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# Calcium influx through 'L'-type channels into rat anterior pituitary cells can be modulated in two ways by protein kinase C (PKC-isoform selectivity of 1,2-dioctanoyl *sn*-glycerol?)

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The depolarisation-induced influx of $^{45}Ca^{2+}$ into anterior pituitary tissue and $GH_3$ cells through 'L'-type, nimodipine-sensitive channels was investigated. In anterior pituitary prisms, phorbol esters, activators of protein kinase C, caused an enhancement of $K^+$-induced $^{45}Ca^{2+}$ influx. However, in the $GH_3$ anterior pituitary cell line, phorbol esters inhibited $K^+$-induced $^{45}Ca^{2+}$ influx. The modulation by phorbol esters in both tissues was stereo-specific and time- and concentration-dependent. The diacylglycerol analogue, 1,2-dioctanoyl *sn*-glycerol was able to mimic the phorbol ester-induced enhancement of calcium influx into anterior pituitary pieces, but was ineffective in $GH_3$ cells. 1,2-Dioctanoyl *sn*-glycerol may selectively activate an isoform of protein kinase C which is responsible for enhanced 'L'-type $Ca^{2+}$-channel activity.

Phorbol ester; Diacylglycerol; Protein kinase C isoform; $Ca^{2+}$ influx; Rat anterior pituitary cell

## 1. INTRODUCTION

Depolarisation of cells can cause an influx of $Ca^{2+}$ into the cells through 'L'-type, voltage-sensitive calcium channels which are known to be a site of action for a number of second-messenger-activated enzymes [1,2]. These nimodipine-sensitive channels can be multiply phosphorylated by protein kinase C (PKC) [2]. In some preparations, including $GH_3$ cells, PKC activity leads to reduced 'L'-type calcium channel activity [3–7]. Cardiac 'L'-type calcium channels can show both an enhancement and an inhibition of their activity upon PKC activation [8], whereas in other preparations PKC activation leads to an enhancement of 'L'-channel activity [9–12]. At the present time, at least seven different isoforms of PKC have been described with distinct cellular expression [13]. Some of the PKC-isoforms show distinctly different activation by agents such as phospholipids, arachidonic acid, other fatty acids and calcium [14–16] and show some substrate selectivity (for reviews see [13,16]).

The present experiments investigate the effects of PKC activators on the depolarisation-induced influx of $^{45}Ca^{2+}$ in two different models, the $GH_3$ clonal rat anterior pituitary cell line and rat anterior pituitary tissue prisms.

*Abbreviations:* PKC, protein kinase C; EGTA, ethyleneglycol-bis-(β-aminoethyl ether)*N,N,N',N'*-tetraacetic acid; PDBu, phorbol 12,13-dibutyrate; 4α-PDD, 4α-phorbol 12,13-didecanoate; 4β-PDD, 4β-phorbol 12,13-didecanoate; DOG, 1,2-dioctanoyl *sn*-glycerol; OAG, 1-oleoyl 2-acetyl *sn*-glycerol.

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## 2. EXPERIMENTAL

### 2.1. *Materials and chemicals*

All standard laboratory chemicals were of Analar grade and purchased from BDH Ltd. (Glasgow, UK). Staurosporine was bought from Novabiochem (UK) Ltd., (Nottingham, UK). $GH_3$ cells and foetal bovine serum were obtained from Flow Laboratories (Irvine, UK). Medium F-10 Ham was supplied by Gibco-BRL (Paisley, UK) and radioactive $^{45}CaCl_2$ was supplied by Amersham International PLC (Amersham, UK) (spec. act. = 17 mCi/mg). All other materials were purchased from the Sigma Chemical Co. (Poole, UK).

### 2.2. *Tissue preparation*

Male Wistar-COB rats (>250 g) supplied by Charles River UK Ltd. (Margate, UK) were maintained under controlled lighting (lights on from 05.00 to 19.00 h) and temperature (22°C) and allowed free access to diet 41B (Oxoid Ltd., Basingstoke, UK) and tap water. Animals were killed by cervical dislocation and anterior pituitaries were rapidly dissected out and hemisected for use in calcium influx studies. $GH_3$ cells were grown in medium F-10 Ham supplemented with 15% foetal bovine serum, 1 mM L-glutamine, 100 U/ml penicillin and 0.1 mg/ml streptomycin in a humidified atmosphere of 95% air/5% $CO_2$ at 37°C. Cells were harvested by agitation, washed by resuspension/centrifugation (100 × *g*, 10 min, 25°C) and prepared for calcium influx measurement as described below.

