Effect of guanosine triphosphate on the release and uptake of Ca^{2+} in saponin-permeabilized macrophages and the skeletal-muscle sarcoplasmic reticulum

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The effects of GTP, with or without polyethylene glycol (PEG), on the release and uptake of Ca^{2+} were examined by using saponin-treated macrophages and sarcoplasmic reticulum isolated from skeletal muscles. The application of GTP in concentrations in the range 0.1–10 μM induced a gradual, small but sustained release of Ca^{2+} from the saponin-treated macrophages. The addition of PEG to GTP markedly enhanced the GTP-mediated Ca^{2+} release. GTP at the same concentration ranges used for Ca^{2+} release decreased the amount of Ca^{2+} uptake, at a steady state, but stimulated the rate of Ca^{2+} accumulation in the presence of oxalate, the Ca^{2+} -precipitating anion. The addition of PEG abolished the GTP-evoked stimulation of Ca^{2+} accumulation in the presence of oxalate. The stimulating effect on the rate of Ca^{2+} accumulation by GTP and its elimination by PEG were not due to changes in the permeability of oxalate by either GTP or PEG, or both. The Ca^{2+} -releasing effect of GTP without PEG was enhanced by eliminating the uptake activity by decreasing the content of ATP. These results indicate that GTP has an inherent activity to release Ca^{2+} from non-mitochondrial intracellular stores of saponin-treated macrophages, and PEG enhances the GTP-mediated Ca^{2+} release, partly owing to its eliminating effect on GTP-stimulated Ca^{2+} uptake activity. These effects of GTP observed with saponin-permeabilized macrophages were not apparent in the isolated skeletal-muscle sarcoplasmic reticulum.

INTRODUCTION

The accumulation and release of Ca^{2+} by the intracellular organelles, in particular the endoplasmic reticulum (ER), is important for regulating the cytosolic free Ca^{2+} concentration within cells (Berridge & Irvine, 1984; Rasmussen & Barrett, 1984). In a variety of cell types, the active Ca^{2+} -accumulating activities of ER have been extensively studied by using chemically permeabilized cells (Hirata & Koga, 1982; Wakasugi *et al.*, 1982; Hirata *et al.*, 1983a; Burgess *et al.*, 1983) or biochemically isolated ER-rich microsomes (microsomal fractions) (Pershad Singh *et al.*, 1980; Prentki *et al.*, 1984a; Dawson & Irvine, 1984). The same preparations have also been used for studies of Ca^{2+} -releasing activities, particularly the Ca^{2+} release mediated by inositol 1,4,5-trisphosphate (InsP_3) (Streb *et al.*, 1983; Joseph *et al.*, 1984; Suematsu *et al.*, 1984; Hirata *et al.*, 1984a; Dawson & Irvine, 1984; Prentki *et al.*, 1984a,b).

Dawson (1985) reported that the rat liver microsomes were practically insensitive to InsP_3 with respect to the release of Ca^{2+} , but became responsive in the presence of GTP. The effect of GTP seemed to require the hydrolysis of GTP and possibly the transfer of the γ -phosphate group from GTP on to the protein(s) (Dawson *et al.*, 1986). In contrast, Gill's group reported that GTP by itself without InsP_3 can induce a remarkable release of Ca^{2+} in the microsomal fraction or permeabilized neuroblastoma N1E-115 cells, and the releasing effects of InsP_3 and GTP were approximately additive (Ueda *et al.*,

1986; Gill *et al.*, 1986). Henne & Söling (1986) confirmed Gill's data, using the ER-rich fraction of rat liver and guinea-pig parotid glands.

The effect of GTP on the release of Ca^{2+} or an enhancing effect on the InsP_3 -mediated Ca^{2+} release essentially required the simultaneous presence of 3–5% polyethylene glycol (PEG) in the ER-rich microsomes (Dawson, 1985; Dawson *et al.*, 1986; Ueda *et al.*, 1986; Henne & Söling, 1986). With the permeabilized N1E-115 cells the presence of PEG was not obligatory, but enhanced to a greater extent the Ca^{2+} release mediated by GTP (Gill *et al.*, 1986).

