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# MgATP-dependent Glucose 6-Phosphate-stimulated $Ca^{2+}$ Accumulation in Liver Microsomal Fractions

## EFFECTS OF INOSITOL 1,4,5-TRISPHOSPHATE AND GTP*

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**$Ca^{2+}$ release triggered by inositol 1,4,5-trisphosphate ($IP_3$) and/or GTP has been studied with rough and smooth microsomes isolated from rat liver. Microsomes were loaded with $Ca^{2+}$ in the presence of MgATP and in the presence or in the absence of glucose 6-phosphate (glucose-6-P) which markedly stimulated the MgATP-dependent $Ca^{2+}$ accumulation in rough and smooth microsomes (5- and 10-fold, respectively). Upon addition of $IP_3$ (5 $\mu$M), rough and smooth microsomes rapidly release a part (not exceeding 20%) of the $Ca^{2+}$ previously accumulated both in the absence and in the presence of glucose-6-P. Under the same experimental conditions, inositol 1,3,4,5-tetrakisphosphate was ineffective in triggering any $Ca^{2+}$ release. Upon addition of GTP (10 $\mu$M) both the microsomal fractions progressively release the $Ca^{2+}$ previously accumulated in the presence of glucose-6-P, when 3% polyethylene glycol was also present. In the absence of polyethylene glycol, GTP released $Ca^{2+}$ from rough microsomes only, and GTP plus $IP_3$ caused a $Ca^{2+}$ release which was the sum of the $Ca^{2+}$ releases caused by GTP and $IP_3$ independently. Both $IP_3$ and GTP, added to microsomes at the beginning of the glucose-6-P-stimulated $Ca^{2+}$ uptake, reduced the $Ca^{2+}$ accumulation into rough and smooth microsomes without modifying the initial rate (3 min) of $Ca^{2+}$ uptake. Also in these conditions, the effects of GTP and $IP_3$ were merely additive. These results indicate that both rough and smooth liver microsomes are responsive to $IP_3$ and GTP with respect to $Ca^{2+}$ release and that $IP_3$ and GTP likely act independently.**

It is widely accepted that a variety of cellular processes is regulated by rapid changes in the cytosolic free $Ca^{2+}$ concentration, and this in turn has prompted intensified interest in the mechanisms that control the cytosolic free $Ca^{2+}$ levels. With regard to the liver cell, many authors have suggested that the endoplasmic reticulum plays a relevant role in the regulation of cytosolic free $Ca^{2+}$ concentration in resting conditions (1–3) as well as under physiological stimuli (4–9). This organelle, in fact, possesses a $Ca^{2+}$ transport activity from cytosol to lumen (10–16) and very likely releases $Ca^{2+}$ into the cytosol under stimulating conditions ((7, 17) see Refs. 18 and 19 for reviews).

Previous studies from our laboratory demonstrated that glucose-6-P[1] markedly stimulates the MgATP-dependent $Ca^{2+}$ uptake by both isolated liver microsomes (16) and endoplasmic reticulum of permeabilized hepatocytes (20). Thus, it was suggested that, in the liver cell, the important metabolite glucose-6-P would regulate the cytosolic $Ca^{2+}$ level by supporting active $Ca^{2+}$ translocation from cytosol to endoplasmic reticulum interstices (21). Recently it has been shown that glucose-6-P also stimulates the $Ca^{2+}$ uptake by endoplasmic reticulum of permeabilized pancreatic islets (22), which suggests a regulatory role for glucose-6-P in the calcium homeostasis of islet cells (22).

