GTP enhances inositol trisphosphate-stimulated Ca^{2+} release from rat liver microsomes

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Low concentrations of GTP (10–50 μM) greatly enhance the inositol 1,4,5-trisphosphate stimulated Ca^{2+} release from rat liver microsomal vesicles. The effect of GTP depends on the presence of low concentrations of polyethylene glycol in the incubation medium. Guanylyl imidodiphosphate is ineffective at mimicking the GTP effect and inhibits the action of GTP added subsequently.

Calcium *Inositol 1,4,5-trisphosphate* *GTP* *Microsome* *Polyethylene glycol* *Polyphosphoinositide*

1. INTRODUCTION

It is now well established that a wide variety of hormones and neurotransmitters exert their effects via mobilisation of intracellular Ca^{2+}. In many cases it has been shown that Ca^{2+} mobilising stimuli are associated with enhanced breakdown of inositol phospholipids [1]. In several permeabilised cell preparations the water-soluble product of polyphosphoinositide hydrolysis, inositol 1,4,5 trisphosphate (IP_3), causes release of Ca^{2+} from an intracellular store [2–7], thought to be the endoplasmic reticulum. IP_3-stimulated Ca^{2+} release from subcellular fractions containing endoplasmic reticulum has been observed for a variety of preparations [8–11]. In the case of liver microsomal preparations, however, the release observed is small [8] and it was suggested that during homogenisation two populations of fragments from the endoplasmic reticulum were formed, one sensitive to IP_3 and one insensitive. All attempts in this laboratory to separate these two vesicle populations have failed, and indeed, have resulted in the total loss of IP_3 sensitivity. The percentage of Ca^{2+} releasable by IP_3 was, however, slightly increased by the presence in the assay medium of a low concentration (3%, w/v) of the membrane fusogen polyethylene glycol ([12]; A.P.D., unpublished). It is reported here that, in the presence of 3% polyethylene glycol, GTP enormously enhances the response of rat liver microsomal fractions to IP_3.

2. MATERIALS AND METHODS

2.1. *Materials*

Ca^{2+}-sensitive electrode membranes, containing the neutral ionophore ETH 1001 were a kind gift from Dr D. Ammann (ETH Zentrum, Zurich). Inositol, 1,4,5-trisphosphate was a gift from Dr R.F. Irvine (AFRC, Babraham, Cambridge). ^{45}Ca (0.5 mCi/μmol) was purchased from Amersham and GTP, GPPNP and polyethylene glycol (average M_r 8000) were from Sigma.

2.2. *Membrane preparations*

The 'mitochondrial fraction' and the heavy microsomal fraction, as defined by Reinhart and Bygrave [13], were prepared as described [8] from the livers of fed, female albino rats, except for the experiment shown in fig.4, which used a preparation from male rats. The mitochondrial fraction sediments at $8000 \times g$ for 10 min and the heavy microsomal fraction at $35\,000 \times g$ for 20 min.

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2.3. *Measurement of Ca^{2+} movements*

Ca^{2+} uptake and release was measured with a Ca^{2+}-sensitive electrode, using an excess of ATP of Mg^{2+} so that free ATP buffered Ca^{2+} in a working range of about 8×10^{-7} M. The incubation medium contained, in a volume of 5 ml: 150 mM sucrose, 50 mM KCl, 3% (w/v) polyethylene glycol, 10 mM Hepes-KOH, pH 7.0, 5 mM ATP (Na salt), 2 mM $MgCl_2$, 10 mM creatine phosphate, 0.1 mg/ml creatine kinase, 1 mM dithiothreitol, 1 μg/ml oligomycin and 4 μM ruthenium red. The temperature was 30°C. Uptake was started by the addition of microsomal vesicles.

Measurement of Ca^{2+} movements using ^{45}Ca was carried out under the same conditions, except that the total volume was 1 ml and contained in addition 0.15 μCi ^{45}Ca. Uptake was started by the addition of 3.5 mg mitochondrial fraction protein and at the given time intervals 50-μl aliquots were removed and filtered through 0.45 μm Millipore filters, pre-wetted with 150 mM sucrose, 50 mM KCl, 3% polyethylene glycol, 10 mM Hepes-KOH, pH 7.0. The samples were immediately washed on the filters with two, ice-cold 1-ml aliquots of the same medium as used to pre-wet the filters. The filters were placed in scintillation vials containing Pico-Fluor 15 (Packard) and counted using a scintillation counter. Total Ca^{2+} was measured on the residues of the incubation mixtures by atomic absorption spectrophotometry after precipitation of the protein with trichloroacetic acid. Total Ca^{2+} was found to be 37 μM. Ca^{2+} uptake figures are corrected for the small (0.5 nmol/mg) passive binding of Ca^{2+} to the vesicles and filters in the absence of an energy source.

