GUANOSINE 3':5'-CYCLIC MONOPHOSPHATE AND CONTRACTION IN VASCULAR SMOOTH MUSCLE

by

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ABSTRACT

Presently, the literature regarding vascular smooth muscle contraction is coloured with many contradictory observations and conclusions. However, like many physiological systems, the biochemical pathways and functional events of vascular smooth muscle contraction vary between individual species and/or tissues. Therefore, determination of a ubiquitous excitation-contraction coupling mechanism is unlikely; variations between receptor classes, receptor density, excitation-contraction coupling pathways and the efficiency of the receptor-pathway interaction contribute to the various observations and conclusions.

The inositol 1,4,5-trisphosphate (IP_3) second messenger cascade regulates the mobilization of intracellular Ca^{2+}, and subsequently contraction, in vascular smooth muscle. However, phospholipase C-mediated production of IP_3 appears to be controlled by tissue-specific regulatory factors. This study examines the effects of three such factors, the presence of extracellular Ca^{2+}, the sensitivity of the associated G-protein and inhibition by 8-bromoguanosine 3':5'-cyclic monophosphate (8-bromo-cGMP), in isolated rat caudal artery. Concentration-response curves were constructed for phenylephrine and isometric contractions measured in isolated tissues. In addition, phosphatidylinositol turnover was assessed using anion exchange chromatography.

The effects of 8-bromo-cGMP on phenylephrine-induced contractions and phosphatidylinositol hydrolysis were compared to those of felodipine, a dihydropyridine Ca^{2+}-channel antagonist, and ryanodine, a putative depletor of intracellular Ca^{2+} stores in rat caudal artery. Pertussis toxin was used to determine the identity of the G-protein

regulating phenylephrine-induced contraction. Further, the effects of felodipine and ryanodine on contraction were determined in rat thoracic aorta to compare the contribution of extracellular and intracellular Ca^{2+} to contraction between a large conduit vessel and a small conduit vessel.

The results of this investigation suggest that phospholipase C-activated phosphatidylinositol hydrolysis in the rat caudal artery is dependent on extracellular Ca^{2+}, mediated, in part, through dihydropyridine sensitive Ca^{2+} channels. Phospholipase C activity is not directly inhibited by 8-bromo-cGMP. However, the nucleotide may regulate vascular smooth muscle contraction by inhibition of Ca^{2+} release from IP_3-mediated intracellular stores, but it is unlikely that 8-bromo-cGMP affects ryanodine-sensitive stores. None of the G-proteins coupled to the α_1-adrenoceptor mediated excitation-contraction pathway in rat caudal artery appear to be sensitive to pertussis toxin. Rat aortic tissue does not rely on intracellular Ca^{2+} to the same extent that rat caudal artery does, confirming the tissue specificity of α_1-adrenoceptor agonist induced excitation-contraction in vascular smooth muscle.

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LIST OF ABBREVIATIONS

WORD	ABBREVIATION
Adenosine 3':5' cyclic monophosphate	cAMP
Adenosine trisphosphate	ATP
8-Bromoguanosine 3':5'-cyclic monophosphate	8-bromo-cGMP
Diacylglycerol	DAG
Effective concentration for 50 % of maximum response	EC_{50}
Ethylene glycol-bis (β-amino-ethyl ether) N, N, N', N'-tetraacetic acid	EGTA
Guanosine 3':5'-cyclic monophosphate	cGMP
Guanosine diphosphate	GDP
Guanosine triphosphate	GTP
Hill coefficient	*n*
Inositol 4,5-bisphosphate	Ins $(4,5)P_2$
Inositol 1,3,4,5-tetrakisphosphate	Ins $(1,3,4,5)P_4$
Inositol 1,3,4-trisphosphate	Ins $(1,3,4)P_3$
Inositol 1,4,5-trisphosphate	IP_3
Inositol 2,4,5-trisphosphate	Ins $(2,4,5)P_3$
Phosphatidylinositol	PI
Phosphatidylinositol 4,5-bisphosphate	PIP_2
Phospholipase C	PLC
Standard error of the mean	S.E.

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DEDICATION

This work is dedicated to a dear friend and mentor, Dr. Fred Einstein.

1. INTRODUCTION

1.1. Vascular Smooth Muscle Contraction

The vasculature is a closed system of vessels responsible for transporting nutrients and oxygen to cells while simultaneously removing metabolic waste products. The circulatory system is a dynamic organ, capable of adapting to changes in individual tissue needs and blood volume. The etiology of several cardiovascular disease states, including hypertension, has been associated with a failure of the vasculature to adequately adapt to changes. More specifically, rigorous regulation of the total peripheral resistance of the vascular circulation is critical to homeostasis. The most important determinant of total peripheral resistance is blood vessel diameter. Vessel diameter is regulated by local chemical and extrinsic neural and hormonal mechanisms that can induce both dilation and constriction.

Constriction of conduit blood vessels is a function of the smooth muscle surrounding the vessels. Extracellular calcium (Ca^{2+}) was first identified as a critical factor in smooth muscle contraction in 1961, when Bohr and Goulet recorded a decrease in epinephrine-induced tension in rabbit and dog aorta and rabbit mesoappendix resistance vessels when Ca^{2+} was removed from the bathing medium. Even at this time, Bohr and Goulet (1961) were able to appreciate the complexity and variability of the intracellular signal transduction mechanisms responsible for smooth muscle contraction. Since 1961, many of the details of excitation-contraction coupling have been established; however, the mechanisms mediating vascular smooth muscle tone remain poorly understood.

Vascular smooth muscle tone is regulated by pharmacomechanical and electromechanical coupling mechanisms under the control of the autonomic nervous system (Somlyo & Somlyo, 1994). Each smooth muscle fibre is a spindle shaped cell containing two contractile proteins: actin, a thin filament anchored to the plasma membrane through cytoplasmic dense bodies, and myosin, a thick filament whose mobile head forms transient cross-bridges with actin (Somlyo *et al.*,1985). The extent of overlap between the two contractile proteins, and degree of muscle tension, is determined by cross-bridge cycling (Bagby & Corey-Kreyling, 1985; Mulvany, 1985).

During contraction, the intracellular Ca^{2+} concentration increases from approximately 0.1 μM to 5 μM (Hartshorne, 1982). As the Ca^{2+} concentration rises, it binds the cytosolic Ca^{2+}-binding protein, calmodulin, to form a regulatory Ca^{2+}-calmodulin complex that activates the catalytic subunit of myosin light-chain kinase (MLCK) (Rüegg *et al.*, 1985). MLCK then phosphorylates serine-19 on the regulatory light chain of myosin (MLC_{20}); both the electric and steric effects of phosphorylation activate myosin's ATPase activity and motility (Sweeny *et al.*, 1994). A four-state cross-bridge model described by Hai and Murphy (1988) successfully predicts the mechanical properties and energetics of smooth muscle contraction. Cycling between the four states requires adenosine triphosphate (ATP), but the primary regulatory mechanism is the Ca^{2+}-dependent phosphorylation of MLC_{20} (Walker *et al.*, 1994).

1.2. Sources of Ca^{2+} Utilized During Contraction

Cross-bridge cycling is regulated through the Ca^{2+}-calmodulin-dependent phosphorylation of MLC_{20}; therefore, increasing the free cytosolic Ca^{2+} concentration is crucial for contraction. Ca^{2+} can enter from the extracellular space through voltage-

gated and ligand-gated channels (Karaki *et al.*, 1984) or Ca^{2+} can be released from intracellular storage sites within the sarcoplasmic reticulum, mobilized through the activity of intracellular second messengers (Brown *et al.*, 1984; Streb *et al.*, 1984). Two functionally and spatially distinct Ca^{2+} pools have been identified within the sarcoplasmic reticulum of vascular smooth muscle cells; one controlled by inositol 1,4,5-trisphosphate (IP_3) and the other sensitive to inhibition by ryanodine (Yamazawa *et al.*, 1992; Tribe *et al.*, 1994). The relative contribution of each Ca^{2+} source is tissue-specific, dependent on variations in receptor classes and density, excitation-contraction coupling mechanisms and the efficiency of the receptor-signal transduction interaction (Vila *et al.*, 1993).

Berridge (1983) observed a rapidly increased concentration of phosphatidylinositol (PI) metabolites in insect salivary glands upon stimulation with 5-hydroxytryptamine. Since activation of plasmalemma receptors had previously been associated with the release of internal Ca^{2+} (Schulz & Stolze, 1980; Exton, 1981), Berridge (1983) suggested that IP_3 was a second messenger capable of mobilizing stored Ca^{2+}. Subsequently, results from rabbit pulmonary artery (Somlyo *et al.*, 1985), rat thoracic aorta (Chiu *et al.*, 1987), rat cerebellar microsomes (Stauderman *et al.*, 1988) and rat pancreatic acinar cells (Thévenod *et al.*, 1989) have demonstrated a close correlation between the generation of IP_3 and the release of intracellular Ca^{2+}. The effects of IP_3 are not blocked by vanadate, an inhibitor of the sarcoplasmic reticulum Ca^{2+}-ATPase re-uptake pump; therefore, IP_3 acts by increasing the Ca^{2+} permeability of the sarcoplasmic reticulum, not by inhibiting the sequestering of free Ca^{2+} (Somlyo *et al.*, 1985). However, IP_3 may increase intracellular Ca^{2+} to a lesser

extent by inhibiting extrusion from the cytosol by the sarcolemmal Ca^{2+}-ATPase (Popescu *et al.*, 1986).

In 1958, duodenal smooth muscle was reported to be irreversibly contracted by the plant alkaloid ryanodine (Hillyard & Procita, 1958). Subsequent studies, reported, however, that ryanodine inhibited agonist-induced contractions in smooth muscle (Kanmura *et al.*, 1988). It now appears that low concentrations of ryanodine are contractile, while higher concentrations inhibit contraction (Berridge, 1993). Recently, the putative mechanism of action of ryanodine in smooth muscle was proposed. Ryanodine inhibits smooth muscle contraction by stimulating a slow Ca^{2+} efflux from an intracellular store; as a result, the store is depleted and subsequent agonist stimulation futile (Kanmura *et al.*, 1988; Hwang & van Breemen, 1987; Julou-Schaeffer & Freslon, 1988). The intracellular Ca^{2+} store that is sensitive to blockade by ryanodine, does not appear to be mobilized by the second messenger IP_3 (Seiler *et al.*, 1987); however, cyclic adenosine 5'-diphosphoribose (cADPR), a metabolite of β-nicotinamide adenine dinucleotide (β-NAD), has been tentatively identified as the endogenous second messenger capable of mobilizing intracellular Ca^{2+} from the ryanodine sensitive pools (White *et al.*, 1993; Galione *et al.*, 1993; Mészáros *et al.*, 1993).

Studies using various antagonists of IP_3- and ryanodine-sensitive Ca^{2+} release have provided evidence that the two intracellular Ca^{2+} release pathways are functionally and spatially independent. IP_3-induced release is blocked by cinnarizine, flunarizine, tetraethylammonium and the local anaesthetics benzocaine and lidocaine (Berridge & Irvine, 1989; Seiler *et al.*, 1987). IP_3-mediated Ca^{2+} mobilization is not affected by nifedipine, diltiazem, verapamil, dantoline, methylenedioxyindene,

ϖ-conotoxin or ryanodine (Seiler *et al.*, 1987; Shah & Pant, 1988). However, pre-treatment with ryanodine does inhibit cADPR-induced release (Seiler *et al.*, 1987; Iino *et al.*, 1988; Yamazawa *et al.*, 1992; White *et al.*, 1993); and, although heparin completely blocks IP_3-induced Ca^{2+} release, it does not affect ryanodine sensitive release (Berridge, 1993; White *et al.*, 1993). The ryanodine sensitive store, can be mobilized by caffeine (Berridge, 1993); however, the IP_3 sensitive store is blocked, not activated, by caffeine in *Xenopus* oocytes and rat cerebellar microsomes (Mignery *et al.*, 1990; Parker & Ivorra, 1991; Brown *et al.*, 1992). Although caffeine-induced Ca^{2+} release is insensitive to changes to the membrane potential, release from the IP_3 sensitive pool is regulated by potential dependent PI hydrolysis (Ganitkevich & Isenberg, 1993).

The IP_3 sensitive pool appears to sequester Ca^{2+} when the cytosolic concentration is high (~ 10^{-6} M); however, the IP_3 insensitive pool is the higher affinity Ca^{2+} buffer, capable of adjusting low intracellular Ca^{2+} concentrations (~ 10^{-7} M) (Thévenod *et al.*, 1989). In brain microsomes, cADPR and IP_3 released approximately 20 and 60 % of the stored Ca^{2+}, respectively (White *et al.*, 1993). Also, Ca^{2+} can induce Ca^{2+} release from the ryanodine sensitive stores (Iino, 1989), but micromolar Ca^{2+} inhibits Ca^{2+} release from IP_3 sensitive stores by indirectly decreasing the affinity of the agonist for its low affinity receptor (Benevolensky *et al.*, 1994). And finally, ryanodine does not affect PI hydrolysis as neither basal nor 8-arginine vasopressin-induced IP_3 synthesis in A7r5 cultured aortic smooth muscle cells were blocked in the presence of this antagonist (Berman *et al.*, 1994). Therefore, IP_3 and ryanodine appear

to regulate functionally and spatially distinct Ca^{2+} pools within the same cell (Yamazawa *et al.*, 1992).

There is some evidence, however, to suggest that IP_3 can induce Ca^{2+} release from a ryanodine sensitive pool. Yamazawa and co-workers (1992) recorded an IP_3-mediated rise in intracellular Ca^{2+} that was reduced by approximately 50 % after treatment with ryanodine. In skinned guinea pig taenia caeci, the amount of Ca^{2+} released by the application of IP_3, or IP_3 and caffeine, was approximately twice as large as that released by caffeine alone (Iino *et al.*, 1988). Finally, ryanodine blocked Ca^{2+} accumulation in both caffeine and IP_3 sensitive stores (Kanmura *et al.*, 1988). These results indicate that two Ca^{2+} pools may co-exist within some excitable cells; one sensitive to IP_3, and the other sensitive to both IP_3 and ryanodine (Yamazawa *et al.*, 1992). Alternatively, an excitable tissue may contain only one Ca^{2+} pool which is sensitive to both second messengers. Undoubtedly, the functional and spatial characteristics of intracellular Ca^{2+} stores vary between tissues.

