Guanosine 5′-triphosphate releases calcium from rat liver and guinea pig parotid gland endoplasmic reticulum independently of inositol 1,4,5-trisphosphate

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GTP releases calcium from rat liver microsomes and guinea pig parotid gland microsomal subfractions independently of the presence of inositol 1,4,5-trisphosphate (IP_3). Non-hydrolyzable guanine nucleotide analogues have no effect and inhibit the effect of GTP. The mechanism of GTP-mediated calcium release differs from IP_3-mediated calcium release as indicated by the following findings: (i) GTP-induced calcium release depends on the presence of compounds which increase the viscosity of the medium (polyethylene glycol, polyvinylpyrrolidone, or bovine serum albumin); (ii) GTP-mediated calcium release is much slower; (iii) GTP-mediated calcium release is strongly temperature-dependent, whereas IP_3-mediated calcium release is not; (iv) GTP-mediated calcium release is much more sensitive to a decrease of intravesicular free calcium than IP_3-mediated calcium release.

Calcium release *GTP* *Inositol 1,4,5-trisphosphate* *Microsome* *(Parotid gland, Liver)*

1. INTRODUCTION

Many receptor-mediated effects involve the release of calcium from intracellular stores [1]. It is assumed that the trigger for this process is inositol 1,4,5-trisphosphate (IP_3) [2–13]. Recent studies [14–18] indicate that GTP might be involved in the receptor-mediated activation of a phosphodiesterase which splits phosphatidylinositol 4,5-bisphosphate into diacylglycerol and IP_3. Dawson [19] reported that rat liver endoplasmic reticulum (ER) vesicles were almost insensitive to IP_3 with respect to calcium release, but became responsive in the presence of GTP. This effect of GTP seemed to require hydrolysis of GTP and possibly transfer of the γ-phosphate group from GTP onto protein(s) [20]. In contrast, we show here that GTP catalyzes calcium release from hepatic as well as from parotid gland ER vesicles in an IP_3-independent manner and that the mechanism of 5′-GTP-mediated calcium release differs from that mediated by IP_3. Consequently, GTP may be involved not only indirectly in the regulation of intracellular free calcium via control of IP_3 formation, but also by directly affecting release of calcium from intracellular pools.

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2. MATERIALS AND METHODS

2.1. *Materials*

Male SPF Wistar rats (180–200 g body wt) and male guinea pigs of the Pirbright White strain (200–250 g body wt) were obtained from Mus-Rattus (Brunnthal, FRG) and Winkelmann (Dernbach, FRG), respectively.

GTP (type III, order no. G 5881, lot no.74 F-7275) was obtained from Sigma (Munich). It was almost pure on the basis of reversed-phase HPLC. Material taken directly from the HPLC-separated GTP peak had the same activity with respect to calcium release as the unpurified Sigma preparation. HPLC-purified guanosine 5′-*O*-

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(3-thiotriphosphate) (GTPγS) and γ-phenyl-GTP were kindly provided by Professor Fritz Eckstein (Max Planck Institut für Exp. Medizin, Göttingen, FRG). All other guanine nucleotides and their analogues were purchased from Boehringer Mannheim (Mannheim). All other chemicals were of the highest purity available and came from E. Merck (Darmstadt).

2.2. *Methods*

2.2.1. Release of calcium from isolated rat liver microsomes

Rat liver was homogenized with a 4-fold volume of 0.3 M sucrose, 20 mM Hepes-KOH, pH 7.2. After centrifugation for 10 min at 1000 × *g*, the supernatant was spun for 10 min at 12000 × *g*. The 12000 × *g* supernatant was spun for 60 min at 100000 × *g*, the sediment (microsomes) resuspended in the homogenization medium to give about 20 mg protein/ml and used for calcium release experiments. Microsomes (1 mg protein) were incubated at 37°C in 1 ml of the following medium (final concentrations): KCl, 130 mM; $MgCl_2$, 2 mM; K phosphate, 2 mM; Hepes-KOH, 20 mM (pH 7.0); ATP, 2.5 mM; creatine phosphate, 5 mM; creatine kinase, 40 U/ml; benzamidine, 1 mM; polyethylene glycol (M_r 8000), 3% (w/v), and NaN_3 10 mM to block interference with mitochondrial calcium metabolism. The concentration of free Ca^{2+} was monitored with a Ca^{2+}-selective electrode which was prepared and calibrated according to [28] using the neutral carrier ETH 1001. The reactions were started by addition of the microsomes to the medium. When the calcium uptake reaction had proceeded to a new steady state calcium release was initiated as described in the figure legends.

