ADRENOCEPTOR SUBTYPES IN THE BOVINE TAIL VEIN

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Marina V. Ioudina and Donald C. Dyer

ABSTRACT

Limited research has been done concerning the functional role of α_1-adrenoceptors in vein. The bovine tail vein possesses a higher affinity for norepinephrine (Ioudina and Dyer, 2000 a) than the bovine tail artery and this could be explained, in part, by a difference in α-adrenoceptor types and/or subtypes involved in contractile responses to adrenergic agonists in these vessels. The purpose of this study was to identify functional α_1-adrenoceptor subtypes in the isolated endothelium denuded bovine (steer) tail vein, incorporating the novel α_{1B}-adrenoceptor selective antagonist L-765,314 in this endeavor. Adrenergic agonists, norepinephrine, phenylephrine and A61603, caused concentration dependent contraction of the isolated bovine tail vein with following potency (pD_2) range: A61603 (7.0 ± 0.09) > norepinephrine (6.5 ± 0.09; n=4) > phenylephrine (5.9 ± 0.13; n=8). The dissociation constant (K_A) for norepinephrine was (0.40 ± 0.07 μM; n=4) and for A61603 was (0.52 ± 0.11 μM; n=4). Contractile responses to phenylephrine and A61603 were significantly inhibited (pK_B) by prazosin (8.8 and 8.7), BMY 7378 (6.7 and 6.6), WB 4101 (8.8 and 9.4), L-765,314 (7.0 and 6.4) and 5-methylurapidil (6.0 and 7.0), respectively. The potencies of WB 4101 and 5-methylurapidil, were higher against A61603 than to phenylephrine while L-765,314 was more potent in inhibiting phenylephrine-induced contractions than those of A61603-induced contractions.

Chloroethylclonidine (50 μM) after 10 min incubation significantly inhibited the maximum (but did not affect EC_{50}) contractile response to phenylephrine but not that to A61603. However, incubation with 50 μM chloroethylclonidine for 20 min or 30 min or with 100 μM chloroethylclonidine for 30 min caused a significant inhibition of the maximal

contractile response to both agonists. Our data suggests that α_1-adrenoceptors play a significant role in contractile responses to adrenergic agonists in the bovine tail vein via activation of both α_{1A}- and α_{1B}-adrenoceptor subtypes.

Keywords: α_1-adrenoceptors, α_{1A}- and α_{1B}-adrenoceptor subtypes, vein, vascular smooth muscle, bovine species

INTRODUCTION

According to modern classification there are two types of α-adrenoceptors (α_1 and α_2) and both are G-protein coupled receptors (Docherty, 1998). The presence of α-adrenoceptors has been reported in a number of species, such as: rat (tail artery, Lachnit et al., 1996; Villalobos-Molina and Ibarra, 1996; hind limb, Zhu et al, 1997; mesenteric artery, Stassen et al, 1998; Piascik et al., 1997; aorta, Villalobos-Molina and Ibarra, 1996; carotid artery, Villalobos-Molina and Ibarra, 1996; spleen, Buckner et al., 1996; heart, Zhang et al., 1999; vas deferens, Buckner et al., 1996; Honner and Docherty, 1999; cerebral cortex, Chess-Williams et al., 1996; kidney, Chess-Williams et al., 1996), human (vas deferens, Furukawa et al., 1995; prostate, Teng et al., 1994; saphenous vein, Connaughton and Docherty, 1990; Smith et al., 1992; Gavin et al., 1997; Roberts et al., 1992; gastric and ileocolic arteries, Guimaraes et al., 1998), dog (saphenous vein, Nunes and Guimaraes, 1993; Low et al, 1999; mesenteric artery, Daniel et al., 1995), rabbit (ear artery, Fagura et al., 1997), equine (digital vein, Elliot, 1997), porcine (thoracic aorta and marginal ear vein, Wright et al., 1995) and cattle (tail artery, Hill and Dyer, 1997).

Both types of α-adrenoceptors (α_1 and α_2) take part in contractile responses in vascular tissues. Thus, activation of α-adrenoceptors vascular smooth muscle cell membrane causes contraction (Hill and Dyer, 1997; Buckner et al., 1996; Lachnit et. al., 1996; Villalobos-Molina et.al., 1997), while activation of α-adrenoceptors on the membrane of the endothelial cell modulates the contractile response of vascular smooth muscle via endothelium-derived factors (MacLean et al., 1993; Zschauer et al., 1997). α_2-Adrenoceptors, located on the sympathetic neuron terminal membrane affect the contractile

response to nerve stimulation via regulation of neurotransmitter release (Trendelenburg et.al. 1997). The present study was designed to examine the role α_1-adrenoceptors play in contraction of bovine tail vein smooth muscle and to evaluate the α_1-adrenoceptor subtypes present in this tissue.

Three native (α_{1A}-, α_{1B}- and α_{1D}-) α_1-adrenoceptor subtypes are recognized by modern classification and three α_1-adrenoceptor (α_{1a}-, α_{1b}- and α_{1d}-) subtypes have been cloned (Langer, 1998). The presence of all α_1-adrenoceptor subtypes have been demonstrated in vascular smooth muscle. Pharmacological characterization of α_{1A}-adrenoceptor-induced contractile responses in vascular smooth muscle (Fagura et al., 1997; Lachnit et. al., 1996; Furukawa et al., 1995; Ioudina and Dyer, 2000c; Marshall et all, 1995) was accomplished using commercially available α_{1A}-adrenoceptors selective agonists, A61603 ((±N-[5-(4,5-dihydro-1 H-imidazol-2yl)-2-hydroxy-5,6,7,8-tetrahydronaphthalen-1-yl] methanesulphonamide hydrobromide) and/or oxymetazoline (Buckner et al., 1996) and antagonists, 5-methylurapidil and/or WB 4101 ((2-(2,6-dimethoxyphenoxyethyl) aminomethyl-1,4 benzodioxane) (Zhu et al., 1997; Buckner et al., 1996). Characterization of functional α_{1D}-adrenoceptors (Villalobos-Molina and Ibarra, 1996; Buckner et al., 1996; Ioudina and Dyer, 2000b) became possible because of the availability of an α_{1D}-adrenoceptor subtype selective antagonist, BMY 7378 ((8-(2-(4-(2-methoxyphenyl)-1-piperazinyl)ethyl)-8-azaspiro(4,5)decane-7,9-dione dihydrochloride)). However, pharmacological characterization of α_{1B}-adrenoceptors is more difficult because of the absence of commercially available selective agonists and antagonists. Chloroethylclonidine, an alkylating agent, has been used to discriminate the functional α_{1B}-adrenoceptors (Testa et al., 1995; Hattori and Kanno, 1997) from the α_{1A}-adrenoceptor subtype because of its higher alkylation rate for α_{1B}-adrenoceptors (Xiao and Jeffries, 1998). However, chloroethylclonidine does not distinguish between α_{1B}- and α_{1D}-adrenoceptor subtypes (Xiao and Jeffries, 1998) and this might become a problem when α_{1B}- and α_{1D}-adrenoceptors are both present in a tissue. A novel α_{1B}-adrenoceptor subtype selective competitive antagonist, L-765,314 (4-amino-2-[4-[1-(benzyloxycarbonyl)-2(S)-[[(1,1-dimethylethyl)amino] carbonyl]-piperazinyl]-6,7-dimethoxyquinazoline), related to prazosin, has been recently

synthesized (Patane, et al., 1998). The affinity (pK_i) of L-765.314 to the α_{1B}-adrenoceptor subtype was about 10 times greater than to α_{1D}-adrenoceptors and more than 100 times greater than to α_{1A}-adrenoceptors (Patane, et al., 1998). Thus, L-765.314 appears to be a useful pharmacological tool for the characterization of α_1-adrenoceptors due to its high affinity for α_{1B}-adrenoceptors.

We have studied adrenergic receptors in the bovine tail artery (Hill and Dyer, 1997; Ioudina and Dyer, 2000a,b) and vein (Ioudina and Dyer, 2000 a, c). Adrenergic agonists cause contraction of the bovine tail artery by activation of predominantly α_1-adrenoceptors (Hill and Dyer, 1997) primarily by an action on α_{1A}-adrenoceptors (Ioudina and Dyer, 2000 b). However, the role of α_1-adrenoceptor subtypes in the bovine tail vein has not been determined. In contrast to the artery, the bovine tail vein possesses a higher affinity to norepinephrine (Ioudina and Dyer, 2000 a) and this may be explained, in part, by the presence of different α-adrenoceptor types (α_2-adrenoceptors) in the vein (Ioudina and Dyer, 2000 c). In contrast to the artery, only a limited number of studies has been reported about the functional role of the α_1-adrenoceptors in vein (Schulz and Westfall, 1982; Leech and Faber, 1996; Elliot, 1997; Marshall et al., 1996; Hu and Dyer, 1997; Low et al., 1999).

The purpose of this study was to identify and characterize functional α_1-adrenoceptor subtypes in the isolated endothelium denuded bovine (steer) tail vein, incorporating the new and novel α_{1B}-adrenoceptor selective antagonist L-765.314 in this endeavor.

MATERIALS AND METHODS

Tissue preparation

Bovine tails (steer) were obtained from a local abattoir and transported to the laboratory on ice. The caudal vein was dissected from the tail 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; dextrose, 7.88; and 0.03 mM EDTA, which was continually aerated (95% O_2 : 5% CO_2). The vein was cut into rings 4-5 mm long. The endothelium was carefully removed by rotating a wooden stick inside the lumen. The tissue

rings were suspended in 10-ml tissue baths filled with aerated Krebs' solution at 38.5^0 C by passing two stainless steel wires through the lumen of each ring. One wire was fixed near the bottom of the tissue bath while the second wire was connected to the force transducer. Isometric contraction was continually recorded by using a MacLab system connected to a computer. An initial tension of 4 g was applied to the vein rings and the tissues permitted to relax during the 60- min equilibration period. All tissues were incubated with 0.36 mM iproniazid during equilibration period in order to inhibit monoamine oxidase (MAO). Prior to beginning the experiments a baseline tension of 1 g was established. Initially each tissue was contracted by 120 mM KCl in order to establish tissues viability. The absence of relaxation to 1 μM acetylcholine in rings contracted by 120 mM KCl was used to confirm endothelium removal. Tissues which developed a tension of less than 2 g in response to 120 mM KCl or which relaxed in response to acetylcholine were discarded. The vein segments were then washed with fresh Krebs' solution every 10 minutes for 30 minutes until the basal tension of 1g was re-established. Vein rings were treated with 10 μM tropolone, 1 μM cocaine and 10 μM corticosterone for 15 minutes in order to block catecholamine-*O*-methyltransferase, neuronal (uptake$_1$) and tissue (uptake$_2$) uptake, respectively (Furchgott and Garcia, 1968; Levin and Furchgott, 1970; Hill and Dyer, 1997) prior to obtaining a concentration-response relationships to an α-adrenoceptor agonist. All concentrations of agonists, norepinephrine (10 nM - 0.1 mM), phenylephrine (10 nM - 0.1 mM) and A61603 (1 nM - 0.1 mM), were added to the bath cumulatively in half-log increments. Concentration-response relationships to an agonist were obtained twice, before and after incubation with one concentration of an antagonist. The maximal tension developed to an agonist obtained during the first concentration-response relationship was set as the 100% response for that tissue. For every experiment a paired tissue not exposed to an antagonist was carried through the procedure to permit correction for time-dependent changes in responses to the agonist, as recommended by Furchgott (1972) and as used in our laboratory (Zhang and Dyer, 1990; Hill and Dyer, 1997).

Analysis of responses to agonists

The dissociation constants (K_A) of the non-subtype selective agonist, norepinephrine, and the α_{1A}-selective agonist, A61603, were determined for the bovine tail vein using the procedure described by Furchgott and Burzstyn (1967) and as used in our laboratory (Hill and Dyer, 1997). Briefly, the vein rings were prepared as described above and a concentration- response relationship to norepinephrine (10 nM - 100 μM) or A61603 (1 nM - 30 μM) was obtained. Following wash out and return to basal tone the tissues were incubated with 0.3 μM dibenamine, an irreversible α-adrenoceptor antagonist, for 20 min in order to inactivate a fraction of the α-adrenoceptors. After incubation with dibenamine all tissues were extensively washed with fresh Krebs' solution 4-5 times over 30 min before obtaining a second concentration-response relationship to an agonist.

Analysis of responses to antagonists

Antagonist potencies ($pK_B = -\log K_B$) were calculated from a single concentration of an antagonist. For this purpose the concentration-response relationship to an agonist, phenylephrine (10 nM - 100 μM) or A61603 (1 nM - 30 μM), was obtained before and after 1 hour incubation with one concentration of an antagonist: WB 4101 (10 nM), BMY 7378 (1 μM), 5-methylurapidil (30 μM), L-765,314 (1 μM) or prazosin (30 nM).

Experiments with chloroethylclonidine

The irreversible α-adrenergic antagonist, chloroethylclonidine, was used to study α_{1B}-adrenoceptors (Villalobos- Molina et al., 1997; Zhu et al., 1997; Marshall et al., 1995). Tissues were prepared as described above. An initial concentration-response relationship to phenylephrine was obtained for all vein rings and the maximum contraction was set as the 100% response. Following washout and return to baseline tension the vessels segments were incubated with either 50 μM of chloroethylclonidine for 10, 20 or 30 min or with 100 μM of chloroethylclonidine for 30 min. Then all tissues were rinsed extensively with fresh Krebs' solution 4 - 5 times for 30 min. A second concentration-response relationship was then acquired for phenylephrine and A61603. A tissue not exposed to chloroethylclonidine was

carried through the experiment and served as a time-matched control. An EC_{50} value was determined only for control tissues and for tissues treated with 50 mM chloroethylclonidine for 10 min.

Data calculation.

The effective molar concentration of an agonist required to produce 50% maximal contraction, i.e. EC_{50} ($-\log EC_{50} = pD_2$) was calculated from its concentration-response relationship curve using the GraphPad Prism computer program (GraphPad Software Inc. San Diego, CA). The effective concentration of an agonist required to produce 30% (EC_{30}) of maximal contraction were calculated by using linear function between the two closest points which overlap the 30% response.

The dissociation constant (K_A) for the receptor-agonist complex was calculated as described Furchgott and Burzstyn (1967). Briefly: a double reciprocal plot of equi-effective concentrations of an agonist before (1/[A]) and after (1/[A]') treatment with dibenamine corrected for the time-dependent changes was made. The dissociation constant (K_A) was calculated by using the equation: K_A = slope - 1/intercept (Ruffolo, 1982). The agonist affinity can be calculated as a reciprocals of K_A value ($1/K_A$) (Furchgott, 1972).

Antagonist potency (pK_B value) for antagonists was obtained as described by Furchgott (1972). EC_{30} values were calculated as described above from the concentration-response curves in the absence [A] and in the presence [A'] of each competitive antagonist concentration. The concentration ratio, CR = [A']/[A] (Furchgott, 1972), and the log (CR -1) was calculated after correction for the time-dependent changes in sensitivity to the agonist. The K_B value was calculated as: K_B = [B]/CR-1, where [B] is the concentration of the antagonist (Furchgott, 1972) and expressed as pK_B ($-\log K_B$).

Statistical Analysis

The results are presented as mean ± S.E.M. and *n* is the number of animals used for each experiment. Student's unpaired t-test was performed to define the differences between

two group means. ANOVA test was performed in order to compare differences among the groups. A P value of 0.05 or less was considered significant.

Drugs

The following drugs were used: (−)-norepinephrine bitartrate, cocaine hydrocholride, corticosterone 21-acetate, ipronizid (phosphate salt), acetylcholine chloride, phenylephrine hydrochloride, idazoxan (Sigma Chemical, St. Louis, MO), A61603 ((±N-[5-(4,5-dihydro-1 H-imidazol-2yl)-2-hydroxy-5,6,7,8-tetrahydronaphthalen-1-yl] methanesulphonamide hydrobromide), (Tocris, Ballwin, MO), chloroethylclonidine, WB 4101 ((2-(2,6-dimethoxyphenoxyethyl) aminomethyl-1,4 benzodioxane), 5-methylurapidil, BMY 7378 (8-(2-(4-(2-methoxyphenyl)-1-piperazinyl)ethyl)-8-azaspiro(4,5)decane-7,9-dione dihydrochloride) (Research Biochemical International, Natick, MA), prazosin (Pfizer, Brooklyn, NY), tropolone (Aldrich, Milwaukee, WI), dibenamine HCl (Smith, Kline and French, Philadelphia, PA), L-765,314 (4-amino-2-[4-[1-(benzyloxycarbonyl)-2(S)-[[(1,1-dimethylethyl)amino] carbonyl]-piperazinyl]-6,7-dimethoxyquinazoline) (Merck, Rahway, NJ). Prazosin was initially prepared in lactic acid and then diluted in isotonic saline solution. Norepinephrine and phenylephrine solutions were prepared in isotonic saline which was acidified with hydrochloric acid (0.0001 N, final concentration). Dibenamine and corticosterone were dissolved in ethanol and no more than 10 µl was added to a 10-ml bath. All other drugs were dissolved in isotonic saline.

RESULTS

Adrenergic agonists, norepinephrine, phenylephrine and A61603, caused concentration dependent contraction of the isolated bovine tail vein. The potency (pD_2) of phenylephrine in the bovine tail vein was (5.9 ± 0.13; n=8) and was significantly different from the potency of norepinephrine (6.5 ± 0.09; n=4) and A61603 (7.0 ± 0.09; n=4). Thus, the order of agonist potency (pD_2 values) order was: A61603 > norepinephrine >

phenylephrine. The dissociation constant (K_A) of norepinephrine (0.40 ± 0.07 μM; n=4) was not different from that of A61603 (0.52 ± 0.11 μM; n=4) (Fig.1).

Antagonists, prazosin, BMY 7378, WB 4101, L-765,314 and 5-methylurapidil, inhibited contractile responses to phenylephrine and A61603 (Fig. 2) and their potency (pK_B) is presented in Table 1. The potencies of antagonists, WB 4101 and 5-methylurapidil, were higher against A61603 than to phenylephrine. L-765,314 was more potent in inhibiting phenylephrine-induced contractions than those of A61603-induced contractions.

