The mechanism of action of GTP on Ca^{2+} efflux from rat liver microsomal vesicles

Alan P. DAWSON,*† Graham HILLS† and John G. COMERFORD*

*School of Biological Sciences, University of East Anglia, Norwich NR4 7TJ, U.K., and †John Innes Institute, Colney Lane, Norwich NR4 7UH, U.K.

1. Guanosine 5'-[γ -thio]triphosphate (GTP[S]), if added before GTP, blocks both Ca^{2+} efflux promoted by GTP and the effect of GTP on enhancement of inositol 1,4,5-trisphosphate (IP_3)-promoted Ca^{2+} release from preloaded microsomal vesicles. If, however, GTP[S] is added after GTP, it does not reverse the Ca^{2+} efflux promoted by GTP, nor does it inhibit IP_3 -promoted Ca^{2+} release. 2. The effect of GTP in enhancing IP_3 -promoted Ca^{2+} release is maintained after washing the microsomal vesicles free of added GTP. After this treatment, enhancement of IP_3 -promoted Ca^{2+} efflux can be observed in the absence of poly(ethylene glycol). 3. Electron microscopy shows that during GTP treatment of microsomal vesicles there is rapid production of very large vesicular structures, apparently produced by fusion of smaller vesicles. 4. Light-scattering changes are detectable during the fusion process. 5. Both Ca^{2+} efflux promoted by GTP and the enhancement of IP_3 -promoted Ca^{2+} release seen in the presence of GTP can probably be attributed to GTP-dependent vesicle fusion.

INTRODUCTION

Inositol trisphosphate (IP_3) has been shown to be effective in mobilizing Ca^{2+} from intracellular stores in permeabilized cells (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) and isolated microsomal fractions ('microsomes') from many different sources (Dawson & Irvine, 1984; Prentki *et al.*, 1984a, 1985; Streb *et al.*, 1984; Muallem *et al.*, 1985; Authi & Crawford, 1985; O'Rourke *et al.*, 1985). Although it was found that IP_3 caused release of Ca^{2+} from rat liver microsomal fractions (Dawson & Irvine, 1984), the amount of releasable Ca^{2+} was a very small percentage of the Ca^{2+} accumulated. However, if GTP was added to the assay medium, in the presence of 5% poly(ethylene glycol) (PEG), the amount of Ca^{2+} released by IP_3 was greatly increased (Dawson, 1985; Dawson *et al.*, 1986). It was also found that addition of GTP in the absence of IP_3 caused a slow efflux of accumulated Ca^{2+} from microsomes. We suggested that a protein, phosphorylated by GTP, had to bind to the IP_3 receptor site or to a Ca^{2+} channel to render the Ca^{2+} channel IP_3 -sensitive. Observations by other workers make this proposal unlikely. Gill *et al.* (1986) and Ueda *et al.* (1986) found, using permeabilized N1E-115 neuroblastoma cells and microsomal fractions derived from them, that GTP by itself caused rapid and extensive Ca^{2+} release, whereas IP_3 -promoted Ca^{2+} release was not significantly altered by the presence of GTP. Similar observations were made by Henne & Söling (1986) on parotid-gland heavy microsomes. Furthermore, in the latter case it was found that the effects of changes of temperature, PEG concentration and intravesicular Ca^{2+} were very different for GTP-dependent and IP_3 -dependent Ca^{2+} efflux, suggesting that the mechanisms are not directly connected.

It seems likely that the GTP-dependent Ca^{2+} efflux observed by us and by Gill *et al.* (1986), Ueda *et al.* (1986) and Henne & Söling (1986) all reflect the same general mechanism of GTP action. The most fundamental difference between the different systems lies in the enhancement of IP_3 -promoted Ca^{2+} efflux, which is seen in liver microsomes, but not in any of the other systems. However, permeabilized N1E 115 cells, microsomes prepared from them, and parotid microsomes show very substantial IP_3 -dependent Ca^{2+} efflux in the absence of GTP, but this is not the case of liver microsomes. We originally proposed (Dawson & Irvine, 1984) that, during homogenization of liver endoplasmic reticulum, some of the vesicles formed contain IP_3 receptors (and are therefore IP_3 -sensitive) and some do not. The IP_3 -sensitivity of the final vesicle population would depend on the density and distribution of IP_3 receptors in the plane of the endoplasmic-reticulum membrane and on the average microsomal vesicle size, since these parameters would determine the chance of any one vesicle containing an IP_3 receptor. The initial rationale for including PEG in the assay medium was an attempt to promote vesicle fusion (Dawson, 1985), since an increase in the average vesicle size should, on the above hypothesis, increase the percentage of IP_3 -releasable Ca^{2+} . Paiement (1984) has shown that GTP can cause fusion of endoplasmic-reticulum vesicles, although the conditions used (0.84 mM-GTP for 2 h) were very different from those used in the experiments discussed above (20 μM -GTP for a few minutes). However, a mechanism involving GTP-induced fusion of vesicles appears to be consistent with the present experimental data. It would be expected to increase IP_3 -sensitivity in systems where IP_3 receptors were present in only a small percentage of vesicles, but not in systems where a high percentage of the vesicle population was IP_3 -sensitive. Also, it could promote Ca^{2+} efflux, in a GTP-dependent

