GTP mobilization of Ca^{2+} from the endoplasmic reticulum of islets

Comparison with *myo*-inositol 1,4,5-trisphosphate

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The effect of the guanine nucleotide GTP on Ca^{2+} release from the endoplasmic reticulum of digitonin-permeabilized islets was investigated. Maximal and half-maximal Ca^{2+} release were observed at 5 μM - and 2.5 μM -GTP respectively. GTP caused a rapid release of Ca^{2+} from the endoplasmic reticulum, which was complete within 1 min. GTP-induced Ca^{2+} release was structurally specific and required the hydrolysis of GTP. The combination of maximal concentrations of GTP (10 μM) and *myo*-inositol 1,4,5-trisphosphate (IP_3) (10 μM) resulted in an additive effect on Ca^{2+} release from the endoplasmic reticulum. GDP (100 μM), which inhibits GTP-induced Ca^{2+} release, did not affect IP_3 -induced Ca^{2+} release. Furthermore, GTP-induced Ca^{2+} release was not independent on submicromolar free Ca^{2+} concentrations, unlike IP_3 -induced Ca^{2+} release. These observations suggest that mechanistically GTP-induced Ca^{2+} release is different from IP_3 -induced Ca^{2+} release from the endoplasmic reticulum.

INTRODUCTION

Changes in intracellular Ca^{2+} concentrations are recognized as having a major role in insulin secretion (Wollheim & Sharp, 1981; Prentki & Wollheim, 1984; Wollheim & Pozzan, 1984; McDaniel *et al.*, 1985; Hellman, 1985; Wollheim & Biden, 1986). The endoplasmic reticulum has a prominent role in the regulation of intracellular Ca^{2+} concentrations in islet β -cells under basal and insulin-stimulatory conditions (for review see Turk *et al.*, 1987). Our laboratory has described several biochemical mechanisms which contribute to intracellular Ca^{2+} homeostasis under physiological conditions: (1) a Ca^{2+} -sequestering pump associated with a Ca^{2+} -ATPase localized to the endoplasmic reticulum (Colca *et al.*, 1982, 1983*a,b*); (2) a Ca^{2+} -sequestering mechanism localized to the endoplasmic reticulum, which is regulated by glucose 6-phosphate and has been implicated in glucose-induced insulin release (Wolf *et al.*, 1986*c*); and (3) Ca^{2+} mobilization or efflux from the endoplasmic reticulum (Wolf *et al.*, 1985, 1986*a,b,d*). Although the precise molecular mechanisms are not known, we and others have shown that IP_3 , a proposed second messenger (Berridge & Irvine, 1984), mobilizes Ca^{2+} from the endoplasmic reticulum of islet β -cells (Wolf *et al.*, 1985, 1986*a*) and of insulinoma cell lines (Prentki *et al.*, 1984; Biden *et al.*, 1984; Joseph *et al.*, 1984).

Several reports have shown that the guanine nucleotide GTP could induce Ca^{2+} release from rat liver microsomal fractions and from the endoplasmic reticulum of the NIE-115 neuronal cell line and the guinea-pig parotid gland (Dawson, 1985; Dawson *et al.*, 1986; Gill *et al.*, 1986; Ueda *et al.*, 1986; Henne & Söling, 1986). However, the relationship between GTP-induced and IP_3 -induced Ca^{2+} release from the endoplasmic reticulum is not clear

(Baker, 1986). We have examined the role of guanine nucleotides in Ca^{2+} mobilization from the endoplasmic reticulum of islet β -cells, using a characterized digitonin-permeabilized-islet model (Colca *et al.*, 1985; Wolf *et al.*, 1985, 1986*a,b,c*).

EXPERIMENTAL

Materials

Male Sprague–Dawley rats (180–200 g) were purchased from Sasco (O'Fallon, MO, U.S.A.). Collagenase (CLS IV) was obtained from Cooper Biomedical (Freehold, NJ, U.S.A.). $^{45}\text{CaCl}_2$ (sp. radioactivity 800 mCi/mmol), $^3\text{H}_2\text{O}$, Protosol and Aquasol were from New England Nuclear (Boston, MA, U.S.A.). [α - ^{32}P]GTP was from Amersham. GTP[S] (tetralithium salt) and GDP[S] (trilithium salt) were obtained from Sigma Chemical Co. (St. Louis, MO, U.S.A.). IP_3 was generously given by Dr. William Sherman, Department of Psychiatry, Washington University School of Medicine, and was prepared as previously described (Wolf *et al.*, 1985). All other chemicals were obtained from Sigma.

