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Stereospecific mobilization of intracellular Ca^{2+} by inositol 1,4,5-trisphosphate

Comparison with inositol 1,4,5-trisphosphorothioate and inositol 1,3,4-trisphosphate

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The stereo specificity of *myo*-inositol 1,4,5-trisphosphate [Ins(1,4,5)P_3] to mobilize Ca^{2+} from an intracellular store has been examined in permeabilized rat pituitary-tumour GH_3 and Swiss 3T3 cells. A comparison of D-Ins(1,4,5)P_3 with the synthetic enantiomer L-Ins(1,4,5)P_3 and the racemate DL-Ins(1,4,5)P_3 clearly demonstrates the marked stereospecificity of the response. Whereas D-Ins(1,4,5)P_3 released 30–50% of non-mitochondrially-bound Ca^{2+} with a EC_{50} (concentration producing 50% of maximal response) of 200 nM, the L isomer was both substantially less potent and efficacious. A high concentration of the L isomer (10 μM) did not significantly shift the dose–response curve for the D isomer in Swiss 3T3 cells, suggesting that the less active isomer is probably a very weak agonist. Other studies revealed, in contrast with previous work, that the other naturally occurring isomer, D-Ins(1,3,4)P_3, was essentially inactive in releasing Ca^+, whereas a novel 5-phosphatase-resistant analogue, DL-*myo*-inositol 1,4,5-trisphosphorothioate, was a relatively potent full agonist in GH_3 cells. These data reveal, for the first time, the stereoselectivity of the intracellular receptor associated with Ca^{2+} release. They also provide evidence for the activity of the novel phosphorothioate analogue of Ins(1,4,5)P_3, but suggest that D-Ins(1,3,4)P_3 is not involved in cellular Ca^{2+} mobilization.

INTRODUCTION

It is now well established that D-Ins(1,4,5)P_3, an immediate product of receptor-mediated phosphatidylinositol 4,5-bisphosphate hydrolysis, can release Ca^{2+} from non-mitochondrial ATP-dependent cellular stores and as such is considered a fundamental second messenger in many cells [1]. Furthermore, the ability to mobilize Ca^{2+} appears to reside in the *vic*-D-4,5-phosphate pairing of inositol phosphates, and such specificity is indicative of a recognition site located on the endoplasmic reticulum or other specialized Ca^{2+}-storing organelles [2].

The characteristics of this 'receptor' are not well understood, since until recently there has been a limited availability of synthetic structural analogues of D-Ins(1,4,5)P_3. However, evidence for such a recognition site has come from binding studies with radiolabelled Ins(1,4,5)P_3 in particulate preparations of adrenal cortex [3,4], hepatocytes and neutrophils [5,6]. More recently a very high density of specific [^{3}H]Ins(1,4,5)P_3-binding sites has been described in rat cerebellum [7,8,9]. Our own studies [8] have demonstrated that the latter sites are stereospecific, with D-Ins(1,4,5)P_3 displaying some 2000-fold higher affinity than the synthetic L enantiomer. Furthermore, the positional specificity was considerable, with D-Ins(2,4,5)P_3, and particularly D-Ins(1,3,4)P_3, displaying a substantially lower affinity for these sites [8]. In view of apparent fundamental diferences in the specificity of this binding site and the Ca^{2+}-release site (see [9]), we have re-evaluated Ca^{2+} release from permeabilized cells and establish the stereo- and positional specificity of this response in GH_3 and Swiss 3T3 cells. We also report the activity of a novel 5-phosphatase-resistant phosphorothioate analogue of Ins(1,4,5)P_3 [13].

