GTP- and Inositol 1,4,5-Trisphosphate-activated Intracellular Calcium Movements in Neuronal and Smooth Muscle Cell Lines*

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Recent evidence has revealed that a highly sensitive and specific guanine nucleotide regulatory process controls intracellular Ca\(^{2+}\) release within N1E-115 neuroblastoma cells (Gill, D. L., Ueda, T., Chueh, S. H., and Noel, M. W. (1986) Nature 320, 461-464). The present report documents GTP-induced Ca\(^{2+}\) release within quite distinct cell types, including the DDT, MF-2 smooth muscle cell line. GTP-induced Ca\(^{2+}\) release has similar GTP sensitivity and specificity among cells and rapidly mobilizes up to 70% of Ca\(^{2+}\) specifically accumulated within a nonmitochondrial Ca\(^{2+}\)-pumping organelle within permeabilized DDT, MF-2 cells. Maximal GTP-induced release of Ca\(^{2+}\) is observed to be greater than inositol 1,4,5-trisphosphate (IP\(_3\))-induced Ca\(^{2+}\) release (the latter being approximately 30% of total releasable Ca\(^{2+}\)). After maximal IP\(_3\)-induced release, further IP\(_3\) addition is ineffective, whereas subsequent addition of GTP further releases Ca\(^{2+}\) to equal exactly the extent of Ca\(^{2+}\) release observed by addition of GTP in the absence of IP\(_3\). Thus, IP\(_3\) releases Ca\(^{2+}\) from the same pool as GTP, whereas GTP also releases from an additional pool. The effects of GTP appear to be reversible since simple washing of GTP-treated cells restores their previous Ca\(^{2+}\) uptake properties. Electron microscopic analysis of GTP-treated membrane vesicles reveals their morphology to be unchanged, whereas treatment of vesicles with 3% polyethylene glycol, known to enhance GTP-mediated Ca\(^{2+}\) release, clearly induces close coalescence of membranes. In the presence of 4 mM oxalate, GTP induces a rapid and profound uptake, as opposed to release, of Ca\(^{2+}\). The findings suggest that GTP-activated Ca\(^{2+}\) movement is a widespread phenomenon among cells, which can function on the same Ca\(^{2+}\) pool mobilized by IP\(_3\), and although activating Ca\(^{2+}\) movement by a mechanism distinct from IP\(_3\), does so via a process that does not appear to involve fusion between membranes.

The processes involved in calcium signaling within cells have been the focus of much recent attention, especially with regard to the production and action of the Ca\(^{2+}\)-mobilizing messenger, inositol 1,4,5-trisphosphate. Many studies now show that IP\(_3\) mediates Ca\(^{2+}\) release from an internal organelle believed to be endoplasmic reticulum (1-3). Recent evidence has revealed that in addition to the release of Ca\(^{2+}\) activated by IP\(_3\), a highly sensitive and specific guanine nucleotide regulatory process functions to promote Ca\(^{2+}\) release from a similar intracellular Ca\(^{2+}\) pool (4, 5). Using either permeabilized cells of the neuronal cell line N1E-115 (4) or microsomal vesicles derived therefrom (5), our experiments have revealed that the latter process promotes a rapid and substantial release of Ca\(^{2+}\) accumulated within permeabilized cells or vesicles via internal (ATP + Mg\(^{2+}\)-dependent Ca\(^{2+}\)-pumping activity characterized earlier (6). Activation of Ca\(^{2+}\) release is induced by submicromolar GTP concentrations and shows high specificity for guanine nucleotides (4, 5). The release process appears to be dependent on terminal phosphate hydrolysis of GTP. This is concluded from the following observations: first, nonhydrolyzable GTP analogues are ineffective in inducing Ca\(^{2+}\) release; second, such analogues block the action of GTP; third, GTP-mediated Ca\(^{2+}\) release is competitively inhibited by GDP indicating that the two nucleotides can bind to a common site (4). At present it is unclear whether the activation process involves terminal phosphate transfer to water (as in the context of a GTPase reaction), or whether a kinase-mediated process transfers phosphate to another substrate molecule. Evidence for the former was recently presented by Nicchitta et al. (7), whereas evidence for a GTP-induced protein phosphorylation possibly associated with Ca\(^{2+}\) release was reported by Dawson et al. (8).

