



# The role of capacitative $\text{Ca}^{2+}$ influx in the $\alpha_{1B}$ -adrenoceptor-mediated contraction to phenylephrine of the rat spleen

Richard P. Burt, \*Christopher R. Chapple & <sup>1</sup>Ian Marshall

Department of Pharmacology, University College London, Gower Street, London WC1E 6BT and \*Department of Urology, The Royal Hallamshire Hospital, Glossop Road, Sheffield S10 2JF

1 The mechanism of contraction to phenylephrine in the rat spleen (mediated via  $\alpha_{1B}$ -adrenoceptors) has been studied in functional experiments.

2 The concentration-dependent contraction of the rat spleen to cumulative additions of phenylephrine ( $\text{pD}_2$   $4.8 \pm 0.1$ ) was not significantly reduced by the selective protein kinase C (PKC) inhibitor, calphostin C ( $10^{-6}$  M) or potentiated by the DAG kinase inhibitor, R59022 ( $10^{-6}$  M).

3 Contraction of the rat spleen in normal Krebs solution containing  $\text{Ca}^{2+}$  (2.5 mM) to a single concentration of phenylephrine ( $3 \times 10^{-4}$  M) produced a maximal response consisting of an initial phasic component and a more slowly developing tonic component. However in  $\text{Ca}^{2+}$ -free Krebs solution (containing EGTA), phenylephrine ( $3 \times 10^{-4}$  M) produced only a phasic contraction which was reduced to  $46 \pm 3\%$  maximum response to phenylephrine in normal Krebs solution.

4 In some tissues after the contraction to phenylephrine ( $3 \times 10^{-4}$  M) in  $\text{Ca}^{2+}$ -free Krebs solution (containing EGTA), the phenylephrine was washed out and the tissue was allowed to recover. After 2 h, upon addition of  $\text{Ca}^{2+}$  (2.5 mM) to the Krebs solution (EGTA now removed) a tonic contraction developed in the tissue ( $97 \pm 4\%$  maximum response to phenylephrine).

5 Cyclopiazonic acid produced a tonic contraction of the rat spleen with a maximum effect at  $10^{-5}$  M ( $202 \pm 8\%$  maximum response compared with that to phenylephrine). The contraction to CPA ( $10^{-5}$  M) was reduced in  $\text{Ca}^{2+}$ -free Krebs solution containing EGTA ( $30 \pm 4\%$  of the maximum response to phenylephrine). One hour after the end of the contraction in  $\text{Ca}^{2+}$ -free Krebs solution (EGTA now removed), upon addition of  $\text{Ca}^{2+}$  (2.5 mM) to the Krebs solution a tonic contraction developed in the tissue ( $263 \pm 12\%$  maximum response to phenylephrine).

6 In  $\text{Ca}^{2+}$ -free Krebs solution, after the spleen had been incubated with cyclopiazonic acid for 30 min, the subsequent contraction to phenylephrine ( $3 \times 10^{-4}$  M) was reduced from  $46 \pm 3\%$  to  $9 \pm 2\%$  maximum response to phenylephrine.

7 Cumulative contractions to phenylephrine and the contraction to cyclopiazonic acid ( $10^{-5}$  M) in the spleen were not significantly affected by nifedipine ( $10^{-6}$  M). The non-selective  $\text{Ca}^{2+}$  channel blocker, SK&F 96365 ( $3 \times 10^{-5}$  M) reduced the maximum response for the cumulative additions of phenylephrine to  $35 \pm 1\%$  and the contraction to CPA ( $10^{-5}$  M) from  $202 \pm 8\%$  to  $108 \pm 8\%$  maximum response to phenylephrine.

8 The tyrosine kinase inhibitors genistein ( $3 \times 10^{-5}$  M) and tyrphostin 23 ( $10^{-4}$  M), reduced the maximum response to phenylephrine in the spleen to  $51 \pm 4\%$  and  $44 \pm 5\%$  respectively and the maximum contraction to cyclopiazonic acid ( $3 \times 10^{-6}$  M) in the spleen from  $132 \pm 6\%$  to  $82 \pm 5\%$  and  $80 \pm 7\%$  maximum response to phenylephrine respectively without affecting contractions to  $\text{K}^+$ .

9 In conclusion, these results are consistent with the contraction of the rat spleen to phenylephrine consisting of an initial phasic contraction due to release of intracellular  $\text{Ca}^{2+}$  and a larger tonic contraction due to capacitative  $\text{Ca}^{2+}$  influx through non-voltage-gated  $\text{Ca}^{2+}$  channels and which may involve a tyrosine kinase. This suggests that inositol triphosphate but not diacylglycerol is involved in the contraction.

**Keywords:** Rat spleen;  $\alpha_1$ -adrenoceptors; phenylephrine; cyclopiazonic acid; nifedipine; SK&F 96365; tyrosine kinase; intracellular  $\text{Ca}^{2+}$  stores; capacitative  $\text{Ca}^{2+}$  influx

## Introduction

The  $\alpha_1$ -adrenoceptors are G-protein coupled receptors that mediate their cellular responses such as contraction of smooth muscle by raising  $[\text{Ca}^{2+}]_i$  (Minneman & Esbenshade, 1994). They are linked to the activation of phospholipase C upon stimulation, which hydrolyses the lipid precursor phosphatidylinositol 4,5-bisphosphate to inositol 1,4,5-trisphosphate ( $\text{IP}_3$ ) and diacylglycerol (DAG).  $\text{IP}_3$  can mobilize  $\text{Ca}^{2+}$  from intracellular stores, raising the cytosolic  $[\text{Ca}^{2+}]_i$ , while DAG can stimulate protein kinase C (PKC) (Berridge, 1993).

