The mechanism of action of GTP on Ca^{2+} efflux from rat liver microsomal vesicles

Measurement of vesicle fusion by fluorescence energy transfer

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1. GTP-promoted fusion between microsomal vesicles was studied by using fluorescence-resonance-energy transfer between the fluorescent membrane probes octadecanoyl-aminofluorescein and octadecyl-rhodamine.
2. The fluorescence increase after GTP addition does not require the presence of ATP, is unaffected by changes in free $[\text{Ca}^{2+}]$ in the range $10\text{ }\mu\text{M}$ – 1 nM , but requires Mg^{2+} , although higher Mg^{2+} concentrations are inhibitory.
3. In terms of requirements for poly(ethylene glycol), dependence on GTP concentration and inhibition by high Mg^{2+} concentrations, there is excellent correlation between rate of increase in fluorescence and rate of GTP-promoted Ca^{2+} efflux measured under Ca^{2+} transport conditions.
4. The observations support our previous conclusions that GTP-induced membrane fusion plays a major role in causing GTP-promoted Ca^{2+} efflux from microsomal vesicles.

INTRODUCTION

In previous work on the Ca^{2+} -release mechanisms of rat liver microsomal fractions (microsomes), we have found that low concentrations of GTP are required, in the presence of poly(ethylene glycol) (PEG), before significant InsP_3 -promoted Ca^{2+} release can be observed (Dawson, 1985; Dawson *et al.*, 1986). Under these circumstances, GTP by itself causes slow Ca^{2+} mobilization, which can, under some conditions, be very extensive (Dawson *et al.*, 1987). In other cell types, and microsomes derived from them, e.g. N1E 115 cells (Gill *et al.*, 1986; Ueda *et al.*, 1986), parotid (Henne & Söling, 1986) and pancreatic islets (Wolf *et al.*, 1987), the effects of GTP are rather different. Ca^{2+} efflux promoted by GTP is rapid and extensive, does not enhance the already considerable InsP_3 -promoted Ca^{2+} release, and under some circumstances does not require PEG (Wolf *et al.*, 1987). These observations, together with the differential temperature-sensitivity and Ca^{2+} -concentration-dependence of GTP and InsP_3 -promoted Ca^{2+} release (Henne & Söling, 1986), apparently rule out a direct effect of GTP on the InsP_3 -sensitive Ca^{2+} channel. Although the requirement for GTP is apparently absolutely specific, the requirement for PEG is not. Other water-structuring colloids, e.g. bovine serum albumin and polyvinylpyrrolidone, can substitute for PEG (Henne & Söling, 1986).

Following the work of Paiement and co-workers (Paiement, 1984; Paiement *et al.*, 1987), who found that GTP can cause fusion of endoplasmic-reticulum vesicles, we have proposed (Dawson *et al.*, 1987) that GTP-dependent vesicle fusion provides a plausible explanation of the effects of GTP on Ca^{2+} release, at least as regards rat liver microsomal vesicles. We showed, using electron microscopy of fixed, stained, samples taken from Ca^{2+} -release assays, that over the time period of GTP-

dependent Ca^{2+} release very large vesicles (approx. $5\text{ }\mu\text{m}$ in diameter) appeared in the incubation mixture. Furthermore, a small but measurable change in light-scattering by the microsomal vesicles took place on GTP addition (Dawson *et al.*, 1987). Both the formation of large vesicles and the light-scattering changes were dependent on the presence of PEG and were blocked by the addition of GTP[S], as is also the case for GTP-dependent Ca^{2+} release.

Neither electron microscopy nor light-scattering measurements are ideal for detailed investigation of GTP-induced vesicle fusion. The former technique gives very poor time resolution, as well as presenting difficulties in quantifying the results. The latter, where PEG is present, is complicated by scattering changes brought about by changes in aggregation state of the vesicles. In addition, both techniques will only measure fusion resulting in a considerable change in vesicle size. They would not, for example, discriminate between a situation where vesicles were not fusing and one where they were rapidly fusing and de-fusing (or between GTP-promoted fusion and GTP-inhibited de-fusion).