### 2.3. *Calcium influx measurements*

Each fresh, hemisected anterior pituitary was sliced into four equal parts and incubated in separate polypropylene tubes in 0.5 ml of 'calcium uptake medium' (concentrations in mM: NaCl 154, KCl 5.4, $CaCl_2$ 1.5, D-glucose 11, HEPES 6, pH adjusted to 7.4 with Tris base and with 0.05% essential fatty acid-free bovine serum albumin). Washed, harvested $GH_3$ cells were diluted to a density of $5 \times 10^6$

cells/ml in 'calcium uptake medium' and aliquoted at 0.5 ml/tube. Both cellular preparations were preincubated (30 min, 37°C, $O_2$ atmosphere) before a 10 min incubation (37°C, $O_2$) with drugs or solvent alone. Cells were then exposed to low $K^+$-(5.4 mM) or high $K^+$-(60 mM) calcium uptake medium containing 4 $\mu$M $^{45}CaCl_2$ (≈3 $\mu$Ci/tube, specific). After 30 s (37°C), $^{45}Ca^{2+}$ uptake was halted by quenching with 3 ml of ice-cold 2 mM EGTA ($Ca^{2+}$-free) calcium uptake medium and tissue was separated by vacuum-filtration through Millipore SCWP cellulose acetate/nitrate filters (8 $\mu$m pore size) underlaid by GF/B filters on Millipore 1225 sampling manifolds (Millipore UK Ltd., Harrow, UK). Samples were washed once immediately with 3 ml ice-cold EGTA calcium uptake medium and then a further three times for 2 min each. Cellulose filters were then counted by liquid scintillation counting. Preliminary experiments revealed that these conditions gave the optimal signal-to-noise ratio and that stimulus-induced influx of $^{45}Ca^{2+}$ in excess of basal controls was maximal within 30 s, suggesting that it represented specific response-triggered flux rather than adsorption or steady accumulation by storage pools.

### 2.4. *Data analysis*

Concentration–response curves were analysed by a non-linear, iterative, individually-weighted curve-fitting program ('P-fit'; Biosoft, Cambridge, UK). The values quoted represent the calculated mean $EC_{50}$ (effective concentration which produces 50% of the maximal response), with errors representing standard errors of the mean.

## 3. RESULTS

Fig. 1 shows that the high-$K^+$-stimulated influx of $^{45}Ca^{2+}$ into both pituitary pieces and $GH_3$ cells was inhibited by the dihydropyridine, nimodipine in a concentration-dependent fashion. The $IC_{50}$ values for nimodipine were 3 ± 2 and 5 ± 2 nM ($n$ = 4) for anterior pituitary prisms and $GH_3$ cells, respectively. Maximal inhibition of calcium influx was seen with ≥100 nM nimodipine. In both pituitary pieces and $GH_3$ cells a small proportion (≈15%) of the response was resistant to block by nimodipine, perhaps occurring through some route other than dihydropyridine-sensitive 'L'-channels.

