Kor J Physiol (1995) 29(2), 225-232

Phorbol Ester-Induced Periodic Contraction in Isolated Rabbit Jugular Vein

Jae-Cheol Ryu, Dong-Keun Jung and Sang-Ho Lee

Department of Physiology, College of Medicine, Pusan National University, Pusan 602-739

=ABSTRACT=

The present study was conducted to evaluate the effect of phorbol 12,13-dibutyrate (PDBu) on the contraction of rabbit jugular vein in vitro. PDBu concentrations of greater than 10 nM induced a periodic contraction which was composed of rapid contraction, plateau and slow relaxation. The frequency of periodic contraction increased as PDBu concentration increased. The PDBu-induced contraction was inhibited by staurosporine (100 nM), it was not changed by tetrodotoxin (1 μM). In Ca^{2+}-free medium, PDBu induced a sustaining contraction, but not periodic contraction. Addition of Ca^{2+} to medium evoked periodic contraction which was inhibited by nifedipine. PDBu concentrations of greater than 0.1 μM increased $^{45}Ca^{2+}$ uptake without changing $^{45}Ca^{2+}$ efflux.

Charybdotoxin and apamin, Ca^{2+}-activated K^+ channel blockers, did not affect the PDBu-induced periodic contraction, whereas tetraethylammonium (TEA) abolished the periodicity. Pinacidil (10 μ M), a potassium channel activator, blocked PDBu induced periodic contraction, which was recovered by glybenclamide (10 μM). In high potassium solution, PDBu did not produce the periodic contraction.

These results suggest that the PDBu-induced periodicity of contraction is modulated by voltage dependent Ca^{2+} channel and ATP-sensitive K^+ channel.

Key Words: Phorbol ester, Periodic contraction, Jugular vein

INTRODUCTION

Several agonist receptors have been identified in various venous smooth muscles. These include receptors for serotonin (Bodelsson et al, 1992), adrenaline (Reese & Mattews, 1986; Cheung, 1988; Jim et al, 1988) and prostaglandin E (Lawrence & Jones, 1992). The activities of some receptors in vascular smooth muscle are known to be associated with phosphoinositide turnover (Berridge & Irvine, 1984; Reese & Mattews, 1986). The hydrolysis of phosphoinositide by phospholipase C forms inositol triphosphate and diacylglycerol (DAG) which activates protein kinase C (PKC) (Villalobos-Molina et al, 1982; Hashimoto et al, 1986; Rapoport, 1987). The activation of PKC induces protein phosphorylation, and thereby regulates various cellular functions (Nishizuka, 1986; Nishizuka, 1988).

Tumor promoting phorbol esters mimic the endogenous DAG in activating PKC due to structural similarity (Castagna et al, 1982; Nishizuka, 1986). Since phorbol ester is not readily metabolized, it is widely used to study the action of PKC (Bell, 1986). Although numerous studies (Chatterjee & Tejada,

1986; Jiang & Morgan, 1987; Rasmussen et al, 1987) have shown that the phorbol ester-induced activation of PKC induces contraction of vascular smooth muscle, the underlying mechanism has not been clearly elucidated. Furthermore, most studies involving phorbol esters have been carried out in the arterial, but not in the venous smooth muscle.

We therefore conducted the present series of experiments to investigate the effect of phorbol 12,13-dibutyrate (PDBu) on the contraction of venous smooth muscle, using rabbit jugular vein preparations. The results indicate that PDBu induces periodic contractions which may be associated with an activation of PKC.

METHODS

Measurement of contraction

New Zealand White rabbits either of sex (2 ~ 4 kg) were sacrificed by a blow to the neck. The external jugular veins were isolated, cleaned of adherent fat and connective tissues and cut into rings of 3 mm width.

Each ring was suspended under a tension of 0.25 g between Z-shaped hooks in a 4 ml organ bath containing Krebs-Hanselite solution (KHS, in mM: NaCl 115, KCl 4.7, $CaCl_2$ 2.5, $MgCl_2$ 1.2, KH_2PO_4 1.2, $NaHCO_3$ 25, and glucose 10). The bath solution was gased with a mixture of 95% O_2-5% CO_2, and was maintained at 37°C. The preparation was equilibrated for 2 hours during which the resting tension was adjusted to 0.25 g. The tension was measured by means of a Grass FT-03 isometric transducer and recorded on a Grass Polygraph recorder. Each preparation was initially tested for the maximal contractile response using 120 mM KCl. A high K^+ solution was obtained by replacing Na^+ with an equivalent amount of K^+. Each trace shown in figures is a representative trace, typical of at least four experiments.

