

Rapid formation of inositol 1,3,4,5-tetrakisphosphate following muscarinic receptor stimulation of rat cerebral cortical slices

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Carbachol stimulation of muscarinic receptors in rat cortical slices prelabelled with *myo*-[2-³H]inositol caused the rapid formation of a novel inositol polyphosphate. Evidence derived from its chromatographic behaviour, and from the structure of the products formed in partial dephosphorylation experiments, suggests that it is probably D-*myo*-inositol 1,3,4,5-tetrakisphosphate. An enzyme in human red cell membranes specifically removes the 5-phosphate from it to form inositol 1,3,4-trisphosphate. It is suggested that inositol 1,3,4,5-tetrakisphosphate is likely to be a second messenger, and that it is the precursor of inositol 1,3,4-trisphosphate and possibly of inositol 1,4,5-trisphosphate.

INTRODUCTION

Considerable progress has been made recently in our understanding of the function of stimulated inositol turnover, in particular with the identification of PtdIns(4,5)P₂ as a primary target for receptor-stimulated hydrolysis (Michell *et al.*, 1981; Berridge, 1983, 1984), and the subsequent demonstration that Ins(1,4,5)P₃ is the likely intracellular message for calcium mobilization (Streb *et al.*, 1983; Berridge & Irvine, 1984). Measurement of Ins(1,4,5)P₃ in stimulated tissues has been complicated by the finding that another inositol trisphosphate, Ins(1,3,4)P₃, is formed which has entirely different metabolic kinetics (Irvine *et al.*, 1984a, 1985; Burgess *et al.*, 1985). The source and function of Ins(1,3,4)P₃ have so far remained unidentified.

Stimulation of a variety of neurotransmitter receptors in brain slices, in common with receptors in many other tissues, causes formation of inositol phosphates (Brown *et al.*, 1984; Daum *et al.*, 1984; Gonzalez & Crews, 1984). During a more detailed study of muscarinic stimulation in cortical slices, we are able not only to identify the two isomers of InsP₃, but also preliminary experiments suggested a more polar inositol phosphate was being rapidly formed. Here we present evidence that this is an entirely novel inositol phosphate, Ins(1,3,4,5)P₄.

MATERIALS AND METHODS

Tissue preparation

Cerebral cortical slices (350 µm × 350 µm) were prepared from male Sprague–Dawley rats and preincubated for 60 min in Krebs–Henseleit buffer as previously described (Brown *et al.*, 1984). Aliquots (50 µl) of packed slices (1–2 mg of protein) were then incubated at 37 °C in flat-bottomed vials with *myo*-[2-³H]inositol (Amersham International), 5 µCi/vial (approx. 1.5 µM) for 60 min in a total volume of 300 µl. Reactions were terminated with 300 µl of 1 M-trichloroacetic acid, and tissue was

sedimented by centrifugation. The acid extracts were washed with 5 × 2 vol. of water-saturated diethyl ether and adjusted to pH 7–8 with NaHCO₃. In some experiments for preparative purposes these procedures were scaled up 15-fold.

Separation of radiolabelled inositol phosphates

For preparative purposes, for preliminary examinations and for partial dephosphorylation experiments, the inositol phosphates were separated on Dowex-1 anion exchange columns (8% cross-linked, mesh size 200–400, formate form; Berridge *et al.*, 1983). A clear separation of InsP₃ and InsP₄ was achieved by elution of the former from columns (2 cm × 0.5 cm) with 10 ml of 0.1 M-formic acid/0.8 M-ammonium formate, followed by elution of the latter with 10 ml of 0.1 M-formic acid/1.0 M-ammonium formate. This separation method was repeatedly checked by the various analytical procedures described below, and by using standards of [³H]InsP₄ from stimulated brain slices and [³²P]Ins(1,4,5)P₃, and it provides a convenient way of separating the two inositol polyphosphates after prior elution of inositol, InsP and InsP₂ (Berridge *et al.*, 1983).