2.2.2. Release of calcium from 'light' and 'heavy' microsomes prepared from guinea pig parotid gland acini

Acini were prepared according to [29] and washed 3 times with the following medium (final concentrations): KCl, 130 mM; $MgCl_2$, 2 mM; K phosphate, 2 mM; Hepes-KOH, 20 mM; EGTA, 0.5 mM; dithiothreitol, 1 mM; benzamidine, 1 mM. They were homogenized in the same medium by 5 strokes in a Potter glass/glass homogenizer. The 1000 × *g* supernatant was spun for 10 min at 12000 × *g* and the resulting pellet (heavy microsomes) resuspended in the washing medium without EGTA. The supernatant was spun for 60 min at 100000 × *g*, and the pellet (light microsomes) resuspended in the same medium as the 12000 × *g* pellet. The medium in the electrode chamber and the performance of the calcium release measurements were as given for the studies with rat liver microsomes.

3. RESULTS AND DISCUSSION

Isolated rat liver ER vesicles which have accumulated calcium in the presence of ATP respond to the addition of GTP alone with a concentration-dependent calcium release (fig.1a). This effect requires the presence of polyethylene glycol (PEG) (see below), but not of IP_3.

Isolated guinea pig parotid gland ER vesicles exhibit a dose-response relationship similar to that of rat liver ER vesicles and saponin-permeabilized guinea pig parotid acini display a dose-dependent calcium release which can already be observed at 10 μM (not shown). Although the free calcium concentration reached at steady state in the presence of ATP is similar for heavy microsomes (12000 × *g* pellet) and light microsomes (100000 × *g* pellet), namely about 0.2 μM, the amount of calcium released in the presence of GTP is considerably higher with heavy microsomes as compared to light microsomes (fig.1b). The GTP effect required the presence of PEG (fig.1c). This does not seem to be a specific effect of PEG, as other compounds increasing viscosity such as albumin or polyvinylpyrrolidone (PVP) exerted a similar effect (fig.1c). Irrespective of the amount of calcium released, the initial velocity of IP_3-mediated calcium release was always faster than that of GTP-induced calcium release (fig.3b). The effect of GTP on calcium release is rather specific: The following compounds were without any measurable effect on calcium release: 5′-GDP (up to 100 μM); GMP-PNP (up to 100 μM); GTPγS (up to 100 μM); GDPβS (up to 100 μM); 3′,5′-cGMP (up to 500 μM); γ-phenyl-GTP (up to 100 μM); 5′-CTP (up to 500 μM); 5′-UTP (up to 500 μM). Only 5′-ITP had a slight effect at concentrations above 200 μM. Analysis of the purity of the compound by HPLC, however, revealed that this compound contained substantial amounts of 5′-GTP (not shown). Both GTPγS (fig.2a) as