The GTP-mediated Ca^{2+} release has been noted in cells from liver, parotid gland and neuroblastoma. However, there is apparently no documentation of the effect of GTP on Ca^{2+} -accumulating activities, of any types of cells. The present study was undertaken to examine the effect of GTP on Ca^{2+} accumulation as well as Ca^{2+} release by saponin-treated macrophages, with special reference to the effect of PEG. The effect of GTP on the sarcoplasmic reticulum of skeletal muscle was also examined, as these are typical vesicles concerned with regulating the cytoplasmic free Ca^{2+} concentration in the excitation–contraction coupling mechanism and are used widely in studies on the mechanism of Ca^{2+} release.

EXPERIMENTAL

Materials

^{45}Ca (35.09 mCi/mg) and [^{14}C]oxalic acid

Abbreviations used: ER, endoplasmic reticulum; InsP_3 , inositol 1,4,5-trisphosphate; GTP[S], guanosine 5'-[γ -thio]triphosphate; PEG, polyethylene glycol.

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(3.7 mCi/mmol) were obtained from New England Nuclear. GTP, GDP, 5'-GMP, 2'(3')-GMP, UTP, ITP and CTP were obtained from Yamasa Shoyu Co., and were used without further purification. Guanosine 5'-[γ -thio]triphosphate (GTP[S]) was from Boehringer Mannheim. PEG (average M_r 6500) was from Wako Pure Chemical Industries Co. Hexokinase (type III) was from Sigma. Other reagents were of the highest purity grade available.

Methods

Preparations of saponin-permeabilized macrophages, skeletal-muscle sarcoplasmic reticulum and human erythrocyte inside-out vesicles. Guinea-pig peritoneal macrophages were treated with saponin as described previously (Hirata & Koga, 1982; Hirata *et al.*, 1983a,b). Skeletal-muscle sarcoplasmic reticulum was prepared from rabbit hind-leg muscle as described by Ogawa (1970). Human erythrocyte inside-out vesicles were prepared by the method of Waisman *et al.* (1981). The protein concentration was determined by the method of Lowry *et al.* (1951), with bovine serum albumin as a standard.

Preparation of microsomal fraction of macrophages. Macrophages, incubated with 1 mM-di-isopropyl fluorophosphate for 10 min at 0 °C to inactivate endogenous proteinases, as described by Bennett *et al.* (1982), were suspended in 0.1 M-KCl/20 mM-Tris/maleate buffer (pH 6.8). Tris/maleate buffer, which was made by adding approx. 0.4 M-maleic acid to 0.2 M-Tris to give pH 6.8

at 37 °C and diluting to 20 mM, was used throughout. The cell suspension was homogenized in a motor-driven homogenizer with a Teflon pestle. The homogenates were subsequently centrifuged at 1600 *g* for 10 min, 8000 *g* for 10 min and 100000 *g* for 40 min. The final pellet was once washed with 0.1 M-KCl/20 mM-Tris/maleate buffer (pH 6.8). The washed pellet was designated as the microsomal fraction, and exhibited an ATP-dependent accumulation of Ca^{2+} of about 15 nmol/mg of protein, and an InsP_3 - or GTP-induced release of Ca^{2+} .