The rise in  $[\text{Ca}^{2+}]_i$  due solely to mobilization of  $\text{Ca}^{2+}$  from intracellular stores by  $\text{IP}_3$  is transient, lasting only a few min-

utes as the stores have a limited capacity and the plasma membrane  $\text{Ca}^{2+}$  pump soon restores  $[\text{Ca}^{2+}]_i$  to resting levels. However the rise in  $[\text{Ca}^{2+}]_i$  associated with mobilization of  $\text{Ca}^{2+}$  from intracellular stores is usually larger and more sustained due to a linked influx of extracellular  $\text{Ca}^{2+}$ . One possibility to explain this influx is by the production of DAG and activation of PKC.  $\text{IP}_3$  can also be phosphorylated to inositol 1,3,4,5-tetrakisphosphate ( $\text{IP}_4$ ) which may enable entry of extracellular calcium in some cells when  $\text{IP}_3$  is also present (Morris *et al.*, 1987). In other cells the influx of extracellular  $\text{Ca}^{2+}$  does not appear to require either the presence of  $\text{IP}_4$  or  $\text{IP}_3$  once the intracellular  $\text{Ca}^{2+}$  stores have been depleted (Irvine, 1992; Putney, 1986; 1990). This form of  $\text{Ca}^{2+}$  influx was called capacitative  $\text{Ca}^{2+}$  entry by Putney (1986). Stimulation

<sup>1</sup> Author for correspondence.

of the influx of extracellular  $\text{Ca}^{2+}$  as a consequence of the depletion of intracellular  $\text{Ca}^{2+}$  stores has been demonstrated using compounds that do not produce inositol phosphates but deplete intracellular  $\text{Ca}^{2+}$  stores by inhibiting the endoplasmic reticulum  $\text{Ca}^{2+}$ -ATPase pump (Jacob, 1990).

The rat spleen has been shown to contract to phenylephrine via the  $\alpha_{1B}$ -adrenoceptor (Aboud *et al.*, 1993; Burt *et al.*, 1995b). Comparison of phenylephrine with cyclopiazonic acid (CPA), which depletes  $\text{Ca}^{2+}$  from intracellular stores by inhibiting the sarcoplasmic reticulum  $\text{Ca}^{2+}$ -ATPase in striated muscle (Seidler *et al.*, 1989) and smooth muscle (Deng & Kwan, 1991), suggests that this contraction involves capacitative  $\text{Ca}^{2+}$  influx. A preliminary account of these results has been presented (Burt *et al.*, 1995a).

## Methods

Male Sprague Dawley rats (350–450 g) were stunned and killed by cervical dislocation. The spleen was removed and bisected longitudinally into two strips. They were then suspended in 5 ml tissue baths containing Krebs solution of the following composition (mM):  $\text{Na}^+$  143,  $\text{K}^+$  5.9,  $\text{Ca}^{2+}$  2.5,  $\text{Mg}^{2+}$  1.2,  $\text{Cl}^-$  128,  $\text{HCO}_3^-$  25,  $\text{HPO}_4^{2-}$  1.2,  $\text{SO}_4^{2-}$  1.2 and glucose 11, at 37°C and bubbled with 95%  $\text{O}_2$ /5%  $\text{CO}_2$  under 1.0 g resting tension and equilibrated for 90 min. Changes in isometric tension were measured with Grass FT.03 transducers and recorded by Biopac Systems Inc. MP100WS for Windows.

A contraction to phenylephrine ( $3 \times 10^{-4}$  M) which produced a maximal response was measured in each tissue. All tissues were then left for 2 h before any further responses were measured due to the long recovery time of this tissue following a contraction to phenylephrine. When  $\text{Ca}^{2+}$ -free Krebs solution was used this contained 1 mM EGTA unless stated otherwise. When cumulative additions of either phenylephrine ( $10^{-7}$  M– $10^{-3}$  M) or  $\text{K}^+$  ( $2 \times 10^{-2}$  M– $10^{-1}$  M) were used, a concentration-effect curve was recorded 2 h after the initial contraction to phenylephrine ( $3 \times 10^{-4}$  M) and then repeated after a further 2 h as either a control or under the relevant conditions for the experiment.

Contractions induced by cumulative additions of phenylephrine ( $10^{-7}$  M– $10^{-3}$  M) were measured in the presence of calphostin C ( $10^{-6}$  M, 1 h incubation) and R 59022 ( $10^{-6}$  M, 30 min incubation). Calphostin C was used in a bright light environment as this is essential to its activity (Bruns *et al.*, 1991). Some control responses were measured in the presence of dimethylsulphoxide (DMSO), which was used to dissolve the calphostin C.

Contractions induced by cumulative additions of phenylephrine ( $10^{-7}$  M– $10^{-3}$  M) were measured in  $\text{Ca}^{2+}$ -free Krebs solution. In other tissues the response to a single addition of phenylephrine ( $3 \times 10^{-4}$  M) was measured either in normal Krebs solution or in  $\text{Ca}^{2+}$ -free Krebs solution in which the tissue had been equilibrated for 30 min after 90 min recovery in normal Krebs solution following the initial phenylephrine contraction.

Twenty minutes after the single addition of phenylephrine in  $\text{Ca}^{2+}$ -free Krebs solution, the agonist was washed out for another 120 min still in  $\text{Ca}^{2+}$ -free Krebs solution with EGTA being removed from the Krebs solution for the last 30 min.  $\text{Ca}^{2+}$  (2.5 mM) was then added to the Krebs solution and the response to this measured. As a control, in other tissues 20 min after the addition of phenylephrine in normal Krebs solution the agonist was washed out for 90 min in normal Krebs solution and then equilibrated for 30 min in  $\text{Ca}^{2+}$ -free Krebs solution with the EGTA being removed for the last 15 min.  $\text{Ca}^{2+}$  (2.5 mM) was then added to the Krebs solution and the response to this measured.

CPA was added to some tissues in normal Krebs solution ( $10^{-7}$  M– $3 \times 10^{-5}$  M, one concentration per tissue). In other tissues the effect of CPA ( $10^{-5}$  M) was measured in  $\text{Ca}^{2+}$ -free Krebs solution. One hour after the end of the contraction to

CPA in  $\text{Ca}^{2+}$ -free Krebs solution and with the EGTA removed for the final 30 min,  $\text{Ca}^{2+}$  (2.5 mM) was then added to the Krebs solution and the response to this measured.

Some tissues were incubated with CPA ( $10^{-5}$  M) in  $\text{Ca}^{2+}$ -free Krebs solution for 30 min and the response to a subsequent addition of phenylephrine ( $3 \times 10^{-4}$  M) was then measured.

Contractions induced by cumulative additions of phenylephrine ( $10^{-7}$  M– $10^{-3}$  M) and the contraction to CPA ( $10^{-5}$  M) were measured in the presence of nifedipine ( $10^{-6}$  M, 20 min equilibration) or SK&F 96365 ( $3 \times 10^{-5}$  M, 20 min equilibration).