In earlier studies, Dawson et al. (8, 9) observed that GTP could promote the effectiveness of IP\(_3\) in inducing Ca\(^{2+}\) release from liver microsomes, whereas GTP or IP\(_3\) alone induced little or no release of Ca\(^{2+}\). In contrast, our results with either permeabilized N1E-115 cells (4) or isolated microsomal vesicles (5), reveal that GTP promotes substantial Ca\(^{2+}\) release without any exogenously added IP\(_3\). Moreover, we recently reported that a number of important distinctions exist between the actions of IP\(_3\) and GTP in releasing intracellular Ca\(^{2+}\) (10). First, IP\(_3\)-activated Ca\(^{2+}\) release is unaffected by either GDP or GTPS, both of which block GTP-activated Ca\(^{2+}\) release. Second, polyethylene glycol promotes GTP-activated release but does not alter the action of IP\(_3\). Third, IP\(_3\)-activated Ca\(^{2+}\) release is clearly modified by the free Ca\(^{2+}\) concentration being completely inhibited at 10 \(\mu M\) Ca\(^{2+}\); in contrast, GTP induces Ca\(^{2+}\) release independent of the free Ca\(^{2+}\) concentration. Fourth, the actions of IP\(_3\) and GTP show very distinct temperature dependence: GTP-induced Ca\(^{2+}\) release is completely blocked by decreasing the temperature from 37 to 4 °C, whereas the rate of IP\(_3\)-induced Ca\(^{2+}\) release is decreased by only 20% at 4 °C, a result consistent with the observations of Smith et al. (11). Several of these distinctions between the actions of GTP and IP\(_3\) have also been reported.
by Henne and Söling (12) using liver and parotid microsomes, and by Jean and Klee (13) using microsomes from NG108-15 neuroblastoma X glioma hybrid cells. In summary, the temperature sensitivity of IPi- and PI-induced Ca2+ release are consistent with its probable direct activation of a Ca2+ channel in ER. In contrast, GTP appears to effect release by a temperature-sensitive process probably involving enzymic hydrolysis of the terminal phosphate from GTP.

Although the modes of activation of Ca2+ release by IP3 and GTP are clearly distinct, their effects are similar; indeed, differences in their activation mechanisms do not preclude possible coupling between their actions, as indicated by Dawson (8, 9). Thus, one goal of the studies described here is to ascertain more information on the relationship between the actions of GTP and IP3, particularly, whether the two effectors operate upon the same pool or distinct pools of Ca2+. A second aim of the studies reported here is to gain further information on the actual movements of Ca2+. The activation of GTP-dependent Ca2+ release probably involves an enzymic GTP hydrolytic mechanism. But what is the actual Ca2+ ion translocation process induced by GTP? Does the mechanism involve channel activation, or, alternatively, could some form of permeability by trypan blue exclusion, and treatment was terminated when 95% of cells were rendered permeable to the dye. Cells were saponin treated at a concentration of approximately 2 × 106 cells/ml, and the time for treatment varied according to cell type, generally between 5–10 min for either NIE-115 cells or DDT, MF-2 cells. After permeabilization cells were kept slowly stirring at 4 °C and were used in experiments within 2 h.

**Results and Discussion**

**Materials and Methods**

*Chemicals and Reagents.*—Crude microsomal fractions were prepared from NIE-115 cells at 10% fetal bovine serum; cells were passaged every 7 days, and medium changes were avoided since they interfered with the conversion of nucleoside diphosphates and since they were found to be unnecessary in view of the minimal decline of ATP concentrations during experiments.

**Electron Microscopy**—Thin section electron microscopy was performed on samples of crude microsomal vesicles (prepared from NIE-115 cells exactly as described in Ref. 5) which had been treated under identical conditions to cells in the Ca2+ uptake procedure described above. Samples of the treated vesicles were fixed by adding one volume of suspended vesicles (at approximately 0.5 mg of membrane protein/ml) in uptake medium to an equal volume of 2% glutaraldehyde in 20 mM phosphate buffer, pH 7.2. After 60 min at 4 °C, vesicles were pelleted at 12,000 × g for 5 min, and treated with 1% buffered osmium tetroxide for 15 h at 4 °C. The same procedure was used for control samples using a graded acetone series and embedded in Epon 812 epoxy plastic. Thin sections were cut and stained with uranyl acetate and lead citrate prior to visualization in a Siemens 1A electron microscope.

