Polyethylene glycol-stimulated microsomal GTP hydrolysis

Relationship to GTP-mediated Ca^{2+} release

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It has recently been observed that GTP mediates Ca^{2+} release from internal Ca^{2+} stores. In contrast to effects on permeabilized cells, GTP-dependent Ca^{2+} release in isolated microsomes requires the presence of polyethylene glycol (PEG). We have investigated the effects of PEG on microsomal GTPase activity and report that PEG stimulates a high-affinity (K_m = 0.9 μM) GTPase. The effects of PEG reflect an increase in the V_{max} of this activity; no effects on K_m were observed. The concentration dependence for PEG-dependent stimulation of the high-affinity GTPase exactly mimicked that for GTP-dependent Ca^{2+} release. The stimulation of GTP hydrolysis by PEG was specific for the microsome fraction; only small effects were obtained with plasma membrane or cytosol fractions. As observed for GTP-dependent Ca^{2+} release, the microsomal PEG-stimulated GTPase was competitively inhibited by the GTP analog GTPγS (K_i = 60 nM). It is proposed that the PEG-stimulated GTPase may represent an intrinsic activity of the guanine nucleotide binding protein involved in the regulation of reticular Ca^{2+} fluxes.

GTP hydrolysis *Ca^{2+} release* *Polyethylene glycol* *Microsome*

1. INTRODUCTION

$InsP_3$ is known to induce Ca^{2+} release from various subcellular fractions believed to be associated with the endoplasmic reticulum [1-4]. In liver, attempts to determine the subcellular site of $InsP_3$ action, as well as the mechanism of $InsP_3$-sensitive Ca^{2+} release, have been hampered by the loss of $InsP_3$ sensitivity which occurs following fractionation [3,5].

It has recently been observed that addition of PEG and GTP to hepatic microsomes renders them sensitive to the Ca^{2+}-releasing effect of $InsP_3$ [5]. In addition, it has been shown that GTP in the presence of PEG also induces reticular Ca^{2+} release [5,6]. Further studies on this phenomenon in the NIE-115 neuroblastoma cell line have indicated that although the presence of PEG is required to observe GTP-dependent Ca^{2+} release in the microsomal fraction, washed, saponin-permeabilized cells show substantial GTP-dependent Ca^{2+} release in the absence of PEG [7,8]. Polyvinylpyrrolidone or bovine serum albumin will also substitute for PEG [9].

This newly described Ca^{2+}-release mechanism is very specific for GTP (K_m = 0.9 μM) and cannot be mimicked by ATP, ITP, CTP, UTP, GDP, GMPPNP or GTPγS [5,7-9]. These data demonstrate that a GTP-binding protein may be directly involved in modulating the activity of a reticular Ca^{2+} channel. The regulation of ion channels by GTP-binding proteins, either directly or indirectly,

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Abbreviations: PEG, polyethylene glycol; $InsP_3$, inositol 1,4,5-trisphosphate; GMPPNP, guanosine 5'-[β,γ-imido]triphosphate; GTPγS, guanosine 5'-*O*-(3-thiotriphosphate); AppNHp, adenyl imidodiphosphate; PVP, polyvinylpyrrolidone

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is not without precedent; a role for GTP-binding proteins in the regulation of plasma membrane K^+ and Ca^{2+} currents has been proposed by a number of laboratories [10–12].

Here, we have characterized the high-affinity GTPase activity of hepatic microsomes and report that this activity is stimulated by PEG. The results of this study form the basis for the proposal that GTP-dependent Ca^{2+} release is mediated by a GTP-binding protein which, in common with other known GTP-binding proteins, contains an intrinsic GTPase activity.

