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The role of diacylglycerol and activation of protein kinase C in α_{1A}-adrenoceptor-mediated contraction to noradrenaline of rat isolated epididymal vas deferens

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1 The mechanism of contraction to noradrenaline (pEC_{50} 5.6±0.1) in the rat epididymal vas deferens (mediated via α_{1A}-adrenoceptors) has been studied in functional experiments.

2 Contractions to noradrenaline at 10^{-6}M were potentiated by the diacylglycerol (DAG) kinase inhibitor R 59022 (3×10^{-7}M) from 49±4% to 63±3% maximum response and the time taken from initiation of contraction to the maximum response was reduced from 16±2 s to 9±1 s. The same contractions were not significantly potentiated by the DAG lipase inhibitor, U-57,908, 10^{-5}M (51±2% control and 53±4% in the presence of U-57,908) nor was the time taken from initiation of contraction to the maximum response significantly altered (17±1 s control and 16±1 s in the presence of U-57,908).

3 Concentration-dependent contractions to noradrenaline (NA) were reduced by staurosporine (10^{-7}M) and the selective protein kinase C inhibitor, calphostin C (10^{-6}M) from 68±2% (NA, 3×10^{-6}M) to 28±2% and 20±2% respectively and from 94±2% (NA, 3×10^{-5}M) to 50±2% and 44±2% respectively. Contractions to K^+ (40±2% maximum response to NA) were also significantly reduced by staurosporine (10^{-7}M) (35±2%) but not by calphostin C (43±3%).

4 The phorbol ester, phorbol-12,13-dibutyrate (PDBu), produced a phasic, concentration-dependent contraction (10^{-7}M-10^{-4}M) which was 41±2% of the maximum response to NA at 10^{-4}M PDBu. The contraction to PDBu (10^{-5}M) was reduced by calphostin C (10^{-6}M) from 33±5% to 4±1% maximum response to NA.

5 Non-cumulative contractions to NA (10^{-8}M-10^{-4}M) were abolished in Ca^{2+}-free Krebs solution containing EGTA (1 mM) and were reduced in the presence of nifedipine (10^{-6}M) in normal Krebs solution by 91±2% at 10^{-4}M NA. The contraction to PDBu (10^{-5}M, 33±5% maximum response to NA) was also abolished in Ca^{2+}-free Krebs solution containing EGTA (1 mM) or by the presence of nifedipine (10^{-6}M) in normal Krebs solution.

6 When NA (10^{-4}M) was added to vasa deferentia in Ca^{2+}-free Krebs solution containing EGTA (1 mM), following its wash out (and with EGTA later removed from the Krebs solution), readdition of Ca^{2+} (2.5 mM) to the Krebs solution produced no response. Cyclopiazonic acid (10^{-5}M), which can deplete Ca^{2+} from intracellular stores, also produced no contraction. Therefore influx of extracellular Ca^{2+} is not a consequence of depletion of intracellular Ca^{2+} stores (capacitative Ca^{2+} influx).

7 Pre-incubation of tissues for 30 min with either cyclopiazonic acid (10^{-5}M) or ryanodine (10^{-4}M), which can both deplete intracellular Ca^{2+} stores, did not reduce the contractions to NA (3×10^{-6}M). Pre-incubation of vasa deferentia with cyclopiazonic acid (1 or 3 min, when any rise in $[Ca^{2+}]_i$ produced by cyclopiazonic acid might still exist) did not potentiate the contraction to PDBu (10^{-5}M). Thus mobilization of intracellular Ca^{2+} may not be required for the activation of protein kinase C involved in these contractions.

8 In conclusion, the contraction of the rat epididymal vas deferens to NA mediated by α_{1A}-adrenoceptors appears to depend upon activation of protein kinase C by diacylglycerol, resulting in the influx of extracellular Ca^{2+} through voltage-gated Ca^{2+} channels. There was no evidence for a role of inositol trisphosphate in the contraction to noradrenaline in this tissue.

Keywords: Rat vas deferens; α_1-adrenoceptors; noradrenaline; phorbol ester; calphostin C; protein kinase C; R 59022; nifedipine; extracellular Ca^{2+}; cyclopiazonic acid

Introduction

Stimulation of α_1-adrenoceptors can mediate contraction of smooth muscle by raising $[Ca^{2+}]_i$ (for review see Minneman & Esbenshade, 1994). These G-protein coupled receptors are linked to the activation of phospholipase C which hydrolyses phosphatidylinositol 4,5-bisphosphate (PIP_2), to inositol 1,4,5-trisphosphate (IP_3), and diacylglycerol (DAG). IP_3 can mobilize Ca^{2+} from intracellular stores (Berridge, 1993), while DAG can activate protein kinase C (PKC) (Lee & Severson, 1994).

The rise in $[Ca^{2+}]_i$ through mobilization of Ca^{2+} from intracellular stores by IP_3 is transient but it can be associated with a more prolonged rise in $[Ca^{2+}]_i$ due to influx of extracellular Ca^{2+} which is stimulated in some cells by the depletion of intracellular Ca^{2+} stores (Fasolato *et al.*, 1994). This mechanism, sometimes called capacitative Ca^{2+} entry (Putney, 1986; 1990), has been shown to be involved in the α_{1B}-adrenoceptor mediated contraction of the rat spleen to phenylephrine (Burt *et al.*, 1995b).

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Alternatively, contraction of some smooth muscle tissues has been shown to involve stimulation of PKC (Lee & Severson, 1994). PKC was first discovered by Inoue *et al.* (1977) and was then shown to require both Ca^{2+} and phospholipid for its activation (Takai *et al.*, 1979). Activation of α_1-adrenoceptors might therefore result in contraction of smooth muscle via activation of PKC by DAG.

The aim of the present experiments was to investigate the role of IP_3, DAG and PKC activation in the α_{1A}-adrenoceptor mediated contraction of the rat epididymal vas deferens to noradrenaline (Aboud *et al.*, 1993; Burt *et al.*, 1995a). The results suggest that noradrenaline contractions are mediated via DAG activation of PKC leading to an influx of extracellular Ca^{2+}.

Methods

Male Sprague Dawley rats between 350–450 g were stunned and killed by cervical dislocation. The vasa deferentia were removed into Krebs solution (see below), associated blood vessels and mesentery were dissected away and were then bisected so that only the epididymal portion (15–20 mm in length) was used. The tissues were suspended in 5 ml tissue baths containing Krebs solution of the following composition (mM): Na^+143, K^+5.9, Ca^{2+}2.5, Mg^{2+}1.2, Cl^-128, HCO_3^-25, HPO_4^{2-} 1.2, SO_4^{2-} 1.2 and glucose 11, at 37°C and bubbled with 95% O_2/5% CO_2. The vasa deferentia were placed under 0.5 g resting tension and equilibrated for 1 h. Changes in isometric tension were measured with Grass FT.03 transducers and recorded by Biopac Systems Inc. MP100WS for Windows.

