GTP-induced fusion of isolated pancreatic microsomal vesicles is increased by acidification of the vesicle lumen

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Using the 'fusogen' polyethyleneglycol (PEG), Dawson et al. [1] have concluded that both guanosine triphosphate (GTP)-induced calcium efflux and the enhancement of IP_3-promoted calcium release from rat liver microsomal vesicles could be attributed to a GTP-dependent vesicle fusion. We have studied GTP-induced fusion of microsomal vesicles from rat exocrine pancreas using light scatter and fluorescence dequenching methods. In the presence of PEG (3%), GTP (10 μM) induced a decrease in light scatter and an increase in fluorescence in the fluorescence dequenching assay (GTP-effect) indicating fusion of the vesicles. Guanosine 5'-*O*-(3-thiotriphosphate) (10 μM) had no effect on its own and inhibited the GTP-induced signals. Preincubation of the vesicles with adenosine triphosphate (ATP) (4 mM) increased the GTP-effect by 80%, whereas bafilomycin B_1, a specific inhibitor of vacuolar type H^+-ATPases, and the protonophore CCCP (10 μM) inhibited only the ATP-dependent part of the GTP-effect. Inhibitors of the vacuolar type H^+-ATPase, which are also SH-alkylating reagents such as *N*-ethylmaleimid (100 μM) and the tyrosine-, cysteine- and lysine-reactive reagent 7-chloro-4-nitrobenz-2-exa-1,3-diazole (10 μM) [2,3], abolished the GTP-effect in the absence or presence of ATP. We conclude that GTP induces fusion of pancreatic microsomes which is increased by an H^+ gradient established by a vacuolar type H^+-ATPase.

Proton-gradient; Energy transfer; Light scatter; Acridine orange; H^+-ATPase; Bafilomycin B_1

1. INTRODUCTION

Electron microscopy studies have indicated that guanosine triphosphate (GTP) induces fusion of liver microsomal vesicles in the presence of polyethyleneglycol (PEG) [1]. In studies on fluorescence energy transfer as a measure for vesicle fusion and on Ca^{2+} transport, Comerford and Dawson found similar dependencies of both vesicle fusion and Ca^{2+} release on the PEG- and GTP-concentrations [4] and on limited proteolysis of rat liver microsomes [5] indicating a relationship between both GTP-induced Ca^{2+} release and vesicle fusion and a participation of proteins in these events. In line with the interpretation that GTP can induce membrane fusion, Mullaney et al. have proposed that GTP could induce Ca^{2+} conveyance between different Ca^{2+} pools in vivo, whereas in vitro GTP possibly leads to a Ca^{2+} leakage by promoting Ca^{2+} conveyance between closed vesicles and non-closed membranes [6]. In contrast Kleinecke et al. suggested that vesicle fusion is not necessary for GTP-induced Ca^{2+} release in liver microsomal vesicles [7].

Previous results from our laboratory indicate the existence of two intracellular Ca^{2+} pools in pancreatic acinar cells. Inositol-1,4,5-trisphosphate (IP_3) releases Ca^{2+} from a pool, which is filled with Ca^{2+} via a Ca^{2+}/H^+-countertransporter at the expense of an H^+ gradient, that is established by an ATP-dependent vacuolar type H^+-pump. The other Ca^{2+} pool which is IP_3-insensitive is filled by a vanadate inhibitable Ca^{2+}-ATPase [8]. In order to obtain information if the H^+ gradient might play a role in GTP-induced fusion of membrane vesicles from pancreatic acinar cells, we have compared results on GTP-induced changes in fluorescence and light scatter with ATP-dependent H^+ uptake into vesicles as determined by the acridine orange technique [9].

We found that GTP-induced fusion was increased in the presence of an H^+ gradient across the vesicle membrane established by the vacuolar type H^+-ATPase [10] and decreased in the presence of the protonophore CCCP or inhibitors of the H^+-ATPase. We conclude from our data that intravesicular acidification might play an important role in GTP-induced fusion of pancreatic microsomes.