1.3. IP_3-Mediated Ca^{2+} Release

Many of the physiological and biochemical characteristics of the IP_3-mediated intracellular Ca^{2+} store have been investigated. The IP_3 receptor consists of four identical 313 kDa subunits (Furuichi *et al.*, 1989; Mignery *et al.*, 1990, Supattapone *et al.*, 1988). Each subunit contains an N-terminal cytosolic extension responsible for IP_3 binding and regulation by ATP and phosphorylation, and eight transmembrane spanning regions which form the Ca^{2+} channel through which stored Ca^{2+} is released (Mignery & Südhof, 1990; Ehrlich & Watras, 1988; Mignery *et al.*, 1990). Immunogold labelling has identified IP_3 receptors on both central and peripheral sarcoplasmic

reticulum (Nixon *et al.*, 1994); however, only part of the organelle, the calciosome, may actually be involved in Ca^{2+} release (Meldolesi *et al.*, 1990).

The IP_3 receptor responds to ligand binding by undergoing a conformational change and increasing the opening frequency of the associated Ca^{2+} channel (Mignery & Südhof, 1990; Berridge, 1993). Channel activation appears highly co-operative; at least four independently bound IP_3 molecules may be required to open each channel (Meyer *et al.*, 1990). Four hundred Ca^{2+} molecules are estimated to be released for every molecule of IP_3 bound (Stauderman *et al.*,1988).

The IP_3 receptor recognizes the D-isomer of IP_3 more readily than the L-isomer (Berridge & Irvine, 1989). However, release of Ca^{2+} can also be elicited by inositol 2,4,5-trisphosphate ($Ins(2,4,5)P_3$) and inositol 4,5-bisphosphate ($Ins(4,5)P_2$). Based on EC_{50} values, the relative potencies are $IP_3 > Ins(2,4,5)P_3 > Ins(4,5)P_2$ in rat cerebellum (Stauderman *et al.*, 1988).

In the absence of extracellular K^+, or in the presence of tetraethylammonium, IP_3-mediated Ca^{2+} release was reduced, although, other monovalent cations ($Na^+ >> Tris^+ > Li^+$) could substitute effectively (Berridge & Irvine, 1989; Shah & Pant, 1988). Ca^{2+} alone could not stimulate K^+ influx; therefore, K^+ conductance was not a result of Ca^{2+} efflux, but was required for release and may neutralize the charge displacement resulting from the release of bound Ca^{2+} (Shah & Pant, 1988).

Following inositol phosphate production, the reaccumulation of Ca^{2+} coincided with the degradation of IP_3, indicating that metabolism is the primary mechanism for terminating the effects of the second messenger (Stauderman *et al.*, 1988; Berridge & Irvine, 1989). Repeated additions of IP_3 to rat cerebellar microsomes did not

desensitize the receptor nor diminish the release of Ca^{2+}. However, the receptor did desensitize in response to Ins(2,4,5)P_3 and Ins(4,5)P_2 (Stauderman *et al.*, 1988). Stauderman and co-workers proposed two explanations: (1) if Ins(2,4,5)P_3 and Ins(4,5)P_2 are not metabolized as quickly as IP_3, the Ca^{2+} channel will remain open and refilling will be impossible, or (2) Ins(2,4,5)P_3 and Ins(4,5)P_2 cannot be metabolized to Ins(1,3,4,5)P_4 or Ins(1,3,4)P_3, metabolites of IP_3 which may enhance the reaccumulation of stored Ca^{2+}.

1.4. Phospholipase C

The phosphoinositide-specific phospholipase C (PLC) isozymes are a series of catalytic proteins that hydrolyze phosphatidylinositol 4,5-bisphosphate (PIP_2) to water soluble IP_3 and membrane bound diacylglycerol (DAG). IP_3 induces Ca^{2+} release from intracellular stores, while DAG activates protein kinase C. PLC can be activated by receptor occupation, increased intracellular Ca^{2+} or Na^+, or increased extracellular K^+ (Akhtar & Abdel-Latif, 1978; Eberhard & Holz, 1988; Martin *et al.*, 1986; Kendall & Nahorski, 1985).

PLC activation in vascular smooth muscle appears to be coupled to the α_1-adrenoceptor. Prazosin, an α_1-adrenoceptor antagonist, inhibited noradrenaline-induced PI hydrolysis in rat aorta (Rapoport, 1987; Manolopoulos *et al.*, 1991), guinea pig cerebral cortical synaptoneurosomes (Gusovsky *et al.*, 1986) and rat caudal artery (Cheung *et al.*, 1990). Noradrenaline-induced PI hydrolysis in rat caudal artery was insensitive to rauwolscine, an α_2-adrenoceptor antagonist (Cheung *et al.*, 1990). In addition, in rat portal vein, noradrenaline-induced PI hydrolysis was shown to be insensitive to chlorethylchlonidine, an irreversible α_{1b}-adrenoceptor alkylating agent

(Lepretre *et al.*, 1994). Although the α_2-adrenoceptor agonist UK 14304 stimulated PI accumulation in rat caudal artery, its effects failed to plateau with increasing concentrations and were sensitive to prazosin rather than rauwolscine (Cheung *et al.*, 1990). The α_1-adrenoceptor antagonists did not directly affect intracellular Ca^{2+} stores as these antagonists did not affect acetylcholine- and caffeine-induced Ca^{2+} release (Lepretre *et al.*, 1994). PLC activation, in both vascular and nonvascular tissues, appears to be coupled to adrenoceptor occupation through an unidentified pertussis toxin insensitive G-protein (Martin *et al.*, 1986; Cheung *et al.*, 1990; Eberhard & Holz, 1988; Vallar *et al.*, 1987; Lepretre *et al.*, 1994; Muntz *et al.*, 1993; Nichols *et al.*, 1989).

While some tissues appear to require extracellular Ca^{2+} as a PLC activator or activation-dependent co-factor, PI hydrolysis in other tissues seems to be Ca^{2+} independent. For example, noradrenaline induced a transient rise in IP_3 and a phasic contraction of rabbit mesenteric artery in a Ca^{2+}-free solution (Itoh *et al.*, 1992); but, acetylcholine-induced stimulation of PI hydrolysis in rabbit iris smooth muscle was increased from 16 % above unstimulated preparations in the absence of extracellular Ca^{2+} to 32 % when Ca^{2+} was included in the extracellular medium (Akhtar & Abdel-Latif, 1978).

PLC activity has also been investigated by measuring intracellular Ca^{2+} release and Ca^{2+} efflux; although less direct than measuring inositol phosphate accumulation, these techniques are validated by the close correlation between PLC hydrolysis and intracellular Ca^{2+} release (Somlyo *et al.*, 1985; Stauderman *et al.*, 1988; Chiu *et al.*, 1986; Thévenod *et al.*, 1989). Results from these studies also demonstrate the variable dependence of PLC on extracellular Ca^{2+} amongst excitable tissues. Thrombin

caused a rapid elevation of intracellular Ca^{2+} in the absence of extracellular Ca^{2+} in human platelets (Nakashima *et al.*, 1986). In addition, noradrenaline-stimulated Ca^{2+} efflux and contraction were transiently increased in rabbit aorta in the absence of extracellular Ca^{2+} (Meisheri *et al.*, 1986). However, in guinea pig cerebral cortical synaptoneurosomes (Gusovsky *et al.*, 1986), permeablized RINm5F cells (Vallar *et al.*, 1987) and rat cerebellar cortical slices (Kendall & Nahorski, 1985), removal of extracellular Ca^{2+}, or addition of a potent dihydropyridine inhibitor, completely abolished agonist-, Bay-K-8644- (a dihydropyridine Ca^{2+} channel blocker) and K^+-induced inositol phosphate accumulation. Furthermore, stimulation of noradrenaline-induced PI hydrolysis in rat caudal artery was enhanced by exogenous Ca^{2+} in a concentration-dependent manner (Cheung *et al.*, 1990), and thyrotropin-releasing hormone-mediated activation of PLC in permeablized GH_3 cells was maximized by the presence of Mg^{2+}, ATP and extracellular Ca^{2+} (Martin *et al.*, 1986). Finally, Ca^{2+}-induced contractions in permeabilized rabbit mesenteric artery were not impaired by felodipine (Hagiwara *et al.*, 1993), noradrenaline-stimulated PI accumulation in canine femoral artery was not affected by diltiazem (Eskinder *et al.*, 1989), and in rat portal vein myocytes, oxodipine did not affect noradrenaline-induced inositol phosphate accumulation (Lepretre *et al.*, 1994). These latter results suggest that in rabbit mesenteric artery, canine femoral artery and rat portal vein myocytes, PLC does not require an influx of Ca^{2+} through voltage sensitive channels for activation. However, Ca^{2+} influx may have occurred through dihydropyridine insensitive channels, as observed in rabbit urethral smooth muscle. Garcia-Pascual and co-workers (1993) found that endothelin-1-induced inositol phosphate accumulation and contraction in

rabbit urethra were abolished in a Ca^{2+}-free medium. However, pre-treatment with nifedipine did not reduce PI hydrolysis, and contraction was sensitive to Ni^{+2}. Therefore, PLC isozymes may differ with respect to their sensitivity to extracellular Ca^{2+} and the means by which Ca^{2+} is acquired. According to Rhee and Choi (1992), the activities of all PLC isozymes are dependent on Ca^{2+}. Therefore, differences between tissues may reflect the variation in the Ca^{2+} sensitivities of the isozymes.

1.5. cGMP Regulated Vasodilation

Researchers who first recorded the association between increased guanosine 3':5'-cyclic monophosphate (cGMP) levels and increased muscle tone suggested that the nucleotide functioned as mediator of contraction (Dunham *et al.*, 1974). Soon after, however, Gruetter and co-workers (1975) observed that inhibitors of nitric oxide-induced relaxation also blocked cytosolic guanylate cyclase, the enzyme responsible for producing cGMP from guanosine triphosphate (GTP). This led Gruetter to suggest that cGMP was involved in vasodilation, not contraction. During contraction, cGMP apparently acted as a negative feedback inhibitor of induced Ca^{2+} influx to prevent over-stimulation (Schultz *et al.*, 1977). In 1980, Furchgott and Zawadzki demonstrated that relaxation of vascular smooth muscle required the presence of functional endothelial cells, and that acetylcholine, acting at muscarinic receptors on the endothelial cells stimulated the release of a substance that caused relaxation. Since then, it has been shown that endothelium-dependent relaxation in vascular smooth muscle is mediated through cGMP, and nitric oxide is the diffusible endothelium-derived relaxing factor which stimulates production of cGMP by soluble guanylate cyclase (Furchgott *et al.*, 1984; Rapoport & Murad, 1983; Furchgott, 1988; Ignarro *et*

al., 1988). However, to date, the mechanism of action of cGMP in vasodilation in vascular smooth muscle has not been identified.

Some of the mechanisms presently considered to explain the effects of cGMP in vascular smooth muscle relaxation include: (1) stimulation of a sarcolemmal Ca^{2+}-ATPase (Popescu *et al.*, 1985), (2) activation of the $Na^+/K^+/Cl^-$ co-transporter (O'Donnell & Owen, 1986), (3) activation of the Na^+/Ca^{2+} exchanger (Furukawa *et al.*, 1991), (4) inhibition of Ca^{2+} translocation across the plasma membrane (Collins *et al.*, 1985; Ishikawa *et al.*, 1993; Collins *et al.*, 1986; Méry *et al.*, 1991; Tohse & Sperelakis, 1991), (5) acceleration of Ca^{2+} uptake into the sarcoplasmic reticulum (Twort & van Breemen, 1988), (6) inhibition of Ca^{2+} release from intracellular storage sites (Collins *et al.*, 1986), (7) phosphorylation of myosin light chain kinase (Nishikawa *et al.*, 1984), (8) phosphorylation of cGMP-dependent cAMP phosphodiesterase (Méry *et al.*, 1991), or (9) phosphorylation of the contractile machinery (Baltensperger *et al.*, 1990). Considering the significance of its role in the regulation of vascular muscle tone, it is unlikely a single mechanism is responsible for the effects of cGMP; rather, several mechanisms probably contribute to cGMP-dependent vasodilation.

If, as Collins and co-workers suggested, cGMP inhibits intracellular Ca^{2+} release, there are three mechanisms by which cGMP could operate, (1) blocking production of IP_3 through PLC, (2) blocking IP_3 binding or activation of the sarcoplasmic reticulum receptor, or (3) hastening IP_3 inactivation or metabolism. A cGMP dependent binding protein, cGMP-regulated ion channel or cGMP-binding cyclic nucleotide phosphodiesterase, may mediate the effects of the nucleotide (Lincoln & Cornwell, 1993).

Conflicting results have been reported for the effects of cGMP on PI hydrolysis. 8-Bromoguanosine 3':5'-cyclic monophosphate (8-bromo-cGMP), a stable, membrane permeable analogue of endogenous cGMP, inhibited PI accumulation induced by noradrenaline and thrombin in rat aorta and human platelets, respectively (Rapoport, 1986; Nakashima *et al.*, 1986). Furthermore, PI hydrolysis in human platelets was inhibited by sodium nitroprusside, which elevates cGMP (Takai *et al.*, 1981). However, the vasorelaxant osthole increased cGMP levels in rat aorta without affecting inositol phosphate formation (Ko *et al.*, 1992). 8-Bromo-cGMP did not block noradrenaline-induced PI hydrolysis in canine femoral artery (Eskinder *et al.*, 1989). Finally, dibutyryl-cGMP did not modify the carbachol-induced formation of inositol phosphates in rat gastric mucosal cells (Puurunen *et al.*, 1987).

1.6. Experimental Objectives

The regulatory effects of cGMP on the biochemical IP_3 second messenger cascade of vascular smooth muscle contraction were investigated. The rat caudal artery was the preparation used in this study. It has been previously demonstrated that agonist-mediated PI hydrolysis is 10-100 times greater in the rat caudal artery than the larger, more frequently employed rat thoracic aorta (Labelle & Murray, 1990). Therefore, according to these researchers, the rat caudal artery appears to be the better preparation to investigate the relationship between PI hydrolysis and contraction.

Specifically, the objectives of this study were, (1) to determine whether or not PLC activity in rat caudal artery is dependent on extracellular Ca^{2+}, (2) to determine whether or not a pertussis toxin sensitive G-protein mediates α_1-adrenoceptor contraction, (3) to establish whether or not cGMP blocks IP_3 production, (4) to

determine whether or not rat caudal artery contains two functionally distinct intracellular Ca^{2+} stores, one sensitive to ryanodine, the other to IP_3, and (5) to compare the relative contributions of intracellular and extracellular Ca^{2+} to contraction in rat caudal artery and rat thoracic aorta.

Phenylephrine-induced PI accumulation and contraction were measured in denuded rat caudal artery in the presence and absence of extracellular Ca^{2+}. The effects of 8-bromo-cGMP on phenylephrine-induced contraction and PI hydrolysis were investigated in rat caudal artery. The effects of the nucleotide were compared to those of felodipine, a dihydropyridine Ca^{2+}-channel antagonist, and ryanodine, a putative depletor of intracellular Ca^{2+} stores. The effect of pertussis toxin on phenylephrine-induced contractions was investigated in rat caudal artery. Finally, comparative contractile studies in rat aortic rings were carried out in the presence and absence of felodipine and ryanodine.