In all tissues a contraction to noradrenaline (10^{-4}M) was measured followed by a non-cumulative concentration-effect curve to noradrenaline (10^{-8}M – 10^{-4}M). In some tissues this was then followed 30 min later by a second concentration-effect curve, either as a control or under appropriate experimental conditions. In other tissues, after the first concentration-effect curve to noradrenaline, responses to noradrenaline at 1×10^{-6}M, 3×10^{-6}M or 3×10^{-5}M were then repeated at either 30 min or 60 min intervals until responses were reproducible. Only one concentration and one time interval was used for each tissue. A final response to noradrenaline was then measured either as a control or under appropriate experimental conditions. The effect of prazosin (10^{-7}M) on contractions to K^+, 60 mM (by addition of KCl solution to the tissue bath) was measured in some tissues. Contractions to K^+ (60 mM) were then measured, always in the presence of prazosin (10^{-7}M), at 30 min or 60 min intervals until the responses were reproducible. They were then used as controls where appropriate for the experiments involving noradrenaline. Control responses to noradrenaline (3×10^{-6}M and 3×10^{-5}M) and K^+ (60 mM) were also measured in the presence of dimethylsulphoxide (DMSO, 0.1%), which was used to dissolve staurosporine, calphostin C, R59022, U-57,908 and cyclopiazonic acid.

The contraction to noradrenaline (10^{-6}M) was measured either in the presence of the DAG kinase inhibitor, R 59022 (3×10^{-7}M, 30 min incubation) or the DAG lipase inhibitor, U-57,908 (10^{-5}M, 30 min incubation). Contractions to noradrenaline (3×10^{-6}M and 3×10^{-5}M) and the contraction to K^+ (60 mM) were measured either in the presence of the non-selective protein kinase inhibitor staurosporine (10^{-7}M, 30 min incubation) or the selective PKC inhibitor, calphostin C (10^{-6}M, 1 h incubation). Calphostin C was used in a bright light environment as this is essential for its activity (Bruns *et al.*, 1991).

The phorbol ester, phorbol-12,13-dibutyrate (PDBu), was added to some tissues (10^{-7}M – 10^{-4}M, one concentration per tissue) in normal Krebs solution 40 min after the initial noradrenaline concentration-effect curve. The response to PDBu (10^{-5}M) was also measured in the presence of calphostin C (10^{-6}M, 1 h incubation). PDBu (3×10^{-5}M) was pre-incubated with some tissues for 2 h before a repeat response to either noradrenaline (10^{-4}M) or K^+ (60 mM) was measured.

Contractions induced by non-cumulative additions to noradrenaline (10^{-8}M – 10^{-4}M) were measured in Ca^{2+}-free Krebs solution containing EGTA (1 mM). After the initial concentration-effect curve (in normal Krebs solution) the tissue was allowed to recover for 15 min in normal Krebs solution followed by 15 min equilibration in Ca^{2+}-free Krebs solution (containing EGTA, 1 mM) before the start of the second curve with concentrations given in ascending order. In other tissues the effect of a single addition of noradrenaline (10^{-4}M) was measured immediately following the 15 min equilibration in Ca^{2+}-free Krebs solution (containing EGTA, 1 mM) to see if the response to a high concentration of noradrenaline was affected by a gradual depletion of intracellular Ca^{2+} which could not be replenished during the non-cumulative additions of noradrenaline. A concentration-effect curve to noradrenaline (in normal Krebs solution) was also measured in the presence of nifedipine (10^{-6}M, 20 min equilibration). Some responses to PDBu (10^{-5}M) were also measured either in Ca^{2+}-free Krebs solution (containing EGTA, 1 mM) or in the presence of nifedipine (10^{-6}M, 20 min equilibration in normal Krebs solution).

To see if any influx of extracellular Ca^{2+} was stimulated by depletion of intracellular Ca^{2+} stores, 20 min after the single addition of noradrenaline (10^{-4}M) in Ca^{2+}-free Krebs solution the agonist was washed out for another 40 min still in Ca^{2+}-free Krebs solution (containing EGTA, 1 mM) so that the concentrations of any second messengers generated by α_1-adrenoceptor stimulation were no longer raised. However, the intracellular Ca^{2+} stores should remain depleted as there was no extracellular Ca^{2+} available to enter and refill them. EGTA was removed from the Krebs solution for the last 20 min. Ca^{2+} (2.5 mM) was then added to the Krebs solution and any response to this was measured. The endoplasmic reticulum Ca^{2+}-ATPase inhibitor, cyclopiazonic acid (10^{-5}M) was also added to some tissues in normal Krebs solution 40 min after the initial concentration-effect curve to noradrenaline.

In other tissues the effect of cyclopiazonic acid (10^{-5}M, 30 min incubation) or ryanodine (10^{-4}M, 30 min incubation) was measured on the contraction to noradrenaline (3×10^{-6}M) in normal Krebs solution. The effect of pre-incubating some tissues with cyclopiazonic acid (10^{-5}M) for either 1 or 3 min was also measured on the response to PDBu (10^{-5}M).

Data analysis

The results were calculated as percentage maximum response of the initial concentration-effect curve to noradrenaline. Responses were then plotted as the mean of at least four separate experiments with vertical bars representing standard error of the mean (s.e.mean). Error bars appear on figures only when they exceed the symbol size. Curve fitting for the calculation of pEC_{50} values by non linear regression was performed using InPlot (GraphPAD Software, San Diego, Calif., U.S.A.). Statistical significance of differences between control and test means was tested for on raw data by a paired *t* test except for comparison of means for the PDBu responses where an unpaired *t* test was used. A *P* value of less than 0.05 was considered to indicate a statistically significant difference. Statistical analysis was performed using InStat (GraphPAD Software, San Diego, Calif., U.S.A.).