Resonance energy transfer between two suitable fluorescent probes has been used successfully to measure fusion events between artificial phospholipid vesicles (e.g. Gibson & Loew, 1979; Vanderwerf & Ullman, 1980) and between plasma membranes of intact cells (Keller *et al.*, 1977). The latter workers used 5-(*N*-octadecanoyl)-aminofluorescein (F18) as donor probe and octadecyl-rhodamine (R18) as acceptor. As well as having appropriate spectral properties, these probes are rapidly incorporated into the bilayer of pre-existing membranes, and thereafter exchange only very slowly, making them suitable for use with natural membranes. In the studies reported here, we have used these two probes to examine some properties of GTP-induced fusion of rat liver microsomal vesicles, and relate these

properties to GTP-promoted Ca^{2+} release. Also, fluorescence changes owing to membrane fusion can be measured under conditions independent of Ca^{2+} movements. Thus we have been able to measure the effects of GTP on membrane fusion under a much wider range of experimental conditions, in terms of cation and nucleotide requirement, than has previously been possible by using measurements of Ca^{2+} efflux.

EXPERIMENTAL

Materials

Ca^{2+} -sensitive electrode membranes containing the neutral ionophore ETH1001 were purchased from Glasblaserie W. Möller, Zurich, Switzerland. PEG (average M_r 8000), phosphocreatine and creatine kinase were from Sigma. ATP, GTP, GTP[S] and dithiothreitol were from BCL, Lewes, Sussex, U.K. The fluorescent probes F18 and R18 were from Molecular Probes Inc., Eugene, OR, U.S.A.

Methods

Microsomal preparations. The microsomal fraction sedimenting at 35000 g for 20 min was prepared from the livers of fed 250 g male rats as described by Dawson & Irvine (1984). Protein concentration was measured by the method of Lowry *et al.* (1951), using bovine serum albumin as standard.

Ca^{2+} uptake and release. These were routinely measured with a Ca^{2+} -sensitive electrode as previously described (Dawson *et al.*, 1987), except that oligomycin and Ruthenium Red were omitted from the assay medium. As long as free Ca^{2+} concentrations of less than 1 μM are used, the presence of these inhibitors is unnecessary.

GTP-promoted Ca^{2+} release was measured under the conditions given in Fig. 1(b) of Dawson *et al.* (1987). However, in experiments where the Mg^{2+} concentration was varied, 80 μM -EGTA was included in the assay medium to maintain a relatively constant Ca^{2+} -buffering capacity. CaCl_2 was added such that the final steady-state pCa was 6.74.

Fluorescence measurements. The fluorimeter used for kinetic experiments was that described by Gains & Dawson (1976), except that the filter on the excitation beam was replaced by a monochromator (10 nm band-pass). The band-pass of the analyser monochromator was also 10 nm. The apparatus uses front-face illumination to minimize interference from any light-scattering changes and has a stirred reaction vessel, thermostatically maintained at 30 °C.

Emission spectra were recorded with a Perkin-Elmer 204 fluorescence spectrophotometer.

Incorporation of fluorescent probes. The probes were made up as stock solutions in ethanol, at a concentration of 3.4 mM for R18 and 4.1 mM for F18. Routinely, microsomal membranes (6–7 mg of protein) were suspended in a total volume of 3.5 ml of 150 mM-sucrose/50 mM-KCl/10 mM-Hepes/KOH (pH 7.0)/2 mM-dithiothreitol/0.2 mM-EGTA. The suspension was then divided into two equal halves. R18 (4 μl of stock solution) was added to one half and F18 (2 μl of stock solution) was added to the other. In both cases, the probe was added while the suspension was mixed vigorously on a vortex

mixer. The suspensions were incubated at 30 °C for 5 min to allow incorporation of probes (see below), after which time the two halves were recombined with thorough mixing and added to the fluorimeter cell, which already contained 3.5 ml of 150 mM-sucrose/50 mM-KCl/10 mM-Hepes/KOH (pH 7.0)/10% (w/v) PEG/0.2 mM- MgCl_2 . Since microsomal membranes tend to form aggregates in the presence of PEG, it is necessary to mix the two labelled membrane samples before adding them to a PEG-containing medium, so that the formation of aggregates with mixed probe populations will be favoured. Energy transfer from F18 to R18 was measured, as described by Keller *et al.* (1977), by monitoring the fluorescence increase at 600 nm by using an excitation wavelength of 460 nm. Further additions were made through a small hole in the lid into the stirred cell.