$IP_3$ has been proposed as a second messenger for various extracellular stimuli because of its ability to mobilize $Ca^{2+}$ from intracellular stores (likely endoplasmic reticulum) into the soluble cytoplasm in many cell types, including the hepatocyte ((23) see Ref. 24 for a review). In fact, it has been demonstrated that $IP_3$ mobilizes $Ca^{2+}$ from a nonmitochondrial pool in permeabilized hepatocytes (8, 18, 20, 25), and an $IP_3$-induced $Ca^{2+}$ release has also been observed in some isolated microsomal preparations from rat liver (26, 27). However, attempts to prepare purified vesicles by subcellular fractionation of rat liver capable of releasing $Ca^{2+}$ on addition of $IP_3$ have been only partially successful.

The original studies by Dawson and Irvine (26) indicated that $IP_3$ is effective on endoplasmic reticulum vesicles which contaminate crude mitochondrial fractions. In purified microsomal preparations a small effect of $IP_3$ was reported by Dawson and Irvine (26), although no effect was observed by Joseph *et al.* (17). On the other hand, a rapid and transient $Ca^{2+}$-mobilizing effect of $IP_3$ in a microsomal preparation derived from rough endoplasmic reticulum has been shown by Muallem *et al.* (27). It was subsequently reported (28) that microsomal preparations which were almost insensitive to $IP_3$, with respect to $Ca^{2+}$ release, became responsive in the presence of GTP. However, Henne and Söling (29) have recently shown that GTP promotes $Ca^{2+}$ release from hepatic (as well as from parotid gland) microsomes in an $IP_3$-independent manner. In addition, Chueh and Gill (30) recently reported an $IP_3$-independent GTP-induced $Ca^{2+}$ release from endoplasmic reticulum in nonhepatic cells.

In view of the present interest in the role of $IP_3$ in $Ca^{2+}$ mobilization from endoplasmic reticulum, we further investigated the effect of $IP_3$ and/or GTP on microsomal vesicles isolated from rat liver.

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[1] The abbreviations used are: glucose-6-P, glucose 6-phosphate; $IP_3$, inositol 1,4,5-trisphosphate; MOPS, 3-(*N*-morpholino)propanesulfonic acid; PEG, polyethylene glycol; $IP_4$, inositol 1,3,4,5-tetrakisphosphate.

We used microsomal fractions corresponding to rough as well as to smooth liver endoplasmic reticulum, both of which actively accumulate $Ca^{2+}$ under glucose-6-P stimulation. This experimental condition (*i.e.* MgATP-dependent $Ca^{2+}$ accumulation in the presence of glucose-6-P) might better reflect the situation in the intact liver, in which glucose-6-P may act in affecting the reticular $Ca^{2+}$ levels. It is known, that glucose-6-P is actually available in the liver cell both in resting (31, 32) and under stimulated conditions (31, 33, 34).

The results presented here show that $Ca^{2+}$ accumulated into microsomal vesicles under glucose-6-P stimulation can be released by both $IP_3$ and GTP independently. This suggests that the glucose-6-P stimulation might play a physiological role by allowing for accumulation of high concentrations of $Ca^{2+}$ in the endoplasmic reticulum, which can subsequently be released by $Ca^{2+}$-releasing mediators such as $IP_3$ and GTP.

## EXPERIMENTAL PROCEDURES

*Preparation of Liver Microsomal Fractions*—Microsomal fractions were isolated from the livers of male 12-h fasted Sprague-Dawley rats weighing 180–220 g. Two microsomal fractions, one mainly corresponding to rough endoplasmic reticulum ("rough microsomes") and the other to smooth endoplasmic reticulum ("smooth microsomes"), were prepared essentially as described by Dallner (35, 36).