Drugs and solutions

Noradrenaline bitartrate and nifedipine were obtained from Sigma. Staurosporine, calphostin C, R 59022 [6-(2-(4-((*p*-fluorophenyl)phenylmethylene)-1-piperidinyl) ethyl)-7-methyl-5H- thiazolo(3,2-a)pyrimidine-5-one], U-57,908 [1,6-bis- (cyclohexyloximinocarbonylamino)-hexane], phorbol-12,13-dibutyrate, ryanodine and cyclopiazonic acid were obtained from Calbiochem. Prazosin hydrochloride was donated by Pfizer

Central Research, Kent. Noradrenaline was dissolved in distilled water, nifedipine was dissolved in ethanol and then diluted in distilled water and both were prepared fresh each day. Ryanodine was dissolved in distilled water and frozen. All other compounds were dissolved as stock solutions in DMSO and frozen, with subsequent dilutions made in DMSO except for prazosin hydrochloride, R 59022 and U-57,908, which were made in distilled water.

Results

Noradrenaline (10^{-4}M) produced a maximal contraction in the rat epididymal vas deferens (2.72 ± 0.12 g, $n=8$, mean ± s.e.mean, Figure 1) and the non-cumulative concentration-effect curves were reproducible (pEC_{50} 5.6 ± 0.1 for the first curve and 5.6 ± 0.1 for the second curve). Contractions to K^+ (60 mM) were reduced by prazosin (10^{-7}M) from $53 \pm 2\%$ to $40 \pm 2\%$ indicating some neuronal release of noradrenaline and therefore subsequently prazosin (10^{-7}M) was added prior to all other additions of high K^+ solution. DMSO (0.1%) had no effect on the contractions to noradrenaline or K^+.

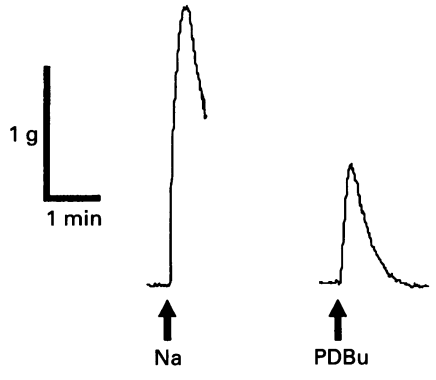

Figure 1 Typical recordings of a contraction to noradrenaline (NA, 10^{-4}M) and a contraction to PDBu (10^{-4}M) in the rat epididymal vas deferens.

Contractions to noradrenaline at 10^{-6}M were potentiated by the DAG kinase inhibitor, R 59022 (3×10^{-7}M) from $49 \pm 4\%$ to $63 \pm 3\%$ maximum response ($P<0.05$) (Figure 2a) and the time taken from initiation of contraction to the maximum response was reduced from 16 ± 2 s to 9 ± 1 s ($P<0.05$) (Figure 2a). Contractions to K^+ (60 mM) were not potentiated by R 59022, 3×10^{-7}M (control, $38 \pm 2\%$ maximum response to noradrenaline and $35 \pm 2\%$ maximum response in the presence of R 59022, representing a significant reduction, $P<0.05$) and the time taken from initiation of contraction to the maximum response was not significantly different (6.0 ± 0.6 s control and 5.8 ± 0.5 s in the presence of R 59022). Contractions to noradrenaline were not significantly potentiated by the DAG lipase inhibitor, U-57,908, 10^{-5}M ($51 \pm 2\%$ control and $53 \pm 4\%$ in the presence of U-57,908) and the time taken from initiation of contraction to the maximum response was not significantly different (17 ± 1 s control and 16 ± 1 s in the presence of U-57,908) (Figure 2b).

The control contraction to noradrenaline at 3×10^{-6}M was $68 \pm 2\%$ maximum response and was reduced to $28 \pm 2\%$ of the maximum ($P<0.05$) by staurosporine (10^{-7}M) and to $20 \pm 2\%$ of the maximum ($P<0.05$) by calphostin C (10^{-6}M) (Figure 3a). The control contraction to noradrenaline at 3×10^{-5}M was $94 \pm 2\%$ maximum response and was reduced to $50 \pm 2\%$ of the maximum ($P<0.05$) by staurosporine (10^{-7}M) and to $44 \pm 2\%$ of the maximum ($P<0.05$) by calphostin C (10^{-6}M) (Figure 3b). The control contraction to K^+ (60 mM) was $40 \pm 2\%$ maximum response to noradrenaline and was $35 \pm 2\%$ of the maximum ($P<0.05$) in the presence of staurosporine (10^{-7}M) and $43 \pm 3\%$ of the maximum ($P>0.05$) in the presence of calphostin C (10^{-6}M).

The phorbol ester, PDBu, produced a phasic contraction of the rat epididymal vas deferens returning to baseline within 90 s (Figure 1). The PDBu contraction was also concentration-dependent (10^{-7}M – 10^{-4}M) and was $41 \pm 2\%$ of the maximum response to noradrenaline at 10^{-4}M PDBu (Figure 4). The response to PDBu was not reproducible and so only one addition of PDBu was made to each tissue. The submaximal