**Results and Discussion**

**Cell Specificity of GTP-mediated Calcium Release**—Using permeabilized cells of the NIE-115 neuronal cell line we have conducted a detailed examination of the Ca2+ accumulation and release properties of an intracellular nonmitochondrial
clearly indicate that a fraction of this accumulated Ca\(^{2+}\) is released by IP\(_{3}\). Half-maximal Ca\(^{2+}\) release is effected with 0.5 \(\mu\)M IP\(_{3}\), a sensitivity very similar to values reported in a number of other systems (1-3). However, not seen before was the extraordinarily sensitive and profound release of Ca\(^{2+}\) induced by GTP we observed using either permeabilized N1E-115 cells (4, 5) or microsomal membrane vesicles derived from these cells (5). In contrast, the studies of Dawson et al. (8, 9) using liver microsomes have shown that GTP, while significantly enhancing the effectiveness of IP\(_{3}\) in inducing Ca\(^{2+}\) release, by itself induced only a very modest effect.

In view of these differences, it was important to establish whether the effectiveness of GTP in directly induced Ca\(^{2+}\) release was an anomaly, perhaps restricted to the N1E-115 neuronal cell line. Experiments undertaken on a quite unrelated cell type, the DDT;MF-2 smooth muscle cell line derived from hamster vas deferens (18), suggest this is not the case. Thus, as shown in Fig. 1, a sensitive, specific, and substantial GTP-dependent release of Ca\(^{2+}\) is observed using permeabilized DDT;MF-2 cells loaded with Ca\(^{2+}\) via intracellular Ca\(^{2+}\) pumping activity. Such release is very similar to that observed with N1E-115 neuroblastoma cells, with pronounced effectiveness of submicromolar GTP concentrations, even in the presence of 1 mM ATP. Maximally effective GTP levels (5 \(\mu\)M or above) effect a rapid release of approximately 70% of the ionophore-releasable Ca\(^{2+}\). In addition to the DDT;MF-2 cell line, we have measured almost identical effects of GTP on Ca\(^{2+}\) release from permeabilized rat BChE-1 smooth muscle cells and human WI-38 normal embryonic lung fibroblasts. Using microsomal membrane vesicle fractions prepared from DDT;MF-2 cells by methods similar to those described for N1E-115 cell-derived microsomes (5), 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 and Soling (12) have reported very similar effects on release of accumulated Ca\(^{2+}\) induced by GTP; in this study, GTP-activated Ca\(^{2+}\) movements were followed using Ca\(^{2+}\) electrodes. The observations of Jean and Klee (13) on GTP- and IP\(_{3}\)-mediated Ca\(^{2+}\) release from microsomes derived from NG108-15 neuroblastoma X glioma hybrid cells are also very consistent with each of the above studies.

Therefore, it appears that the effectiveness of GTP in directly activating Ca\(^{2+}\) release is a widespread phenomenon, not restricted to particular cell types or to the use of specific cellular or subcellular preparations. However, it does appear from the observations of Dawson (8, 9) using liver microsomes, and from preliminary studies of Thomas and Rubin (21) using permeabilized hepatocytes, that the liver responds somewhat differently, GTP giving a slower and less substantial release of Ca\(^{2+}\) relative to those systems mentioned above, and more significantly, a GTP-mediated potentiation of the effectiveness of IP\(_{3}\). Recent preliminary observations of our own using liver microsomal membrane fractions confirm certain of these observations on the actions of GTP. Possible explanations for such differences are commented upon in the accompanying paper (14) in the light of our more recent observations and conclusions regarding the possible mechanism of GTP-activated Ca\(^{2+}\) movements.