Measurement of $^{45}Ca^{2+}$ uptake

$^{45}Ca^{2+}$ uptake by venous strips was measured according to Meisheri et al (1980). After 90 min stabilization in a Na-HEPES buffer, tissues were incubated for 5 min in a medium containing $^{45}Ca^{2+}$ (1 μCi/ml) in the presence or absence of 0.1 μM PDBu. The Na-HEPES buffer contained (in mM) NaCl 120, KCl 5.0, $CaCl_2$ 2.0, $MgCl_2$ 1.2, glucose 10 and HEPES 10, and the pH was adjusted to 7.4 with Tris. Upon completion of the incubation, tissues were washed in 100 ml of ice-cold La-HEPES buffer for 5 sec and then placed in 4 ml of the same buffer for 45 min to remove the extracellularly bound $^{45}Ca^{2+}$. The La-HEPES buffer contained (in mM) NaCl 120, KCl 5.0, $MgCl_2$ 1.2, $LaCl_3$ 10, glucose 10 and HEPES 10 (pH 7.4 with Tris). The tissues were then blotted, weighed and incubated overnight in 200 μl of perchloric acid-H_2O_2 mixture (1 : 1). After the tissue was dissolved, the radioactivity of $^{45}Ca^{2+}$ was counted in a liquid scintillation counter (Packard Tricarb 300C). The amount of net $^{45}Ca^{2+}$ uptake was expressed as μmole/kg/5 min.

Measurement $^{45}Ca^{2+}$ efflux

Segments of vein were loaded with $^{45}Ca^{2+}$ by immersing them in Na-HEPES buffer containing $^{45}Ca^{2+}$ (1 μCi/ml) for 2 hours. Measurement of $^{45}Ca^{2+}$ efflux was made at 37°C using the method described by Henrion et al (1992). Tissues were rinsed with a Na-HEPES buffer containing 2 mM EGTA and transferred at 5-min intervals to sequential 2 ml aliquots of the Na-HEPES buffer containing 2 mM EGTA for 1 hour. Tissues were then blotted, weighed, dissolved in perchloric acid-H_2O_2 mixture (1 : 1) by overnight incubation. The activity of the tissues and the efflux media was measured in a liquid scintillation counter (Packard Tricarb 300C). PDBu was added to the efflux medium after 30 min efflux and then the change in efflux was measured. At 5 min intervals the efflux rate

coefficient was calculated.

Drugs and chemicals

PDBu, tetrodotoxin and staurosporine were purchased from Sigma Co.(St. Louis, MO). $^{45}CaCl_2$ (12.40 mCi/mg) was obtained from New England Nuclear (Boston, MA, USA). All other reagents were of analytical grade. Stock solutions of PDBu and staurosporine were dissolved in dimethyl sulfoxide (DMSO).

Statistical analysis

All results are presented as the mean±SE. The difference between two mean values was evaluated by Student's t-test (unpaired comparison). The difference was considered statistically significant when $p<0.05$.

RESULTS

PDBu-induced contraction

Fig. 1 demonstrates the effect of PDBu on the contractility of rabbit jugular vein rings. PDBu concentrations of greater than 10 nM induced periodic contractions each lasting about 4 min. The frequency of contraction was progressively increased as the concentration of PDBu increased. Similar results were observed in the preparation in which the endothelium was mechanically removed (data not shown), indicating that the PDBu effect was independent of endothelium. DMSO, which was used as a solvent, did not induce vascular contraction.

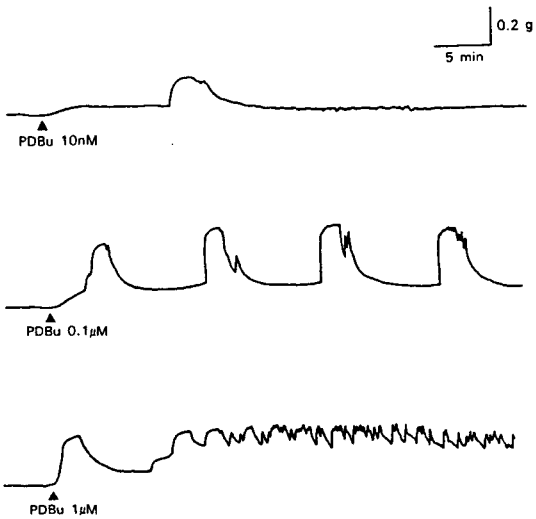

Fig. 1. Typical tracings of the contractile response of rabbit jugular vein ring to various concentrations of PDBu.

