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GTP-dependent Ca^{2+} release from rat liver microsomes

Vesicle fusion is not required

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The GTP-dependent calcium release from rat liver microsomes is known to be promoted in the presence of colloids like polyethyleneglycol (PEG), polyvinylpyrrolidine, or albumin. Dawson et al. [(1987) Biochem. J. 244, 87–92] using the 'fusogen' PEG have concluded that both GTP-induced calcium efflux and the enhancement of $InsP_3$-promoted calcium release in the presence of GTP could be attributed to a GTP-dependent vesicle fusion. Here, using the more physiological colloid albumin we report that GTP-induced calcium release from rat liver microsomes may not be linked to vesicle fusion.

GTP-dependent Ca^{2+} release; GTP-γ-S; Aggregation; Fusion; Polyethyleneglycol; (Rat liver microsome)

1. INTRODUCTION

The effect of the second messenger $InsP_3$ to trigger calcium release from the endoplasmic reticulum seems to be well established [1,2]. In addition, it has been observed in a variety of cells that under certain conditions Ca release from the endoplasmic reticulum can also be induced by GTP [3–7]. Dawson and co-workers [3,4] were the first to report that in the presence of 5% polyethyleneglycol GTP can enhance $InsP_3$-mediated calcium release. Later it was shown that GTP independently, in the absence of $InsP_3$, was able to promote calcium release from the endoplasmic reticulum [7–9]. This effect, however, was still dependent on the presence of additional compounds like PEG, polyvinylpyrrolidone, or bovine serum albumin [7]. Dawson and Irvine [2] have proposed a GTP-dependent fusion of different subpopulations of microsomal vesicles some of which contain $InsP_3$ receptors. Indeed Dawson et al. [10] using electron microscopy and light scattering to follow changes in vesicle size and number have noted the production of very large vesicular structures produced by fusion of smaller vesicles in the presence of GTP (and PEG) and concluded that both GTP-induced calcium efflux and the enhancement of $InsP_3$-promoted Ca release in the presence of GTP could be attributed to a GTP-dependent vesicle fusion. The purpose of this study was to investigate whether the GTP-dependent Ca release is dependent on vesicle fusion, as observed in the presence of PEG [10,11], or whether vesicle fusion under this special condition parallels the release of calcium without being causally linked to this process.

Here, we compared the effects of GTP on calcium release, vesicle aggregation and fusion in the presence of bovine serum albumin with those obtained in the presence of PEG. The results indicate that GTP-mediated calcium release is not dependent on vesicle fusion.

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Abbreviations: PEG, polyethyleneglycol; PVP, polyvinylpyrrolidine; DTT, dithiothreitol; $InsP_3$, inositol 1,4,5-trisphosphate

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2. MATERIALS AND METHODS

Male Wistar rats (b.w. 180–220 g) were obtained from Mus-Rattus, Brunnthal, FRG, and fed ad libitum before use. The animals were decapitated, the abdominal cavity was opened and the liver rapidly excised. The liver was minced and homogenized in 9 parts (v/w) of a medium consisting of 250 mM sucrose, 5 mM Hepes-KOH, pH 7.0, 1 mM DTT, 0.5 mM EGTA using a Potter glass/Teflon homogenizer (Thomas, Philadelphia, size C) and three strokes at 900 rpm. The homogenate was centrifuged at 1000 × g_{max} for 5 min. The supernatant was removed and recentrifuged for 10 min at 7750 × g_{max}. The supernatant from this step was centrifuged at 35000 × g_{max} for 20 min. The resulting sediment was resuspended in about the same volume of homogenization buffer and centrifuged again at 35000 × g_{max} for 20 min. The supernatant was decanted and the pellet suspended to a concentration of 20–30 mg protein/ml in a medium identical to the homogenization buffer, but containing 10 mM KCl instead of EGTA.

