Biochimica et Biophysica Acta, 945 (1988) 185–194
Elsevier

BBA 74185

Characteristics of GTP-mediated microsomal Ca^{2+} release

Suresh K. Joseph, Howard L. Rice and Christopher V. Nicchitta

Department of Biochemistry and Biophysics, University of Pennsylvania School of Medicine, Philadelphia, PA (U.S.A.)

(Received 31 May 1988)

Key words: GTP; Calcium ion release; Inositol trisphosphate; Microsome

Guanosine triphosphate (GTP) can release Ca^{2+} and enhance responses to D-*myo*-inositol 1,4,5-trisphosphate (IP_3) in crude liver microsomes in the presence of polyethylene glycol (PEG) (Dawson et al. (1986) Biochem. J. 234, 311–315). The mechanism of these responses has been further investigated. GTPγS which antagonizes the actions of GTP on microsomes, does not promote Ca^{2+} re-uptake when added after the completion of GTP-mediated Ca^{2+} release. However, the effects of GTP could be reversed by washing or dilution of the microsomes. Addition of PEG to the incubation medium promoted the aggregation of microsomes. Electron microscopy provided no evidence for the fusion of microsomal vesicles in the presence or absence of GTP. In the presence of PEG, GTP produced an alteration of the permeability properties of the microsomal membrane as indicated by increased leakage of an intraluminal esterase, a reduction in the mean buoyant density of the vesicles, and a decrease in the latency of mannose 6-phosphate hydrolysis. All three effects developed relatively slowly, whereas the effects of GTP on Ca^{2+} fluxes occurred more rapidly (complete within 15 min). A low permeability to mannose 6-phosphate was restored upon washing away the GTP. These results suggest that non-specific permeability changes may underly the effects of GTP on Ca^{2+} release and that, under certain conditions, GTP can reversibly modulate the permeability of a transmembrane 'pore' in microsomal membranes that can pass ions and macromolecules. The possibility that such a pore serves to link IP_3-sensitive vesicles with other Ca^{2+}-containing compartments is discussed.

Introduction

Inositol trisphosphate (IP_3) is a messenger molecule which functions to mobilize internal stores of Ca^{2+} in response to certain types of cellular stimuli for reviews, see Refs. 1–3. Its Ca^{2+} mobilizing action can be readily demonstrated in permeabilized cell systems. Subcellular fractions of varying degrees of purity ('microsomes') have been shown to retain responsiveness to IP_3 [4–8]. However, liver microsomes show a negligible response to IP_3 [6,9]. Dawson [10] was the first to observe that incubation of liver microsomes with polyethylene glycol (PEG) and GTP markedly enhanced their responsiveness to IP_3. Subsequently, Gill and colleagues demonstrated that GTP itself induced a rapid release of Ca^{2+} from permeabilized N1E-115 neuroblastoma cells and microsomes derived from this cell line [11,12]. Further work using neuronal cells [13,14], parotid [15] and pituitary [16] microsomes has established

Abbreviations: IP_3, D-*myo*-inositol 1,4,5-trisphosphate; GTPγS, guanosine 5′-*O*-(3-thio)triphosphate; Gpp[NH]p, guanosine 5′-[β,γ-imido]triphosphate; PEG, polyethylene glycol; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; CHAPS, (3-[3-cholamidopropyl)dimethylammonio]-1-propane sulfonate; M-6-*P*, mannose 6-phosphate.

Correspondence: S.K. Joseph, Department of Biochemistry and Biophysics, University of Pennsylvania School of Medicine, Philadelphia, PA 19104, U.S.A.

that GTP- and IP_3-mediated Ca^{2+} release occur via independent mechanisms. A common feature of these effects of GTP on intracellular Ca^{2+} fluxes is that they are specific to GTP and occur despite the presence of a large excess of ATP. Non-hydrolyzable analogs do not mimic the effect of GTP but can inhibit GTP action [10,11]. A second distinctive feature of GTP-mediated Ca^{2+} release is the absolute requirement for PEG observed in isolated microsomes [10,12,15–17] or the stimulatory effect of PEG seen in permeabilized cells [11,12]. In parotid microsomes, PEG can be effectively replaced by albumin or polyvinylpyrrolidone [15].

The nature of the mechanism responsible for the observed effects of GTP and its physiological relevance remain to be determined. Studies on the permeability properties of purified rough liver microsomes have shown that GTP can make such vesicles leaky if adhering ribosomes are first removed [18–20]. These effects were also specific to GTP, occurred at low concentrations of GTP even in the presence of an excess of ATP and were absent in smooth microsomes or in rough microsomes that had not been stripped. Such a non-specific increase in membrane permeability could account for GTP-mediated Ca^{2+} release. Using the degree of latency of mannose 6-phosphate hydrolysis as an index of the leakiness of the microsomal membrane barrier, we have recently provided evidence to support the view that Ca^{2+} release from a stripped rough microsomal preparation is closely correlated with an increased leakiness of the membrane [21]. In this study we have extended these results to crude liver microsomes that have not been subjected to ribosomal stripping. Specifically, we have investigated: (a) the possible relationship between GTP-dependent membrane permeability changes and Ca^{2+} release; (b) the extent to which these changes are reversible; and (c) the effect of PEG on the microsomal membrane.