Abbreviations used: GTP[S], guanosine 5'-[γ -thio]triphosphate; IP_3 , D-*myo*-inositol 1,4,5-trisphosphate; PEG, poly(ethylene glycol).

† To whom reprint requests should be addressed.

fashion, by two possible mechanisms: by allowing Ca^{2+} leakage during the fusion process and by decreased Ca^{2+} -uptake capacity in the larger (and therefore more osmotically sensitive) vesicles formed.

In this paper we present evidence which supports a mechanism involving GTP-induced vesicle fusion as a basis for the effects of GTP on Ca^{2+} efflux from rat liver microsomes.

EXPERIMENTAL

Materials

Ca^{2+} -sensitive electrode membranes containing the neutral ionophore ETH1001 were purchased from Glasblaserie W. Moller, Zurich, Switzerland. IP_3 was kindly given by Dr. R. F. Irvine, A.F.R.C., Babraham, Cambridge, U.K. [^{14}C]GTP (450 mCi/mmol) was from Amersham International. PEG (average M_r 8000), phosphocreatine, creatine kinase, nocodazole and cytochalasin B were from Sigma. ATP, GTP, GTP[S] and dithiothreitol were from BCL, Lewes, Sussex, U.K.

Methods

Microsomal preparation. The microsomal fraction sedimenting at 35000 g for 20 min was prepared from the livers of fed 250 g male 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 as described previously (Dawson, 1985; Dawson *et al.*, 1986). An excess of ATP over Mg^{2+} was used to buffer free Ca^{2+} in the sub-micromolar range. Variations in free Ca^{2+} were achieved by addition of CaCl_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% (w/v) PEG, 5 mM-ATP (Na_4 salt), 2 mM- MgCl_2 , 1 mM-dithiothreitol, 5 mM-phosphocreatine, 20 units of creatine kinase, 5 μg of oligomycin and 2 μM -Ruthenium Red. The temperature was 30 $^{\circ}\text{C}$ and the reaction was started by the addition of microsomes (approx. 6 mg of protein).

Electron microscopy. Microsomal vesicles were incubated at a protein concentration of 1.1 mg/ml in the Ca^{2+} -electrode vessel, in the assay medium described above. When added, GTP[S] was present initially at 90 μM . At suitable points in the time course, 0.5 ml samples were withdrawn and added to tubes containing 40 μl of 25% (v/v) glutaraldehyde, the contents of the tube being mixed by gentle inversion. Samples were post-fixed in 2% (w/v) osmic acid; dehydration was carried out in increasing concentrations of ethanol, and specimens were transferred to L. R. White Resin (London Resin Co., Woking, Surrey, U.K.) by way of a graded series of ethanol/resin concentrations. The resin was polymerized for 16 h at 60 $^{\circ}\text{C}$ and sectioned on a Reichert Ultracut E microtome. The sections were stained with 2% uranyl acetate and lead acetate. Electron microscopy was performed in a JEOL 1200 EX instrument. Fields were selected, and photographs taken, by G.H., who was unaware of the experimental protocol.

Light-scattering. Light-scattering measurements were carried out in an apparatus similar to that described by Selwyn *et al.* (1970). Light transmitted through the stirred thermostatically controlled (30 $^{\circ}\text{C}$) reaction vessel was detected by an ORP 12 photoresistor, and signal processing was carried out on-line by a BBC Master series microcomputer.