2.1. *Measurement of calcium release*

Isolated rat liver microsomes (1.5–2.5 mg/ml) were incubated at 30°C in a thermojacketed chamber in a medium containing 150 mM sucrose, 50 mM KCl, 2 mM $MgCl_2$, 1 mM DTT, 5 mM creatine phosphate, 5 mM ATP, 20 mU/ml creatine kinase, 1 μg/ml oligomycin, 2 μM ruthenium red, 5 mM Hepes, pH 7.0, and the additions as specified in the text. Calcium release was followed by means of a calcium selective electrode (Radiometer), filled with the neutral carrier ETH 1001. After thermoequilibration the reaction was started by addition of microsomes to the incubation. Further additions were made when a steady-state calcium level was manifest. For calibration a pulse of 5 nmol Ca^{2+} was added after each run.

2.2. *Measurements of light scattering*

Light scattering was recorded using an Eppendorf photometer (filter 400–600 nm) equipped with a thermoconstant cuvette holder and a stirring device. Light scattering was followed under the identical conditions given above for the measurement of calcium release.

2.3. *Electron microscopy*

The sampling and handling of specimens for evaluation by electron microscopy was performed as outlined in [10]. Briefly, an aliquot of 0.5 ml taken from the incubation at the timepoints indicated was mixed with 40 μl of 25% (v/v) glutaraldehyde and centrifuged for 10 min. The samples were post-fixed in 2% (w/v) osmium tetroxide. Dehydration was carried out in increasing concentrations of ethanol. The samples were imbedded in epon/araldite (Poly/Bed 812, Polysciences, Inc.) and sectioned using an ultramicrotome.

2.4. *Other methods*

The activity of the rotenone-insensitive NADH-cytochrome-*c* reductase was measured according to [12]. Protein was determined with Coomassie blue [13].

2.5. *Materials*

All chemicals and biochemicals were of the highest purity available. GTP (type III) was obtained from Sigma, GTP-γ-S was a kind gift from Dr F. Eckstein (Max-Planck-Institut für Experimentelle Medizin, Göttingen). The bovine serum albumin came from Miles (fraction V). It was purified before use by gel chromatography (Sephadex G-10).

3. RESULTS AND DISCUSSION

Fig.1 shows the GTP (20 μM)-induced calcium release and changes in light scattering by rat liver microsomes in the presence of PEG or albumin. As we have shown before [7], GTP-induced calcium release is not only promoted in the presence of PEG, but also in the presence of albumin (left panel). The right panel depicts the matched recording of changes in light scattering observed under this condition. The comparison shows a lack of GTP-induced light scattering in the presence of albumin although a sustained release of calcium is observed under these conditions (fig.1B). The change in light scattering observed in the presence of PEG was comparable to that reported by [10] and could be suppressed by 90 μM GTP-γ-S (fig.1A). It could be excluded that the lack of GTP-dependent light scattering in the presence of albumin was due to a different 'opalescence' of the medium. The quench due to albumin (3 g/100 ml) of GTP-induced light scattering in the presence of PEG (5 g/100 ml) was 26 ± 5 (5)%.

Fig.2 shows the corresponding electron micrographs. The presence of PEG causes an aggregation of vesicles to form large clusters. Under these conditions following the addition of GTP the appearance of the vesicles is changed, in that many large vesicles with a mean diameter of 1.5 μm arise, as has been first observed by [10]. However in the presence of albumin, the mean diameter of the microsomal vesicles remains unchanged if one compares their size before and after the addition of GTP (fig.2B).

Again the effect of GTP on vesicle size and number in the presence of PEG is effectively blocked by GTP-γ-S. Dawson and co-workers [10,11] have proposed that a GTP-induced membrane fusion plays a major role in causing GTP-promoted calcium efflux from microsomal vesicles. However, these properties cannot be assigned to albumin as a water-structuring colloid, which promotes apparently calcium release in the presence of GTP, without giving rise to membrane fusion, as is inferred by the lack of GTP-induced increase in light scattering and by the absence of a significant change in vesicle diameter with the ap-