Experimental

Methods. Liver or insulinoma microsomes were prepared by differential centrifugation as described by Joseph et al. [9]. The isolation buffer contained 0.25 M sucrose, 5 mM Na-Hepes (pH 7.2) and 0.5 mM EGTA. Microsomes from rat fore-brain were prepared by the method of Butler and Morell [22].

Assays. Microsomes (1 mg protein/ml) were incubated at 30 °C in a basic incubation medium that contained 0.15 M sucrose, 50 mM KCl, 20 mM Tris-Hepes (pH 7.2), 3 mM MgATP, 0.3 mM $MgCl_2$, 2.5 μM antimycin A, 2.5 μM Ruthenium red, 20 mM phosphocreatine and 10 U/ml creatine kinase. Ca^{2+} electrode studies were performed in a final volume of 0.2 ml and the electrode traces were calibrated as described by Prentki et al. [23]. Unless otherwise stated $^{45}Ca^{2+}$ flux studies were carried out in the basic assay medium supplemented with 0.5 mM EGTA, 1 μCi $^{45}CaCl_2$/ml and sufficient $^{40}CaCl_2$ to produce a free Ca^{2+} concentration of 140 nM as measured with a Ca^{2+} electrode. After different periods of incubation, 75-μl samples were processed on Millipore filters (0.45 μm, HAWP) using a Hoeffer filtration apparatus. The filters were washed twice with 5 ml of a buffer containing 0.25 M Sucrose, 50 mM KCl and 20 mM Tris-Hepes (pH 7.2) and then counted in Ecolume (ICN Radiochemicals). Mannose 6-[^{32}P]phosphate ([^{32}P]M-6-*P*) was prepared essentially as described by Arion et al. [24] and purified on QAE-Sepharose [21]. Hydrolysis of M-6-*P* was assayed as release of [^{32}P]P_i. Radiolabelled M-6-*P* was separated from [^{32}P]P_i on 0.5-ml columns of Dowex AG1-X8 (formate) anion exchange resin. M-6-*P* was eluted with three 2-ml aliquots of 80 mM ammonium formate/100 mM formic acid/20% (v/v) ethanol and [^{32}P]P_i was eluted with 2 ml of 300 mM ammonium formate/100 mM formic acid/20% (v/v) ethanol. Radioactivity was determined by Cerenkov counting. Esterase activity (carboxyl ester hydrolase; EC 3.1.1.1) was assayed with *o*-nitrophenylacetate as substrate [25]. Low concentrations of protein were assayed with a commercial protein assay (Pierce Co.) using a protein standard (Sigma). The concentration of protein in the microsomal stocks was determined by a biuret procedure [26]. RNA was determined by the method of Fleck and Munro [27]. Electron microscopy of several, randomly selected, thin-sections of microsomal pellets were examined after sedimenting aliquots of the microsomal suspension at 100000 × *g* and fixing with a

solution of 2% (v/v) glutaraldehyde in 0.1 M sodium cacodylate buffer.

Results

Comparison of GTP effects on microsomes from liver, insulin-secreting tumor and rat brain

In agreement with the initial observations of Dawson [10], Fig. 1A shows that Ca^{2+} accumulated by liver microsomes can be released by GTP only when PEG was included in the medium. This release was slow and occurred after a short delay (Fig. 1A, trace iv). When this release had completed, the further addition of IP_3 initiated a rapid Ca^{2+} release. IP_3 added before GTP did not produce Ca^{2+} release. However, the addition of GTP after IP_3 provoked a rapid Ca^{2+} release that occurred without a noticeable lag (Fig. 1A, trace iii). The total Ca^{2+} released under these conditions approximates the sum of Ca^{2+} released by GTP and IP_3 separately. GTPγS added prior to GTP blocked the ability of GTP to release Ca^{2+} and to render the microsomes responsive to IP_3 (data not shown). Fig. 1B shows a similar set of experiments performed on microsomes isolated from an insulin-secreting tumor. In this case GTP also induced a slow release of Ca^{2+} but only in the presence of PEG. However, the responsiveness of tumor microsomes to IP_3 was unaffected by GTP or PEG. This was not due to the presence of endogenous GTP as indicated by HPLC analysis of perchloric acid extracts (data not shown). Microsomes from rat brain also showed an IP_3 response that did not require GTP or PEG (Fig. 1C). In contrast to microsomes from liver or insulinoma, GTP did not cause Ca^{2+} release from brain microsomes in the presence or absence of PEG.

