J. exp. Biol. 139, 105–133 (1988)
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INTRACELLULAR CALCIUM TRANSLOCATION: MECHANISM OF ACTIVATION BY GUANINE NUCLEOTIDES AND INOSITOL PHOSPHATES

BY DONALD L. GILL, JULIENNE M. MULLANEY AND TARUN K. GHOSH

Department of Biological Chemistry, University of Maryland School of Medicine, Baltimore, MD 21201, USA

Summary

The movements of Ca^{2+} within cells in response to external stimuli are complex. Internal Ca^{2+} release activated by inositol 1,4,5-trisphosphate ($InsP_3$) is now widely established. However, the mechanism of $InsP_3$-induced Ca^{2+} release, the identity and control of the $InsP_3$-sensitive Ca^{2+} pool and its relationship to other internal and external Ca^{2+} pools all remain uncertain. We have characterized a highly sensitive and specific guanine nucleotide-regulatory mechanism that induces rapid and profound movements of intracellular Ca^{2+} *via* a mechanism distinct from that activated by $InsP_3$. Using permeabilized neural or smooth muscle cells, application of submicromolar concentrations of GTP induces rapid release of Ca^{2+} from a compartment that contains within it the $InsP_3$-releasable Ca^{2+} pool. Although of similar GTP-sensitivity as G-protein-activated events, the apparent dependence on GTP hydrolysis and blockade by GTPγS suggest a mechanism distinct from those mediated by known G-proteins. Recent experiments in the presence of oxalate reveal rapid and profound GTP-activated *uptake* of Ca^{2+} *via* a mechanism with identical nucleotide sensitivity and specificity to GTP-induced Ca^{2+} release. These results were interpreted to suggest that GTP induces a transmembrane conveyance of Ca^{2+} between different compartments distinguished by oxalate permeability; GTP-induced release probably occurs *via* a similar mechanism except involving transfer between closed compartments and nonclosed membranes (perhaps the plasma membrane). Recently, it has been revealed that GTP activates a translocation of Ca^{2+} into the Ca^{2+} pool from which $InsP_3$ induces release. This is an important observation suggesting that the GTP-activated Ca^{2+} translocation process may control entry into and hence the size of the $InsP_3$-releasable Ca^{2+} pool. Indeed, it is possible that GTP-induced Ca^{2+} release observed in permeabilized cells reflects a reversal of the pathway that functions in intact cells to permit external Ca^{2+} entry into the $InsP_3$-releasable pool. This type of process could mediate the longer-term secretory or excitatory responses to external receptors which are known to be dependent on external Ca^{2+}.

Calcium signalling events in cells

It is now well recognized that Ca^{2+} plays a pivotal regulatory role within cells,

Key words: calcium, inositol phosphates, GTP, endoplasmic reticulum.

both as an intracellular mediator of receptor-activated signalling, and in the control of a multitude of cellular processes notable among which is the secretory event. The recent elucidation of the mechanisms coupling cell-surface receptors to Ca^{2+} mobilization in cells, based on the early observations of Hokin & Hokin (1953), has now established in principle the relationship between receptor-induced phosphoinositide breakdown and inositol phosphate-mediated Ca^{2+} release (Berridge & Irvine, 1984; Gill, 1985; Majerus *et al.* 1986; Berridge, 1987). In spite of the fact that much is now known about the phosphoinositide signalling pathway, it should be noted that the regulation of Ca^{2+} within cells involves a complex set of events. Thus, Ca^{2+} signalling occurs through the subtle alteration of one or more of an array of distinct transport mechanisms, located in a number of discrete organelles, and influenced by numerous intracellular regulatory systems. It is the purpose of this chapter to review some intriguing recent developments concerning the control of intracellular Ca^{2+} movements and their possible relationship to what has been ascertained on the processes that mediate Ca^{2+} signalling events within cells. In the first section, certain of the characteristics of Ca^{2+} regulatory organelles and their role in Ca^{2+} signalling are considered.

Cellular sites of calcium regulation

The transfer of Ca^{2+} across membranes within cells is controlled by a number of distinct classes of active or passive transport mechanisms (see Carafoli, 1987). The cytosol of most mammalian cells contains approximately $0{\cdot}1\,\mu mol\,l^{-1}$ free Ca^{2+} under resting conditions, compared with the low millimolar free Ca^{2+} concentration outside cells. This 10 000-fold gradient of free $[Ca^{2+}]$ across the plasma membrane is actively maintained *via* ATP-dependent Ca^{2+} pumping, and perhaps also *via* the Na^+/Ca^{2+} exchanger (Gill, 1982*a*). Ca^{2+} translocation *via* voltage-sensitive Ca^{2+} channels is a well-established route of entry of extracellular Ca^{2+} into excitable cells and perhaps many other cell types (Miller, 1987). Moreover, it is clear now that activation of such channels can be finely controlled by intracellular messenger-mediated phosphorylation events (Tsien *et al.* 1986; Miller, 1987). In addition, many have considered that Ca^{2+} entry across the plasma membrane may be *directly* mediated by activation of channels distinct from voltage-sensitive Ca^{2+} channels (Gill, 1982*a*; Tsien *et al.* 1986; Miller, 1987). The existence and characterization of such channels has not been conclusively described. However, it seems clear that at least the prolonged responses to many Ca^{2+}-coupled receptors are dependent on external Ca^{2+} and may involve entry of Ca^{2+} across the plasma membrane (Putney, 1986), as discussed later.

It has become increasingly clear that, in addition to the plasma membrane, internal organelles also play an important role in the maintenance of cytosolic $[Ca^{2+}]$. Mitochondria are known actively to accumulate Ca^{2+} (see Hansford, 1985) *via* a process dependent on the membrane potential existing across the internal membrane. However, from most observations it appears that mitochondria can only accumulate Ca^{2+} when free Ca^{2+} levels are high, that is, at or above $1–10\,\mu mol\,l^{-1}$; thus it is unlikely that they contribute directly either to the

maintenance of physiological cytosolic Ca^{2+} levels or to the induction of Ca^{2+}-signalling events within cells. In contrast, it appears certain that other Ca^{2+}-accumulating organelles within cells are active in both respects. Thus, endoplasmic reticulum (ER) in a variety of cell types has been observed to sequester large quantities of Ca^{2+} (Henkart, Reese & Brinley, 1978; McGraw, Somlyo & Blaustein, 1980; Wakasugi *et al.* 1982; Burton & Laveri, 1985). Using permeabilized nonmuscle cells, it is clear from a number of different studies that nonmitochondrial organelle(s) exist which accumulate Ca^{2+} *via* high-affinity (ATP + Mg^{2+})-dependent Ca^{2+} pumping activity (see, for example, Burgess *et al.* 1983; Gill & Chueh, 1985). Such internal Ca^{2+} pumps are analogous in function to those of the plasma membrane. However, a number of features distinguish the internal and plasma membrane pumping activities (Gill & Chueh, 1985). Interestingly, these distinguishing characteristics are remarkably consistent with those features which serve to distinguish sarcolemmal and sarcoplasmic reticulum (SR) Ca^{2+} pumps in muscle tissue (Carafoli, 1987). Thus, it has been suggested that ER in nonmuscle cells may fulfil at least some of the specialized Ca^{2+} regulatory functions ascribed to SR in muscle. However, although analogies exist with respect to Ca^{2+} accumulation, it is becoming increasingly apparent that the Ca^{2+} release mechanisms of SR and ER are quite distinct. It should also be noted that whereas the SR is a structurally identifiable organelle with a clearly defined Ca^{2+}-regulatory function, the role of ER in Ca^{2+} signalling within nonmuscle tissues is considerably more tenuous. Thus, the involvement of ER in Ca^{2+}-mobilizing events is concluded from indirect evidence with, as yet, no proven localization of these mechanisms to this specific organelle. Indeed, recent evidence presented by Volpe *et al.* (1988) suggests that Ca^{2+}-accumulating organelles which are *distinct* from ER may be involved in Ca^{2+}-regulatory responses in cells. These organelles have been termed 'calciosomes' and their existence and function are described in detail in the chapter by Pozzan in this volume (Pozzan *et al.* 1988). In spite of the imprecise identity of Ca^{2+}-releasing organelles, ER is frequently referred to as being the organelle from which Ca^{2+} release occurs in response to inositol phosphates, the actions of which are discussed next.

Role of inositol phosphates in calcium signalling

Considerable advances in the understanding of the nature of Ca^{2+}-signalling events within cells have been derived from elucidation of the pathways for metabolism and action of the inositol phosphates derived from receptor-mediated phospholipase C activation (Berridge, 1987). An overview of the role of phosphoinositide metabolism in signal transduction and the control of secretion is given in the chapter by Putney in this volume (Putney, 1988). It is currently held that an important direct product of phosphoinositide breakdown is inositol 1,4,5-trisphosphate (together with its 1,2-cyclic derivative), and that this molecule has proven effectiveness in releasing intracellular Ca^{2+} in a large variety of cells. The metabolism of this product is complex. Although it is not the purpose of the present chapter to describe the intricate processes involved in formation and

breakdown of each of the products, brief mention of the major derivatives is given here since certain of these may also have roles in modifying Ca^{2+} movements in cells. Ins(1,4,5)P_3 undergoes either phosphorylation or dephosphorylation. 5′-Phosphatase activity in cells cleaves InsP_3 to the less active Ins(1,4)P_2 product. Alternatively, 3′-kinase activity can phosphorylate InsP_3 to produce inositol 1,3,4,5-tetrakisphosphate (InsP_4), which is itself a substrate for the 5′-phosphatase, producing in this case inositol 1,3,4-trisphosphate. Whereas the latter molecule has very much less Ca^{2+}-releasing activity than Ins(1,4,5)P_3, the InsP_4 molecule has been reported to exert indirect effects on Ca^{2+} mobilization (Irvine & Moor, 1986, 1987; Morris, Gallacher, Irvine & Petersen, 1987). Thus InsP_4 was observed by Irvine & Moor (1986, 1987) to induce Ca^{2+}-mediated effects in oocytes; these effects appear to be dependent on the presence of InsP_3 and also to require external Ca^{2+}. Interpretation of the results may imply that InsP_4 induces the entry of Ca^{2+} into the InsP_3-releasable pool, perhaps from outside the cell (Michell, 1986; Irvine & Moor, 1987). In a recent report, Morris *et al.* (1987) described a similar synergism between the effects of InsP_3 and InsP_4 on activation of K^+ channels in lacrimal gland; similar conclusions on the possible permissive effect of InsP_4 on the action of InsP_3-mediated Ca^{2+} mobilization were presented. More direct synergistic effects of InsP_3 and InsP_4 on Ca^{2+} have been reported by Spät *et al.* (1987). Thus, it was observed that the extent of InsP_3-mediated Ca^{2+} release from liver microsomal membrane vesicles was significantly increased in the presence of InsP_4. At present, although it seems likely that InsP_4 does exert effects, it is unclear whether it may directly control Ca^{2+} fluxes, whether it modifies the InsP_3-induced release process, or whether it has indirect effects through alteration of the metabolism of InsP_3, for example by competing with InsP_3 at the 5′-phosphatase level.