Fig. 2A presents the effect of staurosporine, a PKC inhibitor, on the PDBu-induced periodic contraction. Addition of 100 nM staurosporine resulted in a complete cessation of the periodic contraction. In the preparations pretreated with staurosporine PDBu caused no contraction (data not shown). These results suggest that PKC was involved in the PDBu induction of periodic contraction.

Fig. 2B illustrates the effect of tetrodotoxin, a sodium channel blocker (Narahashi et al, 1964), on the PDBu-induced periodic contraction. At 1 μM, tetrodotoxin had no apparent effect, suggesting that the PDBu induction of periodic contraction was due to myogenic response.

Effect of extracellular Ca^{2+} on the PDBu-induced contraction

In the Ca^{2+}-free Krebs-Hanselite solution, PDBu

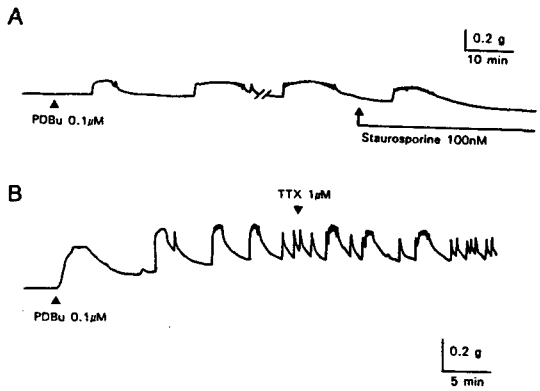

Fig. 2. Effects of staurosporine(A) and tetrodotoxin(B) on the PDBu-induced contraction of rabbit jugular vein ring. TTX, tetrodotoxin.

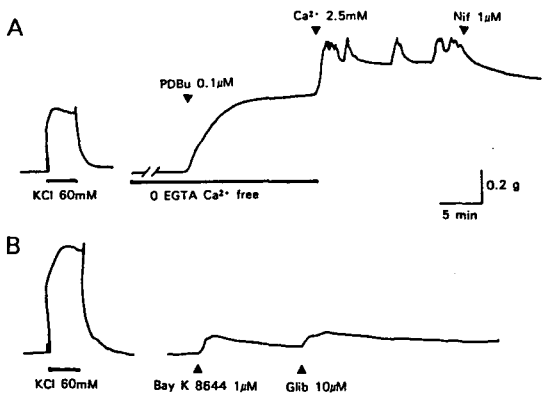

Fig. 3. Effect of extracellular Ca^{2+} on the PDBu-induced contraction of rabbit jugular vein ring. (A) Ca^{2+} free medium without EGTA: PDBu induced a sustained contraction. Addition of 2.5 mM $CaCl_2$ induced periodic contraction, which was eliminated by nifedipine (Nif). (B) normal Ca^{2+} medium: a Ca^{2+} channel activator Bay K 8644 and an ATP-sensitive K^+ channel blocker glybenclamide (Glib) did not induce periodic contraction.

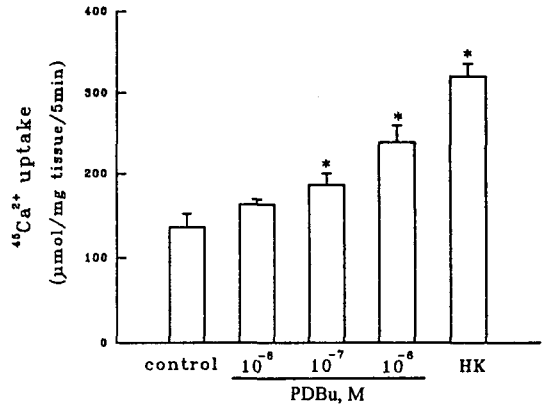

*Fig. 4. Effect of PDBu on the $^{45}Ca^{2+}$ uptake by rabbit jugular vein. PDBu was added 10 min prior to the measurement of $^{45}Ca^{2+}$ uptake in control tissues. HK: high potassium (120 mM) medium. Mean ± SE. n = 5. *, P<0.05, compared with control.*

produced a sustained, rather than periodic, contraction (Fig. 3A). Under this condition, an addition of $CaCl_2$ (2.5 mM) evoked periodic contractions which can be inhibited by nifedipine (1 μM). These indicate that the influx of extracellular Ca^{2+} is involved in the PDBu induction of periodic contraction. However the Ca^{2+} channel activator Bay K 8644 and ATP-sensitive K^+ channel blocker glybenclamide could not induce the periodic contraction in Ca^{2+} containing medium (Fig. 3B).