**Subcellular Specificity of GTP-activated Calcium Release—** It is widely held that IP\(_{3}\) releases Ca\(^{2+}\) from a nonmitochondrial intracellular organelle presumed to be the ER (1-3). This view is based largely on circumstantial evidence that ER is known to be a major Ca\(^{2+}\)-sequestering organelle able to accumulate Ca\(^{2+}\) via a high affinity pump (2, 6). Our previous studies on the GTP-activated Ca\(^{2+}\) release process (4, 5) suggest that Ca\(^{2+}\) is also released from a nonmitochondrial organelle since release was observed in the presence of mitochondrial inhibitors and under free Ca\(^{2+}\) conditions (0.1 \(\mu\)M) which do not permit significant mitochondrial uptake. However, it was important to determine whether the effect of GTP on release was specific to a nonmitochondrial Ca\(^{2+}\) pool, or whether Ca\(^{2+}\) accumulated within mitochondria could also be released or affected in any way by GTP. As shown in Fig. 2, it is clear that GTP does not alter mitochondrial Ca\(^{2+}\). In this experiment, Ca\(^{2+}\) uptake and release were undertaken at a high free Ca\(^{2+}\) concentration (10 \(\mu\)M), using permeabilized N1E-115 cells that were loaded under otherwise standard conditions in the presence of ATP. In the presence of the

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\(^1\) S. H. Chueh, J. M. Mullaney, T. K. Ghosh, and D. L. Gill, unpublished observations.
Experimental Procedures.

Condition permitting uptake into both mitochondrial and nonmitochondrial cells were permeabilized and loaded with Ca²⁺ as described under "Experimental Procedures," except that the total Ca²⁺ concentration during Ca²⁺ flux incubations was 10 µM, and EGTA was not present. Both uptake and release were undertaken either in the presence of 10 µM oligomycin (△, △) or without oligomycin (○, ○), the latter condition permitting uptake into both mitochondrial and nonmitochondrial pools. At the end of a 10-min uptake period, either 10 µM GTP (○, ○) or control buffer (△, △) was added to vials. Samples of the mixture were withdrawn at the intervals shown after these additions followed by rapid filtration and washing as described under "Experimental Procedures."

Mitochondrial ATPase inhibitor oligomycin at 10 µM, mitochondrial Ca²⁺ uptake is completely abolished (6), and under such conditions 10 µM GTP effects more than 50% release of accumulated Ca²⁺ (Fig. 2, lower curves). Without oligomycin, considerably higher (approximately 4-fold) Ca²⁺ uptake is observed since mitochondria can accumulate Ca²⁺ at the higher free Ca²⁺ level included in the experiment. However, upon addition of GTP, only approximately 12% of the accumulated Ca²⁺ is released (Fig. 2, upper curves). In fact, this represents an almost identical amount of GTP-mediated Ca²⁺ release (approximately 3 nmol/10⁶ cells) to that observed in the presence of oligomycin. Therefore, the effects of GTP are indeed specific to a nonmitochondrial Ca²⁺-sequestering pool.

It should be noted that a similar experiment cannot be undertaken to examine the effects of IP₃ because, as we showed in a recent report (10), at this concentration of free Ca²⁺ (10 µM) the effect of IP₃ is completely inhibited. Such inhibition reflects a potentially important Ca²⁺-dependent feedback on IP₃-mediated release which may have much significance in the physiological role of IP₃ within cells.

We previously observed (5) that IP₃ and GTP were without any effects on the movement of Ca²⁺ across isolated inverted plasma membrane vesicles derived from neural tissue (18-20). However, it is important to note that there may be a flaw in this more conventional approach to determining subcellular specificity by isolating discrete fractions enriched in certain organelles. Thus, as described in the accompanying report (14), it is possible that interactions between more than one type of membrane may be involved in the Ca²⁺ release-inducing effects of GTP; hence, isolation and separation of organelles may reduce interactions between specific membranes and therefore prevent the effects of GTP. Although we have no direct proof that ER is a source of GTP-releasable Ca²⁺, interpretation of the effects of oxalate, a known permeator of the ER membrane, may indicate that the ER is indeed a site of action of GTP, as described later.