Reversibility of the responses to GTP

It has been shown that the addition of GDP to permeabilized N1E-115 cells results in a re-accumulation of Ca^{2+} released by GTP [11]. This was accomplished by adding ADP to block the conversion of GDP to GTP catalyzed by contaminating nucleoside diphosphokinase activity. In our hands

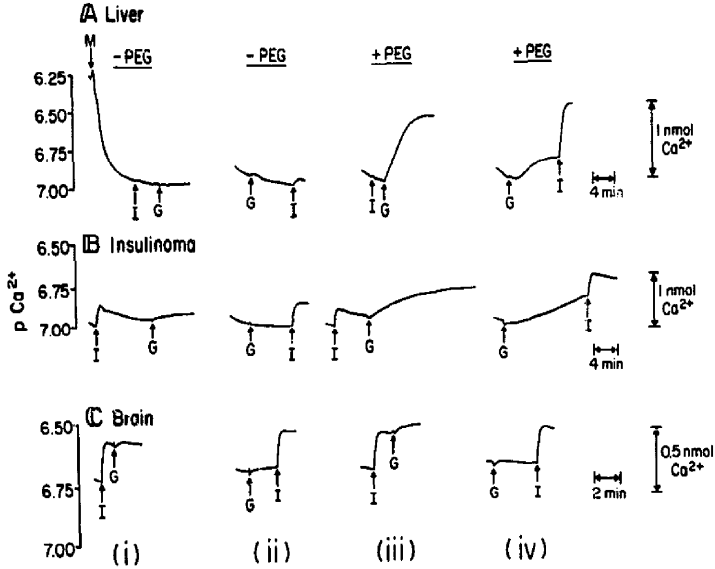

Fig. 1. Characteristics of Ca^{2+} release from rat liver (A), Syrian hamster insulinoma (B) and rat brain (C) microsomes. Microsomes were prepared from the three tissues by differential centrifugation and incubated at a final concentration of 1 mg protein/ml in the basic incubation medium described in the Experimental section. Changes in Ca^{2+} were registered with a Ca^{2+} electrode. IP_3 (I) and GTP (G) were added at 10 μM and 50 μM, respectively. PEG (M_r = 8000) was added at a final concentration of 3% (w/v).

ADP was ineffective in inhibiting this conversion in liver microsomes (data not shown) and therefore this experimental approach could not be used. Consequently, adding GTPγS (an inhibitor of the Ca^{2+} release process) or removing added GTP by washing or dilution were examined as alternative procedures for reversing GTP effects on Ca^{2+} permeability. Results using the first of these approaches are shown in Fig. 2. In this experiment GTP (10 μM) was added after microsomes had accumulated $^{45}Ca^{2+}$ for 10 min. As observed with the Ca^{2+} electrode, $^{45}Ca^{2+}$ was released after a short lag (Fig. 2, open circles). Addition of a 10-fold excess of GTPγS during the lag phase inhibited the Ca^{2+} releasing effect of GTP. GTPγS added during Ca^{2+} release halted the process within 1 min but the released Ca^{2+} was not re-accumulated. Failure to re-accumulate Ca^{2+} was also observed when the GTPγS was added after the release had completed. Experiments were performed to monitor the binding of 10 μM [α-^{32}P]GTP under the conditions used in Fig. 2 (data not shown). These results indicated that GTPγS added at 25 min displaced 85% of the bound label within 10 min. From these data we conclude that the GTP-mediated increase in Ca^{2+} permeability can be halted, but not reversed, when GTPγS displaces GTP from a binding site in the microsomal membrane. Restoration of the original Ca^{2+} permeability of the microsomes may require that this binding site be empty or occupied by GDP.

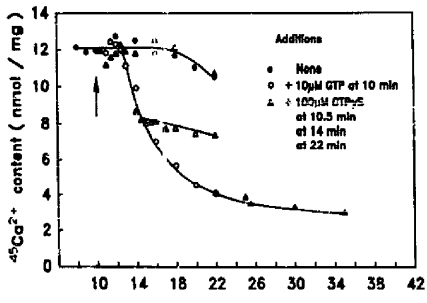

Fig. 2. The effect of GTPγS on GTP-mediated Ca^{2+} release. Liver microsomes were incubated in the medium described in Fig. 1 supplemented with PEG and an EGTA/Ca^{2+} buffer adjusted to buffer the free [Ca^{2+}] at 140 nM. The uptake of $^{45}Ca^{2+}$ was allowed to proceed to steady state and at the arrow GTP (10 μM) was added to promote Ca^{2+} release. GTPγS (100 μM) was added at various times after GTP.

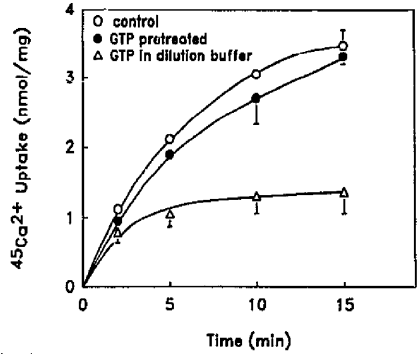

Fig. 3. Reversal of the GTP responses by dilution. Liver microsomes were incubated in the presence (●———●) or absence (○———○) of GTP (5 μM) at 10 mg protein/ml in the basic incubation medium used in Fig. 1 supplemented with PEG (3%; w/v) and EGTA (0.5 mM). After 15 min the microsomes were diluted 50-fold in the same medium without GTP (●———●, ○———○) or containing 5 μM GTP (Δ———Δ). 10 min later $^{45}Ca^{2+}$ was added to bring the free Ca^{2+} concentration to 140 nM and uptake was monitored by a filtration assay. The data shown are the mean ± S.E. of triplicate determinations from a representative experiment.