Studies by Muallem, Schoeffield, Pandol & Sachs (1985) suggest that the action of InsP_3 on the release of Ca^{2+} from what is believed to be ER occurs *via* a process that resembles activation of a channel. This conclusion has been drawn from a number of observations including the remarkably temperature-insensitive activation of Ca^{2+} release in response to InsP_3 (Smith, Smith & Higgins, 1985; Chueh & Gill, 1986). Direct electrophysiological evidence for an InsP_3-activated channel has not yet been published; however, promising results have been discussed and more definitive studies are expected. Studies using labelled InsP_3 have identified a binding site for InsP_3 within cells, with kinetics and specificity similar to that for activation of Ca^{2+} release (Baukal *et al.* 1985; Spät *et al.* 1986; Worley *et al.* 1987). The isolation of an InsP_3-binding protein, which was purified by heparin affinity-chromatography, has recently been reported by Supattapone, Worley, Baraban & Snyder (1988). Indeed, our own recent evidence (Ghosh *et al.* 1988), which shows a profound antagonistic effect of heparin on the action of InsP_3 on Ca^{2+} release from within cells, strongly suggests that the binding protein isolated by Supattapone *et al.* is the physiological receptor for InsP_3. Thus, whereas studies on the molecular structure and mechanism of the site of action of InsP_3 are in their infancy, it is likely that much will come to light in the near future.

Calcium release activated by guanine nucleotides

There have been a number of recent observations on a guanine-nucleotide-activated process that appears directly to activate profound and rapid movements of Ca^{2+} within many different types of cells. Below is a description of the effects of GTP on Ca^{2+} movements, their relationship to the actions of InsP_3, and the possible mechanism of activation of GTP-induced Ca^{2+} translocation. In this section we will consider the characteristics of the fluxes of Ca^{2+} activated by GTP.

Identification of the GTP effect

During some of the earlier experiments on the action of InsP_3 in inducing Ca^{2+} release, permeabilized cell systems of several different types were found to be particularly useful for observing the effects of InsP_3 (Streb, Irvine, Berridge & Schulz, 1983; Burgess *et al.* 1984). In contrast, isolated microsomal membrane fractions presented some problems in permitting observations on the effects of InsP_3 (Dawson & Irvine, 1984). Such difficulties probably reflected either the lability of the InsP_3-activated release process under lengthy vesicle purification procedures, and/or a low yield of intact vesicles derived from the InsP_3-sensitive intracellular organelle. Dawson and his colleagues were approaching this problem using liver microsomes in which they had observed small effects of InsP_3 (Dawson & Irvine, 1984). In attempting to augment this response, Dawson (1985) observed that GTP enhanced the effectiveness of InsP_3, and that this effect was promoted by polyethylene glycol. Undertaking similar experiments with microsomes isolated from cultured N1E-115 neuroblastoma cells, we observed a rather different response (Ueda, Chueh, Noel & Gill, 1986). With these microsomes, addition of InsP_3 effected release of a small fraction (approximately 10 %) of releasable Ca^{2+}. When GTP and InsP_3 were added simultaneously, a much larger release of Ca^{2+} was observed. However, in contrast to the results of Dawson, it was observed that GTP alone was highly effective in releasing Ca^{2+} (Ueda *et al.* 1986). The effect of GTP was rapid and profound, more than 50 % of total accumulated Ca^{2+} being released from the microsomal membrane vesicles within a few seconds. As described below, the nucleotide specificity and sensitivity of the GTP effect were remarkable. The high GTP-sensitivity was considered possible since during their isolation the microsomes had undergone considerable washing and hence were largely devoid of endogenous nucleotides. With this in mind, it was reasoned that the permeabilized cell preparations used extensively in prior Ca^{2+} flux analyses (Gill & Chueh, 1985), having been subjected to fewer washing procedures, would be a less suitable preparation on which to observe GTP-induced Ca^{2+} fluxes. However, this prediction was incorrect, and in fact the permeabilized cell preparations became the system of choice on which most of the characteristics of GTP-activated Ca^{2+} movements were determined. Using permeabilized N1E-115 neuroblastoma cells loaded with Ca^{2+} to equilibrium, the EC_{50} for GTP was observed to be below 1 μmol l^{-1}, GTP releasing between 50 and 70 % of accumulated Ca^{2+} within 30 s (Fig. 1A). The effect GTP was observed to be

almost as rapid as that of the ionophore A23187, although the extent of release was not as complete, an observation that suggested heterogeneity of Ca^{2+}-accumulating compartments (see below).

Nucleotide-sensitivity and nucleotide-specificity of calcium release

The release of Ca^{2+} activated by guanine nucleotides observed using either permeabilized cells (Gill, Ueda, Chueh & Noel, 1986) or microsomes derived from cells (Ueda *et al.* 1986) has remarkably high sensitivity to GTP. The K_m for GTP measured in permeabilized N1E-115 cells is $0{\cdot}75\,\mu mol\,l^{-1}$. The effect also has very considerable nucleotide-specificity. Release was not observed with GMP, cyclic GMP, (either 2′,3′ or 3′,5′), or with the nonhydrolysable analogues of GTP, GTPγS or GppNHp (see Fig. 1). The latter is an important observation since it suggests a divergence in guanine nucleotide-specificity from that of the known G-proteins which are known to be much more effectively stimulated by nonhydrolysable GTP analogues. Other nucleoside triphosphates including ITP, UTP and CTP have no effect on Ca^{2+} movements, these nucleotides being largely ineffective even when added at concentrations up to $1\,mmol\,l^{-1}$ (Gill *et al.* 1986). Submicromolar GTP concentrations function to release Ca^{2+} in the presence of millimolar ATP concentrations (required to maintain constant Ca^{2+} pumping activity), indicating the exceptional specificity of the GTP-activated release process. It was observed that GDP does induce Ca^{2+} release, but only after a significant lag of about 30 s (Fig. 1A); thereafter it releases Ca^{2+} to approximately the same extent as GTP. Results clearly indicate that this effect results from conversion of GDP to GTP *via* nucleoside diphosphokinase (NDPK) activity (Ueda *et al.* 1986; Gill *et al.* 1986). Thus, the effect of GDP is blocked by ADP (Fig. 1B) which effectively competes for the nucleoside diphosphate site on NDPK (Kimura & Shimada, 1983). In fact GDP itself does not induce Ca^{2+} release; thus, GDPβS (which is not easily phosphorylated to GTPβS by NDPK) has no effect on Ca^{2+} release (Fig. 1B). Moreover, not only is GDP without Ca^{2+}-releasing effects of its own, but it actually blocks the action of GTP, as shown in Fig. 1C; (note that, at $100\,\mu mol\,l^{-1}$, GDP saturates NDPK activity and remains present for a longer period to compete with GTP). Further experimentation (in the presence of high [ADP] to prevent conversion of GDP to GTP) revealed that the inhibitory effect of GDP was competitive with respect to GTP with a K_i of approximately $3\,\mu mol\,l^{-1}$ (Gill *et al.* 1986); GTPγS also blocks the effect of GTP, but rather surprisingly, GppNHp does not (Fig. 1C). This differential inhibitory action of the nonhydrolysable analogues has been a useful criterion for defining the specificity of the GTP-activated process and is referred to again later. The lack of direct action of GTPγS and its inhibitory effect on the action of GTP are evidence that GTP hydrolysis is required for the activation of Ca^{2+} release. In fact, a very slow release activated by GTPγS (Chueh & Gill, 1986) may be consistent with slow cleavage of the phosphorothioate residue which is known to occur (Eckstein, 1985). Further evidence for a GTP hydrolytic process being involved in activation of Ca^{2+} release derives from the competitive effect of GDP which indicates that either GTP or