EXPERIMENTAL

Materials

GH_3 cells and Swiss 3T3 cells were initially gifts from Dr. Barry Brown (University of Sheffield, Sheffield, U.K.) and Dr. Colin Taylor (Department of Zoology, University of Cambridge, Cambridge, U.K.) respectively. Cells were cultured in 175 cm^2 flasks in Ham's F10 medium supplemented with 10% (v/v) foetal-calf serum, and penicillin (100 i.u./ml) streptomycin (100 μg/ml), fungizone (5 μg/ml) and 2 mM-glutamine were used when cells were confluent. DL- and L-Ins(1,4,5)P_3 were synthesized from DL- or D-1,2,4-tri-*O*-benzyl-*myo*-inositol [11] by using a phosphite-chemistry approach [12]. DL-Ins(1,4,5)PS_3 was synthesized as described in [13]. D-Ins(1,4,5)P_3 and $^{45}CaCl_2$ were from Amersham International, and ATP, saponin and Quin-2 were obtained

Abbreviations used: D- and L-InsP_3, D- and L-*myo*-inositol trisphosphates, with assignments of phosphate locants where appropriate [e.g. D-Ins(1,4,5)P_3, D-*myo*-inositol 1,4,5-trisphosphate]; PBS, Dulbecco's phosphate-buffered saline; DL-Ins(1,4,5)PS_3, DL-*myo*-inositol 1,4,5-trisphosphorothioate; EC_{50}, concentration producing 50% of maximal response.

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from Sigma. Two separate lots of D-Ins(1,3,4)P_3 (h.p.l.c.-pure) were kindly provided by Dr. Robin Irvine (AFRC, Babraham, Cambridge, U.K.).

$^{45}Ca^{2+}$-release experiments

These experiments were modified from those described by Gershengorn *et al.* [14]. GH_3 and Swiss 3T3 cells were harvested in 20 ml of Dulbecco's phosphate-buffered saline (PBS; Gibco) containing 0.02% EDTA, centrifuged at 500 *g* for 1 min, then resuspended in culture medium and incubated for 60 min. All cells were then collected by centrifugation, washed in PBS twice before resuspension in a 'cytosol-like' buffer (120 mM-KCl/5 mM-ATP/6 mM-$MgCl_2$/5 mM-sodium succinate/20 mM-Hepes, pH 6.9). The free Ca^{2+} concentration of this solution was buffered (and simultaneously measured fluorimetrically) to between 100 and 500 nM by the addition of various amounts of Quin-2 free acid.

After incubation for 10 min at 24 °C, cells were collected by centrifugation and resuspended in cytosol buffer at 5×10^6 cells/ml. Saponin (100 μg/ml) was added, and after exactly 1 min (at which time > 99% of cells were permeable to Trypan Blue, i.e. viable), the cells were centrifuged at 500 *g* and resuspended in cytosol buffer containing 2 μg of oligomycin/ml (to suppress $^{45}Ca^{2+}$ uptake into mitochondria) and 2 μCi of $^{45}Ca^{2+}$/ml.

Cells were incubated for 20 min to actively load non-mitochondrial stores with $^{45}Ca^{2+}$. A 100 μl portion of cell suspension was then added to 100 μl of buffer containing inositol phosphates in microcentrifuge tubes. After 1 min (unless otherwise stated), 500 μl of a silicone-oil mixture [Dow–Corning 556/550, 3:2 (v/v)] was added and the cells centrifuged through the oil at 16000 *g* for 2 min to separate them from the buffer. Buffer and oil were removed and tubes allowed to drain for 30 min. Cell pellets were dissolved in 100 μl of Lumasolve (May and Baker Chemicals, Dagenham, Essex, U.K.) and counted for radioactivity in 1 ml of scintillation fluid (Optiphase-X; Pharmacia). Duplicate determinations were performed for each concentration of inositol phosphates, and the results are expressed as the percentage of $^{45}Ca^{2+}$ released relative to the $^{45}Ca^{2+}$ content of control cells. Data of dose–response curves were analysed by using computer-assisted curve-fitting (ALLFIT program) [15] to determine EC_{50} values and slopes of curves.

H.p.l.c. separation of inositol phosphates and their isomers after incubation with permeabilized cells was performed by using an ammonium phosphate gradient as recently described [16].