For a more detailed analysis, including separation of the InsP₃ isomers, a modification of an h.p.l.c. ion-exchange system (Irvine *et al.*, 1985) was used. The eluant was increased linearly from 0 to 0.75 M-ammonium formate buffered to pH 3.7 with orthophosphoric acid over 5 min to elute lower inositol phosphates, and then the buffer held at this concentration for 2 min. Ins(1,3,4)P₃ and Ins(1,4,5)P₃ were then eluted (distinct from each other) by a linear increase over 6 min to 1.0 M-ammonium formate/phosphoric acid, pH 3.7 (cf. Irvine *et al.*, 1985). After a further 5 min at this strength, the buffer was then increased over 10 min to a final concentration of 1.7 M-ammonium formate/phosphoric acid, pH 3.7, and held there for 6 min before returning to water. InsP₄ was found to be eluted at about 1.5 M-ammonium formate/phosphoric acid, pH 3.7.

Abbreviations used: InsP, InsP₂, InsP₃, InsP₄, InsP₅ and InsP₆, inositol mono-, bis-, tris-, tetrakis-, pentakis- and hexakis-phosphates respectively, with assignment of phosphate locants where appropriate [e.g. Ins(1,3,4)P₃]; PtdIns, PtdInsP, PtdInsP₂ and PtdInsP₃, phosphatidylinositol and its mono-, bis- and tris-phosphorylated derivatives.

Standards

[³²P]Ins(1,4,5)P₃ was prepared as in Downes *et al.* (1982). [³H]InsP₄ was prepared in bulk from carbachol-stimulated brain slices by Dowex ion-exchange chromatography (as above), spiked with 200 nmol of non-radioactive Ins(1,4,5)P₃ to aid desalting, and then desalted by elution with 1.5 M-LiCl from a Dowex (chloride) column followed by removal of the LiCl with ethanol (Tomlinson & Ballou, 1961; Burgess *et al.*, 1984). Its purity was checked by h.p.l.c. and ionophoresis.

A mixture of InsP₅ and InsP₄ was prepared by partial dephosphorylation of InsP₆ (phytic acid) at pH 4, exactly as in Desjoberg & Petek (1956). Non-radiolabelled Ins(1,4,5)P₃ was prepared as in Irvine *et al.* (1984b).

Alditols were obtained as in Irvine *et al.* (1984a).

Separation of inositol phosphates

Separation of InsP₆, InsP₅, InsP₄ and InsP₃ was achieved by paper chromatography (Desjoberg & Petek, 1956) or by ionophoresis in 0.1 M-sodium oxalate, pH 1.5 (Seiffert & Agranoff, 1965).

Identification of alditols after periodate degradation of inositol phosphates

Periodate oxidation, borohydride reduction and alkaline phosphatase dephosphorylation of radiolabelled inositol phosphates was exactly as in Irvine *et al.* (1984a) except that only 0.25 ml of 0.1 M-sodium periodate and 5 mg of sodium borohydride were used. In all experiments > 80% recovery of a radiolabelled alditol from the original inositol tetrakis- or tris-phosphate was achieved. Identification of alditols was by co-chromatography with internal standards in two entirely different separation systems, ionophoresis in 0.1 M-NaOH, and chromatography in ethyl acetate/pyridine/boric acid-saturated water (12:5:4, by vol.) (Irvine *et al.*, 1984a). In all instances two or three separate runs in each system with all probable standards were employed so that the identification of any ³H-labelled alditols, although indirect, was unambiguous.

Partial dephosphorylation of InsP₄

This was achieved in two ways. Firstly, by alkaline phosphatase at pH 9.0. Trial-and-error experiments were performed using various dilutions and incubation times with calf intestine alkaline phosphatase (Boehringer Mannheim), separating InsP₄ and InsP₃ by Dowex anion-exchange chromatography (see above). Once conditions were optimized (25–30% conversion of InsP₄ to InsP₃) a bulk preparation was made; alkaline phosphatase (calf intestine, grade II; Boehringer Mannheim) was diluted 50-fold in 0.1% bovine serum albumin, and 50 µl of this diluate was incubated for 15 min at pH 9.0 (Tris/maleate buffer) with 2 mM-Mg²⁺ in a final volume of 2 ml. The InsP₃ formed was separated by ion exchange and desalted as above, before periodate, borohydride and alkaline phosphatase treatment, or analysis by ionophoresis.