Measurement of probe binding. After labelling of membranes with F18 or R18 as described above, but before mixing of the two membrane populations or addition of them to a PEG-containing medium, the membranes were rapidly separated from the aqueous phase by centrifugation in a Beckman TL-100 table-top ultracentrifuge at 100000 g for 2 min at 4 °C. Free and bound probes were measured by fluorescence of the supernatant and of the pellet, after samples of each had been diluted with 5% (w/v) Triton X-100.

RESULTS

Fig. 1(a) shows a typical trace of fluorescence against time for membranes prelabelled with F18 and R18 as described under 'Methods'. On mixing of the membranes in the fluorimeter cell, there is a slow rate of fluorescence increase. This may be due to continued incorporation of a small amount of unbound probe, or to slow exchange of the probes between the membrane populations. However, on addition of GTP there is a rapid and substantial increase in fluorescence, which is essentially

Fig. 1. Time course of fluorescence increase after GTP addition

(a) Microsomes were labelled with R18 and F18 as described in the text: 3.5 mg of microsomal protein was labelled with 13.6 nmol of R18 and another 3.5 mg with 8.2 nmol of F18. At the arrow marked 'GTP', 57 μM -GTP was added; (b) as for (a), except that the R18-labelled microsomes contained 6.8 nmol of R18 in 3.5 mg of microsomal protein; (c) as for (a), except that 90 μM -GTP[S] was added at the arrow marked 'GTP[S]'.

Fig. 2. Change in fluorescence emission spectrum caused by GTP addition

—, Microsomes were treated with R18 and F18 as described for Fig. 1(a). The emission spectrum was recorded, and then, 5 min after the addition of $57 \mu\text{M}$ -GTP, the spectrum was recorded again. The curve plotted is the difference between the two spectra. ----, Difference in emission spectrum for microsomes labelled simultaneously with R18 and F18 minus microsomes labelled separately with R18 and F18. For simultaneous labelling, 7 mg of microsomal protein was treated with a mixture of 13.6 nmol of R18 and 8.2 nmol of F18. For separate labelling, 3.5 mg of microsomal protein was labelled with 13.6 nmol of R18 and mixed with 3.5 mg of microsomal protein previously labelled with 8.2 nmol of F18. Volumes and other conditions were as described in the text.

complete in about 4 min. The quantities of probe used in experiments of this type are circumscribed by a variety of considerations. Because the fluorescence increase due to resonance energy transfer is a function of $(1/r)^6$ (where r is the average inter-probe distance), the response is dependent on the concentration of probe in the membrane. This is illustrated by the trace shown in Fig. 1(b), where the R18 concentration is decreased by a factor of 2 compared with Fig. 1(a). However, the probe concentrations have to be sufficiently low for the probes to be essentially completely incorporated into the membranes and to avoid any impairment of membrane function. The concentration of probes used here was such that, after 5 min at 30°C , 90% of F18 and 95% of R18 were bound, these quantities remaining unchanged for up to 15 min incubation. Furthermore, ATP-driven Ca^{2+} uptake and both GTP- and InsP_3 -promoted Ca^{2+} release were unaltered in microsomal fractions treated with these probe concentrations for up to 15 min. Fig. 1(c) shows that, in agreement with data for Ca^{2+} release, the fluorescence increase caused by GTP is completely inhibited by GTP[S].

Fig. 3. Effect of PEG on fluorescence increase caused by GTP

Microsomes (3.5 mg of protein in each case) were labelled with F18 and R18 as described in the text, except that the PEG concentration was varied to give the final concentration shown. The rate of fluorescence increase is the initial rate after the addition of $57 \mu\text{M}$ -GTP, corrected for the slow fluorescence increase before GTP addition.

Fig. 2 shows the change in fluorescence emission spectrum after incubation with GTP. Also included in Fig. 2 is the difference in emission spectrum between microsomal fractions where both probes have been incorporated in the same membranes and those where the probes are in separate membranes. The two difference spectra are essentially identical. This strongly supports the view that GTP causes the probes, originally present in different bilayers, to mix in the same bilayer.