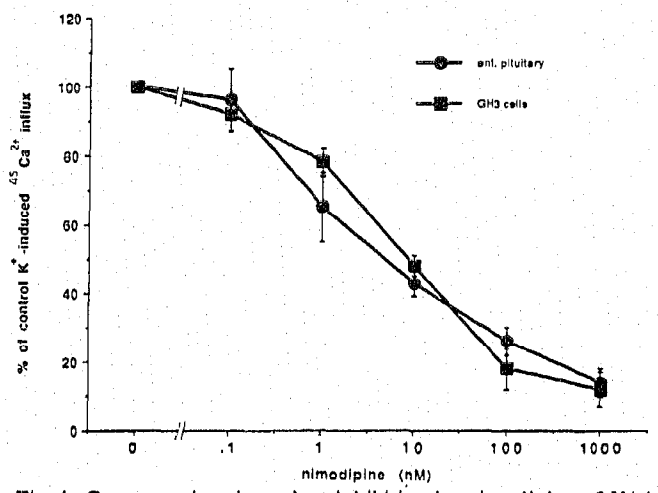


Fig. 1. Concentration-dependent inhibition by nimodipine of $K^+$-induced $^{45}Ca^{2+}$ influx into anterior pituitary prisms and $GH_3$ cells. Typically, basal accumulation of $^{45}Ca^{2+}$ accounted for around 50 fmol $^{45}Ca^{2+}$/hemi-pituitary or $10^6$ $GH_3$ cells/min of which non-specific adsorption to filter and cell surfaces accounted for 34 fmol $^{45}Ca^{2+}$/min. Stimulation with 60 mM $K^+$ increased accumulation to around 120 fmol $^{45}Ca^{2+}$/hemi-pituitary or $10^6$ $GH_3$ cells/min (approximately 1600 dpm per assay). $^{45}Ca^{2+}$ represented 1 part in 375 of the total $Ca^{2+}$ concentration. Anterior pituitary prisms (●) or $GH_3$ cells (■) were preincubated with nimodipine (or solvent alone for control measurements) for 10 min before exposure to 60 mM $K^+$-containing medium with radioactive $Ca^{2+}$. After 30 s, $^{45}Ca^{2+}$ influx was halted as described in section 2. The data represent the means ±SEM of 4 determinations.

Preincubation for 10 min with phorbol 12,13-dibutyrate (PDBu) and 4$\beta$-phorbol 12,13-didecanoate (4$\beta$-PDD) before 30 s exposure to high-$K^+$ medium and measurement of $^{45}Ca^{2+}$ influx caused marked changes in the depolarisation-response seen in both tissue preparations. In anterior pituitary tissue, PDBu and 4$\beta$-PDD (3–1000 nM) enhanced calcium influx in a concentration-dependent manner ($EC_{50}$ of 55 ± 22 nM for PDBu enhancement (Fig. 2)). The enhancement of $K^+$-induced $^{45}Ca^{2+}$ influx into anterior pituitary prisms seen with 4$\beta$-PDD, was not mimicked by its inactive enantiomer 4$\alpha$-PDD at the same concentrations (3–1000 nM). In the presence of maximally-effective concentrations of PDBu, or 1000 nM 4$\beta$-PDD, $K^+$-induced $^{45}Ca^{2+}$ influx was ≈280% of control. In contrast, in $GH_3$ cells, PDBu or 4$\beta$-PDD (3–1000 nM) pretreatment resulted in a concentration-dependent inhibition of the $K^+$-induced influx of calcium ($IC_{50}$ of 17 ± 12 nM for PDBu inhibition (Fig. 3)) which also showed stereoselectivity with the 4-position isomers of PDD. The maximal inhibition of calcium influx seen with either PDBu or 4$\beta$-PDD was ≈50% of total depolarision-evoked $Ca^{2+}$ influx.

The time-courses of the two opposing influences of PDBu in the two preparations are shown in Fig. 4. Both in anterior pituitary prisms and $GH_3$ cells, PDBu (300 nM) induced nearly half of its maximal response with total incubation times of 30 s (i.e. present only in the $^{45}Ca^{2+}$ influx measurement period). Inclusion of the