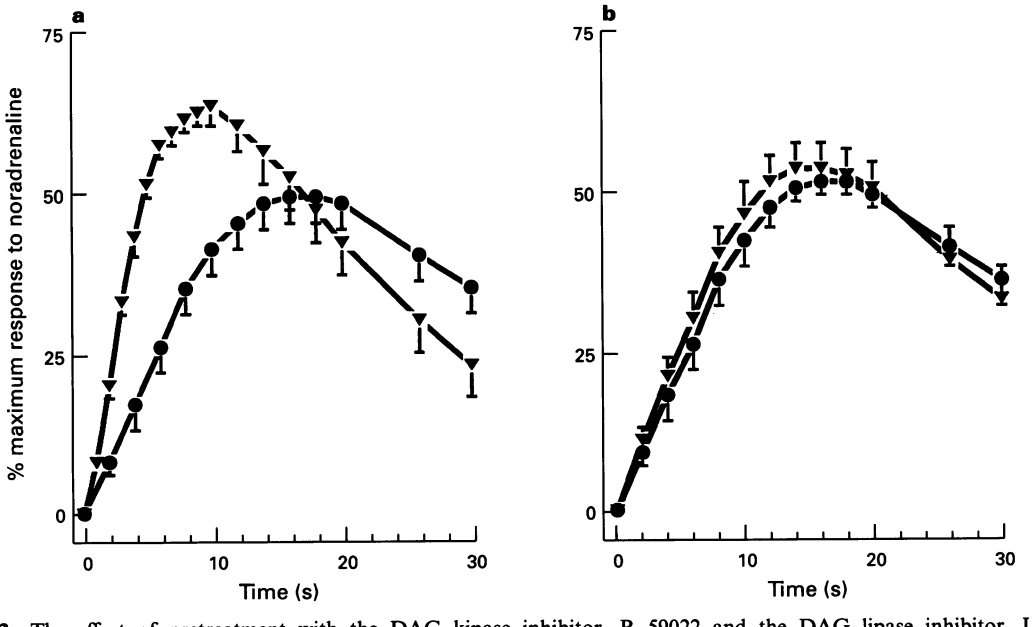

Figure 2 The effect of pretreatment with the DAG kinase inhibitor, R 59022 and the DAG lipase inhibitor, U-57,908 on contractions to noradrenaline (expressed as a percentage of the maximum response to noradrenaline) in the rat epididymal vas deferens. (a) Control response to noradrenaline 10^{-6}M (●); + R 59022, 3×10^{-7}M (▼). (b) Control response to noradrenaline 10^{-6}M (●); + U-57,908, 10^{-5}M (▼). Each plot represents the mean with s.e.mean of 4 separate experiments. The abscissa scale represents the time in s from initiation of the contraction.

contraction to PDBu (10^{-5}M) was significantly reduced by the selective PKC inhibitor, calphostin C (10^{-6}M) from 33±5% to 4±1% ($P<0.05$) maximum response to noradrenaline (Figure 5a). The response to noradrenaline 10^{-4}M, was reduced when the tissue was pre-incubated with PDBu (3×10^{-5}M) for 2 h from 100% to 25±2% maximum response (Figure 5b) but the response to K^+ 60 mM was not significantly affected by this treatment (control, 44±3% maximum response to noradrenaline and 42±2% after PDBu treatment).

The contractions to noradrenaline (10^{-8}M–10^{-4}M) were completely abolished in Ca^{2+}-free Krebs solution containing EGTA (1 mM) (Figure 6a), as was the response to a single addition of noradrenaline (10^{-4}M) in Ca^{2+}-free Krebs solution containing EGTA (1mM) (results not shown). The responses to noradrenaline were also greatly reduced in the presence of nifedipine (10^{-6}M) (Figure 6a). The contraction to PDBu (10^{-5}M) was abolished in Ca^{2+}-free Krebs solution containing EGTA (1 mM) and in the presence of nifedipine (10^{-6}M) in normal Krebs solution (Figure 6b).

Following the single addition of noradrenaline (10^{-4}M) in Ca^{2+}-free Krebs solution containing EGTA (1 mM), when the agonist had been washed out and the EGTA removed from the Krebs solution, the readdition of Ca^{2+} to the Krebs solution did not produce a response. Cyclopiazonic acid (10^{-5}M) also failed to produce a response.

The response to noradrenaline at 3×10^{-6}M (67±2% maximum response) was not significantly affected when the tissues were pre-incubated for 30 min with cyclopiazonic acid 10^{-5}M (65±2% maximum response) or ryanodine 10^{-4}M (68±2% maximum response). The time for noradrenaline to reach a maximal effect at 3×10^{-6}M (17±1 s) was also not significantly affected by the 30 min pre-incubation with either cyclopiazonic acid or ryanodine (18±1 s and 17±1 s respectively). The contraction to PDBu (10^{-5}M, 33±5% maximum response to noradrenaline) was not increased when cyclopiazonic acid was added to the tissues either 1 or 3 min prior to the addition of PDBu (31±2% and 32±2% maximum response to noradrenaline respectively).

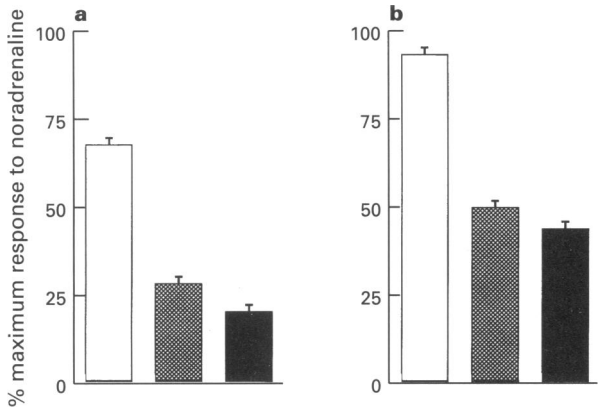

Figure 3 The effect of staurosporine and calphostin C on contractions to noradrenaline (expressed as a percentage of the maximum response to noradrenaline) in the rat epididymal vas deferens. (a) Control response to noradrenaline 3×10^{-6}M (open column); +staurosporine 10^{-7}M (cross-hatched column); +calphostin C 10^{-6}M (solid column). (b) Control response to noradrenaline 3×10^{-5}M (open column); +staurosporine 10^{-7}M (cross-hatched column); +calphostin C 10^{-6}M (solid column). Each column represents the mean with s.e.mean of 4 separate experiments.

Discussion

The potential role of DAG and IP_3 in the contraction to noradrenaline of the rat epididymal vas deferens, mediated by α_{1A}-adrenoceptors (Aboud *et al.*, 1993; Burt *et al.*, 1995a), has been studied in functional experiments.

α_1-Adrenoceptor stimulation has been shown to increase the formation of inositol phosphates in various tissues including the rat vas deferens (Fox *et al.*, 1985) and this should therefore be accompanied by DAG formation. DAG can be metabolized in cells via two main pathways, being converted either to phosphatidic acid by DAG kinase (Bishop & Bell, 1986; Kanoh *et al.*, 1993) or to arachidonic acid by DAG lipase (Severson & Hee-Cheong, 1986). The activity of one or both of these enzymes is therefore at least partly responsible for attenuating PKC activity in response to DAG formation. To see

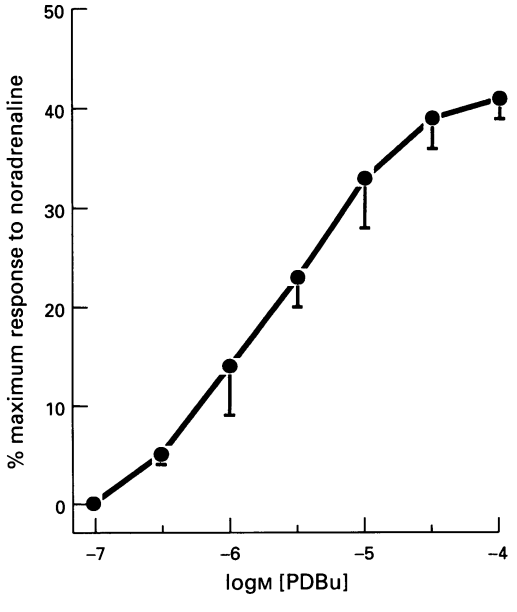

Figure 4 Concentration-response curve to non-cumulative additions of PDBu (expressed as a percentage of the maximum response to noradrenaline) in the rat epididymal vas deferens (●). The plot represents the mean with s.e.mean of 4 separate experiments for each of the 7 concentrations of PDBu (28 tissues).