Fig. 3 shows the effect of PEG concentration on GTP-dependent fluorescence increase. In agreement with data for the effect of PEG on GTP-promoted Ca^{2+} release and GTP stimulation of InsP_3 -promoted Ca^{2+} release (Dawson *et al.*, 1986), the fluorescence increase requires $> 2\%$ (w/v) PEG and is maximal at 5% (w/v) PEG. Fig. 4 shows the effect of GTP concentration on Ca^{2+} release and on the fluorescence increase. Under the standard conditions that we have used for measurement of Ca^{2+} release (5 mM-ATP , 2 mM-Mg^{2+}), both Ca^{2+} release and fluorescence increase show identical GTP-concentration-dependence, with half-maximal response at about $3 \mu\text{M}$ -GTP. However, Fig. 4 also shows data for the fluorescence increase caused by GTP in the absence of ATP. In this experiment, $100 \mu\text{M-Mg}^{2+}$ was present, equivalent to the free Mg^{2+} present in a medium containing 5 mM-ATP and 2 mM-Mg^{2+} . At this Mg^{2+} concentration (but see below), the response of fluorescence to GTP addition is identical with that caused by GTP in the presence of ATP and Mg^{2+} .

The effect of changing Mg^{2+} concentration is shown in Fig. 5. GTP-promoted fluorescence increase is Mg^{2+} -dependent, although in the absence of chelating agents the requirement can be largely fulfilled by endogenous Mg^{2+} . Chelation of endogenous Mg^{2+} by either EDTA or ATP essentially abolishes the effect of GTP on fluorescence. When the rate of fluorescence increase is plotted as a function of free Mg^{2+} , very similar profiles are observed, irrespective of the presence or absence of ATP. In both cases, fluorescence increase is stimulated by free Mg^{2+} concentrations of up to about $100 \mu\text{M}$, and progressively inhibited by concentrations higher than this. Since Ca^{2+} uptake by microsomal fractions is a

Fig. 4. Effect of GTP concentration on Ca^{2+} efflux and rate of fluorescence increase

(●), Conditions were as for Fig. 1(a), except that two 3 mg samples of microsomal membranes were used. (○), as for (●), except that all assays contained 5 mM-ATP and 2 mM- MgCl_2 , giving a free Mg^{2+} concentration of 100 μM . In all cases, the fluorescence increase is the initial rate corrected for the slow background rate. (Δ), Ca^{2+} release, measured with a Ca^{2+} electrode as described in the text. The rate of Ca^{2+} efflux is calculated as the amount of Ca^{2+} (nmol/mg) released in 4 min after the addition of the stated concentration of GTP.

Fig. 5. Effect of free Mg^{2+} concentration on GTP-induced fluorescence increase and on GTP-promoted Ca^{2+} efflux

(○), (●), Initial rate of GTP-induced fluorescence increase. Conditions were as described in Fig. 1(a), except that 3 mg of F18-labelled and 3 mg of R18-labelled membranes were used. In addition, for the points marked (○), 5 mM-ATP was present before addition of GTP. For the points (●), the free Mg^{2+} is assumed to be the total added Mg^{2+} . For (○), free Mg^{2+} is calculated and total added Mg^{2+} ranges from 0 to 6.5 mM. The point marked (□) is the rate of fluorescence increase in the absence of added Mg^{2+} and ATP but in the presence of 100 μM -EDTA. (Δ), Rate of GTP-promoted Ca^{2+} efflux, measured with a Ca^{2+} electrode as described in the text. The rate of efflux is taken as the reciprocal time (min^{-1}) after the addition of 20 μM -GTP for the efflux of 2 nmol of Ca^{2+} /mg of microsomal protein. The protein concentration in the assay was 1.1 mg/ml.