Effect of PDBu on $^{45}Ca^{2+}$ fluxes in rabbit jugular vein

In view of the foregoing results, we next directly evaluated the effect of PDBu on Ca^{2+} uptake by rabbit jugular vein. As summarized in Fig. 4, PDBu concentrations of greater than 10^{-7} M significantly enhanced the net Ca^{2+} uptake. The same concentrations of PDBu, however, showed no apparent effect on Ca^{2+} efflux (Fig. 5). Thus influx of Ca^{2+} must have been increased by PDBu.

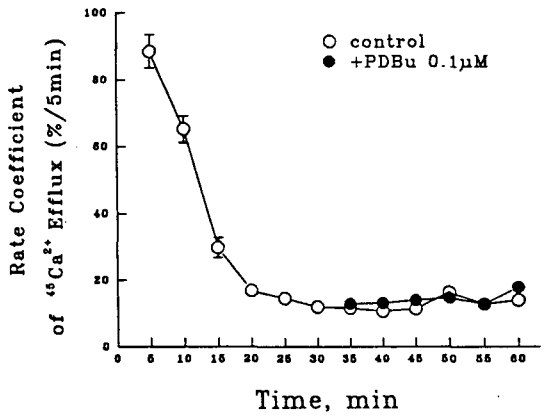

Fig. 5. Rate coefficient of $^{45}Ca^{2+}$ efflux from rabbit jugular vein plotted against efflux time. PDBu (0.1 μM) was added to the efflux medium after 30 min efflux. Each point represents the mean of 4 experiments and vertical bar represents ± SE.

Effects of K^+ channel modulators on PDBu-induced periodic contraction

Ca^{2+}-activated K^+ channels have been classified into Maxi K^+, Int K^+ and Small K^+ channels (Haylett

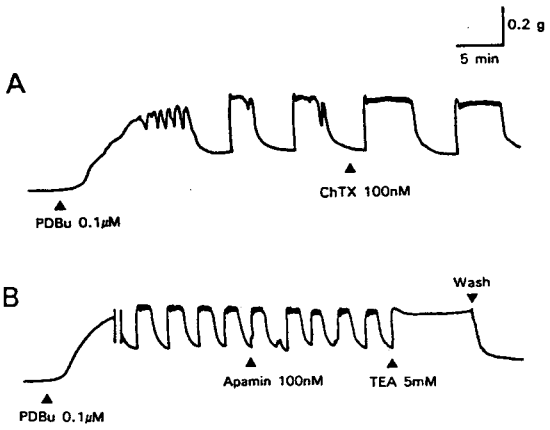

Fig. 6. Effects of potassium channel blockers charybdotoxin (A), apamin and tetraethylammonium (B) on the PDBu-induced periodic contraction of rabbit jugular vein ring. ChTX, charybdotoxin; TEA, tetraethylammonium.

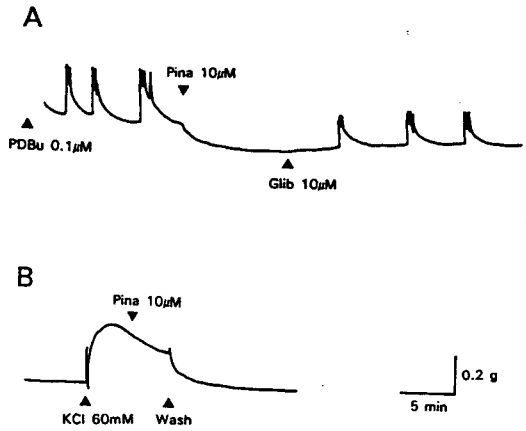

Fig. 7. Antagonistic effect of potassium channel activator pinacidil (Pina) and blocker glybenclamide (Glib) on the PDBu-induced periodic contraction (A) and KCl(60 mM)-induced contraction (B).