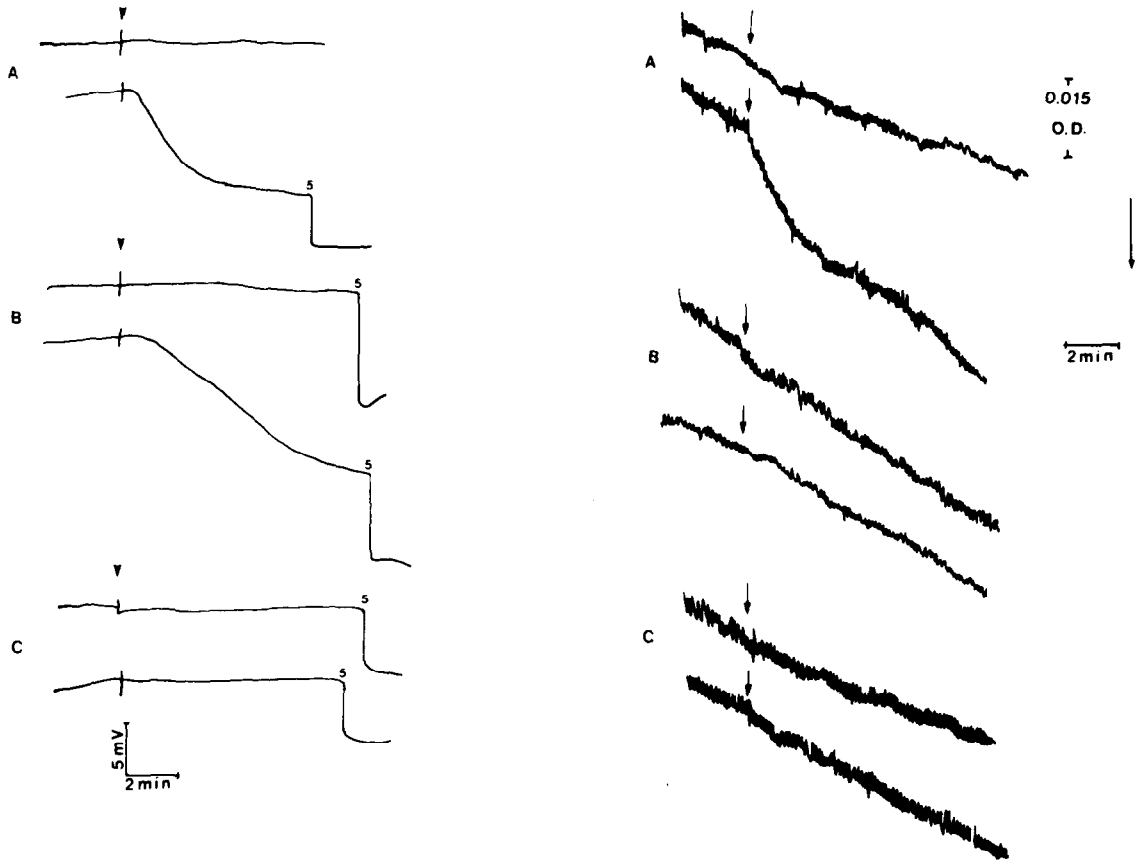

Fig.1. GTP-induced calcium release (left panels) and light scattering (right panels) of rat liver microsomes in the presence of PEG or BSA. Isolated rat liver microsomes (1.2 mg/ml) were incubated in a medium containing 150 mM sucrose, 50 mM KCl, 2 mM $MgCl_2$, 1 mM DTT, 5 mM creatine phosphate, 5 mM ATP, 20 mU/ml creatine kinase, 1 μg/ml oligomycin, 2 μM ruthenium red, 5 mM Hepes, pH 7.0, and the additions as specified. GTP was added (arrows) to give a concentration of 20 μM. Calcium release was followed by means of a calcium selective electrode (Radiometer). Light scattering was measured in an Eppendorf photometer (400–600 nm). Downward deflections (arrow) correspond to calcium release (left panels) or swelling (right panel). In all upper traces, GTP-γ-S (90 μM) was added 4 min prior to the addition of GTP. (A) PEG (5 g/100 ml), (B) BSA (3 g/100 ml), (C) control.

pearance of multilamellar structures, as seen in the presence of PEG.