Contractions to cumulative additions of phenylephrine,  $\text{K}^+$  and the contraction to CPA ( $3 \times 10^{-6}$  M) were measured in the presence of either genistein  $3 \times 10^{-5}$  M, or tyrphostin 23  $10^{-4}$  M, (each incubated with the tissue for 1 h). Control responses to phenylephrine and  $\text{K}^+$  were also measured in the presence of DMSO, which was used to dissolve the genistein and tyrphostin 23.

## Data analysis

The results were calculated as percentage maximum response of the initial phenylephrine contraction or as percentage maximum response of the first cumulative concentration-effect curve for either phenylephrine or  $\text{K}^+$  when one of these was used. Responses were then plotted as the mean of four separate experiments with vertical bars representing standard error of the mean (s.e.mean). Curve fitting for the calculation of  $\text{pD}_2$  values by non linear regression was performed using InPlot (GraphPAD Software, San Diego, Calif., U.S.A.).

## Drugs and solutions

(–)-Phenylephrine hydrochloride and nifedipine were obtained from Sigma. Calphostin C, R 59022 [6-(2-(4-(*p*-fluorophenyl) phenylmethylene)-1-piperidinyl)ethyl)-7-methyl-5H-thiazolo(3,2-*a*)pyrimidine-5-one], cyclopiazonic acid, SK&F 96365, genistein and tyrphostin 23 were obtained from Calbiochem. Phenylephrine and SK&F 96365 were dissolved in distilled water and nifedipine was dissolved in ethanol and then diluted to working concentrations in distilled water. All other compounds were dissolved in DMSO. Phenylephrine and nifedipine were prepared fresh each day and all other compounds were stored frozen.

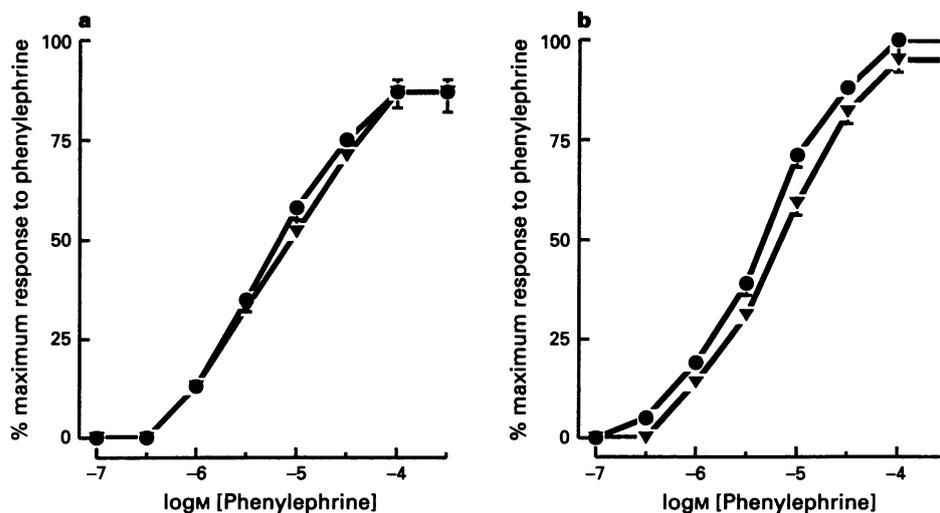
## Results

Phenylephrine ( $3 \times 10^{-4}$  M) produced a maximal response in the rat spleen ( $0.27 \pm 0.02$  g). The cumulative contractions to phenylephrine were reproducible ( $\text{pD}_2$   $4.8 \pm 0.1$ ,  $96 \pm 2\%$  maximum response compared with the initial phenylephrine contraction), as were those to increasing [ $\text{K}^+$ ] ( $115 \pm 4\%$  maximum response to phenylephrine).

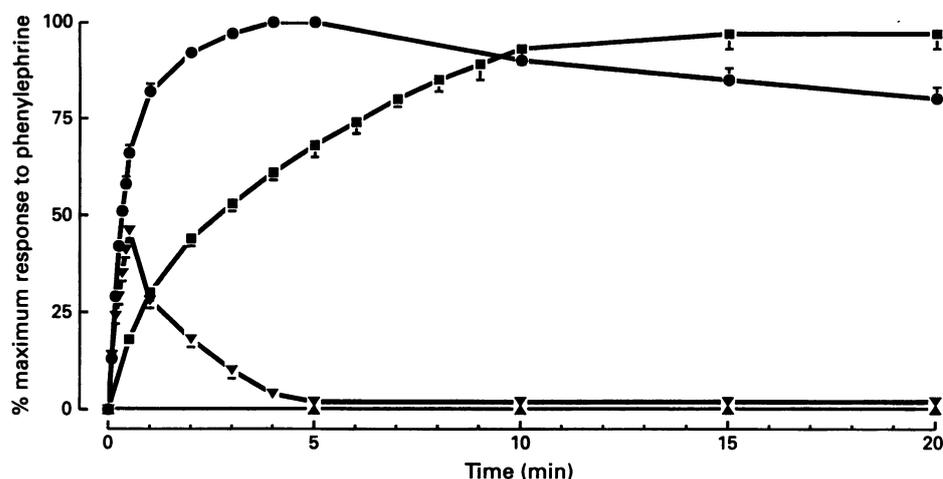
The contractions to cumulative additions of phenylephrine in the spleen were not significantly affected by the selective PKC inhibitor calphostin C  $10^{-6}$  M, (Figure 1a) or the DAG kinase inhibitor, R 59022  $10^{-6}$  M, (Figure 1b).

The contraction to a single addition of phenylephrine ( $3 \times 10^{-4}$  M) in normal Krebs solution consisted of an initial phasic contraction and a more slowly developing tonic contraction (Figure 2). The contractions to cumulative additions of phenylephrine were completely abolished in  $\text{Ca}^{2+}$ -free Krebs solution (results not shown). However a single addition of phenylephrine ( $3 \times 10^{-4}$  M) in  $\text{Ca}^{2+}$ -free Krebs solution produced a phasic contraction ( $46 \pm 3\%$  maximum response), returning to baseline in 5 min (Figure 2).

To see if influx of extracellular  $\text{Ca}^{2+}$  was stimulated by depletion of intracellular  $\text{Ca}^{2+}$  stores, after the addition of phenylephrine ( $3 \times 10^{-4}$  M) in  $\text{Ca}^{2+}$ -free Krebs solution, the agonist was washed out for 2 h still in  $\text{Ca}^{2+}$ -free Krebs solu-



**Figure 1** (a), The effect of calphostin C on contractions to cumulative additions of phenylephrine in the rat spleen. Control in the presence of DMSO (●), +calphostin C  $10^{-6}$  M (▼). (b) The effect of R 59022 on contractions to cumulative additions of phenylephrine in the rat spleen. Control (●), + R 59022  $10^{-6}$  M (▼). Each plot represents the mean with s.e.mean of 4 separate experiments.