2. MATERIALS AND METHODS

2.1. Phosphatidylinositol Hydrolysis in Caudal Artery

Male Sprague-Dawley rats (300-400 grams) were anaesthetized with sodium pentobarbital (65 mg kg^{-1}) i.p.. The caudal artery was dissected free and cleaned of connective tissue in Krebs-bicarbonate buffer of the following composition (in mM): NaCl, 120; KCl, 4.6; glucose, 11; $MgCl_2$, 1.2; $CaCl_2$, 2.5; KH_2PO_4, 1.2; $NaHCO_3$, 25.3. The pH of the buffer following saturation with a 95 % O_2 : 5 % CO_2 gas mixture was 7.4. The composition of the Ca^{2+}-free buffer was the same, except that $CaCl_2$ was omitted and replaced with ethylene glycol-bis(β-amino-ethyl ether) N, N, N', N'-tetraacetic acid (EGTA) (2 mM). The endothelial cell layer was removed from cleaned arteries by inserting a wire through the lumen and rubbing gently.

Phosphatidylinositol hydrolysis was assayed as described previously by Cheung *et al.* (1990). Arteries were cut into 1 cm segments and incubated in buffer at 37°C for 60 minutes; the incubating buffer was changed every 20 minutes. The tissues were transferred into 2 of ml fresh buffer containing 6 μCi ml^{-1} [^{3}H] *myo*-inositol to load for 90 minutes. Loaded tissues were washed five times with ice-cold Krebs before being put into individual assay tubes containing 10 mM LiCl in 300 μl buffer at 37°C. It has previously been demonstrated that Li^+ enhances the accumulation of inositol phosphates by inhibiting metabolism by monophosphatase; maximum enhancement resulted from 10 mM Li^+ with an EC_{50} of 0.2 mM (Cheung *et al.*, 1990). Ca^{2+}-free Krebs-bicarbonate buffer containing EGTA (2 mM) was used to wash, and later incubate those tissues that would be stimulated by phenylephrine in the absence of extracellular

Ca^{2+}. Individual assay tubes contained felodipine (10 nM), 8-bromo-cGMP (10 μM) or double distilled water (9 μl), and tissues were allowed to incubate for 20 minutes before phenylephrine (0.3-30.0 μM) from serial dilutions or double distilled water (9 μl) was added. Individual tubes were gassed continuously with a mixture of 95 % O_2 : 5 % CO_2.

Stimulation with phenylephrine was stopped after 45 minutes by adding 300 μl of ice-cold trichloroacetic acid (1M) to each sample. Tubes were left on ice for 30 minutes and then vortexed. Aliquots (500 μl) were transferred to clean assay tubes and washed with 2 volumes of water-saturated diethyl-ether five times. After the final wash, residual ether was rapidly evaporated by blowing air across the surface of the sample. Part of each sample (400 μl) were then transferred to a clean tube and $NaHCO_3$ (100 mM) added to adjust the pH to 7-8. Aliquots (400 μl) were then applied to Dowex-1 (x8) anion-exchange columns (formate form, 100-200 mesh, 1 ml). Columns were washed with 12 ml of unlabeled *myo*-inositol (5 mM). Tritiated inositol phosphates were then eluted with 12 ml of 0.1 M formic acid/ 1 M ammonium formate. Two volumes of Scinti-Safe 30 % scintillant were added to the two 6 ml aliquots collected and the radioactivity was counted in a Packard 1600TR liquid scintillation counter. The efficiency of the counter was 67 %. At the completion of each experiment, each tissue was blotted and weighed to normalize the radioactive counts per mg wet weight.

2.2. Contractile Studies in Caudal Artery

Caudal arteries were isolated as described for the phosphatidylinositol hydrolysis studies, except that the tissues were cleaned in ice-cold buffer and cut into

0.6 cm lengths. Tissues were mounted in 20 ml organ baths at 37°C under a force of 9.8 mN and gassed continuously with a mixture of 95 % O_2 : 5 % CO_2. Contractions were measured through a force transducer and recorded on a Grass Model 7 Polygraph. The tissues were equilibrated for 60 minutes. Phenylephrine (1 μM) was then used to contract the tissues and acetylcholine (10 μM) was applied to ensure the functional endothelial response had been removed. Tissues were left for 30 minutes before a control concentration-response curve to phenylephrine (0.01 μM-100 μM) was constructed. Following construction of control concentration-response curves, felodipine (1 & 10 nM), ryanodine (3 & 10 μM), 8-bromo-cGMP (10 μM), distilled water (6 or 20 μl) or a combination of felodipine and 8-bromo-cGMP or ryanodine and 8-bromo-cGMP were left in contact with the tissues for 30 minutes to be followed by construction of another concentration-response curve to phenylephrine in the presence of the antagonists or water. Tissues were allowed to equilibrate for 60 minutes between concentration-response curves. When responses were generated in the absence of extracellular Ca^{2+}, Ca^{2+}-free buffer was in contact with the tissues for 15 minutes before a concentration-response curve to phenylephrine was constructed.

2.3. Contractile Studies with Pertussis Toxin in Caudal Artery

Caudal arteries were isolated and mounted as described for the contractile studies in caudal artery detailed above. Following the control concentration-response curve to phenylephrine, tissues were allowed to rest for 30 minutes and then pertussis toxin (100 ng/ml) or distilled water (40 μl) was added to the bath. The tissues were incubated for 2 hours and then washed 5 times. A second concentration-response

curve was then generated for phenylephrine following the pretreatment with pertussis toxin.

2.4. Contractile Studies Thoracic Aorta

Rats were anaesthetized as described for the phosphatidylinositol hydrolysis studies in caudal artery detailed above, and the thoracic aortae rapidly removed and placed in Krebs-bicarbonate buffer. Aortae were cleaned of extraneous connective tissue and the endothelial layer removed by inserting a 20 gauge needle into the lumen and gently rubbing. Aortas were cut into 0.5 cm segments, and mounted in 20 ml organ baths at 37°C under a force of 9.8 mN and gassed continuously with a mixture of 95 % O_2 : 5 % CO_2. Tissues were allowed to equilibrate for 60 minutes. Phenylephrine (10 μM) was then used to contract the tissues and acetylcholine (10μM) was applied to ensure the functional endothelial response had been removed. Tissues were left for 30 minutes before a concentration-response curve to phenylephrine (0.1 nM-10 μM) was constructed. Following construction of control concentration-response curves, felodipine (1 nM & 10 nM), ryanodine (3 μM and 10 μM) or distilled water (80 μl) were added and left in contact with the tissues for 30 minutes to be followed by construction of another concentration-response curve to phenylephrine in the presence of felodipine or ryanodine. Tissues were allowed to equilibrate for 60 minutes between concentration-response curves.

2.5. Data and Statistical Analysis

In the absence of an antagonist, PI hydrolysis was maximum in the presence of 10 μM phenylephrine. Therefore, in experiments involving the use of an antagonist,

results are expressed as a percentage of the maximum PI turnover induced by 10 μM phenylephrine in untreated control tissues. Each experiment was run parallel to two such controls, of which the average radioactivity per mg wet weight was calculated.

Results from contractile studies were calculated as a percentage of maximum contraction induced by phenylephrine in the absence of antagonists. Percent maximum, Hill coefficient and EC_{50} values were calculated for individual curves using a program executed on an IBM compatible microcomputer (Wang & Pang, 1993). These parameters were determined by fitting the percent contractile response at increasing concentrations of phenylephrine, [PE], by non-linear least squares to the relation Y= a + bX, where Y = response and $X = [PE]^n / ([PE]^n + [EC_{50}]^n)$ with *n* fixed at "floating" integral values to obtain the best fit.

Comparison of PI hydrolysis between control and corresponding experiments in the presence of an antagonist were made using an unpaired Student's t-test. For the results of the contractile studies, an analysis of variance block design was used for comparisons between control and treated tissue values for % maximum, Hill coefficient and EC_{50}. For multiple comparisons, Duncan's multiple range test was used to compare between means. For all cases, a probability of error of less than 0.05 was selected as the criterion for statistical significance.

2.6. Chemicals

8-Bromoguanosine 3':5'-cyclic monophosphate sodium salt (8-bromo-cGMP), L-phenylephrine HCl and ethylene glycol-bis(β-amino-ethyl ether) N, N, N', N'-tetraacetic acid (EGTA) were purchased from Sigma Chemical Co. (Ca., USA). *Myo*-[2-^{3}H(N)]-inositol (17.0 Ci mmol^{-1}), ryanodine and salt free pertussis toxin were purchased from

Amersham (Ont., Canada), Calbiochem (Ca., USA) and List Biological Laboratories (Ca., USA), respectively. Felodipine was a gift from Hässle (Sweden). With the exception of felodipine, all drug solutions were prepared in double distilled water. A 10 mM felodipine stock solution was made in 80 % ethanol; dilutions were made with double distilled water. All other chemicals were purchased from Fischer Scientific (B.C., Canada).

3. RESULTS

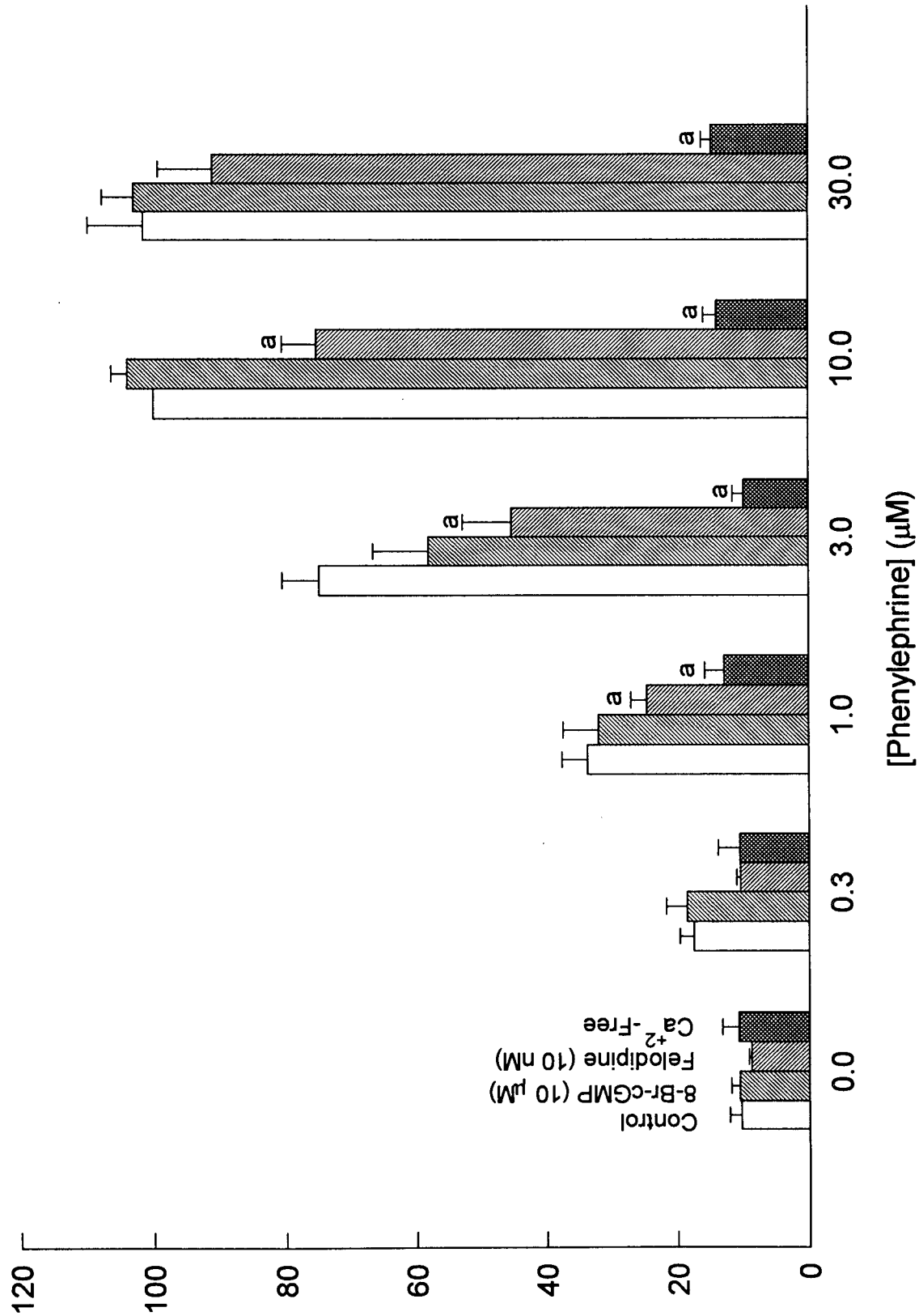
3.1. Phosphatidylinositol Hydrolysis in Caudal Artery

Phenylephrine (0.3-30 μM) increased PI hydrolysis in a concentration dependent manner. A maximum accumulation, approximately 10-times greater than basal accumulation, was achieved at a concentration of 10 μM phenylephrine (Figure 1). The addition of phenylephrine greater than 30 μM significantly decreased PI hydrolysis when compared to maximal PI accumulation (results not shown). Similar effects have been observed in rat caudal artery with supermaximal concentrations of noradrenaline (Labelle & Murray, 1990).

Phenylephrine-induced PI hydrolysis was not affected by 8-bromo-cGMP (10 μM) and maximal accumulation remained approximately 10-fold above basal dpm mg wet weight^{-1} (Figure 1). In contrast, felodipine (10 nM) caused a noticeable decrease in PI hydrolysis at all concentrations of phenylephrine tested (Figure 1). This decrease was found to be statistically significant ($n = 6$; $p < 0.05$) at 1, 3 and 10 μM phenylephrine. Maximum accumulation was only 7-fold above basal dpm mg wet weight^{-1}. Maximum hydrolysis was restored by 30 μM phenylephrine, although accumulation remained noticeably below the control value. We also found that PI accumulation induced by phenylephrine could be completely abolished in the absence of Ca^{2+} (Ca^{2+}-free buffer containing 2 mM EGTA). It was noted that basal PI accumulation was not affected by 8-bromo-cGMP, felodipine or Ca^{2+}-free buffer.

Figure 1. Phosphatidylinositol accumulation in rat caudal artery in the presence and absence of 8-bromo-cGMP, felodipine or Ca^{2+}-free buffer.