Methods

Islet isolation and permeabilization. Isolated pancreatic islets were obtained by collagenase digestion, followed by separation and purification on a discontinuous Ficoll gradient (McDaniel *et al.*, 1983, 1984). Freshly isolated islets (2000–3000 per experiment) were then permeabilized with digitonin by incubation in a modified Hepes/Krebs buffer (25 mM-Hepes, pH 6.8, 115 mM-NaCl, 24 mM- NaHCO_3 , 5 mM-KCl, 1 mM- MgCl_2 , 1 mM-EGTA, 0.1% bovine plasma albumin) at 37 °C with 20 μg of digitonin (Sigma)/ml for 20 min. Permeabilized islets were washed three times in Tris

Abbreviations used: IP_3 , *myo*-inositol 1,4,5-trisphosphate; GTP[S], guanosine 5'-[γ -thio]triphosphate; GDP[S], guanosine 5'-[β -thio]diphosphate.

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buffer (50 mM-Tris/HCl, pH 6.8, 100 mM-KCl, 5 mM-MgCl₂, 0.1% bovine plasma albumin) before use (Colca *et al.*, 1985; Wolf *et al.*, 1985, 1986a,b).

Ca²⁺ efflux in permeabilized islets. Permeabilized islets (30 per 1.5 ml polyallomer Beckman Microfuge tube) were loaded for 30 min at room temperature with 5 μ Ci of ⁴⁵Ca²⁺ and 5 μ Ci of ³H₂O as a cell volume marker in 95 μ l of a Pipes buffer mimicking intracellular conditions (100 mM-Pipes, pH 7.0, 100 mM-KCl, 7 mM-MgCl₂, 5 mM-ATP, 2.25 μ g of Ruthenium Red/ml). The free Ca²⁺ concentration of this buffer was set at 0.2 μ M by including EGTA at a concentration of 0.096–0.138 mM, depending on the specific radioactivity of ⁴⁵CaCl₂ (500–1000 mCi/mmol) as previously described (Wolf *et al.*, 1986a). We have previously shown that a Ca²⁺ steady-state maintained by the endoplasmic reticulum is reached after a 30 min incubation (Wolf *et al.*, 1985, 1986a,b,c). Ca²⁺ efflux was then initiated by the addition of 5 μ l of the appropriate effluxing agent in 200 mM-Pipes, pH 7.0. Tubes were also included which were incubated in parallel in the absence of ATP. Ca²⁺ efflux was stopped at the appropriate time by centrifugation (15 s, 10000 g). The supernatant of each tube was removed and the islet pellet was processed as previously described (Wolf *et al.*, 1985, 1986a). Calculations were performed by computing the volume associated with ⁴⁵Ca²⁺ and subtracting the volume occupied by ³H₂O, thus measuring the volume corresponding to the uptake of ⁴⁵Ca²⁺ by the islet. Non-ATP-dependent ⁴⁵Ca²⁺ uptake (which represents ⁴⁵Ca²⁺ binding to the plasma membrane and secretory granules) is then subtracted. Since Ruthenium Red is present to inhibit mitochondrial ATP-dependent ⁴⁵Ca²⁺ uptake (the other major ATP-dependent Ca²⁺ pool in islets) (McDaniel *et al.*, 1985; Prentki & Wollheim, 1984) and the free Ca²⁺ is maintained at 0.2 μ M, the final result (pmol of Ca²⁺/islet) is a reflection of the ATP-dependent ⁴⁵Ca²⁺ content of the endoplasmic reticulum (Wolf *et al.*, 1986a).

Other methods. GTP degradation was measured quantitatively by incubating 250 permeabilized islets with [α -³²P]GTP under conditions similar to those for GTP-induced Ca²⁺ efflux. Labelled guanine nucleotides were then extracted with 15% (w/v) trichloroacetic acid, processed and analysed by anion-exchange h.p.l.c. as previously described (Turk *et al.*, 1986). Free Ca²⁺ concentrations were maintained by including EGTA and verified with a Ca²⁺-specific electrode as previously described (Wolf *et al.*, 1986a). The preparation of ATP stocks and Ruthenium Red has been described (Wolf *et al.*, 1986a). Guanine nucleotides were dissolved in 200 mM-Pipes, pH 7.0, and titrated to pH 7.0 with 1 M-KOH.