In addition when studying the sedimentation behavior of microsomal membranes in the presence of PEG (0–5 g/100 ml), polyvinylpyrrolidine (0–5 g/100 ml) or albumin (0–5 g/100 ml), there was a marked discrepancy between PEG or PVP and albumin (fig.3). In these experiments the disappearance of the membrane marker enzyme rotenone-insensitive NADH-cytochrome-*c* reductase was followed due to sedimentation after centrifugation for 2 min in an Eppendorf centrifuge. Whereas under these conditions 50% of the total activity was already recovered in the sediment at 2.5 g/100 ml PEG or PVP (fig.3), almost no sedimentation occurred in the presence of albumin, indicating that aggregation of vesicles develops in the presence of PEG (and PVP) but not in the presence of albumin. This aggregation could be a conditio sine qua non for vesicle fusion, as proposed by [10,11], but as shown here does not seem to be a prerequisite for the release of calcium from vesicular stores by GTP. The exact mechanism of GTP-mediated Ca^{2+} release remains obscure. Nishitta et al. [14] have observed that GTP-dependent calcium release in stripped rER preparations did not require the presence of PEG, although the rate of calcium release was stimulated by the latter. They found a correlation with a GTP-sensitive increase in membrane permeability

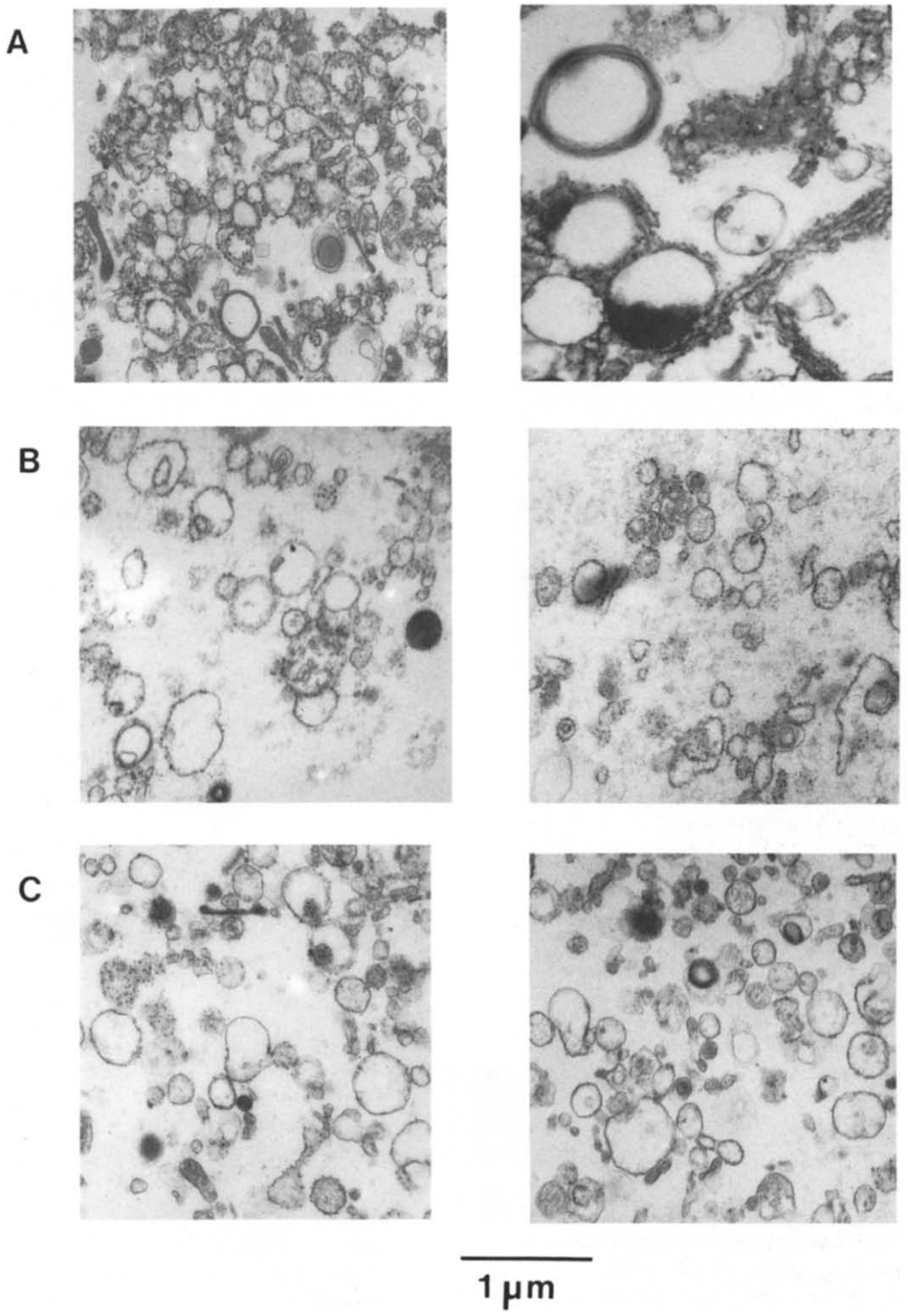

Fig.2. Electron micrographs of microsomal vesicles. Isolated rat liver microsomes (4.8 mg/ml) were incubated as given in the legend to fig.1. After 9–16 min (plateau), a sample (0.5 ml) was taken out for electron microscopy and GTP (20 μM) was added to the incubation. 5 min later a second aliquot of 0.5 ml was taken and mixed with glutaraldehyde as described in section 2. (A) Before and after GTP in the presence of PEG (5 g/100 ml); (B) before and after GTP, in the presence of albumin (3 g/100 ml); (C) before and after GTP-γ-S (90 μM) in the presence of PEG (5 g/100 ml).

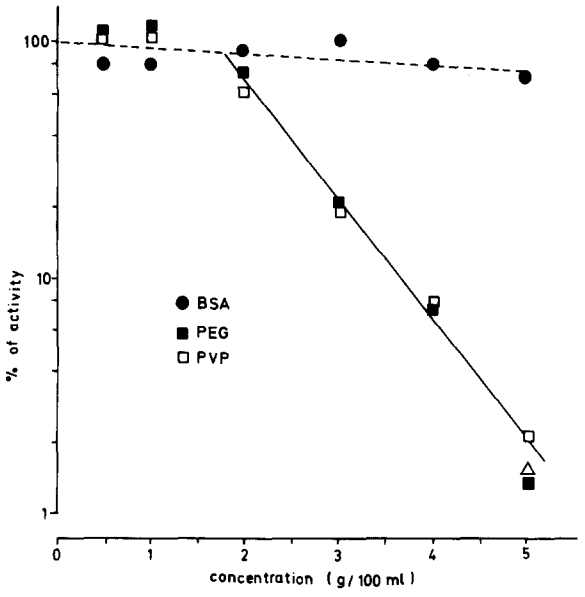