2. MATERIALS AND METHODS

2.1. *Materials*

A23187 and nigericin were obtained from Calbiochem (Frankfurt, FRG); acridine orange was from Merck (Darmstadt, FRG). Adenosine triphosphate (ATP), GTP, Na_3VO_4, bovine serum albumin (BSA), creatine phosphate, and 7-chloro-4-nitrobenz-2-oxa-1,3-diazole (NBD-Cl) were purchased from Sigma (München, FRG). Bafilomycin B_1 was a gift from Bayer AG (Wuppertal, FRG). Collagenase of *Clostridium histolyticum* was from Worthington

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(Freehold, NJ, USA). Carbonylcyanide-*m*-chlorophenylhydrazone (CCCP), valinomycin, guanosine 5'-*O*-(3-thiotriphosphate) (GTPγS), and creatine kinase were obtained from Boehringer (Mannheim, FRG). Octadecylrhodamine (R 18) was from Molecular Probes Inc. (Eugene, OR, USA). PEG (average M_r 6000) was purchased from Fluka (Neu Ulm, FRG).

2.2. *Methods*

2.2.1. Preparation of pancreatic microsomal vesicles

Rat pancreatic microsomal vesicles were prepared from isolated pancreatic acinar cells [11] as described previously [12] by centrifugation of cell homogenate at 11000 × *g*. The fluffy layer on top of the 11000 × *g* pellet, which is enriched by about two-fold in endoplasmic reticulum (ER) with respect to the homogenate [12], was removed and vesicles were resuspended in a mannitol-buffer (pH 7) at a concentration of 30 mg protein/ml, were then frozen in liquid nitrogen and stored until use. Protein was determined according to Bradford [13] using BSA as standard.

2.2.2. Light scattering

Membrane vesicles (0.3 mg protein) were suspended in 2 ml of buffer A, containing (in mM): KCl 155, Hepes 5, $MgCl_2$ 2, $CaCl_2$ 0.38, EDTA 0.2, oligomycin 0.01 and PEG 3% (w/v), pH adjusted to 7.0 with Tris at 30°C at continuous stirring. Light scattering was recorded using a Perkin Elmer LS-5 Luminescence spectrometer at a wavelength of 640 nm and at a detection angle of 90°.

2.2.3. Fluorescence dequenching assay

Loading of microsomes with octadecylrhodamine (R 18) was carried out similarly as described previously [4,14]. In this assay a self-quenching concentration of R 18 is incorporated into the lipid phase of the membranes of vesicles. After mixing these vesicles with unlabeled vesicles, fusion leads to a decrease in the concentration of the dye in the membrane, thus to a lesser degree of self-quenching (= dequenching) and to an increase in fluorescence. Half of the vesicles (1.8 mg protein) was labeled with R 18 (10 μM) in buffer A, whereas the other half remained unlabeled. Following an incubation of 5 min at 30°C both samples were combined. Fluorescence was then monitored at an emission wavelength of 600 nm and an excitation wavelength of 460 nm at continuous stirring at 30°C. GTP, ATP, GTPγS, CCCP, bafilomycin B_1, and NEM were added from 100 × concentrated stock solutions.

2.2.4. Measurement of proton-gradient formation with acridine orange

Measurement of H^+ uptake was carried out as described previously [10] using acridine orange (AO). The difference in absorbance at 493–540 nm was recorded in an Aminco DW-2 UV/vis spectrophotometer (Silverspring, MD). H^+ transport was initiated by adding 4 mM Mg-ATP in the presence of an ATP-regenerating system [10] and in the presence or absence of the indicated substances. When a steady state H^+ gradient had been reached, the protonophore nigericin (1 μM) was added to dissipate the H^+ gradient over the vesicle membrane.

2.2.5. Statistics

Values are given as means ± SE. Significances were calculated with the paired *t*-test.