**Figure 2** The effect of removing extracellular  $\text{Ca}^{2+}$  on the contraction to phenylephrine in the rat spleen and the response to re-addition of  $\text{Ca}^{2+}$  after washout. Contraction to phenylephrine ( $3 \times 10^{-4}$  M) in normal Krebs solution (●), in  $\text{Ca}^{2+}$ -free Krebs solution containing EGTA 1 mM (▼), and the following contraction to the addition of  $\text{Ca}^{2+}$  2.5 mM, 2 h after the phenylephrine has been washed out and the EGTA has been removed from the Krebs solution (■). The effect of re-addition of  $\text{Ca}^{2+}$  2.5 mM after a contraction to phenylephrine ( $3 \times 10^{-4}$  M) in normal Krebs solution, followed by 90 min washout in normal Krebs solution and then 30 min equilibration in  $\text{Ca}^{2+}$  free Krebs solution (▲). Each plot represents the mean with s.e.mean of 4 separate experiments. The abscissa scale shows time in min after the addition of phenylephrine or the readdition of  $\text{Ca}^{2+}$ .

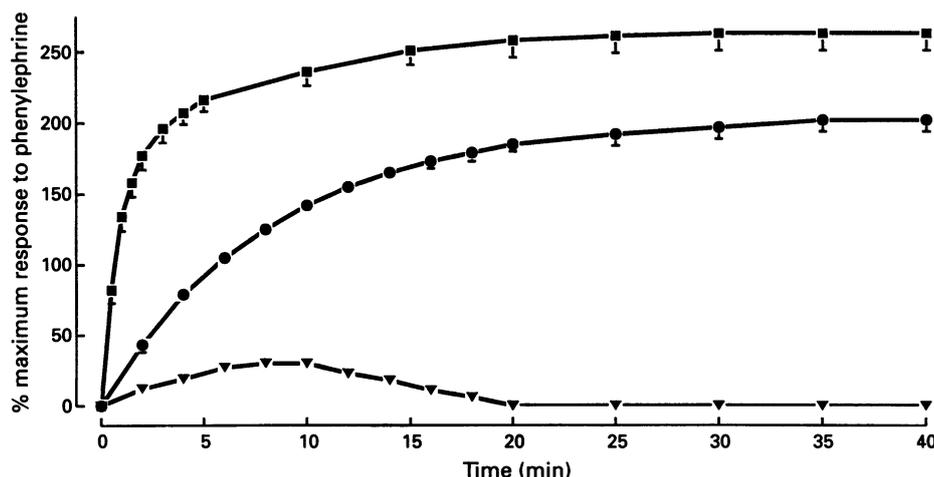
tion. At this time concentrations of any second messengers generated by  $\alpha_1$ -adrenoceptor stimulation would no longer be raised but the intracellular  $\text{Ca}^{2+}$  stores should remain depleted as there was no extracellular  $\text{Ca}^{2+}$  to enter and refill them.  $\text{Ca}^{2+}$  (2.5 mM) was then added to the Krebs solution after this 2 h period and this produced a slowly developing tonic contraction in each tissue reaching  $97 \pm 4\%$  maximum response (Figure 2). After a contraction to phenylephrine in normal Krebs solution and following 90 min washout with normal Krebs solution and then 30 min equilibration in  $\text{Ca}^{2+}$ -free Krebs solution, no response was produced by the addition of  $\text{Ca}^{2+}$  (2.5 mM) (Figure 2).

The endoplasmic reticulum  $\text{Ca}^{2+}$ -ATPase inhibitor CPA produced a concentration dependent slowly developing tonic contraction in normal Krebs solution ( $10^{-7}$ – $10^{-5}$  M,  $\text{pD}_2$   $5.7 \pm 0.1$ ). The maximum response to CPA was reached at  $10^{-5}$  M, 35 min after addition ( $202 \pm 8\%$  maximum response to phenylephrine, Figure 3). The response to CPA ( $10^{-5}$  M) in

$\text{Ca}^{2+}$ -free Krebs solution was much smaller and not as well maintained ( $30 \pm 4\%$  maximum response to phenylephrine), returning to baseline after 20 min (Figure 3). When  $\text{Ca}^{2+}$  (2.5 mM) was added to the Krebs solution 1 h after the end of the contraction in  $\text{Ca}^{2+}$ -free Krebs solution and with the EGTA removed, a tonic contraction developed in the tissue ( $263 \pm 12\%$  maximum response to phenylephrine, Figure 3).

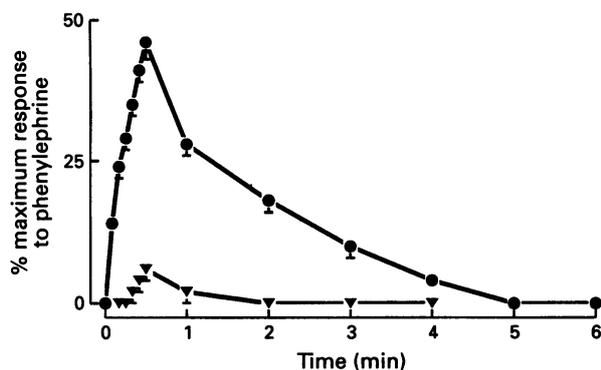
To see if contractions to phenylephrine and CPA in  $\text{Ca}^{2+}$ -free Krebs solution might be due to release of intracellular  $\text{Ca}^{2+}$  from the same store, some tissues were pre-incubated with CPA ( $10^{-5}$  M) in  $\text{Ca}^{2+}$ -free Krebs solution. The subsequent contraction to phenylephrine ( $3 \times 10^{-4}$  M) was reduced to  $9 \pm 2\%$  maximum response to phenylephrine (Figure 4).