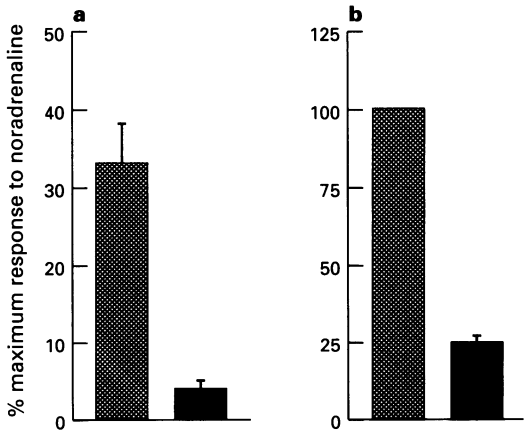

Figure 5 The effect of calphostin C on the contraction to PDBu and the effect of a 2h pre-incubation with PDBu on the response to noradrenaline (expressed as a percentage of the maximum response to noradrenaline) in the rat epididymal vas deferens. (a) Control response to PDBu 10^{-5}M (cross-hatched column); +calphostin C 10^{-6}M (solid column). (b) Control response to noradrenaline 10^{-4}M (cross-hatched column); +PDBu 3×10^{-5}M (solid column). Each column represents the mean with s.e.mean of 4 separate experiments.

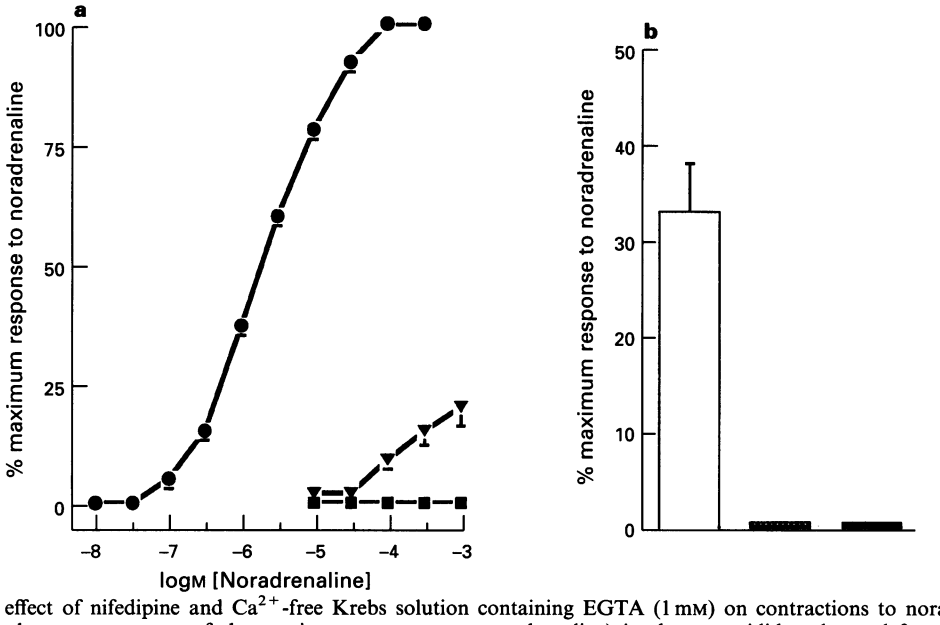

Figure 6 The effect of nifedipine and Ca^{2+}-free Krebs solution containing EGTA (1 mM) on contractions to noradrenaline and PDBu (expressed as a percentage of the maximum response to noradrenaline) in the rat epididymal vas deferens. (a) Control concentration-response curve for non-cumulative additions of noradrenaline in normal Krebs solution (●), in the presence of nifedipine 10^{-6}M (▼), or in Ca^{2+}-free Krebs solution containing EGTA (1 mM) (■). (b) Control contraction to PDBu 10^{-5}M (open column); in the presence of nifedipine 10^{-6}M (cross-hatched column), or in Ca^{2+}-free Krebs solution containing EGTA (1 mM) (solid column). Each plot or column represents the mean with s.e.mean of 4 separate experiments.

if the PKC activity involved in the noradrenaline contraction of this tissue was stimulated by formation of DAG the effect of inhibitors of DAG kinase and DAG lipase were investigated. The DAG kinase inhibitor, R 59022 (de Chaffoy de Corcelles *et al.*, 1985) significantly increased the magnitude and rate of the noradrenaline contraction but not the K^+ contraction, while the DAG lipase inhibitor, U-57,908 (Yang *et al.*, 1991) had no significant effect. This suggests that the PKC activity involved in the noradrenaline contraction is stimulated by production of DAG, which in this tissue is metabolized by DAG kinase but not DAG lipase. However as the PDBu contraction in the vas was also phasic (see below) this suggests that other mechanisms are involved in attenuating the noradrenaline response as well as DAG metabolism.

As DAG can stimulate PKC, the role of this enzyme was investigated. The PKC inhibitor, calphostin C (over 100 fold selectivity for PKC over other protein kinases, Kobayashi *et al.*, 1989) significantly reduced contractions to noradrenaline in the epididymal vas deferens at a concentration which did not inhibit the contractions to K^+. This suggests that PKC activation is involved in the contraction of the rat epididymal vas deferens to noradrenaline. The relatively non-selective protein kinase inhibitor staurosporine (Tamaoki *et al.*, 1986) significantly reduced the noradrenaline contractions and also significantly reduced the K^+ contractions but to a lesser extent. This is in agreement with Shimamoto *et al.* (1993), who reported that contractions in the rat aorta to both phenylephrine and K^+ were reduced by staurosporine while only those to phenylephrine were inhibited by calphostin C. Abraham & Rice (1992) found the protein kinase inhibitor, iso-H7, reduced both noradrenaline and K^+-induced contractions in the rat vas deferens. They suggested that PKC was therefore a common site of activation in both contractions resulting from the increased $[Ca^{2+}]_i$ in both responses. However the results with calphostin C (see above) suggest the effect of iso-H7 on contractions to K^+ in the rat vas deferens (Abraham & Rice, 1992) was not due to PKC inhibition.