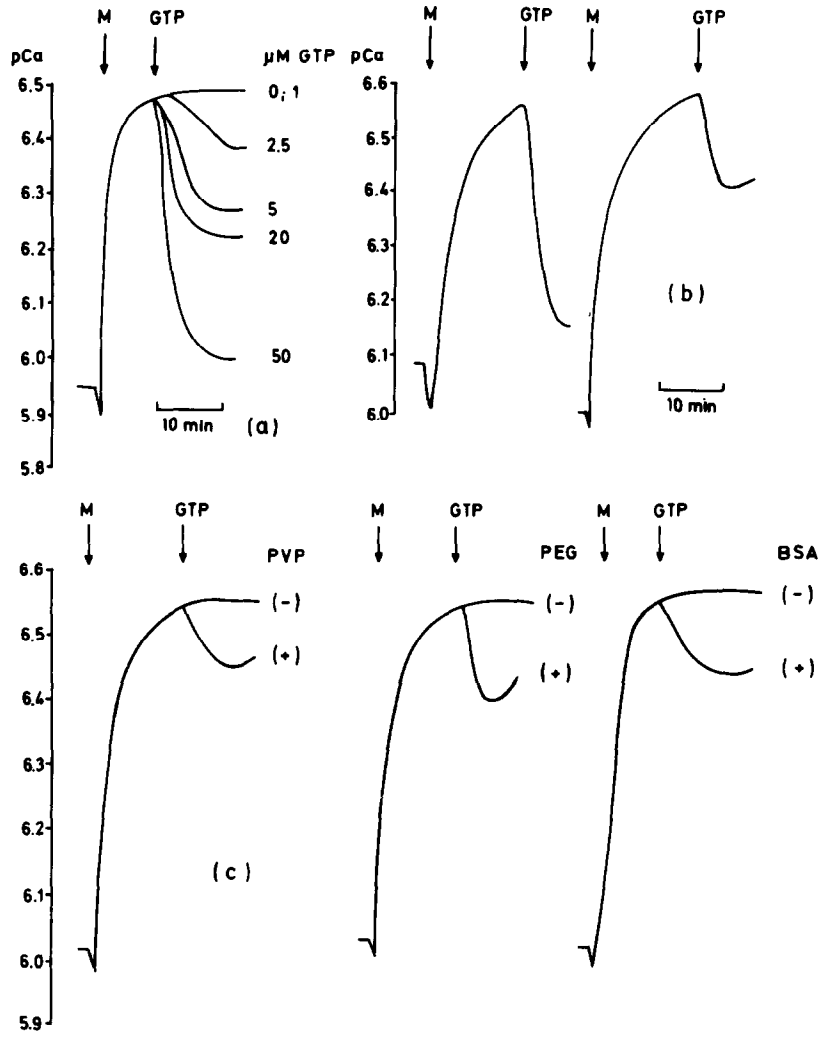


Fig.1. (a) GTP-mediated release of calcium from rat liver microsomes. Microsomes were isolated and loaded with calcium as given in section 2. Microsomes were added at (M) followed by the indicated concentrations of GTP. (b) Effects of addition of GTP (final concentration 5 μM) on calcium release from heavy (left) or light (right) microsomes prepared from isolated guinea pig parotid gland acini. The microsomal fractions were added at (M). The amount of protein added was 350 μg for the heavy as well as for the light microsomal fractions. Preparation of acini and microsomal fractions is described in section 2. (c) Effects of PVP, PEG (M_r 8000), or bovine serum albumin (BSA) on GTP-mediated release of calcium from heavy guinea pig parotid gland microsomes. The concentration of the 3 different compounds tested was in each case 3% (w/v). The concentration of GTP was 5 μM. (−), GTP was added without addition of PVP, PEG, or BSA. Otherwise the experiments were performed as in (b).

well as GDPβS (fig.2b) inhibited the GTP-mediated Ca release. GTPγS at 25 μM virtually abolished the effect of 5 μM GTP (fig.2a).

As isolated ER vesicles are spontaneously leaky for calcium, a net release may be mediated by increasing the efflux of calcium as well as by inhibiting the ATP-dependent calcium uptake. We examined, therefore, whether GTP or IP_3 had an effect on ATP-driven calcium uptake. The initial rate of ATP-driven calcium uptake by isolated parotid gland ER vesicles was determined by first adding unlabelled calcium (100 μM final concentration) in the presence of 2.5 mM ATP under the conditions given for fig.1. When the free Ca^{2+} con-

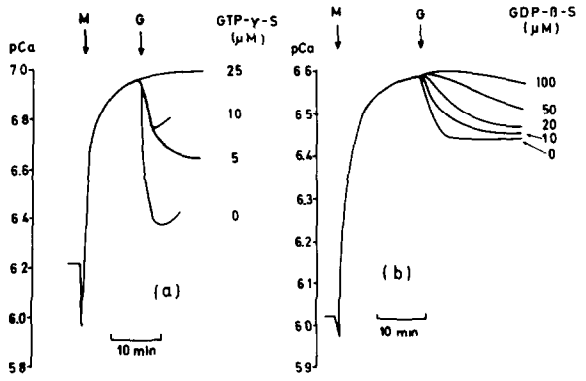


Fig.2. Inhibition of GTP-mediated calcium release from guinea pig parotid gland heavy microsomes by GTPγS and GDPβS. Heavy microsomes prepared from isolated acini were added at (M). GTPγS (a) or GDPβS (b) was added to give the final concentrations indicated and the release of calcium was started by addition of GTP (5 μM final concentration). In (a) GTP and GTPγS were added together at (G). In (b) GDPβS was added 5 min before GTP was added at (G).