Relationship between GTP- and IP₃-releasable Calcium Pools—Perhaps as important as establishing the actual identity of the source(s) of Ca²⁺ released in response to IP₃ and GTP, is the determination of whether the two agents activate release from the same or different Ca²⁺ pools. In addition, this determination had fundamental significance to the question of whether coupling between the actions of GTP and IP₃ could exist in cells. Thus, although our recent studies clearly establish that GTP and IP₃ activate Ca²⁺ release via distinct mechanisms (10), Dawson's results using liver microsomes indicate a potentiating action of GTP on IP₃-induced Ca²⁺ release (8, 9). It became clear to us that while the activation processes for Ca²⁺ release in response to GTP or IP₃ are distinct, coupling between their actions at some level was still a possibility; thus, of major significance to the question of whether or how such coupling could occur was to establish if IP₃ and GTP have a common site of action.

The question of whether IP₃ and GTP activate the same or different Ca²⁺ pools is directly addressed in the study depicted in Fig. 3; however, the answer is not as straightforward as might be predicted. In this experiment, the effects of sequential addition of maximally effective concentrations of IP₃ (10 µM) and GTP (10 µM) were examined in permeabilized N1E-115 cells. When IP₃ is added to fully Ca²⁺-loaded cells (Fig. 3A), approximately 30% of the accumulated Ca²⁺ is rapidly lost, a fractional release which is very constant for these cells. After maximal release is attained, if a further addition of IP₃ is made, little further Ca²⁺ is released. This result suggests that it is unlikely that IP₃ degradation is a limiting factor in Ca²⁺ release. Indeed, if this were the case, Ca²⁺ reaccumulation would be expected after maximal release, as has been observed in other systems where significant IP₃ metabolism presumably does occur (22-24). Under our conditions, using washed permeabilized cells in the presence of more than 1 mM free Mg²⁺, significant IP₃ breakdown due to particulate IP₃ 5-phosphatase activity would be expected (3). However, two other factors probably contribute to diminished IP₃ degradation: First, IP₃ 3-kinase activity, predominantly a cytosolic enzyme (3), would be largely lost in washed, permeabilized cells, and second, the dilute cell suspension used (approximately 2 × 10⁶ cells/ml) would lessen the effectiveness of IP₃-modifying enzymes. As shown in Fig. 3A, if GTP is added after maximal IP₃-induced Ca²⁺ release has occurred, than additional release of Ca²⁺ is observed until finally about 55% of the originally accumulated Ca²⁺ is released. Significantly, this degree of Ca²⁺ release is almost exactly the same as that observed when GTP is the initial addition, without any IP₃ (Fig. 3B). In this case, a further addition of GTP gives little additional Ca²⁺ release; moreover, addition of IP₃ at this time gives almost no greater release than that seen with GTP. It is clear that no change in the releasability of accumulated Ca²⁺ has occurred since A23187 added at any stage of the experiment causes similar.
release of Ca\(^{2+}\) down to the passive equilibration level.

These results suggest that the releasable Ca\(^{2+}\) accumulated within permeabilized N1E-115 cells exists in three distinguishable subcompartments. First, there appears to be a pool of Ca\(^{2+}\) which is releasable with either GTP or IP\(_3\), second, a Ca\(^{2+}\) pool which is releasable only with GTP, and third, a pool which cannot be released with either effector. The fact that comparable results have been obtained using the DDT, MF-2 cell line lends support to this conclusion. Thus, it is apparent that, although in both cell types the GTP-releasable pool differs from the IP\(_3\)-releasable pool in being larger, at least a significant proportion of accumulated Ca\(^{2+}\) lies within a pool which can be released by both agents. In other words, it appears that all of the Ca\(^{2+}\) within the IP\(_3\)-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 IP\(_3\)- and GTP-activated Ca\(^{2+}\) release processes and permits us to consider the existence of a possible coupling event linking their modes of action.