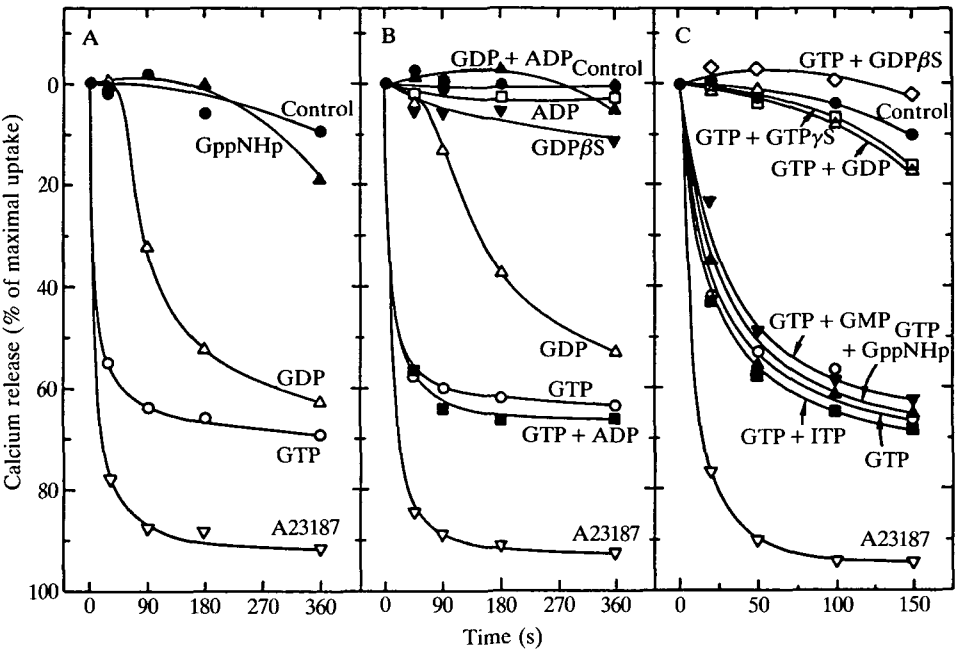

Fig. 1. Influence of different guanine nucleotides on the release of Ca^{2+} from permeabilized N1E-115 cells. Cells were loaded for 4 min with labelled Ca^{2+} under 'cytosolic-like' conditions (140 mmol l^{-1} KCl, 10 mmol l^{-1} NaCl, 2·5 mmol l^{-1} $MgCl_2$, 0·1 μmol l^{-1} free Ca^{2+}, 1 mmol l^{-1} ATP, Hepes-KOH, pH 7·0) at which time the following additions were made: (A) control buffer (●), 10 μmol l^{-1} GTP (○), 20 μmol l^{-1} GppNHp (▲), 20 μmol l^{-1} GDP (△), or 5 μmol l^{-1} A23187 (▽); (B) control buffer (●), 10 μmol l^{-1} GTP (○), 10 μmol l^{-1} GDP (△), 1 mmol l^{-1} ADP (□), 10 μmol l^{-1} GTP with 1 mmol l^{-1} ADP (■), 10 μmol l^{-1} GDP with 1 mmol l^{-1} ADP (▲), 10 μmol l^{-1} GDPβS (▼) or 5 μmol l^{-1} A23187 (▽); (C) control buffer (●), 3 μmol l^{-1} GTP (○), 3 μmol l^{-1} GTP with 100 μmol l^{-1} GppNHp (▲), 3 μmol l^{-1} GTP with 100 μmol l^{-1} GDP (△), 3 μmol l^{-1} GTP with 100 μmol l^{-1} GMP (▼), 3 μmol l^{-1} GTP with 100 μmol l^{-1} ITP (■), 3 μmol l^{-1} GTP with 100 μmol l^{-1} GTPγS (□), 3 μmol l^{-1} GTP with 100 μmol l^{-1} GDPβS (◇), 5 μmol l^{-1} A23187 (▽). The addition of each of these agents or combinations of agents as shown were all made at zero-time. Release was terminated at the times shown by La^{3+}-quenching and rapid filtration to determine the amount of Ca^{2+} remaining in the permeabilized cells. See Gill, Ueda, Chueh & Noel (1986) and Gill & Chueh (1985) for details of the experimental conditions.

GDP can bind to the same site; presumably, the inhibitory effect of GDP arises through prevention of GDP dissociation after hydrolysis of GTP at the Ca^{2+} release-activating site.

Specificity among cells and organelles

Since in our early studies the observed effect of GTP on release of Ca^{2+} from the N1E-115 neuroblastoma cells was so profound, it was important to establish whether this effect was perhaps an anomaly restricted to this particular cell line

used. Using a quite unrelated cell type, the DDT_1MF-2 smooth muscle cell line derived from hamster vas deferens (Norris, Gorski & Kohler, 1974), experiments suggested this was not the case. Thus, a sensitive, specific and substantial GTP-dependent release of Ca^{2+} was observed using permeabilized DDT_1MF-2 cells loaded with Ca^{2+}, with pronounced effectiveness of as low as $0{\cdot}1\ \mu mol\ l^{-1}$ GTP in the presence of $1\ mmol\ l^{-1}$ ATP (Chueh *et al.* 1987). In addition to the DDT_1MF-2 cell line, we have measured almost identical effects of GTP on Ca^{2+} release using permeabilized cells from the rat BC_3H-1 smooth muscle cell line and from the human WI-38 normal embryonic lung fibroblast cell line. Using microsomal membrane vesicle fractions prepared from DDT_1MF-2 cells by methods similar to those described for N1E-115 cell-derived microsomes (Ueda *et al.* 1986), we have observed GTP effects on Ca^{2+} release almost identical to those seen with permeabilized cells. Furthermore, using microsomes derived from guinea pig parotid gland, Henne & Söling (1986) have observed very similar effects on release of accumulated Ca^{2+} induced by GTP. The observations of Jean & Klee (1986) on GTP- and $InsP_3$-mediated Ca^{2+} release from microsomes derived from NG108-15 neuroblastoma X glioma hybrid cells are also consistent with our findings.

The GTP-induced Ca^{2+} release process is specific to a nonmitochondrial Ca^{2+}-sequestering organelle, which may be ER or a subfraction thereof (we frequently refer to it as being ER simply for convenience). Importantly, rather clear experiments demonstrate that no effects of guanine nucleotides or $InsP_3$ can be observed on Ca^{2+} fluxes across mitochondrial or plasma membranes (Ueda *et al.* 1986; Chueh *et al.* 1987). The observation that less than 100 % of Ca^{2+} release from ER is effected by GTP or $InsP_3$ suggests that only a subcompartment of ER contains the activatable efflux mechanisms. Although we have no direct proof that ER is a source of GTP-releasable Ca^{2+}, interpretation of the effects of oxalate (described later), a known permeator of the ER membrane (Gill & Chueh, 1985), may indicate that ER is indeed a site of action of both GTP and $InsP_3$ (Chueh *et al.* 1987; Mullaney, Chueh, Ghosh & Gill, 1987; Mullaney, Yu, Ghosh & Gill, 1988). Moreover, we now know that GTP indeed modifies the movements of Ca^{2+} associated with the $InsP_3$-releasable Ca^{2+} pool and hence that GTP and $InsP_3$ can act on the same Ca^{2+} pool, as described below.

GTP reversibly activates calcium release

One of the most important areas of investigation concerns determination of the nature of the Ca^{2+} translocation process activated by GTP. With regard to this mechanism, either of two distinct possibilities appeared likely: first, GTP could activate a channel process to permit the flow of Ca^{2+} out of the organelle(s) into which Ca^{2+} is sequestered; second, GTP could activate a fusion between organelle membranes resulting in the release or transfer of Ca^{2+}. In the latter case, it would be very unlikely that such a process would be reversible, that is, that the two fused membranes could be returned to the unfused state with the same original enclosed volume. Recently, we reported that GDP at least partially reverses the pr[illegible] effectiveness of GTP suggesting some degree of reversibility of the action of GTP

(Gill *et al.* 1986). Since then, a more definitive indication of the reversibility of the effect of GTP has come from a simpler study involving washing of cells after GTP-activation (Chueh *et al.* 1987). Thus, it has been observed that after activation of the GTP-dependent Ca^{2+} release process (with up to $100\,\mu mol\,l^{-1}$ GTP), the effectiveness of GTP can be substantially (more than 70%) reversed by simple washing of the GTP-treated permeabilized cells with GTP-free medium. In such experiments, cells that had been treated with GTP under conditions that activate Ca^{2+} release were thoroughly washed; after this treatment Ca^{2+} uptake proceeded to an extent approaching that of untreated cells, that is, the ability of ER to accumulate Ca^{2+} was largely restored. Moreover, such GTP-pretreated, washed cells responded again to a further application of GTP, indicating that the release process can be reactivated by GTP. It would be difficult to reconcile this reversibility with a membrane fusion process activated by GTP; in other words, the effects of a direct membrane fusion event would be unlikely to be reversed by washing and result in the restoration of almost normal Ca^{2+} retention, as observed. It should be noted, however, that structural and biophysical measurements undertaken by Dawson and coworkers suggest that fusion of membranes *can* follow GTP treatment of microsomal vesicles (Dawson, Hills & Comerford, 1987; Comerford & Dawson, 1988). At present this question is unresolved.

Close membrane association promotes the action of GTP

Electron microscopic analysis of membrane vesicles treated with GTP has suggested that the action of GTP, although not necessarily involving membrane fusion, may be promoted by close association between membranes. It is now well established that the effects of GTP on Ca^{2+} release are promoted by 1–3% polyethylene glycol (PEG) (Chueh & Gill, 1986; Ueda *et al.* 1986; Gill *et al.* 1986). Thus, although in the absence of PEG, GTP induces a significant release of Ca^{2+}, this effect is substantially increased in the presence of PEG. The effect of PEG is to increase both the sensitivity to GTP and the maximal release induced by it. Although PEG is a known fusogen when present above 25% w/v (Hui, Isac, Boni & Sen, 1985), we believe that the effect of PEG in enhancing Ca^{2+} release is unlikely to involve membrane fusion. Thus, our recent studies have analysed by electron microscopy the appearance of isolated microsomal membrane vesicles derived from N1E-115 cells after GTP-treatment with or without PEG (Chueh *et al.* 1987). We observed that GTP was without any effect on vesicle appearance, whereas 3% PEG induced a very clear coalescence of vesicles into tightly associated conglomerates with very few free or unattached vesicles. The effect of PEG was not visibly altered by GTP. It may therefore be concluded that GTP itself does not induce any observable alteration in vesicle structure or association. However, the striking effectiveness of PEG is good evidence to suggest that the effect of GTP in inducing Ca^{2+} movements is promoted by a condition that clearly increases close associations between membranes. This may be an important clue to the action of GTP, as discussed in detail below. Thus, we consider that close association between membranes might be sufficient to permit the GTP-induced

event which could involve formation of some type of junctional process between membranes, perhaps permitting the flow of Ca^{2+}; thereafter, it is possible that under certain conditions membrane fusion may occur.

Relationship between InsP_3- and GTP-activated calcium movements

InsP_3 *and GTP function via distinct mechanisms*

A further major problem to be addressed is the relationship between the actions of InsP_3 and GTP, and whether the processes activated by each agent involve any common mechanism. As described in a recent report, a number of clear distinctions exist between the actions of InsP_3 and GTP on Ca^{2+} release (Chueh & Gill, 1986). First, InsP_3-mediated release is unaffected by either GDP or GTPγS, both of which block the action of GTP on Ca^{2+} release, as described above. Second, PEG, which considerably promotes GTP-activated release (as described above), does not alter the action of InsP_3; indeed, the lack of effect of PEG on InsP_3-induced Ca^{2+} release suggests that InsP_3 functions *via* a mechanism that does not require close membrane interactions. A third distinction between the actions of InsP_3 and GTP is the temperature-dependency of their effects. Thus, the effect of InsP_3 is remarkably insensitive to temperature changes, the rate of InsP_3-induced Ca^{2+} release being reduced by only 20 % when the temperature is decreased from 37 °C to 4 °C; this contrasts with the complete abolition of the effectiveness of GTP at the lower temperature (Chueh & Gill, 1986). The latter result is consistent with GTP activating release *via* a process involving an enzymic step, perhaps an enzymic hydrolysis of GTP, whereas the action of InsP_3 is unlikely to involve an enzymic step. (As discussed above, this temperature-independence of the action of InsP_3 is highly suggestive of a process involving direct activation of a channel.) A fourth major distinction between the actions of InsP_3 and GTP concerns their Ca^{2+}-dependency. Thus, InsP_3-induced Ca^{2+} release, in contrast to that induced by GTP, is modified by the free Ca^{2+} concentration. Ca^{2+} uptake and release were normally measured at a free Ca^{2+} concentration of 0·1 μmol l^{-1}. When the free Ca^{2+} concentration is increased to 1 μmol l^{-1}, the effect of InsP_3 is reduced by 50 %; at 10 μmol l^{-1} free Ca^{2+} the action of InsP_3 is completely abolished. In contrast, GTP induces identical fractional Ca^{2+} release over this entire range of free Ca^{2+} concentration.