Secondly, human red blood cell membranes (Downes *et al.*, 1982; 2 mg of protein) were incubated at pH 7.5 in a final volume of 2 ml with 2 mM-Mg²⁺ as described in Irvine *et al.* (1984a), and they were found to convert the InsP₄ almost quantitatively (> 80% in 1 h) to InsP₃. This InsP₃ was separated and desalted as described above before analysis by h.p.l.c. or periodate, borohydride and alkaline phosphatase treatment (as above).

RESULTS

General properties of the polar inositol polyphosphate

The observation that a more polar inositol polyphosphate could easily be separated by ion-exchange chromatography from the inositol trisphosphates (see the Materials and methods section) not only gave us a means of purifying it in large quantities, but in itself suggested this polar inositol polyphosphate may not be an inositol trisphosphate. This suggestion was strengthened by the findings (a) that to elute the polar inositol polyphosphate from a Partisil SAX-10 h.p.l.c. column, an elution medium of about 50% greater strength than that used for inositol trisphosphates was required and (b) that partial dephosphorylation of the polar inositol polyphosphate by alkaline phosphatase or human red cell membranes (see below) yielded compounds which by their ionophoretic and chromatographic behaviour are inositol trisphosphates.

That this more polar inositol polyphosphate is a tetrakisphosphate was confirmed by running it in two separation procedures which distinctly separate InsP₃, InsP₄ and InsP₅ (see the Materials and methods section); the separations were carried out with internal InsP₆, InsP₅ and InsP₃ markers and also with internal [³²P]Ins(1,4,5)P₃. In both ionophoresis and paper chromatography the novel compound co-chromatographed with InsP₄. A potential difficulty in making this identity certain emerged in the observation that in addition to the strong InsP₅, InsP₄ and InsP₃ spots visible on ionophoresis of a phytic acid partial hydrolysate, minor spots are also evident caused by some separation of isomers (Seiffert & Agranoff, 1965); the radiolabelled inositol phosphate in question migrated so that it overlapped mostly with the strong InsP₄ spot, but partly with a minor spot migrating just behind whose identity was not certain. To ensure it really was migrating with InsP₄, we performed some experiments in which the length of the ionophoresis run was extended so that InsP₆ and InsP₅ ran off the end of the paper; in these experiments InsP₄ isomers ran as a distinct group with a 4 cm gap clearly separating them from the InsP₃ isomers. The radiolabelled novel inositol phosphate still migrated as InsP₄ under these conditions, mostly overlapping with the major InsP₄ spot, and partly with a minor spot migrating just behind. Paper chromatography gave no hint of InsP₄ isomer separation in our hands, and the radioactive compound co-migrated exactly with the InsP₄ marker.

If the putative InsP₄ was treated extensively with alkaline phosphate, the only radiolabelled product recovered was inositol, as judged by the two separation methods given in the Materials and methods section and also chromatography in propan-1-ol/ethyl acetate/water (24:13:7, by vol.). This evidence shows that our novel compound is an inositol tetrakisphosphate, and, notwithstanding the remote possibility of an isomerization of the radiolabelled *myo*-inositol (see Irvine *et al.*, 1984a), we assume that it is a *myo*-inositol tetrakisphosphate.

