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Effects of 8-bromoguanosine 3':5'-cyclic monophosphate on phenylephrine-induced phosphatidylinositol hydrolysis and contraction in rat caudal artery

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1 The effects of 8-bromoguanosine 3':5'-cyclic monophosphate (8-bromo-cyclic GMP) on phenylephrine-induced contractions and phosphatidylinositol (PI) hydrolysis were investigated in rat isolated 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 purpose of this investigation was to examine the regulatory effects of cyclic GMP on receptor-mediated signal transduction in vascular smooth muscle.

2 Phenylephrine induced a concentration-dependent increase in PI hydrolysis that reached a maximum at 10 μM phenylephrine. Pre-incubation with felodipine (10 nM) significantly reduced PI turnover, but did not affect basal hydrolysis. Similarly, removal of extracellular Ca^{2+} (2 mM ethylene glycol-*bis*(β-amino-ethyl ether) *N*, *N*, *N'*, *N'*-tetraacetic acid (EGTA)) blocked phenylephrine-induced PI hydrolysis, but did not affect basal turnover. In contrast, 8-bromo-cyclic GMP (10 μM) did not affect phenylephrine-induced PI hydrolysis, nor did it affect basal turnover.

3 Phenylephrine induced concentration-dependent contractions that were inhibited by each of 8-bromo-cyclic GMP (10 μM), felodipine (1 nM and 10 nM) and ryanodine (3 μM and 10 μM). In addition, removal of Ca^{2+} from the physiological salt solution (2 mM EGTA) completely abolished contractions elicited by phenylephrine.

4 Phenylephrine-induced contractions were not further affected by felodipine and 8-bromo-cyclic GMP applied concomitantly than by equivalent concentrations of felodipine alone. However, ryanodine and 8-bromo-cyclic GMP applied together significantly inhibited phenylephrine-induced contractions in comparison to ryanodine alone.

5 These results suggest that phospholipase C-activated PI hydrolysis in the rat caudal artery is dependent on extracellular Ca^{2+}, mediated, in part, through dihydropyridine-sensitive Ca^{2+} channels. Inhibition of contraction by felodipine may be brought about through indirect inhibition of IP_3 production and subsequent attenuation of intracellular Ca^{2+} release. 8-Bromo-cyclic GMP does not inhibit PI hydrolysis; it 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-cyclic GMP affects ryanodine-sensitive stores.

Keywords: Calcium; cyclic GMP; felodipine; inositol 1,4,5-trisphosphate; phenylephrine; phospholipase C; ryanodine; vascular smooth muscle

Introduction

The increase in cytosolic Ca^{2+} that induces and sustains contraction in vascular smooth muscle can be inhibited by several mechanisms, including an increase in guanosine 3':5'-cyclic monophosphate (cyclic GMP) (Gruetter *et al.*, 1979; Rapoport & Murad, 1983) by guanylate cyclase (Furchgott *et al.*, 1984). The mechanism by which elevated levels of cyclic GMP regulates cytosolic Ca^{2+} is not completely understood, although several hypotheses have been proposed. Cyclic GMP may reduce Ca^{2+} influx (Collins *et al.*, 1985) through L-type Ca^{2+} channels (Ishikawa *et al.*, 1993) or reduce Ca^{2+} release from intracellular stores (Collins *et al.*, 1986). It may activate extrusion of Ca^{2+} through a Ca^{2+}-ATPase in the plasma membrane (Rashatwar *et al.*, 1987) or initiate sequestration by the sarcoplasmic reticulum (Twort & van Breemen, 1988). One, or more likely, a combination of these and perhaps other mechanisms regulate vascular smooth muscle contraction under the direction of cyclic GMP.