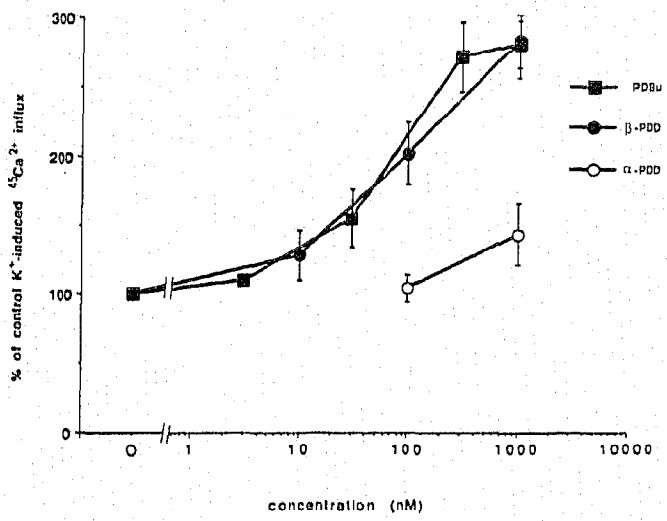


Fig. 2. Concentration-dependent enhancement by phorbol of $K^+$-induced $^{45}Ca^{2+}$ influx into rat anterior pituitary prisms. Rat anterior pituitary prisms were preincubated for 10 min with PDBU (■), 4$\beta$-PDD (●) or 4$\alpha$-PDD (○) before exposure to 60 mM $K^+$-containing medium, $^{45}Ca^{2+}$ influx was measured as described in section 2. None of the compounds had any effects on basal $^{45}Ca^{2+}$ influx at the concentrations used. The data represent the means ±SEM from 4–8 determinations.

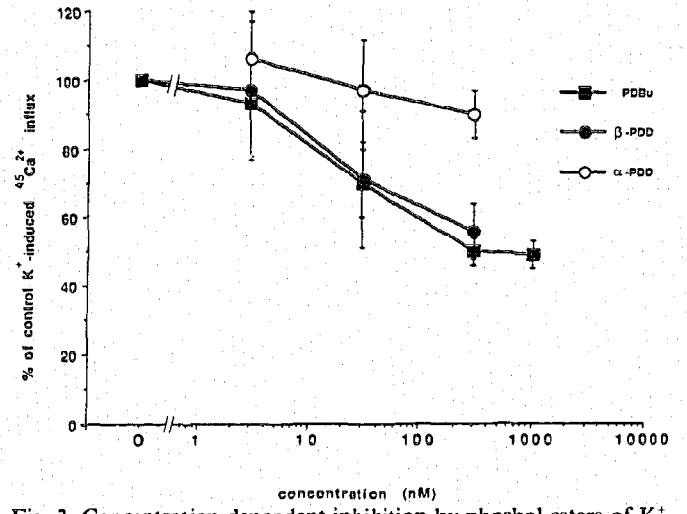


Fig. 3. Concentration-dependent inhibition by phorbol esters of $K^+$-induced $^{45}Ca^{2+}$ influx into $GH_3$ cells. $GH_3$ cells were preincubated for 10 min with PDBU (■), 4$\beta$-PDD (●) or 4$\alpha$-PDD (○) before exposure to 60 mM $K^+$-containing medium. $^{45}Ca^{2+}$ influx was measured as described in section 2. None of the compounds had any effects on basal $^{45}Ca^{2+}$ influx at the concentrations used. The data represent the means ±SEM from 4–8 determinations.

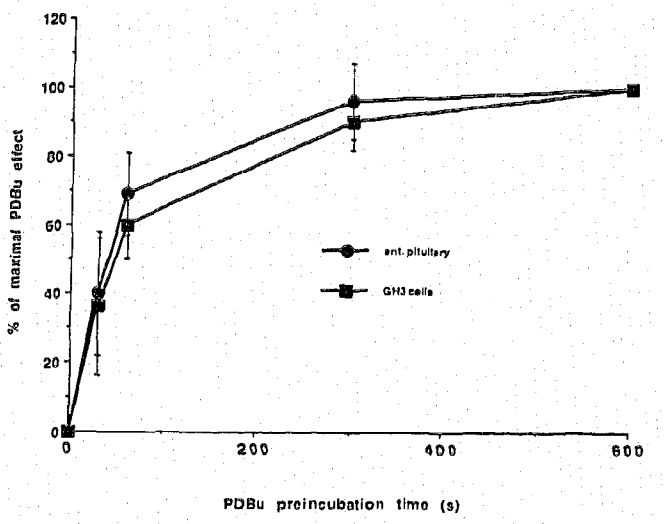


Fig. 4. Time-course of the PDBu modulation of $^{45}Ca^{2+}$ influx into rat anterior pituitary prisms and $GH_3$ cells. Anterior pituitary prisms (●) and $GH_3$ cells (■) were preincubated with 300 nM PDBu for the indicated time before exposure to 60 mM $K^+$. The preincubation time with PDBu is inclusive of the 30 s $^{45}Ca^{2+}$ influx measurement period. The data represent the means ±SEM of 6 determinations.