3. RESULTS

3.1. *Light scattering*

Fig. 1 shows that in the presence of PEG (3%) addition of GTP (10 μM) to vesicles causes a decrease in light scatter of 9 ± 3.6% of the base before addition of GTP (control, $n = 13$). In the absence of PEG no GTP-effect was observed. Guanosine diphosphate (GDP)

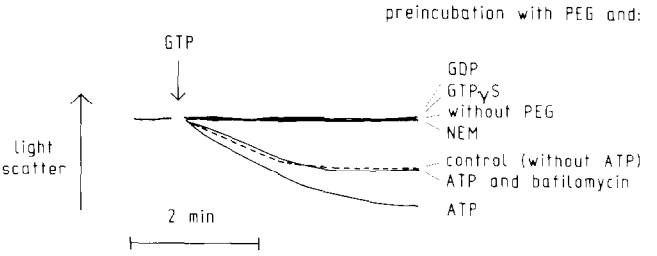

Fig. 1. GTP-induced fusion of vesicles as measured with light scatter in the absence or presence of ATP (4 mM, preincubation for 10 min), bafilomycin B_1 (1 nM, for 10 min), NEM (100 μM, for 2 min), GDP (10 μM, for 2 min), GTPγS (10 μM, for 2 min) or PEG (3%, w/v, for 15 min); addition of GTP (10 μM) where indicated.

(10 μM) and GTPγS (10 μM) did not induce any change in light scatter (not shown) and prevented the GTP-effect (Fig. 1). Preincubation of vesicles with the tyrosine-, cysteine- and lysine-reactive reagent NBD-Cl (10 nM, not shown) or the SH-group-alkylating reagent NEM (100 μM) for 2 min also abolished the GTP-effect (Fig. 1). In the presence of ATP (4 mM) the change in light scatter induced by GTP was increased significantly by 81 ± 24% of the control value (Fig. 1, $P < 0.005$, $n = 7$) whereas in the presence of ATP and the protonophore CCCP (preincubation with 10 μM for 2 min) this increase was prevented and the GTP-effect under these conditions was only 91 ± 25% of the control value (not shown, $P < 0.02$). Preincubation with the H^+-ATPase inhibitor bafilomycin B_1 [15] also decreased the ATP-dependent part of the GTP effect to 110 ± 27% of the control value ($P < 0.02$; Fig. 1), indicating that an H^+-ATPase is involved in the ATP-dependent part of the GTP-effect (see below).

The size of the GTP-effect was not changed at a vesicle inside positive potential difference over the membrane generated in the presence of a K^+ gradient (vesicle outside 155 mM KCl, vesicle inside 10 mM KCl and 290 mM mannitol) and in the presence of the K^+ ionophore valinomycin (10 μM) (not shown).

3.2. *Fluorescence dequenching*

A GTP-effect was also seen in fluorescence dequenching experiments: addition of GTP to membranes, half of which were labeled with R 18, resulted in an increase in fluorescence by 21 ± 7.5% ($n = 6$; Fig. 2a). The GTP-effect was the same when we used microsomes which were all labeled with R 18 (not shown). We assume that different degrees of loading with R 18 to different vesicle populations had taken place, which after fusion leads to equilibration of the dye concentration and hence to fluorescence dequenching.

As in light scatter experiments (Fig. 1) preincubation with GTPγS (10 μM) or NEM (100 μM) for 2 min abolished the GTP-induced fluorescence dequenching in vesicles half of which had been labeled with R 18

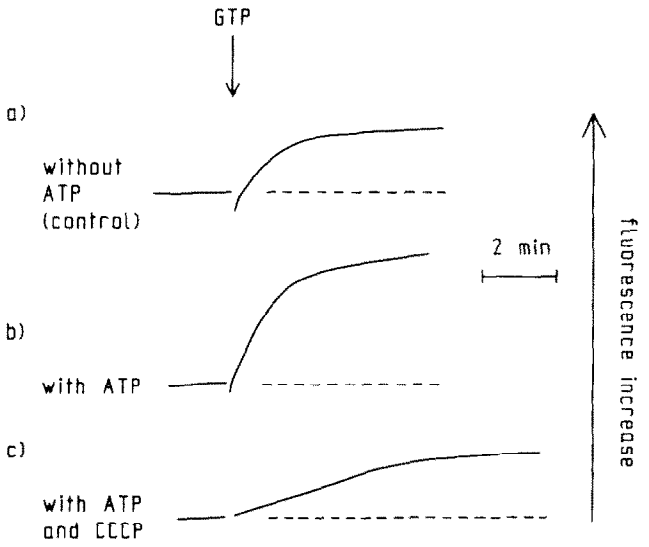

Fig. 2. GDP-induced fluorescence increase in the 'fluorescence dequenching'-assay; one half of the vesicles was labeled with R 18, the other half was unlabeled; (a) in the absence or (b) in the presence of ATP (4 mM, preincubation for 15 min) and (c) in the presence of ATP (4 mM) plus CCCP (10 μM, preincubation for 15 min); addition of GTP (10 μM) where indicated.