RESULTS

Fig. 1 shows the effect of changing the free Ca^{2+} concentration in the assay medium on Ca^{2+} efflux promoted by GTP and by IP_3 (in the presence of GTP). When the starting free Ca^{2+} concentration is relatively high (about 1 μM), Ca^{2+} is accumulated to > 30 nmol of Ca^{2+} /mg of protein. Addition of GTP results in a large efflux of Ca^{2+} , of the order of 20 nmol/mg of protein in

Fig. 1. Effect of free Ca^{2+} concentration on GTP-promoted and IP_3 -promoted Ca^{2+} release

The Figures show Ca^{2+} -electrode traces of Ca^{2+} uptake and release, measured with the experimental system described under 'Methods'. The microsomal protein concentration was 1.3 mg/ml. In (a) the starting pCa was adjusted to 6.14 by the addition of 50 μM - CaCl_2 . For (b), starting pCa 6.74, no exogenous Ca^{2+} was added. The reaction was started by the addition of microsomes at $t = 0$. The arrows marked 'GTP' and ' IP_3 ' show the addition of 20 μM -GTP and of 2 μM - IP_3 . In each case, the right-hand ordinate shows the intra-vesicular Ca^{2+} accumulated from the medium, calculated from the deflection given by the addition of known amounts of Ca^{2+} . Each trace is typical of eight experiments on different batches of microsomes.

Fig. 1(a), but subsequent addition of IP₃ causes the release of only a further 2.5 nmol of Ca²⁺/mg of protein. However, when the starting free Ca²⁺ concentration is low (0.2 μ M), uptake is considerably decreased (17 nmol of Ca²⁺/mg of protein) and efflux promoted by GTP is decreased to 3 nmol/mg of protein. In contrast, IP₃-releasable Ca²⁺ is increased to approx. 10 nmol of Ca²⁺/mg of protein.

Although in these experiments the situation is complex, since both intra- and extra-vesicular Ca²⁺ are changing, it is clear that the effect of one or both of these parameters is very different on GTP-promoted Ca²⁺ efflux compared with the effect on IP₃-promoted efflux, in agreement with the observations of Henne & Söling (1986) on parotid-gland heavy microsomes. It should be noted that under the conditions of Fig. 1 the effect of IP₃ in the absence of GTP is very small, causing the release of 0.4 nmol of Ca²⁺/mg of protein under both conditions. In the present context, the results shown in Fig. 1 are useful in the design of experiments. For measurement of GTP-promoted Ca²⁺ efflux the most suitable conditions are those shown in Fig. 1(a), whereas IP₃-stimulated efflux is better seen under those used in Fig. 1(b).

If Ca²⁺ efflux promoted by GTP, and the subsequent

enhancement of IP₃-promoted Ca²⁺ efflux, were due to vesicle fusion, it would be expected that the effect of GTP would, at least in the short term, be irreversible. Fig. 2 shows results from an experiment designed to test this. In agreement with the observations by Henne & Söling (1986), 90 μ M-GTP[S] causes complete inhibition of Ca²⁺ efflux promoted by 20 μ M-GTP (Fig. 2b) and also blocks the enhancement of IP₃-promoted Ca²⁺ efflux. The small Ca²⁺ release observed after IP₃ addition in Fig. 2(b) (0.4 nmol/mg of protein) is the same as that seen in the absence of GTP[S] and GTP under the same conditions (results not shown). However, Fig. 2(c) shows that, if GTP[S] is added after GTP-promoted Ca²⁺ efflux is complete, there is, firstly, no significant reversal of the Ca²⁺ efflux and, secondly, no inhibition of IP₃-stimulated Ca²⁺ efflux. The effects of GTP[S] on IP₃-stimulated Ca²⁺ efflux are more clearly seen in Figs. 2(d), 2(e) and 2(f), where the experiment was carried out at a lower free Ca²⁺ concentration. Again, although addition of GTP[S] before GTP blocks the enhancement of IP₃-promoted Ca²⁺ release (Fig. 2e), the addition of GTP[S] after GTP does not inhibit IP₃-promoted Ca²⁺ release (Fig. 2f).