The inhibition of InsP_3-mediated Ca^{2+} release with levels of Ca^{2+} above the physiological resting concentration (1 μmol l^{-1}) is a significant observation indicating that the InsP_3 release process is under negative feedback control from the level of Ca^{2+}, a potentially important regulatory response (Chueh & Gill, 1986). Interestingly, recent work from Worley *et al.* (1987) indicates that binding of labelled InsP_3 to its putative membrane receptor has almost identical Ca^{2+}-sensitivity, suggesting that the feedback effect may exist at the InsP_3 binding step. This also provides evidence that the InsP_3 binding site identified by Worley *et al.* (1987) is the site of action of InsP_3. Much more compelling evidence to link t[illegible] binding and action of InsP_3 has recently arisen from analysis of the effects of the

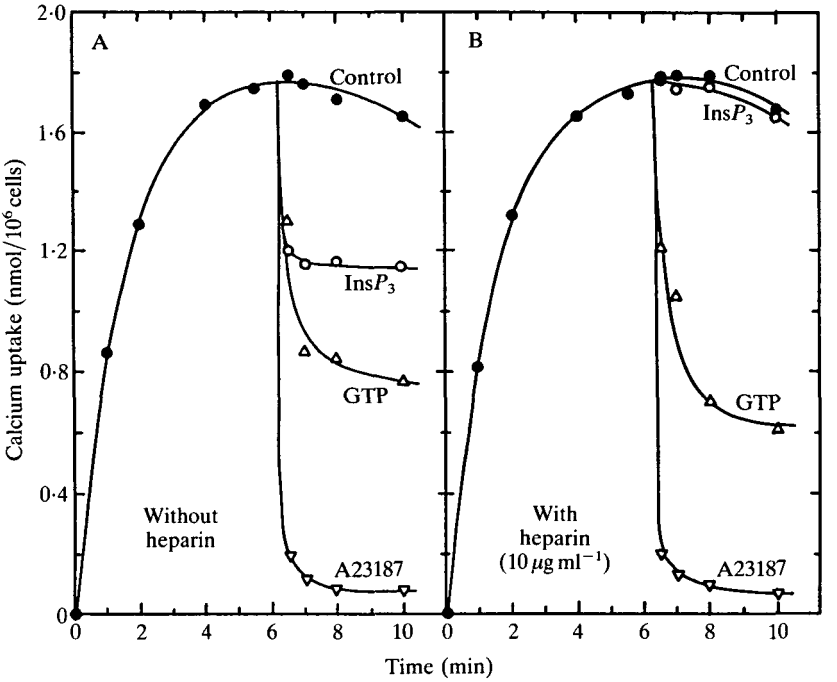

Fig. 2. Specificity of the blockade by heparin of InsP_3-activated Ca^{2+} release from permeabilized DDT_1MF-2 smooth muscle cells. Permeabilized cells were incubated under standard conditions of ATP-dependent $^{45}Ca^{2+}$ accumulation either in the presence (A) or absence (B) of heparin (4–6 kDa). After exactly 6 min of uptake, the following additions were: 10 μmol l^{-1} InsP_3 (○), 10 μmol l^{-1} GTP (△), 5 μmol l^{-1} A23187 (▽), or control buffer (●). At the indicated times, samples (100 μl) were withdrawn from vials and $^{45}Ca^{2+}$ remaining within cells was determined after rapid La^{3+}-quenching and filtration as described in Fig. 1 and by Ghosh, Mullaney & Gill (1988).

glycosaminoglycan, heparin, which has been shown not only potently to inhibit InsP_3 binding (Worley *et al.* 1987) but also to bind to and provide a high degree of purification of a specific InsP_3-binding protein, as recently described by Supattapone, Worley, Baraban & Snyder (1988). In very recent experiments we have observed that heparin is a powerful antagonist of the action of InsP_3 in inducing Ca^{2+} release from either permeabilized cells or isolated membrane vesicles (Ghosh *et al.* 1988). Thus, heparin blocks InsP_3-induced Ca^{2+} release with a K_i of 3 nmol l^{-1}, suggesting a much higher affinity for the site than any known inositol phosphate. Moreover, heparin was shown to inhibit competitively the action of InsP_3, and also to reverse the InsP_3-activated Ca^{2+} release and permit immediate re-uptake of Ca^{2+}. As shown in Fig. 2, the effect of heparin was highly specific towards the action of InsP_3. Thus, heparin altered neither Ca^{2+} pumping activity nor the equilibrium uptake level (hence heparin did not alter any passive Ca^{2+} fluxes that contribute to the attainment of equilibrium). There was also no effect of

heparin on the releasability of Ca^{2+} in response to the ionophore A23187, indicating that heparin did not change the state of accumulated Ca^{2+}. Importantly, GTP-activated Ca^{2+} release was not affected by heparin. In other experiments, even heparin concentrations as high as $100\,\mu g\,ml^{-1}$ were without effect on the action of GTP. This is yet further convincing evidence for the distinction between the mechanisms of Ca^{2+} release activated by InsP_3 and GTP. The reversible and competitive effect of heparin on the action of InsP_3 indicates that when heparin displaces the InsP_3 molecule from its site of action the release process is immediately terminated, suggesting that activation of the putative InsP_3-responsive Ca^{2+} channel is intimately related to occupation of the InsP_3-binding site. This conclusion supports the prior available evidence mentioned above suggesting direct channel activation by InsP_3, in contrast to the action of GTP which involves a quite distinct process.

Several of the distinctions between the actions of GTP and InsP_3 (other than the effect of heparin) have also been reported by Henne & Söling (1986) using either liver- or parotid-derived microsomes, and by Jean & Klee (1986) using microsomes derived from NG108-15 neuroblastoma X glioma hybrid cells. It is concluded that the rapidity, relative temperature insensitivity and reversibility of InsP_3-induced Ca^{2+} release are all consistent with its probable direct activation of a Ca^{2+} channel, a conclusion in agreement with the observations of others (Muallem *et al.* 1985; Smith *et al.* 1985). In contrast, GTP appears to effect release by a temperature-sensitive process which probably involves the enzymic hydrolysis of the terminal phosphate from GTP.

Compartments of calcium responsive to InsP_3 and GTP

Both the InsP_3- and GTP-induced Ca^{2+} release processes function on a similar intracellular Ca^{2+}-sequestering compartment. Yet, the size of the releasable pools of Ca^{2+} are distinct. In the N1E-115 cell line, for example, the pool of Ca^{2+} released by GTP is approximately twice the size of the InsP_3-releasable pool, as shown in Fig. 3. Thus, using permeabilized N1E-115 cells, following maximal Ca^{2+} release by GTP, InsP_3 is ineffective in releasing further Ca^{2+} (Fig. 3B); however, following maximal release by InsP_3 (approximately 30 % of accumulated Ca^{2+}), GTP does effect a further release of Ca^{2+} (Fig. 3A), in fact, down to the level GTP could induce when added alone (that is, approximately 60 % of accumulated Ca^{2+}). These results suggest that three compartments exist; one sensitive to both GTP and InsP_3, another releasing Ca^{2+} only in response to GTP, and a third not releasing Ca^{2+} in response to either agent. Thus, it is apparent that although the GTP-releasable pool differs from the InsP_3-releasable pool in being larger, at least a significant proportion of accumulated Ca^{2+} lies within a pool which can be released by either of the two agents. In other words, it appears that all the Ca^{2+} within the InsP_3-sensitive Ca^{2+} pool is also releasable by the GTP-activated process, even if additional GTP-releasable Ca^{2+} also exists. This implies a probable proximal relationship between the InsP_3- and GTP-activated Ca^{2+}

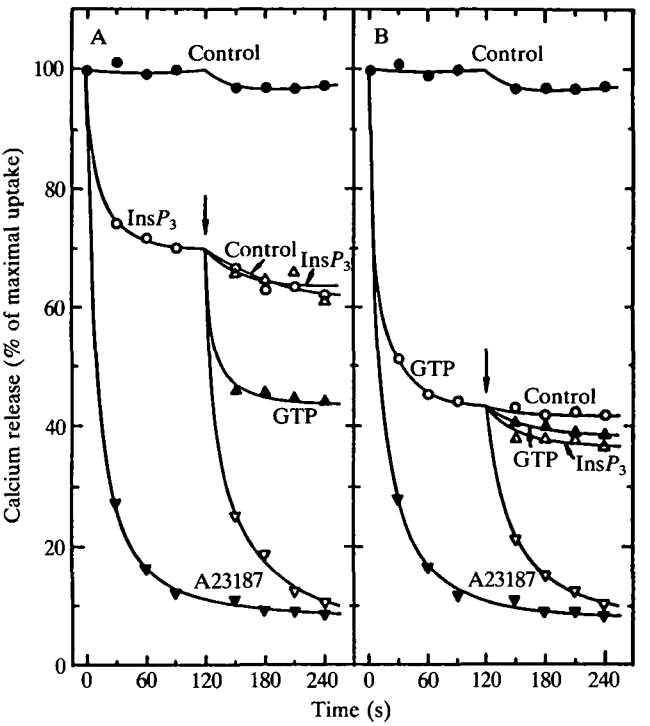

Fig. 3. Effects of sequential addition of $InsP_3$ and GTP on Ca^{2+} release from permeabilized N1E-115 neuroblastoma cells. Ca^{2+} release was measured after loading for 5 min in the presence of 0·1 μmol l^{-1} free Ca^{2+}, under the standard conditions (see Fig. 1). (A) Immediately following uptake, release was observed after addition of either 10 μmol l^{-1} $InsP_3$ (○), 5 μmol l^{-1} A23187 (▼) or control buffer (●); after 120 s of release in the presence of $InsP_3$, measurement of release was continued after further additions of either 10 μmol l^{-1} $InsP_3$ (△), 10 μmol l^{-1} GTP (▲), 5 μmol l^{-1} A23187 (▽) or control buffer (○). (B) Immediately following uptake, release was observed after addition of either 10 μmol l^{-1} GTP (○), 5 μmol l^{-1} A23187 (▼) or control buffer (●); after 120 s of release in the presence of GTP, release was continued after further addition of either 10 μmol l^{-1} $InsP_3$ (△), 10 μmol l^{-1} GTP (▲), 5 μmol l^{-1} A23187 (▽) or control buffer (○). In each case, samples of the Ca^{2+}-loaded permeabilized cell suspension were removed followed by rapid filtration and washing as described by Chueh *et al.* (1987).

release processes, and permits us to consider the existence of possible coupling events linking their modes of action.