(not shown). ATP (4 mM), which alone did not induce any fluorescence increase, enhanced the GTP-effect by 109 ± 45% ($P < 0.005$; Fig. 2b). Both bafilomycin B_1 (1 nM, not shown) and CCCP (10 μM) inhibited the ATP-dependent part of the GTP-effect to 130 ± 24% and 120 ± 18% of the control, respectively ($P < 0.01$; Fig. 2c).

The GTP-effect was independent of extracellular Ca^{2+} (as tested between 5 pM to 38 μM) and was not inhibited by the Ca^{2+} ionophore A23187 (10 μM, not shown).

3.3. *Measurement of proton-gradient formation with acridine orange*

The data obtained with bafilomycin B_1 (Fig. 1) and CCCP (Fig. 2c) suggest participation of an H^+-ATPase in the ATP-dependent part of the GTP-effect. We have therefore directly measured the formation of an H^+ gradient with the AO technique.

Fig. 3a shows the formation of an H^+ gradient upon addition of ATP (4 mM), which was dissipated by the H^+/K^+ ionophore nigericin (1 μM). Preincubation of vesicles with bafilomycin B_1 (1 nM) for 2 min abolished H^+ uptake (Fig. 3a). The protonophore CCCP also

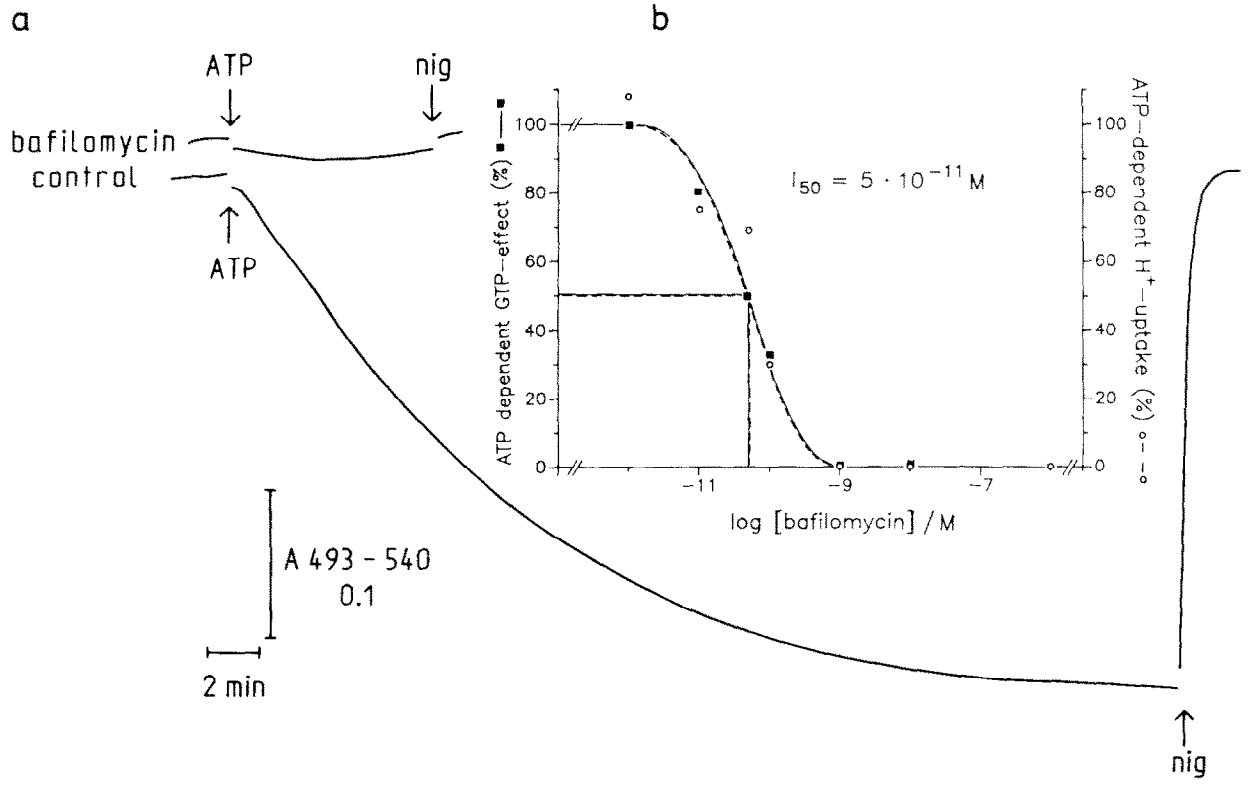

Fig. 3. (a) H^+ uptake by vesicles as measured with acridine orange absorbance in the absence or presence of bafilomycin B_1 (1 nM, preincubation for 2 min); ATP (4 mM Mg-ATP, pH 7.0) and nigericin 1 μM were added where indicated (one out of 6 similar experiments). (b) Correlation of H^+ uptake (as measured with acridine orange) with the ATP-dependent part of the GTP-effect (as measured with light scattering at the same experimental conditions in the presence of 4 mM ATP and an ATP-regenerating system) in the absence or presence of different concentrations of bafilomycin B_1. The maximal sizes of the ATP-dependent part of the GTP-effect and of ATP-dependent H^+ uptake at steady state in the absence of bafilomycin B_1 are expressed as 100%.

prevented the formation of an H^+ gradient (not shown, see [10]).