Assays of Ca^{2+} uptake and Ca^{2+} release by saponin-treated macrophages, skeletal sarcoplasmic reticulum and erythrocyte inside-out vesicles. The preparations (4×10^6 cells/ml for saponin-treated macrophages; 50 $\mu\text{g}/\text{ml}$ for both skeletal sarcoplasmic reticulum and erythrocyte inside-out vesicles) were preincubated at 37 °C for 2 min in solution containing 0.1 M-KCl, 20 mM-Tris/maleate buffer (pH 6.8), 10 mM- NaN_3 , 3 mM- MgCl_2 , 0.12 mM- CaCl_2 (containing 2 μCi of $^{45}\text{Ca}^{2+}/\text{ml}$) and 1 mM- or 0.44 mM-EGTA; free Ca^{2+} concentration was calculated to be 0.14 μM or 0.37 μM respectively, on the assumption that an apparent affinity constant of EGTA for Ca^{2+} is $1 \times 10^6 \text{ M}^{-1}$ at pH 6.8 (Harafuji & Ogawa, 1980). The Ca^{2+} uptake was started by adding 2 mM-ATP. At an appropriate time, a sample of the reaction mixture was passed through a glass-fibre filter (for saponin-treated macrophages; Whatman GF/C, pore size 1.2 μm) or a Millipore filter (for sarcoplasmic reticulum and inside-out vesicles; pore size 0.45 μm). The filter was washed twice with 2 ml of the above solution without ^{45}Ca , dried and

Fig. 1. Effects of GTP, with or without PEG, on Ca^{2+} release from saponin-treated macrophages (a) and skeletal sarcoplasmic reticulum (b).

Saponin-treated macrophages (4×10^6 cells/ml) were incubated in a solution containing 0.1 M-KCl, 20 mM-Tris/maleate buffer (pH 6.8), 10 mM- NaN_3 , 3 mM- MgCl_2 , 2 mM-ATP and 0.14 μM free Ca^{2+} (containing ^{45}Ca) at 37 °C. For skeletal sarcoplasmic reticulum (50 $\mu\text{g}/\text{ml}$), NaN_3 was omitted and the free Ca^{2+} concentration was raised to 0.37 μM . To some mixtures (□, ■) 3% PEG was further added. At the time indicated by the abscissa, a sample of the reaction mixture was filtered through a glass-fibre or Millipore filter. The filter was treated as described in the Experimental section. At the time indicated by the arrow, GTP was added to give a final concentration of 10 μM (●, ■). As a control the same volume of distilled water was added (○, □). In (b), powdered caffeine was added to give a final concentration of 10 mM (▲). Δ , Experiment in the absence of ATP. Points with bars represent means $\pm$ S.E.M. for five to seven separate experiments; points without bars represent the mean of three separate experiments.

Fig. 2. Effects of nucleotides other than GTP on Ca^{2+} release

The incubation medium containing PEG was the same as that described in Fig. 1. At the time indicated by the arrow, various nucleotides were added. Each point represents the mean of three separate experiments. (a) Guanine nucleotides: ●, GTP (10 μM); ▼, GTP[S] (100 μM); ▲, GDP (10 μM); ■, 5'-GMP (100 μM). (b) Nucleoside triphosphates: ●, GTP (10 μM); ▼, CTP (100 μM); ▲, ITP (100 μM); ■, UTP (100 μM).

then counted for radioactivity. The amount of Ca^{2+} accumulation was thus determined. GTP or other reagents in 0.01 vol. of the reaction mixture was added. At an appropriate time, a sample of the mixture was passed through a filter, which was then treated as described above. Thus the Ca^{2+} remaining in the preparations was determined. Oxalic acid was included in the reaction mixture for Ca^{2+} uptake in some experiments, in which saponin-treated macrophages or the sarcoplasmic reticulum were decreased to 2×10^6 cells or 10 $\mu\text{g}/\text{ml}$ respectively.

Assays of [^{14}C]oxalic acid uptake by macrophage microsomes. Macrophage microsomes (108–130 $\mu\text{g}/\text{ml}$) were incubated in the same solution as for the Ca^{2+} uptake for 20 min at 37 $^{\circ}\text{C}$, except that 5.5 mM-oxalate (containing 1.5 μCi of [^{14}C]oxalate/ ml) was included. The mixture was passed through a Millipore filter to determine the [^{14}C]oxalate associated with macrophage microsomes.