GTP-activated calcium translocation: possible mechanism of action of GTP

Several parameters of the GTP-activated process have together suggested to us a model which may explain the translocation of Ca^{2+} which is observed. Before considering this model, another important result must be considered. We recently observed that GTP can induce an entirely opposite effect on Ca^{2+} movements in

the presence of oxalate; that is, GTP induces *uptake* as opposed to *release* of Ca^{2+} when oxalate is present. Although this observation at first appeared anomalous, it has provided an important piece of evidence in formulating our model for the action of GTP.

Calcium uptake activated by GTP

Effects of GTP in the presence of oxalate

From the evidence described above, it was suggested that a simple GTP-mediated membrane fusion event was not entirely consistent with the observed release of Ca^{2+} induced by GTP. However, to investigate this problem experiments were designed to determine whether Ca^{2+} precipitated with oxalate could be released from within permeabilized cells upon application of GTP. As shown in our previous studies (Gill & Chueh, 1985) and established in many different cell types (Henkart *et al.* 1978; McGraw *et al.* 1980; Wakasugi *et al.* 1982; Burton & Laveri, 1985), the ER is permeable to anions including oxalate and phosphate which can diffuse into the ER lumen and hence promote a large increment in Ca^{2+} uptake due to formation of insoluble Ca^{2+}/oxalate or phosphate complexes. To investigate further how GTP activates Ca^{2+} release, we tested to see if oxalate-precipitated Ca^{2+} within ER could be released by GTP; a negative result would again militate against a simple membrane fusion event accounting for release and would instead argue in favour of a more selective channel mechanism, through which precipitated Ca^{2+} would not be expected to pass. However, as shown in Fig. 4, a marked *increase* in Ca^{2+} uptake was observed in the presence of oxalate, a remarkable and entirely opposite effect to that observed in the absence of oxalate. The effect is observed with concentrations of oxalate ($2\,mmol\,l^{-1}$) that have very little effect on uptake of Ca^{2+} in the absence of GTP (Fig. 4C). When oxalate is present at a concentration inducing linear uptake of Ca^{2+} (Fig. 4E), GTP still activates an additional increase in the rate of uptake. This phenomenon is not restricted to particular cell types, thus an identical effect of GTP on Ca^{2+} uptake in the presence of oxalate was observed using either N1E-115 neuroblastoma or DDT_1MF-2 smooth muscle cells.

GTP activates calcium uptake and release by the same mechanism

Considering the paradoxically opposite effects of GTP in the presence and absence of oxalate, it was important to establish whether the two actions of GTP are mediated *via* the same or different mechanisms. It is now clear from a large number of observations that a single GTP-activated mechanism mediates both effects (Mullaney *et al.* 1987). For example, the GTP-dependence of Ca^{2+} uptake induced in the presence of oxalate is almost identical to that of the release induced without oxalate. Thus, the K_m for GTP for Ca^{2+} uptake (with oxalate) is $0{\cdot}9\,\mu mol\,l^{-1}$, which is very close to the value of $0{\cdot}75\,\mu mol\,l^{-1}$ derived from Ca^{2+} release data, as described above. Further studies have revealed that the uptake of

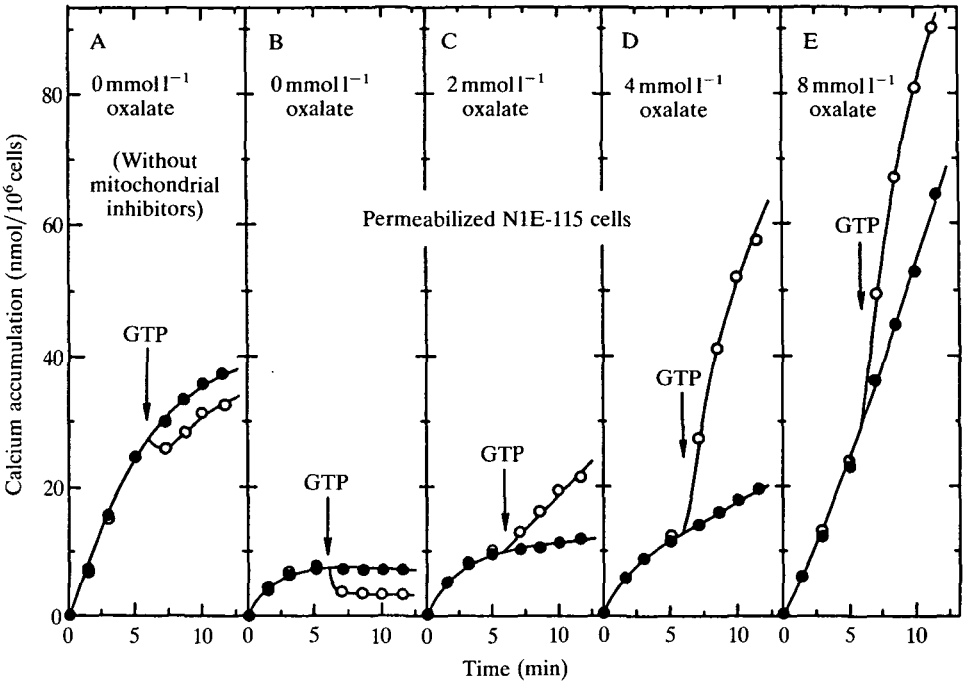

Fig. 4. GTP-activated movements of Ca^{2+} in permeabilized N1E-115 cells in the presence of increasing concentrations of oxalate. Experimental conditions were as described by Chueh *et al.* (1987) and Mullaney, Chueh, Ghosh & Gill (1987). These were essentially the same as in Fig. 1 with the exceptions that EGTA was absent and total $CaCl_2$ was 30 μmol l^{-1}. Experiments were undertaken either in the absence of mitochondrial inhibitors (A) or in the presence of 5 μmol l^{-1} ruthenium red and 10 μmol l^{-1} oligomycin (B–E). Potassium oxalate was either absent (A and B) or present from the beginning of uptake at a final concentration of 2 (C), 4 (D) or 8 mmol l^{-1} (E). After 6 min of uptake, 10 μmol l^{-1} GTP (○) or control buffer (●) was added to the permeabilized cell suspensions. Samples from the incubation vials were taken at the times shown, and Ca^{2+} remaining within cells was determined by rapid La^{3+}-quenching and filtration as described by Chueh *et al.* (1987).

Ca^{2+} induced by GTP in the presence of oxalate is promoted by PEG in a manner very similar to GTP-activated Ca^{2+} release without oxalate (Mullaney *et al.* 1987). Thus, although both GTP-activated release and uptake are observable in the absence of PEG, both effects are considerably augmented in the presence of 3 % PEG. In addition to these similarities between Ca^{2+} uptake and release induced by GTP, the nucleotide specificity profiles of the two processes closely coincide. A particularly important observation in this regard is that both effects of GTP show the same differential specificity towards the actions of nonhydrolysable GTP analogues, as shown in Fig. 5. Thus, GTP-activated Ca^{2+} uptake in the presence of oxalate is activated by neither GTPγS (Fig. 5A) nor GppNHp (Fig. 5B). However, whereas 100 μmol l^{-1} GTPγS almost completely blocks the action of

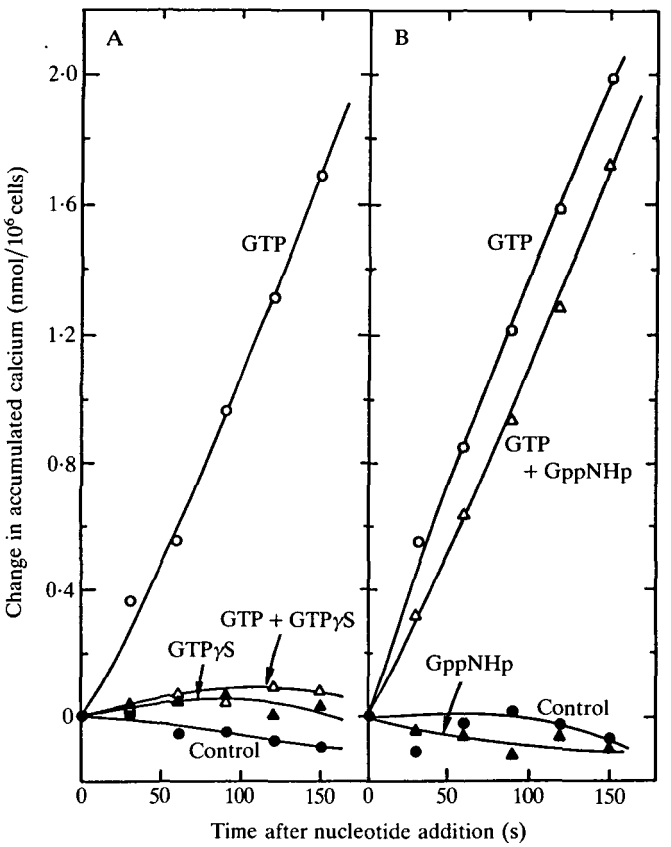

Fig. 5. Specificity of effects of nonhydrolysable analogues of GTP on Ca^{2+} uptake into permeabilized DDT_1MF-2 cells. Uptake of Ca^{2+} was measured under the conditions described in Fig. 1, with 10 μmol l^{-1} oligomycin, 4 mmol l^{-1} potassium oxalate, and 3 % polyethylene glycol present throughout the incubations. Uptake of Ca^{2+} into cells was allowed to proceed for 7 min. At this time, each incubation vial received an addition of 1 mmol l^{-1} ADP (to prevent any subsequent conversion of GDP to GTP) together with the following: (A) 5 μmol l^{-1} GTP (○), 10 μmol l^{-1} GTPγS (▲), 5 μmol l^{-1} GTP together with 100 μmol l^{-1} GTPγS (△) or control buffer (●); (B) 5 μmol l^{-1} GTP (○), 10 μmol l^{-1} GppNHp (▲), 5 μmol l^{-1} GTP together with 100 μmol l^{-1} GppNHp (△) or control buffer (●). Uptake in the presence of these additions above that observed immediately prior to additions is plotted. Ca^{2+} accumulation within cells was determined by removing and rapidly filtering cells at the times indicated by procedures as described in Mullaney, Chueh, Ghosh & Gill (1987).