Metabolism of InsP₄ in stimulated brain slices

Fig. 1 shows a typical result of an experiment to measure the rates of appearance of Ins(1,4,5)P₃, Ins(1,3,4)P₃ and InsP₄ in cortical slices stimulated with a 10⁻³ M concentration of the cholinergic agonist carbachol. Stimulation of each of these polyphosphates was suppressed if slices were preincubated with the

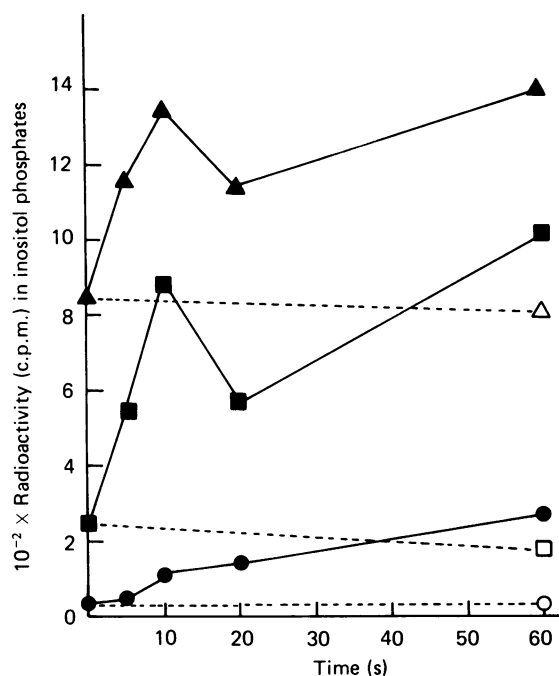


Fig. 1. Time course of inositol tris- and tetrakisphosphate formation in carbachol-stimulated brain slices

Brain slices labelled with *myo*-[2-³H]inositol were stimulated with carbachol (10⁻³ M) for various times as described in the Materials and methods section. InsP₃ isomers and InsP₄ were separated by h.p.l.c. Each data point is the result of the pooling of three separate incubations [to ensure sufficient radioactivity in Ins(1,3,4)P₃]. The data are from one experiment, though very similar data were obtained in another experiment analysed by h.p.l.c., and in two further experiments using Dowex ion-exchange chromatography (which separates InsP₃ and InsP₄, but not the InsP₃ isomers). ○, △, □, controls; ●, ▲, ■, carbachol-treated; ○, ●, Ins(1,3,4)P₃; △, ▲, Ins(1,4,5)P₃; □, ■, Ins(1,3,4,5)P₄.

muscarinic antagonist, atropine (10⁻⁵ M). It is apparent that Ins(1,4,5)P₃ and InsP₄ are both produced rapidly (within 5 s) but that there may be a lag before Ins(1,3,4)P₃ rises, as in other tissues (Irvine *et al.*, 1985; Burgess *et al.*, 1985), although, because of the low radioactivity in Ins(1,3,4)P₃, we cannot be sure of this. Also, the specific activities of InsP₃ and InsP₄ are not known, so we cannot compare absolute rates of synthesis. The essential point of Fig. 1 is that [³H]InsP₄ is rapidly produced after carbachol stimulation of cortical slices.

Structure of InsP₄

In investigating the distribution of phosphates around the inositol ring (and we shall assume a *myo*-inositol ring for the arguments in this section) we have used the same methods as previously (Irvine *et al.*, 1984a). These are, in turn, adapted from the techniques of Ballou and co-workers (Grado & Ballou, 1961; Tomlinson & Ballou, 1961). Periodate oxidation splits the bond between two carbon atoms bearing hydroxyls, whereas the presence of a phosphate group on one or both of the carbon atoms protects the bond from attack. So, periodate treatment of an inositol polyphosphate, followed by reduction and dephosphorylation and identification of the resulting

alditol, yields information about the distribution of phosphates in the inositol ring.

