The effect of GTP on inositol 1,4,5-trisphosphate-stimulated Ca^{2+} efflux from a rat liver microsomal fraction

Is a GTP-dependent protein phosphorylation involved?

Alan P. DAWSON, John G. COMERFORD and Derek V. FULTON

School of Biological Sciences, University of East Anglia, Norwich NR4 7TJ, U.K.

1. GTP, when added to a rat liver microsomal fraction that had previously been allowed to accumulate Ca^{2+} , causes a slow release of Ca^{2+} , which is greatly enhanced by addition of inositol trisphosphate (IP_3). 2. The Ca^{2+} release caused by IP_3 under these conditions is very much greater than that observed in the absence of GTP. 3. The effect of GTP is dependent on the presence of polyethylene glycol in the incubation medium and is not due to inhibition of the Ca^{2+} -accumulation system. 4. The response to GTP is time-dependent, particularly at low ($4\ \mu\text{M}$) GTP concentrations, and cannot be mimicked by ATP, ITP, CTP, UTP and GDP. 5. Studies with $[\gamma\text{-}^{32}\text{P}]\text{GTP}$ show that, during incubation with microsomal fractions, the terminal phosphate of GTP is transferred to two protein species, of M_r 38000 and 17000. 6. These protein phosphorylations are still present when an excess of unlabelled ATP is included in the incubation mixture, but appear to be unaffected by the presence or absence of IP_3 and polyethylene glycol. 7. As a working hypothesis, it is suggested that a protein, phosphorylated by GTP, has to bind to the microsomal membranes before IP_3 can stimulate Ca^{2+} release, and that, *in vitro*, the binding of this protein is favoured by the presence of polyethylene glycol.

INTRODUCTION

It is now well established that a wide variety of hormones and neurotransmitters exert their effects, at least in part, by mobilization of intracellular Ca^{2+} . In many cases, Ca^{2+} -mobilizing stimuli are associated with enhanced breakdown of inositol phospholipids (Michell, 1975; Michell *et al.*, 1981), and elevation of phosphoinositide and diacylglycerol concentrations in the cytosol. In several permeabilized cell preparations (Streb *et al.*, 1983; Burgess *et al.*, 1984; Joseph *et al.*, 1984a,b; Hirata *et al.*, 1984; Prentki *et al.*, 1984b; Biden *et al.*, 1984), the water-soluble product of polyphosphatidylinositol hydrolysis, inositol 1,4,5-trisphosphate (IP_3), has been shown to cause Ca^{2+} release from a non-mitochondrial store, which is thought to be endoplasmic reticulum. Microsomes, derived from endoplasmic reticulum, have, for several cell types, been shown to respond to IP_3 by releasing part of their accumulated Ca^{2+} . This has been shown for microsomes from liver (Dawson & Irvine, 1984), an insulinoma (Prentki *et al.*, 1984a) and pancreatic acinar cells (Streb *et al.*, 1984). However, for liver microsomes, substantial Ca^{2+} release was only obtained from the microsomal fraction sedimenting with the mitochondria, the so-called 'mitochondrial fraction' as defined by Reinhart & Bygrave (1981). For microsomal fractions sedimented at higher g values, IP_3 -stimulated Ca^{2+} release was very small (Dawson & Irvine, 1984; Joseph *et al.*, 1984b). We suggested, on the basis of the Ca^{2+} -release and -uptake properties of the various microsomal fractions from rat liver, that, during homogenization the endoplasmic reticulum was fragmented into IP_3 -sensitive and IP_3 -insensitive vesicles, the latter predominating in the smaller vesicle populations.

However, attempts to separate these hypothetical populations caused the total loss of IP_3 sensitivity. We therefore tried the reverse strategy of trying to fuse the vesicles back together, by the use of the membrane fusogen polyethylene glycol (PEG) (Boni *et al.*, 1984). Low concentrations (3–5%, w/v) of PEG in the Ca^{2+} -uptake medium caused a small increase in the percentage of intravesicular Ca^{2+} released by IP_3 and were therefore routinely incorporated into experimental media.