RESULTS

Effect of GTP and related nucleotides on Ca^{2+} release

Fig. 1 shows the effect of GTP on the release of Ca^{2+} in saponin-permeabilized macrophages (a) or skeletal sarcoplasmic reticulum (b), in the presence or absence of PEG (3%, w/v). The accumulation of Ca^{2+} in the presence of NaN_3 at a free Ca^{2+} concentration of 0.14 μM originates from the ER pool of saponin-treated cells (Hirata & Koga, 1982; Hirata *et al.*, 1983a,b, 1984a). The application of GTP, after the Ca^{2+} accumulation had reached a plateau, gradually induced a small release of Ca^{2+} in the absence of PEG, i.e. about 0.2 or 0.36 nmol of $\text{Ca}^{2+}/4 \times 10^6$ cells was released 5 or 10 min after the addition of 10 μM -GTP, respectively. The addition of 3% PEG in the solution for Ca^{2+} uptake decreased the amount accumulated from 2.4 to

2.0 nmol/ 4×10^6 cells. PEG at 10% (w/v) further decreased the Ca^{2+} accumulation, to about 1.3 nmol/ 4×10^6 cells (results not shown). The application of GTP in the presence of 3% PEG induced a remarkable release of Ca^{2+} ; the Ca^{2+} content was decreased from 2.0 to 0.8 nmol/ 4×10^6 cells at 5 min after the addition of 10 μM -GTP. In contrast with findings with the saponin-treated macrophages, the skeletal sarcoplasmic reticulum was not responsive to GTP, even in the presence of PEG, whereas the addition of caffeine, well known to induce the release of Ca^{2+} through activation of the Ca^{2+} -induced Ca^{2+} -release mechanism (Endo, 1977), induced a rapid release of Ca^{2+} (Fig. 1b). The erythrocyte inside-out vesicles, typical vesicles originating from the plasma membrane, were also insensitive to GTP with PEG (results not shown).

Fig. 3. Dose-response relation of GTP- or GDP-induced Ca^{2+} release

Ca^{2+} release with PEG was relative to that 5 min after the addition of 30 μM -GTP. Almost equal amounts of Ca^{2+} were released by 30 μM -GTP and -GDP. ●, GTP (means $\pm$ S.E.M. for seven separate experiments); ■, GDP (means of three separate experiments).

Fig. 2 shows the effect of guanine nucleotide or nucleoside triphosphate on the release of Ca^{2+} from saponin-treated macrophages in the presence of PEG. GTP[S] up to $100\ \mu\text{M}$ had no effect on the Ca^{2+} release, thereby indicating the requirement for the hydrolysis of the γ -phosphate to exert the effect, as noted by Dawson (1985) and Gill *et al.* (1986). GDP was as effective as GTP (Fig. 2*a*), but a time lag of 1 min was required. GMP (5'-isomer; $100\ \mu\text{M}$) also induced a remarkable release of Ca^{2+} only after a time lag of 3 min (Fig. 2*a*), whereas the 2'(3')-isomer up to $300\ \mu\text{M}$ was without effect (results not shown). Neither guanosine nor guanine at $100\ \mu\text{M}$ released Ca^{2+} (results not shown). Only ITP among the nucleoside triphosphates examined (UTP, CTP and ITP) had a moderate effect, at a concentration of $100\ \mu\text{M}$ (Fig. 2*b*). H.p.l.c. analysis of ITP on a TSK-SAX column showed a maximal 1.5% contamination with GTP, and there was no contamination with GDP or GMP. Fig. 3 shows the dose-response relation of the GTP- or GDP-induced Ca^{2+} release. The half-maximal effect was observed at about $1.1\ \mu\text{M}$ -GTP and $1.3\ \mu\text{M}$ -GDP. These results support the data of Gill *et al.* (1986) and Henne & Söling (1986), in that GTP without PEG induces only a small release of Ca^{2+} , whereas the Ca^{2+} release by GTP is markedly enhanced in the presence of PEG.