Statistical analysis. Data were analysed by one-way ANOVA, followed by multiple comparison between means with the least-significant-difference test. All experiments were performed at least three times in triplicate unless otherwise indicated.

RESULTS

The guanine nucleotide GTP mobilized Ca²⁺ from the endoplasmic reticulum of digitonin-permeabilized islets. Fig. 1 shows the GTP dose/response curve for Ca²⁺

Fig. 1. Dose/response curve of GTP-induced Ca²⁺ release from the endoplasmic reticulum of digitonin-permeabilized islets

Permeabilized islets were loaded with ⁴⁵Ca²⁺ as described in the Experimental section. Ca²⁺ efflux from the endoplasmic reticulum was then measured after 10 min in the presence of GTP (0–50 μ M). Non-ATP-dependent Ca²⁺ content of the endoplasmic reticulum was subtracted for each condition. Ca²⁺ release is expressed as the ATP-dependent Ca²⁺ content of the endoplasmic reticulum in the absence of GTP minus the ATP-dependent Ca²⁺ content of the endoplasmic reticulum in the presence of GTP. Control ATP-dependent Ca²⁺ content of the endoplasmic reticulum was 0.93 ± 0.04 pmol/islet. Results are shown as means $\pm$ S.E.M. of Ca²⁺ released (pmol/islet) from the endoplasmic reticulum for 18–26 observations per each condition for three experiments.

release (10 min) from the endoplasmic reticulum. The non-ATP-dependent Ca²⁺ content of islets was not affected by GTP. Maximal and half-maximal Ca²⁺ release were observed at 5 μ M- ($P < 0.001$) and 2.5 μ M- ($P < 0.05$) GTP respectively. The time course of GTP-induced Ca²⁺ release from the endoplasmic reticulum is illustrated in Fig. 2. GTP (10 μ M) induced maximal Ca²⁺ release within 1 min ($P < 0.02$), followed by progressive Ca²⁺ re-uptake over the next 20 min.

The specificity of GTP-induced Ca²⁺ release from the endoplasmic reticulum was next examined (Table 1). The non-hydrolysable analogue of GTP, GTP[S], had no effect on Ca²⁺ release at 10 and 50 μ M. GDP at 10 μ M did not elicit any Ca²⁺ release from the endoplasmic reticulum. However, 50 μ M-GDP mobilized Ca²⁺ as effectively as did GTP ($P < 0.001$). This latter effect can be ascribed to phosphorylation of GDP by a nucleoside kinase in the presence of ATP to form GTP (Gill *et al.*, 1986). Nucleoside kinase is effectively inhibited by 100 μ M-GDP. Ca²⁺ release was not observed in the presence of 100 μ M-GDP ($P < 0.001$). Furthermore, GDP[S], a GDP analogue which is neither hydrolysed nor phosphorylated, and GMP did not mobilize Ca²⁺ from the endoplasmic reticulum. Thus GTP-induced Ca²⁺ release is highly specific. The fact that GTP[S] did not induce any Ca²⁺ release from the endoplasmic reticulum indicated that Ca²⁺ release was dependent on GTP being either hydrolysed or providing a high-energy phosphate group in a phosphorylation reaction. Interestingly, the combination of GTP with GDP or GDP[S] inhibited GTP-induced Ca²⁺ release from the endoplasmic reticulum (Table 1), suggesting that hydrolysis of

Fig. 2. Time course of GTP-induced Ca²⁺ release from the endoplasmic reticulum of digitonin-permeabilized islets

Permeabilized islets were loaded with ⁴⁵Ca²⁺ as described in the Experimental section. Ca²⁺ efflux was initiated by the addition of GTP (10 μM; ○) or not (control; ●), and the ATP-dependent Ca²⁺ content of the endoplasmic reticulum was determined at 0, 1, 2, 3, 4, 10 and 20 min after initiation of Ca²⁺ efflux. Non-ATP-dependent Ca²⁺ content was subtracted from the data. Results are shown as means ± S.E.M. of ATP-dependent Ca²⁺ content (pmol/islet) of the endoplasmic reticulum from 12–18 observations per each condition for three experiments.