& Jenkinson, 1990). Of these, the Maxi K^+ channel has been widely identified in vascular smooth muscles (Benham et al, 1982; Latorre & Miller, 1983; Shoemaker & Worrell, 1991) and is suggested to be involved in vasorelaxation (Inoue et al, 1986; Sadoshima et al, 1988; Willams et al, 1988). Fig. 6 shows that the periodic contraction induced by PDBu was not prevented by charybdotoxin (ChTX), a Maxi K^+ channel blocker (Habermann, 1984), although its duration was slightly prolonged (Fig. 6A). This result also shows that the PDBu effect was not apparently altered by apamin, a Small K^+ channel blocker (Miller et al, 1985) (Fig. 6B). However, addition of TEA (5 mM) caused a sustained contraction without changing the amplitude.

The K^+ channel activator pinacidil (10 μM) relaxed the PDBu-induced periodic contraction, and this effect was reversed by glybenclamide, an ATP-sensitive K^+ channel blocker (Fig. 7A). The same concentration of pinacidil had no effect on the high K^+ (60 mM)-induced contraction (Fig. 7B). This suggests that the periodic contraction induced by PDBu is mediated by the ATP-sensitive K^+ channel.

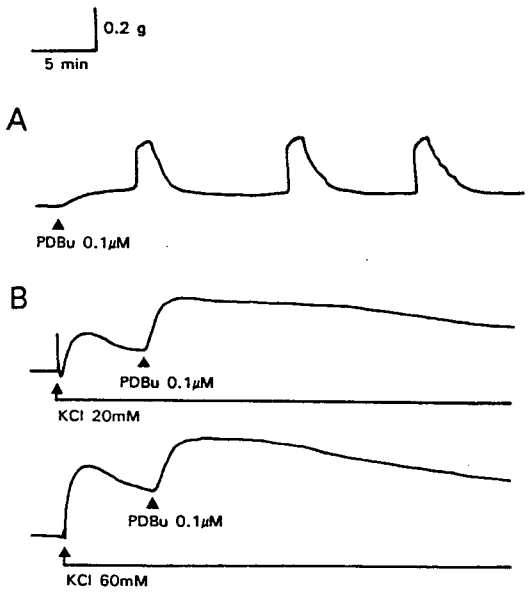

Fig. 8. Effect of membrane hypopolarization produced by high potassium solution on the PDBu-induced contraction. A: normal KHS. B: high potassium KHS.

Effect of high K^+ medium on the PDBu-induced periodic contraction

Fig. 8 compares the effect of PDBu in normal and high K^+ media. In normal KHS, PDBu induced typical periodic contractions. In comparison, in high K^+ media, PDBu induced a sustained contraction, not a periodic contraction. These indicate that the periodic contraction could not be observed when membrane potential was reduced.

DISCUSSION

The present study clearly demonstrates that PDBu induced periodic contractions of isolated rabbit jugular vein (Fig. 1). To the best of our knowledge, this is the first demonstration of the specific action of a phorbol ester in venous smooth muscle. Another study involving canine saphenous vein (Jim et al, 1988) reported that phorbol esters induced a sustained contraction only, as observed in arterial smooth muscles (Forder et al, 1985; Nakaki et al, 1985).

The results of the present study indicate that extracellular Ca^{2+} plays an important role in the PDBu-induced periodic contraction. In the absence of Ca^{2+}, PDBu evokes a sustained contraction, not a periodic contraction. However, addition of Ca^{2+} induces periodic contraction (Fig. 3A). Extracellular Ca^{2+} most probably acts through the voltage dependent Ca^{2+} channel, since the PDBu-induced periodicity was eliminated by nifedipine (Fig. 3). However, direct activation of the channel by Bay K 8644 did not induce the same type of contraction, suggesting that simple increase in Ca^{2+} influx does not induce the periodic contraction. The fact that the PDBu-induced periodicity was completely inhibited by staurosporine suggests strongly that PKC activation was involved (Fig. 2).

Several studies (Fish et al, 1988; Loirand et al, 1990) have shown that phobol esters increase Ca^{2+} influx into vascular smooth muscles. The present study also showed that PDBu increased Ca^{2+} uptake in rabbit jugular vein preparation without changing ^{45}Ca efflux (Fig. 4 and 5). This implies that Ca^{2+} influx induced by PDBu was mediated by PKC. This Ca^{2+} influx may be responsible for the rising phase of the periodic contraction.