To examine if there is a correlation between the ATP-dependent part of the GTP-effect and formation of an H^+ gradient, we have determined H^+ uptake into vesicles with the AO-technique and the GTP-effect by light scatter in the presence of different concentrations of bafilomycin B_1 (Fig. 3b). Both ATP-dependent H^+ uptake (right ordinate) and ATP-dependent GTP-effect (left ordinate) were completely inhibited by 1 nM bafilomycin B_1; 50% inhibition of both was observed at 50 pM bafilomycin B_1.

Addition of GTP (10 μM) to vesicles at H^+ uptake conditions did not show any change in AO-absorbance (not shown).

4. DISCUSSION

Previous studies have examined if GTP-induced vesicle fusion is involved in GTP-induced Ca^{2+} release in microsomal vesicles [1,4,7]. Dawson et al. [1], using electron microscopy and light scattering to follow changes in vesicle size and number, have noted the production of large vesicle 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 IP_3-promoted Ca^{2+} release in the presence of GTP could be attributed to a GTP-dependent vesicle fusion. In contrast, Kleineke et al. [7] have suggested that aggregation of vesicles in the presence of PEG or PVP could be a conditio sine qua non for GTP-induced vesicle fusion as had been proposed by Dawson and collaborators [1,4] but does not seem to be a prerequisite for GTP-induced Ca^{2+} release [7]. Whereas in all studies with subcellular fractions the effects of GTP on Ca^{2+} translocation could not be seen without the presence of additional compounds like PEG [16], PVP or bovine serum albumin [17], GTP-induced Ca^{2+} translocation could be observed without the need of PEG or of other fusogens in permeabilized cells [6,18]. It has been proposed that the action of PEG at 1–3% in promoting GTP-activated Ca^{2+} movements is due to formation of close appositions between membranes [6]. It could be that this close apposition of membranes is maintained in non-disrupted cells as compared to isolated microsomes. We have recently shown that Ca^{2+} uptake into IP_3-sensitive and IP_3-insensitive Ca^{2+} pools is mediated by a Ca^{2+}/H^+-exchanger at the expense of an H^+ gradient which is established by a vacuolar type H^+-ATPase [10]. In the present study we have focussed on the question if GTP-induced vesicle fusion is influenced by intravesicular acidification.