The alternative approach of using dilution to remove added GTP is illustrated in Fig. 3. Microsomes were first pretreated in the absence of external Ca^{2+} with 5 μM GTP for 15 min followed by a 50-fold dilution to reduce the GTP concentration to 0.1 μM GTP, a value below the $K_{0.5}$ for Ca^{2+} release (0.8 μM) [11]. The permeability of the vesicles was assessed after 10 min by measuring the ability to sequester added Ca^{2+}. Vesicles that were exposed to GTP during both the preincubation and dilution steps showed an increased Ca^{2+} permeability, as judged by a slower rate and extent of $^{45}Ca^{2+}$ accumulation (Fig. 3, triangles). However, vesicles that were preincubated with GTP and diluted into a GTP-free medium showed a Ca^{2+} permeability that was similar to control vesicles (Fig. 3, closed vs. open circles).

In another series of experiments (Fig. 4), microsomes were pretreated with GTP in the absence of Ca^{2+} and subjected to two sequential washing steps to remove the added GTP. Permeability to Ca^{2+}, as measured by $^{45}Ca^{2+}$ uptake, was clearly altered by GTP when measured at the end of a 15

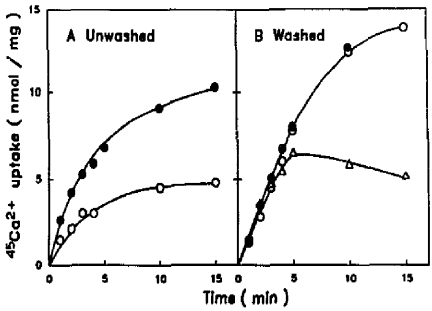

Fig. 4. Reversal of the GTP responses by washing. Liver microsomes (10 mg protein/ml) were incubated in 1.5 ml basic incubation medium supplemented with 3% PEG (w/v) and EGTA (0.5 mM). After incubation for 15 min at 30°C in the absence (●——●) or presence (○——○) of 50 μM GTP. (A) 75-ml aliquots from each condition were diluted 10-fold into the same medium containing $^{45}Ca^{2+}$ and sufficient $^{40}Ca^{2+}$ to produce a free Ca^{2+} concentration of 140 nM. (B) The remaining microsomes were diluted with 20 ml of microsome isolation buffer and washed twice after centrifugation at 65000 × g. The pellets were resuspended and assessed for $^{45}Ca^{2+}$ uptake as described for (A). ●——●, control washed; ○——○, GTP washed; △——△, 50 μM GTP added to GTP washed.

min incubation period (Fig. 4A). However, the Ca^{2+} permeability of washed GTP-pretreated microsomes was identical to control microsomes that had not been incubated with GTP (Fig. 4B). Exposure of the washed, GTP-treated microsomes to a second addition of GTP resulted in an inhibition of Ca^{2+} accumulation after a prolonged lag (Fig. 4B), indicating that washing alone had not removed the GTP-responsiveness of the microsomes. The data shown in Figs. 3 and 4, taken together, suggest that GTP mediates a reversible change in the Ca^{2+} permeability of crude microsomes.

Morphological changes produced by PEG and GTP

It has been reported that the fusion of rough liver microsomes, stripped of adhering ribosomes, can be promoted by incubation with 0.5 mM GTP for 120 min [28,29]. Since PEG is also a membrane fusogen, the possibility that vesicle fusion may underly some of the effects observed in crude microsomal preparations was examined. However, electron microscope analysis of thin sections prepared from microsomal pellets obtained after incubation under various conditions for 15 min gave no evidence for changes in vesicle size produced by PEG, in the presence or absence of GTP (data not shown). Nevertheless, several lines of evidence suggest that PEG does increase the aggregation state of the microsomes. Firstly, the addition of microsomes to a PEG-containing medium produces a light-scattering change that is consistent with an increased aggregation state of the vesicles [30]. It should be pointed out that the aggregates formed in the presence of PEG are sufficiently large as to be directly visualized by phase contrast microscopy (Thomas, A.P., personal communication) or negative contrast electron microscopy [31].

The possibility that GTP may exert additional effects on vesicle structure was explored by measuring the buoyant density of microsomes on Percoll gradients after incubation under various conditions. Vesicles incubated in the presence of PEG migrated as a relatively sharp band (Fig. 5A) compared to vesicles incubated in the absence of PEG (Fig. 5B); a result consistent with PEG promoting an increased aggregation of vesicles. The inclusion of PEG in the Percoll gradient and in the microsomal incubation medium increased the sharpness of the band while only slightly altering the shape of the density gradient formed at equilibrium, as determined with calibrated density marker beads (Fig. 5A). It was observed that GTP shifted this band to a lower mean density but only in the presence of PEG (Fig. 5A vs. 5B). This effect required the addition of GTP to the incubation medium only. In these experiments EGTA (0.5 mM) was present in the incubation medium and the Percoll gradients, thereby excluding the possibility that loss of intravesicular Ca^{2+} could account for the decrease in density promoted by GTP. The density shift could also not be ascribed to a GTP induced loss of ribosomes from the microsomal membranes since direct measurements of RNA indicated no significant differences in ribosomal content (data not shown). The vesicles corresponding to the center of the protein band was removed, sedimented and fixed for electron microscopic analysis. Again no vesicle fusion was observed even after 1 h incubation with GTP and PEG, conditions which yielded a pronounced decrease in vesicle density.