Chloroethylclonidine inhibited contractile responses to phenylephrine and A61603 in the bovine tail vein (Fig. 3). Chloroethylclonidine (50 μM) after 10 min incubation significantly inhibited the maximum (but did not affect EC_{50}) contractile response to phenylephrine but not that to A61603. However, incubation with 50 μM chloroethylclonidine for 20 min (data not shown) or 30 min or with 100 μM chloroethylclonidine for 30 min caused a significant inhibition of the maximal contractile response to both agonists.

DISCUSSION

The purpose of this study was to evaluate the role of α_1-adrenoceptors in contractile responses in the isolated endothelium denuded bovine tail vein by studying the affinity of adrenergic agonists and antagonists. The adrenergic agonists, norepinephrine, phenylephrine and A61603, caused concentration dependent contractions in the endothelium denuded bovine tail vein. A61603 had a significantly greater potency (pD_2) to α-adrenoceptors compared to that of norepinephrine or phenylephrine. Their potency (pD_2) order was: A61603 > norepinephrine > phenylephrine. A high potency for A61603, a selective α_{1A}-adrenoceptors agonist suggests, the presence of α_{1A}-adrenoceptors. The potency (pD_2) order of α_1-adrenergic agonists, A61603, norepinephrine and phenylephrine, determined in the bovine tail vein was similar to the affinity (pK_i) order of the same agonists determined by radioligand binding assay for native α_{1A}- (rat submaxillary) and cloned α_{1a}-adrenoceptors (bovine and human) (Buckner et al., 1996). The other two α_1-adrenoceptor subtypes, α_{1d}-

and α_{1d}-adrenoceptors, had a higher affinity (pK_i) to norepinephrine than to A61603 (Buckner et al., 1996). The agonist-receptor dissociation constant (K_A) was determined using the alkylating agent dibenamine which appears to have similar affinity to all (α_{1A}-, α_{1B}- and α_{1D}-) α_1-adrenoceptor subtypes. This assumption is based on the experimental findings that dibenamine was equally potent in inhibiting α_1-adrenoceptors at both low and high affinity [^{3}H]Prazosin binding sites in bovine prostate tissue (Maruyama et al., 1992). The affinity ($1/K_A$) of A61603 in the endothelium denuded bovine tail vein was not significantly different from that of norepinephrine. Interestingly, the K_A value (0.40 μM) for norepinephrine in the endothelium denuded bovine tail vein was lower than that determined for the endothelium intact bovine tail artery (3.11 μM) (Hill and Dyer, 1997). These findings are in agreement with our previous observations that the bovine tail vein exhibits a higher sensitivity (EC_{50}) to norepinephrine than does the artery (Ioudina and Dyer, 2000 c). The differences in K_A values for norepinephrine in the vein and artery might be explained by differences in the α-adrenoceptor types and subtypes present in each tissue. Contractile responses in the bovine tail artery to adrenergic agonists occurs via activation of predominantly α_1-adrenoceptors (Hill and Dyer, 1997) of which the α_{1A}-adrenoceptor subtype appears to play a significant role (Ioudina and Dyer, 2000 b). In addition, α_2-adrenoceptors play an important role in contractile responses to adrenergic agonists in the bovine tail vein (Ioudina and Dyer, 2000 c) but not in the artery (Hill and Dyer, 1997). The adrenergic agonist potency data suggests the presence of an α_{1A}-adrenoceptor subtype in the bovine tail vein.

In order to investigate the possibility of the presence of other α_1-adrenoceptor subtypes in the tail vein, the potencies (pK_B) of antagonists, WB 4101, BMY 7378, 5-methylurapidil, L-765,314 and prazosin, were determined against phenylephrine and A61603 (Table 1). As expected, the α_{1A}-adrenoceptor subtype selective antagonists, 5-methylurapidil and WB 4101, were significantly more potent in inhibiting A61603-induced contraction (7.02 and 9.40, respectively) than that to phenylephrine (6.01 and 8.76, respectively). These data provide additional evidence for the presence of functional α_{1A}-adrenoceptors in the bovine tail vein.

In order to ascertain if the α_{1D}-adrenoceptor subtype is present in the bovine tail vein we used the potent α_{1D}-adrenoceptor subtype selective competitive antagonist, BMY 7378. The affinity of BMY 7378 determined by radioligand binding methods (pK_i = 8.2 -9.4; Goetz et al., 1995; Zhu et al., 1997) for the cloned α_{1d}-adrenoceptor was similar to those reported from functional studies (pA_2 = 8.9 - 10.1; Goetz et al., 1995; Noble, et al., 1997) for the native α_{1D}-adrenoceptor in rat aorta and was higher than affinities for either α_{1A}- or α_{1B}-adrenoceptors (Goetz et al., 1995; Zhu et al., 1997; Noble, et al., 1997). In the bovine tail vein BMY 7378 inhibited contractile responses to phenylephrine and A61603 with similar potency (pK_B = 6.64 and 6.71, respectively). The low affinity of BMY 7378 suggests the lack of functional α_{1D}-adrenoceptors in the bovine tail vein.

For α_{1B}-adrenoceptor subtype identification we used L-765.314, a novel α_{1B}-adrenoceptor subtype selective antagonist, and chloroethylclonidine. L-765.314 exhibited a significantly higher affinity (pK_B) against phenylephrine than against A61603 (7.0 and 6.4, respectively) induced contraction. The affinity (pK_i) of L-765.314 determined by radioligand binding methodology for the cloned α_{1a}-, α_{1b}- and α_{1d}-adrenoceptors was 6.7, 8.5 and 7.7, respectively (Patane et al.,1998). The difference in L-765.314 antagonism against phenylephrine, a non-subtype selective agonist, versus A61603, an α_{1A}-adrenoceptor subtype selective agonist, suggests the presence of the α_{1B}-adrenoceptor subtype in the bovine tail vein. This result was supported by experiments with chloroethylclonidine. Determination of the presence of α_{1B}-adrenoceptors can be obtained by evaluating the alkylation rate of α_1-adrenoceptors subtypes by chloroethylclonidine (Xiao and Jeffries, 1997). We found that treatment with a low concentration of chloroethylclonidine (50 μM) for 10 min significantly inhibited the maximum contractile response to phenylephrine, but had no effect on that to A61603. However, increasing the concentration of chloroethylclonidine (to 100 μM) or increasing the incubation time (to 20 or 30 min) significantly inhibited the maximum contraction to both phenylephrine and A61603. These data suggest that incubation of the bovine tail vein with 50 μM chloroethylclonidine (10 min) caused inactivation of α_{1B}-adrenoceptors.

In summary, our data suggest that α_1-adrenoceptors play a significant role in contractile responses to adrenergic agonists in the bovine tail vein via activation of α_{1A}- and α_{1B}-adrenoceptor subtypes.

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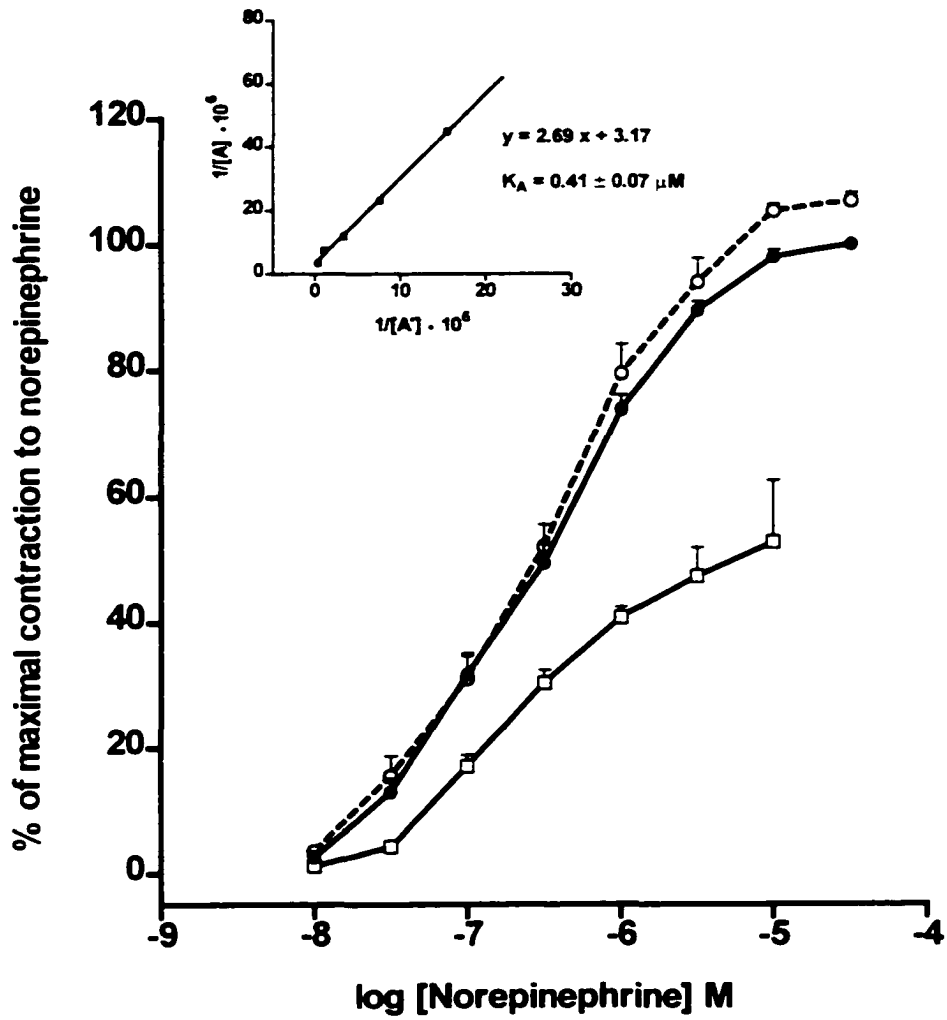
Figure 1. Determination of the dissociation constant K_A for norepinephrine and A61603 acting on α-adrenergic receptors in endothelium denuded bovine tail vein rings. Concentration-response relationships to an agonist were obtained in tissues not exposed to dibenamine (control, ●) tissue and time-matched control tissue (○) and after incubating the tissues with 0.3 μM dibenamine for 20 min (□). A double reciprocal plot of equi-active concentrations before and after incubation was used to determine the K_A of agonists (see Methods).

(**A**). Concentration-response relationship to norepinephrine before and after treatment with dibenamine and a double reciprocal plot of equi-active concentrations was plotted for the K_A determination, (n=4).

(**B**). Concentration-response relationship to A61603 before and after treatment with dibenamine and a double reciprocal plot of equi-active concentrations was plotted for the K_A determination, (n=4).

Each point on the concentration-response curves represents the mean ± S.E.M.

A


% of maximal contraction to norepinephrine
log [Norepinephrine] M
y = 2.69 x + 3.17
K_A = 0.41 ± 0.07 µM
1/[A] . 10^6
1/[A] . 10^6

B

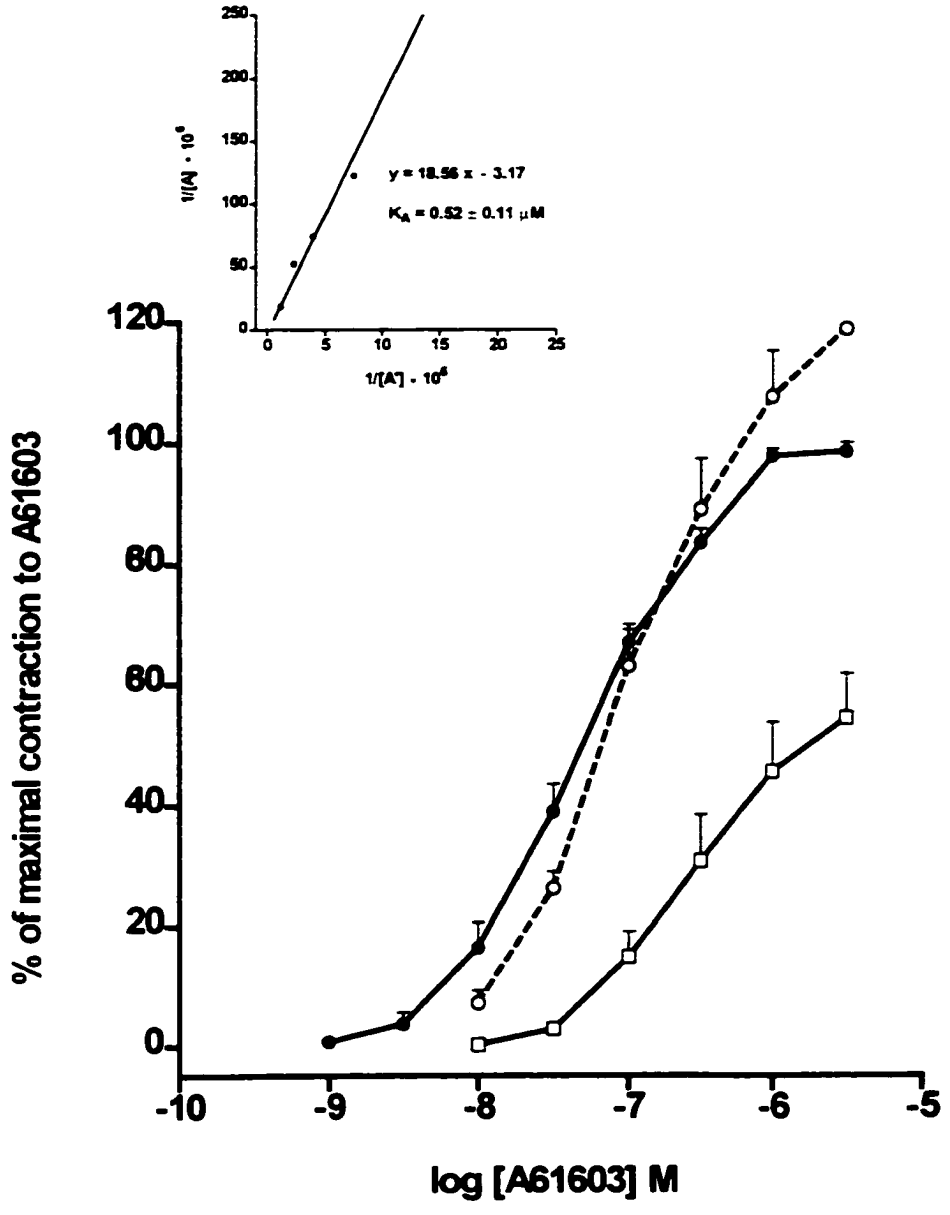


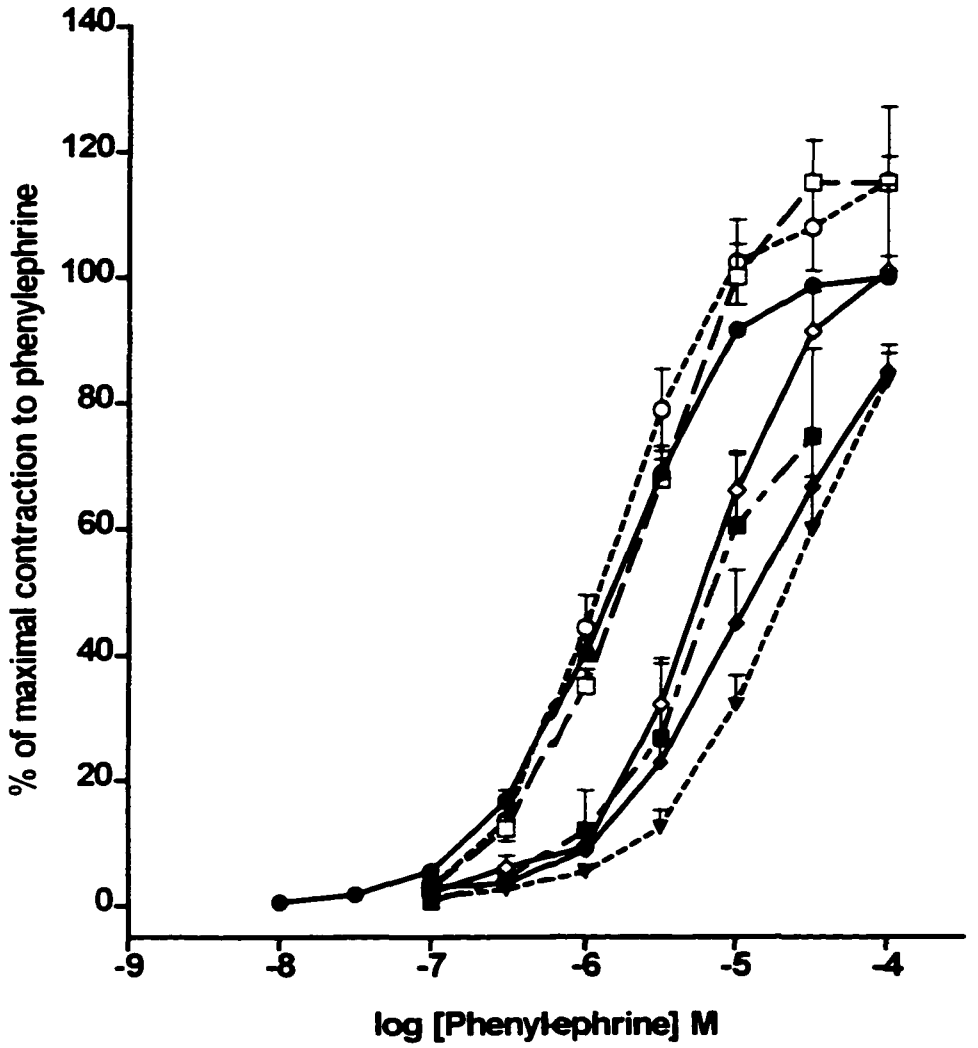
Figure 1. (continued)

Figure 2. Concentration-response relationships to phenylephrine (A) and A61603 (B) in endothelium denuded bovine tail vein rings in the absence of antagonists (control, ●) tissue and time-matched control tissue (○) and in the presence of adrenergic antagonists. Each point on the concentration-response curve represents the mean ± S.E.M.

(**A**). Concentration-response relationships to phenylephrine in the absence and in the presence of 10 nM WB 4101 (◆, n=7); 1 μM BMY 7378 (◇, n=7); 30 μM 5-methylurapidil (□, n=5); 1 μM L-765,314 (■, n=4) and 30 nM prazosin (▼, n=7).