Both light scatter and fluorescent techniques show an increase of the GTP-effect after preincubation with ATP. The increase was abolished if formation of the H^+ gradient had been prevented by the H^+-ATPase inhibitor bafilomycin B_1 or the protonophore CCCP. The SH-alkylating H^+-ATPase inhibitors NEM and the tyrosine-, cysteine- and lysine-reactive reagent NBD-Cl abolished both the ATP-dependent and the ATP-independent part of the GTP-effect. The mechanism of GTP-induced vesicle fusion as described here could involve an NEM-sensitive factor which had been described recently to be involved in cell sorting fusion processes within the Golgi stack of animal cells [19]. The increase of the GTP-effect in the presence of ATP is probably due to formation of an H^+ gradient by the H^+-ATPase present in pancreatic microsomes [10] since it can be abolished by inhibitors of the H^+-ATPase and by protonophores (see Figs 1 and 2). The increase in fusion cannot be attributed to a change in membrane potential by the electrogenic H^+-pump, as the size of the GTP-effect is not affected by a change of the electrical membrane potential in the presence of valinomycin.

Membrane fusion which is activated by low pH is known from other systems as the influenza virus [20], cells expressing glycoproteins from herpes simplex virus

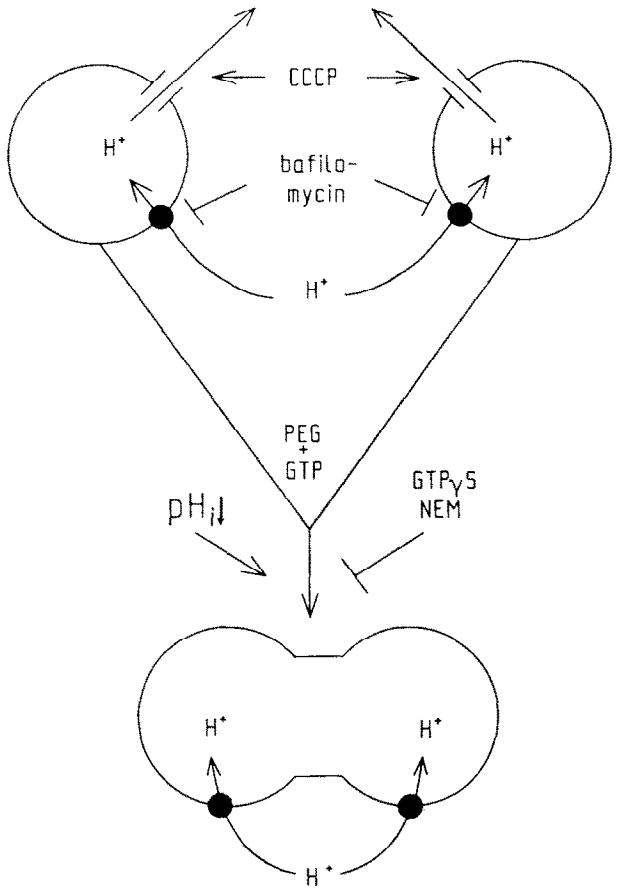

Fig. 4. Model for membrane vesicle fusion of endoplasmic reticulum from rat exocrine pancreas; for explanations see text.

[21] or in clathrin-induced fusion of liposomes [22], but was so far unknown for GTP-induced fusion of pancreatic microsomes.

Our data indicate that intravesicular acidification rather than intravesicular Ca^{2+} concentrations enhance GTP-induced vesicle fusion.

Since addition of GTP did not induce any change in AO absorbance, a GTP-induced fusion of vesicles with different intraluminal pH is unlikely to occur. Our data therefore indicate that the ability for GTP-induced fusion is a property of vesicles containing the H^+-pump. As suggested by our model shown in Fig. 4, fusion can occur in the presence of PEG and in the absence of an H^+ gradient but is enhanced if the H^+-pump is activated with ATP. Substances which lead to a decrease of intravesicular acidification such as the H^+-pump inhibitor bafilomycin B_1 or the protonophore CCCP also decrease GTP-induced fusion. The fusion process is inhibited by the weakly hydrolysable analogues of GTP such as GTPγS and by SH-group reagents such as NBD-Cl or NEM (Fig. 4). According to previous observations that GTP induces Ca^{2+} conveyance between Ca^{2+} pools [6] and to our previous models [8], GTP-induced fusion should take place only between Ca^{2+} pools which contain an H^+-pump in addition to the Ca^{2+}/H^+-exchanger.

Whether this intravesicular acidification has any significance for Ca^{2+} conveyance of intracellular Ca^{2+} pools remains to be investigated.

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