Inositol 1,4,5-trisphosphate (IP_3) is an intracellular second messenger produced by hydrolysis of phosphatidylinositol 4,5-bisphosphate (PIP_2) following activation of phospholipase C (PLC) by an extracellular signal (Berridge, 1983; Berridge & Irvine, 1984). IP_3 activates a receptor-operated channel on the sarcoplasmic reticulum (Berridge, 1993). Ca^{2+} is released through the channel and induces contraction (Somlyo *et al.*, 1985). The IP_3 signal transduction pathway has been identified as a possible target for regulation by cyclic GMP. However, conflicting evidence has been presented with respect to the site of action for this nucleotide. Production of inositol phosphates was reportedly inhibited by 8-bromo-cyclic GMP in human platelets (Nakashima *et al.*, 1986) and in rat thoracic aorta (Rapoport, 1986). These results suggested that cyclic GMP could inhibit PLC hydrolysis. In contrast, Eskinder *et al.* (1989) found that 8-bromo-cyclic GMP did not inhibit noradrenaline-induced PI hydrolysis in canine femoral artery, and Ko *et al.* (1992) found that the vasorelaxant, osthole, increased the level of cyclic GMP in aortic tissue but did not affect inositol monophosphate formation. Moreover, Puurunen *et al.* (1987) reported that dibutyryl-cyclic GMP did not modify the carbachol-induced formation of inositol phosphates in gastric mucosal cell preparations isolated from rats. Of particular interest is a recent report that cyclic GMP could block the IP_3 pathway by indirectly phosphorylating and thus inhibiting the IP_3 receptor at the sarcoplasmic reticulum (Komalavilas &

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Lincoln, 1994). However, Ca^{2+} release from the sarcoplasmic reticulum has been found to be unaffected by cyclic GMP in rat aortic cells (Twort & van Breemen, 1988).

In rat detrusor and urethral muscle, the IP_3-mediated intracellular signalling pathway is dependent on extracellular Ca^{2+} (Garcia-Pascual *et al.*, 1993). Removal of extracellular Ca^{2+} or addition of the Ca^{2+} channel antagonist, nifedipine, has been found to inhibit effectively PI hydrolysis in the rat aorta (Rapoport, 1987). Cyclic GMP has been proposed to regulate Ca^{2+} influx through L-type channels (Collins *et al.*, 1985; Ishikawa *et al.*, 1993), and could, therefore, block IP_3-mediated functional effects by inhibiting the influx of the PLC co-factor, Ca^{2+}. In the present study, we have examined the effects of felodipine (an L-type Ca^{2+} channel antagonist), 8-bromo-cyclic GMP and Ca^{2+}-free buffer on phenylephrine-induced PI hydrolysis in rat caudal artery. In addition, we have compared the effects of 8-bromo-cyclic GMP to those of felodipine, ryanodine (a putative depletor of intracellular Ca^{2+} stores) and Ca^{2+}-free buffer on phenylephrine-induced contractions in the same preparation.

Methods

Measurement of phosphatidylinositol hydrolysis

Male Sprague-Dawley rats (300–400 g) were anaesthetized with sodium pentobarbitone (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 gently rubbing.

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 min; the incubating buffer was changed every 20 min. The tissues were transferred into 2 ml of fresh buffer containing 6 μCi ml^{-1} [^{3}H]-*myo*-inositol to load for 90 min. Loaded tissues were washed five times with ice-cold Krebs solution before being put into individual assay tubes containing 10 mM LiCl in 300 μl buffer at 37°C. 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-cyclic GMP (10 μM) or twice distilled water (9 μl), and tissues were allowed to incubate for 20 min before phenylephrine (0.3–30.0 μM) from serial dilutions or twice 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 min by addition of 300 μl of ice-cold trichloroacetic acid (1 M) to each sample. Tubes were left on ice for 30 min 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) was 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 (×8) anion-exchange columns (formate form, 100–200 mesh, 1 ml). Columns were washed with 12 ml of unlabelled *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.