When [³H]InsP₄ from brain slices was submitted to this periodate, borohydride and alkaline phosphatase treatment, the predominant (> 90%) product was inositol, even if the periodate treatment was allowed to proceed for 5 days at pH 6.5. This means that the InsP₄ has no vicinal hydroxyls. Thus the phosphates must be distributed either as two pairs, or as a single with a group of three. These two alternatives can be distinguished, and the structure of the InsP₄ explored, by partial dephosphorylation to InsP₃ using alkaline phosphatase. Although essentially non-specific in its phosphatase activity on inositol phosphates, alkaline phosphatase greatly favours a solitary phosphate group over a phosphate group adjacent to other phosphates (Tomlinson & Ballou, 1961). Thus conversion of the InsP₄ to InsP₃, followed by periodate, borohydride and alkaline phosphatase treatment, should yield a complex mixture of hexitols if the InsP₄ phosphates are paired, but only one product, a pentitol, if there is a single phosphate with a group of three. The latter was what we found; xylitol was the only detectable (> 90%) labelled product after periodate, borohydride and alkaline phosphatase treatment of the resulting InsP₃. We carried out the ionophoretic separation of alditols in 0.1 M-NaOH (see Irvine *et al.*, 1984a) using both long (4.5 h) runs which clearly separate xylitol from all alditols except arabitol, and short (2 h) runs to ensure that no fast-migrating compounds (e.g. tetritols) were formed. Arabitol and xylitol were then distinctly separated by paper chromatography (Grado & Ballou, 1961; Irvine *et al.*, 1984a) to confirm the identity of the alditol as xylitol. These results indicate a D- or L-(3,4,5) grouping of phosphates in the original InsP₄. [We should note in passing that another InsP₃, Ins(4,5,6)P₃, will also yield xylitol, but in so doing the 2-carbon with its attached hydroxyl and hydrogen will be lost, eventually to be evaporated off as methanol. As the radiolabel of the original *myo*-[³H]inositol is in the 2 position, the > 80% conversion of [³H]InsP₃ to [³H]xylitol in these experiments eliminates Ins(4,5,6)P₃ as a possibility.]

The InsP₄ must therefore, in view of its resistance to periodate, have its fourth phosphate in the D- or L-(1) position. This was confirmed independently by the observation that human erythrocyte ghosts could quantitatively convert the InsP₄ to InsP₃ with < 5% formation of InsP₂, InsP or inositol. On periodate, borohydride and alkaline phosphatase treatment, this InsP₃ yielded labelled altritol with 85% recovery, which is indicative of a D- or L-Ins(1,2,4) or Ins(1,3,4) structure (Irvine *et al.*, 1984a). As with the xylitol (see above), ionophoretic separations of differing length were performed to ensure both the identification of altritol, and that it was the only alditol formed. The InsP₃ under examination also co-chromatographed exactly with Ins(1,3,4)P₃ on h.p.l.c. (Irvine *et al.*, 1985) and, in view of the observations above with regard to the (3,4,5) grouping in the InsP₄, it must therefore be D- or L-Ins(1,3,4)P₃. As well as confirming independently that the InsP₄ has a phosphate in the D- or L- (1) and (4) positions, these experiments reveal that human red cells have an enzyme(s) which in addition to specifically removing the 5-phosphate from Ins(1,4,5)P₃ (Downes *et al.*, 1982) also removes the 5-phosphate from InsP₄ to form Ins(1,3,4)P₃, a compound which is formed in several

stimulated tissues (Irvine *et al.*, 1984; Burgess *et al.*, 1985) including brain (Fig. 1).