Phorbol esters are known to activate PKC by binding to the DAG binding site (Castagna *et al.*, 1982). The effect of the phorbol ester, PDBu, was therefore studied in the rat epididymal vas deferens. PDBu produced a concentration-dependent contraction of the tissue which was phasic, similar to the noradrenaline response, but produced a smaller maximum response (41 ± 2%) compared to noradrenaline. The contraction to PDBu was also significantly reduced by calphostin C confirming that the effect of the phorbol ester is mediated by stimulation of PKC. Abraham & Rice (1992) found that phorbol-12,13-diacetate and PDBu produced contractions less than 6% maximum response to noradrenaline in the rat vas deferens. The reason for this difference is not clear although it is not stated whether they used the whole vas or just the epididymal or prostatic portion. The magnitude of the responses to noradrenaline reported by Abraham & Rice (1992) however suggests a predominantly prostatic portion was used.

Incubation of tissues with phorbol esters can also cause down regulation of PKC (Merkel *et al.*, 1991) and the response to noradrenaline in the rat epididymal vas deferens was significantly reduced by this treatment with PDBu in the present experiments, but it did not affect the K^+ contraction. This also suggests PKC activity is involved in the noradrenaline contraction.

As hydrolysis of PIP_2 by PLC also produces IP_3, the possibility that release of Ca^{2+} from intracellular stores also contributes to the noradrenaline contraction in the rat epididymal vas deferens was studied. Non-cumulative contractions to noradrenaline in this tissue were completely abolished in Ca^{2+}-free Krebs solution and almost completely abolished in the presence of nifedipine in agreement with Han *et al.* (1987). A single high concentration of noradrenaline in Ca^{2+}-free Krebs solution also failed to produce a response showing that the lack of response to noradrenaline was not due to a gradual depletion of Ca^{2+} from intracellular stores (by individual doses of increasing concentration) which could not be replenished and therefore could not initiate contraction. This shows that the contraction is completely dependent on influx of extracellular Ca^{2+} through voltage-gated Ca^{2+} channels and mobilization of intracellular Ca^{2+} does not contribute directly to the contraction. The contraction to PDBu was also completely abolished in Ca^{2+}-free Krebs solution and in the presence of nifedipine, confirming that activation of PKC mediates a contraction via influx of extracellular Ca^{2+} through voltage-gated Ca^{2+} channels.

It has been suggested by Bultmann *et al.* (1993) that the adrenergic component of neurogenic contractions in the rat vas deferens are mediated in part by mobilization of $[Ca^{2+}]_i$ in the smooth muscle as these responses are inhibited in the presence of ryanodine. However ryanodine does not reduce contractions to exogenous noradrenaline in these experiments (see later), in agreement with Bourreau *et al.* (1991). The reason for this difference is not clear.

It might be possible however that Ca^{2+} release from intracellular stores by IP_3 in this tissue, while not being sufficient alone to initiate contraction, might still stimulate capacitative Ca^{2+} influx which could then be partially responsible for the contraction. If this were the case then when the tissue had been stimulated by noradrenaline (and then washed out) in Ca^{2+}-free Krebs solution the intracellular stores should remain depleted so that upon addition of extracellular Ca^{2+}, capacitative Ca^{2+} influx would still occur resulting in a contraction. However there was no response in this tissue to the readdition of Ca^{2+} showing that this mechanism of Ca^{2+} influx does not exist in the response to noradrenaline. This is unlike the rat spleen where readdition of Ca^{2+} after stimulation of α_{1B}-adrenoceptors with phenylephrine did produce a contraction (Burt *et al.*, 1995b). Also, cyclopiazonic acid, which can deplete Ca^{2+} from intracellular stores by inhibiting the sarcoplasmic reticulum Ca^{2+}-ATPase (Seidler *et al.*, 1989; Deng & Kwan, 1991) and therefore stimulate capacitative Ca^{2+} influx in tissues where this mechanism exists, had no effect in the rat epididymal vas deferens at a concentration which produced a maximal contraction in the rat spleen (Burt *et al.*, 1995b). This confirmed that capacitative Ca^{2+} influx cannot be stimulated in the rat epididymal vas deferens.

Both Ca^{2+}-dependent and -independent isoforms of PKC have been identified (Lee & Severson, 1994). It is possible therefore that following hydrolysis of PIP_2, IP_3 which is released into the cytosol, raises $[Ca^{2+}]_i$ producing a translocation of PKC to the membrane (Haller *et al.*, 1990) where DAG, which remains membrane bound, can then also bind to PKC resulting in its full activation. (This mechanism might occur even though noradrenaline in Ca^{2+}-free Krebs solution failed to produce a response as the rise in $[Ca^{2+}]_i$ may not be great enough to initiate contraction). This could also be a reason why activation of PKC by PDBu could not produce the same maximal response as that to noradrenaline. Some tissues were therefore pre-incubated with cyclopiazonic acid for 30 min before addition of noradrenaline. This treatment should deplete Ca^{2+} from intracellular stores and therefore this would not be available for release by IP_3 in the noradrenaline response. The length of the incubation time for cyclopiazonic acid with the tissue should ensure than any rise in $[Ca^{2+}]_i$ produced by this compound had returned to resting levels before addition of the noradrenaline. However pre-incubation with cyclopiazonic acid did not affect either the magnitude or rate of rise of the noradrenaline contraction in this tissue. Other tissues were pre-incubated with cyclopiazonic acid for either 1 or 3 min prior to the addition of PDBu, when any rise in $[Ca^{2+}]_i$ produced by cyclopiazonic acid might still exist. This did not however potentiate the PDBu contraction. Some cells have also been shown to have intracellular Ca^{2+} stores which are insensitive to IP_3 but are sensitive to ryanodine (Sorrentino & Volpe, 1993; Ehrlich *et al.*, 1994) and ryanodine binding sites have been demonstrated in the rat vas deferens (Bourreau *et al.*, 1991). However, ryanodine at a concentration shown to abolish increases in $[Ca^{2+}]_i$ produced by noradrenaline in rabbit mesenteric artery (Itoh *et al.*, 1992), had no effect on the noradrenaline contraction in the rat epididymal vas deferens. These results suggest that release of intracellular Ca^{2+} by IP_3 (or by a ryanodine-sensitive mechanism) is not required for the PKC activity involved in the contraction to noradrenaline. This could mean that the PKC isoform involved in the contraction of the rat epididymal vas deferens belongs to the Ca^{2+}-independent group. Alternatively, DAG/phorbol ester binding to PKC might increase the affinity of the enzyme for Ca^{2+} and therefore a Ca^{2+}-dependent isoform could be involved which does not require a rise in $[Ca^{2+}]_i$ for activation (Castagna *et al.*, 1982).