3. RESULTS

Fig.1 shows results obtained using the mitochondrial fraction as described in [8] as a source of microsomes. Uptake of Ca^{2+} by mitochondria was inhibited by oligomycin and ruthenium red. Fig.1a shows that, after Ca^{2+} uptake is essentially complete, the addition of IP_3 causes the release of about 0.6 nmol Ca^{2+} per mg protein, as reported [8]. Although this appears from the trace to be a large percentage of the accumulated Ca^{2+}, this is not the case since, as will be shown later, most of the Ca^{2+} is accumulated during the first 2 min after the addition of the vesicles. Under these conditions, this part of the trace is lost due to the relatively slow electrode response to the addition of endogenous Ca^{2+} with the vesicles. When the initial release caused by IP_3 has finished, the further addition of 10 μM GTP causes a much greater Ca^{2+} release, which continues for a considerable time. Fig.1b shows the results obtained when the order of addition is reversed. In this case, the addition of 10 μM GTP causes a small, slow Ca^{2+} release but the subsequent challenge with IP_3 results in massive Ca unloading compared to that observed in the absence of GTP. Some idea of the concentration dependence of the GTP effect can be gained from the trace shown in fig.1c, which demonstrates that, altough clearly submaximal, 2 μM GTP has a very significant effect on Ca^{2+} release triggered by IP_3. It should be noted that GTP apparently has no effect in the absence of polyethylene glycol in the incubation medium (not shown).

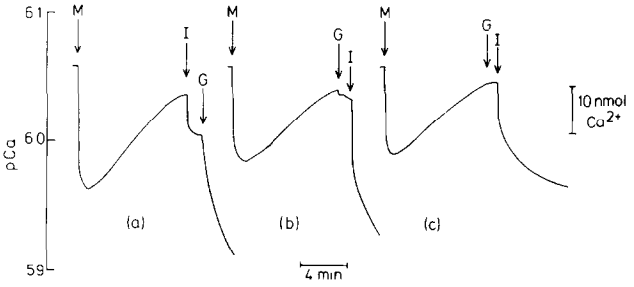

Fig.1. Ca^{2+} uptake and release from the mitochondrial fraction. Experimental conditions were as described in the text. Arrows: M, addition of 15 mg mitochondrial fraction protein; I, 5 nmol IP_3; G, addition of GTP, 50 nmol for traces a and b, and 10 nmol for trace c.

To obtain a better estimate of the percentage of intravesicular Ca^{2+} released under the various conditions, experiments similar to those of fig.1 were carried out using ^{45}Ca and a filtration method to follow Ca^{2+} movements. Fig.2b shows that IP_3 alone causes the release of less than 10% of the intravesicular Ca^{2+}, an amount which is only just detectable given the combined experimental and counting errors. However, as in fig.1, the subsequent addition of GTP causes a large Ca^{2+} release, amounting to about 50% of the total accumulated Ca^{2+}. In fig.2a, the addition of GTP causes a slow release of Ca^{2+} and, subsequently, IP_3 gives a rapid and massive ($\sim$50%) Ca^{2+} release.

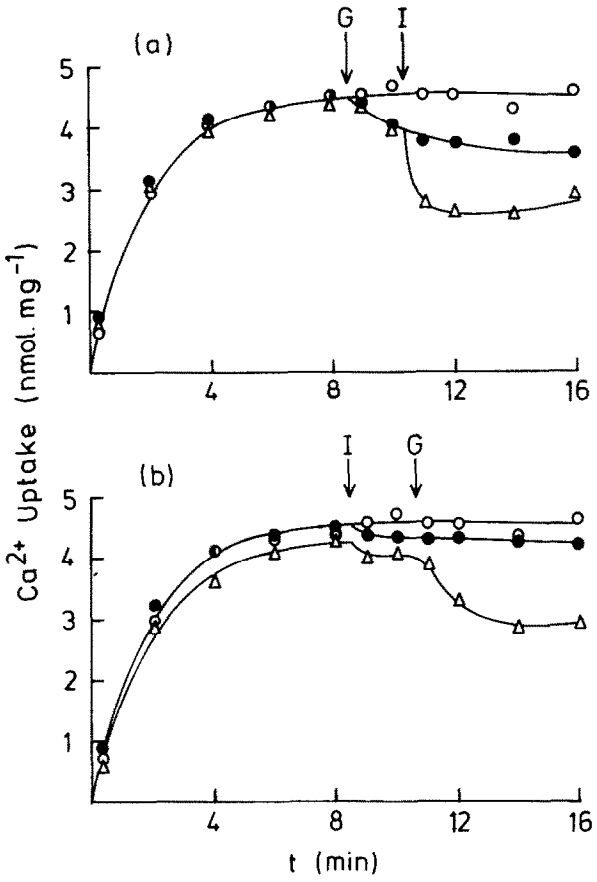

Fig.2. Measurement of Ca^{2+} movements using ^{45}Ca. Experimental conditions were as described in the text. At $t=0$, 3.5 mg mitochondrial fraction protein was added. (a) (○) No further additions, (●) 10 nmol GTP added at G, (△) 10 nmol GTP added at G, 1 nmol IP_3 added at I. (b) (○) No further additions, (●) 1 nmol IP_3 added at I, (△) 1 nmol IP_3 added at I, 10 nmol GTP added at G. Ca^{2+} uptake figures are corrected for the small (0.5 nmol/mg) passive binding of Ca^{2+} to the vesicles and filters in the absence of an energy source.