PKC inhibitor, staurosporine [17], reversed the response to 300 nM PDBu in both tissues. The PDBu effects were inhibited by staurosporine (1–1000 nM) in a concentration-dependent manner, with 30 nM staurosporine reversing 59 ± 12 and 60 ± 17% of the 300 nM PDBu response on $K^+$-induced $^{45}Ca^{2+}$ influx into anterior pituitary prisms and $GH_3$ cells, respectively ($n$ = 8). With the highest concentration of staurosporine used, no significant amount of the PDBu response remained in either preparation ($n$ = 8).

When the diacylglycerol analogue 1,2-dioctanoyl *sn*-glycerol (DOG) was tested on the two systems (Fig. 5), DOG was able to mimic the enhancement by PDBu and 4$\beta$-PDD in anterior pituitary prisms, resulting in an enhancement to ≈220% of control $K^+$-induced influx with 100 $\mu$M DOG. However, even up to a concentration of 100 $\mu$M, DOG was unable to inhibit $K^+$-induced $^{45}Ca^{2+}$ influx into $GH_3$ cells (maximum of 4% inhibition at 100 $\mu$M).

## 4. DISCUSSION

The data presented here show that activation of PKC by phorbol esters can modulate (in a stereo-specific, concentration- and time-dependent manner) the depolarisation-induced influx of $^{45}Ca^{2+}$ into both rat anterior pituitary prisms and $GH_3$ cells. The $K^+$-induced influx of $^{45}Ca^{2+}$ into both anterior pituitary prisms and $GH_3$ cells was mediated mainly through a nimodipine-sensitive 'L'-channel. The remaining 15% of nimodipine-resistant influx represents another voltage-sensitive route which may be a 'T' or 'N'-type $Ca^{2+}$-channel [1] and/or the more recently discovered dihydropyridine-resistant, slow-inactivating, high voltage-activated $Ca^{2+}$-channel described by Mori et al. [18]. However, both this channel (designated the 'BI'-type channel by Mori et al.) and the 'T'-type $Ca^{2+}$-channel are not totally insensitive to block by dihydropyridines [18–20] and the presence in pituitary cells of voltage-activated calcium channels other than 'L'- or 'T'-type is uncertain [6]. Both tissues are known to contain 'L'- and 'T'-type $Ca^{2+}$-channels although the contribution of any transiently-opening $Ca^{2+}$-channels to the total influx seen in this 30 s depolarisation-induced $^{45}Ca^{2+}$ protocol is unknown.

Protein kinase C is known to modulate the activity of other voltage-activated channels, for example $\alpha$, $\beta$- and $\gamma$-PKC can decrease transient $K^+$-currents in *Xenopus* oocytes, whereas only $\alpha$- and $\beta$-PKC reduced transient $Na^+$-currents [21]. Epithelial $Cl^-$-channels can be either up- or down-regulated by activation of PKC with either phorbol esters or diacylglycerols [22]. Such regulation of the channel may be attributable to different PKC-isoforms phosphorylating different sites on the $Cl^-$-channel, one PKC-isoform increasing, and another isoform decreasing channel activity [22]. A similar array of PKC influences after activation with phorbol esters was also seen in cardiac 'L'-type $Ca^{2+}$-channels [8]. In that case, the time-dependent increase followed by a decrease in cardiac 'L'-channel activity may involve modulation by distinct PKC-isoforms. However, here the PDBu-induced enhancement and inhibition of $K^+$-induced $^{45}Ca^{2+}$ influx had similar time-courses in the two preparations (Fig. 4). At least two PKC-phosphorylation sites exist on subunits of 'L'-type $Ca^{2+}$-channels [2] but the functional effect of such subunit-specific PKC-phosphorylation is unknown. The opposing actions of PKC-activation on 'L'-channel influx seen here