centration had reached a steady state, 5 μCi carrier-free calcium was added and the uptake of ^{45}Ca followed every 10 s over 1 min by filtration of aliquots through cellulose acetate filters as in [21]. Under these conditions, the uptake was linear over at least 50 s in the absence and presence of calcium-releasing agents. Neither GTP (5 μM) nor IP_3 (5 μM) had a measurable effect under these conditions (calcium uptake: controls, 85; GTP, 81; IP_3, 80 nmol/mg protein per min).

Neither GTP-mediated nor IP_3-mediated calcium efflux was affected by calcium channel blockers (verapamil up to 10 μM; nifedipine up to 10 μM) (not shown). As shown by Muallem et al. [22] for isolated rat liver ER vesicles, the release of Ca^{2+} is limited by the membrane potential unless it is compensated by the influx of monovalent cations. As indicated in fig.3, this also applies to IP_3- and GTP-mediated release of calcium from parotid gland ER vesicles: in sucrose-containing medium with low (10 mM) concentrations of monovalent cations, neither GTP nor IP_3 induces a substantial release of calcium.

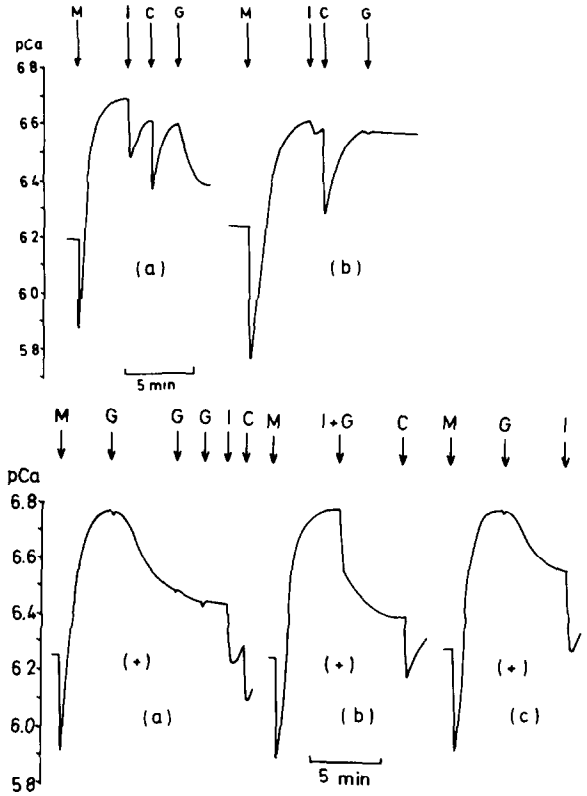
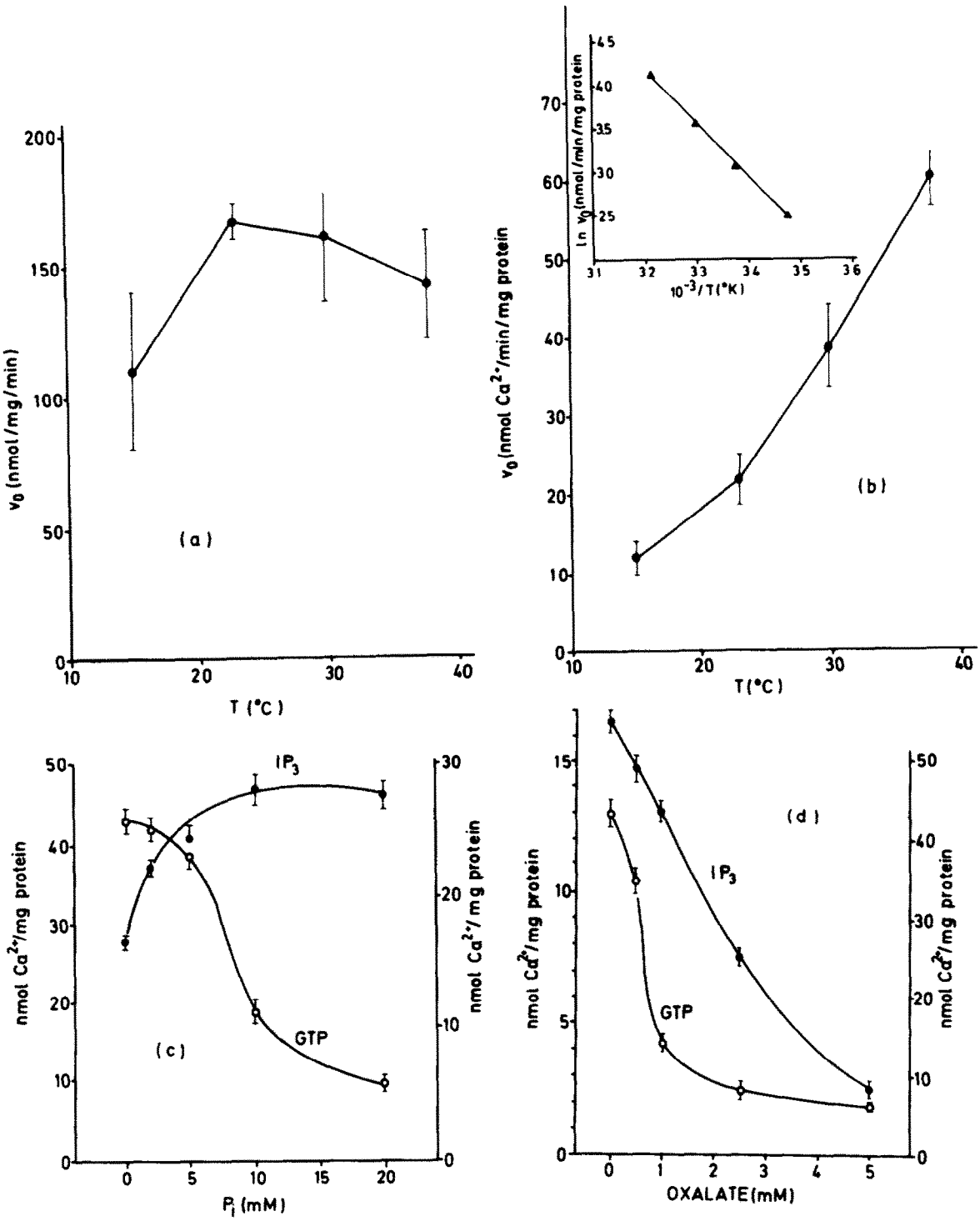