Contractile studies

Caudal arteries were isolated as described above 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. The tissues were equilibrated for 60 min. 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 then left for 30 min before a control cumulative concentration-response curve to phenylephrine (0.01 μM–100 μM) was constructed. Following construction of control concentration-response curves, felodipine (1 and 10 nM), ryanodine (3 and 10 μM), 8-bromo-cyclic GMP (10 μM), distilled water (6 or 20 μl) or a combination of felodipine and 8-bromo-cyclic GMP or ryanodine and 8-bromo-cyclic GMP were left in contact with the tissues for 30 min to be followed by construction of another concentration-response curve to phenylephrine. Tissues were allowed to equilibrate for 60 min 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 min before a concentration-response curve to phenylephrine was constructed. Ca^{2+} was re-introduced by replacing Ca^{2+}-free buffer with normal Krebs solution.

Data and statistical analysis

In the absence of an antagonist, PI hydrolysis reached a maximum at 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. Percentage of maximum, Hill coefficient and EC_{50} values were calculated for individual curves using a programme 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 by Student's unpaired *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.

Chemicals

8-Bromoguanosine 3':5'-cyclic monophosphate sodium salt (8-bromo-cyclic GMP), (–)-phenylephrine HCl and ethylene glycol-*bis*(β-amino-ethyl ether) *N,N,N',N'*-tetraacetic acid (EGTA) were purchased from Sigma Chemical Co. (Ca., U.S.A.). *Myo*-[2-^{3}H(N)]-inositol (17.0 Ci $mmol^{-1}$) and ryanodine were purchased from Amersham (Ont., Canada) and Calbiochem (Ca., U.S.A.), respectively. Felodipine was a gift

from Hässle (Sweden). With the exception of felodipine, all drug solutions were prepared in twice distilled water. A 10 mM felodipine stock solution was made in 80% ethanol; dilutions were made in twice distilled water. All other chemicals were purchased from Fischer Scientific (B.C., Canada).

Results

Phosphatidylinositol hydrolysis

Phenylephrine (0.3–30 μM) increased PI hydrolysis in a concentration-dependent manner. A maximum turnover, approximately 10 times greater than basal d.p.m. mg^{-1} wet weight, was achieved at a concentration of 10 μM phenylephrine (Figure 1). We found that addition of phenylephrine greater than 30 μM significantly decreased PI hydrolysis when compared to maximal PI turnover (data not shown).

Phenylephrine-induced PI hydrolysis was not affected by 8-bromo-cyclic GMP (10 μM) and maximal turnover remained approximately 10 fold above basal d.p.m. mg^{-1} wet weight (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 turnover was only 7 fold above basal d.p.m. mg^{-1} wet weight. We also found that PI turnover 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 turnover was not affected by 8-bromo-cyclic GMP, felodipine or Ca^{2+}-free buffer.

Contractile studies

Concentration-response curves to phenylephrine were displaced to the right in the presence of 8-bromo-cyclic GMP (10 μM) (Figure 2). 8-Bromo-cyclic GMP significantly ($n=6$; $P<0.05$) decreased the maximum tension and increased the EC_{50} of the concentration-response curve without altering the Hill coefficient. These effects were unchanged by 8-bromo-cyclic GMP (10 μM) in a subsequent concentration-response curve to phenylephrine (Table 1).