(**B**). Concentration-response relationships to A61603 in the absence and in the presence of 10 nM WB 4101 (◆, n=6); 1 μM BMY 7378 (◇, n=5); 30 μM 5-methylurapidil (□, n=6); 1 μM L-765,314 (■, n=4) and 30 nM prazosin (▼, n=7).

A


140
120
100
80
60
40
20
0
% of maximal contraction to phenylephrine
-9
-8
-7
-6
-5
-4
log [Phenylephrine] M

B

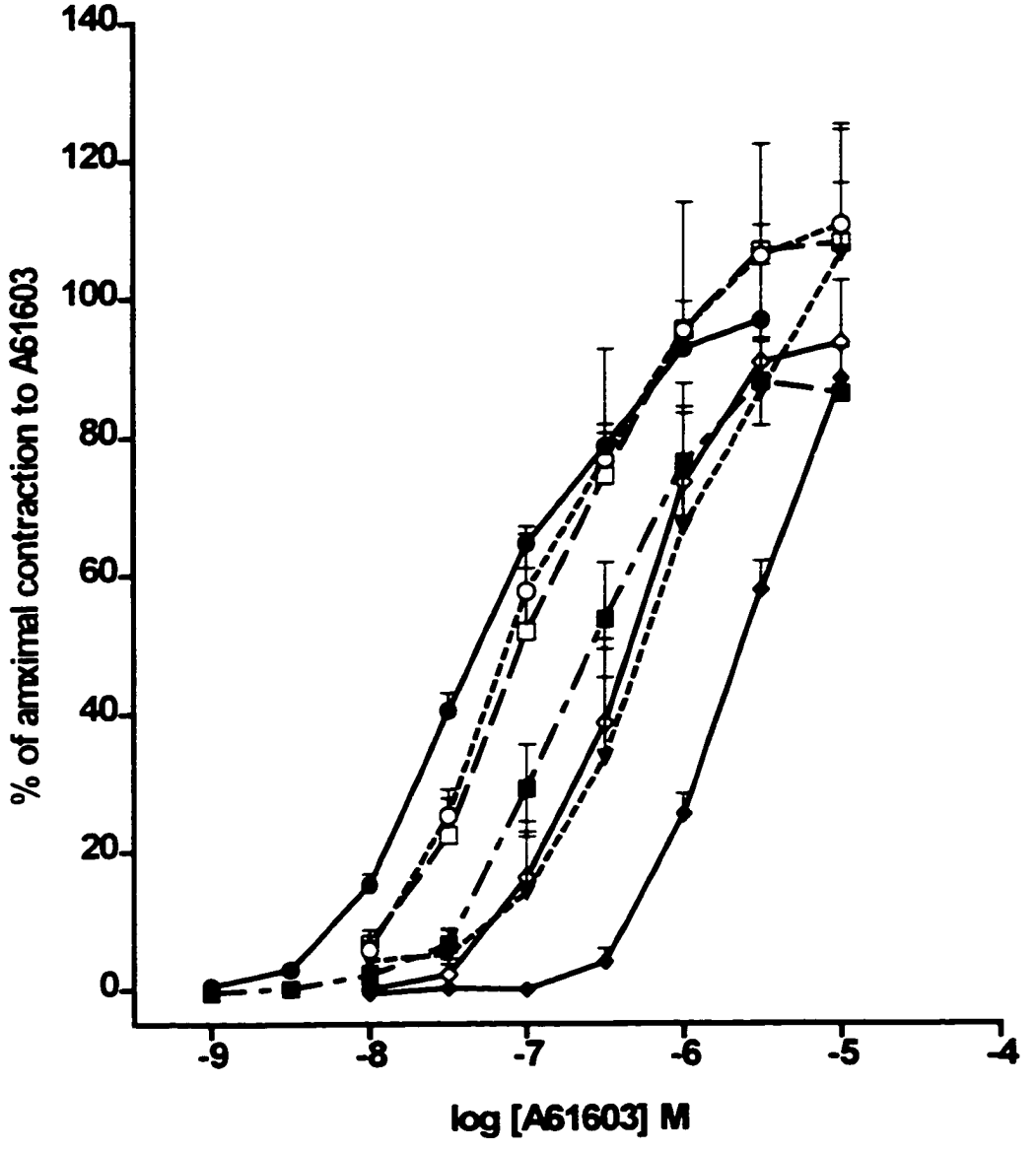


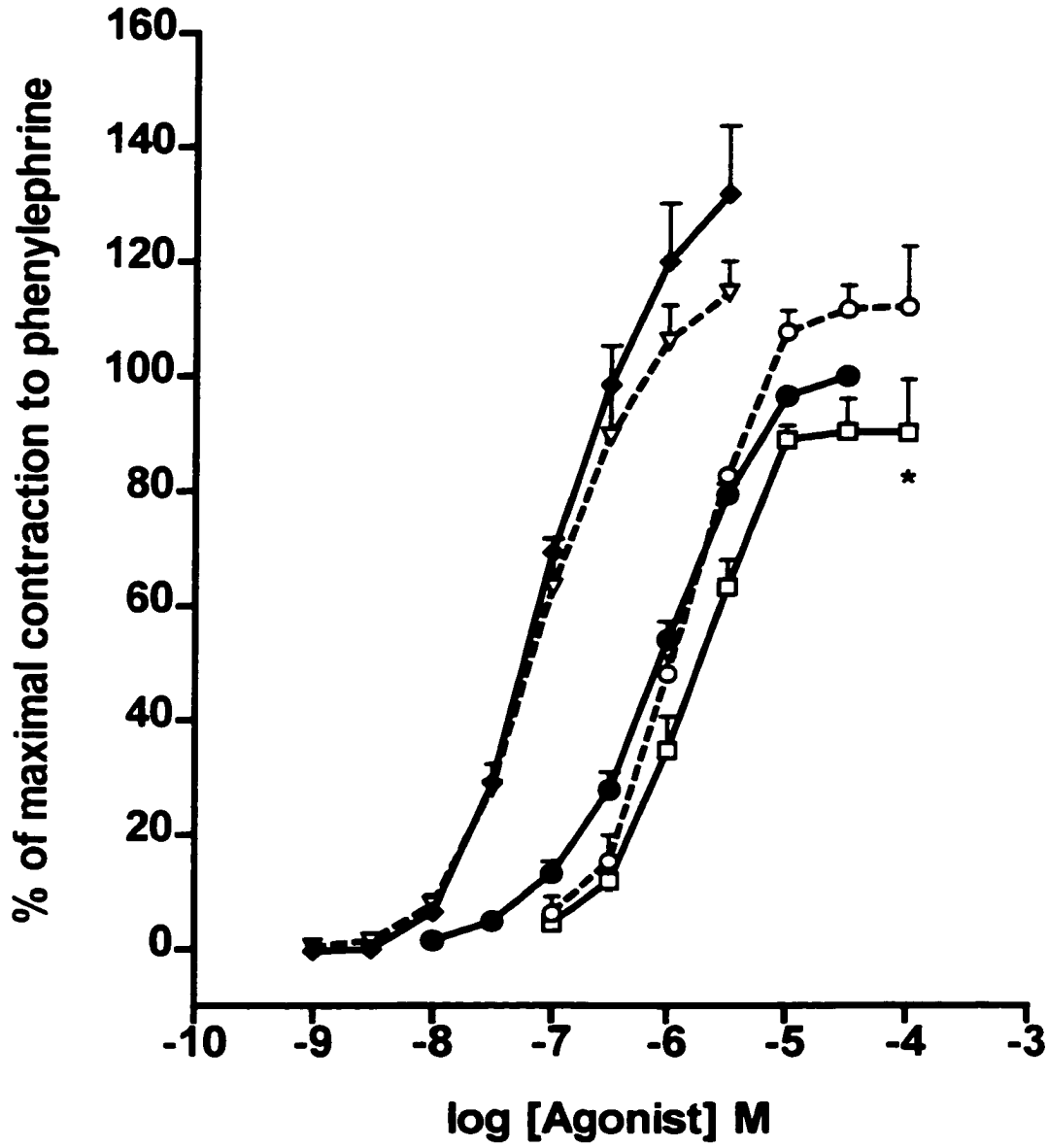
Figure 2. (continued)

Figure 3. Concentration-response relationships in isolated bovine tail vein rings to phenylephrine and A61603 alone and in experiments with chloroethylclonidine. **(A)** The effect of incubation with 50 μM of chloroethylclonidine for 10 min and **(B)** the effect of incubation with 50 μM and 100 μM of chloroethylclonidine for 30 min.

(**A**). The first (●) concentration-response relationship to phenylephrine (control tissue), (○) the second concentration-response relationship to phenylephrine for the untreated tissue (time-matched control), (□) concentration-response relationship to phenylephrine after exposure to chloroethylclonidine (50 μM, 10 min), (◆) concentration-response relationship to A61603 in untreated (control) tissue and (Δ) concentration-response relationship to A61603 in chloroethylclonidine (50 μM, 10 min) treated tissues. Each point on the concentration-response curve represent the mean ± S.E.M, n=4. (*) Maximal contraction to phenylephrine in chloroethylclonidine treated vein rings was different from that in time-matched control tissue.

(**B**). Concentration-response relationship to phenylephrine in (●) control tissue, (○) time-matched control, and after exposure to (▲) 50 μM and (Δ) 100 μM chloroethylclonidine for 30 min. Concentration-response relationship to A61603 in (◆) untreated (control) tissue and in tissues treated with (✳) 50 μM and (◇) 100 μM chloroethylclonidine for 30 min. Each point on the concentration-response curve represent the mean ± S.E.M, n=4.

A


160
140
120
100
80
60
40
20
0
% of maximal contraction to phenylephrine
-10
-9
-8
-7
-6
-5
-4
-3
log [Agonist] M
*

B

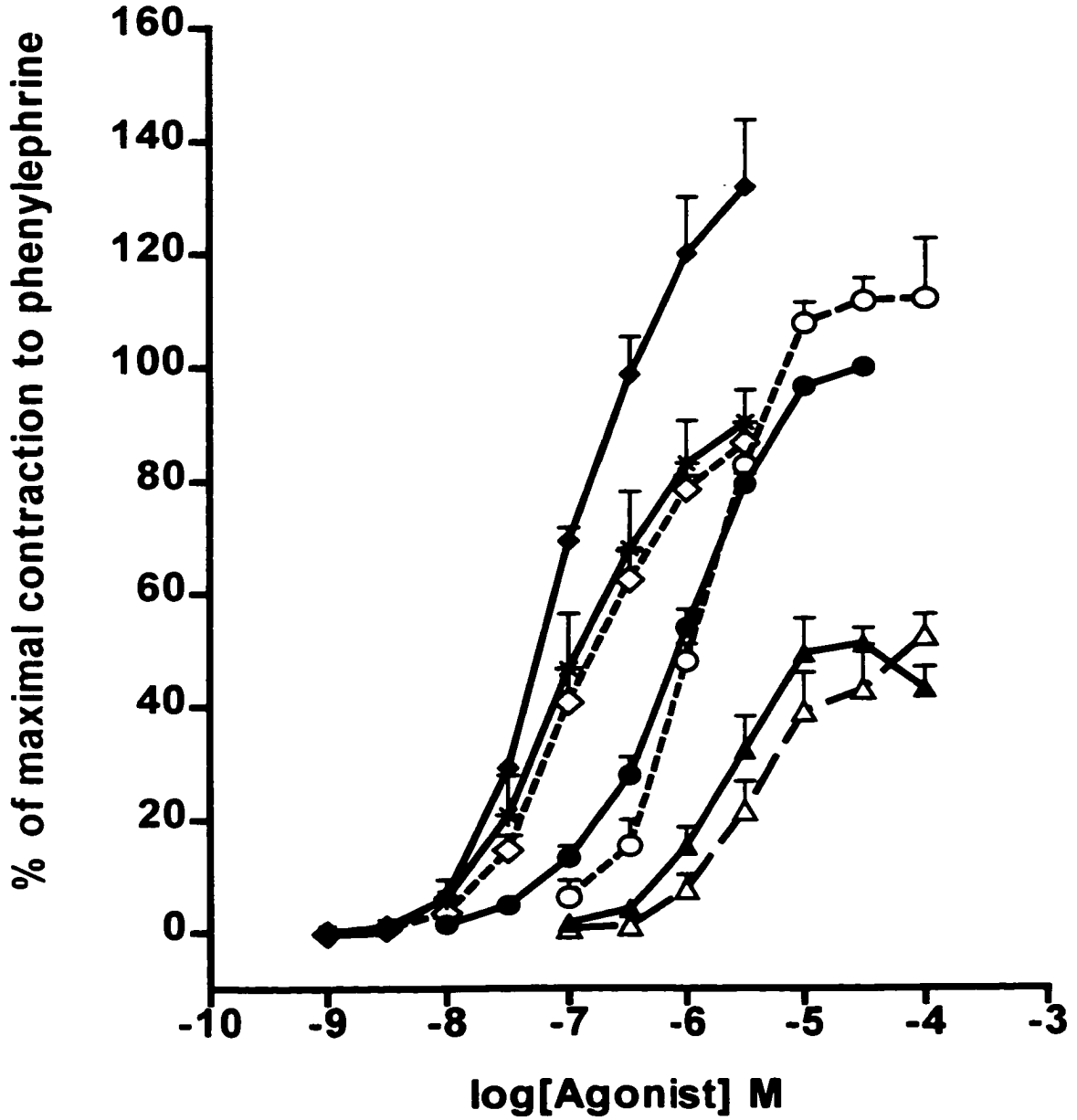


Figure 3. (continued)

Table 1. pK_B values for α_1-adrenoceptor antagonists in the endothelium denuded bovine tail vein.

Agonist	Antagonist	pK_B	*n*
Phenylephrine	WB 4101	8.77 ± 0.16*	7
Phenylephrine	BMY 7378	6.71 ± 0.08	7
Phenylephrine	Prazosin	8.84 ± 0.14	7
Phenylephrine	5-methylurapidil	6.01 ± 0.15*	5
Phenylephrine	L-765.314	7.0 ± 0.12*	4
A61603	WB 4101	9.40 ± 0.11	6
A61603	BMY 7378	6.64 ± 0.11	5
A61603	Prazosin	8.74 ± 0.08	7
A61603	5-methylurapidil	7.02 ± 0.41	6
A61603	L-765.314	6.4 ± 0.02	4

Data are expressed as mean ± SEM of the number (*n*) of animals used. * Mean of pK_B value determined against phenylephrine was significantly different ($P<0.05$) from the mean determined against A61603.

CHAPTER IV. THE ROLE OF α_2-ADRENOCEPTORS IN CONTRACTILE RESPONSES IN THE BOVINE TAIL VEIN AND ARTERY.

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ABSTRACT

The purposes of this study were to compare contractile responses to α_2-adrenoceptor agonists in the isolated endothelium denuded bovine tail artery and vein and to identify the α_2-adrenoceptor subtypes present. UK 14,304, an α_2-adrenoceptor selective agonist, caused concentration-dependent contraction of the bovine tail vein with a pD_2 of 6.9 ± 0.10 and a maximum tension developed 3.3 ± 0.3 g or 62.8 ± 5.2 % (n=9) of that to 120 mM KCl. The maximum tension developed to UK 14,304 in the bovine tail artery was only 6 ± 1.1% (n=4) of that to 120 mM KCl. The potencies (pK_B ± S.E.M) for α_2-adrenoceptor antagonists calculated against UK 14,304 in the bovine tail vein were: rauwolscine (8.2 ± 0.20), WB 4101 (7.8 ± 0.26), idazoxan (7.9 ± 0.13), prazosin (7.7 ± 0.50), yohimbine (7.6 ± 0.27) and ARC 239 (6.4 ± 0.17). The potency of prazosin (7.7) against UK 14,304 in the vein suggests the presence of α_{2B}- and/or α_{2C}-adrenoceptor subtypes rather than the presence of $\alpha_{2A/D}$-adrenoceptors. To discriminate between α_{2B}- and α_{2C}-adrenoceptor subtypes we determined, using pK_B values, the relative potency ratio of pK_B values for ARC 239/rauwolscine in the bovine tail vein and found it to be 0.77. This ratio was not different from a similar ratio (0.83 ± 0.03, n=5) using literature pK_i or pK_D values for α_{2C}-adrenoceptors. Norepinephrine was more potent in producing contractions in the bovine tail vein (pD_2 = 6.5 ± 0.07) than in the tail artery (pD_2 = 6.1 ± 0.16, n=4) $P<0.05$. Idazoxan pK_B values against norepinephrine were (6.6 ± 0.08, n=8) in the bovine tail artery and (6.8 ± 0.12, n=7) in the vein. This was significantly ($P<0.05$) lower than the pK_B (7.9) for UK 14,304 in vein. In conclusion, functional α_2-adrenoceptors in the bovine tail vein play a significant role in contractile

responses to adrenergic agonists. UK 14,304, an α_2-adrenergic agonist, caused contraction in the isolated endothelium denuded bovine tail vein via activation of α_{2C}-adrenoceptors. However, the possibility that other functional α_2-adrenoceptor subtypes (α_{2B} and/or α_{2D}) in the bovine tail vein participate in adrenergic agonist mediated contraction cannot be excluded and needs further study. Contraction mediated by α_2-adrenoceptors in the endothelium denuded bovine tail artery is very small.

Keywords: α_2-adrenoceptor subtypes, vascular smooth muscle, bovine, tail artery, tail vein.