The failure of GTP[S] to reverse the effect of GTP suggested that it might be possible to maintain the effect of GTP on IP₃-stimulated Ca²⁺ release after washing

Fig. 2. Effects of GTP[S] and GTP-promoted and IP₃-promoted Ca²⁺ release

In (a)–(c), conditions were as for Fig. 1(a), except that the microsomal protein concentration was 1.2 mg/ml. Arrows: M, microsomes; GTP, 20 μ M-GTP; GTP[S], 90 μ M-GTP[S]; IP₃, 2 μ M-IP₃ (final concentrations in each case). In (d)–(f), conditions were as for Fig. 1(b). The traces show the final section of records equivalent to those in (a)–(c), but at a lower Ca²⁺ concentration. (d), Control, GTP added 10 min before IP₃; (e), GTP[S] added 2 min before GTP, IP₃ added 10 min after GTP; (f), GTP[S] added 5 min after GTP, IP₃ added 5 min after GTP[S]. Concentrations of GTP, GTP[S] and IP₃ were as for (a)–(c). The upper time bar and Ca²⁺-calibration bars apply to traces (a)–(c) and the lower time and Ca²⁺-calibration bars apply to (d)–(f).

away the GTP. The presence of PEG in the assay medium causes the microsomal vesicles to aggregate, so that they sediment very well under mild centrifugation. Fig. 3 shows the results of an experiment of this type. Fig. 3(a) shows the pretreatment of the vesicles before the washing procedure. After treatment with GTP and IP_3 (point A in Fig. 3a) or IP_3 in the absence of GTP (point B in Fig. 3a), the contents of the reaction vessel were removed and centrifuged at 1500 *g* for 5 min at 4 °C. The supernatant was removed by aspiration, and the walls of the tube and the surface of the pellet were washed by gently running in 5 ml of ice-cold 150 mM-sucrose/50 mM-KCl/10 mM-Hepes/KOH (pH 7.0)/5% (w/v) PEG/1 mM-dithiothreitol. This was then immediately removed with suction, and a further 5 ml of the same buffer was applied. The pellet was gently resuspended by sucking up and down three times into a 5 ml plastic pipette (tip

Fig. 3. Effect of GTP removal on IP_3 -promoted Ca^{2+} release

(a) Conditions were as described for Fig. 1(b). Microsomes (1.1 mg/ml) were added at 'M', and 2 μM - IP_3 at ' IP_3 '. For the continuous trace 20 μM -GTP was added at 'GTP'. The broken trace shows the time course in the absence of GTP. Microsomal membranes were harvested at point A (+GTP) or point B (-GTP) and washed as described in the text. (b) Washed microsomes, harvested at A (continuous trace) or B (broken trace), were added at 'M' to the reaction medium specified for Fig. 1(b). The initial phase of Ca^{2+} uptake before IP_3 addition was identical for the two traces. At ' IP_3 ', 2 μM - IP_3 was added in each case, and at 'GTP', 20 μM -GTP was added to the microsomes harvested at B. (c) As for (b), except that the reaction medium did not contain PEG.

diameter 1 mm). The resulting suspension was centrifuged as before and the supernatant removed. The final pellet was ground up with the addition of 0.1 ml of the same resuspension buffer and transferred completely, with a wide-ended positive-displacement pipette, into the new assay medium. Although for most experiments the washed pellet was used immediately, similar data were obtained from microsomes that had been left for over 1 h at the final pellet stage. Fig. 3(b) shows traces from microsomes treated in this way and put back into an assay medium containing 5% PEG, but in the absence of GTP. It is clear that the amount of Ca^{2+} releasable by IP_3 is very much greater for the membranes pretreated with GTP compared with those where GTP was not added during the pretreatment. The dashed trace in Fig. 3(b) shows, however, that the latter still respond to GTP after the washing procedure. From experiments on four different batches of microsomes, using this protocol, the amounts of Ca^{2+} (nmol/mg of protein) liberated by IP_3 under the conditions of Fig. 3(a) were 7.7 ± 0.7 in the presence of GTP and 0.4 ± 0.1 in the absence of GTP. For the washed vesicles, under the conditions of Fig. 3(b), GTP-pretreated vesicles released 2.8 ± 0.5 and vesicles pretreated without GTP released 0.4 ± 0.1 nmol of Ca^{2+} /mg of protein when challenged by IP_3 .