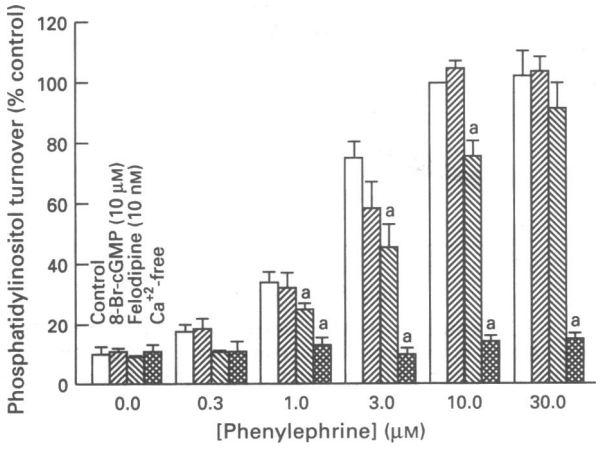

Figure 1 Phosphatidylinositol turnover induced by phenylephrine in the absence (open column) and presence of 10 μM 8-bromo-cyclic GMP (left-to-right hatched column) or 10 nM felodipine (right-to-left hatched column) or Ca^{2+}-free buffer (2 mM EGTA) (cross-hatched column). Percentage turnover was calculated relative to the maximum turnover induced by 10 μM phenylephrine in the absence of an antagonist. Basal turnover for control and treated tissues was 215 ± 20 d.p.m. mg^{-1} wet weight (mean ± s.e., $n=24$). Each column represents the mean of six experiments ± s.e. [a]Significantly different from control ($P<0.05$).

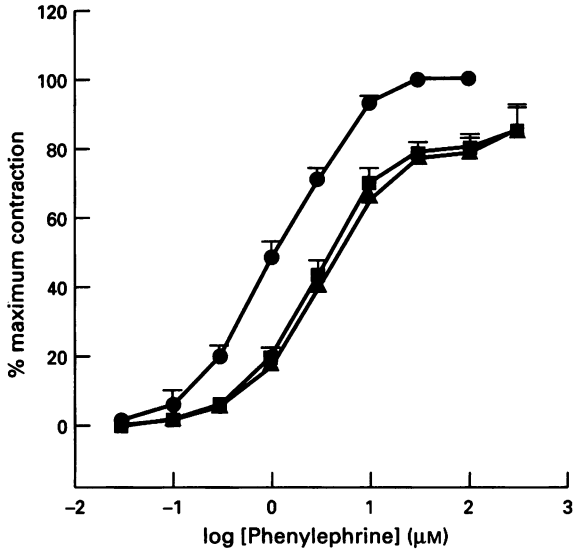

Figure 2 Concentration-response curves to phenylephrine in the absence (●) or in the presence of two consecutive concentrations of 8-bromo-cyclic GMP (10 μM), for the first time (■) and for the second time (▲). Each point represents the mean of six experiments ± s.e.

In the presence of felodipine (1 and 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 76.91 ± 7.78% and 56.63 ± 3.41% of control, respectively. However, the EC_{50} and Hill coefficient values were unchanged in the presence of felodipine (Table 1). Ryanodine (3 and 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 1). 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 1).

Contractions induced by phenylephrine were not further inhibited by simultaneous application of 8-bromo-cyclic GMP and felodipine as compared to felodipine alone (Table 1). 8-Bromo-cyclic GMP did not accentuate the inhibitory action of felodipine at either 1 or 10 nM as compared to felodipine alone (Figure 5). In contrast, concomitant application of 8-bromo-cyclic GMP and ryanodine significantly ($n=6$; $P<0.05$) inhibited contractions induced by phenylephrine in comparison to ryanodine alone (Table 1). Ryanodine alone, at 3 μM and 10 μM, lowered the maximum contraction to 80.71 ± 6.70% and 75.96 ± 7.65% of control, respectively, and when combined with 8-bromo-cyclic GMP these values were further reduced by 29% and 38% of control, respectively (Figures 4 and 6).

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, replacement of Ca^{2+}-free buffer with normal Krebs solution restored contraction to phenylephrine without affecting the maximum tension, Hill coefficient or EC_{50} in comparison to the control (Table 1).