There is much interest in the role of GTP-binding proteins in the coupling of receptor occupancy to phosphoinositide breakdown (for review see Berridge & Irvine, 1984). There is also the observation that GTP analogues affect Ca^{2+} -channel gating in mast cells (Gomperts, 1983). This led us to try GTP as a modulator of IP_3 action on liver microsomes (Dawson, 1985). We found that GTP greatly enhanced IP_3 -stimulated Ca^{2+} release, both in the 'mitochondrial fraction' and in the microsomal fraction sedimented at 35000 g for 20 min (the '35000 g fraction'). In the latter preparation, Ca^{2+} release is very small in the absence of GTP. The effect of GTP was totally dependent on the presence of PEG in the experimental medium and could not be mimicked by the non-hydrolysable GTP analogue guanosine 5'-[β , γ -imido]triphosphate, which inhibited the effect of GTP added subsequently (Dawson, 1985).

The latter observation suggested that metabolism of the GTP might be important. In the present paper we report more data which favour this view and suggest that protein phosphorylation by GTP is a feasible mechanism for the effect of GTP on the system. We also suggest a possible explanation for the requirement for PEG.

Previous work was conducted largely on the 'mito-

Abbreviations used: IP_3 , inositol 1,4,5-trisphosphate; PEG, polyethylene glycol, average M_r 8000.

chondrial fraction', which is a very crude low-speed pellet containing a large quantity of mitochondria as well as endoplasmic reticulum. The present study is on the 35000 *g* fraction, which contains very few mitochondria and therefore provides a more defined experimental system.

EXPERIMENTAL

Materials

Ca^{2+} -sensitive electrode membranes, containing the neutral ionophore ETH1001, were kindly given by Dr. D. Ammann (ETH Zentrum, Zurich, Switzerland). IP_3 was given by Dr. R. F. Irvine (AFRC, Babraham, Cambridge, U.K.). ^{14}C -labelled protein M_r standards, $^{45}\text{Ca}^{2+}$ (0.5 mCi/ μmol) and $[\gamma\text{-}^{32}\text{P}]\text{GTP}$ (13 Ci/mmol) were from Amersham International. PEG (average M_r 8000) was from Sigma, ATP, ITP, CTP, UTP, GTP and dithiothreitol were from BCL, Lewes, Sussex, U.K.

Methods

Microsomal preparation. The microsomal fraction sedimented at 35000 *g* for 20 min was prepared from the livers of fed 250 g rats as described by Dawson & Irvine (1984). Protein concentration was measured by the method of Lowry *et al.* (1951), after dissolving the membranes in SDS; bovine serum albumin was used as a standard.

Ca^{2+} uptake and release. These were routinely measured with a Ca^{2+} -sensitive electrode, with some modification to the method previously described (Dawson, 1985). The electrode system was coupled to a PHM 63 pH-meter (Radiometer), the output of which was passed, via a sensitivity control and back-off system, to a potentiometric recorder. An excess of ATP over Mg^{2+} was used to buffer free Ca^{2+} concentrations at the required value (usually in the range 0.3–0.8 μM free Ca^{2+}). The assay mixture contained, in a volume of 5 ml: 150 mM-sucrose, 50 mM-KCl, 10 mM-Hepes/KOH, pH 7.0, 5 mM-ATP (Na salt), 2 mM- MgCl_2 , 1 mM-dithiothreitol, 20 μM added CaCl_2 , 1 μg of oligomycin/ml, 2 μM -Ruthenium Red and 5% (w/v)

PEG, unless otherwise stated. The temperature was 30 °C, and the reaction was started by the addition of microsomes.

In experiments where it was necessary to start the reaction by the addition of ATP, the addition artefact on the Ca^{2+} electrode made the above method unusable. Accordingly, a filtration method was used. This was essentially as described previously (Dawson, 1985), by using 1 ml of the assay medium described above, with the omission of ATP and containing 0.15 μCi of $^{45}\text{Ca}^{2+}$. Uptake was started by the addition of ATP.