RESULTS

Saponin-treated GH_3 and Swiss 3T3 cells displayed a rapid and ATP-dependent uptake of 100–500 nM-$^{45}Ca^{2+}$ when cells were incubated with oligomycin to block mitochondrial uptake. Uptake under these conditions was half-maximal at about 3 min and reached a steady state by 10–15 min which remained essentially unchanged for at least 40 min. Ionomycin (10 μM) released > 90% of $^{45}Ca^{2+}$ accumulated, suggesting that it is intravesicular. These basic properties of $^{45}Ca^{2+}$ loading are very similar to those detailed by Gershengorn *et al.* [14] using almost identical methods and suggest that a high-affinity non-mitochondrial Ca^{2+} store is being actively loaded under these conditions.

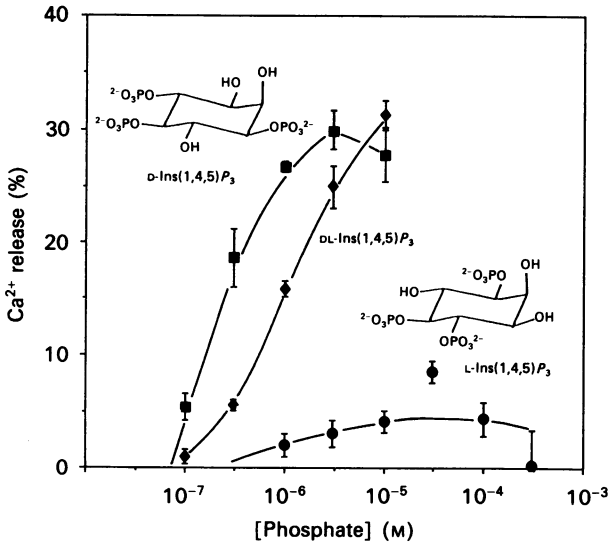

Fig. 1. Dose–response curves for the release of Ca^{2+} from permeabilized GH_3 cells by D- (■), DL- (◆) and L- (●) Ins(1,4,5)P_3

Each point is the mean ± S.E.M. for at least three experiments.

Addition of D-Ins(1,4,5)P_3 to loaded cells resulted in a very rapid and sustained release of $^{45}Ca^{2+}$ from saponin-treated cells. Maximal release was observed at < 30 s, and in both GH_3 and Swiss 3T3 cells this loss was maintained for at least 15 min. Dose–response experiments were therefore terminated at 1 min, but identical data were obtained in one set of experiments in which incubations were allowed to proceed for 5 min. In one set of experiments, D-[^{3}H]Ins(1,4,5)P_3 (0.1–10 μM) was incubated with permeabilized cells and samples were assayed by h.p.l.c. [14]. Minimal (< 10%) metabolism via phosphatase or kinase steps occurred even after 10 min incubation with permeabilized GH_3 cells. The maximal extent of D-Ins(1,4,5)P_3-induced release of $^{45}Ca^{2+}$ was 25–30% in GH_3 cells, but 50–60% in Swiss 3T3 cells.

Data in Figs. 1, 2 and Table 1 reveal the stereospecificity of Ins(1,4,5)P_3-induced $^{45}Ca^{2+}$ release from both GH_3 and Swiss 3T3 cells. The D-(1,4,5)P_3 isomer potently released $^{45}Ca^{2+}$, whereas the synthetic unnatural L-(1,4,5)P_3 isomer [D-Ins(3,5,6)P_3] was very weak in this respect. The synthetic DL racemate displayed about a 4–5-fold lower EC_{50} than the D-isomer (Table 1). Theoretically one would anticipate a 2-fold lower EC_{50} for this racemate. This small discrepancy may relate to inaccuracies in phosphate quantification of commercial inositol phosphates and those synthesized 'in house'. Data in Fig. 2 also indicate that the naturally occurring D-Ins(1,3,4)P_3 isomer is essentially inactive at concentrations as high as 30 μM in Swiss 3T3 cells. Other experiments revealed that D-Ins(1,4)P_2 and InsP_6 are inactive at 30 μM in Swiss cells (Table 1). Although clearly very weak, L-Ins(1,4,5)P_3 did release $^{45}Ca^{2+}$ at some concentrations in excess of 5 μM, although at concentrations above 50 μM it appeared to be inactive. Problems of solubility in the cytosol-like buffer hampered further investigation. In order to determine whether this isomer was a relatively potent partial agonist or a very