Fig.3. (Upper) Effect of K^+ on GTP- and IP_3-mediated release of calcium from parotid gland heavy microsomes. Heavy microsomes (12000 × *g*) were prepared as described in section 2. The medium in the electrode chamber consisted in (a) of the same medium as given in section 2 except that creatine phosphate and creatine kinase were omitted and NaN_3 was replaced by 1 μM antimycin A and 1 μM oligomycin. In (b) the same medium as in (a) was used except that 130 mM KCl was replaced by 0.3 M sucrose. GTP (G) and IP_3 (I) were added to give final concentrations of 5 and 2.5 μM, respectively. At (C) 5 nmol $CaCl_2$ was added. (Lower) Effects of sequential addition of GTP and IP_3 on the release of calcium from heavy microsomes isolated from guinea pig parotid gland acini. After addition of the microsomes (M) in (a) 3 pulses of GTP (G) (5 μM each) were given followed by addition of IP_3 (I) (2.5 μM). (C) indicates the addition of 5 nmol $CaCl_2$. In (b) 2.5 μM IP_3 and 5 μM GTP were added simultaneously, and in (c) 2.5 μM IP_3 was added before the effect of 5 μM GTP was completed. Note the higher initial velocity of IP_3-induced calcium release.

Fig.4. (Upper) Temperature dependence of IP_3-mediated (a) and GTP-mediated (b) release of calcium from guinea pig parotid heavy microsomes. They were prepared and incubated in the electrode chamber as given in section 2. Calcium release at different temperatures was corrected for the effects of temperature on the initial rate of calcium uptake which had been determined separately. An Arrhenius plot of the data given in (b) is presented in the inset. Each point



represents the mean ± SE from 3 experiments. (Lower) Effects of different concentrations of phosphate (c) and oxalate (d) on GTP- and IP_3-mediated release of calcium from isolated parotid gland heavy microsomes. The medium in the electrode chamber was the same as given in section 2 except that phosphate or oxalate was varied as indicated. The concentrations of GTP and IP_3 in all experiments were 5 and 2.5 μM, respectively. Each point represents the mean ± SE from 3 experiments.