Briefly, the livers were perfused *in situ* with 0.25 M sucrose and quickly removed. Fifty percent (w/v) liver homogenates were prepared by four passes in a glass homogenizer (Thomas, size C) tightly fitted with a motor-driven (600 rpm) Teflon pestle. In all experiments samples from the livers of three animals were used to prepare each homogenate. The homogenates were diluted 1:1 (v/v) with 0.25 M sucrose and then centrifuged at 10,000 × *g* for 20 min. Five ml of the 10,000 × *g* supernatant were layered over 0.6 M sucrose (0.7 ml) and 1.3 M sucrose (3 ml), both containing 15 mM CsCl, in 10-ml polycarbonate (Nalgene) tubes, which were then centrifuged in a type 40 Beckman-Spinco rotor at 80,000 × *g* for 120 min. Smooth microsomes (at the 0.6/1.3 M sucrose interface) and rough microsomes (pellet) were separately recovered, diluted, or resuspended (10 ml, final volume) with 100 mM KCl, 20 mM NaCl, 5 mM $MgCl_2$, 1 mM $KH_2PO_4$, and 10 mM MOPS, pH 7.2, and centrifuged at 80,000 × *g* for 45 min. The resulting pellets were resuspended in the KCl-MOPS buffer as above to have about 5 mg of microsomal protein/ml. The microsomal suspensions were maintained at 0–4 °C and used within 18 h.

*Morphologic and Biochemical Characteristics of the Microsomal Fractions*—For electron microscopy examination, the suspensions of rough and smooth microsomes were fixed and stained as reported by other authors (6). Examination by electron microscopy showed that the rough microsomal fraction consists almost entirely of vesicles of various sizes surrounded by ribosomal particles (Fig. 1*A*). The smooth microsomal fraction (Fig. 1*B*) consists of smaller vesicles than the rough ones; the vesicles are rather uniform in size; a few ribosomes are present. Intact or large fragments of mitochondria are absent in both instances.

The amounts of protein, RNA, and phospholipids (determined as reported in Refs. 37, 38, and 39, respectively) present in the two fractions were measured. The enrichment in RNA of rough with respect to smooth microsomes (3.6-fold) as well as the enrichment in phospholipids of smooth with respect to rough microsomes (1.4-fold) as very similar to those reported in the original method for the preparation of the microsomal fractions (35). In order to assess the relative purity of the two microsomal fractions, the activities of glucose-6-phosphatase (40), cytochrome *c* oxidase (6), and 5′-nucleotidase (6) as markers for endoplasmic reticulum, mitochondria, and plasma membrane, respectively, were determined. The results are shown in Table I. The extents of contamination with mitochondria and plasma membrane are lower (mitochondria) or similar (plasma membrane) to those usually reported for purified total microsomes (6, 14, 16). However, in order to minimize the possible contribution of mitochondria to the energy-dependent $Ca^{2+}$ uptake by microsomal fractions, the mitochondrial inhibitors, 5 mM $NaN_3$ (10) or 2 μM ruthenium red (6, 12), were added to all the incubation systems.

In addition to electron microscopy examination, the integrity of the membrane of rough and smooth vesicles was also ascertained by measuring the latency of mannose-6-phosphatase activity, as described by Nilsson *et al.*. (41). The mannose-6-phosphatase activities