Nifedipine ( $10^{-6}$  M) had no significant effect on either the contractions to cumulative additions of phenylephrine (control  $\text{pD}_2$   $4.8 \pm 0.1$ , with nifedipine  $\text{pD}_2$   $4.8 \pm 0.2$ ) or the contraction to CPA ( $10^{-5}$  M) (control  $202 \pm 8\%$ , with nifedipine  $191 \pm 25\%$

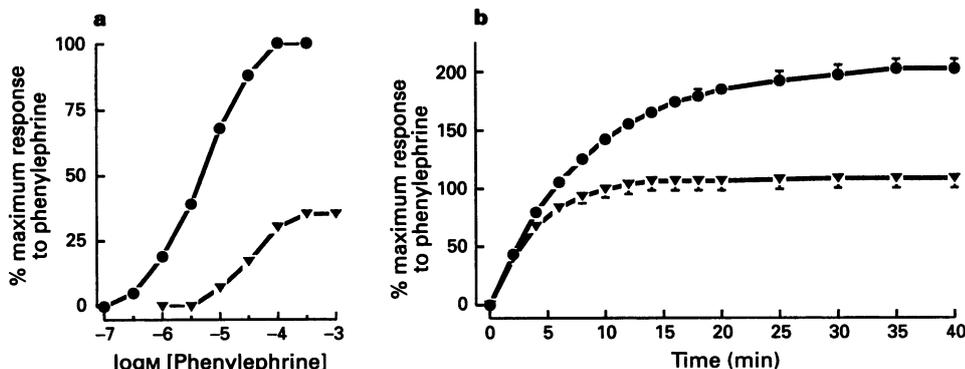


**Figure 3** The effect of removing extracellular  $\text{Ca}^{2+}$  on the contraction to CPA in the rat spleen and the following response to the re-addition of  $\text{Ca}^{2+}$ . Contraction to CPA ( $10^{-5}$  M) in normal Krebs solution ( $\bullet$ ), in  $\text{Ca}^{2+}$ -free Krebs solution containing EGTA 1 mM ( $\blacktriangledown$ ). The contraction to the addition of  $\text{Ca}^{2+}$  (2.5 mM) 1 h after the end of the response to CPA in  $\text{Ca}^{2+}$ -free Krebs solution with the EGTA removed from the Krebs solution ( $\blacksquare$ ). Each plot represents the mean with s.e.mean of 4 separate experiments. The abscissa scale shows time in min after the addition of CPA or the re-addition of  $\text{Ca}^{2+}$ .

maximum response to phenylephrine). SK&F 96365 ( $3 \times 10^{-5}$  M) reduced the maximum response for the cumulative additions of phenylephrine to  $35 \pm 1\%$  (Figure 5a) and the contraction to CPA ( $10^{-5}$  M) from  $202 \pm 8\%$  to  $108 \pm 8\%$  maximum response to phenylephrine (Figure 5b). In 3 out of 12 tissues SK&F 96365 ( $3 \times 10^{-5}$  M) produced a contraction and so the responses in these tissues were not included in the results.



**Figure 4** The effect of CPA pretreatment on the contraction to phenylephrine in  $\text{Ca}^{2+}$ -free Krebs solution in the rat spleen. Control contraction to phenylephrine ( $3 \times 10^{-4}$  M) in  $\text{Ca}^{2+}$ -free Krebs solution containing EGTA 1 mM ( $\bullet$ ), and after 30 min pre-incubation with CPA  $10^{-5}$  M ( $\blacktriangledown$ ). Each plot represents the mean with s.e.mean of 4 separate experiments.



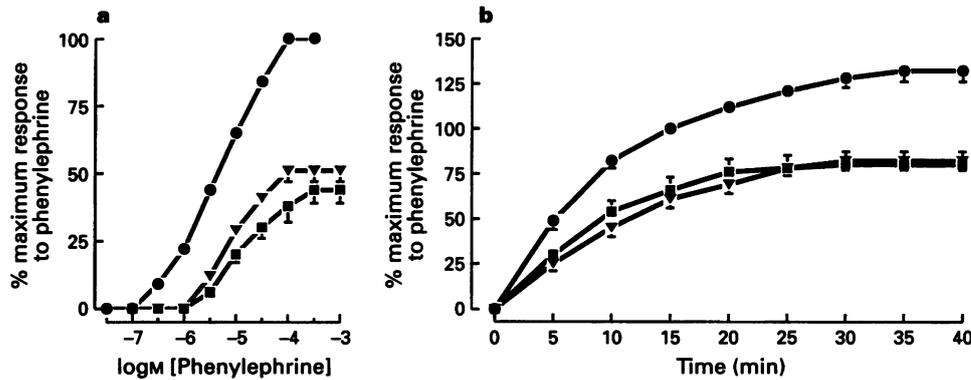
**Figure 5** The effect of the non-selective  $\text{Ca}^{2+}$  channel blocker, SK&F 96365 in the rat spleen on (a), contractions to cumulative additions of phenylephrine and (b), a contraction to CPA ( $10^{-5}$  M). Control ( $\bullet$ ), and in the presence of SK&F 96365  $3 \times 10^{-5}$  M ( $\blacktriangledown$ ). Each plot represents the mean with s.e.mean of 4 separate experiments.

The tyrosine kinase inhibitors genistein ( $3 \times 10^{-5}$  M) and tyrphostin 23 ( $10^{-4}$  M) reduced the maximum response for the cumulative additions of phenylephrine to  $51 \pm 4\%$  and  $44 \pm 5\%$  respectively (Figure 6a). Genistein ( $3 \times 10^{-5}$  M) and tyrphostin 23 ( $10^{-4}$  M) also reduced the maximum response for the contraction to CPA ( $3 \times 10^{-6}$  M) from  $132 \pm 6\%$  to  $82 \pm 5\%$  and  $80 \pm 7\%$  maximum response to phenylephrine respectively (Figure 6b). At these concentrations genistein and tyrphostin 23 had little or no effect on the contractions to increasing  $[\text{K}^+]$  (control  $\text{pD}_2$   $1.4 \pm 0.1$ , with genistein  $\text{pD}_2$   $1.4 \pm 0.1$ , with tyrphostin 23  $\text{pD}_2$   $1.4 \pm 0.1$ ).