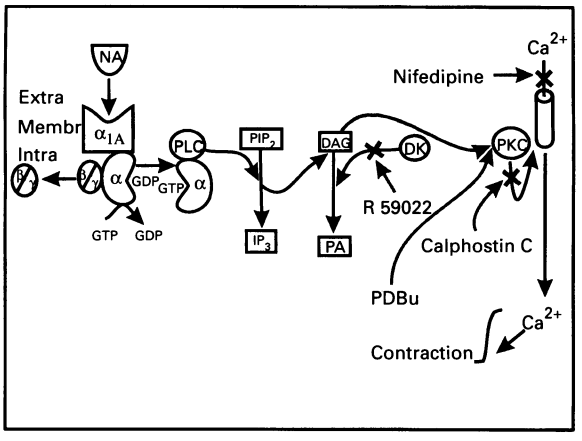

Figure 7 Schematic diagram of the cellular mechanisms proposed to be involved for the α_{1A}-adrenoceptor mediated contraction to noradrenaline (NA) in the rat epididymal vas deferens, which also shows the sites of action for compounds used in this study. DAG: diacylglycerol, PA: phosphatidic acid, DK: DAG kinase, PKC: protein kinase C, GDP: guanosine diphosphate, GTP: guanosine triphosphate, IP_3: inositol 1,4,5-trisphosphate, NA: noradrenaline, PIP_2: phosphatidylinositol 4,5-bisphosphate, PLC: phospholipase C, Membr: cell membrane, Extra: extracellular, Intra: intracellular. α and β/γ are the three subunits of a heterotrimeric G-protein.

The maximum response to PDBu being less than that to noradrenaline could be due to the time PDBu takes to diffuse into the tissue, or PDBu might be a 'partial agonist' compared with DAG for the PKC isoform involved. Different phorbol esters can produce varying biological responses in tissues suggesting that they do have some isoform selectivity (Ryves *et al.*, 1991) and different species of DAG may also have PKC isoform selectivity (Nishizuka, 1986; Ford *et al.*, 1989).

These results show that the α_{1A}-adrenoceptor-mediated contraction of the rat epididymal vas deferens (Aboud *et al.*, 1993; Burt *et al.*, 1995a) involves different cellular mechanisms compared with the α_{1B}-adrenoceptor-mediated contraction of the rat spleen, which does not involve PKC but is mediated via capacitative Ca^{2+} influx (Burt *et al.*, 1995b). It is unlikely however that each subtype is always linked to the same mechanism as the α_{1A}-adrenoceptor mediated contraction of the human prostate (Marshall *et al.*, 1995) does not involve activation of PKC (unpublished observations). Contractions to phenylephrine in the rat aorta mediated predominantly by α_{1D}-adrenoceptors (Goetz *et al.*, 1995) partly involve activation of PKC (Shimamoto *et al.*, 1993). It may be that cellular processes involved after PIP_2 hydrolysis are more tissue rather than receptor subtype-dependent as the NK_2-mediated contraction to neurokinin A in the rat epididymal vas deferens, like that to noradrenaline, may involve activation of PKC by DAG (Burt *et al.*, 1993).

Figure 7 is a schematic diagram showing the cellular mechanisms that it is proposed may be involved in the contraction of the rat epididymal vas deferens to noradrenaline. It shows the α_{1A}-adrenoceptor mediated contraction is dependent on activation of PKC by DAG produced from PLC-mediated hydrolysis of PIP_2, which results in an influx of extracellular Ca^{2+} through voltage-gated Ca^{2+} channels. No evidence for inositol trisphosphate involvement in the contraction was found.

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References

ABOUD, R., SHAFI, M. & DOCHERTY, J.R. (1993). Investigation of the subtypes of α_1-adrenoceptor mediating contractions of rat aorta, vas deferens and spleen. *Br. J. Pharmacol.*, **109,** 80–87.

ABRAHAM, S.T. & RICE, P.J.(1992). Protein kinase C-mediated contractile response of the rat vas deferens. *Eur. J. Pharmacol.*, **218,** 243–249.

BERRIDGE, M.J. (1993). Inositol trisphosphate and calcium signalling. *Nature*, **361,** 315–325.

BISHOP, W.R. & BELL, R.W. (1986). Attenuation of *sn*-1,2-diacylglycerol second messenger: metabolism of exogenous diacylglycerols by human platelets. *J. Biol. Chem.*, **261,** 12513–12519.

BOURREAU, J.P., ZHANG, Z.D., LOW, M., KWAN, Y. & DANIEL, E.E. (1991). Ryanodine and the adrenergic, purinergic stimulation in the rat vas deferens smooth muscle: functional and radioligand binding studies. *J. Pharmacol. Exp. Ther.*, **256,** 1063–1071.

BRUNS, R.F., MILLER, F.D., MERRIMAN, R.L., HOWBERT, J.J., HEATH, W.F., KOBAYASHI, E. & TAKAHASHI, I. (1991). Inhibition of protein kinase C by calphostin C is light-dependent. *Biochem. Biophys. Res. Commun.*, **176,** 288–293.

BULTMANN, R., VON KUGELGEN, I. & STARKE, K. (1993). Effects of nifedipine and ryanodine on adrenergic neurogenic contractions of rat vas deferens: evidence for a pulse-to-pulse change in Ca^{2+} sources. *Br. J. Pharmacol.*, **108,** 1062–1070.

BURT, R.P., CHAPPLE, C.R. & MARSHALL, I. (1993). Differing neurokinin NK_2 receptor signal transduction mechanisms in epididymal and prostatic ends of the rat vas deferens. *Br. J. Pharmacol.*, **108,** 61P.

BURT, R.P., CHAPPLE, C.R. & MARSHALL, I. (1995a). Evidence for a functional α_{1A}-(α_{1C}-)adrenoceptor mediating contraction of the rat epididymal vas deferens and an α_{1B}-adrenoceptor mediated contraction of the rat spleen. *Br. J. Pharmacol.*, **115,** 467–475.

BURT, R.P., CHAPPLE, C.R. & MARSHALL, I. (1995b). The role of capacitative calcium influx in the α_{1B}-adrenoceptor mediated contractions to phenylephrine in the rat spleen. *Br. J. Pharmacol.*, **116,** 2327–2333.

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.

de CHAFFOY de COURCELLES, D., ROEVENS, P. & VAN BELLE, H. (1985). R59022, a diacyl glycerol kinase inhibitor. *J. Biol. Chem.*, **260,** 15762–15770.