Protein phosphorylation. Protein phosphorylation with $[\gamma\text{-}^{32}\text{P}]\text{GTP}$ was carried out under similar conditions to those used for measuring Ca^{2+} movements. The reaction mixture contained, in a volume of 50 μl , 150 mM-sucrose, 50 mM-KCl, 10 mM-Hepes/KOH, pH 7.0, 4 μM -Ruthenium Red, 3 μg of oligomycin/ml, 2 mM- MgCl_2 , 1 mM-dithiothreitol, 5 mM-ATP, 5% PEG, 20 μM added CaCl_2 , and 120 μg of microsomal protein. Phosphorylation was initiated by the addition of 1 nmol of GTP containing 2.5 μCi of $[\gamma\text{-}^{32}\text{P}]\text{GTP}$. IP_3 when present was added at a final concentration of 2 μM . Detailed variations on this general protocol are given in the Figure legends. The reaction was stopped by the addition of 30 μl of the reaction mixture to 20 μl of gel sample buffer containing 0.4 M-dithiothreitol, 8% (v/v) SDS, 1 M-Tris/HCl (pH 6.8), 60% (v/v) glycerol and 0.024% (w/v) Bromophenol Blue. Samples were heated in a boiling-water bath for 2 min, centrifuged briefly, and 30 μl was applied per lane to SDS/polyacrylamide gels (10% or 12.5%) and electrophoresed by the method of Laemmli (1970) as modified by Blattler *et al.* (1972). Autoradiograms of dried gels were developed for 5–7 days, by using enhancer screens.

RESULTS

The effect of GTP on IP_3 -stimulated Ca^{2+} release from the 35000 *g* microsomal fraction is shown in Fig. 1, by using three different experimental procedures. When the microsomes have reached a steady state of Ca^{2+} accumulation, the addition of 1 μM - IP_3 (Fig. 1a) results

Fig. 1. Effect of GTP on Ca^{2+} release from the 35000 *g* microsomal fraction

The reaction mixture (final volume 5 ml) was as described in the Experimental section, with the inclusion of 5% (w/v) PEG. Additions: M, 6 mg of 35000 *g* microsomal fraction protein; I, 5 nmol of IP_3 ; G, 100 nmol of GTP. In trace (c), 100 nmol of GTP was present in the reaction mixture before the addition of microsomes. The small initial deflection caused by GTP is an addition artefact.

in a very small Ca²⁺ release. The addition of GTP (20 μ M) causes, after a short lag, the rapid release of a substantial fraction of the accumulated Ca²⁺. Fig. 1(b) shows that the addition of GTP before IP₃ causes, after a lag of approx. 20 s, a slow, linear, Ca²⁺ efflux. Addition of IP₃ then causes a large immediate Ca²⁺ release. When GTP is present in the incubation medium before addition of microsomes (Fig. 1c), the rate of Ca²⁺ accumulation is unaltered for about the first 60 s, but the final Ca²⁺ accumulation is considerably decreased compared with that observed in the absence of GTP. However, IP₃ addition still results in a rapid, substantial, Ca²⁺ release. A notable feature of these experiments is that there is a lag phase between GTP addition and Ca²⁺ efflux, in both the presence and the absence of IP₃, whereas the addition of IP₃ after GTP results in an immediate Ca²⁺ release.

It is clear from the above results that the effect of GTP cannot be mimicked by ATP, since this is always present to drive Ca²⁺ uptake. By using the protocol of Fig. 1(a), it was found that ITP, UTP and CTP were also ineffective (results not shown). GDP caused Ca²⁺ efflux in the presence of IP₃, but only after a long lag period. This lag could be greatly shortened by addition of nucleoside diphosphate kinase, suggesting that GDP is only effective after conversion into GTP by ATP and endogenous nucleoside diphosphate kinase.

Since GTP in the absence of added IP₃ causes a slow rate of Ca²⁺ efflux, one possible mechanism for its action is inhibition of the Ca²⁺-accumulation system. This is made rather unlikely by the observation in Fig. 1(c). Furthermore, experiments using ⁴⁵Ca²⁺ and the filtration method for measuring Ca²⁺ accumulation showed no effect of GTP on initial rate of Ca²⁺ accumulation even when the microsomes were incubated with GTP for 30 s before starting Ca²⁺ uptake by addition of ATP. Also, 1 mM-vanadate, although promoting Ca²⁺ efflux from Ca²⁺-loaded microsomes, does not enhance the amount of Ca²⁺ released by a subsequent addition of IP₃.