Further characteristics of this effect are described in Table I where the position of the center

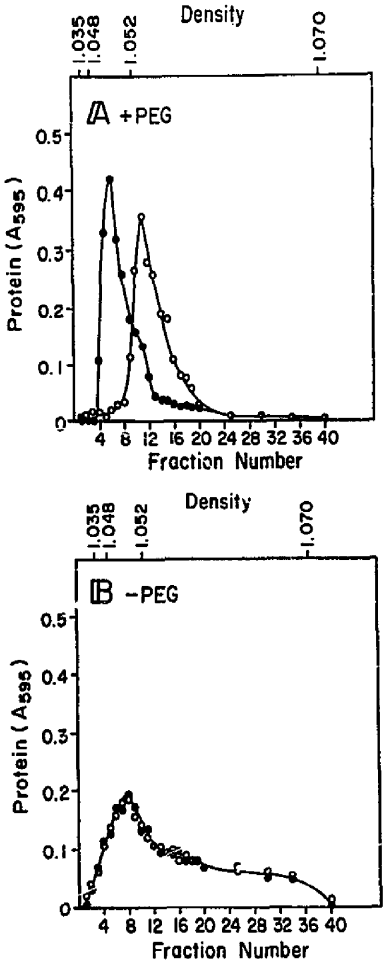

Fig. 5. GTP decreases the mean buoyant density of the microsomal vesicles. Liver microsomes were incubated at 2.5 mg protein/ml in the medium described in Fig. 1 supplemented with 0.5 mM EGTA in the presence (●——●) or absence (○——○) of GTP (50 μM) After 30 min incubation 0.8 ml aliquots were mixed with 10 ml Percoll (30%; v/v) containing 0.25 M sucrose, 0.5 mM EGTA and 10 mM Tris-Hepes (pH 7.2). The tubes were centrifuged at 30600 × *g*, fractionated and assayed for protein. The experiment was run with PEG in the incubation medium and Percoll gradient (Panel A) or in the absence of PEG (Panel B).

of the band has been expressed as a ratio of the distance from the meniscus to the total length of the gradient. The density shift produced by GTP could not be produced by GTPγS which did, however, block the effect of GTP. Gpp[NH]p was less effective as an inhibitor than GTPγS, in agreement with its relatively poor ability to block GTP-mediated Ca^{2+} release [11]. Incubation of the microsomes with GTP at 4°C failed to induce the density shift suggesting that hydrolysis of the nucleotide is involved. A marked temperature dependence for GTP-mediated Ca^{2+} release has already been demonstrated [11,15]. Finally, the difference in density of the GTP-treated microsomes was retained even after the GTP was removed by washing (Table I, Expt. 3). The irreversible nature of the effect of GTP, in contrast to its effect on Ca^{2+} permeability, raises the possibility that the GTP-induced density shift may result from the loss of intravesicular components. To test this possibility the leakage of esterase, an intraluminal enzyme [25] was measured (Fig. 6). Microsomes incubated in the presence of PEG and sedimented showed no significant increase in enzyme activity in the supernatant. The inclusion of GTP, however, produced a rapid and sustained leakage of

TABLE I

CHARACTERISTICS OF THE DENSITY SHIFT INDUCED BY GTP

Experiments were performed using the experimental conditions for Fig. 6 (Panel A). The position of the center of the protein band was expressed as a ratio of the distance from the meniscus to the total length of the gradient.

	R_F $\frac{\text{(distance from meniscus)}}{\text{(total length of gradient)}}$
Expt. 1	
Control	0.15
GTP (10 mM)	0.08
GTPγS (10 mM)	0.17
GTP + GTPγS (100 mM)	0.17
GTP + Gpp[NH]p (100 mM)	0.11
Expt. 2	
Control; 30°C	0.08
GTP 30°C	0.05
Control; 4°C	0.11
GTP; 4°C	0.11
Expt. 3	
Control	0.11
GTP	0.08
Washed control	0.09
Washed GTP	0.06

the enzyme which was maintained over a period of one hour.