INTRODUCTION

There are two types of α-adrenoceptors (α_1- and α_2-) and both are found in vascular smooth muscle (see Docherty, 1998, for review). Both α_1- and α_2-adrenoceptors can participate in vascular contraction to adrenergic agonists (Elliott 1997; Leech and Faber, 1996). α_1-Adrenoceptors appear to play a dominant role in vascular contraction (Hill and Dyer, 1997), but not always (Guimaraes and Nunes, 1990). In general, it is accepted that functional α_2-adrenoceptors can be characterized by their high affinity to the α_2-adrenoceptor agonists, clonidine and UK 14,304, (5-bromo-6-[2-imidazolin-2-ylamino]-quinoxaline bitartrate), and to the α_2-adrenoceptor antagonists, rauwolscine and yohimbine and by a low affinity to the α_1-adrenoceptor antagonist, prazosin (see MacKinnon et al., 1994 for review). The presence of functional α_2-adrenoceptors has been reported in several vascular beds such as equine digital vein (Elliot, 1997), dog mesenteric artery (Daniel et al., 1995), dog mesenteric vein (Paiva et al., 1999), dog saphenous vein (MacLennan et al., 1997), rat femoral vein (Paiva et al., 1999) and human saphenous vein (Roberts et al., 1992; Gavin et al., 1997). According to current classification, functional α_2-adrenoceptors are divided into three subtypes ($\alpha_{2A/2D}$-, α_{2B}-, α_{2C}) (Docherty, 1998) while four recombinant α_2-adrenoceptor subtypes have been cloned and identified as, α_{2A}-, α_{2B}-, α_{2C}- and α_{2D} adrenoceptors (Bylund et al., 1994; Docherty, 1998). The α_{2A}- and α_{2D}-adrenoceptor subtypes appear to be species homologues of the same receptor subtype ($\alpha_{2A/D}$-subtype) (Bylund et al., 1994) but pharmacological differences between α_{2A}- and α_{2D}-adrenoceptor subtypes (Bohmann et al.,

1994; Guimaraes et al.,1998; Ali, et al.,1998) have been reported. α_{2A}-Adrenoceptors are found in humans (Guimaraes et al., 1998), pigs (Trendelenburg et al., 1996) and rabbits (Trendelenburg et al., 1994), while the α_{2D}-adrenoceptor subtype is present in the rat (Ho et al., 1998), mouse (Limberger et al., 1995), guinea pig (Ali, et al., 1998) and cattle (Bylund et al., 1997).

All three subtypes ($\alpha_{2A/2D}$-, α_{2B}-, α_{2C}) of α_2-adrenoceptors have been found in vein smooth muscle. α_{2A}-Adrenoceptors have been identified in dog saphenous vein (MacLennan et al., 1997), dog mesenteric vein (Paiva et al., 1999) while α_{2B}-adrenoceptors (Smith et al., 1992) and α_{2C}-adrenoceptors (Gavin et al., 1997) are found in human saphenous vein. It has been reported that activation of both α_1- and α_2-adrenoceptors causes contraction of human femoral vein (Glusa and Markwardt, 1983), equine digital vein (Elliot, 1997) and porcine carotid arteriovenous anastomoses (Willems et al., 1999). The expression of contractile responses is dependent on the densities of α_1- and α_2-adrenoceptors on the cell membrane of vascular smooth muscle (Wright et al., 1995).

The type of adrenergic receptors in the endothelium intact bovine tail artery has been studied in our laboratory (Hill and Dyer, 1997) and it was reported that contractile responses in the bovine tail artery occur predominantly through activation of α_1-adrenoceptors. There is no information to our knowledge concerning functional α-adrenoceptor types and subtypes in the bovine tail vein and in comparison with those in the tail artery. The bovine tail artery and vein exhibit different sensitivities to norepinephrine (Ioudina and Dyer, 1999) which might be explained in part on differences in adrenoceptor types/subtypes involved in contractile responses in these vessels. The purposes of this study were to compare contractile responses to α_2-adrenoceptor agonists in the bovine tail artery and vein and to identify the α_2-adrenoceptor subtypes.

α-Adrenoceptors (α_1-and α_2-) on the endothelial cell membrane (MacLean, et al., 1993; Zschauer et al., 1997) can modify the contractile responses to adrenergic agonists via endothelium-dependent mechanisms. In order to avoid the possible effect by the endothelium on contractile responses to adrenergic agonists in the bovine tail artery and vein we used endothelium denuded vessels.

MATERIALS AND METHODS

Tissue preparation

Bovine tails (steers) were obtained from a local abattoir and delivered on ice to the laboratory. The median caudal artery and vein were isolated from the tail and placed in an aerated modified Krebs' solution. The composition of the Krebs' solution was (mM): NaCl, 115.21; KCl 4.70; $CaCl_2$, 1.80; $MgSO_4$, 1.16; KH_2PO_4, 1.18; $NaHCO_3$, 22.14; dextrose, 7.88; and 0.03 mM EDTA. The procedure used in preparing the tissue was similar to that described by Hill and Dyer (1997). Artery and vein were cut into rings 4-5 mm long. The endothelium was denuded by careful rotation of a blunt wooden stick inside the vessel lumen. Each vessel ring was placed in a 10 ml tissue bath filled with continually aerated (95% O_2 : 5% CO_2) Krebs' solution maintained at 38.5°C. The tissue rings were mounted between two stainless steel wires passing through the lumen of each ring. One wire was stationary and was attached near the bottom of the tissue bath while the second wire was connected to the force transducer. Isometric contraction was continually monitored by using a MacLab system connected to a computer. The ring segments were initially stretched to a tension of 15g (artery) and 4g (vein) and then allowed to relax over a 60-min period. Baseline tension was adjusted to 2g for artery and to 1g for vein rings before beginning the experiments.

Evaluation of contractile response to UK 14,304 in the bovine vessels

Artery and vein rings were prepared as described above. At the end of the equilibration period the vessel rings were contracted to 120 mM KCl. This contractile response was set as the 100% response to which all other agonist-induced contractile responses were compared. The absence of a relaxation response to the addition of 1μM acetylcholine to the bath was used to confirm endothelium removal. Tissues which relaxed to acetylcholine were not used. Vessel rings were then washed with fresh Krebs' solution every 10 minutes for 30 minutes until the basal tension of 2 g for artery and 1 g for vein was re-established. A concentration-response relationship to UK 14,304 (1 nM - 10 μM) was obtained without or in the presence of an antagonist. UK 14,304 was added cumulatively to

the tissue baths in half-log increments. The vein segments were incubated for 1 hour with one concentration of an α-adrenoceptor antagonist: rauwolscine (0.3 μM), WB 4101 (0.3 μM), idazoxan (0.3 μM), prazosin (0.3 μM), yohimbine (0.3 μM) or ARC 239 (3 μM). A tissue not exposed to an antagonist was used as a control tissue for each experiment.

Evaluation of contractile response to norepinephrine in the bovine vessels

Tissues were prepared as described above. During the initial 60-min equilibration period, artery and vein segments were incubated with 0.36 mM iproniazid in order to inhibit monoamine oxidase (MAO). Absence of a relaxation response to acetylcholine (1μM) in artery and vein rings contracted by KCl (45 and 120 mM, respectively) was used to confirm endothelium removal. The vessel rings that did relax to acetylcholine were not used. Tissues were then extensively washed with fresh Krebs' solution for 30 minutes until the basal tension was re-established. Prior to obtaining a concentration-response relationship to norepinephrine all tissues were treated with 10 μM tropolone, 1 μM cocaine and 10 μM corticosterone for 15 minutes in order to block catecholamine-*O*-methyltransferase, neuronal ($uptake_1$) and tissue ($uptake_2$) uptake, respectively (Furchgott and Garcia, 1968; Levin and Furchgott, 1970; Hill and Dyer, 1997).

Concentration-response relationships to norepinephrine (10 nM - 0.1 mM) were obtained twice, before and 1 hour after incubation with one concentration of idazoxan (1 or 3 μM). The maximum contraction to norepinephrine obtained during the first concentration-response relationship was set as the 100% response for that tissue. One tissue not exposed to idazoxan was carried through each experiment in order to correct for time-related changes in tissue sensitivity to norepinephrine.

Calculation of data

The effective concentration of an agonist which produced 50% of the maximal contraction, i.e. EC_{50} ($-\log EC_{50} = pD_2$) was calculated based on concentration-response relationships to an agonist using non-linear regression (GraphPad computer program; GraphPad Software Inc, San Diego, CA). The effective concentration of an agonist to

produce 25% (EC_{25}) or 30% (EC_{30}) of maximal contraction were calculated by using linear function between two the closest points on either side of the 25% or 30% response. EC_{25} and EC_{30} values were used to calculate antagonist potencies (pK_B) as described by Furchgott (1972). Briefly: EC_{25} or EC_{30} were calculated in the absence [A] and in the presence [A´] of each competitive antagonist concentration from the concentration-response curves. The concentration ratio, CR = [A´]/[A] (Furchgott, 1972), and the log (CR - 1) was calculated after correction for the time-dependent changes in sensitivity to the agonist. The K_B value was calculated as: K_B = [B]/CR-1, where [B] is the concentration of the antagonist (Furchgott, 1972) and expressed as pK_B = -log K_B.

Drugs

The following drugs were used: (−)-norepinephrine bitartrate, cocaine hydrocholride, corticosterone 21-acetate, iproniazid (phosphate salt), acetylcholine chloride, yohimbine, idazoxan (Sigma Chemical, St. Louis, MO), ARC 239 ((2-[2,4-(methoxyphenyl)piperazin-1-yl]-ethyl)-4,4-dimethyl-1,3-(2H,4H)-isoquinolindine dihydrochloride), UK 14,304, (5-bromo-6-[2-imidazolin-2-ylamino]-quinoxaline bitartrate) (Tocris, Ballwin, MO), WB 4101 ((2-(2,6-dimethoxyphenoxyethyl) aminomethyl-1,4 benzodioxane) hydrochloride), rauwolscine (Research Biochemical International, Natick, MA), prazosin (Pfizer, Brooklyn, NY), tropolone (Aldrich, Milwaukee , WI). UK 14,304 was initially dissolved in DMSO and then diluted in isotonic saline. Prazosin was initially prepared in lactic acid and then diluted in isotonic saline solution. Norepinephrine solutions were prepared in isotonic saline which was acidified with hydrochloric acid. All other drugs were dissolved in isotonic saline.

Statistical Analysis

The results are presented as mean ± S.E.M. and *n* is the number of animals used for each experiment. The group differences among the treatments were determined by using ANOVA test. The differences between two group means were examined by using Student's unpaired t-test. A P value of 0.05 or less was considered significant.

RESULTS

UK 14,304, an α_2-adrenoceptor selective agonist, caused concentration-dependent contraction of the bovine tail vein (Fig.1) with a pD_2 of 6.9 ± 0.10 and a maximum tension developed 3.3 ± 0.3 g or 62.8 ± 5.2 % (n=9) of that to 120 mM KCl. Tension developed in response to UK 14,304 in the bovine tail artery was only 0.4 ± 0.12 g and was 6 ± 1.1% (n=4) of that to 120 mM KCl (data not shown).

α_2-Adrenoceptor antagonists, rauwolscine (0.3 μM), WB 4101 (0.3 μM), idazoxan (0.3 μM), prazosin (0.3 μM), yohimbine (0.3 μM) and ARC 239 (3 μM), caused inhibition of contractile responses to UK 14,304 in the bovine tail vein (Fig.1). The antagonist potencies (pK_B values) are presented in Table 1. Potencies for the same antagonists in the bovine tail artery were not obtained because of the small contraction developed to UK 14,304.

Norepinephrine produced concentration-dependent contractions of the bovine tail vessels (Fig. 2) and was more potent in producing contractions in the bovine tail vein (pD_2 = 6.5 ± 0.07, n=8) than in the artery (pD_2 = 6.1 ± 0.16, n=4) $P<0.05$. Idazoxan (1 and 3 μM) (Fig.2) inhibited concentration-dependent contraction to norepinephrine with similar potency (pK_B) in the bovine tail artery (6.6 ± 0.08, n=8) and vein (6.8 ± 0.12, n=7). The antagonist potency of idazoxan was significantly ($P<0.05$) higher against UK 14,304 in vein (pK_B = 7.9 ± 0.12, n=7) as compared to that of norepinephrine.

DISCUSSION

The purpose of this study was to investigate the role of α_2-adrenoceptors in contractile responses in the isolated endothelium denuded bovine tail artery and vein. In order to evaluate the role of α_2-adrenoceptors we used the non-subtype selective α_2-adrenergic agonist, UK 14,304. Based on radioligand binding methods, UK 14,304 seems to have a higher affinity to all cloned α_2-adrenoceptor subtypes (pK_i values: 6.3 - 6.7) as compared to that of norepinephrine (pK_i values: 4.9 - 5.8) (Renouard et al., 1994; Uhlén et al., 1992). In our study UK 14,304 caused a significant contractile response in the endothelium denuded

bovine tail vein with a maximal tension developed of 3.3 g which was 63 % of the 120 mM KCl response. α_2-Adrenoceptor antagonists, prazosin, idazoxan, ARC 239, WB 4101, rauwolscine and yohimbine inhibited contractile responses to UK 14.304. Antagonist potencies were calculated from a single concentration of antagonist and expressed as a pK_B value (Table 1). We found that prazosin inhibited contractile responses to UK 14.304 with a relatively high potency (pK_B = 7.7) which suggests the presence of α_{2B}- and/or α_{2C}-adrenoceptor subtypes rather than α_{2D}-adrenoceptors. A higher potency (pK_i) for prazosin has been demonstrated for α_{2B}- and/or α_{2C}-adrenoceptor subtypes (Uhlén et al., 1994, MacLenann et al., 1997) and a lower (pK_i) for $\alpha_{2A/D}$-adrenoceptors (Trendelenburg et al., 1994, Bohmann et al., 1994, Limberger et al., 1995, Wahl et al., 1996). However, prazosin is unable to discriminate between α_{2B}- and α_{2C}-adrenoceptor subtypes. For this purpose we compared antagonist potency of ARC 239 and rauwolscine. These antagonists exhibit affinity for all α_2-adrenoceptor subtypes. However, ARC 239 has a slightly greater selectivity for α_{2B}-adrenoceptors (Blaxall et al., 1991, Uhlén et al., 1994, Renouard et al., 1994) while rauwolscine is more selective for α_{2C}-adrenoceptors (Blaxall et al., 1991, Uhlén et al., 1994). In order to discriminate between α_{2B}- and α_{2C}-adrenoceptor subtypes we calculated relative potencies (Blaxall et al., 1991, Fuder and Selbach, 1993) of ARC 239 and rauwolscine for α_{2B}- and α_{2C}-adrenoceptors. For example, the pK_B value for ARC 239 (6.4) in the bovine tail vein was relatively small compare to that of rauwolscine (8.2) (Table 1) and the calculated relative potency ratio (ARC 239/rauwolscine) was 0.77. This ratio was compared with similar ratios (ARC 239/ rauwolscine) from the literature. These values were determined from a number of radioligand binding experiments (pK_i or pK_D) for α_{2B}- and α_{2C}-adrenoceptors (Table 2). The calculated mean potency ratio for α_{2C}-adrenoceptors (0.83 ± 0.03, n=5) was significantly lower than that for α_{2B}-adrenoceptors (0.98 ± 0.03, n=6). This is in agreement with the higher selectivity of ARC 239 for α_{2B}-adrenoceptors and of rauwolscine for α_{2C}-adrenoceptors, discussed above. The relative potency ratio of ARC 239/rauwolscine calculated for the bovine tail vein (0.77) was not different from the ratio calculated for literature values for α_{2C}-adrenoceptors. These results suggest the presence of functional α_{2C}-adrenoceptors in the bovine tail vein. No significant correlation of our data

(pK_B values) with antagonist potencies reported in the literature from radioligand binding (pK_i or pK_D values) experiments for known α_2-adrenoceptor subtypes (Uhlén et al., 1992, Uhlén et al., 1994, Blaxall et al., 1991) was found. A lack of potent subtype selective α_2-adrenergic agonists and antagonists makes the identification of receptor subtype difficult especially in functional studies. The majority of commercially available α_2-adrenoceptor antagonists have an affinity for all α_2-adrenoceptor subtypes (Uhlén et al., 1994, MacLenann et al., 1997, Blaxall et al., 1991, Trendelenburg et al., 1994, Limberger et al., 1995, Wahl et al., 1996) but with a limited selectivity for one specific α_2-adrenoceptor subtype (Blaxall et al., 1991, Uhlén et al., 1994, Renouard et al., 1994, MacLenann et al., 1997). We conclude that UK 14,304 caused contractile responses in the bovine tail vein through activation of α_2-adrenoceptors which resemble the α_{2C}-adrenoceptor subtype. However, our data do not permit us to exclude the possibility of α_{2B}- and/or α_{2D}-adrenoceptors participating in the contractile response to an adrenergic agonist.

In the isolated endothelium denuded bovine tail artery UK 14,304 caused a very small contractile response which was only 6% of that to 120 mM KCl. The artery rings developed a much smaller tension in response to UK 14,304 (0.4 g) when compared to vein rings (3.3 g). A small contractile response to α_2-adrenoceptor agonists in the tail artery is in agreement with previously published data (Hill and Dyer, 1997). They found that in intact isolated bovine tail artery medetomidine, a selective α_2-adrenoceptor agonist, caused contraction but with low potency ($pD_2 = 5.6$) and low maximal response. Another α_2-adrenoceptor agonist, B-HT 920 (5,6,7,8-tetrahydro-6-(2-propenyl)4H-thiazolo [4,5-d]azepin-2-amine dihydrochloride) failed to produce a contractile response. In their study idazoxan and rauwolscine did not inhibit the contractile response to medetomidine which suggests that the large concentration of medetomidine required to elicit a contraction may not have been via α_2-adrenoceptors. We found that norepinephrine had a similar potency to produce contractile responses in the endothelium denuded ($pD_2 = 6.1$) tail artery when compared to the endothelium intact ($pD_2 = 5.9$) (Hill and Dyer, 1997) bovine tail artery. The bovine tail vein was more sensitive to norepinephrine ($pD_2 = 6.5$) than the tail artery ($pD_2 = 6.1$). These findings might be explained by differences in the α-adrenoceptor types (α_1 - in

artery; α_1 and α_2 - in vein) contributing to response to norepinephrine in those vessels. A higher norepinephrine potency was reported by Blaylock and Wilson (1995) for those vessel with a larger population of α_2-adrenoceptors. Idazoxan caused inhibition of contractile responses to norepinephrine in the isolated denuded bovine tail artery and vein (Fig. 2). The potency (pK_B) of idazoxan against the non-subtype selective α-adrenoceptors agonist, norepinephrine, was similar in the artery and vein (6.6 and 6.8, respectively) and was significantly lower than that found for idazoxan in vein (pK_B= 7.9) against a selective α_2-adrenoceptors agonist, UK 14.304. Idazoxan is a potent non-subtype selective α_2-adrenoceptors antagonist. The potency (pK_i or pK_D) of idazoxan obtained from radioligand binding studies has been published in a number of reports and varied from 7.3 to 8.7 (Blaxall et al., 1991, McLennan et al., 1997; Renouard et al., 1994) for different α_2-adrenoceptor subtypes. This range encompasses the potency (pK_B = 7.9) of idazoxan against UK 14.304 in the bovine tail vein. Low idazoxan potency (pK_B) in the bovine tail artery and vein against norepinephrine may indicate affinity of idazoxan for α_1-adrenoceptors. We speculate that a high concentration of idazoxan (1 - 3 μM) may cause inhibition of contractile responses to norepinephrine, in part, via inhibition of α_1-adrenoceptors. The role of functional α_1-adrenoceptors in the bovine tail vessels is under current investigation.