Clearly, one possible explanation of the maintenance of GTP activation through the washing procedure is carry-over of GTP into the second assay medium. Accordingly, the experiment shown in Figs. 3(a) and 3(b) was repeated, with 0.5 μCi of [^{14}C]GTP included in the GTP added to the pretreatment assay medium. The carry-over of ^{14}C into the re-assay medium was found to be $1 \pm 0.05\%$ ($n = 4$), equivalent to a GTP concentration of 0.2 μM . This concentration of GTP had no effect, either in causing Ca^{2+} efflux or in stimulating IP_3 -sensitive Ca^{2+} efflux, when incubated for 10 min with microsomes under the conditions of Fig. 3(a). Carry-over of GTP is also ruled out as an explanation by the experiment shown in Fig. 3(c). After pretreatment and washing of the microsomes as above, the vesicles were re-assayed in a medium without PEG. The effect of GTP on enhancement of IP_3 -stimulated Ca^{2+} efflux clearly persisted, even into a medium where addition of GTP has no effect (dashed trace, Fig. 3c). Although the maintenance of the effect in a PEG-free medium is not as great as in media with PEG, it is still considerable. GTP-pretreated microsomes were found, under the conditions of Fig. 3(c), to release 2.0 ± 0.3 nmol of Ca^{2+} /mg of protein ($n = 3$) on addition of IP_3 .

The above experiments show that, once GTP has reacted with the microsomes, the effect on IP_3 -stimulated Ca^{2+} release persists after GTP removal and even into conditions (plus GTP[S] or minus PEG) where the original effects of GTP addition cannot be observed. One possibility is persistence of the phosphorylated form of the 38 kDa protein, which we found was phosphorylated with the γ -phosphate of GTP (Dawson *et al.*, 1986). This is, however, unlikely, for two reasons. Firstly, we have found that the microsomal pellet is dephosphorylated very rapidly during centrifugation and, secondly, addition of a 10-fold excess of unlabelled GTP chases the label out of the protein in < 30 s (A. P. Dawson, unpublished work). That vesicle fusion is a more plausible explanation is shown by electron micrographs of microsomal vesicles before and after GTP treatment (Fig. 4). The presence of PEG causes the vesicles to

Fig. 4. Electron micrographs of microsomal vesicles

Microsomes were incubated as described in Fig. 1(b). For each condition, two typical fields are shown. Samples of the incubation mixture were taken and fixed in glutaraldehyde as described in the text. (a) Left and right, 1 min before GTP addition; (b) left and right, 1 min after the addition of 20 μ M-GTP; (c) left and right, 3 min after GTP addition. The 2 μ m dimension bar (c, right) applies to all the photographs.

aggregate in large clusters. Fig. 4(a) shows typical examples of these. They appear to be a mixture of small vesicles, ranging in size from 0.1 to 0.5 μ m diameter, apparently derived from both rough and smooth endoplasmic reticulum. A few mitochondria are also present. The white areas are probably glycogen granules. At 1 min after the addition of 20 μ M-GTP (Fig. 4b), the appearance of the clusters, in thin section, has changed considerably. There are now many large vesicles present, mainly, apparently, at the periphery of the vesicle clusters. These vesicles appear to be free of ribosomes, and some of them show convoluted or multilayered character. After a further 2 min (Fig. 4c), the vesicles are very large (up to 4 μ m in diameter), and some of them show complex multilayering. In samples where 90 μ M-GTP[S] was present, before GTP addition, no large vesicles were found, and the overall appearance closely resembled that of the membranes before GTP treatment in Fig. 4(a) (results not shown). When PEG was omitted from the assay medium, neither the aggregates of vesicles nor the large vesicles formed after GTP addition could be observed.

Fig. 5. Light-scattering changes caused by GTP

Microsomes (1.2 mg of protein/ml) were incubated, under the experimental conditions of Fig. 1(b), in the light-scattering apparatus for 10 min before the addition of GTP. (a) 20 μ M-GTP was added at the arrow marked 'GTP' and 2 μ M-IP₃ at 'IP₃'; (b) 90 μ M-GTP[S] was added at 'GTP[S]' and 20 μ M-GTP at 'GTP'.

Fusion of vesicles would be expected to produce changes in the light-scattering properties of the microsomal suspension. The experimental situation is complicated, since the aggregates of vesicles formed in the presence of PEG are highly refractile, and changes within the aggregates are largely masked. However, GTP does, under the standard experimental conditions, cause a significant change in light-scattering (Fig. 5). This change is blocked by prior addition of GTP[S]. Furthermore, the light-scattering change occurs over a very similar time scale to that of GTP-induced Ca²⁺ release. However, the light-scattering change cannot be attributed to Ca²⁺ movements themselves, since the addition of IP₃ after GTP does not cause any change in light-scattering.