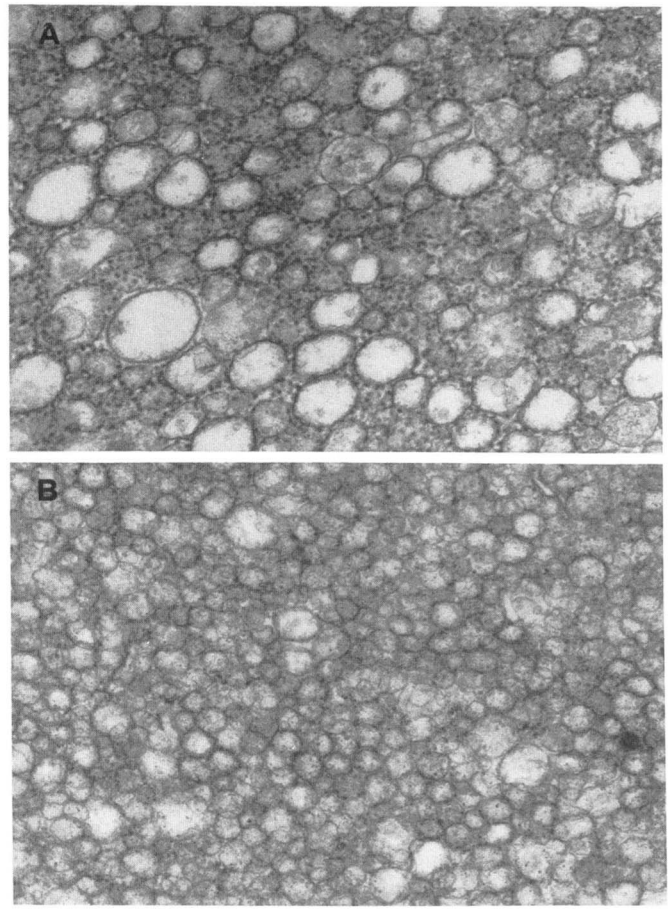


FIG. 1. **Electron micrographs of rough (*A*) and smooth (*B*) microsomal fractions.** The fractions were isolated by sucrose discontinuous gradient centrifugation and prepared for electron microscopy as described under "Experimental Procedures." Magnification × 35,000.

of rough and smooth microsomes were 10.6 ± 1.9 and 10.1 ± 1.5% (means ± S.E. of four typical preparations), respectively, with respect to the activities of fully disrupted microsomal vesicles.

*Measurement of Calcium Uptake and Release*—The microsomal fractions were incubated at 37 °C in a medium which had the following composition (mM): KCl, 100; NaCl, 20.0; $MgCl_2$, 5.0; $KH_2PO_4$, 1.0; MOPS, 10.0, pH 7.2, in the presence of 1 mM ATP plus an ATP-regenerating system (5 mM creatine phosphate and creatine phosphokinase (5 μM units/ml)), 20 μM $CaCl_2$, and 0.1 μCi/ml $^{45}CaCl_2$. When indicated in the individual experiments, 1 mM glucose-6-P was also present. Either 5 mM $NaN_3$ or 2 μM ruthenium red was added to the medium as mitochondrial inhibitor. The incubation was started by the addition of a small volume (1/50 of the volume of the incubation mixture) of the microsomal suspensions to the prewarmed (5 min at 37 °C) complete medium in order to have 0.08–0.1 mg of microsomal protein/ml. The volume of the incubation mixture was 2–5 ml according to different experiments.

In the experiments for testing $IP_3$ or GTP $Ca^{2+}$-releasing activity, two aliquots of the same incubation mixture were quickly transferred (using an automatic prewarmed (37 °C) pipette) into prewarmed (37 °C) tubes which contained either $IP_3$ and/or GTP in a small volume (less than 2% of the total volume of the sample) of 0.15 M KCl or an equal volume of 0.15 M KCl. The samples were further incubated at 37 °C for the indicated periods of time.

Calcium accumulated by microsomes was measured by using a rapid filtration technique (10) as follows. At definite time intervals, 100 μl of the incubation mixture were drawn and placed on 25-mm cellulose nitrate filters (0.2-μm pore diameter, Sartorius). The filters were quickly washed with cold 0.25 M sucrose (5 ml, three times), and the $^{45}Ca^{2+}$ retained on the filters was determined by liquid scintillation spectrophotometry (16). The specific radioactivity of the radioisotope in each incubation was determined by counting the radioactivity of an unfiltered 100-μl sample (16). Each $Ca^{2+}$ uptake assay was cor-