In summary, functional α_2-adrenoceptors in the bovine tail vein play a significant role in contractile responses to adrenergic agonists. UK 14.304, α_2-adrenergic agonist, caused contraction in the isolated endothelium denuded bovine tail vein via activation of α_{2C}-adrenoceptors. However, the possibility that other functional α_2-adrenoceptor subtypes (α_{2B}- and/or α_{2C}-) in the bovine tail vein participate in adrenergic agonist mediated contraction cannot be excluded and needs further study. The role of α_2-adrenoceptors in the bovine tail artery is very small, because UK 14.304 caused only a very small contraction.

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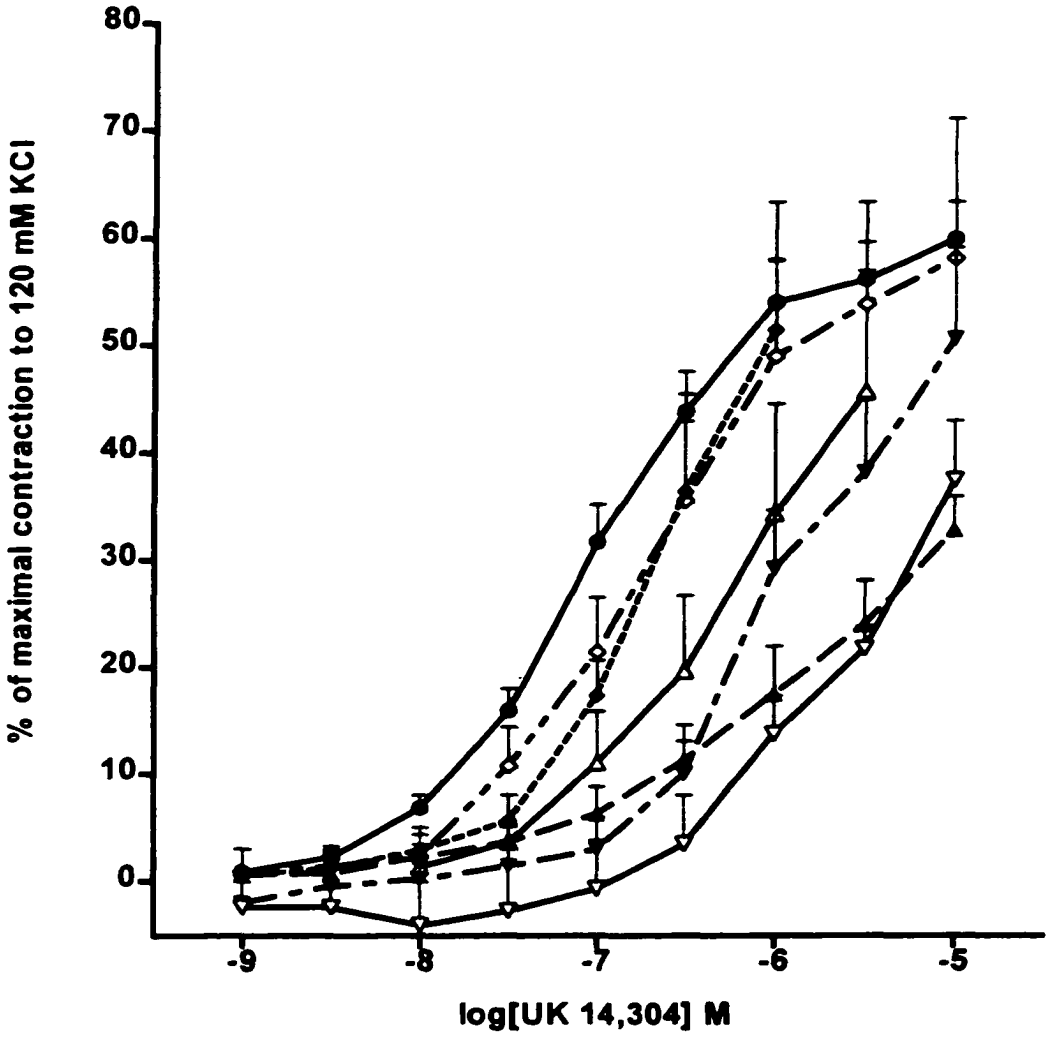
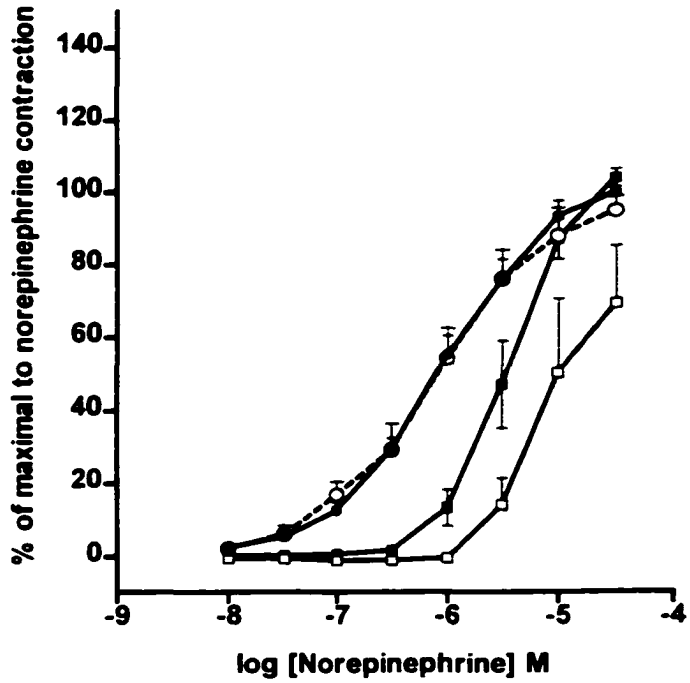


Figure 1. Concentration-response relationships to UK 14,304 in endothelium denuded bovine tail vein rings in the absence of antagonists (control (●) tissue) and in the presence of adrenergic antagonists: 0.3 μM WB 4101 (Δ) (n=4); 0.3 μM rauwolscine (▲) (n=6); 0.3 μM idazoxan (▼) (n=7); 0.3 μM prazosin (◆) (n=5); 3 μM ARC 239, (◇) (n=5) and 0.3 μM yohimbine (▽) (n=5). Each point on the concentration-response curve represents the mean ± S.E.M.

Figure 2. Concentration-response relationships to norepinephrine in endothelium denuded bovine tail vessels in control (●), time-matched control (○) tissue, and in the presence of 1 μM (■) and 3 μM (□) idazoxan. (**A**). Contractile responses in the isolated bovine tail artery, n=4. (**B**). Contractile responses in the isolated bovine tail vein, n=8. Each point on the concentration-response curve represents the mean ± S.E.M.

A


% of maximal to norepinephrine contraction
140
120
100
80
60
40
20
0
-9
-8
-7
-6
-5
-4
log [Norepinephrine] M

B

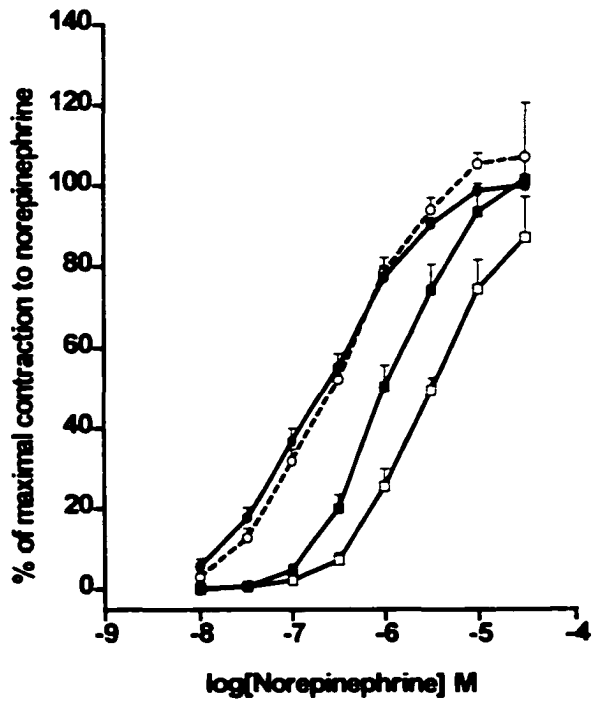

% of maximal contraction to norepinephrine
140
120
100
80
60
40
20
0
-9
-8
-7
-6
-5
-4
log[Norepinephrine] M

Table 1. Antagonist potencies in the isolated endothelium denuded bovine tail vein.

Antagonist	pK_B	Number of animals (n)
WB 4101	7.8 ± 0.26	4
Prazosin	7.7 ± 0.50	5
ARC 239	6.4 ± 0.17	5
Rauwolscine	8.2 ± 0.20	6
Idazoxan	7.9 ± 0.13	7
Yohimbine	7.6 ± 0.27	5

Antagonists potencies were obtained against UK 14.304, an α_2-adrenoceptor selective agonist. Data are expressed as mean ± SEM of the number (*n*) of animal used.

Table 2. Relative potencies of ARC 239 and rauwolscine for α_{2B}- and α_{2C}-adrenoceptors calculated as a ratio of potencies reported from radioligand binding studies.

Subtype of α_2-adrenoceptors and literature source		Relative potency (ARC 239/rauwolscine ratio)
2B, rat neonatal kidney, Uhlén et al., 1992		1.00
2B, rat neonatal lung, Uhlén et al., 1992		0.98
2B, guinea pig cerebral kidney, Uhlén et al., 1995		1.09
2B, rat neonatal lung, Blaxall et al., 1991		0.94
2B, cloned human receptors, Uhlén et al., 1994		0.92
2B, rat neonatal lung, Renouard et al., 1994		0.91
	mean	0.98 ± 0.03 *
2C, rat cerebral cortex, Uhlén et al., 1992		0.83
2C, cloned rat pA2d cells, Uhlén et al., 1992		0.81
2C, guinea pig cerebral cortex receptors, Uhlén et al., 1995		0.87
2C, opossum kidney cells, Blaxall et al., 1991		0.72
2C, cloned human receptors, Uhlén et al., 1994		0.82
	mean	0.83 ± 0.03

The calculated mean ratio of data obtained from the literature and presented in this table for α_{2B}-adrenoceptors and for α_{2C}-adrenoceptors. The relative potency for ARC 239 and rauwolscine (ARC 239/rauwolsine ratio) based on pK_B values obtained in the present study for the isolated bovine tail vein is 0.77. * Mean value is significantly different ($P<0.05$) from that ratio (0.77) found for bovine tail vein.

CHAPTER V. EVALUATION OF NERVE-MEDIATED CONTRACTION IN THE BOVINE TAIL ARTERY IN RESPONSE TO ELECTRICAL FIELD STIMULATION

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ABSTRACT

The purpose of this study was to investigate the possibility of a purinergic component in nerve–mediated contractile responses in a bovine skeletal muscle (tail) artery when subjected to field stimulation. The isolated bovine tail artery was used as the tissue model. We found, that contractile responses of the isolated bovine tail artery subjected to electrical field stimulation were frequency dependent and were abolished by tetrodotoxin (1 mM). The maximum contraction to field stimulation was 43% - 50% of the maximum to that of norepinephrine. Phentolamine and prazosin caused concentration-dependent inhibition of contractile responses to exogenous norepinephrine. Phentolamine (1, 3 and 10 μM) and prazosin (10, 30 and 100 nM) both effectively inhibited frequency-dependent contractions to field stimulation. However, 15% of the maximum contraction to norepinephrine remained at 32 Hz in the presence of the high antagonist concentration.

α,β-MeATP (0.1μM - 10 μM) caused concentration-dependent contractions of the isolated bovine tail artery. The maximum contractile response obtained from a non-cumulative concentration-response relationship to α,β-meATP (102 ± 7.4 %) was significantly higher then that obtained when the α,β-meATP was added to the bath in a cumulative manner (64 ± 5.6). Desensitization of purinergic receptors with α,β-meATP (1 μM) was not long-lasting and did not permit this method to be used as a to inactivate the purinergic receptors. Suramin (300 μM) but not prazosin (100 nM) significantly inhibited contractile responses to α,β-meATP in the bovine tail artery. Suramin (300 μM) did not affect the contraction to KCl but unexpectedly antagonized contractions to norepinephrine.

Contractile responses to field stimulation at 8 - 32 Hz were significantly inhibited by suramin (300 μM) but the degree of inhibition by a combination of prazosin (100 nM) and suramin (300 μM) was not different from inhibition produced by prazosin alone. PPADS (pyridoxalphosphatte-6-azophenyl-2',4'-disulphonic acid) (100 μM) had no effect on either α,β-meATP-evoked contractions nor contractions to field stimulation.

In conclusion, our data suggest that there are functional purinergic (P_{2X}) receptors in the bovine tail artery and their activation causes a contractile response. There is a possibility of norepinephrine/purinergic co-transmission in the bovine tail artery but additional experiments will be required to resolve this problem.

Keywords: bovine tail artery, field stimulation, nerve-mediated contraction, α,β-meATP.

INTRODUCTION

The co-transmission concept, established since the 1970's and well-accepted today, states that norepinephrine can be stored and released from the sympathetic nerve terminal alone or in combination with ATP (adenosine 5'-triphosphate) and/or neuropeptide Y (see Lunberg, 1996). ATP can be stored and released from the adrenergic nerve terminal along with norepinephrine (Lagercrantz and Stjarne, 1974; Burnstock, 1976). ATP released from the nerve terminal (Lagercrantz and Stjarne, 1974; Burnstock, 1976; Levitt and Westfall, 1982; Sedaa, et al., 1990) activates purinergic receptors (see Ralevic and Burnstock, 1998) and causes contraction (Muramatsu et al., 1989; Ziyal et al., 1997; Ziganshin et al., 1994) or relaxation of vascular smooth muscle (Ziganshin et al., 1994; Ziyal et al., 1997). ATP co-transmission has been demonstrated in vascular tissues, such as dog mesenteric artery (Muramatsu, 1986), rabbit ear artery (O'Connor et al., 1990), rabbit aorta (Sedaa et al., 1990), canine splenic artery (Ren et al., 1996) as well as in non-vascular tissues, such as guinea-pig and rat vas deferens (Fedan et al., 1981), guinea-pig taenia coli and rat duodenum (Windscheif et al., 1995). However, information about the co-transmission process in skeletal muscle vessels is limited. For example, ATP co-transmission has been reported in

the rat tail and femoral arteries and dog saphenous vein (Sajag et al., 1990) and in rabbit saphenous artery (Burnstock and Warland, 1987). Limited research has been done concerning the regulation of vascular smooth muscle contraction in cattle (Dzielak, 1983).

Some difficulties exist in determining whether ATP is involved in the co-transmission processes. In general, those problems can be divided into two major categories. The first group of problems is related to the chemical properties of ATP as a neurotransmitter and the second group is related to the number and types of purinergic receptors present in the tissue. For example, neuronally released ATP is a very unstable compound and is rapidly, within 100 milliseconds (Bao and Stjärne, 1993), enzymatically inactivated by adenosine 5'-triphosphatase (ecto-ATPase) to adenosine (Gordon, 1986). Adenosine can cause cellular responses on its own via activation of adenosine receptors and thereby mask the role of ATP (Ralevic and Burnstock, 1998). Current classification divides purinergic receptors into two categories: P1 (adenosine receptors) and P2 (ATP receptors) (Ralevic and Burnstock, 1998). ATP receptors (P2) are subdivided into two classes, P_{2X} and P_{2Y}, where P_{2X} receptors are ATP-gated ion channels and P_{2Y} receptors are G-protein coupled receptors. In addition, seven subtypes of P_{2X} (P_{2X1} - P_{2X7}) and five subtypes of P_{2Y} (P_{2Y1} - P_{2Y5}) receptors have been cloned (see Ralevic and Burnstock, 1998). Lack of subtype selective agonists and antagonists for P_{2X} and P_{2Y} receptors makes it impossible to determine purinergic receptor subtypes by studying contractions in smooth muscle.

Electrical field stimulation (Levitt and Westfall, 1982; Machaly et al., 1988; Evans and Kennedy, 1994) has been commonly employed to stimulate the sympathetic nerve terminal in order to produce effects via neurotransmitter(s) release. ATP release from the sympathetic nerve terminal can be directly studied by measuring [^{3}H]-purine outflow from the sympathetic nerve terminal (Levitt and Westfall, 1982; Sedaa et al., 1990). Measuring contractile responses to field stimulation in isolated tissue can be employed as a method to evaluate whether ATP co-transmission occurs when it is coupled with the use of appropriate pharmacological chemicals.

The purpose of this study was to investigate whether there is a purinergic component in contractile responses in a bovine skeletal muscle artery subjected to field stimulation by using the isolated bovine tail artery as the tissue model.

MATERIALS AND METHODS

Tissue preparation

Bovine tails from steers were obtained from a local abattoir. The tissue was transported to the laboratory on ice where the tail arteries were dissected from the basal part (upper third) of the tail. Dissected vessels were placed in a modified Krebs' solution with 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; dextrose, 7.88 and EDTA, 0.03 mM. Intact vessels were cut into rings 4-5 mm long and each ring was mounted on two stainless steel wires passing through the lumen of the ring. Each ring was placed in a 10 ml tissue bath filled with Krebs' solution at 38.5^0 C and continually aerated by bubbling O_2: CO_2 (95:5) from the bottom of the bath. Indomethacin (2.8 μM) was added to the Krebs' solution to inhibit cyclooxygenase and prevent the synthesis of prostaglandins in all experiments. No attempt was made to remove the endothelium.

One wire was stationary and secured near the bottom of the tissue bath while the second wire was connected to the force transducer. Up to 8 rings were studied per experimental day. Isometric contractions were continually monitored by using a MacLab system connected to a computer. The ring segments were initially stretched to a tension of 15 g and then allowed to relax over a 60-min period. Then the ring segments were adjusted to a basal tension of 2g and maintained at the basal tension for 20 - 30 min before being exposed to drugs.