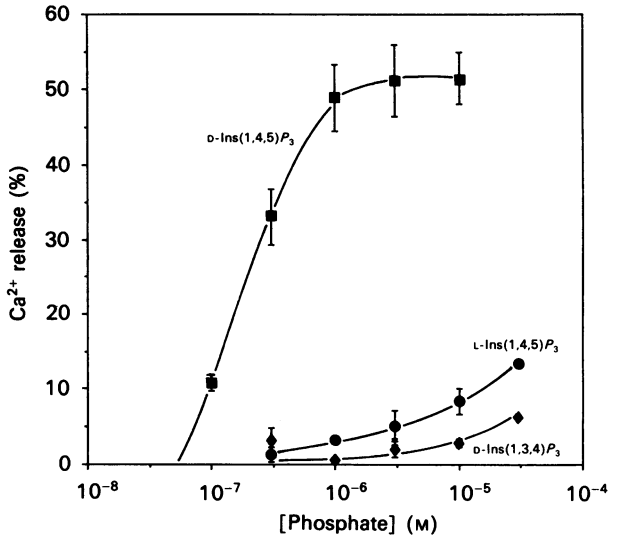

Fig. 2. Dose–response curves for D- (■) and L- (●) Ins(1,4,5)P_3 and D-Ins(1,3,4)P_3 (◆) in Swiss 3T3 cells (identical data were obtained with two samples of the latter)

Each point is the mean ± S.E.M. for at least three experiments.

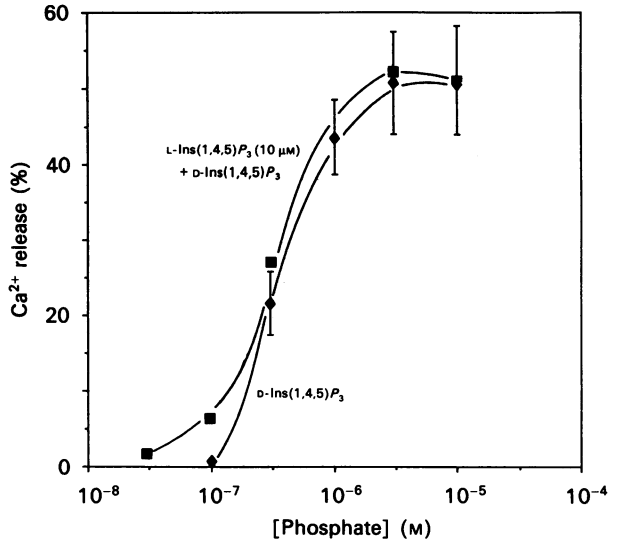

Fig. 3. Dose–response curves for D-Ins(1,4,5,)P_3 in the presence (●) or absence (◆) of L-Ins(1,4,5)P_3 in Swiss 3T3 cells

Each point is the mean ± S.E.M. for at least three experiments.

Table 1. $^{45}Ca^{2+}$ mobilization by inositol phosphates

EC_{50} values were obtained by analysing data using the computer-assisted curve-fitting program ALLFIT. Values are mean ± S.E.M for at least three separate experiments.