2. MATERIALS AND METHODS

2.1. *GTPase assay*

GTPase activity was determined in a buffer consisting of 150 mM sucrose, 50 mM KCl, 10 mM Tris-Hepes (pH 7.2), 2 μM ruthenium red, 1 μg/ml oligomycin, 1.5 mM $MgCl_2$, 1 mM Tris ATP, 2 mM Tris AppNHp, 0.25 μM GTP, 5 mM phosphocreatine, 10 U/ml creatine phosphokinase and 0.5 μCi [γ-^{32}P]GTP/ml in a final volume of 0.1 ml. Assays were conducted at 30°C and initiated by addition of an aliquot of the microsome suspension (final protein concentration 125 μg/ml). At the desired time 0.9 ml of 5% (w/v) charcoal, 1% PEG, 10 mM H_3PO_4, pH 2.3, was added, the sample vortex-mixed and immediately placed on ice. Samples were centrifuged at 8000 × *g* for 1 min and 0.5 ml of the supernatant assayed for $^{32}P_i$. Parallel blanks were performed for each condition and subtracted from the appropriate values. As described, the GTPase assay yielded linear rates of $^{32}P_i$ release for at least 15 min. At 15 min less than 15% of the total added [γ-^{32}P]GTP was hydrolyzed; the calculated rates are thus a reasonable estimate of the initial rate. At concentrations up to 1%, PEG decreased the efficiency of the charcoal trap. To eliminate this artifact 1% PEG was included as a standard component of the quench medium.

Liver microsomes were prepared as described in [3]. The pellet from the final centrifugation step was resuspended at a protein concentration of about 30 mg/ml in medium containing 0.25 M sucrose, 5 mM Tris-Hepes (pH 7.2). Rat liver plasma membranes were prepared by the procedure of Prpic et al. [13]. Synaptic plasma membranes were prepared as described by Gill et al. [14].

2.2. *GTP-mediated Ca^{2+} release*

Microsomes (1 mg/ml) were incubated in a medium (30°C, pH 7.2) containing 50 mM KCl, 100 mM sucrose, 20 mM Tris-Hepes, 1 mM $MgCl_2$, 3 mM MgATP, 10 mM creatine phosphate, 10 U/ml creatine kinase, 0.5 mM EGTA and sufficient $CaCl_2$ to give a free [Ca^{2+}] of 0.140 μM, as measured with a Ca^{2+}-sensitive electrode. In addition the medium contained 1 μCi/ml of $^{45}CaCl_2$ uptake, as measured by Millipore filtration (0.45 μM), reached steady state within 15 min. At this point GTP (630 μM) was added and Ca^{2+} release was measured over a 15 min interval. Data shown are the mean ± SE of triplicate determinations from a representative experiment. All experiments were repeated at least three times and similar results were obtained when Ca^{2+} release was measured with a Ca^{2+} electrode.

2.3. *Materials*

Nucleotides were obtained from Boehringer Mannheim (ATP, GTP, GMPPNP, AMPPNP) or Sigma (GTPγS). [γ-^{32}P]GTP was obtained from Amersham Corp. All other reagents were from commercial sources and were of the highest purity available.

3. RESULTS

Addition of microsomes to the assay medium, in the presence or absence of PEG, resulted in linear rates of GTP hydrolysis for at least 15 min (fig. 1). No lag was observed under either condition. In the presence of PEG (3%, w/v) GTP hydrolysis was stimulated at all time points assayed. Comparison of the amount of GTP hydrolyzed in 15 min indicates that PEG stimulates GTP hydrolysis by approx. 50%. In 15 experiments the mean stimulation of GTP hydrolysis by PEG was 42 ± 4%.

Crude membrane preparations frequently contain a variety of GTPase activities which vary widely in their affinity for GTP [15–17]. To determine whether PEG was regulating the activity of the high- or low-affinity GTPases the effect of PEG on GTP hydrolysis was assayed over a range of GTP concentrations from 0.1 to 1000 μM. As

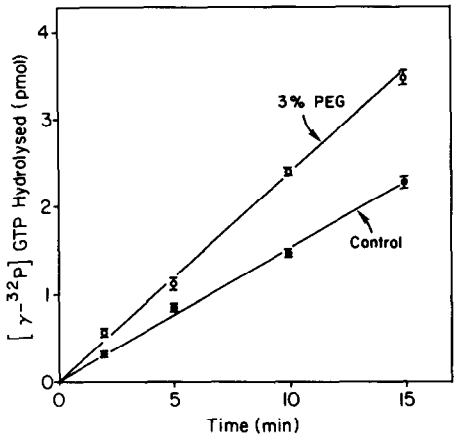

Fig.1. Time course of GTP hydrolysis in the presence and absence of 3% PEG. Hepatic microsomes (0.125 mg/ml) were incubated in the standard assay medium at 30°C for the indicated times. Each point represents the mean ± SE of triplicate incubations.