The presence of 10 μM GTP in the incubation medium at $t=0$ does not inhibit the initial rate of Ca^{2+} accumulation, although it does somewhat reduce the final loading. However, the stimulation of IP_3-promoted efflux is similar to that observed in figs 1b and 2a. This strongly suggests that the effect of GTP is on the efflux mechanism and not on accumulation.

In [8], we found that the lighter microsomal fraction, sedimenting at $35000\times g$ for 20 min, released an extremely small percentage of accumulated Ca^{2+} when IP_3 was added, and a similar observation was made by Joseph et al. [10] also using rat liver microsomes. Fig.3 shows that, although in the absence of GTP Ca^{2+} release is very small, it rises to quite substantial proportions if GTP is added either before or after IP_3.

The above experiments do not discriminate between a direct effect of GTP and an effect resulting from its metabolism, either as a phosphoryl group donor or as a source of other guanosine phosphates. That metabolism of GTP is required is suggested by studies using the non-metabolisable analogue guanylyl imidodiphosphate (GPPNP). Fig.4 shows that the size of the IP_3 response was

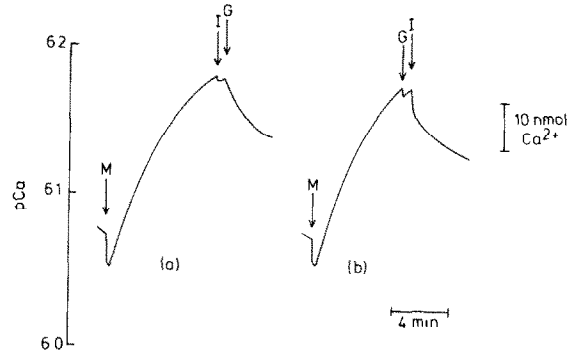

Fig.3. Ca^{2+} uptake and release from the microsomal fraction. Experimental conditions were as for fig.1, except that arrows marked M show the addition of 4.1 mg microsomal fraction protein. I, addition of 5 nmol IP_3; G, 20 nmol GTP added.

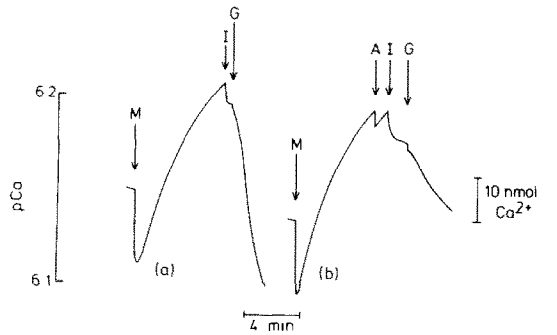

Fig.4. Effect of GPPNP on Ca^{2+} release. Experimental details were as for fig.1, except that the mitochondrial fraction was prepared from male rats. M, 13 mg mitochondrial fraction protein was added; I, 5 nmol IP_3; G, 50 nmol GTP. At A, 1 μmol GPPNP was added. The initial deflection of the trace at A is an addition artefact.

essentially unaffected by the presence of up to 200 μM GPPNP, although the response to a subsequent addition of GTP, using the protocol of fig.1a, was severely inhibited. Cyclic 3′,5′-GMP (50 μM) was also ineffective at promoting Ca^{2+} release (not shown).

4. DISCUSSION

The results described above present a basis for showing large effects of IP_3 on Ca^{2+} release from subcellular fractions of rat liver, although this basis remains, for the moment somewhat empirical. In particular, the requirement for polyethylene glycol to observe the effect of GTP suggests that the phenomenon is complex, requiring possibly, membrane aggregation or a rather specific interaction of membrane proteins. Nevertheless, Ca^{2+} release is still fundamentally dependent on IP_3. The finding that GPPNP is incapable of mimicking the effect of GTP distinguishes it from the effect of guanine nucleotides on the gating of Ca^{2+} channels in mast cells [14] where GPPNP was effective.

Under the assay conditions used here, the IP_3 stimulated Ca^{2+} release becomes very comparable in magnitude to that observed for permeabilised liver cells [4]. There is evidence for the involvement of GTP-binding proteins in signal transduction for Ca^{2+} mobilising receptors, by modulating the link between the receptor and the phosphatidylinositol (4,5)bisphosphate diesterase (review [15]). It therefore seems possible that GTP is involved both in the production of IP_3 and diacylglycerol and in the control of the effects of IP_3 on Ca^{2+} release from endoplasmic reticulum.

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