Effect of GTP with or without PEG on Ca^{2+} uptake

We then examined the effect of GTP, with or without PEG, on the Ca^{2+} -uptake activities of saponin-treated macrophages and skeletal sarcoplasmic reticulum, to study whether the GTP-induced Ca^{2+} release from saponin-treated macrophages, but not from skeletal sarcoplasmic reticulum, is due to inhibition of the Ca^{2+} -pumping activity. Fig. 4 shows the effect of GTP, with or without PEG, on the Ca^{2+} uptake of saponin-treated macrophages (*a*) or the skeletal sarcoplasmic reticulum (*b*), in the absence of a Ca^{2+} -precipitating

anion, oxalate. A plateau amount of Ca^{2+} accumulation was inhibited by $10\ \mu\text{M}$ -GTP without PEG by about 20% at 10 min after the addition of ATP, in the saponin-permeabilized macrophages. The Ca^{2+} accumulation in the presence of PEG was decreased to that seen in the presence of GTP, and simultaneous addition of GTP and PEG markedly inhibited the accumulation of Ca^{2+} . In contrast with the saponin-treated macrophages, $10\ \mu\text{M}$ -GTP, with or without PEG, did not decrease the Ca^{2+} -accumulating activity in skeletal sarcoplasmic reticulum (Fig. 4*b*). Only PEG (3%, w/v), with or without GTP, inhibited the initial rate of the Ca^{2+} accumulation, i.e. Ca^{2+} accumulation without PEG reached a plateau within the first 20 s, whereas that with PEG reached a plateau after 1 min. As shown in Fig. 4(*a*), in the first 30 s, in the saponin-treated macrophages, the amount of Ca^{2+} accumulation without PEG was 10–20% greater in the presence of GTP than in its absence. This enhancing effect of GTP was small but significant, and was consistently observed. Therefore there remains the possibility that the apparent inhibition of Ca^{2+} uptake is due to increases in the release of Ca^{2+} .

We also examined the effects of GTP, with or without PEG, on unidirectional Ca^{2+} uptake by saponin-treated macrophages and skeletal sarcoplasmic reticulum. In these experiments oxalate was added to the incubation medium. The ER of the saponin-treated cells is permeable to oxalate, which serves to trap Ca^{2+} intravesicularly and thus to eliminate the efflux component (Hirata *et al.*, 1983*a*, 1985; Delfert *et al.*, 1986). Fig. 5 shows the effect of GTP, with or without PEG, on the unidirectional Ca^{2+} uptake in the presence of oxalate. Surprisingly, GTP ($10\ \mu\text{M}$) markedly stimulated the unidirectional Ca^{2+} uptake in saponin-treated macrophages (Fig. 5*a*), but not in skeletal sarcoplasmic reticulum (Fig. 5*b*). When the concentration of oxalate was varied to 5, 10 or 20 mM, the enhancing effect by

Fig. 4. Effects of GTP with or without PEG on Ca^{2+} uptake in saponin-treated macrophages (*a*) and skeletal sarcoplasmic reticulum (*b*) in the absence of oxalate

The incubation medium was the same as that described in Fig. 1, except that GTP at $10\ \mu\text{M}$ was included from the start. ○, No addition; ●, GTP ($10\ \mu\text{M}$); □, PEG (3%); ■, GTP plus PEG. Points with bars represent means $\pm$ S.E.M. for five experiments; points without bars represent means of three experiments.

Fig. 5. Effects of GTP with or without PEG on Ca^{2+} uptake in saponin-treated macrophages (a) and skeletal sarcoplasmic reticulum (b) in the presence of oxalate

The incubation medium was essentially the same as that described in Fig. 1. In (a), the amount of saponin-treated macrophages were decreased to 2×10^6 cells/ml and 10 mM-oxalate was included. In (b), protein concentration was decreased to $10 \mu\text{g}/\text{ml}$ and 5 mM-oxalate was included. $\circ$, No addition; $\bullet$, GTP ($10 \mu\text{M}$); $\square$, PEG (3%); $\blacksquare$, GTP plus PEG. $\triangle$, $\blacktriangle$, Experiments in the absence of ATP ($\triangle$, + GTP; $\blacktriangle$, no GTP). Points with bars represent means $\pm$ S.E.M. for seven separate experiments; points without bars represent means of three experiments.