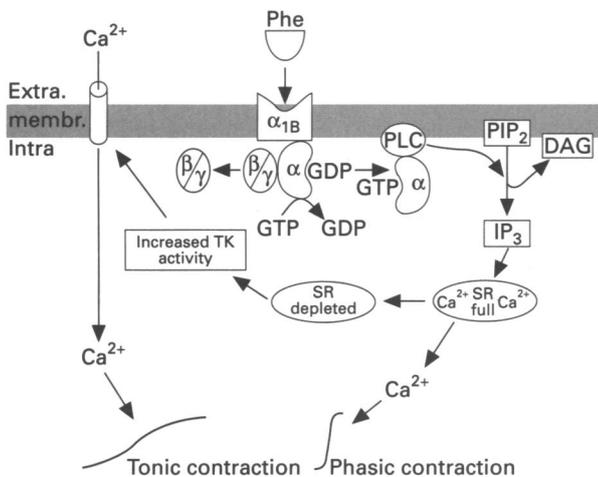
## Discussion

The mechanism of contraction to phenylephrine of the rat spleen which is mediated by  $\alpha_{1B}$ -adrenoceptors (Aboud *et al.*, 1993; Burt *et al.*, 1995b) has been investigated using functional studies.  $\alpha_1$ -Adrenoceptors are G-protein linked receptors which generally mediate their cellular responses via stimulation of PLC and production of inositol phosphates and DAG.

The DAG kinase inhibitor, R 59022 (de Chaffoy de Courcelles *et al.*, 1985), which inhibits metabolism of DAG to phosphatidic acid, did not potentiate the contraction to phenylephrine in the spleen and the selective PKC inhibitor calphostin C (Kobayashi *et al.*, 1989) did not reduce the contraction. R 59022 has been shown to potentiate the contraction and calphostin C to inhibit the same contraction in



**Figure 6** The effect of the tyrosine kinase inhibitors, genistein and tyrphostin 23 on (a), contractions to cumulative additions of phenylephrine in the rat spleen and (b), a contraction to CPA ( $3 \times 10^{-6}$  M) in the rat spleen. Control ( $\bullet$ ), in the presence of genistein  $3 \times 10^{-5}$  M ( $\blacktriangledown$ ), and in the presence of tyrphostin 23  $10^{-4}$  M ( $\blacksquare$ ). Each plot represents the mean of 4 separate experiments with standard error of the mean.



**Figure 7** Schematic diagram of the cellular mechanisms proposed for the  $\alpha_{1B}$ -adrenoceptor-mediated contraction to phenylephrine in the rat spleen. DAG: diacylglycerol, GDP: guanosine diphosphate, GTP: guanosine triphosphate,  $\text{IP}_3$ : inositol 1,4,5-trisphosphate, Phe: phenylephrine,  $\text{PIP}_2$ : phosphatidylinositol 4,5-bisphosphate, PLC: phospholipase C, SR: sarcoplasmic reticulum, TK: tyrosine kinase, Membr: cell membrane, Extra: extracellular, Intra: intracellular;  $\alpha$  and  $\beta/\gamma$  are the three subunits of a heterotrimeric G-protein. Although this scheme shows the increased tyrosine kinase activity in the cytosol this may occur in the membrane.

another smooth muscle when stimulated via a PLC coupled receptor (Burt *et al.*, 1993). This suggests that DAG and PKC are not involved in the contraction of the rat spleen to phenylephrine.

$\text{IP}_3$  can mobilize  $\text{Ca}^{2+}$  from intracellular stores causing a transient rise in  $[\text{Ca}^{2+}]_i$  (Irvine, 1992; Berridge, 1993). The effect of removing  $\text{Ca}^{2+}$  from the Krebs solution was therefore studied to see if the contraction to phenylephrine in the spleen was dependent upon influx of extracellular  $\text{Ca}^{2+}$ . In normal Krebs solution the maximal response to phenylephrine consisted of an initial phasic contraction and a larger fairly well maintained tonic contraction which lasted over 20 min. In  $\text{Ca}^{2+}$ -free Krebs solution a contraction to the same concentration of phenylephrine was reduced by over 50% and consisted of an initial phasic contraction which disappeared within 5 min. This suggests that the contraction to phenylephrine in the rat spleen consists of an initial phasic contraction due to the release of  $\text{Ca}^{2+}$  from intracellular stores by  $\text{IP}_3$  and a larger tonic contraction due to the influx of extracellular  $\text{Ca}^{2+}$ . When cumulative additions of phenylephrine were made in  $\text{Ca}^{2+}$ -free Krebs solution, no response was observed. This is probably due to the intracellular  $\text{Ca}^{2+}$  stores being depleted gradually and so not causing a large enough rise in  $[\text{Ca}^{2+}]_i$  at any time to initiate contraction.

The possibility that the influx of extracellular  $\text{Ca}^{2+}$  in the contraction of the spleen to phenylephrine might be stimulated by the depletion of  $\text{Ca}^{2+}$  from intracellular stores was then investigated. After the contraction to phenylephrine in  $\text{Ca}^{2+}$ -free Krebs solution the phenylephrine was removed and the tissues were allowed to recover for 2 h (in  $\text{Ca}^{2+}$ -free Krebs solution). After this period any rise in concentration of second messengers produced by the stimulation of the  $\alpha_1$ -adrenoceptors or the rise in  $[\text{Ca}^{2+}]_i$  should have returned to resting levels. However, intracellular  $\text{Ca}^{2+}$  stores should remain depleted as there has been no influx of extracellular  $\text{Ca}^{2+}$  to allow them to be refilled. Therefore influx of  $\text{Ca}^{2+}$  should still occur when extracellular  $\text{Ca}^{2+}$  is made available if the depletion of intracellular  $\text{Ca}^{2+}$  stores is the stimulus for  $\text{Ca}^{2+}$  influx. Upon addition of  $\text{Ca}^{2+}$  to the Krebs solution after this 2 h period a well maintained tonic contraction developed in the tissue, equivalent in size to the phenylephrine contraction in normal Krebs solution but without the initial phasic component. This suggests that influx of extracellular  $\text{Ca}^{2+}$  in the spleen is stimulated as a consequence of the depletion of intracellular  $\text{Ca}^{2+}$  stores and that this is the mechanism by which the tonic component of the contraction to phenylephrine in the spleen is produced. When intracellular  $\text{Ca}^{2+}$  stores were allowed to refill after a contraction to phenylephrine by washing the tissue in normal Krebs solution, no response was observed to the addition of  $\text{Ca}^{2+}$  after equilibrating the tissue in  $\text{Ca}^{2+}$ -free Krebs solution. This was consistent with the contraction to  $\text{Ca}^{2+}$  following the phenylephrine contraction in  $\text{Ca}^{2+}$ -free Krebs solution being stimulated by depletion of intracellular  $\text{Ca}^{2+}$  stores and was not an effect of equilibrating the tissue in  $\text{Ca}^{2+}$ -free Krebs solution.