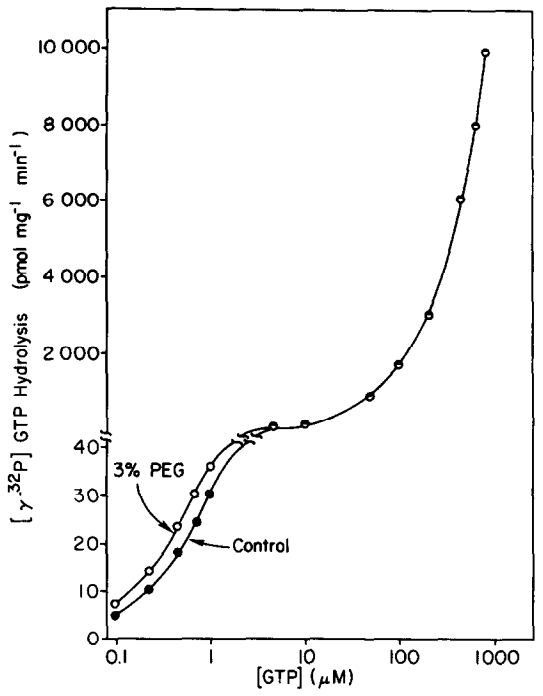

Fig.2. Effect of GTP concentration on the initial rate of GTP hydrolysis. Assays were performed for 15 min as described in section 2. Values are means ± SE of triplicate incubations. For GTP concentrations above 10 μM the concentration of label was increased to 1.0 μCi/ml.

shown in fig.2 the majority of the GTPase activity associated with the microsomal preparation was of low affinity and was unaffected by PEG. The stimulatory effect of PEG was only observed at GTP concentrations below 5 μM.

The relationship between GTP concentration and GTPase activity in the presence and absence of PEG is further detailed in fig. 3. In the presence of 3% PEG, GTP hydrolysis was stimulated at all GTP concentrations between 0.1 and 1 μM (fig.3A). Analysis of the data by a Hanes-Woolf plot (fig.3B) demonstrates that the stimulation of GTP hydrolysis by PEG reflects an increase in the V_{max} of the high-affinity GTPase. No effect on the K_m was observed. In three experiments the mean K_m was 0.9 ± 0.15 μM. This value is in very close agreement with the K_m of GTP for GTP-mediated Ca^{2+} release in microsomes prepared from NIE-15 cells (1 μM) [8] which we confirmed in our laboratory.

In an effort to correlate the observed microsomal PEG-stimulated GTPase activity with the GTP-dependent Ca^{2+} release, the PEG concentration dependencies for the two effects were compared. As shown in fig.4, both the stimulation of GTP hydrolysis and GTP-dependent Ca^{2+} release were dependent on the PEG concentration. For both systems half-maximal activation was observed at 1% PEG and maximal effects at approx. 3% PEG. The activities plateaued at PEG concentrations between 3 and 5%. PEG concentrations greater than 7% were found to inhibit the GTPase activity (not shown).

It has recently been observed that either PVP or bovine serum albumin can substitute for PEG in restoring GTP and $InsP_3$ sensitivity to isolated microsomes [9]. However, these agents appear to be somewhat less effective than PEG at equivalent concentrations. The effects of PVP, as well as other compounds, on microsomal GTPase activity are shown in table 1. PVP at a concentration of 3% stimulated the microsomal GTPase activity by 41%. The stimulation observed, in a paired experiment, in response to PEG was 52%. Neither $InsP_3$ nor the recently discovered inositol metabolite inositol 1,3,4,5-tetrakisphosphate affected either basal or PEG-stimulated microsomal GTPase activity.