Hydrolysis of mannose 6-phosphate (M-6-*P*) by microsomal glucose-6-phosphatase is limited by the slow rate of transport of M-6-*P* across the microsomal membrane [24]. Increased leakiness of the microsomal vesicles therefore results in a stimulation of M-6-*P* hydrolysis. We have recently shown that GTP increases M-6-*P* hydrolysis by stripped rough microsomes and has a similar effect on crude microsomes but only in the presence of PEG [21]. The latency of M-6-*P* hydrolysis, measured using 0.2% (w/v) CHAPS to determine total mannose-6-phosphatase activity, was increased more than 3-fold upon incubation of microsomes with 50 μM GTP for 60 min (Control, $10.1 \pm 0.7\%$; GTP, $36.9 \pm 2.7\%$; $n = 3$). In order to determine if this change was reversible, the GTP-treated microsomes were washed twice to remove the added GTP and their ability to hydrolyse added M-6-*P* was again measured. The change in

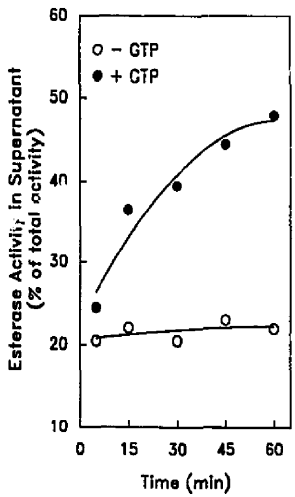

Fig. 6. GTP induces the release of an intraluminal enzyme (esterase). Microsomes were incubated in a medium containing PEG and EGTA in the presence (●——●) or absence (o——o) of 50 μM GTP. At the indicated times aliquots were removed and the aggregated microsomes centrifuged at 8000×*g* [30]. The supernatant was assayed for esterase activity (*o*-nitrophenylacetate as substrate) and protein. Enzyme activity was expressed as a percentage of the total activity measured in an uncentrifuged sample assayed in the presence of 0.2% (v/v) Triton X-100.

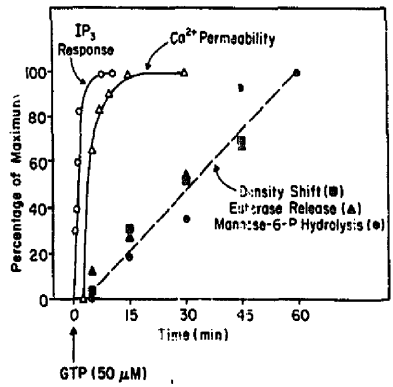

Fig. 7. Comparison of the time courses of GTP effects on crude liver microsomes. The time course of the GTP-induced increase in Ca^{2+} permeability was assessed by the $^{45}Ca^{2+}$ method used in Fig. 3 with the modification that 0.5 mM GTPγS was added with the $^{45}Ca^{2+}$ to quench the action of GTP (cf. Fig. 2). GTPγS was also added to the microsomes prior to measurement of the density shift or the hydrolysis of ^{32}P-labelled M-6-*P*. Hydrolysis of 5 mM labelled M-6-*P* (spec. act. 250 cpm/nmol) was measured over a 30 min interval. Acquisition of the IP_3 response was measured with a Ca^{2+} electrode as shown in Fig. 1.

M-6-*P* hydrolysis produced by GTP was not retained after washing (Control, $14.3 \pm 6.0\%$; GTP, $14.9 \pm 2.7\%$).

The temporal relationships of several of the parameters linked to microsomal permeability changes produced by GTP have been quantified in Fig. 7. Stimulation of M-6-*P* hydrolysis, esterase release and associated density shift all occurred over a similar, but slow time-course. The effect of GTP on Ca^{2+} permeability was more rapid being complete within 15 min. The most rapid effect of GTP was to enhance microsomal responsiveness to IP_3, a significant IP_3 effect was observed within 15 s of GTP addition.

Discussion

The present study and previously published data [11–15] clearly indicate that GTP has profound and specific effects on the permeability properties of endoplasmic reticulum (ER) membranes. The removal of ribosomes from purified rough ER vesicles has been shown to render them particularly susceptible to GTP-induced mem-

brane permeability changes. These include a decreased latency of M-6-*P* hydrolysis, increased core glycosylation of proteins from exogenously added precursors, and the release of intraluminal esterase and nucleoside diphosphatase activity into the incubation medium [17–19]. Ca^{2+} accumulated by stripped rough ER is also released by GTP [20]. The present data show that GTP can produce the same spectrum of permeability changes in ER membranes that have not been subjected to ribosomal stripping, provided PEG is present in the incubation medium. Therefore, the effects of GTP and PEG on Ca^{2+} release seen in several microsomal systems are, in all probability, the result of a non-specific increase in membrane permeability, rather than an activation of a specific Ca^{2+} release system.

The available data (Refs. 30, 31; Fig. 5) suggests that PEG is required to facilitate aggregation of the vesicles and that aggregation acts in the same way as the removal of ribosomes to unmask a mechanism that couples GTP hydrolysis to permeabilization of the ER membrane. The resulting changes affect Ca^{2+} permeability more rapidly (Fig. 7) than other parameters linked to disruption of the membrane barrier, e.g. mannose-6-phosphatase latency, esterase release. All of these changes are specific to GTP and are inhibited by GTPγS suggesting that they are all initiated by a common mechanism. The disparity in the time-courses of the Ca^{2+} permeability change and the other parameters may imply that a small increase in permeability is sufficient to dissipate Ca^{2+} gradients but that progressively bigger changes are required to allow esterase leakage or increased mannose -6-phosphate permeability. The different time-courses could also arise if the individual effects were occurring at different rates in discrete vesicle sub-populations.