GTP was similarly observed in saponin-treated cells, whereas in the sarcoplasmic reticulum the GTP effect was not observed with oxalate concentrations of 0.5, 1, 3 or 10 mM. As shown in Fig. 6, the dose-dependence of GTP-stimulated unidirectional Ca^{2+} uptake was similar to that of Ca^{2+} release, i.e. the half-maximal effect was observed at $1.3 \mu\text{M}$ -GTP. GDP and GMP at 10 and $100 \mu\text{M}$ respectively were effective, but GTP[S] at $100 \mu\text{M}$ was not as effective as $10 \mu\text{M}$ -GTP from the first 5 min, with regard to the unidirectional Ca^{2+} uptake (results not shown).

The addition of PEG (3%) to the medium from the initiation, or later, abolished the stimulating effect by GTP, but not the basal Ca^{2+} accumulation in saponin-treated cells (Fig. 5a). On the other hand, 3% PEG with or without $10 \mu\text{M}$ -GTP slightly decreased the unidirectional Ca^{2+} accumulation by the sarcoplasmic reticulum (Fig. 5b).

The stimulating effect on the unidirectional Ca^{2+} uptake by GTP may be due to enhancement of the Ca^{2+} -pumping activity in the ER of saponin-treated macrophages, but not in the skeletal sarcoplasmic reticulum. Alternatively, it may be that GTP enhances the permeability to oxalate of the ER of saponin-treated macrophages, because the amount of Ca^{2+} accumulated doubled when the concentration of oxalate was elevated from 5 to 10 mM or from 10 to 20 mM. The latter possibility was excluded by examining the permeability to oxalate of the ER, by using [^{14}C]oxalate. For this experiment the microsomal fraction instead of the saponin-treated cells was used, because vesicular structures other than just ER, such as nuclei and mitochondria, were electron-microscopically observed in saponin-treated macrophages (Hirata *et al.*, 1983b, 1984a). As shown in Table 1, GTP did not affect the radioactivity of [^{14}C]oxalate associated with microsomes.

The addition of PEG (3%) enhanced the radioactivity associated with microsomes by 20–30%. These results suggest that GTP stimulates the Ca^{2+} -pumping activity of the ER of saponin-treated macrophages, and PEG diminishes the stimulating effect of GTP.

Effect of elimination of Ca^{2+} -uptake activity on the GTP-induced Ca^{2+} release

Since GTP stimulated not only the Ca^{2+} release but also the Ca^{2+} -uptake activity, we examined the effect of abolishment of Ca^{2+} uptake on the GTP-induced Ca^{2+} release in the absence of PEG in saponin-treated macrophages. As shown in Fig. 7(a), the addition of both

Fig. 6. Dose-response relation of GTP- or GDP-induced stimulation of Ca^{2+} uptake in the presence of oxalate

The stimulation of Ca^{2+} uptake was relative to that 10 min after the addition of $30 \mu\text{M}$ -GTP. Almost equal stimulation was observed with $30 \mu\text{M}$ -GTP and -GDP. $\bullet$, GTP (means $\pm$ S.E.M. for five separate experiments); $\blacksquare$, GDP (mean of three experiments).

Table 1. Permeability of macrophage microsomes to oxalate

Macrophage microsomes (108–130 $\mu\text{g/ml}$) were incubated in a solution containing 0.1 M-KCl, 20 mM-Tris/maleate buffer (pH 6.8), 3 mM-MgCl₂, 2 mM-ATP, 0.14 μM free Ca²⁺ and 5.5 mM-oxalate (containing 1.5 μCi of [¹⁴C]-oxalate/ml) at 37 °C for 20 min. [¹⁴C]Oxalate associated with microsomes was determined by passing the mixture through a Millipore filter (pore size 0.45 μm). Numbers of experiments, with different preparations of microsomes, are given in parentheses.