The slow release promoted by GTP and the enhancement of IP₃-stimulated release appear to reflect the same underlying phenomenon. Evidence for this view comes from the data shown in Figs. 2 and 3. Fig. 2 shows the

Fig. 2. Effect of PEG concentration on Ca²⁺ release from the 35000 g microsomal fraction

Experiments were done using the protocol described in Fig. 1(b), and the experimental conditions were identical, except that the PEG concentration was varied. ○, Rate of Ca²⁺ release (nmol/min per mg of protein) after addition of 100 nmol of GTP; ●, amount of Ca²⁺ released (nmol/mg of protein) in the 60 s after the addition of 5 nmol of IP₃. In all cases, IP₃ was added 2 min after GTP.

effect of changing the concentration of PEG on the slow release of Ca²⁺ after addition of GTP and also on the quantity of Ca²⁺ rapidly released after a challenge with IP₃. It is clear that dependence on PEG concentration is identical for both parameters. A similar conclusion can be drawn from the data in Fig. 3. Although increasing the GTP concentration above 20 μ M has no further effect, suggesting that the system is saturated at this concentration (results not shown), decreasing the GTP concentration from 20 to 4 μ M greatly increases the lag time before Ca²⁺ efflux begins. Addition of IP₃ during this lag causes a rather small immediate Ca²⁺ release, although there is a subsequent ongoing phase of Ca²⁺ efflux. However, if IP₃ addition is delayed until the efflux produced by GTP

Fig. 3. Effect of GTP concentration on Ca²⁺ release from 35000 g microsomes

Conditions were as described for Fig. 1(b). M indicates the addition of 5 mg of microsomal protein and I addition of 5 nmol of IP₃. (a) 100 nmol of GTP added at G; (b) and (c), 20 nmol of GTP added at G. The broken line in (b) shows the course of Ca²⁺ uptake and the effect of IP₃ in the absence of GTP addition.

Fig. 4. Phosphorylation of proteins by $[\gamma\text{-}^{32}\text{P}]\text{GTP}$ in the presence and absence of ATP and IP_3

The phosphorylation medium contained, in a volume of 0.1 ml: 150 mM-sucrose, 50 mM-KCl, 10 mM-Hepes/KOH, pH 7.0, 5% (w/v) PEG, 1 mM-dithiothreitol, 0.3 μg of oligomycin, 3 μM -Ruthenium Red, 5.0 μCi of $[\gamma\text{-}^{32}\text{P}]\text{GTP}$ and 2 nmol of GTP. In addition, there was either 0.1 mM- MgCl_2 (lanes 1–4) or 2 mM- MgCl_2 plus 5 mM-ATP (lanes 5–8). The total Mg^{2+} concentration was adjusted to maintain the same free Mg^{2+} concentration in both incubation mixtures. IP_3 (final concn. 2 μM) was present in the incubation mixtures for lanes 3, 4, 7 and 8. Phosphorylation was started by the addition of 0.36 mg of 35000 g microsomal protein. After 20 s (lanes 1, 3, 5, 7) or 50 s (lanes 2, 4, 6, 8), 30 μl samples were removed and added to 20 μl of gel sample buffer as given in the Experimental section, and applied to a SDS/10%-polyacrylamide gel. The un-numbered lanes on the outside of gel contain radioactive protein M_r markers, run in the absence of PEG, the M_r values of which are indicated on the right-hand side of the Figure. The fogged area bottom left is an orientation mark. The autoradiogram was developed for 5 days from the dried gel.

alone has started, then a much larger immediate Ca^{2+} release is observed. It is also clear that decreasing GTP concentration has, over this concentration range, rather little effect on the rate of Ca^{2+} efflux or the IP_3 response as long as sufficient time is allowed to elapse before IP_3 addition.

The observation of lag periods after GTP addition, the dependence of the length of lag on GTP concentrations, the ineffectiveness of GDP (and guanosine 5'-[β,γ -imido]triphosphate and 3',5'-cyclic GMP; Dawson, 1985) all point to the idea that GTP has to be metabolized in some way before the microsomes are converted into an IP_3 -sensitive form. Perhaps the most obvious mechanism for this is that GTP acts as a phosphoryl donor to a component of the system.