Reversibility of GTP-activated Calcium Release—An important question to answer concerns the nature of the Ca\(^{2+}\) translocation process activated by GTP. 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; alternatively, GTP could activate a fusion or communication between organelle membranes and result in the release or transfer of Ca\(^{2+}\). Whereas the rapidity and temperature insensitivity of IP\(_3\)-activated release are suggestive of channel activation (10-12), the relative temperature sensitivity of the effect of GTP could reflect a quite different process. Perhaps more significantly, the promotion of the effects of GTP by polyethylene glycol (4, 5, 10) strongly suggests that membrane interactions are involved in this process, as addressed below. However, certain observations on the reversibility of the GTP-activated process militate against the view that simple membrane fusion is induced by GTP resulting in Ca\(^{2+}\) release from a closed organelle. Thus, we recently reported that GDP, which competitively blocks the action of GTP, could at least partially reverse the effect of GTP and allow a small reuptake of Ca\(^{2+}\) into the previously GTP-activated permeabilized cells (4). If membrane fusion were the mechanism for Ca\(^{2+}\) liberation, then even partial reversibility of the effect would not be expected. However, the GDP reversal of GTPs effect was never complete and was frequently small perhaps due to rapid nucleotide diphosphokinase-mediated GDP to GTP conversion known to occur within the permeabilized cells (4, 5).

A more definitive indication of the reversibility of the GTP effect has come from a simpler study involving washing of cells after GTP activation, as shown in Fig. 4. In this experiment, DDT, MF-2 cells were pretreated with or without 10 \(\mu M\) GTP, in the presence of the usual uptake medium including polyethylene glycol (to promote a maximal GTP effect)

![Graph showing effects of sequential addition of IP\(_3\) and GTP on Ca\(^{2+}\) release from permeabilized N1E-115 neuroblastoma cells.](Image)
but without labeled Ca\(^{2+}\). If such pretreated cells, without washing, are now included in an uptake assay (that is, in the presence of label), Ca\(^{2+}\) uptake proceeds as shown in Fig. 4A; in this case the cells had not previously been treated with GTP. Thus, inclusion of GTP in the uptake assay prevents uptake into the GTP-releasable pool, just as A23187 prevents uptake into all pools (compare with Fig. 3). Unwashed GTP-pretreated cells, as expected, show no uptake above that seen when GTP is included in the uptake assay, since GTP from the pretreatment has fully activated the release process (Fig. 4B). However, when GTP-pretreated cells are washed three times with GTP-free uptake medium (still containing polyethylene glycol) uptake closer to that seen with cells not pretreated with GTP is observed (Fig. 4D). When these same pretreated and washed cells are included in an uptake assay containing GTP, there is a similar inhibition of uptake, indicating reactivation of the release process. As a control, washed cells not pretreated with GTP behave the same as unwashed cells when included in the uptake assay (Fig. 4C). Similar reversibility by washing has been observed even when cells were pretreated with up to 100 \(\mu\)M GTP.

These results suggest that simple washing with GTP-free medium effectively reverses a previous GTP-mediated activation of Ca\(^{2+}\) release. Even though uptake after washing is not restored completely to 100\% of that observed without pretreatment, the fact that GTP gives a large effect on the washed GTP-pretreated cells indicates that most of the GTP-stimulated release activity has been restored. The lack of complete restoration of uptake after washing probably results from a small amount of residual GTP which dissociates perhaps more slowly from within the permeabilized cells. It would be difficult to reconcile this observed reversibility with a simple membrane fusion process activated by GTP; in other words, the effects of a direct membrane fusion event would not be washed away and result in the restoration of almost normal Ca\(^{2+}\) retention, as observed.