Ca^{2+} influx in vascular smooth muscle is generally modulated by membrane potential (Bolton, 1979; Cauvin et al, 1983; Marin, 1988). Thus the periodic contraction observed in this study is thought to represent the fluctuation of membrane potential.

In the present study, TEA blocked the PDBu-induced periodicity of contraction (Fig. 6B), indicating that K^+ channels were involved in the periodic contraction. Perhaps, the slow relaxing phase of the periodic contraction may have been induced by increased K^+ permeability. Since charybdotoxin and apamine did not change the PDBu-induced periodic contraction (Fig. 6), the K^+ channel involved could not be the Ca^{2+}-activated K^+ channel. In high K^+ medium, PDBu did not induce the periodic contraction (Fig. 8). It is therefore presumed that the periodicity induced by PDBu was due to a change in membrane potential modulated by K^+ permeability.

The PDBu-induced periodicity of contraction was removed by the ATP-sensitive K^+ channel opener pinacidil (Fig. 7A). This effect of pinacidil was eliminated by glybenclamide which inhibits ATP-sensitive K^+ channels. This sugggests that the PDBu-induced periodicity of contraction was due to a change in K^+ permeability associated with the ATP-sensitive K^+ channel.

Finally, it is important to point out that the contraction of vascular smooth muscle is very sensitive to temperature change. Thus, any change in experimental temperature would alter the contractile state. In the present study the temperature of organ chambers was adjusted by the same water bath. Thus, if the vascular tone observed in the present study was as a result of temperature change, the response should be identical for all preparations run sim-

ultaneously. However, as shown in Fig. 1, the PDBu response at a given time was not identical between the three sets of tracing. It is therefore clear that the changes in vascular tone observed in the present study were not due to temperature change.

REFERENCES

Bell RM (1986) Protein kinase C activation by diacylglycerol second messengers. *Cell* **45,** 631-632

Benham CD, Bolton TB, Kitamura K (1982) Single channel currents in collagenase dispersed smooth muscle cells of adult guinea-pigs and rabbits. *J Physiol* **328,** 54-55

Berridge MJ, Irvine RF (1984) Inositol triphosphate, a novel second messenger in cellular signal transduction. *Nature* **312,** 315-321

Bodelsson M, Törnebrandt K, Bertilsson I, Arneklo-Nobin B (1992) Heterogeneity of contractile receptors in human hand veins. *Eur J Pharmacol* **219,** 455-460

Bolton TB (1979) Mechanism of action of transmitter and other substances on smooth muscle. *Physiol Rev* **59,** 606-718

Castagna M, Takai Y, Kaibuchi K, Sano K, Kikkawa U, Nishizuka Y (1982) Direct activation of calcium-activated, phospholipid-dependent protein kinase by tumor-promoting phorbol esters. *J Biol Chem* **257,** 7847-7851

Cauvin C, Saida K, Van Breemen C (1983) Mechanisms of calcium antagonist-induced vasodilation. *Annu Rev Pharmacol Toxicol* **23,** 373-396

Chatterjee M, Tejada M (1986) Phorbol ester-induced contraction in chemically skinned vascular smooth muscle. *Am J Physiol* **251,** C356-C361

Cheung DW (1988) Phorbol 12,13-dibutyrate potentiates α_2-adrenoceptor-mediated contraction in the rat saphenous vein. *Eur J Pharmacol* **158,** 145-148

Fish RD, Sperti G, Colucci WS, Clapham DE (1988) Phorbol ester increases the dihydropyridine-sensitive calcium conductance in a vascular smooth muscle cell line. *Circ Res* **62,** 1049-1054

Forder J, Scriabine A, Rasmussen H (1985) Plasma membrane calcium flux, protein kinase C activation and smooth muscle contraction. *J Pharmacol Exp Ther* **235,** 267-273

Habermann E (1984) Apamin. *Pharmacology and Therapeutics* **25,** 255-270

Hashimoto T, Hirata M, Itoh T, Kanimura Y, Kuriyama H (1986) Inositol 1.4.5-triphosphate activates pharmacomechanical coupling in smooth muscle of the rabbit mesenteric artery. *J Physiol* **370,** 605-618