Fig. 4 shows an autoradiogram of an SDS/polyacrylamide gel in which 35000 g microsomes have been incubated with $[\gamma\text{-}^{32}\text{P}]\text{GTP}$ in the presence and absence of unlabelled ATP and IP_3 . The presence of unlabelled ATP causes the disappearance of three rather weakly labelled bands in the top half of the gel, which can probably therefore be ascribed to non-specific phosphorylations. However, the presence of ATP has no effect on the speed and intensity of labelling of two of the bands,

Fig. 5. Effect of PEG on mobility of the phosphorylated components

The photograph is of an autoradiogram of a SDS/12.5%-polyacrylamide gel. The incubation mixture (lanes 1–4) contained, in a volume of 0.05 ml: 150 mM-sucrose, 50 mM-KCl, 5% (w/v) PEG, 10 mM-Hepes/KOH, pH 7.0, 5 mM-ATP, 2 mM- MgCl_2 , 1 mM-dithiothreitol, 3 μM -Ruthenium Red, 3 μg of oligomycin/ml, 20 μM added CaCl_2 and 0.13 μg of 35000 g microsomal protein. After 3 min, 1 nmol of GTP containing 1.5 μCi of $[\gamma\text{-}^{32}\text{P}]\text{GTP}$ was added. The reaction was stopped as described for Fig. 4 after a further 30 s (lane 1), 60 s (lane 2) or 90 s (lane 3). Lane 4 was similar to lane 3, except that 2 μM - IP_3 was added 30 s before stopping the reaction. Lanes 6 and 7 are as for lanes 3 and 4, except that PEG was omitted from the experimental medium. Lane 5 was as for lane 6, except that, after stopping the reaction, 6 μl of 20% PEG was added to adjust the PEG concentration in the sample applied to the gel to the same value as that for lanes 1–4. Arrows in the right-hand side indicate the mobilities of the M_r markers in the absence of PEG.

one apparently migrating with the M_r -30000 marker and one migrating close to the M_r -14000 marker, just behind the dye front (the gel has in this instance been removed from the dye front onwards to avoid fogging of the film). Both these bands are apparently fully labelled after 20 s of incubation, and IP_3 has no effect on the intensity of labelling. Particularly in the case of the M_r -30000 band, it is apparent that the protein has not run normally on the gel, showing pronounced tailing at the edges of the lanes. Fig. 5 shows that the distortion of the bands is due to the presence of PEG in the sample on the gel, and also

that both protein bands are still labelled by [γ -³²P]GTP in the absence of PEG. Lanes 6 and 7 show that, in the absence of PEG, two labelled bands are produced, migrating with M_r values of 38 000 and 17 000 respectively. Addition of PEG after phosphorylation has been stopped by the addition of sample buffer (lane 5) causes distortion of the M_r -38 000 band in a manner similar to that shown in Fig. 4, and also causes a decrease in the R_F of the M_r -17 000 band. The band pattern produced by the addition of PEG after stopping the phosphorylation appears identical with the pattern produced when PEG is present during the phosphorylation. It is therefore concluded that the true M_r values of the phosphorylated species are 38 000 and 17 000, in both the presence and the absence of PEG.

Both bands that are observed under these sorts of experimental conditions appear to be phosphorylated fast enough to be responsible for the GTP-dependent increase in microsomal Ca²⁺ permeability. However, the absence of any apparent effects of PEG or IP₃ on the phosphorylation pattern clearly raises the question of whether or not either phosphorylated protein is involved in Ca²⁺ release. As yet we have no direct evidence on this point. However, preliminary results (not shown) suggest that, under the experimental conditions used for Fig. 5, followed by rapid centrifugation to separate membranes and supernatant, the M_r -38 000 protein associated with the radioactive band is largely in the supernatant in the absence of PEG, and in the membrane fraction in the presence of PEG. The radioactivity associated with the M_r -17 000 band is approximately evenly divided between pellet and supernatant, in both the presence and the absence of PEG.

DISCUSSION

The evidence presented here confirms and extends our earlier work on the effect of GTP on Ca²⁺ release by microsomes (Dawson, 1985). For the reasons explained above, the data appear to be best explained by the postulate of a reaction which depends on the metabolism of GTP rather than the involvement of a GTP-binding protein. The detection of two protein species that become labelled with ³²P from [γ -³²P]GTP in the presence of unlabelled ATP suggests that the microsomes contain a system for protein phosphorylation with GTP specifically as the phosphoryl donor. Unfortunately, at the moment there is no direct evidence to implicate either species in the IP₃-responsiveness of the microsomes.

However, the observation that addition of GTP by itself causes some Ca²⁺ efflux suggests that whatever effect GTP is having is not intrinsically dependent on added IP₃, although it may be that there are traces of endogenous IP₃ in the preparation which fulfil any IP₃ requirement. It is not unexpected, in view of this, that neither of the radioactive bands observed shows any noticeable effect of IP₃ on intensity of labelling, and this lack of effect cannot be used to exclude them from an involvement in the effect of GTP on Ca²⁺ efflux.