	[¹⁴ C]Oxalate associated with macrophage microsomes (c.p.m./mg of protein)	
	None	PEG (3%)
Control	11372 $\pm$ 1014 (4)	15164 (2)
GTP (20 μM)	12183 $\pm$ 1171 (7)	15002 $\pm$ 1732 (5)

hexokinase and glucose induced a release of Ca²⁺, probably owing to the remarkable decrease in ATP required for Ca²⁺ uptake, and these compounds alone were without effect. When GTP was simultaneously added to the medium under such conditions, the release of Ca²⁺ was progressively enhanced, even in the absence of PEG. As shown in Fig. 7(b), the addition of 1 mM-vanadate, which eliminates the Ca²⁺ uptake (Wang *et al.*, 1979; Prentki *et al.*, 1984b; Hirata *et al.*, 1985), also induced the release of Ca²⁺. However, as compared with the addition of GTP in the presence of both hexokinase and glucose, GTP in the presence of vanadate induced a much lesser amount of Ca²⁺ release.

DISCUSSION

In the present study, we examined the effects of GTP, with or without PEG, on Ca²⁺ uptake as well as Ca²⁺

release by saponin-treated macrophages. Skeletal sarcoplasmic reticulum was also used, because it possesses the typical vesicles for controlling the cytosolic free Ca²⁺ concentration in excitation-contraction coupling mechanism of muscles, and thus has been extensively used for studies of the Ca²⁺-releasing mechanism (Endo, 1977; Volpe *et al.*, 1985). However, the GTP effects were observed with saponin-treated macrophages, but not with the skeletal sarcoplasmic reticulum, thereby suggesting that this may not be applicable to internal Ca²⁺ stores of all types of cells.

GTP at low concentrations induced a small but sustained Ca²⁺ release from the saponin-treated macrophages. The same concentrations of GTP also stimulated the rate of Ca²⁺ accumulation. When PEG was added to the medium, the Ca²⁺ release induced by GTP was markedly enhanced, and the stimulation of Ca²⁺ accumulation by GTP was abolished. The Ca²⁺ release may be in a dynamic balance between the Ca²⁺-accumulating and -releasing activities (Clapper & Lee, 1985). Therefore the gradual and small release of Ca²⁺ by GTP without PEG could be partly due to its enhancing effect on the Ca²⁺-accumulating activity. The PEG effect on the GTP-induced Ca²⁺ release may support the above possibility, because PEG abolished the Ca²⁺-accumulating activity induced by GTP. Furthermore, there is supporting evidence that the Ca²⁺-releasing effect by GTP without PEG was augmented by eliminating the Ca²⁺ uptake through a decrease in ATP, but not through vanadate, as discussed below. These results indicate that GTP inherently possessed the ability to release Ca²⁺ from the ER of saponin-treated macrophages, and that this activity may be modified by PEG by interfering with the Ca²⁺ uptake. However, the enhancement of GTP-mediated Ca²⁺ release by decreased ATP was much smaller than that induced by PEG, irrespective of the finding that PEG inhibited only the stimulated accumulation of Ca²⁺. Therefore, the effect of PEG on the stimulation of GTP-mediated Ca²⁺ release may be partly

Fig. 7. Effect of elimination of Ca²⁺-uptake component on GTP-mediated Ca²⁺ release

At the time indicated by the arrow, both hexokinase (4.5 units/ml) and glucose (10 mM) in (a), or vanadate (1 mM) in (b), was added to the medium. ○, No addition; ●, GTP (10 μM); □, in (a) hexokinase+glucose, or in (b) vanadate; ■, in (a) hexokinase+glucose+GTP, or in (b) vanadate+GTP. Each point represents the mean of three separate experiments.

due to factor(s) other than its abolition of the stimulated rate of Ca^{2+} accumulation by GTP. As to the effect of PEG, Henne & Söling (1986) reported that the enhancement of GTP-mediated Ca^{2+} release does not simply depend on PEG, and that the presence of compounds which increase the viscosity of the medium, such as polyvinylpyrrolidone or bovine serum albumin, also stimulates Ca^{2+} release.