Haylett DG, Jenkinson DH (1990) Calcium-activated potassium channels, in Cook, NS (ed): *Potassium channels.* Ellis Horwood, New York, pp70-95

Henrion D, Laher I, Laporte R, Bevan JA (1992) Further evidence from an elastic artery that angiotensin II amplifies noradrenaline-induced contraction through activation of protein kinase C. *Eur J Pharmacol* **224,** 13-20

Inoue R, Okabe K, Kitamura K, Kuriyama H (1986) A newly identified Ca^{2+} dependent K^+ channel in the smooth muscle membrane of single cells dispersed from the rabbit portal vein. *Pflugers Arch* **406,** 138-143

Jiang MJ, Morgan KG (1987) Intracellular calcium levels in phorbol ester-induced contractions of vascular smooth muscle. *Am J Physiol* **253,** H1365-H1371

Jim KF, Reese JB, Mattews WD (1988) Role of calcium in phorbol-ester-induced contractions of the canine saphenous vein. *J Cardiovascular Pharmacol* 11, 24-28

Latorre R, Miller C (1983) Conduction and selectivity in potassium channels. *J Membr Biol* **71,** 11-30

Lawrence R, Jones RL (1992) Investigation of the prostaglandin E (EP-) receptor subtype mediating relaxation of the rabbit jugular vein. *Br J Pharmacol* **105,** 817-824

Loirand G, Pacaud P, Mironneau C, Mironneau J (1990) GTP-binding preteins mediate noradrenaline effects on calcium and chloride currents in rat portal vein myocytes. *J Physiol* **428,** 517-529

Marin J (1988) Vascular effects of calcium antagonists. Uses in some cerebrovascular disorders. *Gen Pharmacol* **19,** 295-306

Meisheri UD, Palmer RF, Van Breemen C (1980) The effects of amrion on contractility. Ca^{2+} uptake and cAMP in smooth muscle. *Eur J Pharmacol* **61,** 159-165

Miller C, Moczydlowski E, Latorre R, Phillips M (1985) Charybdotoxin, a protein inhibitor of single Ca^{2+}-activated K^+ channels from mammalian skeletal muscle. *Nature* **313,** 316-318

Nakaki T, Roth BL, Chuang DM, Costa E (1985) Phasic

and tonic components in 5-HT_2 receptor-mediated rat aorta contraction: participation of Ca^{2+} channels and phospholipase C. *J Pharmacol Exp Ther* **234,** 442-446

Narahashi T, Moore JW, Scott WR (1964) Tetrodotoxin blockage of sodium conductance increase in lobster giant axons. *J Gen Physiol* **47,** 965-976

Nishizuka Y (1986) Studies and perspectives of protein kinase C. *Science* **233,** 305-312

Nishizuka Y (1988) The molecular heterogeneity of protein kinase C and its implications for cellular regulation. *Nature* **334,** 661-665

Prosser CL (1992) Smooth muscle: diversity and rhythmicity. *NIPS* **7,** 100-105

Rapoport RM (1987) Effects of norepinephrine on contraction and hydrolysis of phosphatidylinositols in rat aorta. *J Pharmacol Exp Ther* **242,** 188-194

Rasmussen H, Takuwa Y, Park S (1987) Protein kinase C in the regulation of smooth muscle contraction. *FASEB J* **1,** 177-185

Reese JB, Mattews WD (1986) α-Adrenergic agonists stimulate phosphatidylinositol hydrolysis in canine saphenous vein. *The Pharmacologist* **28,** 161-163

Sadoshima J, Akaike N, Tomoike H, Nakamura M (1988) Ca-activated K channel in cultured smooth muscle cells of rat aortic media. *Am J Physiol* **255,** H410-H418

Shoemaker RL, Worrell RT (1991) Ca^{2+}-sensitive K^+ channel in aortic smooth muscle of rats. *PSEMB* **196,** 325-332

Villalobos-Molina R, Uc M, Hong E, Garcia-Sainz JZ (1982) Correlation between phosphatidylinositol labeling and contraction in rabbit aorta: Effect of alpha-1 adrenergic activation. *J Pharmacol Exp Ther* **222,** 258-261

Williams DL Jr, Katz GM, Roy-Contancin L, Reuben JP (1988) Guanosine 5'-monophosphate modulates gating of high-conductance Ca^{2+}-activated K^+ channels in vascular smooth muscle cells. *Proc Natl Acad Sci USA* **85,** 9360-9364