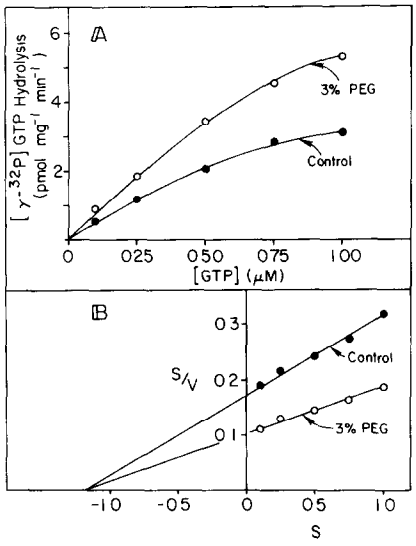

Fig.3. Influence of PEG on GTP hydrolysis: Effect on K_m vs V_{max}. Assays were performed in the standard assay medium for 15 min. (A) Initial rate of GTP hydrolysis as a function of GTP concentration. (B) Hanes-Woolf plot of data presented in panel A. Values represent the mean of triplicate incubations. Standard errors were ≤4% of the mean.

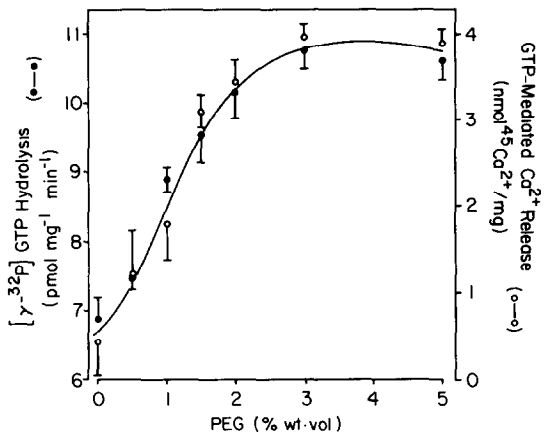

Fig.4. PEG concentration dependence for stimulation of GTP hydrolysis and GTP-dependent Ca^{2+} release. Assays for GTPase activity and GTP-dependent Ca^{2+} release were performed as described in section 2. Each point represents the mean ± SE of triplicate incubations. (•) GTPase activity, (○) GTP-dependent Ca^{2+} release.

Table 1

Regulation of microsomal GTPase activity

Condition	GTPase (pmol·mg^{-1}·min^{-1})	% difference
Control	10.18 ± 0.9	–
+3% (w/v) PEG	15.47 ± 0.16	+ 52
+3% (w/v) PVP-40	14.41 ± 0.07	+ 41
Control + 20 μM IP_3	10.01 ± 0.05	0
+3% PEG, 20 μM IP_3	14.14 ± 0.12	+ 41
Control + 20 μM IP_4	10.02 ± 0.14	0
+3% PEG, 20 μM IP_4	14.15 ± 0.11	+ 41

All assays were performed as described in the legend to fig.2. Values represent means ± SE of triplicate incubations

To determine whether the stimulation of GTP hydrolysis by PEG was specific for the GTP-binding protein(s) of the microsomal fraction, the effect of PEG on GTP hydrolysis by liver and cerebral cortex plasma membranes was investigated. As shown in table 2, PEG stimulated GTP hydrolysis in both fresh and frozen (4 weeks, −70°C) rat liver microsomes. There was a slight stimulation (≤10%) of GTP hydrolysis by PEG with the plasma membrane fractions from both liver and brain. Based upon marker enzyme analysis, however, the plasma membrane fraction is estimated to be 27% contaminated with endoplasmic reticulum [23]. Thus, a 10% stimulation is almost precisely what would be predicted if the PEG effect were specific for microsomal GTP-binding protein. The microsome fraction would also be expected to contain large quantities of protein synthesis initiation factors, which are also capable of hydrolyzing GTP. When microsomes are prepared in 0.5 M KCl these factors are recovered in the soluble fraction [24]. As shown in table 2 microsomes prepared in 0.5 M KCl retained the full PEG-stimulated GTPase response, indicating that the associated GTP-binding protein was not removed by the salt treatment.