5 μmol l^{-1} GTP (Fig. 5A), GppNHp has almost no effect on the action of GTP (Fig. 5B). As mentioned above, it is assumed that the specificity between the two GTP analogues derives from the specificity of the nucleotide-binding site. The noneffectiveness of these analogues in promoting GTP-like effects is in clear distinction to the effects of guanine nucleotides on known G-protein activities

Table 1. *Summary of parameters of GTP-activated calcium release and calcium uptake in the absence and presence of oxalate, respectively*

Parameter or condition	Calcium release (observed without oxalate)	Calcium uptake (observed with oxalate)
K_m for GTP	0·75 μmol l^{-1}	0·9 μmol l^{-1}
10 μmol l^{-1} GDP	Delayed full effect	Delayed full effect
10 μmol l^{-1} GDP (+1 mmol l^{-1} ADP)	No effect	No effect
100 μmol l^{-1} GDPβS	No effect	No effect
10 μmol l^{-1} GTP + 100 μmol l^{-1} GDP (+1 mmol l^{-1} ADP)	GTP effect blocked	GTP effect blocked
10 μmol l^{-1} GTP + 100 μmol l^{-1} GDPβS	GTP effect blocked	GTP effect blocked
10 μmol l^{-1} GTPγS	Slight effect	Slight effect
10 μmol l^{-1} GTP + 100 μmol l^{-1} GTPγS	GTP effect blocked	GTP effect blocked
10 μmol l^{-1} GppNHp	No effect	No effect
10 μmol l^{-1} GTP + 100 μmol l^{-1} GppNHp	GTP effect not blocked	GTP effect not blocked
1–3 % PEG	Stimulated	Stimulated
1 mmol l^{-1} vanadate	No effect	Blocked

Each of the parameters for GTP-activated Ca^{2+} uptake observed in the presence of oxalate refers to data presented by Chueh *et al.* (1987) and Mullaney, Chueh, Ghosh & Gill (1987). The observations relating to Ca^{2+} release (in the absence of oxalate) were published in prior reports (Ueda, Chueh, Noel & Gill, 1986; Gill, Ueda, Chueh & Noel, 1986; Chueh & Gill, 1986). Explanations and details of the conditions described are given in the text.

PEG, polyethylene glycol.

(such as those modulating adenylate cyclase) where nonhydrolysable analogues are maximally or supereffective. The similarity between the GTP-activated release and uptake processes is further exemplified by the effects of GDP on Ca^{2+} movements. Thus, GDP gives a full but delayed uptake response (Mullaney *et al.* 1987) which exactly coincides with its effect on release in the absence of oxalate (Gill *et al.* 1986; see Fig. 1). Moreover, as with release, GDP-mediated uptake is blocked by a high (1 mmol l^{-1}) ADP concentration, indicating that its action arises from conversion to GTP *via* nucleoside diphosphokinase activity; in the presence of ADP, GDP blocks the action of GTP. GDPβS, which does not activate uptake, also blocks the action of GTP exactly as it does on GTP-induced Ca^{2+} release (see Fig. 1).

These data reveal almost complete correlation between parameters affecting GTP-activated uptake and release. A summary of these effects is given in Table 1. Such data provide very strong evidence suggesting that the same GTP-activated process mediates both uptake and release of Ca^{2+} in the presence and absence of oxalate, respectively. The only divergence between the two processes is the effectiveness of vanadate which blocks GTP-induced uptake but does not block GTP-activated release (Mullaney *et al.* 1987), indicating that GTP-activated uptake is dependent on the continuous action of the Ca^{2+} pump. However, as

discussed below, the proposed model for the actions of GTP accounts for this difference.

Indicators of the mechanism of action of GTP

From the above data, a number of clues can be derived which together have suggested to us a model invoking a GTP-mediated conveyance of Ca^{2+} across membranes and perhaps between organelles. Before discussing this conclusion, let us summarize this new information. First, we have observed that a discrete pool of GTP-releasable Ca^{2+} exists in cells, a pool that may incorporate within it a smaller InsP_3-releasable Ca^{2+} pool; yet, despite the overlap between pools, we have provided substantial evidence suggesting distinctions between the mechanisms of GTP and InsP_3 in *activating* Ca^{2+} release (Chueh & Gill, 1986). Second, since PEG promotes both the effects of GTP and a clearly observable membrane coalescence at the same concentration (1–3 %) (Chueh *et al.* 1987), it is probable that activation of Ca^{2+} movements within cells is related to the occurrence of close appositions between membranes. Third, although direct fusion between membranes could account for some of the effects of GTP on Ca^{2+} movements, the observed reversibility of the effects of GTP together with the nonreleasability of oxalate-complexed Ca^{2+} by GTP would argue against a simple GTP-mediated fusion event between membrane surfaces as being the direct cause of Ca^{2+} movements. Fourth, there seems little doubt that the process of GTP-activated Ca^{2+} uptake in the presence of oxalate occurs *via* the same mechanism by which GTP activates release of Ca^{2+}, in spite of the apparent opposite nature of these two GTP-mediated events.

This last piece of information appeared the most perplexing, yet ironically it may provide the most significant clue to the action of GTP. Thus, it is likely that oxalate promotes the uptake of Ca^{2+} into a discrete Ca^{2+}-accumulating pool. As described above, it is well known that the ER membrane is permeable to anions including oxalate and phosphate. Hence passive entry of oxalate permits the formation of clearly observable insoluble complexes within the lumen of ER in cells; the entry of such anions may be mediated *via* a nonselective anion transporter activity analogous to that functioning in the SR membrane of muscle (see Martonosi, 1982). It is also apparent from our previous studies that, whereas Ca^{2+} accumulation in permeabilized cells and isolated microsomal membrane vesicles is oxalate-promoted, the accumulation of Ca^{2+} within purified inverted plasma membrane vesicles *via* the high-affinity plasma membrane Ca^{2+} pump is not enhanced by oxalate (Gill & Chueh, 1985). Since we have shown that these plasma membrane vesicles can indeed accumulate high intravesicular Ca^{2+} concentrations (Gill, Grollman & Kohn, 1981; Gill, 1982*b*; Gill, Chueh & Whitlow, 1984), more than sufficient to be precipitated in the presence of millimolar oxalate concentrations, we conclude that such membranes are largely impermeable to oxalate or phosphate. Thus, there is a good precedent for the existence of membranes through which passage of anions such as oxalate does not

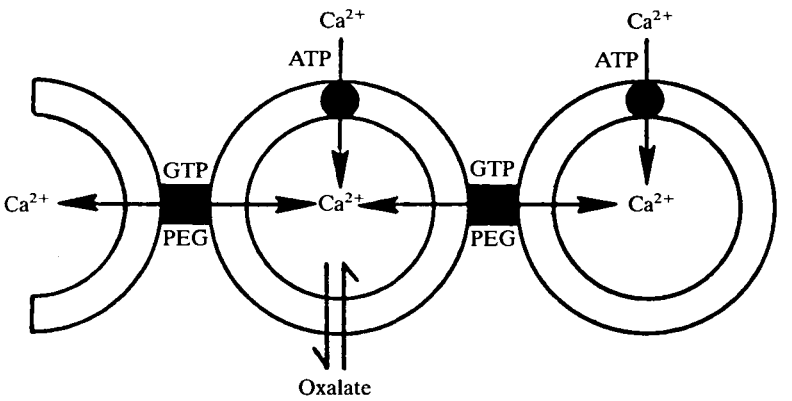

Fig. 6. Hypothetical model explaining the two effects of GTP on Ca^{2+} movements in cells, that is, GTP-mediated Ca^{2+} release and GTP-mediated Ca^{2+} uptake in the absence and presence of oxalate, respectively. Details of the evidence and an explanation of the proposed 'conveyance' of Ca^{2+} between open and closed compartments *via* a transmembrane Ca^{2+} translocation process are given in the text and in Chueh *et al.* (1987) and Mullaney, Chueh, Ghosh & Gill (1987). PEG, polyethylene glycol.

occur, and that organelle membranes may perhaps be distinguished according to their permeability to oxalate.

Model for GTP-activated transmembrane calcium 'conveyance'

With the knowledge that distinct membranes exist which are differentially permeable to oxalate, we propose that in the presence of oxalate, GTP promotes uptake of Ca^{2+} as the result of a GTP-mediated movement of Ca^{2+} from a nonoxalate-permeable pool, which actively pumps Ca^{2+}, to another Ca^{2+}-pumping pool which is freely permeable to oxalate. Thus, it is envisaged that GTP promotes a transmembrane conveyance of Ca^{2+} between such pools by activating some type of junctional process between the two membranes (see Fig. 6). Alternative schemes involving GTP-promoted oxalate-permeability or enhanced Ca^{2+} pumping are possible; but why then should an almost identical GTP-dependent process mediate movement (release) of Ca^{2+} in the absence of oxalate? In the model depicted in Fig. 6, the oxalate-permeable pool is very likely to be the ER or a subcompartment thereof; the nature of the putative nonoxalate-permeable pool is uncertain. Although the plasma membrane has been rendered permeable in our studies, it is possible that separate enclosed membranes derived from the plasma membrane might exist within the cell; such autonomous vesicles would be largely protected from the permeabilizing effects of saponin. The postulated process of junction formation between membranes would obviously be promoted by conditions that favour close appositions between membranes, as occurs in the presence of PEG. The action of GTP is envisaged as a necessary factor in either inducing the formation of junctions or activating the movement of Ca^{2+} through junctional processes arising by either random or PEG-promoted

membrane interactions. Such transfer of Ca^{2+} would be activated by terminal phosphate hydrolysis from GTP; when GTP is washed away, the continued operation of such transfer would be terminated, as indicated by the reversibility experiments described above (Chueh *et al.* 1987).