Inositol phosphate	Cells	EC_{50} (M)
D-Ins(1,4,5)P_3	GH_3	$(2.2 \pm 0.1) \times 10^{-7}$
	3T3	$(2.1 \pm 0.1) \times 10^{-7}$
DL-Ins(1,4,5)P_3	GH_3	$(1.1 \pm 0.1) \times 10^{-6}$
DL-Ins(1,4,5)PS_3	3T3	$(9.6 \pm 0.5) \times 10^{-7}$
L-Ins(1,4,5)P_3	3T3	
D-Ins(1,3,4)P_3	3T3	$\gg 3 \times 10^{-5}$
D-Ins(1,4)P_2	3T3	
D-InsP_6	3T3	

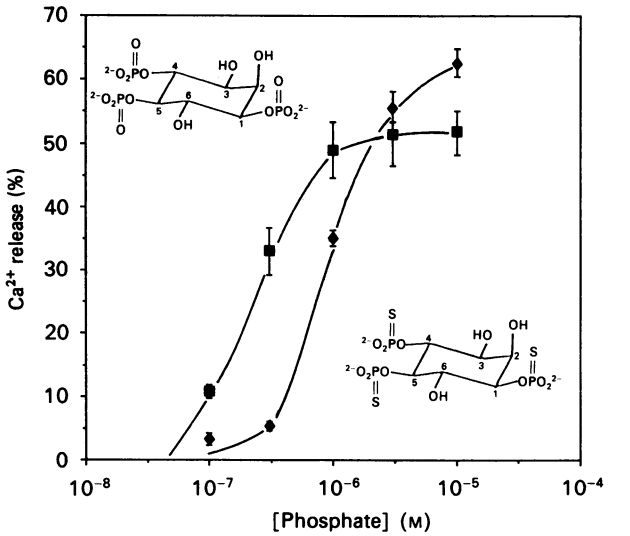

Fig. 4. Dose–response curves for D-Ins(1,4,5)P_3 (■) and DL-Ins(1,4,5)PS_3 (◆) in Swiss 3T3 cells

Each point is the mean ± S.E.M. for at least three experiments.

weak full agonist, experiments were performed in which dose–response curves for the D-isomer were examined in the presence or absence of 10 μM-L-Ins(1,4,5)P_3. The data in Fig. 3 clearly indicate that the two curves were not significantly different, strongly indicating that the L isomer exhibits very low affinity for the Ca^{2+}-release site.

Finally, we examined the effect of a novel synthetic trisphosphorothioate analogue, DL-Ins(1,4,5)PS_3 [13], on $^{45}Ca^{2+}$ release in GH_3 cells. Data in Fig. 4 demonstrate the effectiveness of this novel phosphatase-resistant analogue. Although displaying a 3–4-fold lower EC_{50} value than DL-Ins(1,4,5)P_3, this analogue is clearly a full agonist.

DISCUSSION

It is now generally accepted that D-Ins(1,4,5)P_3 can release Ca^{2+} from a non-mitochondrial intracellular store, perhaps the recently described 'calciosome' [17], in a variety of cells (see Berridge [2], for a recent compilation of data). Furthermore, from the rather limited studies on the structural requirements of inositol phosphates for this effect, this second messenger appears to be the most potent examined. Thus, in studies predominantly performed on the Swiss 3T3 fibroblast cells, whereas D-Ins(1:2-cyclic,4,5)P_3 appeared to be equipotent with D-Ins(1,4,5)P_3, the dephosphorylation product of the latter, namely D-Ins(1,4)P_2, is inactive. Furthermore, although less potent, D-Ins(2,4,5)P_3, D-glycerophosphoinositol 4,5-bisphosphate and D-Ins(4,5)P_2 are nevertheless capable of mobilizing intracellular stored Ca^{2+} at high concentrations [1,2,9,18]. Such data are indicative of a

specificity for inositol phosphates with a *vic*-D-4,5-phosphate pairing, although the report [9] that D-Ins(1,3,4)P_3 is active at releasing Ca^{2+}, albeit 30 times less potently than D-Ins(1,4,5)P_3, is quite inconsistent with this model. Nevertheless, the specificity of the Ca^{2+}-release process has led to the proposal that a receptor protein exists [1], perhaps closely associated with the Ca^{2+} channel or as a separate protein with channel interaction mediated indirectly.