In studies on permeabilized NIE-115 cells and microsomes it was observed that GTPγS was a potent inhibitor of GTP-dependent Ca^{2+} release [8].

Table 2

PEG-stimulated GTP hydrolysis: specificity for the microsomal fraction

Condition	PEG	GTPase activity ($pmol \cdot mg^{-1} \cdot min^{-1}$)	Percent difference
Liver microsomes	−	10.43 ± 0.18	
	+	15.76 ± 0.11	+ 51
Liver microsomes (frozen)	−	8.62 ± 0.08	
	+	12.06 ± 0.06	+ 40
Liver plasma membranes	−	18.72 ± 0.75	
	+	20.58 ± 0.50	+ 10
Cerebral cortex plasma membranes	−	47.01 ± 0.80	−
	+	51.94 ± 0.89	+ 10
Liver microsomes (0.5 M KCl)	−	9.50 ± 0.15	−
	+	14.58 ± 0.13	+ 53

All assays were conducted as described in the legend to fig.2. Plasma membranes were prepared as described in section 2. All values represent means ± SE of triplicate incubations

GppNHp, although less effective that GTPγS, was also capable of inhibiting GTP-dependent Ca^{2+} release [8]. We have observed that GTPγS is also a potent inhibitor of the microsomal PEG-stimulated GTPase (K_i = 60 nM) whereas GppNHp is approx. 30-times less potent (not shown).

4. DISCUSSION

Characterization of the GTPase activities of rat liver microsomes has demonstrated that this fraction contains a high-affinity GTPase which is stimulated in a concentration-dependent manner by PEG. This activity shares a number of characteristics with the GTP-dependent Ca^{2+} release system:

(i) The K_m for GTP of the PEG-stimulated GTPase (0.9 ± 0.15 μM) is equivalent to the K_m for GTP of the Ca^{2+}-release system. It should be noted that the K_m values for these responses are significantly higher than those reported for other known GTP-binding proteins. For example, isoproterenol-stimulated GTPase activity in turkey erythrocyte membranes has a K_m for GTP of 0.1 μM [15]. Purified G_s has a K_m for GTP of 0.31 μM whereas G_i has a K_m for GTP between 0.04 and 0.1 μM [18,19]. The microsomal GTPase also differs from other known GTPases in other respects. When compared to G_s, for example, the microsomal GTPase differs in the ratio of the K_m for GTP to the K_i for GTPγS by over 2-fold, being 15 for the microsomal GTPase and 6.2 for G_s [18].

(ii) The microsomal high-affinity GTPase is stimulated by PEG and the PEG concentration dependence exactly mimics that observed for GTP-dependent Ca^{2+} release. In addition, other agents known to restore GTP-sensitive Ca^{2+} release to microsomes, such as PVP, stimulate the high-affinity GTPase activity.

(iii) The high-affinity microsomal GTPase is very sensitive to inhibition by GTPγS as is the GTP-dependent Ca^{2+}-release system. Both systems also show much reduced sensitivity to GppNHp.

We have been unable to observe any effects of $InsP_3$ or inositol 1,3,4,5-tetrakisphosphate on either control or PEG-stimulated GTP hydrolysis. In addition, we have so far been unable to observe any effects of GTPγS on IP_3 binding, or vice versa, in the presence or absence of PEG (Joseph, S.K., unpublished), indicating that the interaction between the $InsP_3$-binding site and the Ca^{2+}-release mechanism does not involve a functional interaction with a GTP-binding protein in a manner analogous to that observed for adenylate cyclase.

In summary, the present data demonstrate the existence of a microsomal PEG-stimulated GTPase which shares a number of characteristics with the GTP-dependent Ca^{2+} release system. This activity is enriched in the microsome fraction and appears to be present only as a low specific activity contaminant in various plasma membrane preparations. Current experiments are directed towards purifying and further characterizing this novel GTP-binding protein.

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