TABLE I

*Distribution and specific activity of marker enzymes*

Liver homogenate, 10,000 × *g* pellet, and microsomal fractions were prepared as described under "Experimental Procedures." Rough and smooth microsomes were resuspended in the KCl-MOPS buffer but without inorganic phosphorus in order to measure inorganic phosphorus released by 5'-nucleotidase and glucose-6-phosphatase. 5'-Nucleotidase and cytochrome *c* oxidase activities were measured as reported by Reinhart and Bygrave (6). Glucose-6-phosphatase activity was measured as previously described (40). One unit of 5'-nucleotidase as well as of glucose-6-phosphatase activity is defined as 1 nmol of inorganic phosphorus formed/min/mg of protein. One unit of cytochrome *c* oxidase is defined as 1 nmol of cytochrome *c* oxidized/min/mg of protein. Data are means ± S.E. of four experiments. In the case of the 10,000 × *g* pellet means of two experiments are reported.

| Fraction | Enzyme activity | | | | | |
|---|---|---|---|---|---|---|
| | 5'-Nucleotidase | | Cytochrome *c* oxidase | | Glucose-6-phosphatase | |
| | *units/mg* | *% recovery* | *units/mg* | *% recovery* | *units/mg* | *% recovery* |
| Liver homogenate | 63 ± 2 | (100) | 70 ± 3 | (100) | 52 ± 2 | (100) |
| 10,000 × *g* pellet | 94 | 70.6 | 122 | 82.6 | 51 | 46.3 |
| Rough microsomes | 81 ± 9 | 6.2 ± 0.4 | 14 ± 2 | 1.0 ± 0.1 | 232 ± 8 | 21.9 ± 1.8 |
| Smooth microsomes | 114 ± 9 | 6.5 ± 1.1 | 12 ± 1 | 0.6 ± 0.1 | 196 ± 10 | 13.2 ± 1.6 |

rected for nonspecifically bound $Ca^{2+}$ by subtracting any radioactivity remaining on the filters in the absence of ATP from the assay medium (16). $Ca^{2+}$ accumulation by microsomes was calculated by assuming the total $Ca^{2+}$ concentration of the medium to be 20 μM on the basis of added $Ca^{2+}$, without taking into consideration the amount of $Ca^{2+}$ present as contaminant of routine solutions. The concentration of contaminating $Ca^{2+}$ present in the complete medium (including 1 mM glucose-6-P) was 11.0 ± 2.0 μM (mean ± S.E. of five different preparations), as measured by atomic absorption spectroscopy as previously reported (16).

In some experiments the microsomal $Ca^{2+}$ uptake was measured by monitoring the free $Ca^{2+}$ concentration of the incubation mixture with a $Ca^{2+}$ electrode, rather than by measuring the $^{45}Ca^{2+}$ uptake by microsomes. In the conditions employed, a good correlation was observed between the two methods, as assessed by the concomitant measurement of the uptake of $^{45}Ca^{2+}$, added in trace amounts to the incubate.

$Ca^{2+}$ electrodes were constructed as described by Affolter and Sigel (42). Measurements were made with a remote calomel reference electrode (K4040, Radiometer). The $Ca^{2+}$ electrode was calibrated as described by other authors (43).

*Other Analytical Procedures*—Glucose-6-phosphatase activity was assayed by measuring the amount of glucose released from glucose-6-P. Glucose was determined by the glucose-oxidase method as previously reported (16). Protein were determined according to Lowry *et al.* (37) using bovine serum albumin as standard.

*Materials*—ATP, GTP, glucose-6-P, mannose 6-phosphate, phosphocreatine, creatine phosphokinase (Sigma Type III), and PEG ($M_r$ 8000) were from Sigma. $IP_3$ (inositol 1,4,5-trisphosphate) was from Amersham Corp. or from Sigma. The latter contained approximately 20% inositol 2,4,5-trisphosphate. Purified $IP_4$ (inositol 1,3,4,5-tetrakisphosphate) was generously provided by Dr. Robin Irvine, Cambridge, Great Britain. $^{45}CaCl_2$ (1650 Ci/mol) was from Du Pont-New England Nuclear. $Na_3VO_4$ was from Fisher. Ruthenium red (BDH Chemicals) was purified by the method of Fletcher *et al.* (44), and its concentration was determined by measurement of $A_{532}$ by using an $A_{mM}$ of 61.5 (44). All the other chemicals were of analytical grade.