Fig.3. Sedimentation of microsomal vesicles in the presence of varying concentrations of polyethyleneglycol (PEG), polyvinylpyrrolidine (PVP) and bovine serum albumin (BSA). Microsomal vesicles (2.4 mg/ml) were incubated under conditions given in fig.1 in the standard incubation medium and in the presence of varying concentrations of PEG, PVP or BSA as indicated on the abscissa. After 8 min, GTP (20 μM) was added and 4 min later the incubation was terminated by rapid centrifugation using an Eppendorf centrifuge at 12500 × g for 2 min. A small aliquot of the supernatant was used for the determination of the microsomal membrane marker rotenone-insensitive cytochrome-c reductase, as described in section 2. The enzyme activity recovered in the supernatant in the absence of PEG, PVP or albumin (0.46 ± 0.09 (4) μmol·min^{-1}·ml^{-1}) was set at 100%. The triangle represents the activity remaining in the supernatant in the presence of albumin after 5 min of centrifugation at 160000 × g_{max} using a Beckman airfuge.

as judged by latency measurements of microsomal mannose 6-phosphatase. Thomas [15] using digitonin-permeabilized hepatocytes to study the GTP effect reported a loss of PEG requirement after extensive washing of the cells, most probably altering by this the membrane organisation leading to anomalous coupling between $InsP_3$ and GTP effector sites. It thus appears that GTP causes permeability changes and presumably also structural changes in certain areas of the membrane obligatory for calcium release, which in the presence of PEG will facilitate aggregation and fusion.

This effect could be tissue specific as in N1E-115 cells the electron microscopic evaluation did not reveal vesicle fusion, in the presence of PEG + GTP, although a close coalescence of membranes was noted [16]. The model proposed by these authors [17,18] includes essentially the formation of close appositions of membranes in the presence of PEG. However, in the liver, this kind of aggregation does not seem to be a mandatory condition for the stimulation of calcium release by GTP.

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