**Electron Microscopic Analysis of GTP- and Polyethylene Glycol-treated Membrane Vesicles**—More direct evidence to
suggest that membrane fusion is not an obvious explanation for the effects of GTP is derived from electron microscopic visualization of membrane vesicles. Moreover, this approach gives an important indication about the type of process occurring when cells are treated with polyethylene glycol; thus, the results reveal that polyethylene glycol promotes a very obvious coalescence of membranes. Electron microscopic studies were performed using isolated microsomal membrane vesicles derived from N1E-115 cells and are shown in Fig. 5. These vesicles have been used extensively in previous studies (5) and have been shown to accumulate Ca\(^{2+}\) via ATP-dependent Ca\(^{2+}\) pumping activity and to release Ca\(^{2+}\) by a polyethylene glycol-promoted GTP-dependent process identical to those activities observed with permeabilized cells (4, 5). Microsomal vesicles were treated under conditions that exactly correspond to those present during Ca\(^{2+}\) uptake and release experiments, either with or without GTP in the presence or absence of 3% polyethylene glycol (at this concentration polyethylene glycol maximally promotes the effects of GTP; see Ref. 10). As can be seen from Fig. 5, A and B, polyethylene glycol induces a very obvious coalescence between the membrane vesicles. Thus, whereas in Fig. 5A it is clear that vesicles are dispersed, the polyethylene glycol-treated vesicles in Fig. 5B exist almost totally as closely associated aggregates; in fact, it was difficult to locate any unattached membrane vesicles in this sample. While vesicles appear closely associated with each other, there is no obvious evidence that they are no longer intact or that their membranes have fused. Thus, if fusion were a major event under this condition it might be expected that larger membrane sacs would appear, whereas the vesicle profiles appear either the same or perhaps slightly decreased in size. It is important to remember that, as we have previously clearly demonstrated, ATP-dependent Ca\(^{2+}\) uptake into polyethylene glycol-treated membrane vesicles or polyethylene glycol-treated permeabilized cells is almost completely unaltered relative to nonpolyethylene glycol-treated preparations, indicating that polyethylene glycol does not significantly alter either the intravesicular volume or the functional integrity of the membranes (4, 5, 9), despite any differences in the appearance of vesicles induced by polyethylene glycol. When vesicles are treated with GTP in the absence of polyethylene glycol (Fig. 5C), there is no significant alteration in their appearance. Furthermore, when the vesicles are treated with both polyethylene glycol and GTP under conditions that exactly correspond to those known to promote Ca\(^{2+}\) release, the vesicles appear identical to those treated only with polyethylene glycol (compare Fig. 5, B and D). It may thus be concluded that GTP itself does not induce any observable alteration in vesicle structure or association. However, the striking effectiveness of polyethylene glycol is good evidence to suggest that the effect of GTP in inducing Ca\(^{2+}\) movements is promoted by a condition that increases the close association between membranes. This important point is extended below and in the following report (12).

**Oxalate Effects on GTP-mediated Calcium Movements**

The evidence provided in Figs. 4 and 5 suggests that simple membrane fusion is not likely to account for the observed release of Ca\(^{2+}\) induced by GTP. However, in pursuit of a more definitive approach to this question, a dramatic and unexpected result was derived which, as described in the following report (14), has subsequently afforded us a much better understanding of the mechanism of action of GTP. Previous studies have shown that Ca\(^{2+}\) pumping into the ER within permeabilized cells is greatly enhanced when oxalate is present (6). Such a process is well recognized in many cell types and derives from the permeability of the ER membrane to anions, including oxalate and phosphate, which can form insoluble complexes with Ca\(^{2+}\). In the presence of such anions, precipitation of accumulated Ca\(^{2+}\) effectively reduces Ca\(^{2+}\) efflux resulting in a sustained linear rate of uptake, as was observed previously using permeabilized NIE-115 cells (6). We sought to test the releasability of Ca\(^{2+}\)-sequestered within permeabilized cells in the presence of oxalate. If oxalate-precipitated Ca\(^{2+}\) could be released by GTP, it would favor the idea of some nonselective "emptying" event, as expected from a process such as membrane fusion. On the other hand, if GTP could not effect release of the Ca\(^{2+}\)-oxalate precipitate, this would support a more selective efflux event being mediated by GTP. As shown in Fig. 6, in fact neither of these two predictions occurred. Instead, in the presence of oxalate, a rapid and profound increase in the accumulation of Ca\(^{2+}\) was observed upon addition of GTP in the presence of oxalate. In this experiment, permeabilized DDT, MF-2 cells were used, and the free Ca\(^{2+}\) concentration was increased to 30 μM to sustain the larger accumulation of Ca\(^{2+}\) occurring in the presence of oxalate (see Ref. 14). Under such conditions, in the absence of oxalate, 10 μM GTP induces greater than 50% release of accumulated Ca\(^{2+}\) (Fig. 6A), a result typical of those described in the above experiments. If 10 mM oxalate were included in the same assay, a linear rate of Ca\(^{2+}\) uptake would be attained, approximately equaling the initial rate of uptake observed without oxalate (not shown, but see Ref. 6). With oxalate at a lower concentration (4 mM), only a slight en-