Although preliminary evidence for such a recognition site has come from radioligand binding studies [3–6], more recent studies on a very rich density of such sites in rat cerebellum have revealed at least some of the structural requirements for binding [8]. In particular, we were able to show the marked stereospecificity of the site, with the L-Ins(1,4,5)P_3 isomer being some 2000-fold weaker than the D isomer. Furthermore, we were surprised to observe the virtual inactivity of D-Ins(1,3,4)P_3 at this site, despite the report of its ability to release Ca^{2+} from permeabilized Swiss 3T3 cells [19].

In the present studies we have been able to estabish permeabilized cell preparations that display ATP-dependent high-affinity uptake of $^{45}Ca^{2+}$ into non-mitochondrial intracellular stores and have developed an assay that allows a marked Ca^{2+} response to inositol phosphates to be reproducibly observed. The maximal extent of Ca^{2+} release differed between the GH_3 and Swiss 3T3 cells, and although at present it is difficult to offer reasoned arguments for this, preliminary studies in other cells suggest it is a property of the cell rather than the process of permeabilization. For example, identical studies in the human neuroblastoma SK-N-SH cell have revealed more than 70% release of $^{45}Ca^{2+}$ by D-Ins(1,4,5)P_3 (J. McBain, J. Baird, J. Strupish & S. R. Nahorski, unpublished work). However, the present results clearly establish, for the first time, the marked stereoselectivity of Ca^{2+} release from both GH_3 and 3T3 cells, with the L-Ins(1,4,5)P_3 isomer being a very weak agonist. Studies in which relatively high concentrations of this isomer are co-incubated with the D-(1,4,5)P_3 isomer also established that it also exhibits very low affinity for this site (i.e. it is not a relatively potent partial agonist or antagonist). Thus the present data is consistent with that obtained in the cerebellum binding assay [8] and supports the original suggestion [1] of a 4,5-*trans*,*vic*-phosphate substitution in a D-*myo*-inositol phosphate as a prerequisite for binding and Ca^{2+} release. Also consistent with this model, the present results reveal that h.p.l.c.-pure D-Ins(1,3,4)P_3 is inactive in releasing Ca^{2+} from Swiss 3T3 cells. Although consistent with the very low affinity displayed by this isomer in binding assays [8], these results conflict with those obtained in the same cells by Irvine *et al.* [9]. The only technical difference between the studies appear to be that, in the present work, cells were used in suspension rather than plated on culture trays. It is difficult to imagine that this difference could alter the specificity of the response, and without confirmation of this, we would prefer to conclude that, in our hands, both L-Ins(1,4,5)P_3 and D-Ins(1,3,4)P_3 are essentially inactive at the cerebellar binding site and Ca^{2+}-release site in GH_3 and Swiss 3T3 cells. Preliminary data from work with other cells lines and primary cultures are consistent with the present results, and identical data were obtained with two lots of D-Ins-(1,3,4)P_3.

Although we cannot be certain that the sites labelled in cerebellum are identical with those involved in Ca^{2+} release {attempts to examine Ca^{2+} release from cerebellar microsomal fractions have been hampered by technical problems, but a low density of stereospecific [^{3}H]Ins-(1,4,5)P_3 sites can be detected in Swiss 3T3 cells (A. Willcocks, J. Strupish & S. R. Nahorski, unpublished work)}, it is striking that the rank order of various inositol trisphosphates is identical. This even extends to the novel compound *myo*-inositol 1,4,5-trisphosphorothioate recently synthesized in these laboratories [13]. It was hoped that replacement of phosphate groups by phosphorothioate groups would confer resistance to attack by phosphatases [19,20]. We have recently established that this analogue is resistant to Ins(1,4,5)P_3 5-phosphatase, yet binds with only a 6-fold lower affinity than Ins(1,4,5)P_3 to cerebellar membranes [20]. The present study also reveals that, although slightly weaker than the natural metabolite, the phosphorothioate is, nevertheless, a full agonist with respect to releasing Ca^{2+} from permeabilized Swiss 3T3 cells. A similar activity has been very recently reported in *Xenopus* oocytes [21]. One would anticipate that this novel compound, in view of its resistance to 5-phosphatase, should offer considerable potential in the investigation of phosphoinositide-linked receptor responses. This should be exploited in systems that, unlike the present permeabilized-cell preparations, display rapid metabolism of D-Ins(1,4,5)P_3.