Phosphatidylinositol accumulation in rat caudal artery induced by phenylephrine in the absence (open) and presence of 8-bromo-cGMP (left-to-right hatched) or 10 nM felodipine (right-to-left hatched) or Ca^{2+}-free buffer (2 mM EGTA) (cross hatched). Percent accumulation calculated relative to the maximum accumulation induced by 10 μM phenylephrine in the absence of an antagonist. Basal accumulation for control and treated tissues was 215 ± 20 dpm mg wet weight^{-1} (mean ± S.E.; n = 24). Each column represents the mean of six experiments ± S.E. [a] Significantly different from control ($p < 0.05$).


Phosphatidylinositol Accumulation (% Control)
120
100
80
60
40
20
0
Control
8-Br-cGMP (10 μM)
Felodipine (10 nM)
Ca+2-Free
0.0
0.3
1.0
3.0
10.0
30.0
[Phenylephrine] (μM)
a

3.2. Contractile Studies in Caudal Artery

Concentration-response curves to phenylephrine were displaced to the right in the presence of 8-bromo-cGMP (10 μM) (Figure 2). 8-Bromo-cGMP significantly ($n = 6$; $p < 0.05$) decreased the maximum tension and increased the EC_{50} of the concentration-response curve; the Hill coefficient was not affected. These effects were unchanged by 8-bromo-cGMP (10 μM) in a subsequent concentration-response curve to phenylephrine (Table I).

In the presence of felodipine (1 & 10 nM) contractions induced by phenylephrine were attenuated (Figure 3). Felodipine at 1 nM and 10 nM significantly ($n = 6$; $p < 0.05$) reduced the maximum tension to 77 ± 8 % and 57 ± 3 % of control, respectively. However, the EC_{50} and Hill coefficient values were unchanged in the presence of felodipine (Table I). Ryanodine (3 & 10 μM) also inhibited phenylephrine-induced contractions (Figure 4), and it significantly ($n = 6$; $p < 0.05$) reduced the maximum response and increased the EC_{50} value without affecting the Hill coefficient (Table I). Addition of ryanodine did not affect baseline tension. Maximum response, Hill coefficient and EC_{50} were not affected following addition of distilled water over time (Table I). The maximum tension of the control curve in the time study was 2.69 ± 0.47 mN (mean ± S.E.; $n = 6$).

Contractions induced by phenylephrine were not further inhibited by simultaneous application of 8-bromo-cGMP and felodipine as compared to felodipine alone (Table I; Figure 5). In contrast, concomitant application of 8- bromo-cGMP and ryanodine significantly ($n = 6$; $p < 0.05$) inhibited contractions

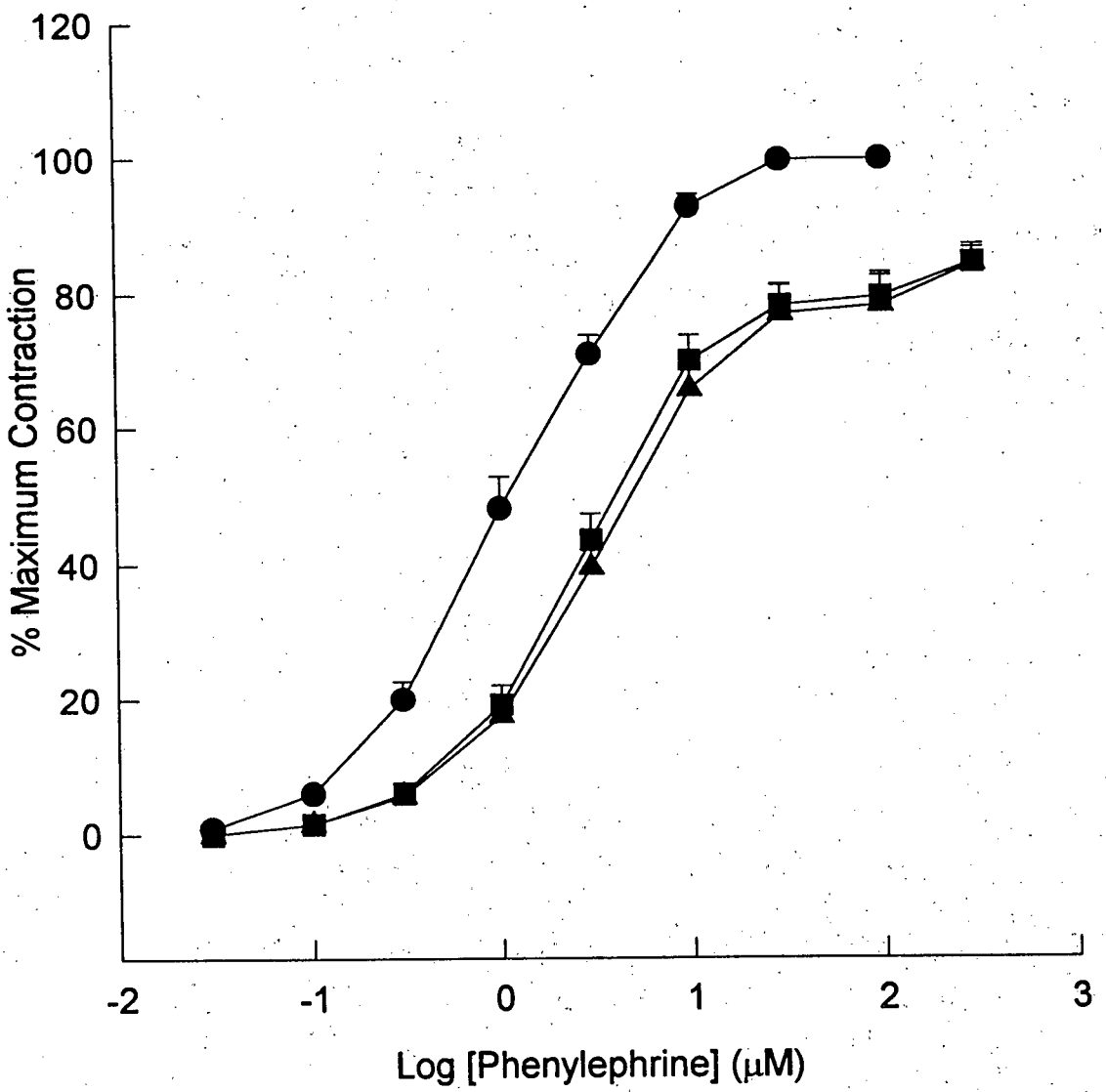


Figure 2. Contractions in rat caudal artery in the absence and presence of 8-bromo-cGMP.

Cumulative concentration-response curves in rat caudal artery to phenylephrine in the absence (circles) or in the presence of 8-bromo-cGMP (10 μM) for the first time (squares) and for the second time (triangles). Each point represents the mean of six experiments ± S.E. Maximum tension in the absence of antagonist was 4.17 ± 0.68 mN (mean ± S.E.; n = 6).

Table I. Phenylephrine-induced concentration-response curve results obtained from rat caudal artery preparations in the absence and presence of various antagonists.

EC_{50}, Hill coefficient (*n*) and % maximum response values obtained from individual concentration-response curves in rat caudal artery preparations. Each value represents the mean of six experiments ± S.E.

Groups	EC_{50} (μM)	*n*	% Maximum
Control	1.25 ± 0.18	1.10 ± 0.06	102 ± 1
8-Bromo-cGMP (10 μM)	2.65 ± 0.35[a]	1.32 ± 0.07[a]	81 ± 3[a]
8-Bromo-cGMP (10 μM)	3.16 ± 0.49[a]	1.25 ± 0.06	80 ± 5[a]
Control	1.49 ± 0.28	0.92 ± 0.04	104 ± 1
Felodipine (1 nM)	3.51 ± 0.98	1.03 ± 0.10	77 ± 8[a]
Felodipine (10 nM)	2.63 ± 0.45	1.11 ± 0.09	57 ± 3[ab]
Control	1.29 ± 0.19	1.10 ± 0.10	102 ± 1
Ryanodine (3 μM)	1.96 ± 0.27[a]	1.03 ± 0.06	81 ± 6[a]
Ryanodine (10 μM)	2.17 ± 0.32[a]	1.03 ± 0.08	76 ± 8[a]
Control	0.89 ± 0.18	0.92 ± 0.04	104 ± 1
Fel (1 nM) + 8-br (10 μM)	2.60 ± 0.25[a]	1.21 ± 0.05	74 ± 4[a]
Fel (10 nM) + 8-br (10 μM)	3.83 ± 0.28 [ab]	1.25 ± 0.05	59 ± 5[ab]
Control	1.62 ± 0.36	1.15 ± 0.03	102 ± 1
Ry (3 μM) + 8-br (10 μM)	3.53 ± 0.81	1.20 ± 0.09	52 ± 7[ac]
Ry (10 μM) + 8-br (10 μM)	3.52 ± 0.70	1.37 ± 0.13	38 ± 9[ac]
Control	0.95 ± 0.12	1.16 ± 0.10	101 ± 1
Ca^{2+}-free	N	N	N
+ Ca^{2+} (2.5 mM Ca^{2+})	1.01 ± 0.12	1.20 ± 0.08	103 ± 3
Control	1.48 ± 0.03	1.01 ± 0.07	103 ± 1
Distilled H_2O (6 μl)	1.56 ± 0.31	1.10 ± 0.05	102 ± 4
Distilled H_2O (20 μl)	1.52 ± 0.25	1.05 ± 0.07	103 ± 6

Fel = felodipine; Ry = ryanodine; [a]Significantly different from control, $p < 0.05$; [b]Significantly different from the first concentration of drug, $p < 0.05$; [c]Significantly different from the same concentration of ryanodine alone, $p < 0.05$; N = no measurable increase in contraction above resting tension.

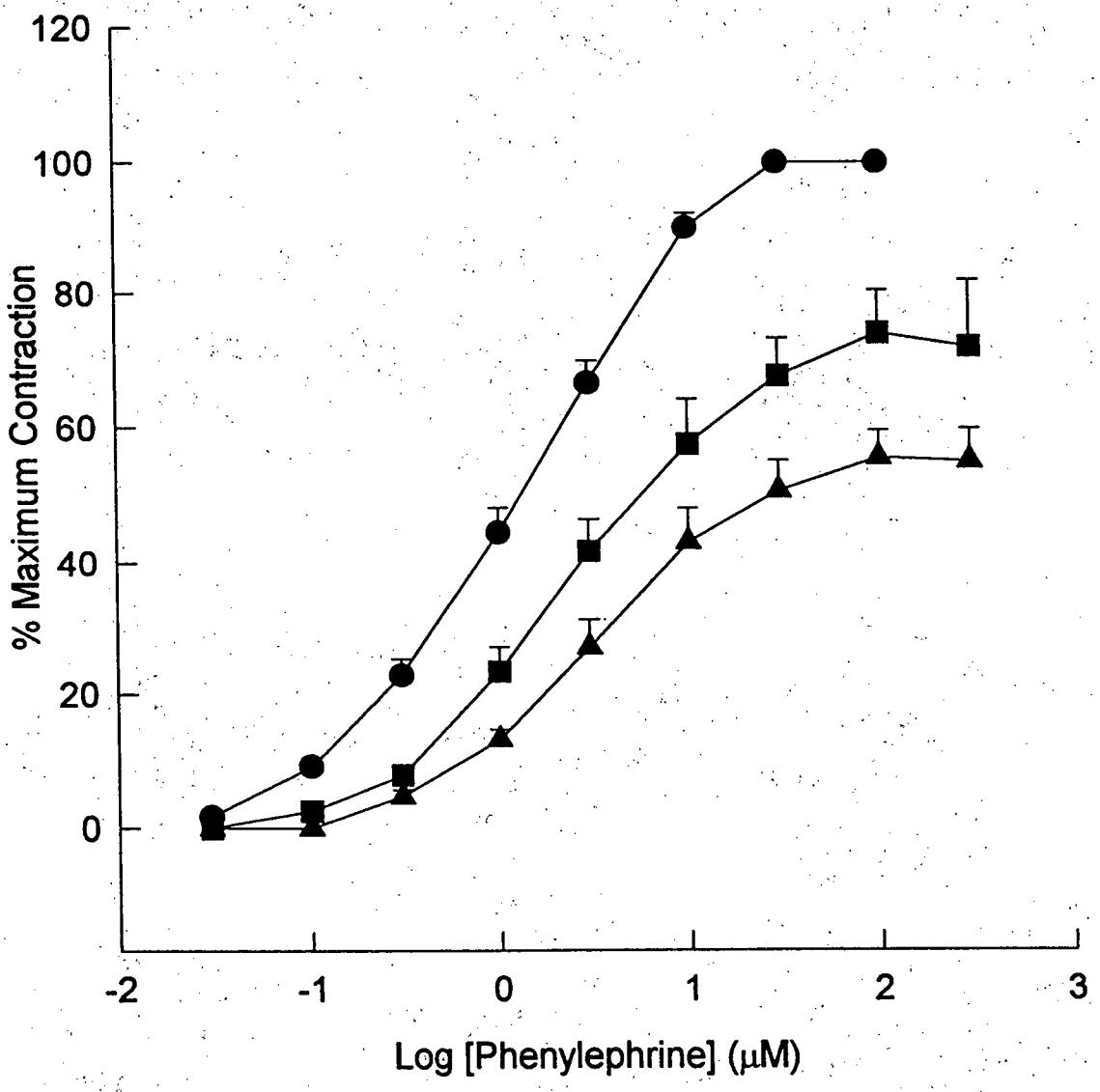


Figure 3. Contractions in rat caudal artery in the absence and presence of felodipine.

Cumulative concentration-response curves in rat caudal artery to phenylephrine in the absence (circles) or in the presence of felodipine, 1 nM (squares) and 10 nM (triangles). Each point represents the mean of six experiments ± S.E. Maximum tension in the absence of antagonist was 3.04 ± 0.22 mN (mean ± S.E.; n = 6).

induced by phenylephrine in comparison to ryanodine alone (Figure 6). Ryanodine (3 μM & 10 μM) alone lowered the maximum contraction to 81 ± 6 % and 76 ± 8 % of control, respectively, and when combined with 8-bromo-cGMP these values were further reduced by 29 % and 38 % of control, respectively (Table I).

Attempts to produce contractions with phenylephrine in Ca^{2+}-free buffer were unsuccessful (Figure 7). Addition of up to 300 μM phenylephrine did not produce contraction. However, re-introduction of Ca^{2+} into the bath restored contraction to phenylephrine without affecting the maximum tension, Hill coefficient or EC_{50} in comparison to the control (Table I).

3.3. Contractile Studies with Pertussis Toxin in Caudal Artery

Concentration-response curves to phenylephrine were not affected by pertussis toxin (100 ng/ml) (Figure 8). Maximum tension, EC_{50} and Hill coefficient were unchanged by pertussis toxin (Table II). Maximum tension, EC_{50} and Hill coefficient were not affected by the addition of distilled water over time (Table II). The maximum tension of the control curve in the time study was 2.19 ± 0.29 mN (mean ± S.E.; n = 6).