DISCUSSION

The results described above show that the effects of GTP on microsomal Ca²⁺ movements are irreversible, in that they persist after removal of GTP or blockage of further action by GTP by GTP[S]. Further, during incubation of microsomes with GTP, very large vesicles are produced, in agreement with the observations by Paiement (1984). Since it was predicted from our original hypothesis (Dawson & Irvine, 1984; Dawson, 1985) that vesicle fusion would lead to enhanced IP₃-sensitivity, it is clearly very tempting to assume a causal relationship between vesicle fusion and the irreversible effects of GTP on IP₃-promoted Ca²⁺ release. Such a relationship is consistent with the experimental data and, in particular, with the observation that both vesicle fusion and the effect of GTP on Ca²⁺ release can be blocked by the omission of PEG or the addition of GTP[S]. However, we have no evidence as yet that IP₃-promoted Ca²⁺ release is taking place specifically from the large vesicles, nor have we any detailed data on the relative time courses of appearance of the large vesicles and of IP₃-sensitivity.

Although fusion of microsomal vesicles in the presence of GTP has been reported previously (Paiement, 1984), the observations reported above indicate that, under our conditions, fusion can occur with remarkable speed (within 4 min) and at low GTP concentrations. It seems likely that the rapidity of the process is due to the presence of PEG, since this causes the vesicles to

aggregate and therefore to achieve very high local concentrations with membranes in close apposition. In terms of the physiological relevance of the phenomenon, Henne & Söling (1986) found that, for GTP-dependent Ca^{2+} efflux from parotid microsomes, high concentrations of bovine serum albumin could substitute for PEG, which more accurately mimics the situation *in vivo*. In agreement with Ueda *et al.* (1986), we can find no evidence for the involvement of cytoskeletal elements as yet, since enhancement of IP_3 sensitivity is, in our system, not inhibited by cytochalasin B or nocodazole (A. P. Dawson, unpublished work). The molecular mechanism involved in vesicle fusion is difficult to visualize, although it appears that GTP hydrolysis is involved, since the formation of large vesicles is blocked by GTP[S]. Also, we cannot at this stage rule out a role for the proteins phosphorylated by GTP in the microsomal fraction (Dawson *et al.*, 1986). The Ca^{2+} efflux which occurs after the addition of GTP is likely to reflect some aspect of the fusion event, but whether it is due to an increase in membrane permeability or to transient lysis of the vesicles is not clear.

As discussed by Paiement (1984) and by Warren (1985), disassembly and re-fusion of endoplasmic reticulum seems to be an essential part of the cell cycle. It seems likely that the observations described in the present paper may provide an experimental basis for investigation of one aspect of this process.

We thank Dr. R. F. Irvine for kindly supplying IP_3 , the Wellcome Trust for financial support, and D. Fulton for technical assistance.

REFERENCES

- Authi, K. S. & Crawford, N. (1985) *Biochem. J.* **230**, 247–253
 Biden, T. J., Prentki, M., Irvine, R. F., Berridge, M. J. & Wollheim, C. B. (1984) *Biochem. J.* **223**, 467–473
 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
 Dawson, A. P., Comerford, J. G. & Fulton, D. V. (1986) *Biochem. J.* **234**, 311–315
 Gill, D. L., Ueda, T., Chueh, S.-H. & Noel, M. W. (1986) *Nature (London)* **32**, 461–464
 Henne, V. & Söling, H.-D. (1986) *FEBS Lett.* **202**, 267–273
 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
 Lowry, O. H., Rosebrough, N. J., Farr, A. L. & Randall, R. J. (1951) *J. Biol. Chem.* **193**, 265–275
 Muallem, S., Schoeffield, M., Pandol, S. & Sachs, G. (1985) *Proc. Natl. Acad. Sci. U.S.A.* **82**, 4433–4437
 O'Rourke, F. A., Halenda, S. P., Zavoico, G. B. & Feinstein, M. B. (1985) *J. Biol. Chem.* **260**, 956–962
 Paiement, J. (1984) *Exp. Cell Res.* **151**, 354–366
 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
 Prentki, M., Corkey, B. E. & Matschinsky, F. M. (1985) *J. Biol. Chem.* **260**, 9185–9190
 Selwyn, M. J., Dawson, A. P., Stockdale, M. & Gains, N. (1970) *Eur. J. Biochem.* **14**, 120–126
 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. & Schultz, I. (1984) *J. Membr. Biol.* **81**, 241–253
 Ueda, T., Chueh, S.-H., Noel, M. W. & Gill, D. L. (1986) *J. Biol. Chem.* **261**, 3184–3192
 Warren, G. (1985) *Trends Biochem. Sci.* **10**, 439–443

Received 2 December 1986/19 January 1987; accepted 30 January 1987