Discussion

The results of this investigation demonstrate that phenylephrine-induced PI hydrolysis and contractions in the caudal artery are dependent upon extracellular Ca^{2+}. Felodipine-sensitive Ca^{2+} channels appear to regulate, in part, the influx of extracellular Ca^{2+} for PLC activation. As 8-bromo-cyclic GMP does not block production of inositol phosphates its

Table 1 EC_{50} Hill coefficient (n_H) and % maximum response values obtained from individual concentration curves in caudal artery preparations

Groups	*EC_{50} (μM)*	*n_H*	*% maximum*
Control	1.25±0.18	1.10±0.06	102±1
8-Br-cGMP (10 μM)	2.65±0.35[a]	1.32±0.07	81±3[a]
8-Br-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-cGMP (10 μM)	2.60±0.25[a]	1.21±0.05	74±4[a]
Fel (10 nM)+8-Br-cGMP (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
Ryan (3 μM)+8-Br-cGMP (10 μM)	3.53±0.81	1.20±0.09	52±7[ac]
Ryan (10 μM)+8-Br-cGMP (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
Normal Krebs	1.01±0.12	1.20±0.08	103±3
Control	1.48±0.03	1.01±0.07	103±1
Twice distilled water (6 μl)	1.56±0.31	1.10±0.05	102±4
Twice distilled water (20 μl)	1.52±0.25	1.05±0.07	103±6

Each value represents the mean of six experiments ± s.e.
Fel = felodipine; Ryan = 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.

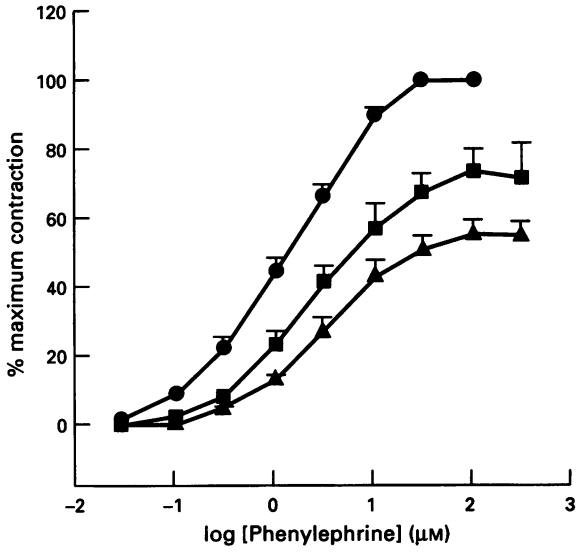

Figure 3 Concentration-response curves to phenylephrine in the absence (●) or in the presence of felodipine, 1 nM (■) and 10 nM (▲). Each point represents the mean of six experiments ± s.e.

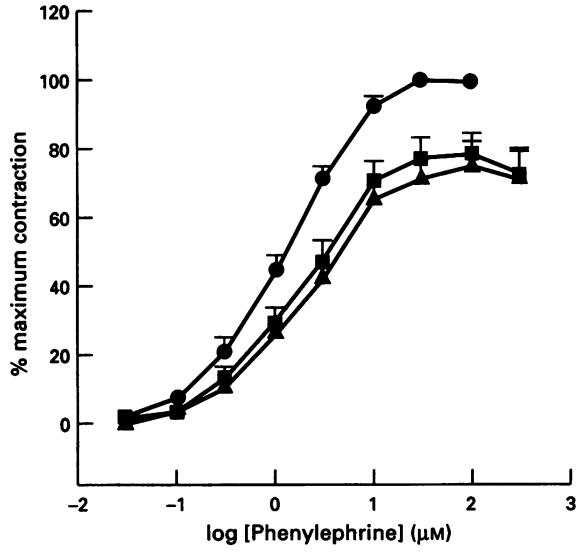

Figure 4 Concentration-response curves to phenylephrine in the absence (●) or in the presence of ryanodine, 3 μM (■) and 10 μM (▲). Each point represents the mean of six experiments ± s.e.

regulation of contraction is not mediated through inhibition of PI hydrolysis in rat caudal artery.