Among the guanine nucleotides other than GTP, GDP and 5'-GMP, but not 2'(3')-GMP, were effective for the Ca^{2+} release and uptake. The dose-dependence of GDP was much the same as that of GTP, but a much higher dose was required with GMP. Gill *et al.* (1986) reported that the effectiveness of GDP was possibly due to the conversion of GDP into GTP by nucleoside diphosphokinase activity as contamination in saponin-permeabilized NIE-115 cells. Such may be indeed the case with saponin-treated macrophages, since h.p.l.c. analysis of GDP and GMP on a TSK-SAX column showed little contamination with GTP, and a time lag of 1–3 min was always required to evoke the Ca^{2+} release. We did not go further into this point. ITP, but not UTP and CTP, at 100 μM evoked a moderate release of Ca^{2+} . This may be explained by the contamination with GTP in commercially available ITP, because maximally a 1.5% contamination of ITP with GTP was estimated, as determined by h.p.l.c. on a TSK-SAX column.

The mechanism of action of GTP is unknown. As initially noted by Dawson (1985), and confirmed by Gill *et al.* (1986), Henne & Söling (1986) and by the present study, the effect of GTP on the remarkable release of Ca^{2+} requires the hydrolysis of the γ -phosphate of GTP. The stimulation of Ca^{2+} uptake in the presence of oxalate by GTP also seemed to require hydrolysis of the terminal phosphate, because GTP[S] did not evoke the stimulation. Furthermore, the dose-response relationship of GTP-mediated stimulation of Ca^{2+} uptake was much the same as that of GTP-mediated Ca^{2+} release. Therefore, the GTP-evoked effects on Ca^{2+} uptake and release are possibly demonstrated through the same site(s), or sites closely related. Thus the site at which GTP acts may be an ATPase protein of the ER of saponin-treated macrophages, or closely related site(s), since an ATP-dependent Ca^{2+} uptake is mediated through ATPase activity. As to this point, the following result should be noted. The enhancement of GTP-evoked Ca^{2+} release without PEG was demonstrated with the addition of hexokinase and glucose, but not of vanadate. Vanadate has been shown to inhibit many ATPases (Simons, 1979), including ATPases of Ca^{2+} -accumulating vesicles (Wang *et al.*, 1979; Caroni & Carafoli, 1981; Prentki *et al.*, 1984b; Hirata *et al.*, 1985). These inhibitory effects probably occur as a result of interference with the binding of ATP by acting as an ATP analogue (Simons, 1979; Caroni & Carafoli, 1981).

The physiological significance of the GTP-mediated Ca^{2+} release in a stimulus-response coupling in intact cells is unknown. The increase in the cytosolic free Ca^{2+} concentration induced by an external stimulation appears to be rapid. Such is the case in chemotactic-peptide-stimulated macrophages, i.e. the addition of chemotactic peptide increased the cytosolic free Ca^{2+} within 20 s, and the content returned to the basal value within 6 min, as determined by loading with quin2 (Hirata *et al.*, 1984b). On the contrary, the GTP-mediated Ca^{2+} release without PEG was slow and small. The

remarkable release of Ca^{2+} in the presence of PEG was observed within 1 min. Therefore, the Ca^{2+} release mediated by GTP may play a physiological role in intact cells, on the assumption that some factor(s) affecting the same effect as PEG are present in intact cell or that there is a compartmentation of intracellular GTP pools (Henne & Söling, 1986). The former assumption may be supported by the finding by Henne & Söling (1986) that PEG could be replaced by compounds raising the viscosity of the medium, e.g. 3% (w/v) bovine serum albumin. In macrophages, the concentration of cytoplasmic soluble proteins is estimated to be 40–60 $\mu\text{g}/10^6$ cells (Hirata *et al.*, 1983a), and thereby 2–3% (w/v) under the estimation of cell volume, 2 $\mu\text{l}/10^6$ macrophages (Hirata *et al.*, 1984b). GTP without PEG decreased the accumulation of Ca^{2+} at a steady state. Thus we assume that the GTP effect plays an important role in the long-term regulation of Ca^{2+} distribution among the intracellular organelles.

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