Field stimulation experiments

The tension developed in the isolated artery rings in response to field stimulation were obtained as a frequency-response relationship (Duckles and Silverman, 1980; Ren and

Burnstock, 1997) with a frequency range of 1 - 32 Hz. Field stimulation was delivered by a Grass SD9 stimulator which was connected to a Med-Lab Stimu-Splitter II (Med Lab Instruments, Fort Collins, Co) to create a constant current between a pair of electrodes in the tissue bath. A constant current method has been proposed as the best technique to use in field stimulation experiments in order to stimulate the nerve terminals and not the smooth muscle (Duckles and Silverman, 1980). Tissues were stimulated for 20 sec at each frequency with an electrical pulse duration of 1 msec at a constant current of 150 mA in the tissue bath. A constant current of 150 mA was maintained through the stimulation period and was manually adjusted when required.

An initial series of experiments were conducted to confirm that: 1) the chosen electrical parameters caused contraction due to neurotransmitter(s) release, and were not the result of direct stimulation of vascular smooth muscle, and 2) repeated field stimulation did not change the tissues excitability to norepinephrine or to field stimulation over the experimental time period.

Tissues were prepared as described above. An initial concentration-response relationship to norepinephrine was followed by a 30-min washout period. A set of three consecutive frequency-response relationships (1 - 32 Hz) to field stimulation was then obtained. A 15-min interval between two frequency-response relationships was sufficient and allowed the tissues to relax to the baseline tension (2 g) before the next stimulation session occurred. Then artery rings were washed with fresh Krebs' solution and 2 or 3 tissues per experiment were incubated (1 hour) with 1 mM tetrodotoxin in order to block the voltage-gated Na^+-channels. A second set of three consecutive frequency-response relationships to field stimulation was then obtained and this was followed by a concentration-response relationship to norepinephrine.

The following experiments were conducted for the purpose to investigate and compare the role of adrenergic and purinergic components in contractile responses to field stimulation.

Frequency-response relationships to field stimulation (1 - 32 Hz) were obtained before and after 1-hour incubation with one concentration of phentolamine (0.1, 1, 3 or

10 μM) and prazosin (10, 30 or 100 nM). An artery ring not exposed to an antagonist, which served as a time-matched control tissue, was carried through each experiment. Contractile responses to field stimulation were compared to a concentration-response relationship to norepinephrine (10 nM - 100 μM) which was obtained twice, before and after determining a frequency-response relationship to field stimulation.

Frequency-response relationships to field stimulation (1 - 32 Hz) were obtained before and 1-hour after incubation with one concentration of PPADS (100 μM) (pyridoxalphosphatte-6-azophenyl-2',4'-disulphonic acid), prazosin (100 nM), suramin (300 μM) or a combination of prazosin and suramin (100 nM and 300 μM, respectively).

Contractile responses to α,β-meATP

A stable ATP analogue, α,β-meATP, was used to ascertain if it would contract the isolated bovine tail artery in order to confirm the presence of functional purinergic receptors in this blood vessel.

Tissues were prepared as described above. The maximum contractile response obtained during the determination of a concentration-response relationship to norepinephrine (10 nM - 100 μM) was set as the 100% response and was used for comparison to contractile responses to α,β-meATP. We compared cumulative vs. non-cumulative concentration-response relationship to α,β-meATP. A cumulative concentration-response relationship to α,β-meATP (0.1μM - 10 μM) was obtained by adding it in half-log increments to the tissue bath. For the non-cumulative concentration-response relationship, α,β-meATP (0.1μM - 10 μM) was added to the tissue bath in single increasing concentrations, where every successive concentration was one-half log higher than preceding concentration. A twenty-minute washout interval was sufficient between the two consecutive concentrations for tissue tension to return to basal conditions (2 g).

In order to evaluate the effectiveness of desensitization of purinergic receptors by the cumulative application of α,β-meATP we used a single concentration of α,β-meATP (1 μM). Immediately after obtaining the cumulative concentration-response to α,β-meATP tissues

were extensively washed three times over a 5 min period and then 1 μM α,β-meATP was added to the bath. An addition 1 μM α,β-meATP was added to the bath 20 min later.

The effects of the purinergic antagonists, suramin and PPADS, as well as an α_1-adrenergic antagonist, prazosin, were evaluated for their effects on contractile responses to α,β-meATP. Tissues were prepared as described above. After the equilibration period, all tissues were challenged with a single (3 μM) concentration of α,β-meATP, which produced a standardized contractile response. The tissues were then washed with fresh Krebs' solution for 30 min before adding the antagonists: prazosin (100 nM), suramin (300 μM), a combination of prazosin and suramin (100 nM and 300 μM, respectively) or PPADS (100 μM). A tissue not exposed to an antagonist was carried through each experiment and it served as a time-matched control tissue. Cumulative concentration-response relationships to α,β-meATP (0.1 μM - 10 μM) were obtained 1 hour after incubation with antagonists. The effect of suramin on contractile responses to norepinephrine and KCl were also obtained 1 hour after incubation with 300 μM suramin.

Statistical Analysis

The results are presented as mean ± S.E.M. and *n* is the number of animals used for each experiment. EC_{50} values for agonists, were calculated from their concentration-response relationships using the GraphPad Prism computer program (GraphPad Software Inc, San Diego, CA). The group differences among the treatments were analyzed by using ANOVA. Student's unpaired t-test was performed to define the differences between two group means. Values are given as means ± S.E.M. A P value of 0.05 or less was considered significant.

Drugs

The following drugs were used in this study: norepinephrine, phentolamine, suramin, α,β-meATP, indomethacin, tetrodotoxin (Sigma Chemical, St. Louis, MO), PPADS (pyridoxalphosphatte-6-azophenyl-2',4'-disulphonic acid) (Research Biochemical International, Natick, MA), prazosin (Pfizer, Brooklyn, NY). Indomethacin was dissolved in 10% Na_2CO_3 and solutions were made fresh daily. Prazosin was initially dissolved in lactic

acid and then diluted in isotonic saline solution. Norepinephrine solutions were prepared in isotonic saline which was acidified with hydrochloric acid. All other drugs were dissolved in isotonic saline.

RESULTS

Contractile responses in the isolated bovine tail artery to electrical field stimulation were frequency dependent (Fig. 1A). Tetrodotoxin (1 mM), significantly inhibited contractile responses to field stimulation (Fig. 1A) but had no effect on the concentration-response relationship to exogenous norepinephrine (Fig. 1B). Repeated field stimulation did not change the tissue excitability to norepinephrine or to field stimulation during the course of the experiment (Fig. 1). The maximum contraction to field stimulation was found to be 43% - 50% (Fig. 2A and Fig. 3A) of the maximum to norepinephrine.

Phentolamine (1, 3 and 10 μM) and prazosin (10, 30 and 100 nM) significantly decreased contractile responses to field stimulation (Fig. 2A and Fig. 3A, respectively) and to norepinephrine (Fig. 2B and Fig. 3B, respectively). Phentolamine and prazosin caused concentration-dependent inhibition of contractile responses to norepinephrine. However, inhibition of contractile responses to field stimulation by prazosin and phentolamine was not concentration-dependent. Different concentrations of phentolamine (1, 3 and 10 μM) and prazosin (10, 30 and 100 nM) were equally potent in inhibiting frequency-dependent contractions to field stimulation, and approximately 15% of the maximum contraction to norepinephrine remained at 32 Hz (Fig.2A and Fig 3A, respectively) in the presence of these antagonists.

α,β-MeATP (0.1μM - 10 μM) caused concentration-dependent contractions of the isolated bovine tail artery (Fig. 4). The maximum contractile response obtained from the non-cumulative concentration-response relationship to α,β-meATP was significantly higher (102 ± 7.4 %) then that obtained from the cumulative (64 ± 5.6%) concentration-response relationship (n=4). A single concentration (1 μM) of α,β-meATP did not cause contraction of the tail artery when it was applied immediately after the washout period following the

cumulative concentration-response relationship to α,β-meATP. After an additional 20-min interval an addition 1 μM of α,β-meATP in the bath produced a contraction of the artery (data not shown).

Suramin (300 μM) but not prazosin (100 nM) significantly inhibited contractile responses to α,β-meATP in the bovine tail artery (Fig. 5). PPADS (100 μM) did not inhibit α,β-meATP-evoked contractions in the bovine tail artery (data not shown). The same concentration of suramin (300 μM) did not affect the contraction to KCl (Fig. 6A) but unexpectedly antagonized contractions to norepinephrine (Fig. 6B).

Contractile responses to field stimulation at 8 - 32 Hz were significantly inhibited by suramin (300 μM) in the bovine tail artery rings (Fig. 7). However, the degree of inhibition by a combination of prazosin (100 nM) and suramin (300 μM) was not different from the inhibition produced by prazosin alone (Fig. 7). PPADS (100 μM) did not affect the contractile response to field stimulation (data not shown).

DISCUSSION

We used field stimulation to study contractile responses to sympathetic nerve terminal stimulation and to evaluate the possibility that purinergic co-transmission processes occur in the isolated bovine tail artery. An electrical field stimulation with a constant current between two parallel platinum electrodes was used in all experiments (Duckles and Silverman, 1980). Tetrodotoxin (1 mM), a voltage-gated Na^+- channel blocker, abolished the contractile responses to electrical field stimulation in the bovine tail artery but had no effect on contractile responses to norepinephrine. These findings indicated that the contractions observed were due to neurotransmitter(s) release and were not the result of direct stimulation of vascular smooth muscle.

If norepinephrine is the only neurotransmitter released from sympathetic nerve terminals then an equivalent response will be produced by both nerve stimulation and exogenous norepinephrine when an equivalent number of adrenergic receptors are occupied. To test this hypothesis we compared contractile responses to norepinephrine and field

stimulation in the presence of α-adrenergic antagonists, prazosin and phentolamine. Contractile responses to field stimulation were frequency-dependent with the maximum response being about 43 - 50% of that to the maximum obtained to norepinephrine (100 μM). Prazosin and phentolamine, as expected, significantly inhibited contractile responses to exogenous norepinephrine and caused a right shift of the concentration-response relationship curve for norepinephrine (Fig. 2A and 3A). Prazosin (30 and 100 nM) and phentolamine (3μM and 10 μM) completely inhibited contractile responses to exogenous norepinephrine at concentrations lower than the EC_{50} of norepinephrine in time-matched control tissue (Fig. 2 and 3). However, responses to field stimulation at 32 Hz in the presence of prazosin (30 and 100 nM) and phentolamine (3μM and 10 μM) were about 15% of the maximal contraction to norepinephrine. These data suggest that prazosin and phentolamine were more potent in inhibiting contractile responses to exogenous norepinephrine than those to field stimulation in the bovine tail artery. Based on these findings we concluded that there was a possibility of more than one neurotransmitter, besides norepinephrine, being involved in the contractile response to sympathetic nerve terminal stimulation. We then sought to determine whether purinergic (ATP) co-transmission occurred in this tissue.

There are no reports to our knowledge about the presence of purinergic receptors in the bovine tail artery. Contractile responses to the purinergic agonist α,β-meATP, a synthetic stable ATP analogue, which activates purinergic (P_{2X}) receptors have been demonstrated in rat mesenteric artery (Windschief et al., 1994), rabbit ear artery (O'Connor et al., 1990) and rat urinary bladder (Bo and Burnstock, 1990). We found that α,β-meATP caused concentration-dependent contraction of the bovine tail artery, which suggests the presence of functional purinergic (P_{2X}) receptors. A comparison of concentration-response relationships to α,β-meATP when applied in single concentrations versus cumulatively indicated that some desensitization occurred during the cumulative approach for obtaining a concentration-response relationship. The maximal response to cumulatively applied α,β-meATP was about 40% less than when it was applied in a non-cumulatively manner. After obtaining a cumulative concentration response relationship to α,β-meATP, followed by a 5-min washout period, the addition of a single concentration (1 μM) of α,β-meATP did not

produce a contraction. However, at 20 min, α,β-meATP (1 μM) did produce a contraction. Thus, the desensitization was not long-lasting. Based on these findings, desensitization did not prove to be an effective method to inactivate purinergic receptors in the isolated bovine tail artery. In contrast to our findings, Ziyal and co-workers (1997) did not find differences between cumulative and non-cumulative concentration response relationships to α,β-meATP in rabbit saphenous artery.

Contractile responses to α,β-meATP were significantly inhibited by suramin (300 μM) but not by prazosin (100 nM) in the bovine tail artery. These findings were expected and in agreement with others (Bao and Stjärne, 1993; Leff et al., 1990; Ziyal et al., 1997; Morris, 1999). It has been shown that suramin, as a purinergic (P_{2X}) competitive antagonist, inhibited contractile responses to α,β-meATP in the isolated rabbit ear artery (pA_2 = 4.79) (Leff et al., 1990) and rabbit saphenous artery (pA_2 = 4.79) (Ziyal et al., 1997). Similar to that reported earlier (Bao and Stjärne, 1993), suramin (300 μM) did not affect contractions to KCl in the isolated bovine tail artery. However, we found that suramin (300 μM) slightly, but significantly, inhibited contraction to norepinephrine. These findings are not in agreement with previously published reports about suramin's effect on contractile responses to exogenous norepinephrine. For example, 30 μM suramin did not affect contractile responses to submaximal (10 μM) concentration of norepinephrine in guinea pig small cutaneous artery (Morris, 1999) and 500 μM suramin did not interfere with the concentration-response relationship to norepinephrine (10 nM - 100 μM) in rat tail artery (Bao and Stjärne, 1993). The antagonism by suramin to low concentrations of norepinephrine was not expected and cannot be clearly explained. There are no reports, to our knowledge, about suramin inhibition of contractile responses to norepinephrine. However, it has been recently discovered that suramin (100 μM) is a G protein inhibitor and inhibits the activation of the G_α subunit (Freissmuth et al., 1996; Hohenegger et al., 1997). Norepinephrine is known to produce contraction of bovine tail artery through activation of α-adrenoceptors (predominantly α_1-adrenoceptors, Hill and Dyer, 1997) which are coupled to G proteins (for review see Docherty, 1998). The possibility of cross-talk interactions between G_i- and G_o-coupled receptors has been discussed in the literature (Selbie and Hill, 1997). We

speculate that inhibition of the activation of the G_α subunit by suramin would be expected to inhibit contractile responses to norepinephrine in the bovine tail artery. These findings clearly need further study.

PPADS, a purinergic (P_{2X}) receptor antagonist, has been reported to effectively inhibit the contractile responses to α,β-meATP in some vascular tissue preparations, such as isolated rabbit ear artery (Ziganshin et al., 1994), isolated splenic artery (Ren and Burnstock, 1997) and perfused rat mesenteric artery (Windscheif et al., 1994). However, PPADS (100 μM) did not inhibit contractile responses to α,β-meATP or field stimulation and could not be used as a chemical tool to study purinergic receptors in the bovine tail artery.

We studied contractile responses to field stimulation in the presence of 300 μM suramin alone and in combination with 100 nM prazosin. Suramin (300 μM) inhibited frequency-dependent contraction in the bovine tail artery (Fig. 7) and inhibition became significant at higher frequencies (8 -32 Hz). Unfortunately, suramin's effect on field stimulation may not be the result of inhibition of only purinergic receptors because it inhibited contractions to norepinephrine and because it may inhibit G-protein mediated contraction (Freissmuth et al., 1996; Hohenegger et al., 1997). Suramin (300 μM) in combination with prazosin (100 nM) significantly, but not completely, inhibited contraction to field stimulation and it was not different from the inhibitory effect of prazosin (100 nM) alone. These findings suggest the possibility of ATP co-transmission in sympathetic nerves in the bovine tail artery. To solve this issue the measurement of ATP release under field stimulation of sympathetic nerve terminals using the methods described by Levitt and Westfall (1982) may be helpful. The possibility that neuropeptide Y may be acting as a sympathetic co-transmitter should be also examined. It has been reported recently that neuropeptide Y and norepinephrine are sympathetic co-transmitters and mediate contraction in guinea pig small cutaneous artery (Morris, 1999) while in human saphenous vein, ATP, neuropeptide Y and norepinephrine are involved in contractile responses to field stimulation (Racchi et al., 1999).

In conclusion, our data suggest that there are functional purinergic (P_{2X}) receptors in the bovine tail artery and their activation causes contractile responses. The lack of potent

selective purinergic antagonists has not permitted us to conclude that ATP is definitely a co-transmitter but data were obtained which point in this direction. The development of potent and selective purinergic antagonists and the measurements of ATP release in response to field stimulation will help to resolve the issue of whether ATP acts as a co-transmitter in the bovine tail artery.

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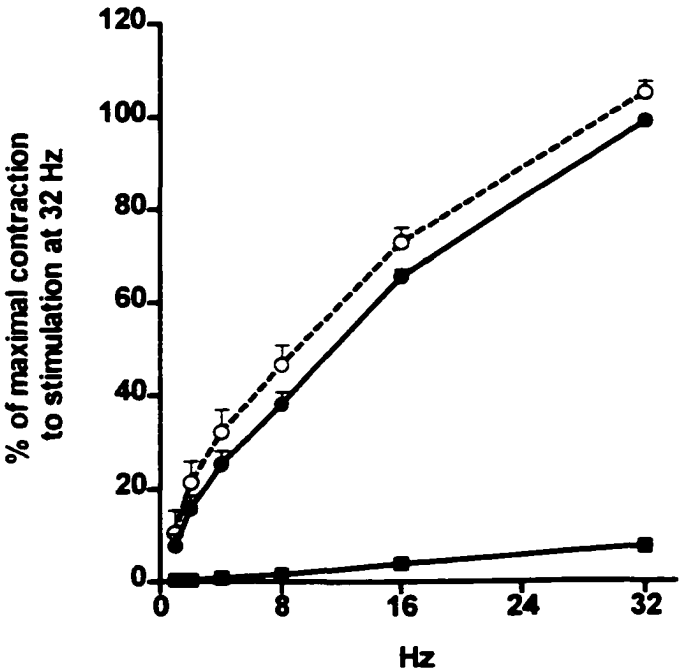
Figure 1. Contractile responses to (**A**) field stimulation and (**B**) exogenous norepinephrine in the bovine tail artery in control (●), time-matched control tissues (○) and in the presence of 1mM tetrodotoxin (■). **A.** Each line is a mean of three consecutively (with 15-min interval between every two frequency-response relationships) obtained frequency-response relationships (1 – 32 Hz) to electrical field stimulation. Data arrived from 4 animals. **B.** Concentration-response relationships to exogenous norepinephrine obtained for the same tissues (see **A.** above) prior to and after field stimulation. Each point represents mean ± S.E.M., n=4.