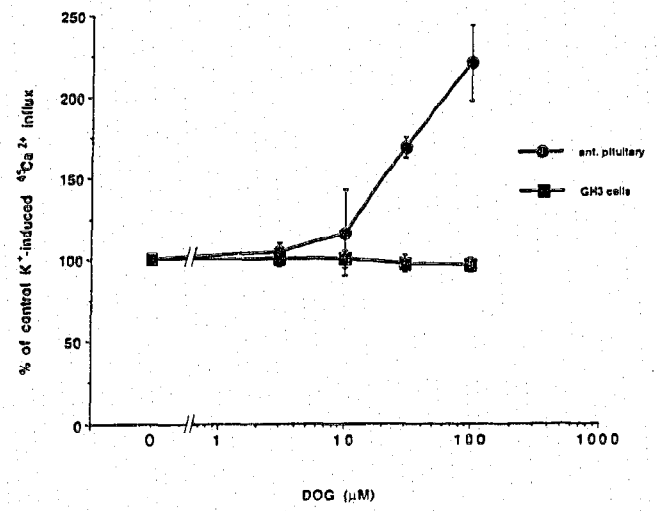


Fig. 5. Concentration-dependent enhancement by DOG of $K^+$-induced $^{45}Ca^{2+}$ influx into rat anterior pituitary prisms and inability of DOG to modulate $K^+$-induced $^{45}Ca^{2+}$ influx into $GH_3$ cells. Anterior pituitary prisms (●) and $GH_3$ cells (■) were preincubated for 10 min with the indicated concentration of DOG before exposure to 60 mM $K^+$-containing medium. $^{45}Ca^{2+}$ influx was measured as described in section 2. There was no effect of 100 $\mu$M DOG on basal $^{45}Ca^{2+}$ influx, or on non-specific adsorption of $^{45}Ca^{2+}$ to filter blanks. The data represent the means ±SEM of 4 determinations.

may be due to different PKC-isoforms acting in opposing ways on 'L'- channel activity. The identity of these putative, opposing PKC-isoforms is unknown, as is the complete PKC-isoform content of the two tissues (although both tissues contain at least $\alpha$-, $\beta$- and $\varepsilon$-PKC, but not $\gamma$-PKC (Simpson, J., MacEwan, D.J., Mitchell, R., Johnson, M.S., Thomson, F.J. and Parker, P.J., unpublished). The exact relative quantity of each of these PKC-isoforms within the two tissues is also uncertain. Naor [23] determined that the $\alpha$- and $\beta$-PKC content of the two tissues varied considerably, with $GH_3$ cells containing 83% $\alpha$-PKC and anterior pituitary containing 58% $\alpha$-PKC (the remainder being $\beta$-PKC), but these results do not take account of $Ca^{2+}$-independent PKC isoforms [13]. The differences in absolute amount of particular PKC isoform(s) in anterior pituitary prisms and $GH_3$ cells may account for the functional differences seen here between the two preparations with PDBu-induced modulations of $Ca^{2+}$-channel activity.

Interestingly, Lacerda et al. [8] were unable to elicit the phorbol ester-mediated inhibitory regulation of 'L'-channels using the synthetic diacylglycerol, DOG. Likewise, DOG was only able to activate the PKC-induced enhancement of 'L'-channels in anterior pituitary pieces and was ineffective on the PKC-induced inhibition of 'L'-channel activity in $GH_3$ cells (this effect is not due to differential degradation of DOG between the two tissues (MacEwan, D.J. and Mitchell, R., unpublished)). If indeed the dual modulation of 'L'-type $Ca^{2+}$-channels seen in cardiac myocytes and in the present study is due to differences in PKC-isoform action, then DOG, unlike PDBu, may only be capable of activating particular PKC-isoforms, including that responsible for enhanced 'L'-channel activity. The phorbol esters, PDBu and 4$\beta$-PDD are capable of activating both the PKC-elicited enhancement and inhibition of depolarisation-induced $^{45}Ca^{2+}$ influx. Protein kinase C-independent actions of phorbol esters and diacylglycerol analogues have been reported [24,25], however, the concentration of phorbol esters necessary to see the effects are very high (>5 $\mu$M) and occur with PKC-inactive isomers, unlike the stereospecificity of action shown here (Figs. 2 and 3). The diacylglycerol analogue, OAG was reported to depress $Ca^{2+}$-channel activity dependent on its PKC actions [24], but DOG at the same concentrations could not mimic OAG at depressing $Ca^{2+}$-currents [24] and furthermore, did not act to reduce $^{45}Ca^{2+}$ influx here (Fig. 5) suggesting that non-PKC activity may only occur with OAG and not DOG.