DENG, H.W. & KWAN, C.Y. (1991). Cyclopiazonic acid is a sarcoplasmic reticulum Ca^{2+}-pump inhibitor of rat aortic muscle. *Acta Pharmacol. Sin.*, **12,** 53.

EHRLICH, B.E., KAFTAN, E., BEZPROZVANNAYA, S. & BEZPROZVANNY, I. (1994). The pharmacology of intracellular Ca^{2+}-release channels. *Trends Pharmacol.*, **15,** 145–149.

FASOLATO, C., INNOCENTI, B. & POZZAN, T. (1994). Receptor-activated Ca^{2+} influx: how many mechanisms for how many channels? *Trends Pharmacol. Sci.*, **15,** 77–83.

FORD, D.A., MIYAKE, R., GLASER, P.E. & GROSS, R.W. (1989). Activation of protein kinase C by naturally occurring ether-linked diglycerides. *J. Biol. Chem.*, **264,** 13818–13824.

FOX, A.W., ABEL, P.W. & MINNEMAN, K.P. (1985). Activation of α_1-adrenoceptors increases ^{3}H-inositol metabolism in rat vas deferens and caudal artery. *Eur. J. Pharmacol.*, **116,** 145–152.

GOETZ, A.S., KING, H.K., WARD, S.D.C., TRUE, T.A., RIMELE, T.J. & SAUSSY, D.L. (1995). BMY 7378 is a selective antagonist of the D subtype of α_1-adrenoceptors. *Eur. J. Pharmacol.*, **272,** R5–R6.

HALLER, H., SMALLWOOD, J.I. & RASMUSSEN, H. (1990). Protein kinase C translocation in intact vasculature smooth muscle strips. *Biochem. J.*, **270,** 375–381.

HAN, C., ABEL, P.W. & MINNEMAN, K.P. (1987). α_1-Adrenoceptor subtypes linked to different mechanisms for increasing intracellular Ca^{2+} in smooth muscle. *Nature*, **329,** 333–335.

INOUE, M., KISHIMOTO, A., TAKAI, Y. & NISHIZUKA, Y. (1977). Studies in a cyclic nucleotide-independent protein kinase and its proenzyme in mammalian tissues. *J. Biol. Chem.*, **252,** 7910.

ITOH, T., KAJIKURI, J. & KURIYAMA, H. (1992). Characteristic features of noradrenaline-induced Ca^{2+} mobilization and tension in arterial smooth muscle of the rabbit. *J. Physiol.*, **457,** 297–314.

KANOH, H., SAKANE, F., SHIN-ICHI, I. & WADA, I. (1993). Diacylglycerol kinase and phosphatidic acid phosphatase-enzymes metabolizing lipid second messengers. *Cell Signalling*, **5,** 495–503.

KOBAYASHI, E., NAKANO, H., MORIMOTO, M. & TAMAOKI, T. (1989). Calphostin C (UCN-1028c), A novel microbial compound, is a highly potent and specific inhibitor of protein kinase C. *Biochem. Biophys. Res. Commun.*, **159,** 548–553.

LEE, M.W. & SEVERSON, D.L. (1994). Signal transduction in vascular smooth muscle: diacylglycerol second messengers and PKC action. *Am. J. Physiol.*, **267,** C659–678.

MARSHALL, I., BURT, R.P. & CHAPPLE, C.R. (1995). Noradrenaline contractions of human prostate mediated by α_{1A}-(α_{1c}-)adrenoceptor subtype. *Br. J. Pharmacol.*, **115,** 781–786.

MERKEL, L.A., RIVERA, L.M., COLUSSI, D.J. & PERRONE, M.H. (1991). Protein kinase C and vascular smooth muscle contractility: effects of inhibitors and down-regulation. *J. Pharmacol. Exp. Ther.*, **257,** 134.

MINNEMAN, K.P., & ESBENSHADE, T.A. (1994). α_1-Adrenergic receptor subtypes. *Annu. Rev. Pharmacol. Toxicol.*, **34,** 117–133.

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

PUTNEY, J.W., Jr. (1986). A model for receptor-regulated calcium entry. *Cell Calcium.*, **7,** 1–12.

PUTNEY, J.W., Jr. (1990). Capacitative calcium entry revisited. *Cell Calcium*, **11,** 611–624.

RYVES, W.J., EVANS, A.T., OLIVER, A.R., PARKER, P.J. & EVANS, F.J. (1991). Activation of the PKC-isotypes α, β_1, γ, δ and ε by phorbol esters of different biological activities. *FEBS Lett.*, **288,** 5–9.

SEIDLER, N.W., JONA, I., VEGH, M. & MARTONOSI, A. (1989). Cyclopiazonic acid is a specific inhibitor of the Ca^{2+}-ATPase of sarcoplasmic reticulum. *J. Biol. Chem.*, **264,** 17816.

SEVERSON, D.L. & HEE-CHEONG, M. (1986). Diacylglycerol lipase and kinase activities in rabbit aorta and coronary microvessels. *Biochem. Cell Biol.*, **64,** 976–983.

SHIMAMOTO, Y., SHIMAMOTO, H., KWAN, C-Y. & DANIEL, E.E. (1993). Differential effects of putative protein kinase C inhibitors on contraction of rat aortic smooth muscle. *Am. J. Physiol.*, **264,** H1300–1306.

SORRENTINO, V. & VOLPE, P. (1993). Ryanodine receptors: how many and why? *Trends Pharmacol. Sci.*, **14,** 98–103.

TAKAI, T., KISHIMOTO, A., IWASA, Y., KAWAHARA, Y., MORI, T. & NISHIZUKA, Y. (1979). Calcium-dependent action of a multifunctional protein kinase by membrane phospholipids. *J. Biol. Chem.*, **254,** 3692.

TAMAOKI, T., NOMOTO, H., TAKAHASHI, I., KATO, Y., MORIMOTO, M. & TOMITA, F. (1986). Staurosporine, a potent inhibitor of phospholipid/Ca^{2+} dependent protein kinase. *Biochem. Biophys. Res. Commun.*, **135,** 397–402.

YANG, S-G., SAIFEDDINE, M., CHUANG, M., SEVERSON, D.L. & HOLLENBERG, M.D. (1991). Diacylglycerol lipase and the contractile action of epidermal growth factor-urogastrone: evidence for distinct signal pathways in a single strip of gastric smooth muscle. *Eur. J. Pharmacol.*, **207,** 225–230.

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