![Fig. 6. GTP-induced movements of Ca\(^{2+}\) in permeabilized DDT, MF-2 cells in the presence and absence of oxalate. Ca\(^{2+}\) fluxes were measured under the the conditions described under "Experimental Procedures" with the exception that total Ca\(^{2+}\) was present at 30 μM, and EGTA was not added. No oxalate was present in experiment A, whereas 4 mM K-oxalate was present throughout experiment B. Uptake was measured from zero time (that is, the time of cell addition), and aliquots were removed at the times shown. After 5 min of uptake, either 10 μM GTP (O) or control buffer (●) was added. Aliquots of cells were rapidly filtered and washed as described under "Experimental Procedures."](image-url)
hancement of the final rate of Ca\(^{2+}\) uptake is observed (Fig. 6B). However, in the presence of 4 mM oxalate, upon the addition of 10 mM GTP, a rapid and dramatic increase in the rate of Ca\(^{2+}\) accumulation is observed, as shown in Fig. 6B. This result certainly does indicate again that simple GTP-induced fusion of membranes is an unlikely mechanism to account for the effects of GTP. Investigation of this apparently paradoxical effect of GTP in promoting sustained Ca\(^{2+}\) uptake and its relationship to GTP-mediated Ca\(^{2+}\) release forms the substance of the following report (14).

Concluding Remarks—Two major areas of investigation have been addressed in this report. The initial area concerns the cellular and subcellular specificity of the GTP-activated Ca\(^{2+}\) pool and its relationship to the pool of Ca\(^{2+}\) released by IP\(_3\). Three important conclusions are drawn from these studies. First, we have observed that the process of GTP-mediated Ca\(^{2+}\) release is a general phenomenon, not restricted to one particular cell type. Nor is its existence restricted to the use of GTP, can itself induce membrane fusion, this type of studies using purified membrane fractions may yield misleading results (this point is emphasized in the following report). Even though localization of the site of action of GTP to a particular organelle has not yet been achieved, the effects of oxalate do provide at least indirect evidence for the involvement of ER. Thus, it is known that the ER is permeable to Ca\(^{2+}\) and that when oxalate is introduced into a variety of different cell types, extensive and very visible Ca\(^{2+}\) oxalate precipitates can be observed within ER (25-28). The third conclusion concerns the relationship between pools of Ca\(^{2+}\) releasable in response to IP\(_3\) and GTP. Thus, evidence supports the view that at least a function of accumulated Ca\(^{2+}\) resides in a pool that is releasable by either IP\(_3\) or GTP. However, it seems clear that the GTP-activatable pool is larger, and hence may include an additional discrete component. This is an important inference with considerable relevance to the data and conclusions presented in the following report (14).

The second area addressed in the present report concerns the nature of the actual translocation process activated by GTP which results in the observed movements of Ca\(^{2+}\). We have concluded from three quite distinct approaches that release of Ca\(^{2+}\) is unlikely to occur by simple fusion of membrane resulting in the release of Ca\(^{2+}\) from a closed organelle. However, it should be pointed out that we do not as yet have definitive proof against the involvement of a fusion process. Indeed, since polyethylene glycol, which promotes the action of GTP, can itself induce membrane fusion, this type of process appeared an attractive hypothesis. However, such fusion which may involve a combination of membrane surface dehydration and bilayer disruption occurs only in the presence of polyethylene glycol concentrations of 25% or higher (29). It should also be pointed out that studies from Pajem and co-workers (30-32) have shown that GTP itself can induce what appears to be fusion between ER and nuclear membranes within cells. However, in these studies the effective GTP levels were approximately three orders of magnitude higher than those shown to be effective in inducing Ca\(^{2+}\) movements in the present studies. Thus, at present it seems more plausible to consider that polyethylene glycol enhances the effectiveness of GTP by promoting a close association between membranes, as has been observed in the present report. As discussed in detail in the following report (14), it is envisaged that GTP activates a conveyance of Ca\(^{2+}\) across and/or between different membranes. Such a model adequately explains most of the observations so far reported by ourselves and others on the movements of Ca\(^{2+}\) induced by GTP.

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