We observed substantial qualitative differences between the effects of GTP and IP_3. (i) When calcium is released from isolated ER vesicles by sufficiently effective concentrations of GTP, a second application of GTP will not initiate further release of calcium (fig.3, lower). If IP_3 is now added, calcium is immediately released. If IP_3 and GTP are applied simultaneously, the initial velocity of calcium release reflects mainly the effect of IP_3 whereas the subsequent slower release seems to be determined by the action of GTP. The total amount of calcium released is similar to that released when GTP and IP_3 are added sequentially (fig.3, lower). The same can be observed if IP_3 is given first and GTP second (not shown). (ii) Over the temperature range 38–23°C, IP_3-mediated calcium release remains almost unaffected and exhibits only a minor decrease between 23 and 15°C (fig.4a). On the other hand, GTP-mediated calcium release decreases continuously over the whole temperature range tested (fig.4b). An Arrhenius plot gave an E_A for GTP-dependent calcium release of 53.7 ± 2.4 kJ/mol. This indicates that in contrast to the IP_3-mediated calcium release, GTP-catalyzed calcium release is strongly energy-dependent. (iii) The intra/extravesicular $[Ca^{2+}]$ gradient affects the two kinds of calcium release in different ways: increasing concentrations of phosphate strongly inhibit GTP-mediated calcium release (fig.4c). IP_3-mediated calcium release on the other hand is not inhibited at all. On the contrary, phosphate concentrations up to 20 mM lead to an increase of IP_3-mediated calcium release (fig.4c). With oxalate instead of phosphate, GTP- as well as IP_3-mediated calcium release is inhibited (fig.4d). However, whereas IP_3-mediated calcium release is only inhibited by 30% at 1 mM oxalate, the GTP-mediated calcium release is already inhibited by 70% at this concentration. The GTP-mediated release is almost completely abolished at 2.5 mM oxalate where the IP_3-mediated release is only inhibited by 55% (fig.4d).

These findings indicate that the mechanism by which GTP leads to a release of calcium differs from that of IP_3 and can, therefore, not be explained by GTP-catalyzed in vitro formation of IP_3. In addition, we have found that pretreatment of parotid gland ER vesicles with up to 2 mM neomycin, an inhibitor of phospholipase C-catalyzed hydrolysis of phosphatidylinositol 4,5-bisphosphate, did not block the GTP-mediated calcium release (not shown).

At present, the mechanism of action of GTP awaits clarification. Our data and those presented by Gill's group obtained with microsomes and permeabilized cells from a neuroblastoma cell line [23,24] indicate that hydrolysis of GTP is involved, which separates this effect clearly from the role of GTP in receptor-mediated control of adenylate cyclase, where hydrolysis is not required. A second site of action of GTP independent of its role in the regulation of a polyphosphoinositide-specific phosphodiesterase has been postulated by Borrowman et al. [25] on the basis of experiments with permeabilized leucocytes. According to their concept, however, this second site should be responsive to non-hydrolyzable GTP analogues (e.g. GTPγS) and therefore cannot be the GTP-mediated calcium release described here. A participation of GTP in calcium release from the ER in intact cells would require a strong compartmentation of intracellular guanine nucleotide pools in order to avoid a permanent activation of the GTP-induced calcium release mechanism: in rat liver cells the total cytosolic concentrations of GTP and GDP are about 320 and 90 μM, respectively [26,27] – conditions which in vitro would lead to full activation of the GTP-stimulated calcium, release from isolated rat liver ER vesicles. However, these considerations also apply to other types of GTP-dependent regulation (e.g. chain initiation and elongation during the translation process; modulation of the function of N_s and N_i in the regulation of adenylate cyclase). It seems highly likely, therefore, that the total guanine nucleotide concentrations do not reflect the concentrations of the free nucleotides. Further work is needed to learn how the intracellular compartmentation of guanine nucleotides might be regulated.

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