A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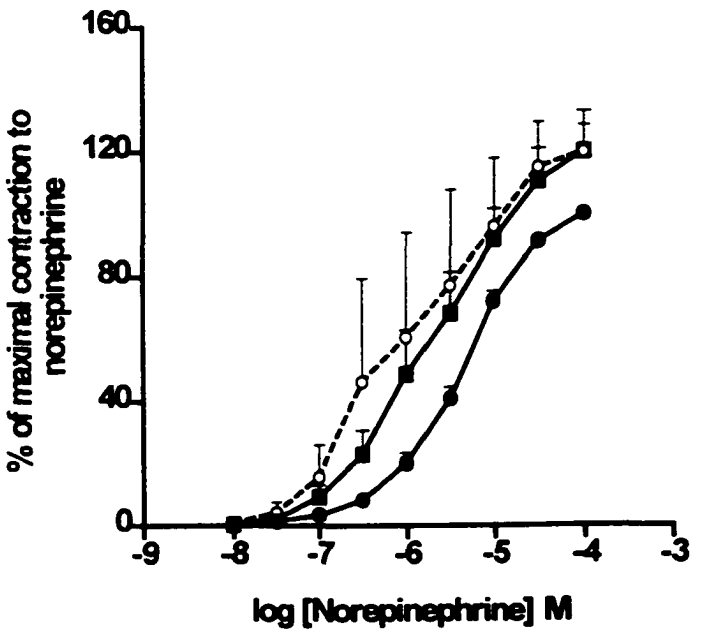
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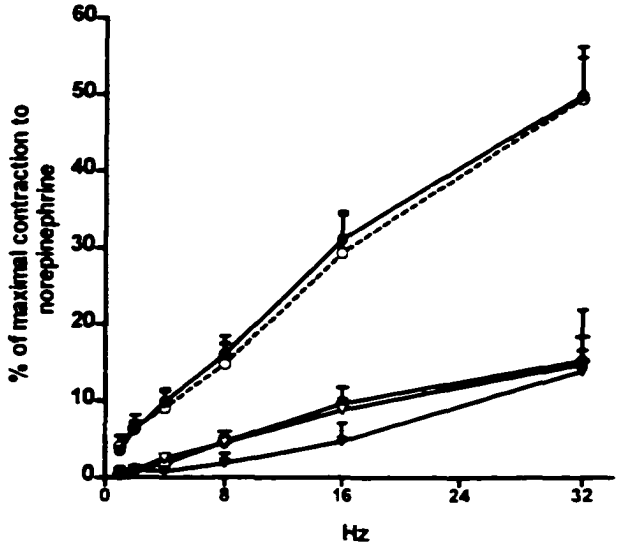
A



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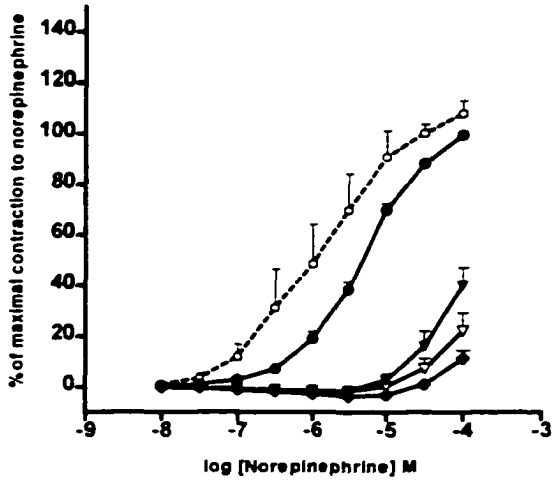
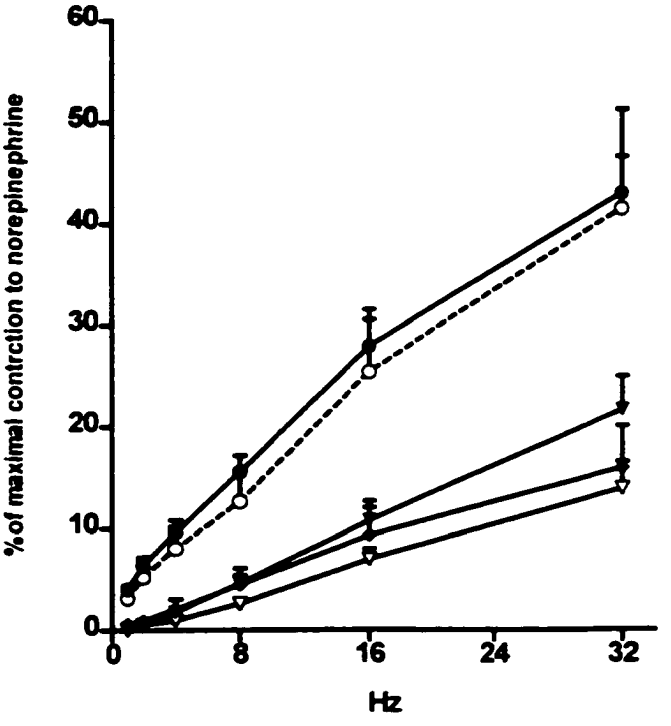


Figure 2. Contractile responses to (**A**) field stimulation and (**B**) exogenous norepinephrine in the bovine tail artery in (●) control, (○) time-matched control tissues and in the presence of (▼) 1 μM, (▽) 30 μM and (◆) 100 μM phentolamine. **A.** Frequency-response relationship to field stimulation in the bovine tail artery. Data plotted as percent of maximal contraction to norepinephrine. Data obtained from 7 -13 animals. Each point represents the mean ± S.E.M. **B.** Concentration-response relationships to exogenous norepinephrine obtained prior to and after field stimulation. Each point represents mean ± S.E.M., n=5 - 7.

Figure 3. Contractile responses to (**A**) field stimulation and (**B**) exogenous norepinephrine in the bovine tail artery in (●) control, (○) time-matched control tissues and in the presence of (▼) 10 nM, (▽) 30 nM, and (◆) 100 nM prazosin. **A.** Frequency-response relationship to field stimulation in the bovine tail artery. Data plotted as percent of maximal contraction to norepinephrine. Each point represents mean ± S.E.M., n=7. **B.** Concentration-response relationships to exogenously added norepinephrine obtained prior to and after field stimulation. Each point represents mean ± S.E.M., n=7.

A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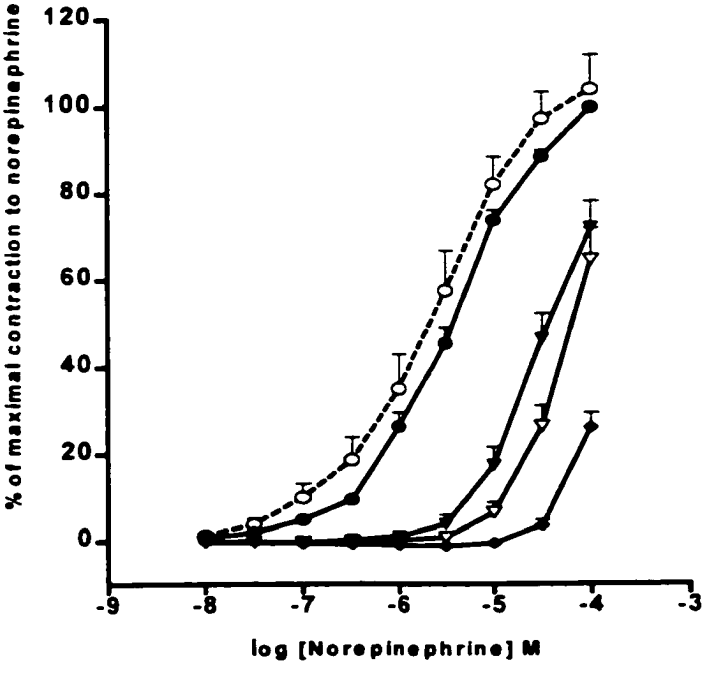
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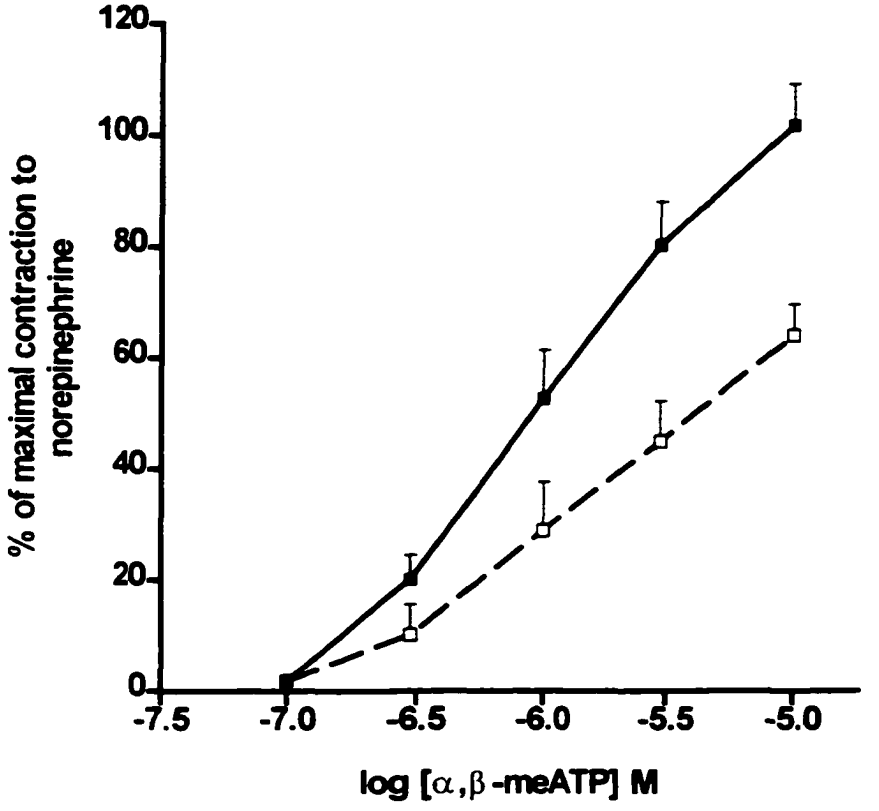


Figure 4. Concentration-response relationship to α,β-meATP applied cumulatively (□) and in single increasing concentrations (non-cumulatively, see Methods) (■) in the isolated bovine tail artery. Data plotted as percentage of maximum contraction to norepinephrine. Each point represents mean ± S.E.M., n=4.

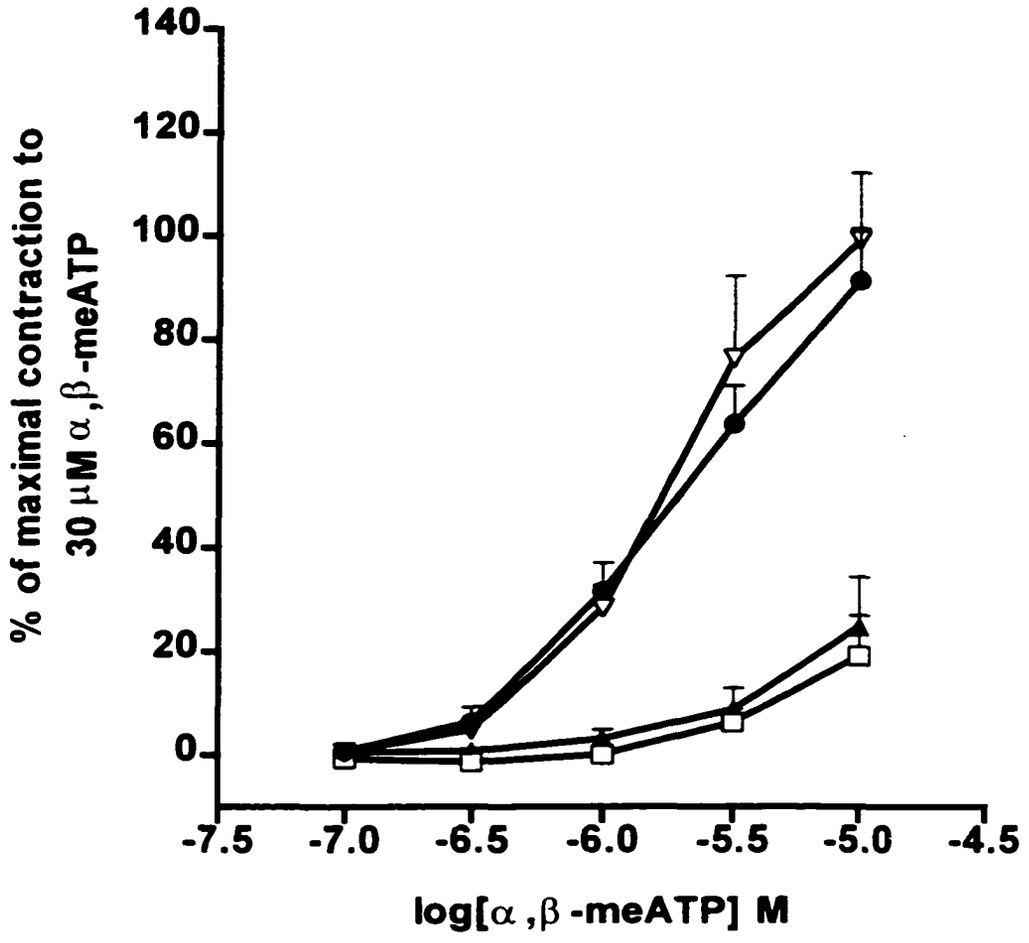
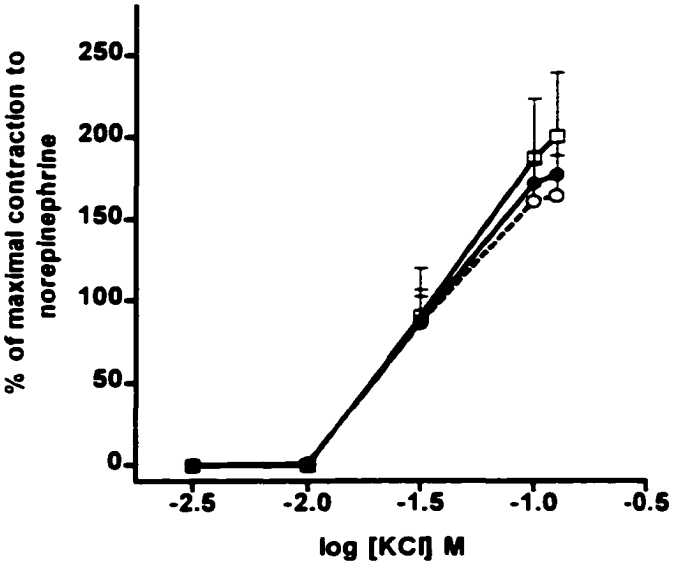


Figure 5. Concentration-response relationships to α,β-meATP in the isolated bovine tail artery in the absence of antagonists (control (●) tissue) and in the presence of (▽) 100 nM prazosin, (□) 300 μM suramin and (▲) combination of 100 nM prazosin and 300 μM suramin. Data plotted as a percentage of the maximum contraction to 30 μM α,β-meATP. Each point represents mean ± S.E.M., n=6.

A



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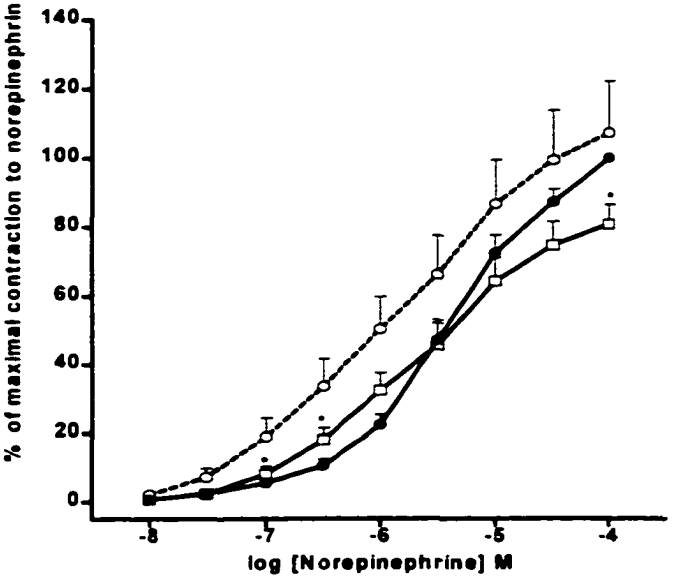

Figure 6. Concentration-response relationships to KCl (**A**) and to norepinephrine (**B**) in the isolated bovine tail artery in (●) control tissue, (○) time-matched control tissue and in the presence of (□) 300 μM suramin. Data plotted as a percentage of the maximum contraction to norepinephrine. Each point represents mean ± S.E.M., n=5. (*) - Indicates responses in the presence of suramin (300 μM) are significantly different from those in the time-control tissues ($P < 0.05$).