3.4. Contractile Studies in Thoracic Aorta

Concentration-response curves to phenylephrine were displaced to the right in the presence of felodipine (10 nM) (Figure 9). Felodipine (10 nM) significantly ($n = 4$; $p < 0.05$) lowered the maximum tension and increased the EC_{50} (Table III). The Hill coefficient was not affected by pretreatment with felodipine. Felodipine (1 nM) did not affect the concentration-response curves to phenylephrine.

Ryanodine (3 μM) did not significantly ($n = 4$; $p < 0.05$) affected the concentration-response curve to phenylephrine, maximum tension, EC_{50} and Hill coefficient remained unchanged (Figure 10; Table III). Ryanodine (10 μM) increased the maximum tension without affecting EC_{50} and Hill coefficient; however, the time control showed a similar, but insignificant trend (Figure 10; Table III). Maximum tension, EC_{50} and Hill coefficient were not affected by the addition of distilled water over time (Table III). The maximum tension of the control curve in the time study was 10.47 ± 0.93 mN (mean ± S.E.; $n = 4$).

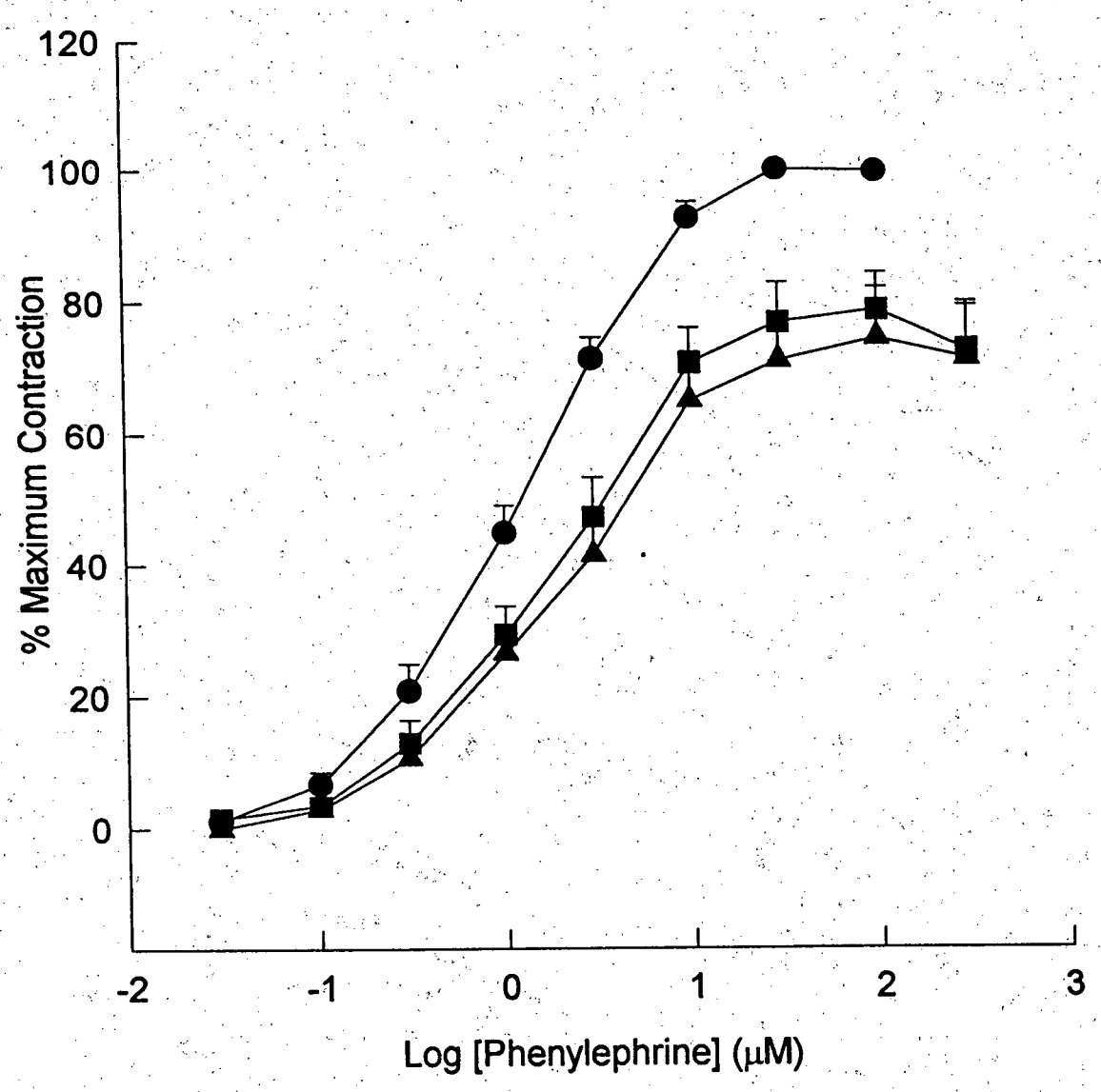


Figure 4. Contractions in rat caudal artery in the absence and presence of ryanodine.

Cumulative concentration-response curves in rat caudal artery to phenylephrine in the absence (circles) or in the presence of ryanodine, 3 μM (squares) and 10 μM (triangles). Each point represents the mean of six experiments ± S.E. Maximum tension in the absence of antagonist was 4.70 ± 0.71 mN (mean ± S.E.; n = 6).

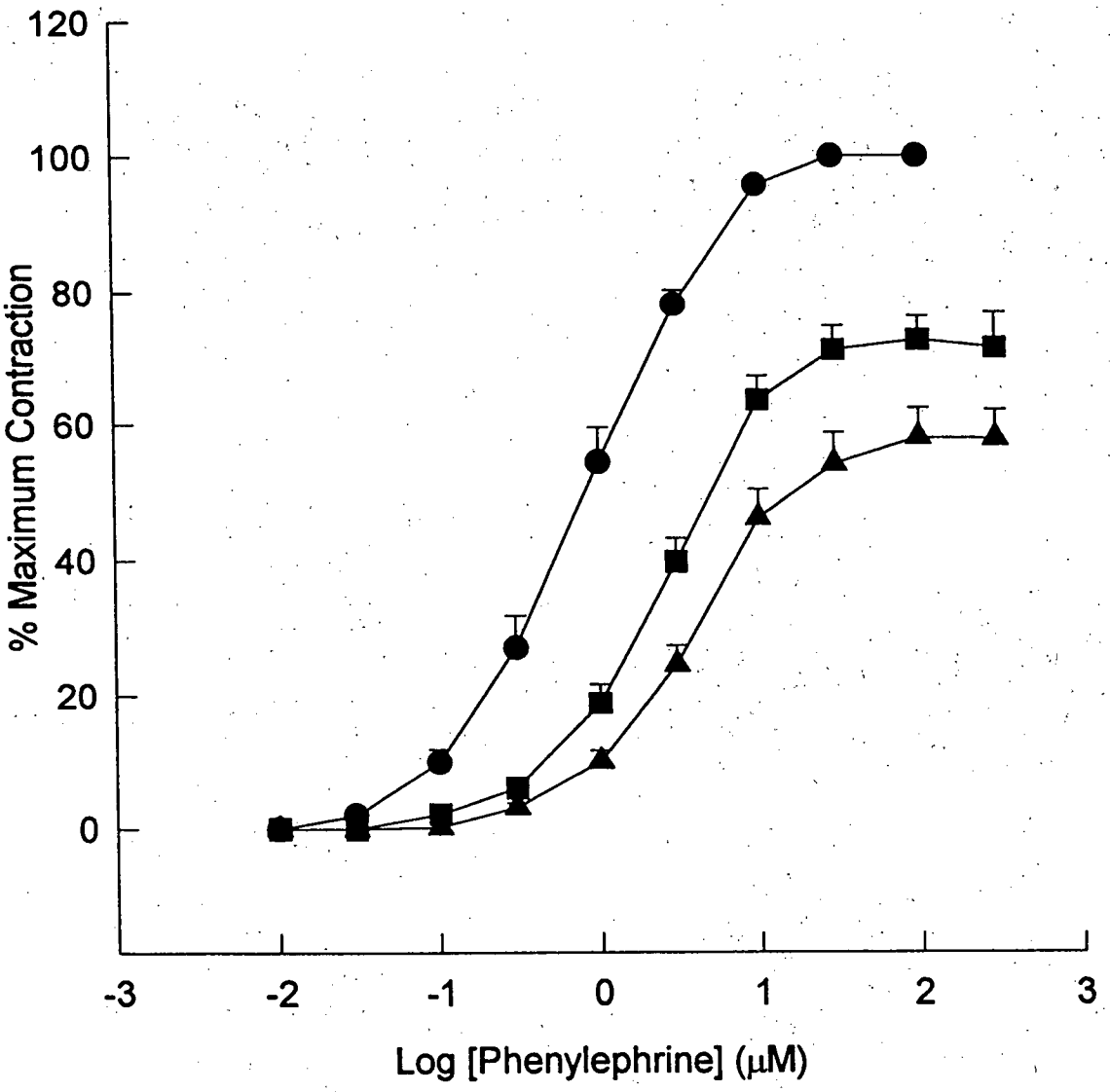


Figure 5. Contractions in rat caudal artery in the absence and presence of felodipine plus 8-bromo-cGMP.

Cumulative concentration-response curves in rat caudal artery to phenylephrine in the absence (circles) and in the presence of 1 nM felodipine and 10 μM 8-bromo-cGMP (squares) or 10 nM felodipine and 10 μM 8-bromo-cGMP (triangles). Each point represents the mean of six experiments ± S.E. Maximum tension in the absence of antagonists was 5.29 ± 0.97 mN (mean ± S.E.; n = 6).

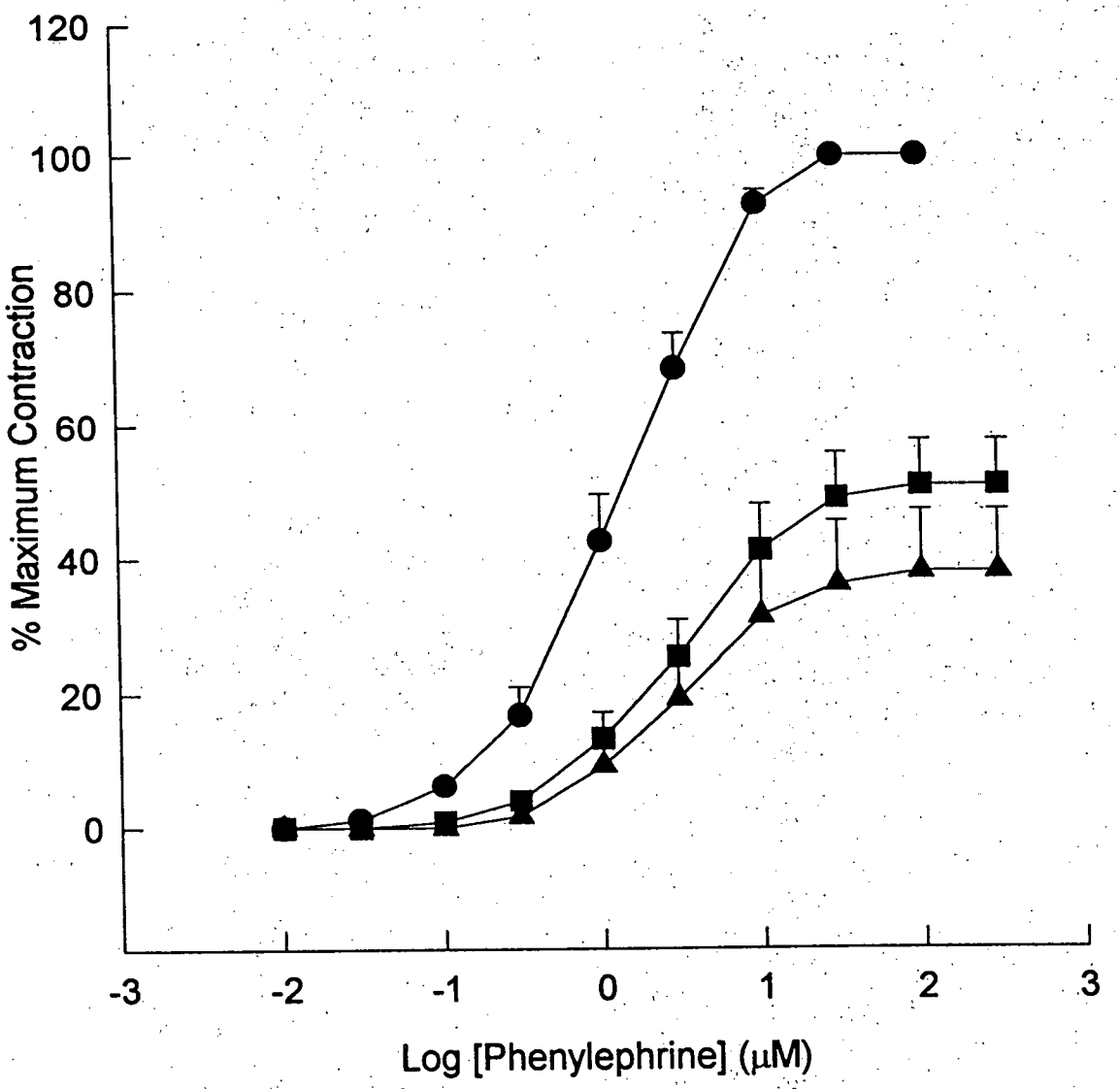


Figure 6. Contractions in rat caudal artery in the absence and presence of ryanodine plus 8-bromo-cGMP.

Cumulative concentration-response curves in rat caudal artery to phenylephrine in the absence (circles) and in the presence of 3 μM ryanodine and 10 μM 8-bromo-cGMP (squares) or 10 μM ryanodine and 10 μM 8-bromo-cGMP (triangles). Each point represents the mean of six experiments ± S.E. Maximum tension in the absence of antagonists was 3.79 ± 0.51 mN (mean ± S.E.; n = 6).