An obvious problem is how GTP-mediated Ca^{2+} release could be accounted for by the same model. It seems entirely possible that the same type of junctional connections could be formed between intact organelles such as ER and nonclosed membranes, perhaps the plasma membrane. In this case, transmembrane conveyance of Ca^{2+} would result in release of Ca^{2+} to the medium (see Fig. 6). If such a conveyance of Ca^{2+} to the outside could be mediated by GTP how could GTP induce a build-up of Ca^{2+} within the oxalate-permeable pool? If the hypothetical GTP-activated junctional processes transmit only small solutes between pools (as gap junctions between cells are known to do), then a precipitate of the Ca^{2+}–oxalate complex would not be expected to be transferred. Thus, in the experiments described above, oxalate and Ca^{2+} have been permitted to equilibrate within the oxalate-permeable pool; addition of GTP may result in a substantial 'injection' of Ca^{2+} from a nonoxalate-permeable (but, nevertheless, Ca^{2+}-pumping) pool; this Ca^{2+} would be immediately precipitated owing to the excess oxalate present. When GTP and oxalate are added simultaneously at the beginning of uptake, GTP causes a prolonged inhibition of Ca^{2+} uptake due to activation of the release process. With time and in the presence of sufficient oxalate, there is a gradual increase in uptake followed eventually by a sustained uptake which proceeds at a rate approaching the maximal rate of uptake observed when GTP is added after oxalate (see below). The initial phase of this type of response is presumably due to the continued release of Ca^{2+} to the exterior, thus preventing sufficient build-up of Ca^{2+} to that critical level at which precipitation with oxalate occurs. Irrespective of when oxalate is added, the Ca^{2+}-conveyance model predicts that Ca^{2+}-pumping activity is essential to sustain GTP-activated Ca^{2+} uptake in the presence of oxalate, a prediction clearly confirmed by the blocking action of vanadate.

A significant problem that has dominated the physiological implications of the GTP-activated Ca^{2+} movements we have described, is how the high levels of GTP within cells ($0{\cdot}1$–$0{\cdot}3\,mmol\,l^{-1}$) can be reconciled with the extreme sensitivity of the GTP-activated process; thus, it was argued that under physiological conditions, the intracellular pool acted upon by GTP would be permanently depleted (Baker, 1986). However, by implicating a transfer of Ca^{2+} only between actively pumping organelles (and possibly with the outside of the cell), there would not be any collapse of existing gradients. Thus, the release that is observed with GTP may only reflect an artificially imposed, diminished external Ca^{2+} level that is a consequence of using permeabilized cells. In other words, such release could actually represent reversed movement of Ca^{2+} through a system that normally exists to convey Ca^{2+} perhaps to replenish the intracellular pool. The implication here is that such interpool communication may normally exist between organelles in intact cells but be reversed when cells are broken and GTP washed away.

Alternatively, the functioning of such Ca^{2+}-communication between organelles may be regulated *in situ* by another cytosolic factor.

GTP-induced loading in the InsP_3-releasable calcium pool

With the above model in mind, perhaps the most relevant problem to be addressed was the relationship between the pools of Ca^{2+} modified by GTP and that Ca^{2+} pool sensitive to InsP_3. This area of investigation has produced some important results. One initial step was to ascertain whether InsP_3 releases Ca^{2+} from an oxalate-permeable or oxalate-impermeable pool. This question is largely answered by the data shown in Fig. 7. In permeabilized cells from the DDT_1MF-2 smooth muscle and the N1E-115 neuroblastoma cell lines, InsP_3 in the absence of oxalate reduces Ca^{2+} uptake by 50 and 30%, respectively (Fig. 7A,C), effects entirely consistent with the extent of Ca^{2+} release observed following InsP_3 addition to Ca^{2+}-loaded cells, as described above. In the presence of oxalate a sustained increase in the rate of ATP-dependent Ca^{2+} accumulation is observed (Fig. 7B,D) consistent with formation of the insoluble Ca^{2+}/oxalate complex and hence a reduced rate of Ca^{2+} efflux (Gill & Chueh, 1985; Mullaney *et al.* 1987). Importantly, 10 μmol l^{-1} InsP_3 (a maximally effective concentration) completely eliminates the increment in Ca^{2+} uptake induced by oxalate in permeabilized DDT_1MF-2 cells (Fig. 7B) indicating that InsP_3 activates Ca^{2+} release from an oxalate-permeable pool. Although not completely abolishing oxalate-enhanced Ca^{2+} uptake, the effectiveness of InsP_3 is very similar using permeabilized N1E-115 cells (Fig. 7D); hence in these cells, whereas InsP_3 does release Ca^{2+} from an oxalate-permeable pool, a small fraction of this pool may be unresponsive to InsP_3. As stated above, it is well established that the ER membrane is permeable to anions including oxalate, hence permitting clearly observable precipitation of Ca^{2+} within the ER lumen when oxalate is presented intracellularly. Thus, these data, although not providing definitive proof, are consistent with the view that the source of InsP_3-mobilizable Ca^{2+} is the ER or at least a subcompartment thereof.

Although, as described above, there are clear distinctions between the mechanisms by which InsP_3 and GTP activate Ca^{2+} movements, the data shown in Fig. 8 clearly establish a link between the actions of the two effectors. When added from the start of uptake, GTP and InsP_3 inhibit the accumulation of Ca^{2+} in a nonadditive manner (Fig. 8A) consistent with the extent of release described above. In the presence of oxalate, the action of GTP is very different from that of InsP_3 (Fig. 8B). Thus, whereas InsP_3 merely inhibits accumulation, GTP shows a biphasic effect. This effect of GTP is interpreted to support further the model in Fig. 6 since it shows that the two opposing GTP-activated movements of Ca^{2+} directly compete for access of Ca^{2+} to a common compartment. Thus, although initially Ca^{2+} release occurs resulting from interactions between closed and open compartments, thereafter, as the threshold of accumulated Ca^{2+} reaches that precipitable by oxalate, release of complexed Ca^{2+} is prevented and Ca^{2+} continues to accumulate at a higher rate reflecting the combined pumping activity of intact pools between which Ca^{2+} movement has been activated by GTP. Most

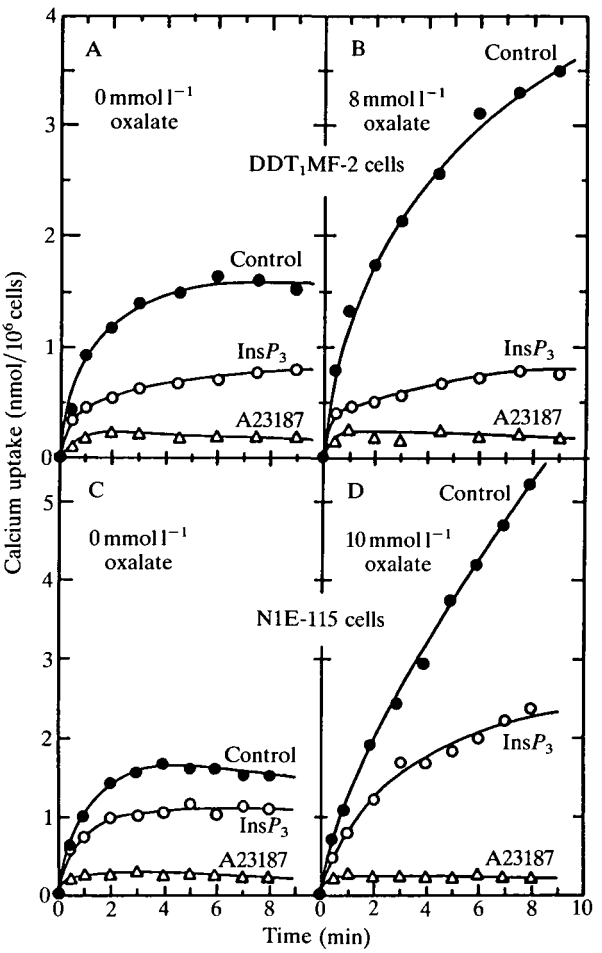

Fig. 7. InsP_3-mediated inhibition of oxalate-dependent Ca^{2+} uptake into permeabilized DDT_1MF-2 smooth muscle cells (A,B) or N1E-115 neuroblastoma cells (C,D). Uptake of Ca^{2+} was started at time zero by addition of ATP and labelled Ca^{2+} to gently stirred cells in uptake medium and was terminated by rapid filtration of samples of cells removed at the indicated times, as described for Fig. 1. Oxalate was either absent from the uptake medium (A,C) or was present from the start of uptake at either 8 (B) or 10 mmol l^{-1} (D). Uptake was measured either under standard conditions (●) or in the presence of 10 μmol l^{-1} InsP_3 (○) or 5 μmol l^{-1} A23187 (△) added to incubation vials in each case at time zero. The medium contained 50 μmol l^{-1} $CaCl_2$ buffered to 0·1 μmol l^{-1} free Ca^{2+} with EGTA; further experimental details are given in Mullaney, Chueh, Ghosh & Gill (1988).

importantly, the GTP-induced enhanced Ca^{2+} uptake phase is almost completely abolished when InsP_3 is added together with GTP (Fig. 8B) indicating that InsP_3 releases Ca^{2+} from the same pool into which GTP activates Ca^{2+} accumulation. These results obtained using permeabilized N1E-115 neuroblastoma cells have been repeated using permeabilized DDT_1MF-2 smooth muscle cells. It should be

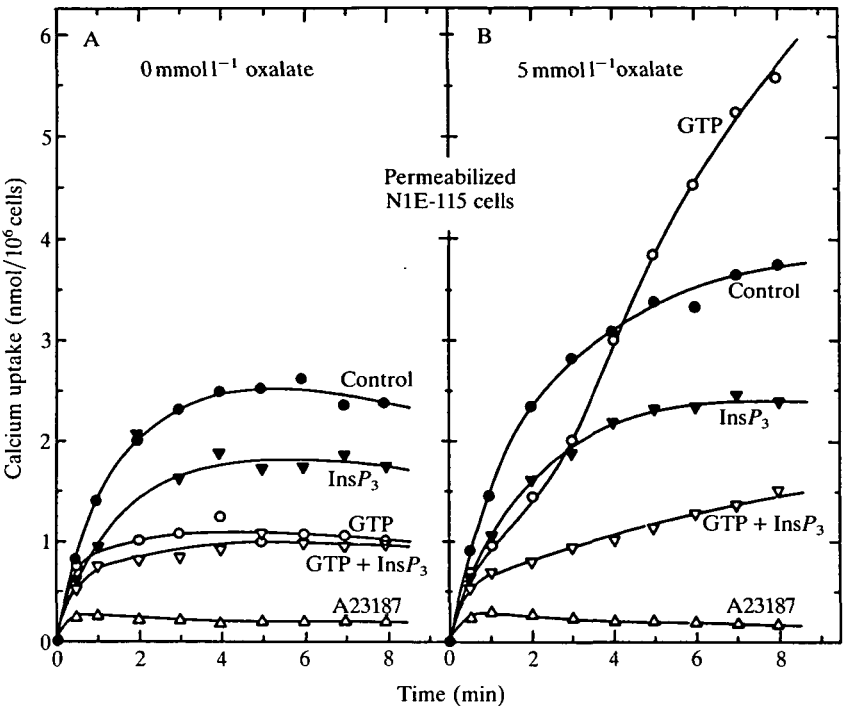

Fig. 8. InsP_3-induced reversal of the GTP-activated Ca^{2+} uptake phase in permeabilized N1E-115 neuroblastoma cells. ATP-dependent uptake of Ca^{2+} was measured at the indicated times after addition of ATP and labelled Ca^{2+} to cells as described in Fig. 7 (see Mullaney, Yu, Ghosh & Gill, 1988). Incubations were conducted either in the absence of oxalate (A) or in the presence of 5 mmol l^{-1} oxalate (B). Uptake proceeded under otherwise standard conditions (●), or after addition of either 10 μmol l^{-1} GTP (○), 10 μmol l^{-1} InsP_3 (▼), 10 μmol l^{-1} GTP together with 10 μmol l^{-1} InsP_3 (▽) or 5 μmol l^{-1} A23187 (△). Additions of oxalate, InsP_3, GTP and A23187 were all made at time zero.