PLC activation following the stimulation of α_1-adrenoceptors has been reported to be mediated via the activation of G_q/G_{11} guanine nucleotide-binding protein (Lepretre *et al.*, 1994). α_1-Adrenoceptor agonists can, therefore, be expected to induce an increase in cytosolic IP_3 concentration and subsequently initiate IP_3-dependent functional changes. In the rat caudal artery, α_1-adrenoceptor agonist stimulation failed to induce PI hydrolysis and contraction when Ca^{2+} was omitted from the extracellular fluid. This suggests, (1) that PLC activity in this tissue is dependent on extracellular Ca^{2+}, consistent with Cheung *et al.* (1990), and (2) that rat caudal artery contraction is critically dependent upon an influx of Ca^{2+}.

Unlike caudal artery α_1-adrenoceptor activation, stimulation of aortic α_1-adrenoceptors in Ca^{2+}-free buffer induces a phasic contractile response that is generally accepted to result from IP_3-induced intracellular Ca^{2+} release (Heaslip & Sickels,

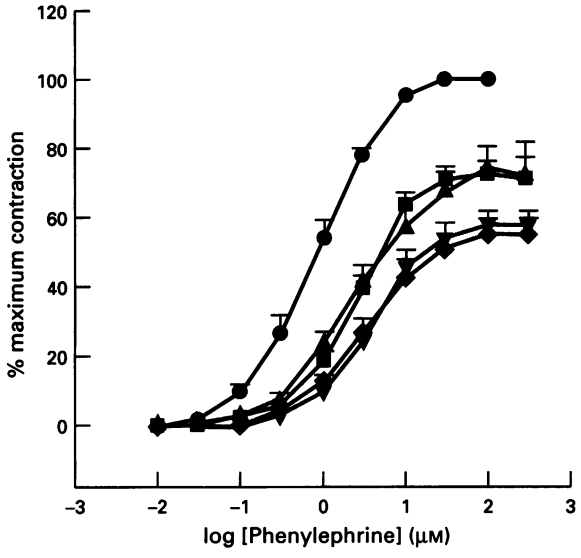

Figure 5 Concentration-response curves to phenylephrine in the absence (●) and in the presence of 1 nM felodipine and 10 μM 8-bromo-cyclic GMP (■) or 10 nM felodipine and 10 μM 8-bromo-cyclic GMP (▼). Concentration-response curves to phenylephrine in the presence of 1 nM felodipine alone (▲) and 10 nM felodipine alone (◆). Each point represents the mean of six experiments ± s.e.

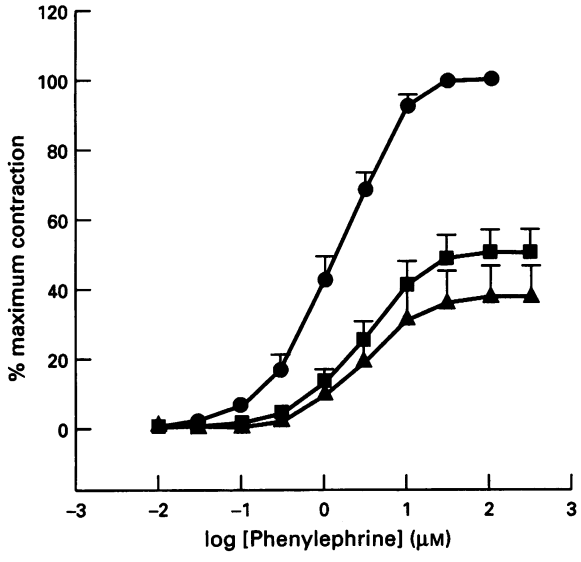

Figure 6 Concentration-response curves to phenylephrine in the absence (●) and in the presence of 3 μM ryanodine and 10 μM 8-bromo-cyclic GMP (■) or 10 μM ryanodine and 10 μM 8-bromo-cyclic GMP (▲). Each point represents the mean of six experiments ± s.e.