Another way to investigate the effects of depleting intracellular  $\text{Ca}^{2+}$  stores in tissues is to use compounds that deplete  $\text{Ca}^{2+}$  from these stores by inhibiting the sarcoplasmic reticulum  $\text{Ca}^{2+}$ -ATPase such as CPA (Deng & Kwan, 1991) without causing a rise in inositol phosphate levels (Demaurex *et al.*, 1992). The effects of CPA on the spleen was therefore studied. CPA produced a concentration-dependent tonic contraction of the spleen with a maximal effect at  $10^{-5}$  M. To see if the tonic contraction to CPA was dependent upon influx of extracellular  $\text{Ca}^{2+}$ , the contraction to CPA ( $10^{-5}$  M) in normal Krebs solution was compared to the response to CPA in  $\text{Ca}^{2+}$ -free Krebs solution. Removal of extracellular  $\text{Ca}^{2+}$  reduced the size of the contraction to CPA and this was not well maintained. However, when the tissue was given time to recover after the contraction to CPA in  $\text{Ca}^{2+}$ -free Krebs solution, a large tonic contraction was still produced in the tissue when  $\text{Ca}^{2+}$  was added to the Krebs solution. This is consistent with a small component of the contraction to CPA in the spleen being due to the release of intracellular  $\text{Ca}^{2+}$ , with a much larger tonic component of the contraction being due to influx of extracellular  $\text{Ca}^{2+}$  stimulated by depletion of in-

tracellular Ca<sup>2+</sup> stores. These results therefore further support this mechanism of Ca<sup>2+</sup> influx existing in the rat spleen and its involvement in the contraction of the spleen to phenylephrine.

The contraction to CPA was different in some respects from the phenylephrine contraction but this can be explained by the difference in the way the two compounds deplete intracellular Ca<sup>2+</sup> stores rather than different cellular mechanisms being involved. Unlike phenylephrine, the CPA contraction did not have an initial phasic component due to the CPA having to first enter the cell and then deplete the Ca<sup>2+</sup> stores by inhibiting the Ca<sup>2+</sup>-ATPase. In this case the Ca<sup>2+</sup> release from intracellular stores by CPA is probably more gradual than that following addition of phenylephrine. Also, the maximum contraction to CPA was about twice the size of that to phenylephrine. This might be because CPA is more efficient in depleting intracellular Ca<sup>2+</sup> stores, resulting in a larger stimulus for Ca<sup>2+</sup> influx.

There is evidence to suggest that in some cells there are IP<sub>3</sub>-sensitive and IP<sub>3</sub>-insensitive (e.g. ryanodine sensitive) intracellular Ca<sup>2+</sup> stores (Sorrentino & Volpe, 1993; Ehrlich *et al.*, 1994). The effect of CPA (10<sup>-5</sup> M) on the contraction to phenylephrine in Ca<sup>2+</sup>-free Krebs solution was therefore investigated to see if they were releasing Ca<sup>2+</sup> from the same intracellular stores. The CPA caused a large reduction in the phasic contraction to phenylephrine in Ca<sup>2+</sup>-free Krebs solution suggesting that they both release Ca<sup>2+</sup> from the same intracellular store, although it is still possible that CPA additionally releases Ca<sup>2+</sup> from an IP<sub>3</sub>-insensitive Ca<sup>2+</sup> store.

Capacitative Ca<sup>2+</sup> influx has been shown not to involve voltage-gated Ca<sup>2+</sup> channels (Hoth & Penner, 1992; Fasolato *et al.*, 1994) but is blocked by the non-selective Ca<sup>2+</sup> channel blocker, SK&F 96365 (Merritt *et al.*, 1990; Demareux *et al.*, 1992; Wayman *et al.*, 1995). The voltage-gated Ca<sup>2+</sup> channel blocker, nifedipine, was found to have no significant effect on contractions to either phenylephrine or CPA in the spleen but SK&F 96365 did inhibit both contractions. This suggests that the influx of extracellular Ca<sup>2+</sup> stimulated by intracellular Ca<sup>2+</sup> store depletion in the rat spleen is also through non-voltage-gated Ca<sup>2+</sup> channels. It is not certain why SK&F 96365 at 3 × 10<sup>-5</sup> M produced a contraction in a few tissues

(which were not included in the results). However, SK&F 96365 has been shown to cause intracellular Ca<sup>2+</sup> release from IP<sub>3</sub>-insensitive stores in some cells (Merritt *et al.*, 1990; Hughes & Schachter, 1994).

Depletion of intracellular Ca<sup>2+</sup> stores has been shown to lead to a rise in tyrosine phosphorylation in human platelets (Sargeant *et al.*, 1994). Also influx of extracellular Ca<sup>2+</sup> stimulated by depletion of intracellular Ca<sup>2+</sup> stores has been shown to be reduced by tyrosine kinase inhibitors in rat pancreatic acinar cells (Yule *et al.*, 1994) and human lymphocytes (Tepel *et al.*, 1994). The two tyrosine kinase inhibitors genistein (Akiyama *et al.*, 1987) and tyrphostin 23 (Gazit *et al.*, 1989) were found to reduce the contractions to phenylephrine and CPA at concentrations which had no significant effect on contractions to increasing [K<sup>+</sup>] in the rat spleen. This suggests that a tyrosine kinase is also involved in the influx of extracellular Ca<sup>2+</sup> stimulated by depletion of intracellular Ca<sup>2+</sup> stores in the rat spleen.

It is not clear if α<sub>1B</sub>-adrenoceptors mediate their cellular responses in all tissues by capacitative Ca<sup>2+</sup> influx as this has not yet been investigated in other tissues where functional α<sub>1B</sub>-adrenoceptors have been characterized. However the α<sub>1A</sub>-adrenoceptor-mediated contraction of the rat epididymal vas deferens (Aboud *et al.*, 1993; Burt *et al.*, 1995b) and human prostate (Marshall *et al.*, 1995) does not involve capacitative Ca<sup>2+</sup> influx (manuscript in preparation).

Figure 7 is a schematic diagram showing the cellular mechanisms proposed to be involved in the contraction of the rat spleen to phenylephrine based on these results. It shows the contraction consisting of an initial phasic component due to release of intracellular Ca<sup>2+</sup> and a tonic component due to capacitative Ca<sup>2+</sup> influx through non-voltage-gated Ca<sup>2+</sup> channels which may involve a tyrosine kinase. This suggests that inositol triphosphate but not diacylglycerol is involved in the contraction.

We thank Pfizer Central Research, Kent, for supporting this work.