noted that InsP_3 does not block the effects of GTP *per se*, since Ca^{2+} accumulation is reduced to a level well below that induced by InsP_3; thus, it may be inferred that although InsP_3 prevents the additional accumulation of Ca^{2+} activated by GTP, it in fact permits the Ca^{2+}-releasing effects of GTP to dominate. These results provide direct evidence for the operation of both GTP- and InsP_3-activatable Ca^{2+} transport mechanisms on the same pool of Ca^{2+}. Most significantly, they suggest that loading of Ca^{2+} within the InsP_3-sensitive pool may be controlled by the GTP-activated Ca^{2+} translocation process.

Conclusions and scheme for the actions of InsP_3 and GTP

The proposed scheme of GTP-activated Ca^{2+} movements accounts for all the observed effects of GTP and oxalate on Ca^{2+} movements. Alternative schemes invoking direct effects of GTP on Ca^{2+} pumping or GTP-enhanced movements of

oxalate are inherently unlikely since they do not account for rapid GTP-mediated Ca^{2+} release. In fact, GTP-mediated Ca^{2+} release occurs in the presence of vanadate and in the absence of ATP (Mullaney *et al.* 1987), that is, in the absence of any pumping activity. Also, in a recent report, Hamachi *et al.* (1987) described similar GTP-enhanced uptake of Ca^{2+} in the presence of oxalate. Although no explanation was offered for the effect, direct experiments revealed no effect of GTP on oxalate movements. Although recent work from Dawson and colleagues (Dawson *et al.* 1987; Comerford & Dawson, 1988) suggests membrane fusion may account for the effects of GTP, as stated above, the observations we have made on reversibility of the effects of GTP and on the electron microscopic structure of microsomal membrane vesicles treated with GTP, together argue against a simple membrane fusion process being activated by GTP. Although GTP hydrolysis is clearly implicated in the process of GTP-activated Ca^{2+} translocation (Chueh & Gill, 1986; Gill *et al.* 1986), it is presently unclear whether terminal phosphate is transferred to water (as in the case of a GTPase reaction), or whether a kinase-mediated mechanism transfers phosphate to another substrate molecule. Evidence for the former was recently presented by Nicchitta, Joseph & Williamson (1986), whereas a GTP-induced protein phosphorylation possibly associated with Ca^{2+} release was claimed by Dawson, Comerford & Fulton (1986).

Based on several important conclusions drawn from the data given in Figs 7 and 8, the scheme described above to account for the effects of GTP can be extended to encompass the action of $InsP_3$. First, from the data in Fig. 8A and data described earlier, it is apparent that the $InsP_3$-releasable Ca^{2+} pool is both smaller than and contained within the GTP-activatable pool. Second, based on the results shown in Fig. 7, the pool from which $InsP_3$ induces Ca^{2+} release is itself permeable to oxalate. Third, and most significant, this $InsP_3$-releasable Ca^{2+} pool is indeed the same pool that can be loaded with Ca^{2+} *via* the GTP-induced Ca^{2+}-translocating process, as shown in Fig. 8B. These observations suggest to us that the $InsP_3$-releasable Ca^{2+} pool *is* the oxalate-permeable subcompartment of the GTP-activatable pool, as depicted in the model shown in Fig. 9. Thus, we assume that the efficient operation of the $InsP_3$-activated Ca^{2+} channel enhances efflux of Ca^{2+} from this pool effectively enough to prevent sufficient build-up of Ca^{2+} to reach the oxalate-precipitable threshold. Interestingly, when the experiment shown in Fig. 8B is conducted with $10\,mmol\,l^{-1}$ oxalate or higher (data not shown), some GTP-dependent build-up of Ca^{2+} does occur at later times in the presence of $InsP_3$, suggesting that by lowering the oxalate threshold, even the rapid release effected by $InsP_3$ is insufficient to prevent a significant build-up of Ca^{2+}.

The direct reversal of the effect of GTP by $InsP_3$ provides a strong argument for considering that indeed both $InsP_3$ and GTP can act upon a common pool of Ca^{2+}. Such conclusions are reminiscent of our earlier 'flux reversal' studies which provided direct proof for the coexistence of specific plasma membrane Ca^{2+} and Na^+ flux mechanisms in a single population of synaptic membrane vesicles (Gill *et al.* 1981; Gill, 1982*a*,*b*). The most significant implication of the scheme shown in

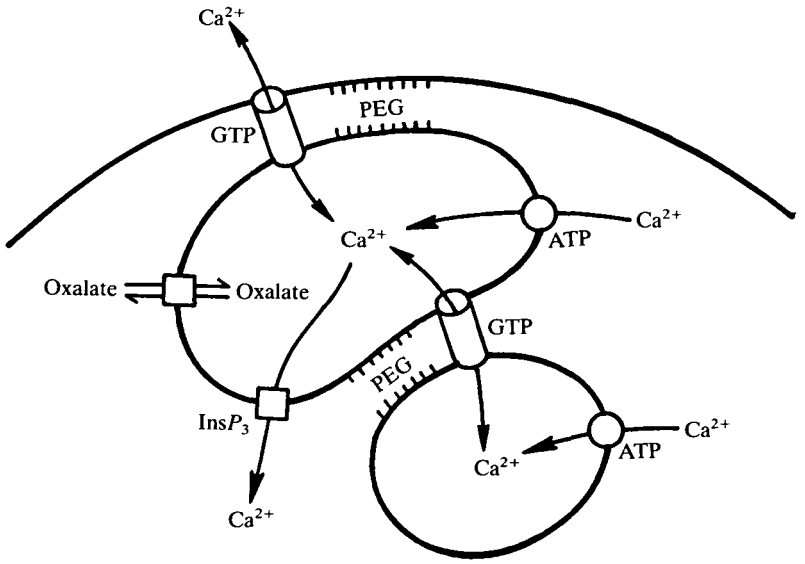

Fig. 9. Proposed scheme for the movements of Ca^{2+} induced by GTP and $InsP_3$. The model proposes that separate ATP-dependent Ca^{2+}-pumping compartments exist which are distinct with respect to both $InsP_3$-releasability and oxalate permeability, and that GTP mediates Ca^{2+} translocation between such compartments, perhaps *via* activation of intermembrane junctional processes. It is further proposed that GTP-activated Ca^{2+} release occurs *via* the same mechanism except involving interactions between the surfaces of closed and nonclosed membranes. As described previously (Chueh & Gill, 1986; Ueda, Chueh, Noel & Gill, 1986; Gill, Ueda, Chueh & Noel, 1986; Chueh *et al.* 1987; Mullaney, Chueh, Ghosh & Gill, 1987), the effects of GTP on both uptake and release of Ca^{2+} are promoted by low concentrations of polyethylene glycol (PEG) (1–3 %) which also promote the formation of close appositions between membrane surfaces (Chueh *et al.* 1987). However, PEG is not essential and the same GTP-activated Ca^{2+} movements are still observable in its absence (Chueh & Gill, 1986; Mullaney *et al.* 1987). Details of the evidence indicating the site of action of $InsP_3$ and the proposed mechanism of GTP in this scheme are described in the text and in Chueh *et al.* (1987); Mullaney *et al.* (1987); Mullaney, Yu, Ghosh & Gill (1988).

Fig. 9 is that a close interrelationship probably exists between the actions of $InsP_3$ and GTP. We had previously speculated that this might be the case (Mullaney *et al.* 1987) but had no proof. The data presented in Figs 7 and 8 provide for the first time direct evidence that both $InsP_3$ and GTP can modify the same Ca^{2+} compartment in spite of their probable distinct mechanisms of action.

It is very possible that the GTP-regulated Ca^{2+}-translocating process may control the size of the $InsP_3$-induced Ca^{2+} signal by permitting $InsP_3$ to release Ca^{2+} from a more extensive internal Ca^{2+} pool. Moreover, the same process may regulate the loading and/or replenishment of Ca^{2+} within the $InsP_3$-releasable pool. Such potential regulation derives much relevance from the considerable recent attention that has been directed towards the possible mechanisms by which the $InsP_3$-releasable Ca^{2+} pool may be replenished from the outside. Thus, Putney

(1986) has suggested that external Ca^{2+} entry may be directed into this pool and hence account for the frequently observed prolonged responses to receptor-induced signals which are dependent on extracellular Ca^{2+}. Recently, Irvine & Moor (1986, 1987) have presented experimental evidence suggesting the possible involvement of inositol 1,3,4,5-tetrakisphosphate ($InsP_4$) in inducing Ca^{2+} entry; in fact, their studies on activation of sea urchin eggs are consistent with the possibility that $InsP_4$ may promote entry of external Ca^{2+} into the $InsP_3$-releasable pool *via* a mechanism remarkably similar to the scheme described here for the movements of Ca^{2+} induced by GTP (Irvine & Moor, 1987). We are currently investigating whether this putative action of $InsP_4$ is related to GTP-activated Ca^{2+} movements and/or whether $InsP_4$ may modulate GTP-induced Ca^{2+} translocation.

We extend our sincerest gratitude to our former colleagues Dr Teruko Ueda and Dr Sheau-Huei Chueh for their very considerable contributions to the work described, and to Mark Noel and Cindy Ebert for their excellent technical assistance. This work was supported by grant number NS19304 from the National Institutes of Health, and grant number DCB-8510225 from the National Science Foundation.

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