Due to its ability to penetrate cells, DOG is often used as an agent to reflect the actions of endogenous diglycerides. Our evidence suggests that DOG may be pharmacologically selecting for one or more PKC-isoforms and therefore caution must be used in interpretation of results arising from use of the agent.

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## REFERENCES

[1] Tsien, R.W., Lipscombe, D., Madison, D.V., Bley, K.R. and Fox, A.P. (1988) Trends Neurosci. 11, 431–438.
[2] Campbell, K.P., Leung, A.T. and Sharp, A.H. (1988) Trends Neurosci. 11, 425–430.
[3] Di Virgilio, F., Pozzan, T., Wollheim, C.B., Vicentini, L.M. and Meldolesi, J. (1986) J. Biol. Chem. 261, 32–35.
[4] Lewis, D.L. and Weight, F.F. (1988) Neuroendocrinology 47, 169–175.
[5] Rosenthal, W., Hescheler, J., Hinsch, K.-D., Spicher, K., Trautwein, W. and Schultz, G. (1988) EMBO J. 7, 1627–1633.
[6] Marchetti, C. and Brown, A.M. (1988) Am. J. Physiol. C206–210.
[7] Rane, S.G., Walsh, M.P., McDonald, J.R. and Dunlap, K. (1989) Neuron 3, 239–245.
[8] Lacerda, A.E., Rampe, D. and Brown, A.M. (1988) Nature 335, 249–251.
[9] DeRiemer, S.A., Strong, J.A., Albert, K.A., Greengard, P. and Kaczmarek, L.K. (1985) Nature 313, 313–316.
[10] Strong, J.A., Fox, A.P., Tsien, R.W. and Kaczmarek, L.K. (1987) Nature 325, 714–717.
[11] Fish, R.D., Sperti, G., Colucci, W.S. and Clapham, D.E. (1988) Circ. Res. 62, 1049–1054.
[12] Velasco, J.M. and Petersen, O.H. (1989) Q.J. Exp. Physiol. 74, 367–370.
[13] Nishizuka, Y. (1988) Nature 334, 661–665.
[14] Shearman, M.S., Naor, Z., Sekiguchi, K., Kishimoto, A. and Nishizuka, Y. (1989) FEBS Lett. 243, 177–182.
[15] Huang, K.-P., Huang, F.L., Nakabayashi, H. and Yoshida, Y. (1988) J. Biol. Chem. 263, 14839–14845.
[16] Parker, P.J., Kour, G., Marais, R.M., Mitchell, F., Pears, C., Schaap, D., Stabel, S. and Webster, C. (1989) Mol. Cell. Endocrinol. 65, 1–11.

[17] Tamaoki, T., Nomoto, H., Takahashi, I., Kato, Y., Morimoto, M. and Tomita, F. (1986) Biochem. Biophys. Res. Commun. 142, 436–440.
[18] Mori, Y., Friedrich, T., Kim, M.-S., Mikami, A., Nakai, J., Ruth, P., Bosse, E., Hofmann, F., Flockerzi, V., Furuichi, T., Mikoshiba, K., Imoto, K., Tanabe, T. and Numa, S. (1991) Nature 350, 398–402.
[19] Bean, B.P. (1985) J. Gen. Physiol. 86, 1–30.
[20] Bean, B.P., Sturek, M., Puga, A. and Hermsmeyer, K. (1986) Circ. Res. 59, 229–235.
[21] Lotan, I., Dascal, N., Naor, Z. and Boton, R. (1990) FEBS Lett. 267, 25–28.
[22] Li, M., McCann, J.D., Anderson, M.P., Clancy, J.P., Liedtke, C.M., Nairn, A.C., Greengard, P. and Welsh, M.J. (1989) Science 244, 1353–1356.
[23] Naor, Z. (1990) Endocrinology 126, 1521–1526.
[24] Hockberger, P., Toselli, M., Swandulla, D. and Lux, H.D. (1989) Nature 338, 340–342.
[25] Doerner, D., Abdel-Latif, M., Rogers, T.B. and Algers, B.E. (1990) J. Neurosci. 10, 1699–1706.