1989; Manolopoulos *et al.*, 1991). However, contractions observed in rat aortic rings by Manolopoulos and co-workers (1991) and Heaslip & Sickels (1989) were recorded in physiological solutions containing 0.4 mM and 1.0 mM EGTA, respectively. According to Cheung *et al.* (1990), agonist-stimulated PI hydrolysis reaches a maximum at 5 mM extracellular Ca^{2+} with an EC_{50} of about 80 μM in the rat caudal artery. Studies carried out by Rapoport (1987) using rat aorta have shown that noradrenaline-induced contraction and PI hydrolysis in Ca^{2+}-free buffer containing 2 mM EGTA could be greatly reduced. The results of the latter study suggest that in rat aorta PLC activity and contraction are dependent in part on an influx of Ca^{2+}. Furthermore, a study of PI hydrolysis and contraction in rabbit mesenteric artery found that noradrenaline induced a phasic contraction and transient rise in IP_3 in a Ca^{2+}-free solution which contained 2 mM EGTA (Itoh *et al.*, 1992). Thus it appears that PI hydrolysis and contraction in the absence of extracellular Ca^{2+} in vascular smooth muscle is tissue- and/or species-specific.

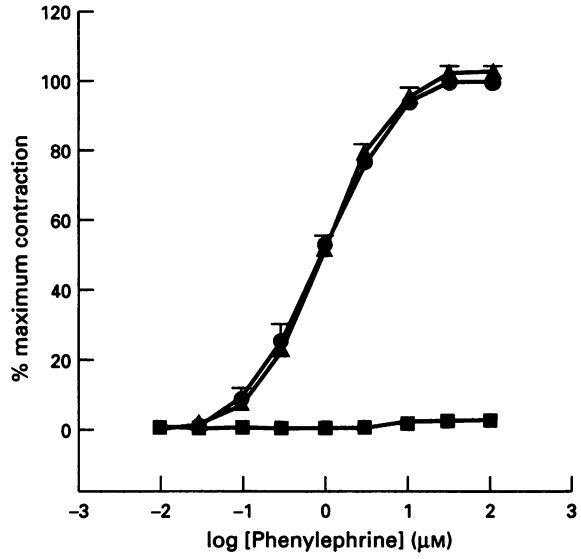

Figure 7 Concentration-response curves to phenylephrine in the presence of Ca^{2+} (●), in the absence of Ca^{2+} (2 mM EGTA) (■) and after Ca^{2+} had been reintroduced (▲). Each point represents the mean of six experiments ± s.e.

Chiu *et al.* (1987) and Legan *et al.* (1985) have also reported that α_1-agonist-induced PLC activation in rat aorta does not depend upon an influx of Ca^{2+} via voltage-operated Ca^{2+} channels as blockade of Ca^{2+} influx by nifedipine does not affect IP_3 accumulation to noradrenaline, phenylephrine or cirazoline. However, these studies investigated only antagonist effects 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 be reported only for three concentrations of the agonist. We also found that a supramaximal concentration of phenylephrine could restore maximum PI turnover in the presence of felodipine.

In the present study, we found that in the presence of felodipine, phenylephrine-induced PI turnover in the rat caudal artery was significantly reduced. This suggests that the influx of extracellular Ca^{2+} required for PLC activation occurred, in part, through voltage-gated dihydropyridine-sensitive Ca^{2+} channels. A model describing α_1-adrenoceptor activation of PLC in the rat caudal artery must consider the cellular depolarization required to open L-type channels. Van Renterghem *et al.* (1988) previously described a mechanism of action for endothelin-1-induced vasoconstriction in the rat aortic cell line A7r5 that effectively addressed the dihydropyridine-sensitivity of the agonist-induced contractions. Van Renterghen *et al.* (1988) suggested that endothelin-1-stimulates, (1) the release of intracellular Ca^{2+} through production of IP_3, and (2) the opening of non-specific cation channels. α_1-Adrenoceptor agonists may operate in a similar, but not identical, manner. α_1-Adrenoceptor activation has also been linked to an increase in chloride ion efflux (Videbaek *et al.*, 1990) which could cause membrane depolarization and activation of voltage-dependent Ca^{2+} channels.