## References

- ABOUD, R., SHAFI, M. & DOCHERTY, J.R. (1993). Investigation of the subtypes of α<sub>1</sub>-adrenoceptor mediating contractions of rat aorta, vas deferens and spleen. *Br. J. Pharmacol.*, **109**, 80–87.
- AKIYAMA, T., ISHIDA, J., NAKAGAWA, S., OGAWARA, S., WATANABE, S., ITOH, N., SHIBUYA, M. & FUKAMI, Y. (1987). Genistein, a specific inhibitor of tyrosine-specific protein kinases. *J. Biol. Chem.*, **262**, 5592–5595.
- BERRIDGE, M.J. (1993). Inositol triphosphate and calcium signalling. *Nature*, **361**, 315–325.
- 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.
- BURT, R.P., CHAPPLE, C.R. & MARSHALL, I. (1993). Differing neurokinin NK<sub>2</sub> receptor signal transduction mechanisms in epididymal and prostatic ends of the rat vas deferens. *Br. J. Pharmacol.*, **108**, Proc. Suppl. 61P.
- BURT, R.P., CHAPPLE, C.R. & MARSHALL, I. (1995a). Capacitative calcium influx is stimulated in contraction of rat spleen to phenylephrine. *Br. J. Pharmacol.*, **114**, 38P.
- BURT, R.P., CHAPPLE, C.R. & MARSHALL, I. (1995b). Evidence for a functional α<sub>1A</sub>- (α<sub>1C</sub>-) adrenoceptor mediating contraction of the rat epididymal vas deferens and an α<sub>1B</sub>-adrenoceptor mediating contraction of the rat spleen. *Br. J. Pharmacol.*, **115**, 467–475.
- de CHAFFOY de COURCELLES, D., ROEVENS, P. & VAN BELLE, H. (1985). R 59022, a diacyl glycerol kinase inhibitor. *J. Biol. Chem.*, **260**, 15762–15770.
- DEMAUREX, N., LEW, D.P. & KRAUSE, K.H. (1992). Cyclopiazonic acid depletes intracellular Ca<sup>2+</sup> stores and activates an influx pathway for divalent cations in HL-60 cells. *J. Biol. Chem.*, **267**, 2318–2324.
- DENG, H.W. & KWAN, C.Y. (1991). Cyclopiazonic acid is a sarcoplasmic reticulum Ca<sup>2+</sup>-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<sup>2+</sup>-release channels. *Trends Pharmacol. Sci.*, **15**, 145–149.
- FASOLATO, C., INNOCENTI, B. & POZZAN, T. (1994). Receptor-activated Ca<sup>2+</sup> influx: how many mechanisms for how many channels? *Trends Pharmacol. Sci.*, **15**, 77–83.
- GAZIT, A., YAISH, P., GILON, C. & LEVITZKI, A. (1989). Tyrphostins 1: synthesis and biological activity of protein tyrosine kinase inhibitors. *J. Med. Chem.*, **32**, 2344–2352.
- HOTH, M. & PENNER, R. (1992). Depletion of intracellular calcium stores activates a calcium current in mast cells. *Nature*, **355**, 353–356.
- HUGHES, A.D. & SCHACHTER, M. (1994). Multiple pathways for entry of calcium and other divalent cations in a vascular smooth muscle cell line (A7r5). *Cell Calcium*, **15**, 317–330.
- IRVINE, R.F. (1992). Inositol phosphates and Ca<sup>2+</sup> entry: toward a proliferation or a simplification? *FASEB J.*, **6**, 3085–3091.
- JACOB, R. (1990). Agonist-stimulated divalent cation entry into single cultured human umbilical vein endothelial cells. *J. Physiol.*, **421**, 55–77.
- 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.
- MARSHALL, I., BURT, R.P. & CHAPPLE, C.R. (1995). α<sub>1A</sub>- (α<sub>1C</sub>-) Adrenoceptor subtype mediates noradrenaline contractions of human prostate. *Br. J. Pharmacol.*, **115**, 781–786.

- MERRITT, J.E., ARMSTRONG, W.P., BENHAM, C.D., HALLAM, T.J., JACOB, R., JAXA-CHAMIEC, A., LEIGH, B.K., MCCARTHY, S.A., MOORES, K.E. & RINK, T.J. (1990). SK&F 96365, a novel inhibitor of receptor-mediated calcium entry. *Biochem. J.*, **271**, 515–522.
- MINNEMAN, K.P. & ESBENSHADE, T.A. (1994).  $\alpha_1$ -Adrenergic receptor subtypes. *Annu. Rev. Pharmacol. Toxicol.*, **34**, 117–133.
- MORRIS, A.P., GALLACHER, D.V., IRVINE, R.F. & PETERSEN, O.H. (1987). Synergism of inositol trisphosphate and inositol tetrakisphosphate in activating  $Ca^{2+}$ -dependent  $K^+$  channels. *Nature*, **330**, 653–655.
- 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.
- SARGEANT, P., FARNDAL, R.W. & SAGE, S. (1994). Calcium store depletion in dimethyl bapta-loaded human platelets increases protein tyrosine phosphorylation in the absence of a rise in cytosolic calcium. *Exp. Physiol.*, **79**, 269–272.
- 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.
- SORRENTINO, V. & VOLPE, P. (1993). Ryanodine receptors: how many and why? *Trends Pharmacol. Sci.*, **14**, 98–103.
- TEPEL, M., KUHNAPFEL, S., THEILMEIER, G., TEUPE, C., SCHLOTTMANN, R. & ZIDEK, W. (1994). Filling state of intracellular  $Ca^{2+}$  pools triggers trans plasma membrane  $Na^+$  and  $Ca^{2+}$  influx by a tyrosine kinase-dependent pathway. *J. Biol. Chem.*, **269**, 26239–26242.
- WAYMAN, C.P., GIBSON, A., MCFADZEAN, I. & TUCKER, J.F. (1995). Calcium store depletion by cyclopiazonic acid activates a non-selective cation current in single smooth muscle cells from the mouse anococcygeus. *Br. J. Pharmacol. Proc. Suppl.*, **114**, 97P.
- YULE, D.I., KIM, E.T. & WILLIAMS, J.A. (1994). Tyrosine kinase inhibitors attenuate 'capacitative'  $Ca^{2+}$  influx in rat pancreatic acinar cell. *Biochem. Biophys. Res. Commun.*, **202**, 1697–1704.

(Received March 3, 1995

Revised June 12, 1995

Accepted June 28, 1995)