Mg^{2+} -requiring process (Dawson & Fulton, 1983), it is not possible to measure the effects of GTP on Ca^{2+} release over the full range of Mg^{2+} concentrations. However, included in Fig. 5 are data for the inhibition of GTP-dependent Ca^{2+} release by higher concentrations of free Mg^{2+} . This shows a very similar concentration-dependence to that for inhibition of fluorescence increase by higher Mg^{2+} concentrations. It should be noted that 0.1 mM-EGTA was present routinely in incubation mixtures, giving a free Ca^{2+} concentration of about 1 nM. However, the omission of EGTA or the addition of Ca^{2+} to give a free concentration of 10 μM had no effect on the initial rate of fluorescence increase caused by GTP addition (results not shown). This seems to rule out a role for Ca^{2+} in the vesicle-fusion phenomenon.

DISCUSSION

The use of resonance energy transfer to measure vesicle fusion has shed light on two aspects of the process. Firstly, it has allowed observations to be made under conditions where Ca^{2+} movements cannot be readily measured. Secondly, it has allowed a more detailed comparison of the properties of GTP-induced membrane fusion with those of GTP-promoted Ca^{2+} release.

Since the addition of GTP increases energy transfer between F18 and R18, it is clearly acting in some way to cause bilayer fusion. The alternative mechanism, where bilayer fusion is happening all the time, but GTP somehow stabilizes the fused membranes, is incompatible with the observations, since GTP would not be required to allow the probes to interact. The initial rate of fluorescence increase is independent of the presence or absence of ATP (as long as free $[\text{Mg}^{2+}]$ is constant), so that the process apparently depends only on GTP, and not on both GTP and ATP. Our present results indicate total specificity for GTP, since other nucleoside triphosphates (ATP, ITP, UTP) are inactive (results not shown). Furthermore, Fig. 4 shows that the presence of 5 mM-ATP does not alter the concentration of GTP (3 μM) required to give the half-maximal rate of fluorescence increase, suggesting a nucleotide-binding site of remarkable specificity. The process appears to be independent of Ca^{2+} , since EGTA addition has no effect on the rate, but to be dependent on Mg^{2+} , being totally inhibited by EDTA and stimulated by the addition of Mg^{2+} in the absence of chelating agents.

Comparison of the properties of GTP-induced fluorescence increase and GTP-promoted Ca^{2+} efflux strongly supports our view (Dawson *et al.*, 1987) that both GTP-promoted Ca^{2+} efflux and the ensuing enhancement of InsP_3 -promoted Ca^{2+} efflux are due to vesicle fusion. Ca^{2+} release and fluorescence increase show identical dependence on GTP concentration, inhibition by high free $[\text{Mg}^{2+}]$, PEG requirement and inhibition by GTP[S]. However, the situation in other cell types, where GTP causes Ca^{2+} release without enhancing InsP_3 sensitivity, is not so clear. Henne *et al.* (1987) have shown that, for parotid microsomal fractions separated on a Percoll gradient, GTP-dependent Ca^{2+} release is much more marked in vesicles sedimenting at higher density, whereas InsP_3 -dependent release is much more pronounced in lower-density fractions. Assuming that the GTP-dependent Ca^{2+} release observed by Henne *et al.* (1987)

is associated with vesicle fusion, these observations raise the possibility that in this case GTP-dependent vesicle fusion is restricted to a particular type of higher-density microsomal vesicle and is not very pronounced in the vesicles containing InsP_3 receptors. Thus, although our data for liver would suggest a role for GTP in controlling (or maintaining) accessibility between the InsP_3 -sensitive Ca^{2+} channels and intracellular Ca^{2+} pools, this may not be generally true for all cell types.

It seems very likely that the fusion process observed by us is fundamentally similar to that originally observed by Paiement (1984), although, apart from the requirements for GTP and Mg^{2+} , the experimental conditions are very different. Furthermore, Paiement *et al.* (1987) observed a decrease in latency of mannose-6-phosphatase after GTP-induced fusion, suggesting an overall increase in permeability of the microsomal vesicle membrane. Such an increase in permeability would be compatible with our observations on Ca^{2+} efflux produced by GTP addition under conditions where membrane fusion occurs. Although the mechanism of the fusion process remains obscure, we have described (Dawson *et al.*, 1986) a protein which is phosphorylated by $[\gamma\text{-}^{32}\text{P}]\text{GTP}$. Furthermore, Nichitta *et al.* (1986) have found a PEG-stimulated GTPase activity which seems to have the properties expected of an enzyme involved in the process.

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