In the present study, we also found that 8-bromo-cyclic GMP reduced maximum phenylephrine-induced tension, yet it did not block PI turnover. This is consistent with previously reported results and indicates that the nucleotide does not in-

teract with PLC directly, nor does it interfere with the enzyme's Ca^{2+} source (Puurunen *et al.*, 1987; Eskinder *et al.*, 1989; Ko *et al.*, 1992). Applying 8-bromo-cyclic GMP and felodipine together, yet failing to induce additive inhibition of contraction, demonstrates that the two inhibitors operate alone the same pathway. Since felodipine but not 8-bromo-cyclic GMP, blocks PI turnover, inhibition of the PI-mediated contractile pathway by the nucleotide appears to occur subsequent to IP_3 production; the IP_3 receptor on the sarcoplasmic reticulum is a likely candidate. Support for this proposal comes from the finding that the purified IP_3 receptor is phosphorylated *in vitro* by cyclic GMP-dependent protein kinases (Komalavilas & Lincoln, 1994), and the discovery of a cyclic GMP-dependent protein kinase substrate that closely resembles the IP_3 receptor (Koga *et al.*, 1994).

A second intracellular Ca^{2+} release pathway, sensitive to ryanodine, has been proposed to exist in brain microsomes (White *et al.*, 1993; Mészáros *et al.*, 1993). Ryanodine activates a sarcoplasmic reticulum receptor, opening a Ca^{2+} channel, depleting the intracellular Ca^{2+} store and inhibiting subsequent receptor activation (Hwang & van Breemen, 1987; Julou-Schaeffer & Freslon, 1988). In the rat aorta, ryanodine (10 μM) inhibited contractile responses to noradrenaline only after Ca^{2+} had been removed from the extracellular fluid (Julou-Schaeffer & Freslon, 1988). This indicated that ryanodine interfered with the release of intracellular Ca^{2+} but maximum contraction was not dependent on this mechanism. In the caudal artery, however, ryanodine reduced the contractile response to phenylephrine in the presence of extracellular Ca^{2+}. This suggests the presence of a ryanodine-sensitive intracellular Ca^{2+} pool in the rat caudal artery that is critical for maximum contraction. The observation that phenylephrine-induced contractions were abolished in the absence of extracellular Ca^{2+} suggests that both intracellular Ca^{2+} release pathways, that through the IP_3 receptor and that through the ryanodine-sensitive receptor, are dependent on extracellular Ca^{2+}. Therefore, it appears that the relative contributions of Ca^{2+} influx, IP_3-mediated intracellular Ca^{2+} release and ryanodine-sensitive Ca^{2+} release to agonist-induced contraction differ significantly between caudal and aortic tissue.

Further, the inhibitory effects of ryanodine were significantly increased with the simultaneous addition of 8-bromo-cyclic GMP, indicating that the two inhibitors operate at distinct sites. This suggests, (1) that intracellular Ca^{2+} release from ryanodine-sensitive sites is not regulated by 8-bromo-cyclic GMP, (2) that the ryanodine-sensitive Ca^{2+} store and IP_3-regulated Ca^{2+} store are functionally distinct in the rat caudal artery, and (3) that the ryanodine-sensitive and IP_3-regulated Ca^{2+} stores are spatially 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).

In summary, PI hydrolysis in the rat caudal artery is dependent on extracellular Ca^{2+} mediated through dihydropyridine-sensitive channels. Contraction is also dependent on extracellular Ca^{2+}. The failure of 8-bromo-cyclic GMP to potentiate felodipine-induced inhibition of contraction and inability to antagonize PLC activity, suggest that although the nucleotide affects the felodipine-sensitive contractile mechanism, its interaction occurs beyond the production of IP_3. Cyclic GMP may indirectly affect the IP_3-regulated receptor on the sarcoplasmic reticulum to prevent intracellular Ca^{2+} release. However, cyclic GMP does not appear to affect the ryanodine-sensitive receptor, which is also coupled to release of intracellular Ca^{2+}.

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