The mechanism by which GTP induces the permeability change remains unknown. Hypotheses that have been suggested include GTP-dependent protein phosphorylation [17] or the GTP-dependent synthesis of phosphatidic acid (a Ca^{2+} ionophore) from endogenous 1,2-diacylglycerol [32]. A third possibility, originally suggested by Paiement et al. [29], is that GTP hydrolysis activates a pore lying underneath the ribosomal complex which normally functions to transfer nascent polypeptide chains into the ER lumen for subsequent processing. Evidence pointing to a specific involvement of GTP in the insertion of membrane proteins into the ER membrane has been obtained using in vitro assays of protein translocation [33,34]. GTP-binding proteins are present in intracellular membranes [35], and this family of proteins has been proposed to be constitutively involved in the normal translocation [36] and processing of proteins from the ER to the Golgi stack [37,38]. Whichever hypotheses proves to be correct, it is clear that the action of GTP on the ER may be related to processes quite distinct from cellular Ca^{2+} homeostasis.

GTP and IP_3-mediated Ca^{2+} release

GTP (in the presence of PEG) greatly enhances the responsiveness of liver microsomes to IP_3. It should be stressed that this is not a universal phenomena (Fig. 1); responses to GTP and IP_3 are independent of each other in several experimental systems (Fig. 1; Refs. 12, 14–16, 39). Recent studies by Henne et al. [40] on parotid microsomes have demonstrated that the two processes actually reside in different membrane fractions. In liver microsomes, it is possible that the preparative procedure yields membrane vesicles that have an IP_3-mediated Ca^{2+} release system but lack a means of accumulating Ca^{2+}. IP_3-mediated release would be facilitated when vesicles containing the release system interact with other vesicles containing Ca^{2+}. Transfer of Ca^{2+} from one type of vesicle to another could occur as a result of fusion of the vesicles. Alternatively, the GTP induced permeability change could be involved in transferring Ca^{2+} between vesicles if the vesicles were in close contact. Dawson and coworkers have provided experimental support for a model involving membrane fusion by showing that: (a) large multilamellar vesicles form within 1 min of incubation in the presence of GTP and PEG [30]; (b) that the same incubation conditions promote the intervesicular transfer of a fluorescence label [41] and (c) that the effects of GTP cannot be reversed either by extensive washing or by GTPγS [30]. In contrast our experiments show no evidence of fusion and the effects of GTP were reversed by washing. Since the functional effects of GTP on Ca^{2+} release were the same in both studies, it can be

concluded that fusion is not a prerequisite event in the mechanism enabling GTP to release Ca^{2+} or to enhance IP_3 responsiveness. A secondary role for GTP-induced membrane fusion cannot be presently excluded. The reversibility of GTP responses and the absence of vesicle fusion has also been noted by Gill and colleagues in microsomes prepared from a neuronal cell line [42]. In addition to demonstrating reversibility of GTP effects on Ca^{2+} permeability, we have shown that the effects of the nucleotide on M-6-*P* permeability are also reversible. These results support the proposal that GTP hydrolysis may regulate the permeability of a transmembrane 'pore' in microsomal membranes. It can be inferred that the size of such a pore must be large to allow the passage of esterases whose molecular weights are in the range of 57–60 kDa [43].

At present, it is difficult to ascribe any physiological significance to the effects of GTP described above. Clearly, in the intact cell the cytosolic GTP must be prevented from permeabilizing the endoplasmic reticulum in the manner observed for isolated microsomes. Indeed, GTP added directly to saponin-treated hepatocyte does not alter the hydrolysis of added M-6-*P* or promote Ca^{2+} release (data not shown). However, Thomas [44] has recently shown that GTP does increase the amount of Ca^{2+} in the IP_3-releasable compartment of the hepatocyte. Mullaney et al. [31] have also provided evidence that GTP facilitates the transfer of Ca^{2+} between different compartments of a neuronal cell. These studies raise the intriguing possibility that GTP may function in the cell to maintain linkages between various IP_3-sensitive and -insensitive compartments.

Acknowledgements

We would like to acknowledge Bob Smith and the electron microscopy core facility of the Diabetes Center for their assistance. John Williamson is acknowledged for providing encouragement, criticism and the facilities to carry out this work. Financial support was provided by NIH grant DK-34804 (S.K.J.). S.K.J. is the recipient of a Career Development Award from the Juvenile Diabetes Foundation International.