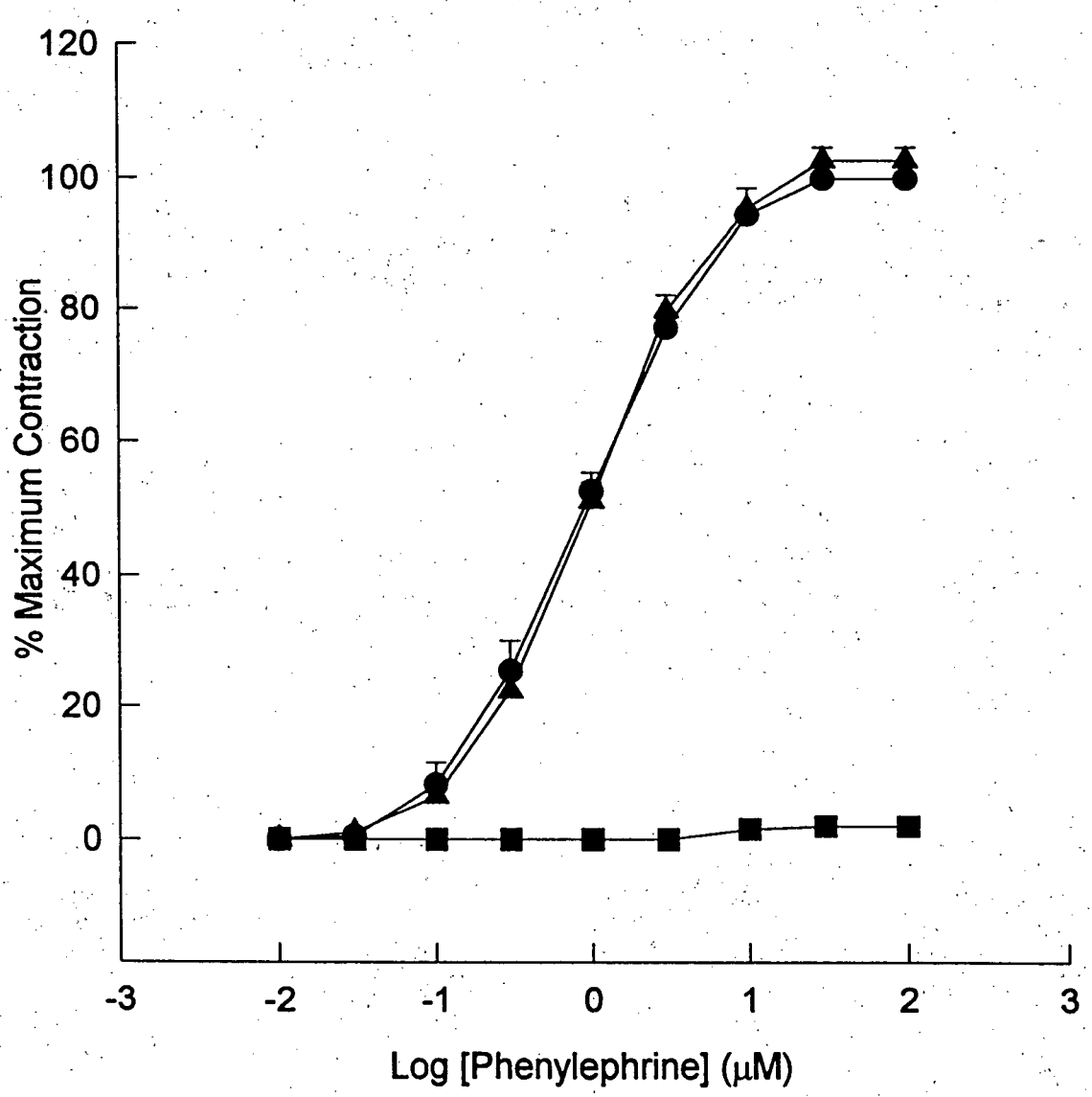


Figure 7. Contractions in rat caudal artery the presence and absence of extracellular Ca^{2+}.

Cumulative concentration-response curves in rat caudal artery to phenylephrine in the presence of Ca^{2+} (circles), in the absence of Ca^{2+} (2 mM EGTA) (squares) and after Ca^{2+} had been reintroduced (triangles). Each point represents the mean of six experiments ± S.E. Maximum tension in the presence of extracellular Ca^{2+} was 4.97 ± 0.63 mN (mean ± S.E.; n = 6).

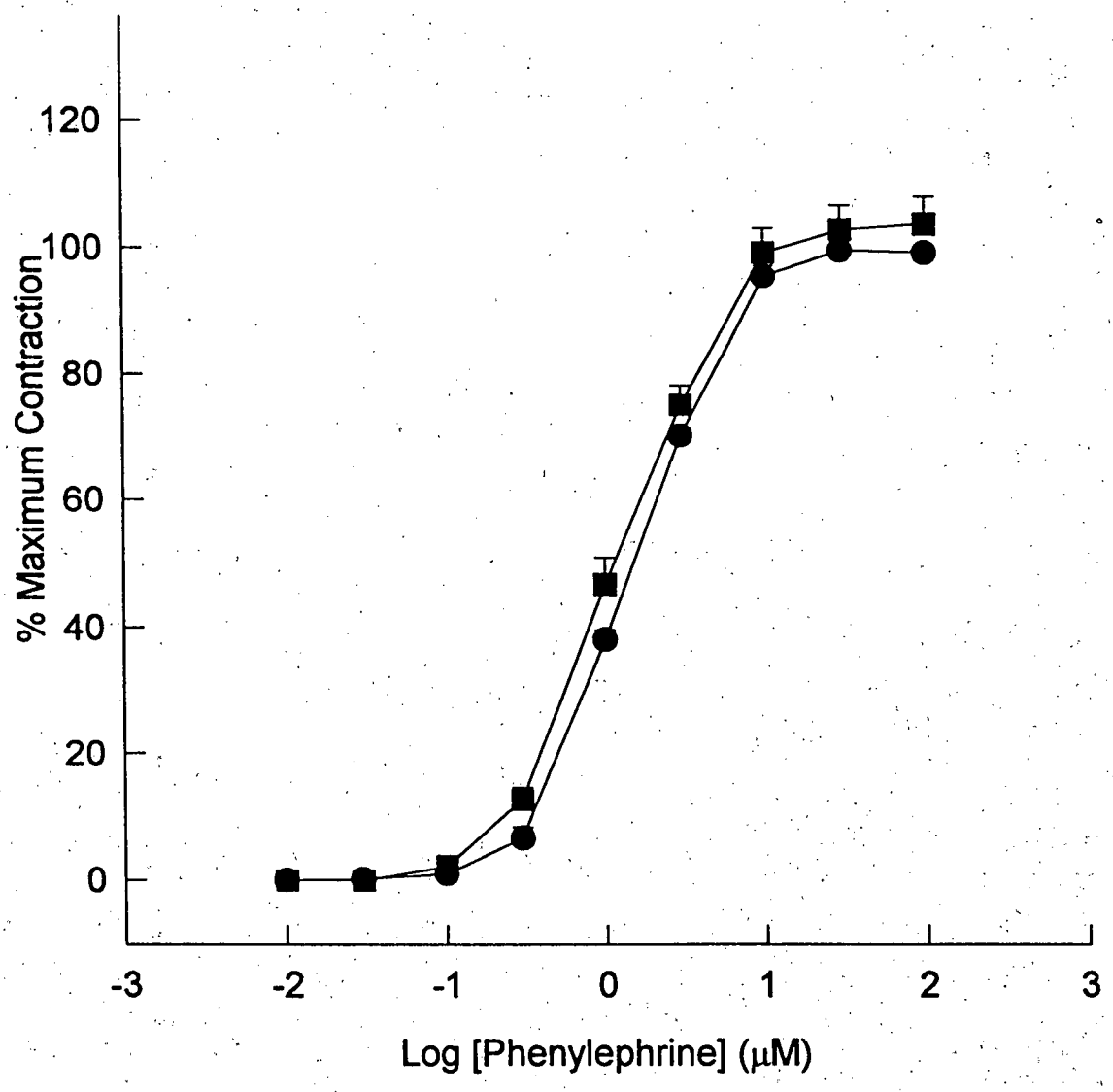


Figure 8. Contractions in rat caudal artery in the absence of pertussis toxin and following pretreatment with pertussis toxin.

Cumulative concentration-response curves in rat thoracic aorta to phenylephrine in the absence (circles) or in the presence of pertussis toxin (100 ng/ml) (triangles). Each point represents the mean of six experiments ± S.E. Maximum tension in the absence of antagonist was 3.76 ± 0.64 mN (mean ± S.E.; n = 6).

Table II. Phenylephrine-induced concentration-response curve results obtained from rat caudal artery preparations in the absence and presence of pertussis toxin.

EC_{50}, Hill coefficient (*n*) and % maximum response values obtained from individual concentration-response curves in rat caudal artery preparations in the presence and absence of pertussis toxin. Each value represents the mean of six experiments ± S.E.

Groups	EC_{50} (μM)	*n*	% Maximum
Control	1.51 ± 0.05	1.49 ± 0.06	100 ± 1
Pertussis toxin (100 ng/ml)	1.28 ± 0.10	1.37 ± 0.04	104 ± 4
Control	1.72 ± 0.21	1.49 ± 0.17	101 ± 1
Distilled H_2O (40 μl)	1.66 ± 0.22	1.48 ± 0.15	106 ± 6

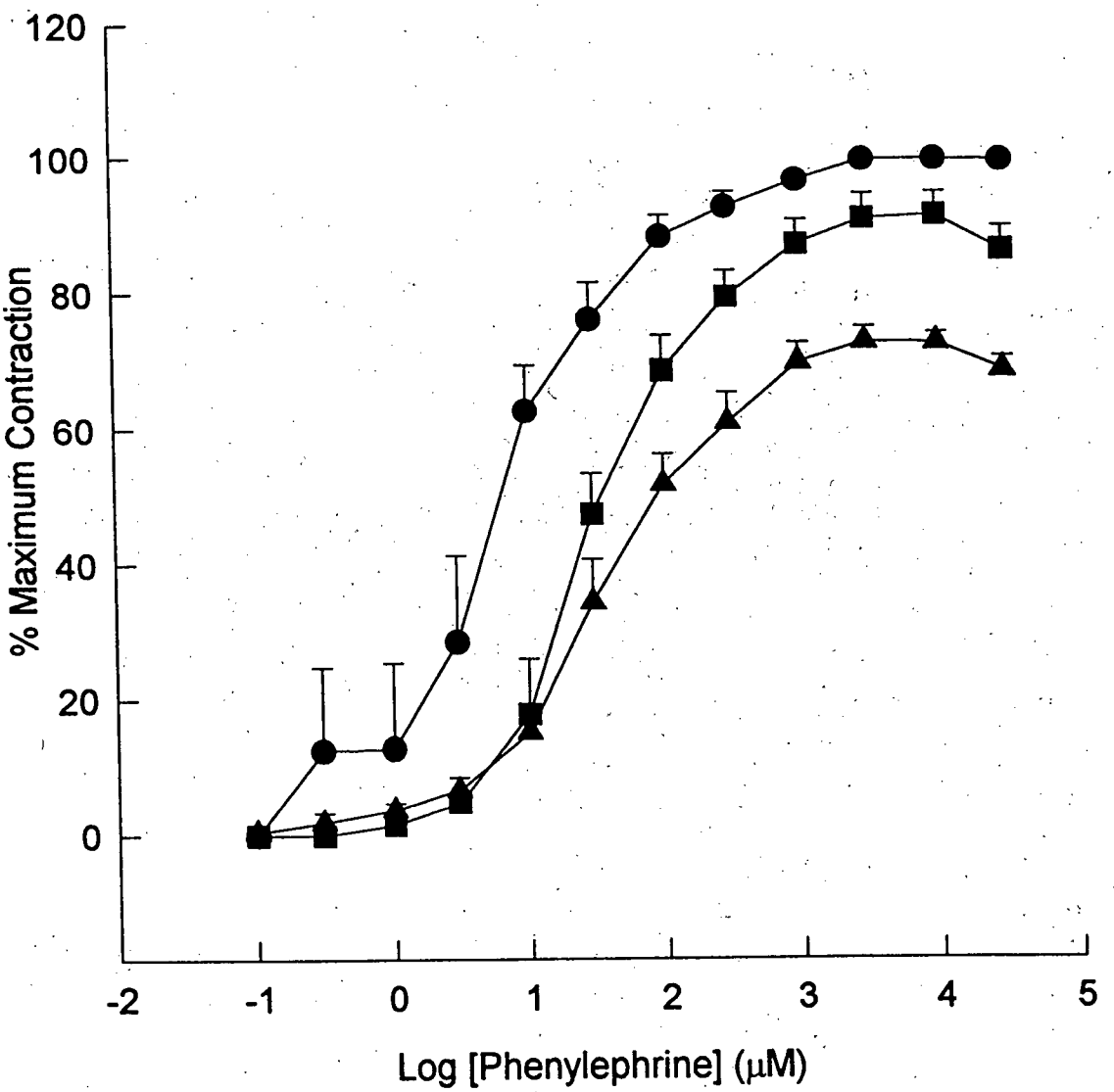


Figure 9. Contractions in rat aortic tissue in the absence and presence of felodipine.

Cumulative concentration-response curves in rat thoracic aorta to phenylephrine in the absence (circles) or in the presence of felodipine, 1 nM (squares) and 10 nM (triangles). Each point represents the mean of four experiments ± S.E. Maximum tension in the absence of antagonist was 11.64 ± 0.51 mN (mean ± S.E.; n = 4).

Table III. Phenylephrine-induced concentration-response curve results obtained from rat aortic tissue in the absence and presence of various antagonists.

EC_{50}, Hill coefficient (*n*) and % maximum response values obtained from individual concentration-response curves in rat thoracic aorta preparations. Each value represents the mean of four experiments ± S.E.