GTP is required for GTP-induced Ca²⁺ release from the endoplasmic reticulum.

Reports in the literature have indicated that GTP enhances IP₃-induced Ca²⁺ release (Dawson, 1985). Others have suggested that GTP- and IP₃-induced Ca²⁺ release are not related (Gill *et al.*, 1986; Ueda *et al.*, 1986; Henne & Söling, 1986). We have compared IP₃-induced and GTP-induced Ca²⁺ release from the endoplasmic reticulum of islets. Fig. 3 indicates that the combination of GTP (10 μM) and IP₃ (10 μM) was completely additive for Ca²⁺ release (0.53 ± 0.04 pmol of Ca²⁺ released/islet, *P* < 0.001 versus control) as compared with either GTP alone (0.23 ± 0.07 pmol of Ca²⁺ released/islet, *P* < 0.05 versus control) or IP₃ alone (0.32 ± 0.06 pmol of Ca²⁺ released/islet, *P* < 0.002 versus control). This observation suggests that different mechanisms are involved in IP₃- and GTP-induced Ca²⁺ release. Furthermore, GDP (100 μM) at a concentration which inhibits GTP-induced Ca²⁺ release did not affect IP₃-induced Ca²⁺ release from the endoplasmic reticulum (Table 1). GTP-induced Ca²⁺ release from the endoplasmic reticulum was also examined at a free Ca²⁺ concentration of 40 μM, since previous studies have demonstrated that IP₃ does not cause any Ca²⁺ release from the endoplasmic reticulum at a free Ca²⁺ concentration of 40 μM (Wolf *et al.*, 1985, 1986a). At a free Ca²⁺ concentration of 40 μM, GTP (5 μM) mobilized 21.7 ± 5.2% of the ATP-dependent Ca²⁺ content of the endoplasmic reticulum (*P* < 0.002 versus control), whereas the Ca²⁺ ionophore A23187 (2 μM) released 54.5 ± 5.6% (*P* < 0.001 versus control).

The effect of GTP on Ca²⁺ release from the endoplasmic reticulum cannot be explained by inhibition of the ATP-dependent Ca²⁺-transport pump of the endoplasmic reticulum, since GTP (10 μM) did not affect the initial rate of Ca²⁺ uptake during the first 1.5 min (results not shown). Studies performed with [α-³²P]GTP

Table 1. Specificity of GTP-induced Ca²⁺ release from the endoplasmic reticulum of digitonin-permeabilized islets

Ca²⁺ release (pmol/islet) was measured as described in the Experimental section in the presence of guanine nucleotides. Control ATP-dependent Ca²⁺ content of the endoplasmic reticulum was 1.11 ± 0.04 pmol/islet.

Agent	Concn. (μM)	Ca ²⁺ released (pmol/islet)
GTP	10	0.35 ± 0.04** (32)
	50	0.34 ± 0.04** (18)
GTP[S]	10	0.05 ± 0.07 (18)
	50	0.07 ± 0.05 (18)
GDP	10	0.12 ± 0.07 (18)
	50	0.37 ± 0.05** (18)
	100	0.08 ± 0.06 (18)
GDP[S]	10	0.12 ± 0.06 (14)
	50	0.04 ± 0.07 (17)
GMP	10	0.03 ± 0.06 (18)
	50	0.07 ± 0.06 (18)
GTP + GDP	10 + 100	0.10 ± 0.04 (18)
GTP + GDP[S]	10 + 50	0.12 ± 0.05 (17)
IP ₃	10	0.32 ± 0.04* (6)
IP ₃ + GDP	10 + 100	0.33 ± 0.04** (12)

P* < 0.005; *P* < 0.001.

(10 μM) indicated that, during a 10 min incubation period, 80% of the GTP was not degraded (results not shown). Furthermore, those studies showed that, in the presence of ATP, there was significant phosphorylation of GDP to GTP.