PEG, as well as having membrane-fusogenic properties, has also been used to induce protein aggregation and to mimic high intracellular protein concentrations in experiments *in vitro* (Medina *et al.*, 1985). The finding that the M_r -38 000 band protein is largely in the supernatant

in the absence of PEG and membrane-associated in its presence suggests that the requirement for PEG may be due to its causing the association of a loosely bound protein with the membranes. *In vivo*, this function could be fulfilled by high protein and membrane binding-site concentrations.

As a working hypothesis, we propose that the microsomal fractions as isolated contain a protein which, in the conditions of assay, can dissociate from the membranes. The binding of this protein, and its phosphorylation by GTP, are required to make the Ca²⁺-efflux system in the microsomal membranes IP₃-sensitive. This hypothesis would appear to be open to experimental test by studying the effects of adding back extracts of microsomal extrinsic proteins. The possible requirement for protein phosphorylation and protein binding to endoplasmic reticulum to create an IP₃-sensitive Ca²⁺-release mechanism would give the potential to control the IP₃/Ca²⁺-release branch of the polyphosphoinositide system in isolation from the diacylglycerol/protein kinase C branch.

We thank Dr. D. Ammann for Ca²⁺-electrode membranes, Dr. R. F. Irvine for gifts of IP₃ and for helpful discussions, and the SERC for financial support for J. G. C.

REFERENCES

- Berridge, M. J. & Irvine, R. F. (1984) *Nature* (London) **312**, 315–321
- Biden, T. J., Prentki, M., Irvine, R. F., Berridge, M. J. & Wollheim, C. B. (1984) *Biochem. J.* **223**, 467–473
- Blattler, D. P., Garner, F., Van Slyke, K. & Bradley, A. (1972) *J. Chromatogr.* **64**, 147–155
- Boni, L. T., Hah, J. S., Hui, S. W., Mukherjee, P., Ho, J. T. & Jung, C. Y. (1984) *Biochim. Biophys. Acta* **775**, 409–418
- Burgess, G. M., Irvine, R. F., Berridge, M. J., McKinney, J. S. & Putney, J. W. (1984) *Biochem. J.* **224**, 741–746
- Dawson, A. P. (1985) *FEBS Lett.* **185**, 147–150
- Dawson, A. P. & Irvine, R. F. (1984) *Biochem. Biophys. Res. Commun.* **120**, 858–864
- Gomperts, B. D. (1983) *Nature* (London) **306**, 64–66
- Hirata, M., Suematsu, E., Hashimoto, T., Hamachi, T. & Koga, T. (1984) *Biochem. J.* **223**, 229–236
- Joseph, S. K., Thomas, A. P., Williams, R. J., Irvine, R. F. & Williamson, J. R. (1984a) *J. Biol. Chem.* **259**, 3077–3081
- Joseph, S. K., Williams, R. J., Corkey, B. E., Matschinsky, F. M. & Williamson, J. R. (1984b) *J. Biol. Chem.* **259**, 12952–12955
- Laemmli, U. K. (1970) *Nature* (London) **227**, 680–685
- Lowry, O. H., Rosebrough, N. J., Farr, A. L. & Randall, R. J. (1951) *J. Biol. Chem.* **193**, 265–275
- Medina, R., Aragon, J. J. & Sols, A. (1985) *FEBS Lett.* **180**, 77–80
- Michell, R. H. (1975) *Biochim. Biophys. Acta* **415**, 81–147
- 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
- Prentki, M., Biden, T. J., Janjic, D., Irvine, R. F., Berridge, M. J. & Wollheim, C. B. (1984a) *Nature* (London) **309**, 562–564
- Prentki, M., Wollheim, C. B. & Lew, P. D. (1984b) *J. Biol. Chem.* **259**, 13777–13782
- Reinhart, P. H. & Bygrave, F. L. (1981) *Biochem. J.* **194**, 541–549
- Streb, H., Irvine, R. F., Berridge, M. J. & Schultz, I. (1983) *Nature* (London) **306**, 67–69
- Streb, H., Bayerdorffer, E., Haase, W., Irvine, R. F. & Shultz, I. (1984) *J. Membr. Biol.* **81**, 241–253.