Groups	EC_{50} (μM)	*n*	% Maximum
Control	6.6 ± 2.4	1.42 ± 0.24	93 ± 1
Felodipine (1 nM)	31.3 ± 7.8	1.47 ± 0.17	86 ± 3
Felodipine (10 nM)	63.0 ± 17.2[a]	0.94 ± 0.12	74 ± 3[a]
Control	7.8 ± 2.0	1.64 ± 0.12	94 ± 1
Ryanodine (3 μM)	14.0 ± 1.0	1.53 ± 0.06	97 ± 2
Ryanodine (10 μM)	13.0 ± 3.0	1.67 ± 0.34	104 ± 2[a]
Control	17.0 ± 8.2	1.78 ± 0.61	103 ± 7
Distilled H_2O (20 μl)	16.0 ± 3.4	1.79 ± 0.04	106 ± 4
Distilled H_2O (20 μl)	22.0 ± 6.0	1.44 ± 0.12	112 ± 5

[a]Significantly different from control, $p < 0.05$

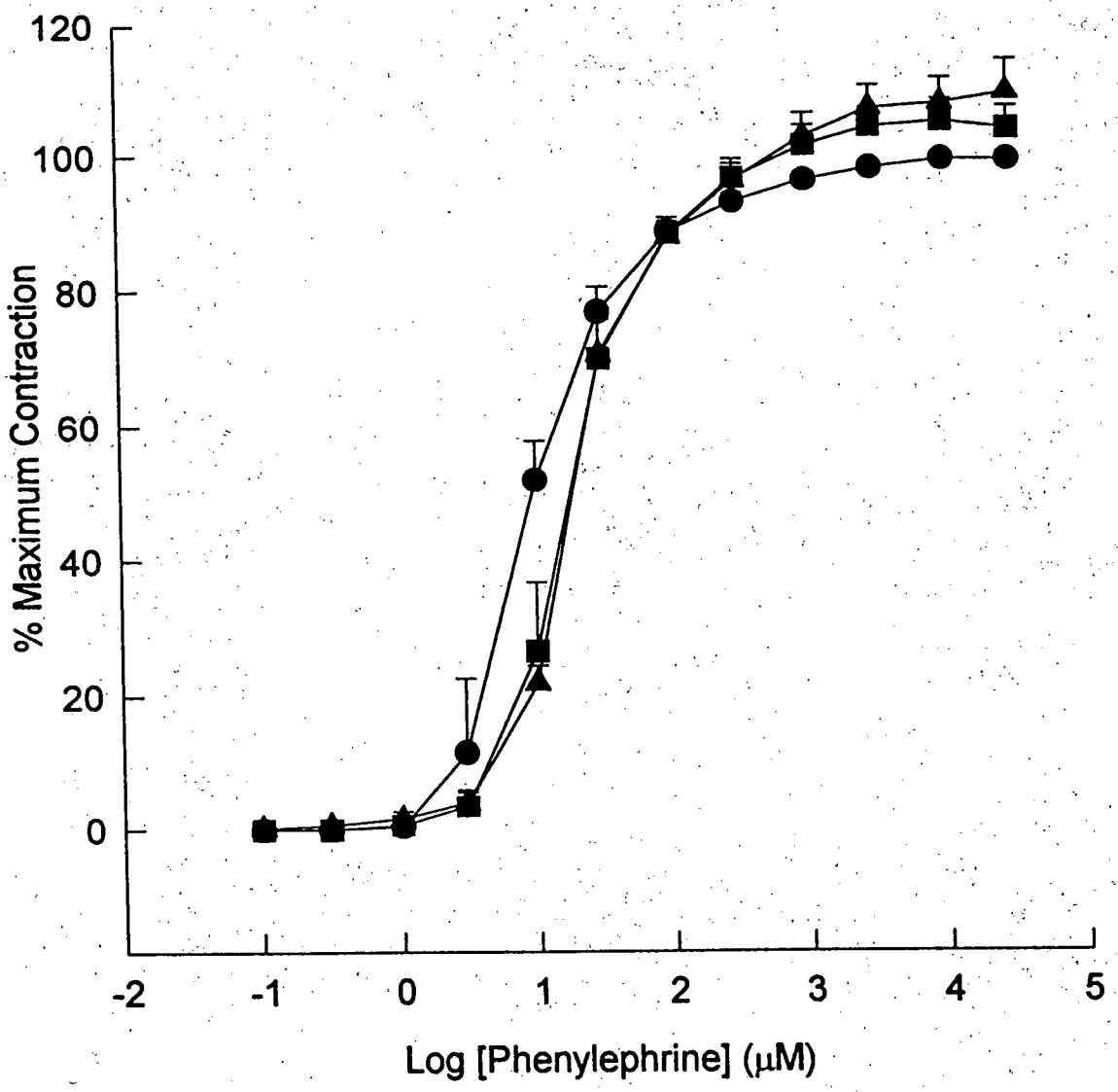


Figure 10. Contractions in rat aortic tissue in the absence and presence of ryanodine.

Cumulative concentration-response curves in rat thoracic aorta to phenylephrine in the absence (circles) or in the presence of ryanodine, 3 μM (squares) and 10 μM (triangles). Each point represents the mean of four experiments ± S.E. Maximum tension in the absence of antagonist was 10.84 ± 1.52 mN (mean ± S.E.; n = 4).

4. DISCUSSION

This investigation found that phenylephrine-induced inositol phosphate accumulation and contraction in rat caudal artery were critically dependent on the presence of extracellular Ca^{2+}. The influx of Ca^{2+} required for phenylephrine-induced inositol phosphate accumulation and contraction was regulated, in a small part, by felodipine sensitive Ca^{2+} channels. Pre-treatment with pertussis toxin did not impair the phenylephrine-mediated excitation-contraction coupling mechanism in rat caudal artery. 8-Bromo-cGMP lowered the maximum phenylephrine-induced tension when applied alone, but did not additively inhibit contraction when applied concomitantly with felodipine. 8-Bromo-cGMP did not block phenylephrine-induced inositol phosphate accumulation. Ryanodine inhibited phenylephrine-induced contractions, and these inhibitory effects were significantly potentiated in the presence of 8-bromo-cGMP.

In rat thoracic aorta, pre-treatment with felodipine weakly, but significantly, reduced the maximum phenylephrine-induced contraction; however, pre-treatment with ryanodine did not affect phenylephrine-induced tension under normal pharmacological conditions.

4.1. Intracellular Versus Extracellular Ca^{2+}

According to a classification scheme derived by Han and co-workers in 1987, α_1-adrenoceptors that induce an influx of extracellular Ca^{2+} are designated α_{1a}, while α_1-adrenoceptors that mediate PI hydrolysis are α_{1b}. More recently, however, Wilson and Minneman (1990) suggested that both α_1 subtypes induce inositol phosphate accumulation, but differ with respect to their mechanisms of activation. According to

Wilson and Minneman (1990), the α_{1a} subtype mediates PLC activity that is induced by Ca^{2+} influx, while the α_{1b} subtype activates a Ca^{2+} insensitive PLC.

The heterotrimeric guanine nucleotide-binding proteins (G proteins) that couple surface receptors to secondary effectors have been classified as members of the G_i/G_o, G_s, G_{12} or G_q families (Simon *et al.*, 1991). Upon stimulation of the receptor, the guanosine diphosphate (GDP) bound to the α subunit of a G protein is exchanged for GTP and the G protein trimer dissociates to the functional G_α subunit and G_β/G_γ complex. Gene transfection studies have demonstrated that α_{1b}-adrenoceptors can interact favourably with all four members of the $G_{\alpha q}$ family, $G_{\alpha 14}$, $G_{\alpha 16}$, $G_{\alpha q}$ and $G_{\alpha 11}$, coupling noradrenaline receptor-binding to inositol phosphate accumulation; however, α_{1a}-adrenoceptors can only interact favourably with $G_{\alpha q}$ and $G_{\alpha 11}$ (Wu *et al.*, 1992). *Bordetela pertussis* toxin catalyzes ADP-ribosylation at the α subunit of sensitive G proteins, uncoupling the protein from its associated receptor and inhibiting activities mediated by the dissociated G_α subunit and G_β/G_γ complex. The G_o/G_i family is sensitive to pertussis toxin, although most tissues are insensitive to bacterial toxins (Wu *et al.*, 1992; Smrcka *et al.*, 1991).

PLC is the enzyme responsible for hydrolyzing membrane phospholipids to intracellular second messengers. Three families of phosphoinositide selective PLC enzymes, β, δ and γ, have been identified and subclassified on the basis of their molecular structure and mechanism of regulation (Rhee & Choi, 1992). Although the activities of all three families are Ca^{2+} dependent, their mechanisms of activation appear to differ (Rhee & Choi, 1992). The β isozymes appear to be activated through G proteins coupling surface receptors (Wu *et al.*, 1992), the γ isozymes may be

activated when phosphorylated by a tyrosine kinase/receptor (Rhee & Choi, 1992), and the δ isozymes may be activated by Ca^{2+} (Hamet *et al.*, 1995).

PLC can be activated by both the G_α subunit or the G_β/G_γ complex (Katz *et al.*, 1992). However, gene transfection studies have demonstrated that certain G protein subunits couple significantly more effectively with specific PLC isozymes. For example, it has been reported that $G_{\alpha q}$ and $G_{\alpha 11}$ activated PLC-β1, but not the -γ1, -δ1 or -β2 isozymes (Wu *et al.*, 1992; Smrcka *et al.*, 1991; Aragay *et al.*, 1992; Park *et al.*, 1992; Lee *et al.* 1992). In addition, $G_{\alpha 16}$, but neither $G_{\alpha q}$ nor $G_{\alpha 11}$, activated PLC-β2 (Schwinn *et al.*, 1991; Lee *et al.*, 1992).

4.2. α_1-Agonist-Induced Contraction in Rat Caudal Artery

Unfortunately, the PLC isozymes in rat caudal artery have not yet been identified. However, the results of this study and results previously reported, strongly suggest that in rat caudal artery, α_{1a}-adrenoceptors are coupled through a $G_{\alpha q}/G_{\alpha 11}$ protein to PLC-β1 and PLC-δ1.

Agonist-induced contractile studies in the presence of the α_{1a}-adrenoceptor antagonist SZL-49 demonstrated that nerve terminals in rat caudal artery are only associated with α_{1a}-adrenoceptors (Piascik *et al.*, 1991). Therefore, PI hydrolysis in rat caudal artery is likely mediated through α_{1a}-adrenoceptors.

Rosenthal and colleagues (1988) noted that a number of systems, adrenocortical cells and rabbit pulmonary artery included, were sensitive to both pertussis toxin and dihydropyridine Ca^{2+} channel blockers. Nicholas and co-workers (1989) reported a similarity between the inhibition patterns of the dihydropyridine Ca^{2+}

channel blocker nifedipine and pertussis toxin on agonist-induced increased diastolic pressure in the pithed rat and suggested that tissues dependent on an influx of Ca^{2+} through voltage gated Ca^{2+} channels would also be sensitive to pertussis toxin. It was demonstrated in this study that pre-treatment with felodipine, but not pertussis toxin, affected phenylephrine-induced contractions in rat caudal artery. Furthermore, it has been reported that pre-treatment with pertussis toxin did not affect noradrenaline-induced PI turnover in rat caudal artery (Cheung *et al.*, 1990). Therefore, the G protein coupling the α_{1a}-adrenoceptor to PLC in rat caudal artery is pertussis toxin insensitive, and a member of one of the G_s, G_q or G_{12} families. According to Wu and co-workers (1992), the α_{1a}-adrenoceptor is effectively coupled to the $G_{\alpha q}$ family members $G_{\alpha q}$ and $G_{\alpha 11}$, and tissues expressing α_1-adrenoceptors almost always simultaneously express $G_{\alpha q}/G_{\alpha 11}$ proteins. Therefore, it is likely that PI hydrolysis in rat caudal artery is mediated through an α_{1a}-adrenoceptor coupled to a $G_{\alpha q}/G_{\alpha 11}$ protein.

The present study demonstrated (Figure 1) that inositol phosphate accumulation in rat caudal artery was critically dependent on the presence of extracellular Ca^{2+}. This is consistent with previously reported results; in rat caudal artery, exogenous Ca^{2+} enhanced noradrenaline-induced PI hydrolysis in a concentration dependent manner (Cheung *et al.*, 1990). Wilson *et al.* (1990) and Suzuki *et al.* (1990) suggested that activation of PLC by α_{1a}-adrenoceptor agonists is mediated through a G protein that activates sarcolemmal Ca^{2+} channels and the resulting Ca^{2+} influx activates PLC. The PLC isozyme most likely activated in this manner is PLC-δ1. Gene transfection studies may fail to identify the indirect coupling between $G_{\alpha q}/G_{\alpha 11}$ and PLC-δ1 if a compatible α_{1a}-adrenoceptor-operated Ca^{2+} channel is not included in the transfection system.

Therefore, PI hydrolysis in rat caudal artery is probably mediated through an α_{1a}-adrenoceptor coupled to a receptor-operated Ca^{2+} channel through a $G_{\alpha q}/G_{\alpha 11}$ protein; Ca^{2+} influx through the receptor-operated channel then activates PLC-δ1.

However, the present study also demonstrated that inositol phosphate accumulation was partially dependent on Ca^{2+} influx through felodipine sensitive Ca^{2+} channels. Inhibition by felodipine is unlikely to have resulted from non-specific intracellular effects as Ca^{2+}-induced contractions in permeabilized smooth muscle cells were not affected by pre-treatment with felodipine (1 nM) (Hagiwara *et al.*, 1993). Therefore, two mechanisms of PI hydrolysis appear to operate simultaneously in rat caudal artery, but differ with respect to the manner of Ca^{2+} influx required for PLC activation. It is possible that a single tissue may contain more than one phosphoinositide selective PLC system; PI turnover in human umbilical vein endothelial cells reportedly contains two different G proteins coupled to histamine and bradykinin receptors (Voyno-Yasenetskaya *et al.*, 1989).

In rat caudal artery, the second inositol phosphate pathway must share the α_{1a}-adrenoceptor with the PLC-δ1 pathway as the α_{1a}-subtype is reportedly the only α_1-adrenoceptor present (Piascik *et al.*, 1991). Similarly, a $G_{\alpha q}/G_{\alpha 11}$ protein likely mediates both the PLC-δ1 pathway and the second pathway. However, the second PLC isozyme is probably PLC-β1, as this is the PLC isozyme most effectively activated by $G_{\alpha q}/G_{\alpha 11}$ (Wu *et al.*, 1992; Smrcka *et al.*, 1991; Aragay *et al.*, 1992; Park *et al.*, 1992; Lee *et al.*, 1992). In the absence of extracellular Ca^{2+}, the second pathway is inactive, as is the PLC-δ1 pathway. Below 0.1 μM Ca^{2+}, PLC-β1 activity is reportedly negligible (Park *et al.*, 1992). Therefore, if the free intracellular Ca^{2+} concentration of a resting myocyte is

approximately 0.1 μM (Hartshorne, 1982), Ca^{2+} must enter the cell before PLC-β1 can operate effectively. These observations can be explained if the α_{1a}-adrenoceptor is coupled to an ion channel whose opening initiates a current that depolarizes the membrane and activates Ca^{2+} influx through felodipine sensitive channels. Subsequently, Ca^{2+} influx through the L-type channels sufficiently increases the intracellular Ca^{2+} concentration to allow activation of PLC-β1 upon α_1-agonist binding.

These observations are effectively summarized by an excitation-contraction mechanism loosely based on models proposed by Van Renterghem *et al.* (1988) and Nichols *et al.* (1989). Van Renterghem *et al.* (1988) described a mechanism of action for endothelin-I in A7r5 cells that addressed the observed dihydropyridine sensitivity of the agonist-induced contractions. Van Renterghem and co-workers suggested that agonist interaction activated Ca^{2+}-sensitive K^+ channels to induce a transient hyperpolarization followed by a sustained depolarization that opened non-specific cation channels, allowing an influx of Ca^{2+} and Mg^{2+}; this depolarization then activated L-type Ca^{2+} channels. Nichols *et al.* (1989) proposed a model for α_1-adrenoceptor mediated vasoconstriction in which the receptor was linked to two distinct G proteins, one sensitive to pertussis toxin and coupled the receptor to Ca^{2+} channels, and the other insensitive to pertussis toxin and involved in the mobilization of intracellular Ca^{2+}. Excitation-contraction coupling in the rat caudal artery appears to involve three α_{1a}-receptor-coupled G proteins; one coupled to a receptor-operated Ca^{2+} channel, another coupled to an ion channel whose opening depolarizes the cell and the last coupled directly to PLC-β1. Under normal pharmacological conditions, α_1-agonist binding simultaneously opens the two receptor-operated channels and readies PLC-β1 for

activation. The membrane is depolarized and Ca^{2+} enters the cell through voltage-gated channels and receptor-operated channels. The elevated intracellular Ca^{2+} concentration activates PLC-δ1 and completes the activation requirements for PLC-β1. However, in the presence of an L-type channel blocker, Ca^{2+} influx is reduced and subsequent activation of the Ca^{2+}-activated PLC-δ1 and Ca^{2+}-dependent PLC-β1 attenuated. In the absence of extracellular Ca^{2+}, neither excitation-contraction coupling system operates as both PLC isozymes rely to some extent on extracellular Ca^{2+}.