References

1 Berridge, M.J. and Irvine, R.F. (1984) Nature 312, 315–321.
2 Williamson, J.R. (1986) Hypertension 8, 140–156.
3 Putney, J.W. (1987) Am. J. Physiol. 252, G149–G157.
4 Streb, H., Bayerdorffer, E., Haase, W., Irvine, R.F. and Schulz, I. (1984) J. Membr. Biol. 81, 241–243.
5 Prentki, M., Biden, T.J., Janjic, D., Irvine, R.F., Berridge, M.J. and Wollheim, C.B. (1984) Nature 309, 562–564.
6 Joseph, S.K., Williams, R.J., Corkey, B.E., Matschinsky, F.M. and Williamson, J.R. (1984) J. Biol. Chem. 259, 12952–12955.
7 O'Rourke, F., Halenda, S.P., Zavoico, G.B. and Feinstein, M. (1985) J. Biol. Chem. 260, 956–962.
8 Clapper, D.L. and Lee, H.C. (1985) J. Biol. Chem. 260, 13947–13954.
9 Joseph, S.K., Thomas, A.P., Williams, R.J., Irvine, R.F. and Williamson, J.R. (1984) J. Biol. Chem. 259, 3077–3081.
10 Dawson, A.P. (1985) FEBS Lett. 185, 147–150.
11 Gill, D.L., Ueda, T., Chueh, S.-H. and Noel, M.W. (1986) Nature 320, 461–464.
12 Ueda, T., Chueh, S.-H., Noel, M.W. and Gill, D.L. (1986) J. Biol. Chem. 261, 3184–3192.
13 Chueh, S.-H. and Gill, D.L. (1986) J. Biol. Chem. 261, 13883–13886.
14 Jean, T. and Klee, C.B. (1986) J. Biol. Chem. 261, 16414–16420.
15 Henne, V. and Söling, H.-D. (1986) FEBS Lett. 202, 267–273.
16 Kiesel, L., Lukacs, G.L., Eberhardt, I., Runnebaum, B. and Spät, A. (1987) FEBS Lett. 217, 85–88.
17 Dawson, A.P., Comerford, J.G. and Fulton, D.V. (1986) Biochem. J. 234, 311–315.
18 Godelaine, D.H., Beufay, H. and Wibo, M. (1977) Proc. Natl. Acad. Sci. USA 74, 1095–1099.
19 Godelaine, D.H., Beufay, H., Wibo, M. and Amar-Costesec, A. (1979) Eur. J. Biochem. 96, 17–26.
20 Godelaine, D.H., Beufay, H., Wibo, M. and Ravset, A.M. (1983) J. Cell. Biol. 97, 340–350.
21 Nicchitta, C.V., Joseph, S.K. and Williamson, J.R. (1987) Biochem. J. 248, 741–747.
22 Butler, M. and Morell, P. (1982) J. Neurochem. 39, 155–164.
23 Prentki, M., Janjic, D. and Wollheim, C.B. (1983) J. Biol. Chem. 258, 7597–7602.
24 Arion, W.J., Wallin, B.K., Carlson, P.W. and Lange, A.J. (1972) J. Biol. Chem. 247, 2558–2565.
25 Beufay, H., Amar-Costesec, A., Feytmans, E., Thines-Sempoux, D., Wibo, M., Robbi, M. and Berthet, H. (1974) J. Cell. Biol. 61, 188–200.
26 Gornall, A.G., Bardawill, C.J. and David, M.M. (1949) J. Biol. Chem. 177, 751–766.
27 Fleck, A. and Munro, H.N. (1962) Biochim. Biophys. Acta 55, 571–583.
28 Paiement, J., Beufay, H. and Godelaine, D. (1980) J. Cell. Biol. 86, 29–37.
29 Paiement, J., Rindress, D., Smith, C.E., Poliquin, L. and Bergeron, J.J.M. (1987) Biochim. Biophys. Acta 898, 6–22.

30 Dawson, A.P., Hills, G. and Comerford, J.G. (1987) Biochem. J. 244, 87–92.
31 Mullaney, J.M., Cheuh, S.-H., Ghosh, T.K. and Gill, D.L. (1987) J. Biol. Chem. 262, 13865–13872.
32 Jean, T. (1988) in Hormones and Cell Regulation, Vol. 12 (Nunez, T. and Carafoli, E., eds.), Inserm Paris/John Libbey, in press.
33 Connolly, T. and Gilmore, R. (1986) J. Cell. Biol. 103, 2253–2261.
34 Hoffman, K.E. and Gilmore, R. (1988) J. Biol. Chem. 263, 4381–4385.
35 Cosin, J., Kimura, S. and Kraus-Friedmann, N. (1988) Biochem. Biophys. Res. Commun. 150, 848–852.
36 Robinson, A. and Austen, B. (1987) Biochem. J. 246, 249–261.
37 Segev, N., Mulholland, J. and Botstein, D. (1988) Cell 52, 915–924.
38 Melancon, P., Glick, B.S., Malhotra, V., Weidman, P.J., Serafini, T., Gleason, M.L., Orci, L. and Rothman, J.E. (1987) Cell 51, 1053–1062.
39 Wolf, B.A., Florholmen, J., Colca, J.R. and McDaniel, M.L. (1987) Biochem. J. 242, 137–141.
40 Henne, V., Piiper, A. and Söling, H.-D. (1987) FEBS Lett. 218, 153–158.
41 Comerford, J.G. and Dawson, A.P. (1988) Biochem. J. 249, 89–93.
42 Chueh, S.H., Mullaney, J.M., Ghosh, T.K., Zachary, A.L. and Gill, D.L. (1987) J. Biol. Chem. 262, 13857–13864.
43 Marks, A.S., Rosenfeld, M.G., Sabatini, D.D. and Kreibich, G. (1986) J. Cell. Biol. 102, 65a (abstr.).
44 Thomas, A.P. (1988) J. Biol. Chem. 263, 2704–2711.