Source of $\text{Ins}(1,3,4,5)\text{P}_4$

The most likely source of $\text{Ins}(1,3,4,5)\text{P}_4$ is phosphodiesteratic hydrolysis of a $\text{PtdIns}(3,4,5)\text{P}_3$. Evidence for PtdInsP_3 has been reported previously (Klenk & Hendricks, 1961; Santiago-Calvo *et al.*, 1963), and though further observations suggested that at least the first of these might have been due to phosphate contamination (Kerr *et al.*, 1963), the evidence of Santiago-Calvo *et al.* (1963) suggests that PtdInsP_3 may exist in brain, albeit at low levels (Seiffert & Agranoff, 1965). We looked for $[^3\text{H}]\text{PtdInsP}_3$ in lipids extracted by acidified chloroform/methanol from the trichloroacetic acid precipitates in some of the present brain slice experiments, using methods similar to those by which we sought $\text{PtdIns}(3,4)\text{P}_2$ in parotid glands (Irvine *et al.*, 1984a) [i.e. a chemical degradation of the lipids to inositol phosphates using deacylation (Clarke & Dawson, 1981) and removal of the glycerol moiety (Brown & Stewart, 1966) followed by examination of the resulting inositol phosphates by h.p.l.c.]; we were unable to detect any InsP_4 in these experiments, and we can say that in our extracts PtdInsP_3 must be less than 1% of the $\text{PtdIns}(4,5)\text{P}_2$ radioactivity. We have, however, no guarantee that the extraction method we used would be effective for recovering PtdInsP_3 . Furthermore, a very rapidly turning over pool of PtdInsP_3 could be difficult to detect, yet be able to generate the levels of InsP_4 that we measure. The only obvious alternative route of synthesis of InsP_4 would be by a phosphorylation of $\text{Ins}(1,4,5)\text{P}_3$, but we think that a lipid precursor [i.e. a phosphorylation of $\text{PtdIns}(4,5)\text{P}_2$ on the 3-hydroxyl] is more likely. Either way, in view of the known structure of $\text{PtdIns}(4,5)\text{P}_2$ as the D-isomer (Grado & Ballou, 1961; Brown & Stewart, 1966; Agranoff, 1978), it is probable that the InsP_4 under study is D-myo-inositol(1,3,4,5) P_4 .

DISCUSSION

Our identification here of $\text{Ins}(1,3,4,5)\text{P}_4$, as with the previous identification of $\text{Ins}(1,3,4)\text{P}_3$ in parotid glands (Irvine *et al.*, 1984a), can only be preliminary in that it is based on indirect methods centered on co-chromatography of radioactivity with authentic standards. Nevertheless, within the limits of such methods, the only structure consistent with all the data is $\text{Ins}(1,3,4,5)\text{P}_4$, most likely the D-isomer. The rapid formation of $\text{Ins}(1,3,4,5)\text{P}_4$ in stimulated brain slices suggests the possibility that, like $\text{Ins}(1,4,5)\text{P}_3$ (Berridge & Irvine, 1984), it has second messenger functions.

The most obvious function would be as an adjunct to $\text{Ins}(1,4,5)\text{P}_3$ in calcium mobilization, or even as a more important message in this respect. However, as the metabolism of $\text{Ins}(1,4,5)\text{P}_3$ in stimulated tissues is itself so strongly consistent with its proposed second messenger role (Berridge *et al.*, 1984; Irvine *et al.*, 1985), and also as $\text{Ins}(1,4,5)\text{P}_3$ is such a potent and specific mobilizer of calcium in permeabilized cells (Streb *et al.*, 1983; Irvine *et al.*, 1984b), a completely separate messenger function of $\text{Ins}(1,3,4,5)\text{P}_4$ must be regarded as at least as likely a possibility.

One thing we can say with some degree of confidence is that $\text{Ins}(1,3,4,5)\text{P}_4$ is the probable precursor of $\text{Ins}(1,3,4)\text{P}_3$. We cannot be sure whether the same enzyme

in red cell membranes as that which hydrolyses $\text{Ins}(1,4,5)\text{P}_3$ (Downes *et al.*, 1982), is responsible for $\text{Ins}(1,3,4,5)\text{P}_4$ hydrolysis, and this question must await purification of the enzyme(s). But the remarkably specific removal of the 5-phosphate from $\text{Ins}(1,3,4,5)\text{P}_4$ to form $\text{Ins}(1,3,4)\text{P}_3$ in these experiments shows that such an enzyme does exist in at least one tissue, and this, allied with our former inability to detect $\text{PtdIns}(3,4)\text{P}_3$ (Irvine *et al.*, 1985; Burgess *et al.*, 1985) or an $\text{Ins}(1,4,5)\text{P}_3$ isomerase (R. F. Irvine & M. J. Berridge, unpublished work), makes the conclusion almost inescapable that $\text{Ins}(1,3,4)\text{P}_3$ is the normal enzymic breakdown product of $\text{Ins}(1,3,4,5)\text{P}_4$. Finally, it should be considered that if $\text{Ins}(1,3,4,5)\text{P}_4$ is derived from a lipid then it could also be the precursor of $\text{Ins}(1,4,5)\text{P}_3$, with the proportion of the InsP_3 isomers generated dependent on the respective activities of appropriate 5- and 3-phosphatases. Such speculation, as well as the intriguing possibility that the hydrolysis of $\text{PtdIns}(3,4,5)\text{P}_3$ could be the event directly controlled by receptors, can only be tested by further experimentation. Whatever the function or catabolism of InsP_4 , the data here open up a new facet, and a new pathway, of inositol metabolism which is an exciting area for experimental exploration.