Membrane potential and intracellular Ca^{2+} concentrations have been strongly correlated in rat mesenteric artery (Nilsson *et al.*, 1994). Changes to the membrane potential of smooth muscle have been found to affect PLC activity; depolarization is positively associated with inositol phosphate accumulation, while hyperpolarization inhibits PI turnover (Itoh *et al.*, 1992). These observations are consistent with the model of excitation-contraction coupling described above. Although depolarization can result from an influx of cations, or efflux of anions, recent evidence favours the latter mechanism in α_1-adrenoceptor-mediated activation of L-type Ca^{2+} channels. Noradrenaline increased Cl^- efflux, while depolarizing and contracting rat mesenteric arteries, but it did not alter the rates of K^+ efflux or Na^+ influx (Videbæk *et al.*, 1990). In guinea-pig mesenteric veins a rapid, noradrenaline-induced depolarization had a reversal potential of -22 mV and was suppressed in a low-chloride solution (Van Helden, 1988). Furthermore, noradrenaline decreased the Cl^- concentration of rat portal veins without effecting Na^+ and K^+ concentration (Wahlström, 1973). It is unlikely that changes to the membrane potential are the result of contraction and relaxation, as drugs that cause relaxation (atrial natriuretic factor, substance P and sodium

nitroprusside) did not induce changes to the membrane potential (Videbæk *et al.*, 1990). Therefore, it appears that the ion channel activated by α_{1a}-adrenoceptor agonists is an anion channel, and Cl^- is the anion whose efflux is responsible for myocyte depolarization. However, altering the membrane potential is unlikely to affect a simple electrostatic response in excitable cells.

4.3. Ryanodine Sensitive Ca^{2+} Store in Rat Caudal Artery

In rat caudal artery, ryanodine inhibited phenylephrine-induced contractions, indicating that a ryanodine sensitive contractile mechanism is present in the tissue and required for maximal α_1-agonist-induced contraction. Because phenylephrine-induced contractions were abolished in the absence of extracellular Ca^{2+}, all the α_1-adrenoceptor-mediated excitation-contraction coupling pathways in caudal artery , including that sensitive to ryanodine, are critically dependent on extracellular Ca^{2+}. The Ca^{2+}-induced Ca^{2+} release mechanism effectively explains these results within the context of the α_1-adrenoceptor excitation-contraction model described above. Under normal pharmacological conditions, α_1-adrenoceptor agonists appear to activate Ca^{2+} influx through receptor-operated and voltage-gated channels. In addition to regulating PLC activity, the increased intracellular Ca^{2+} may initiate Ca^{2+} release from the ryanodine sensitive store. Iino (1989) provided direct evidence for the existence of a Ca^{2+}-induced Ca^{2+} release mechanism in guinea-pig taenia caeci smooth muscle; therefore, it is likely that similar Ca^{2+}-regulated release mechanism exists in caudal artery smooth muscle.

The inhibitory effects of ryanodine in rat caudal artery were significantly increased with the simultaneous addition of 8-bromo-cGMP. This suggests that, (1) intracellular Ca^{2+} release from ryanodine-sensitive stores is not regulated by 8-bromo-cGMP, (2) the ryanodine-sensitive Ca^{2+} store and IP_3-regulated Ca^{2+} store are functionally distinct in the rat caudal artery, and (3) the ryanodine-sensitive and IP_3-regulated Ca^{2+} stores are functionally isolated. Functionally and spatially distinct intracellular Ca^{2+} stores have been identified in vascular smooth muscle cells cultured from arterial myocytes (Tribe *et al.*, 1994).

4.4. α_1-Agonist-Induced Contraction in Rat Thoracic Aorta

Previously reported results and the results from this study suggest that in rat thoracic aorta α_1-adrenoceptors are couple through a pertussis toxin insensitive G_α protein to PLC-δ1. Little work has been done to indicate which G protein couples α_1-adrenoceptors and PLC activity in rat thoracic aorta. Therefore, speculating with respect to the identity of the G protein is impossible. However, while pertussis toxin reportedly did not impair noradrenaline-induced contractions in rat aorta, cholera toxin did (Tabrizchi, 1994).

In rat thoracic aorta, PLC-γ1 and PLC-δ1 isozymes, but not PLC-β1, were identified, and only the PLC-γ1 subtype was activated by angiotensin II-induced tyrosine phosphorylation (Marrero *et al.*, 1994). Previously, two PLC enzymes whose activities increased with increasing Ca^{2+} had been characterized in rat thoracic aorta (Griendling *et al.*, 1991). The results of the current study demonstrate that contraction in rat thoracic aorta is, in a small part, sensitive to inhibition by felodipine. This

suggests that α_1-adrenoceptor mediated contraction in rat thoracic aorta relies heavily on intracellular Ca^{2+}. This is consistent with the previously reported observations. Phenylephrine induced a phasic contraction in rat thoracic aorta in the absence of extracellular Ca^{2+} (Nishimura *et al.*, 1991), and α_1-agonist-induced PLC activation in rat aorta does not depend on an influx of Ca^{2+} through voltage-operated channels as nifedipine did not affect IP_3 accumulation to noradrenaline, phenylephrine or cirazoline (Chiu *et al.* 1987; Legan *et al.* 1985). Noradrenaline-stimulated Ca^{2+} efflux from rabbit aorta was transiently increased in the presence and absence of extracellular Ca^{2+} (Collins *et al.*, 1986). Furthermore, a thromboxane A_2-induced increase in intracellular Ca^{2+} was not affected by removal of extracellular Ca^{2+} and was associated with an increased accumulation of IP_3 (Dorn II & Becker, 1992).

However, other studies have suggested that PLC activity in rat aorta is dependent on extracellular Ca^{2+} to some extent. A study by Rapoport (1987) using rat aorta demonstrated that noradrenaline-induced contraction and PI hydrolysis in a Ca^{2+}-free buffer were greatly reduced, but not completely abolished. Moreover, the addition of noradrenaline to rat aortic rings produced a notably reduced phasic contraction in the absence of extracellular Ca^{2+} (Manolopoulos *et al.*, 1991). Only 38 % of noradrenaline-induced Ca^{2+} influx was insensitive to nisoldipine (Morel & Godfriand, 1991). Finally, noradrenaline induced a phasic contraction and an increase in inositol phosphate accumulation in rat aorta in the absence of extracellular Ca^{2+}, although PI turnover was less than that in a physiological solution containing Ca^{2+} (Heaslip & Sickels, 1989). These results suggest that although PI hydrolysis in rat thoracic aorta

is not critically dependent on extracellular Ca^{2+}, contraction can be maximally induced when the ion is present.

4.5. Ryanodine Sensitive Ca^{2+} Store in Rat Thoracic Aorta

In rat thoracic aorta, ryanodine did not affect α_1-agonist-induced contractions, which suggests that maximal contraction in aortic tissue is not affected through a ryanodine sensitive pathway under normal pharmacological conditions. These results do not rule out the possibility, however, that a ryanodine sensitive contractile pathway in aortic tissue is activated only after the dominant excitation-contraction mechanisms are impaired. This possibility is likely as Julou-Schaeffer & Freslon (1988) found that ryanodine inhibited noradrenaline-induced contraction in rat aorta only after Ca^{2+} had been removed from the extracellular fluid. Similarly, Low and co-workers (1993) reported that phenylephrine-induced contractions were inhibited by ryanodine in a Ca^{2+}-free medium. Furthermore, ryanodine induced a slowly developing rise in aortic tension, yet subsequent addition of noradrenaline induced a contractile response that was not significantly different from that of the control (Julou-Schaeffer & Freslon, 1988). Tabrizchi (1994) reported that noradrenaline-induced contractions in aortic rings could not be impaired by ryanodine unless the animal had been pretreated with the α-adrenoceptor alkylating agent, phenyoxybenzamine. It appears, therefore, that ryanodine inhibits Ca^{2+} release from an intracellular pool in aortic tissue, but maximum contraction following α_1-adrenoceptor activation in rat thoracic aorta is not dependent on release from this pool.

4.6. cGMP and Inositol Phosphate Accumulation

The results of this study demonstrate that 8-bromo-cGMP does not block inositol phosphate accumulation in rat caudal artery. This is consistent with previously reported results (Eskinder *et al.*, 1989; Ko *et al.*, 1992; Puurunen *et al.*, 1987), and indicates that the nucleotide neither interacts with PLC directly, nor interferes with the agonist-induced influx of extracellular Ca^{2+} required for PLC activity.

8-Bromo-cGMP and felodipine failed to induce additive inhibition of contraction when applied together. This suggests that the two inhibitors appear to operate along the same excitation-contraction coupling pathway. Since felodipine, but not 8-bromo-cGMP, blocks inositol phosphate accumulation, the nucleotide's inhibition of the PI-mediated contractile pathway appears to occur subsequent to IP_3 production. Evidence of an interaction between cGMP and the IP_3 receptor has been reported. A cGMP-dependent protein kinase that closely resembles the IP_3 receptor has been discovered (Koga *et al.*, 1994). Furthermore, purified rat cerebellum IP_3 receptors are stoichiometrically phosphorylated at the serine-1755 residue by cGMP-dependent protein kinases (Komalavilas & Lincoln, 1994). Results from bovine trachea and rat aortic smooth muscle cells suggest that the effects of cGMP are mediated through a cGMP dependent kinase (Felbel *et al.*, 1988; Cornwell & Lincoln, 1989).

The results of this study, however, do not rule out the possibility that cGMP hastens the rate of metabolism of IP_3 by inositol 1,4,5-trisphosphate 3-kinase to inositol 1,3,4,5-tetrakisphosphate ($Ins(1,3,4,5)P_4$) (Irvine *et al.*, 1986) or by inositol trisphosphate 5-phosphatase to inositol 1,4-bisphosphate (IP_2). It is unlikely, however, that the rate IP_3 metabolism by these two enzymes is increased significantly by cGMP

as they naturally operate quite rapidly (Irvine *et al.*, 1986; Storey *et al.*, 1984). However, if cGMP had accelerated IP_3 hydrolysis, the assay used in this study to measure inositol phosphate accumulation may not have recorded this effect. The Li^+ used in this study would have prevented recycling of most of the inositol phosphates back to inositol and the assay would have recorded them. However, IP_2 is incapable of releasing Ca^{2+} from the IP_3 sensitive intracellular stores (Stauderman *et al.*, 1988) and Ins(1,3,4,5)P_4 Is reportedly involved in Ca^{2+} re-uptake into the intracellular store (Irvine and Moor, 1987) and activation of Ca^{2+}-dependent K^+ channels (Morris *et al.*, 1987). Therefore, we may have been recording the presence of ineffective IP_3 metabolites which are incapable of eliciting contraction.

4.7. Experimental Design

Many studies have investigated the inhibitory effects of antagonists at a single agonist concentration. The importance of testing antagonist effects over an agonist concentration range is apparent from the felodipine experiments in the present study. Although a qualitative decrease in PI hydrolysis was apparent over the entire phenylephrine concentration range, significant changes could only be reported for three concentrations of the agonist. We also found that a supermaximal concentration of phenylephrine could restore maximum PI turnover in the presence of felodipine. This may be indicative of an α_1-adrenoceptor reserve. Although PI response does not normally demonstrate a significant receptor reserve (Michell & Kirk, 1981), it must be sufficient in the caudal artery to overcome the effects of felodipine.

Berta and co-worker (1986), investigating the influence of extracellular Ca^{2+} on serotonin- and phenylephrine-induced contractions and phosphoinositide metabolism in

rat caudal artery, reported that although phenylephrine-induced contractions were completely abolished by removal of extracellular Ca^{2+}, PI hydrolysis was unaffected by the omission. These results indicated that although extracellular Ca^{2+} was required for contraction, it was not necessary for PLC activity. However, according to Cheung *et al.* (1990), agonist stimulated PI hydrolysis in rat caudal artery reaches a maximum at 5 mM extracellular Ca^{2+} with an EC_{50} of about 80 μM. Therefore, the 0.5 mM EDTA used by Berta to chelate Ca^{2+} may have been insufficient to completely immobilize the Ca^{2+} contributed to the bathing medium by the other ingredients; and, aggressive concentrations of chelating agents are required to ensure a Ca^{2+}-free environment.

4.8. Conclusion

The comparison of phenylephrine-induced contractions in rat caudal artery and rat thoracic aorta performed in this study has demonstrated that there is a significant difference between the relative contributions of intracellular and extracellular Ca^{2+} to the excitation-contraction coupling mechanisms in these two tissues. Contraction in rat caudal artery is critically dependent on extracellular Ca^{2+} mediated, in part, through dihydropyridine-sensitive channels. The contractile mechanism in rat thoracic aorta, however, is not significantly dependent on Ca^{2+} influx through voltage dependent channels nor on Ca^{2+} release from ryanodine sensitive stores under normal physiological conditions. Contraction in rat caudal artery, however, is sensitive to inhibition by ryanodine.

α_1-Agonist-induced contractions in rat caudal artery are not sensitive to pertussis toxin; therefore, the G protein coupling the α_1-adrenoceptor and PLC activity is not a member of the G_i/G_s family.

This study also demonstrated that although 8-bromo-cGMP does not inhibit PLC activity in rat caudal artery, the nucleotide does impair α_1-adrenoceptor-induced contraction. Inhibition of contraction by felodipine and 8-bromo-cGMP occurs along the same excitation-contraction coupling pathway. Since felodipine blocks inositol phosphate accumulation, 8-bromo-cGMP likely blocks IP_3-induced Ca^{2+} release from intracellular stores. However, 8-bromo-cGMP does not appear to affect the ryanodine-sensitive contractile mechanism which mediates Ca^{2+} release from an IP_3-insensitive intracellular pool.

The results of this study confirm what Bohr and Goulet had suspected and predicted in 1961; that the mechanisms of contraction in the conduit vessels of the vascular circulatory system are unique to the individual vessel.

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