In conclusion, therefore, the present studies have provided new data on the stereo- and positional specificity of inositol trisphosphates in releasing Ca^{2+} from intracellular stores. Data still suggest that a 4,5-*trans*,*vic*-phosphate substitution in a D-*myo*-inositol phosphate is required for Ca^{2+} release and that the presence of a 1-phosphate group may enhance binding affinity. The preparation of further inositol phosphate analogues may provide new information on affinity and intrinsic activity at this site (or sites) and help towards its isolation.

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REFERENCES

1. Berridge, M. J. & Irvine, R. F. (1984) Nature (London) **312**, 315–321
2. Berridge, M. J. (1987) Annu. Rev. Biochem. **56**, 159–193
3. Guillemette, G., Balla, J., Baukal, A. J., Spat, A. & Catt, K. (1985) J. Biol. Chem. **262**, 1010–1015
4. Baukal, A. J., Guillemette, G., Rubin, R., Spat, A. & Catt, K. (1985) Biochem. Biophys. Res. Commun. **133**, 532–538
5. Spat, A., Bradford, P. G., McKinney, J. S., Rubin, R. & Putney, J. W. (1986) Nature (London) **319**, 514–516
6. Spat, A., Fabiato, A. & Rubin, R. P. (1986) Biochem. J. **233**, 929–932
7. Worley, P. F., Baraban, J. M., Colvin, J. S. & Snyder, S. H. (1987) Nature (London) **325**, 159–161
8. Willcocks, A. L., Cooke, A. M., Potter, B. V. L. & Nahorski, S. R. (1987) Biochem. Biophys. Res. Commun. **146**, 1071–1078
9. Irvine, R. F., Letcher, A. J., Lander, D. J. & Berridge, M. J. (1986) Biochem. J. **240**, 301–304

10. Gigg, J., Gigg, R., Payne, S. & Conant, R. (1987) J. Chem. Soc. Perkin Trans. I, 423–429
11. Gigg, J., Giff, R., Payne, S. & Conant, R. (1987) J. Chem. Soc. Perkin Trans. 1757–1762
12. Cooke, A. M., Potter, B. V. L. & Gigg, R. (1987) Tetrahedron Lett. **28**, 2305–2308
13. Cooke, A. M., Gigg, R. & Potter, B. V. L. (1987) J. Chem. Soc. Chem. Commun. 1525–1526
14. Gershengorn, M. C., Geros, E., Purrello, V. S. & Rebecski, M. J. (1984) J. Biol. Chem. **259**, 10675–10681
15. De Lean, A., Munson, P. J. & Rodbard, D. (1978) Am. J. Physiol. **235**, E97–E108
16. Dean, N. M. & Moyer, J. D. (1987) Biochem. J. **242**, 361–366
17. Volpe, P., Krause, K.-H., Sadarnitsu, H., Zorgato, F., Pozzan, T., Meldolesi, J. & Lew, P. D. (1988) Proc. Natl. Acad. Sci. U.S.A. **85**, 1091–1095
18. Irvine, R. F., Anggard, E. E., Letcher, A. J. & Downes, C. P. (1985) Biochem. J. **229**, 505–511
19. Hamblin, M. R., Flora, J. S. & Potter, B. V. L. (1987) Biochem. J. **246**, 771–774
20. Nahorski, S. R., Cooke, A. M., Potter, B. V. L., Strupish, J. & Willcocks, A. L. (1988) Br. J. Pharmacol., in the press
21. Taylor, C. W., Berridge, M. J., Brown, K., Cooke, A. M. & Potter, B. V. L. (1988) Biochem. Biophys. Res. Commun. **150**, 626–632

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