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REFERENCES

- Agranoff, B. W. (1978) *Trends Biochem. Sci.* **3**, N283–N285
- Berridge, M. J. (1983) *Biochem. J.* **212**, 849–858
- Berridge, M. J. (1984) *Biochem. J.* **220**, 345–360
- Berridge, M. J. & Irvine, R. F. (1984) *Nature (London)* **312**, 315–321
- Berridge, M. J., Dawson, R. M. C., Downes, C. P., Heslop, J. P. & Irvine, R. F. (1983) *Biochem. J.* **212**, 473–482
- Berridge, M. J., Buchan, P. B. & Heslop, J. P. (1984) *Mol. Cell. Endocrinol.* **36**, 37–42
- Brown, D. M. & Stewart, J. C. (1966) *Biochim. Biophys. Acta* **125**, 413–421
- Brown, E., Kendall, D. A. & Nahorski, S. R. (1984) *J. Neurochem.* **42**, 1379–1387
- Burgess, G. M., Godfrey, P. P., McKinney, J. S., Berridge, M. J., Irvine, R. F. & Putney, J. W. (1984) *Nature (London)* **309**, 63–66
- Burgess, G. M., McKinney, J. S., Irvine, R. F. & Putney, J. W. (1985) *Biochem. J.* **232**, 237–243
- Clarke, N. G. & Dawson, R. M. C. (1981) *Biochem. J.* **195**, 301–306
- Daum, P. R., Downes, C. P. & Young, J. M. (1984) *J. Neurochem.* **43**, 25–32
- Desjoberg, G. R. & Petek, F. (1956) *Bull. Soc. Chim. Biol.* **38**, 871–883
- Downes, C. P., Mussat, M. C. & Michell, R. H. (1982) *Biochem. J.* **203**, 169–177
- Gonzalez, R. A. & Crews, F. T. (1984) *J. Neurosci.* **4**, 3120–3127
- Grado, C. & Ballou, C. E. (1961) *J. Biol. Chem.* **236**, 54–60
- Irvine, R. F., Letcher, A. J., Lander, D. J. & Downes, C. P. (1984a) *Biochem. J.* **223**, 237–243
- Irvine, R. F., Brown, K. D. & Berridge, M. J. (1984b) *Biochem. J.* **222**, 269–272
- Irvine, R. F., Anggård, E. E., Letcher, A. J. & Downes, C. P. (1985) *Biochem. J.* **229**, 505–511
- Kerr, S. E., Kfoury, G. A. & Djibelian, L. G. (1963) *Biochim. Biophys. Acta* **70**, 474–476

- Klenk, E. & Hendricks, U. W. (1961) *Biochim. Biophys. Acta* **50**, 602–603
- Michell, R. H., Kirk, C. J., Jones, L. M., Downes, C. P. & Creba, J. A. (1981) *Philos. Trans. R. Soc. London Ser. B* **296**, 123–137
- Santiago-Calvo, E., Mule, S. J. & Hokin, L. E. (1963) *Biochim. Biophys. Acta* **70**, 91–93
- Seiffert, U. B. & Agranoff, B. W. (1965) *Biochim. Biophys. Acta* **98**, 574–581
- Streb, H., Irvine, R. F., Berridge, M. J. & Schulz, I. (1983) *Nature (London)* **306**, 67–69
- Tomlinson, R. V. & Ballou, C. E. (1961) *J. Biol. Chem.* **236**, 1902–1906

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