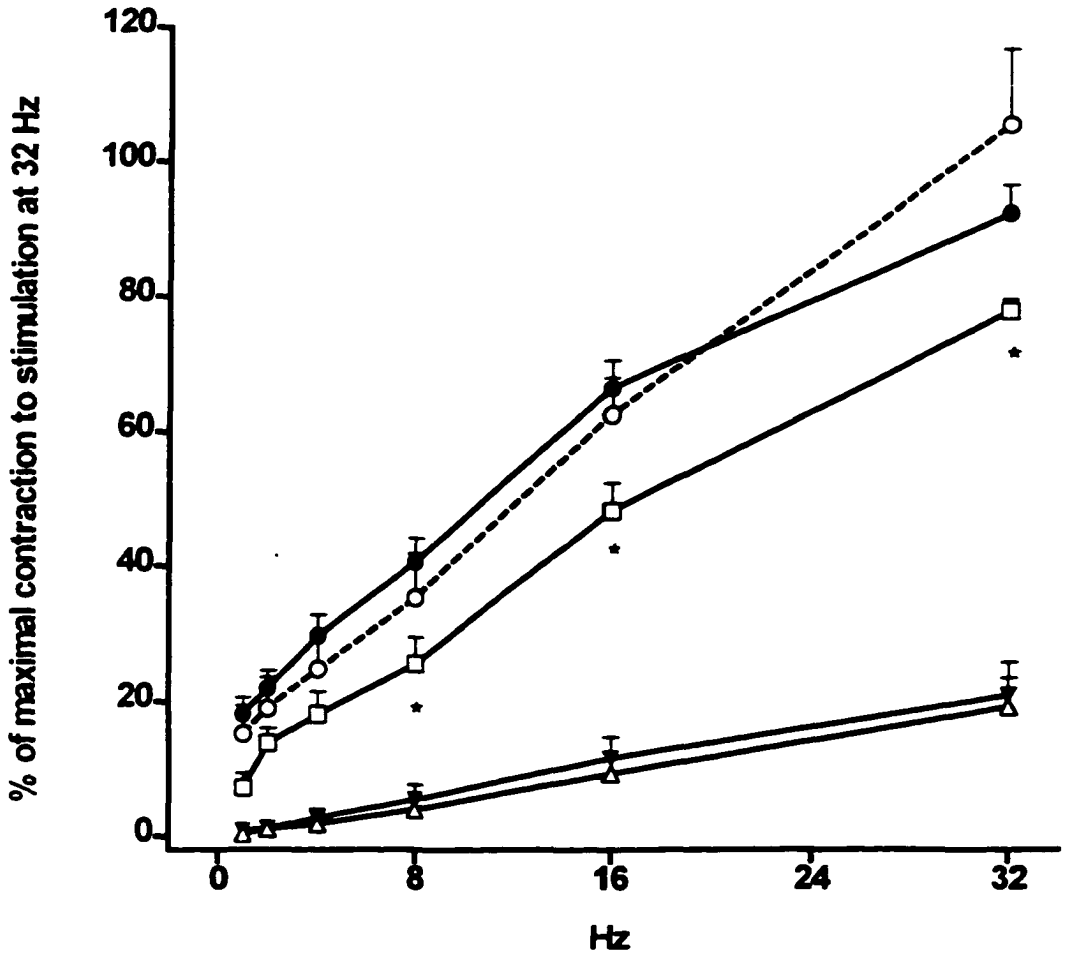

Figure 7. Frequency-response relationships to field stimulation in the isolated bovine tail artery in control tissue (●) and time-matched control tissues (○) and in the presence of (Δ) 100 nM prazosin, (□) 300 μM suramin and (▼) a combination of 100 nM prazosin and 300 μM suramin. Data plotted as a percentage of the maximum contraction to 30 μM α,β-meATP. Each point represents mean ± S.E.M., n=6. (*) - Indicates contractile responses that are statistically different ($P < 0.05$) from those to in time-matched control tissues.

CHAPTER VI. GENERAL CONCLUSIONS

General Discussion

This chapter contains a discussion of all the major conclusions from the present study.

α-Adrenoceptors mediating contractile responses in the bovine tail artery

It was shown earlier that adrenergic agonists induced contractile responses in the isolated bovine tail artery primarily through α_1-adrenoceptors and not through α_2-adrenoceptors (Hill and Dyer, 1997). No attempt was made to identify the α_1-adrenoceptor subtypes involved in vasoconstriction responses in the bovine tail artery. In the present study contractile responses were investigated using isolated tissue bath methods similar to those used by Hill and Dyer (1997). Characterization of α_1-adrenoceptor subtypes was done on the endothelium denuded bovine tail artery in order to avoid the possible effect by the endothelium (MacLean, et al., 1993; Zschauer et al., 1997) on contractile responses to adrenergic agonists.

The presence of α_{1A}-adrenoceptors was demonstrated in experiments with the α_{1A}-adrenoceptor subtype selective agonist, A61603 (Buckner et al., 1996). A61603 caused concentration-dependent contractions and was more potent than the non-selective α_1-adrenoceptor agonists norepinephrine and phenylephrine. The potency order was: A61603 > norepinephrine > phenylephrine, based on a comparison of EC_{50} values. Similar data were found for the bovine cloned α_{1a}-adrenoceptor in radioligand binding experiments (Buckner et al., 1996). The affinity (pK_i) of A61603 for cloned hamster α_{1b}-adrenoceptors or cloned rat α_{1d}-adrenoceptors was less than that for norepinephrine and phenylephrine (Buckner et al., 1996).

The affinity (pA_2) of the α_1-adrenoceptor antagonists (5-methylurapidil, WB 4101, BMY 7378 and ARC 239) against A61603 and phenylephrine in the bovine tail artery was compared with literature (pK_i) values obtained for the same antagonists for cloned receptors.

All four antagonists exhibited competitive antagonism against A61603. There was a significant correlation of pA_2 values from our study with pK_i values reported by Zhu and colleagues (1997) for the cloned α_{1a}-adrenoceptor, but not with cloned α_{1b}- or α_{1d}-adrenoceptors, suggesting the presence of functional α_{1A}-adrenoceptors in the bovine tail artery.

The affinity of BMY 7378, a selective α_{1D}-adrenoceptor antagonist (Goetz et al., 1995; Zhu et al., 1997), was found to be significantly higher against the non-subtype selective agonist phenylephrine than against a selective α_{1A}-adrenoceptor agonist, A61603. A higher potency of BMY 7378 against phenylephrine can be taken as evidence (Fagura et al., 1997; Villalobos-Molina et al., 1997; Zhu et al., 1997) for the presence of functional α_{1D}-adrenoceptors in the bovine tail artery.

Determination of the presence of α_{1B}-adrenoceptors in the bovine tail artery was done in experiments using the irreversible antagonist chlorethylclonidine (O'Rourke et al., 1995). Chloroethylclonidine has been used to distinguish between α_{1A}-adrenoceptors and α_{1B}-adrenoceptors (Teng et al., 1994; Testa et al., 1995; Hattori and Kanno, 1997). The alkylating rate of chlorethylclonidine is significantly lower for α_{1A}-adrenoceptors than that for α_{1B}- or α_{1D}-adrenoceptors (Xiao and Jeffries, 1997). In the bovine tail artery, pretreatment of tissues with a low concentration of chloroethylclonidine (50 μM) for 10 min significantly inhibited contractile responses to phenylephrine but had no effect on the concentration-response relationship to A61603. A higher concentration of chloroethylclonidine and a longer incubation time significantly inhibited contractions produced by both phenylephrine and A61603, probably by an inactivation of all α_1-adrenoceptor subtypes. However, chlorethylclonidine cannot discriminate between α_{1B}- and α_{1D}-adrenoceptor subtypes because it alkylates α_{1B}- and α_{1D}-adrenoceptor subtypes at the same rate (Xiao and Jeffries, 1997). Whether the bovine tail artery possesses functional α_{1B}-adrenoceptor subtypes is unclear since this possibility could not be excluded.

The possible involvement of α_2-adrenoceptors in mediating contractile responses in the isolated endothelium denuded bovine tail artery was studied using the α_2-adrenoceptor selective agonist UK 14,304. UK 14,304 caused very small contractile responses which were

only 6% (or about 0.4 g) of that to 120 mM KCl. Idazoxan, an α_2-adrenoceptor antagonist, inhibited contractile responses to norepinephrine in the isolated endothelium denuded bovine tail artery with potency (pK_B) significantly lower than the affinity (pK_i or pK_D) reported from radioligand binding studies for α_2-adrenoceptors (Blaxall et al., 1991, MacLennan et al., 1997; Renouard et al., 1994). A low pK_B value for idazoxan in the bovine tail artery determined against norepinephrine may be an indication of idazoxan blocking α_1-adrenoceptors and not α_2-adrenoceptors.

These experiments extend the earlier findings of Hill and Dyer (1997) and support their conclusion for lack of a significant α_2-adrenoceptor involvement in adrenergic mediating contractions in the bovine tail artery.

In summary, in isolated endothelium denuded bovine tail arteries, α_1-adrenoceptors but not α_2-adrenoceptors were found to play a role in contractile responses to adrenergic agonists. α_{1A}-Adrenoceptors plays a dominant role in contractile responses to adrenergic agonists compared to that of α_{1D}-adrenoceptors. Evidence for the presence of functional α_{1B}-adrenoceptors is more equivocal but it is likely that α_{1B}-adrenoceptors also co-exist in the bovine tail artery (Table 1).

α-Adrenoceptors mediating contractile responses in the bovine tail vein

In contrasts to artery, a fewer number of studies have reported on the functional role of α_1-adrenoceptors in vein (Schulz and Westfall, 1982; Leech and Faber, 1996; Elliot, 1997; Marshall et al., 1996; Hu and Dyer, 1997; Low et al., 1999).

Investigation of the presence of α_{1A}-adrenoceptors in the bovine tail vein was studied using an experimental design similar to that for the study of α_1-adrenergic receptors in the bovine tail artery. The adrenergic agonists norepinephrine, phenylephrine and A61603 caused concentration dependent contractions in the endothelium denuded bovine tail vein and A61603 had a significantly greater potency (pD_2) than that of norepinephrine or phenylephrine. Their potency (pD_2) order (A61603 > norepinephrine > phenylephrine) indicates the presence of α_{1A}-adrenoceptors (Buckner et al., 1996). However, the

dissociation constant for the receptor-agonist complex (K_A) for norepinephrine in the endothelium denuded bovine tail vein was not significantly different from that for A61603. Interestingly, the affinity ($1/K_A$) of norepinephrine and its pD_2 value were higher in the bovine tail vein than in the bovine tail artery. The differences in affinity of norepinephrine in the vein and artery may be explained by differences in the α-adrenoceptor types and subtypes present in each tissue (Bevan et al., 1988; Bevan et al., 1986).

The potency (pK_B) of α_1-adrenoceptor antagonists, WB 4101, BMY 7378, 5-methylurapidil and prazosin was evaluated against phenylephrine and A61603. In addition, a new and novel α_{1B}-adrenoceptor subtype selective competitive antagonist, L-765,314 (4-amino-2-[4-[1-(benzyloxycarbonyl)-2(S)-[[(1,1-dimethylethyl)amino] carbonyl]-piperazinyl]-6,7-dimethoxyquinazoline) (Patane, et al., 1998) became available for our use and was used to help determine whether α_{1B}-adrenoceptors are present in the tail vein.

The presence of functional α_{1A}-adrenoceptors in the bovine tail vein was strongly indicated by a higher affinity of the α_{1A}-adrenoceptor selective antagonists, 5-methylurapidil and WB 4101 against A61603-mediated contraction than that of phenylephrine-mediated contraction.

There are some differences in the α_1-adrenoceptor subtype(s) composition in the bovine tail vein as compared to that in the artery. In contrast to the artery, no evidence for the presence of the α_{1D}-adrenoceptor subtype was found in the bovine tail vein. This conclusion is based on a low potency of the α_{1D}-adrenoceptor subtype selective antagonist BMY 7378 in inhibiting contractile response to A61603 and phenylephrine in the bovine tail vein. In addition, the presence of α_{1B}-adrenoceptor subtypes in the bovine tail vein was demonstrated by using L-765,314, an α_{1B}-adrenoceptor subtype selective antagonist and chloroethylclonidine. Both antagonists were more potent in inhibiting contractile responses to phenylephrine than those mediated by A61602. In summary, two α_1-adrenoceptor subtypes, α_{1A}- and α_{1B}-adrenoceptors, play a role in contractile responses to adrenergic agonists in the bovine tail vein (Table 1).

Differences in types of functional α-adrenoceptors were found in bovine tail vein in comparison to the tail artery. The presence of the α_2-adrenoceptor involvement in contractile

responses in the isolated endothelium denuded bovine tail vein was determined by using a non-subtype selective α_2-adrenergic agonist, UK 14,304, (Renouard et al., 1994; Uhlén et al., 1992). UK 14,304 produced a significant (about 63% of the maximal contraction to 120 mM KCl) contractile response in the endothelium denuded bovine tail vein.

Table 1. α -Adrenoceptors characterized in the isolated endothelium denuded bovine tail artery and vein.

	Bovine tail vessels	
	Artery	Vein
α_1- adrenoceptors	Contraction	Contraction
α_{1A}	Present, predominant subtype	Present
α_{1B}	Presence can not be excluded	Present
α_{1D}	Present	Not present
α_2- adrenoceptors	Little role in contraction	Contraction
α_{2B}	Not applicable	Presence can not be excluded
α_{2C}	Not applicable	Present
α_{2D}	Not applicable	Presence can not be excluded

α_2-Adrenoceptor antagonists, prazosin, idazoxan, ARC 239, WB 4101, rauwolscine and yohimbine inhibited contractile responses to UK 14,304 and their potency was calculated as a pK_B value. A relatively high potency of prazosin, as an α_2-adrenoceptor antagonist, in inhibiting UK 14,304 induced contraction suggests the presence of α_{2B}- and/or α_{2C}-adrenoceptor subtypes rather than α_{2D}-adrenoceptors (Uhlén et al., 1994, MacLenann et al., 1997; Trendelenburg et al., 1994, Bohmann et al., 1994, Limberger et al., 1995, Wahl et al., 1996). In order to discriminate between α_{2B}- and α_{2C}-adrenoceptor subtypes, the relative potencies (Blaxall et al., 1991, Fuder and Selbach, 1993) of ARC 239 and rauwolscine were compared. The rationale for this comparison is based on the fact that ARC 239 has a slightly greater selectivity for α_{2B}-adrenoceptors (Blaxall et al., 1991, Uhlén et al., 1994, Renouard et al., 1994) while rauwolscine is more selective for α_{2C}-adrenoceptors (Blaxall et al., 1991,

Uhlén et al., 1994). Relative potency was calculated as a ratio of potency based on pK_B values for (ARC 239/ rauwolscine) and compared to the literature values obtained from a number of radioligand binding experiments (pK_i or pK_D) (Blaxall et al., 1991, Uhlén et al., 1992, Uhlén et al., 1994, Uhlén et al., 1995, Renouard et al., 1994) for α_{2B}- and α_{2C}-adrenoceptors. The relative potency ratio of ARC 239/rauwolscine calculated for the bovine tail vein was similar to that calculated for literature values for α_{2C}-adrenoceptor subtypes and this suggests the presence of functional α_{2C}-adrenoceptors in the bovine tail vein. However, the possibility that α_{2B}- and α_{2D}-adrenoceptor subtypes are present in the tail vein cannot be excluded (Table 1).

Investigation of nerve-mediated contraction in the bovine tail artery in response to electrical field stimulation

Investigation of nerve-mediating contraction and an evaluation of possible co-transmission from sympathetic nerve terminals in the bovine tail artery in response to electrical field stimulation was a part of the research project. Electrical field stimulation with a constant current between two parallel platinum electrodes was used in all experiments (Duckles and Silverman, 1980). Contractile responses to field stimulation in the bovine tail artery were blocked by tetrodotoxin (1 mM), a voltage-gated Na^+- channel blocker and this indicated that the contraction observed was due to neurotransmitter(s) release and not the result of direct stimulation of vascular smooth muscle.

The working hypothesis was that if norepinephrine is the only neurotransmitter released from sympathetic nerve terminals then equal responses should be produced when an equal number of adrenoceptors are occupied, regardless of whether the norepinephrine comes from sympathetic neurons or is added exogenously to the tissue bath. Therefore, an equivalent concentration of norepinephrine at the receptor region would produce an equivalent contractile response due to the activation of the same number of receptors. In order to test this hypothesis contractile responses to norepinephrine and field stimulation were compared in the presence of α-adrenergic antagonists, prazosin and phentolamine. Prazosin and phentolamine, as expected, significantly inhibited contractile responses to

exogenous norepinephrine in a concentration-dependent manner and caused a right shift of the concentration-response relationship curve for norepinephrine. However, prazosin and phentolamine were relatively less potent in inhibiting contractile responses to field stimulation than those to exogenously added norepinephrine. These findings suggested that there was a possibility of more than one neurotransmitter, besides norepinephrine, being involved in the contractile response to sympathetic nerve terminal stimulation. The possible involvement of ATP being a co-transmitter was evaluated.

The presence of functional purinergic receptors (P_{2X}) was demonstrated with a synthetic stable ATP analogue, α,β-meATP, which produced concentration-dependent contraction of the bovine tail artery (Windschief et al., 1994; O'Connor et al., 1990; Bo and Burnstock, 1996).

Contractile responses to α,β-meATP were significantly inhibited by the purinergic (P_{2X} receptor) competitive antagonist, suramin (Bao and Stjärne, 1993; Leff et al., 1990; Ziyal et al., 1997; Morris, 1999) but not by prazosin. In addition, suramin slightly but significantly inhibited contractile responses to field stimulation in the bovine tail artery. However, these results were not easy to interpret because suramin, unexpectedly, slightly but significantly inhibited contractions to norepinephrine. Another purinergic (P_{2X}) receptor antagonist PPADS which has been reported to effectively inhibit contractile responses to α,β-meATP in some vascular tissue preparations was evaluated for its ability to antagonize α,β-meATP (Ziganshin et al., 1994; Ren and Burnstock, 1997; Windscheif et al., 1994). However, PPADS did not inhibit contractile responses to α,β-meATP or field stimulation in the bovine tail artery. The desensitization approach of inactivating purinergic receptors (Ralevic and Burnstock, 1998) was attempted but was futile since the desensitization process did not last long enough.

In searching for an explanation of suramin's inhibitory effect on norepinephrine-induced contraction we discovered that suramin has been reported to have an inhibitory effect on the α subunit of G protein (Freissmuth et al., 1996; Hohenegger et al., 1997). Since contractions to norepinephrine are mediated via G protein (and if this occurs in the artery) then suramin would be expected to inhibit contractions to norepinephrine regardless of its source (nerve or exogenously added to the bath).

In conclusion, our data suggest that there are functional purinergic (P_{2X}) receptors in the bovine tail artery and their activation causes contractile responses. The lack of potent selective purinergic antagonists has hampered this research. The possibility of sympathetic ATP co-transmission occurring in the bovine tail artery cannot be excluded and will be more convincing when experiments measuring ATP release under field stimulation are carried out. The possibility of neuropeptide Y involvement in the co-transmission process should be also examined in future studies.

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