DISCUSSION

We have used the digitonin-permeabilized islet model to assess the effect of GTP on Ca²⁺ fluxes maintained by the endoplasmic reticulum. Previous work from our laboratory has demonstrated that Ca²⁺ efflux from the endoplasmic reticulum of islets can be measured with the digitonin-permeabilized-islet model (Wolf *et al.*, 1985, 1986a,b,c). GTP caused rapid release of Ca²⁺ from the endoplasmic reticulum when measured under conditions which mimic intracellular conditions, thus providing to our knowledge the first demonstration of an effect of GTP on Ca²⁺ release from the endoplasmic reticulum of an endocrine cell. GTP-induced Ca²⁺ release from the endoplasmic reticulum was structurally specific and dose-dependent. Furthermore, this effect was observed in the absence of any added poly(ethylene glycol). In previous studies, the latter was required to observe GTP-induced Ca²⁺ release. These observations in the endoplasmic reticulum of islets confirm and extend previously reports of GTP-induced Ca²⁺ release from rat liver microsomal fractions, the endoplasmic reticulum of NIE-115 neuronal cells and the guinea-pig parotid gland (Dawson, 1985; Dawson *et al.*, 1986; Gill *et al.*, 1986; Ueda *et al.*, 1986; Henne & Söling, 1986).

IP₃ is recognized as a second messenger in numerous cells (Berridge & Irvine, 1984) and has been proposed to be involved in glucose-induced and carbamoylcholine-

Fig. 3. Comparison of IP₃- and/or GTP-induced Ca²⁺ release from the endoplasmic reticulum of digitonin-permeabilized islets

Permeabilized islets were loaded with ⁴⁵Ca²⁺ as described in the Experimental section. Ca²⁺ efflux was initiated by the addition of IP₃ (10 μM; ▨), GTP (5 and 10 μM; ▩), IP₃+GTP (10+5, 10+10 μM respectively; ■) and A23187 (2 μM; ▤). Ca²⁺ efflux was carried out for 10 min. Non-ATP-dependent Ca²⁺ content was subtracted from the data. Control ATP-dependent Ca²⁺ content of the endoplasmic reticulum was 1.00 ± 0.10 pmol/islet. Ca²⁺ release was calculated as in Fig. 1 and is expressed as the mean ± S.E.M. (pmol/islet) from 12 observations per each condition for two experiments.

induced insulin secretion (for review see Turk *et al.*, 1987; Wolf *et al.*, 1985; Wollheim & Biden, 1986; Prentki & Wollheim, 1984). Dawson and co-workers have proposed, on the basis of studies with a rat liver microsomal fraction, that IP₃-induced Ca²⁺ release from liver endoplasmic reticulum is mediated by GTP-phosphorylation of a protein which binds to microsomal membranes (Dawson, 1985; Dawson *et al.*, 1986). Studies by Gill and co-workers have suggested in studies performed with the NIE-115 neuronal cell line that GTP-induced Ca²⁺ release is probably related to IP₃-induced Ca²⁺ release (Ueda *et al.*, 1986). However, Henne & Söling (1986) have suggested that GTP-induced Ca²⁺ release is independent of IP₃-induced Ca²⁺ release in microsomal fractions of rat liver and guinea-pig parotid gland. Our studies in islet endoplasmic reticulum indicate that GTP-induced Ca²⁺ release is probably independent of IP₃-induced Ca²⁺ release, since the combination of maximal doses of IP₃ and GTP elicits precisely additive Ca²⁺ efflux from the endoplasmic reticulum, suggesting that different mechanisms of action are involved (Fig. 3). Furthermore, in the presence of a concentration of GDP which totally abolishes GTP-induced Ca²⁺ release, IP₃-induced Ca²⁺ release is not affected. Finally, we have

previously shown (Wolf *et al.*, 1985, 1986a) that IP₃-induced Ca²⁺ release from the endoplasmic reticulum of islets is only observed at submicromolar free Ca²⁺ concentrations. GTP-induced Ca²⁺ release from the endoplasmic reticulum was independent of the free Ca²⁺ concentration, however. These various lines of evidence indicate that GTP- and IP₃-induced Ca²⁺ release occur through different mechanisms.

The mechanism of GTP-induced Ca²⁺ release from the endoplasmic reticulum of islets is not clear, although GTP hydrolysis seems important (Gill *et al.*, 1986). The physiological significance of this observation and its relationship to glucose-induced insulin secretion remain unknown, since very little is known about GTP concentrations in islets (Hedekov, 1980).

In conclusion, we have shown that GTP mobilizes Ca²⁺ from the endoplasmic reticulum of islets and that this effect is mechanistically different from IP₃-induced Ca²⁺ release.

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