## RESULTS

*Stimulatory Effect of Glucose-6-P on MgATP-dependent Calcium Accumulation by Rough and Smooth Microsomes*—The time course of the glucose-6-P-stimulated MgATP-dependent $Ca^{2+}$ uptake by both rough and smooth microsomes is presented in Fig. 2. Glucose-6-P markedly stimulates the energy-dependent $Ca^{2+}$ uptake in rough as well as in smooth microsomes although the effect is more pronounced in smooth microsomes (Fig. 2*B*). Such results essentially confirm our previous ones, both in total microsomes from rat liver (16) and in isolated hepatocytes permeabilized with digitonin (20).

The stimulatory effects of glucose-6-P on $Ca^{2+}$ accumulation by rough and smooth microsomes at different concentrations of glucose-6-P are reported in Fig. 3. A progressive increase in the stimulation of $Ca^{2+}$ accumulation can be observed up to glucose-6-P concentrations of 2 mM in both

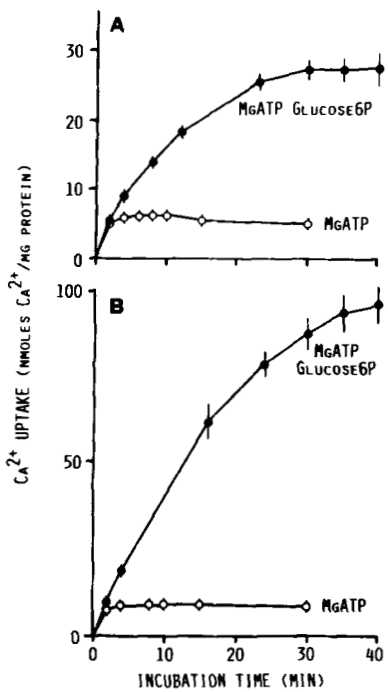


FIG. 2. **Time course of the glucose/glucose-6-P-stimulated MgATP-dependent $Ca^{2+}$ uptake by rough (*A*) and smooth (*B*) microsomes.** The microsomal fractions (0.08–0.1 mg of protein/ml) were incubated at 37 °C in a medium which had the following composition (mM): KCl, 100; NaCl, 20.0; $MgCl_2$, 5.0; $KH_2PO_4$, 1.0; MOPS, 10.0, pH 7.2, containing 1 mM ATP plus an ATP-regenerating system (creatine phosphate, 5 mM and creatine phosphokinase, 5 μM units/ml), 20 μM $CaCl_2$, and 0.1 μCi/ml $^{45}CaCl_2$ in the presence (●) or in the absence (○) of 1 mM glucose-6-P. Ruthenium red (3 μM) was also present as mitochondrial inhibitor. At the indicated times, aliquots of the incubation mixture were drawn to measure $^{45}Ca^{2+}$ taken up by microsomes, and $Ca^{2+}$ uptake was calculated as reported under "Experimental Procedures." The amount of $Ca^{2+}$ nonspecifically bound to microsomes (*i.e.* in the absence of ATP in the incubation mixture) was in all experimental conditions (rough and smooth microsomes, in the presence and in the absence of glucose-6-P) lower than 0.9 μmol of $Ca^{2+}$/mg of protein at any incubation time. Data shown represent means ± S.E. of five experiments.

fractions. Higher concentrations of glucose-6-P (up to 10 mM, data not shown) do not result in a further stimulation. With all the different glucose-6-P concentrations used, the extent of stimulation by glucose-6-P is higher in smooth (Fig. 3*B*) than in rough microsomes (Fig. 3*A*). This difference is the subject of a separate study. What can be observed here is that glucose-6-P is effective in stimulating active $Ca^{2+}$ accumula-

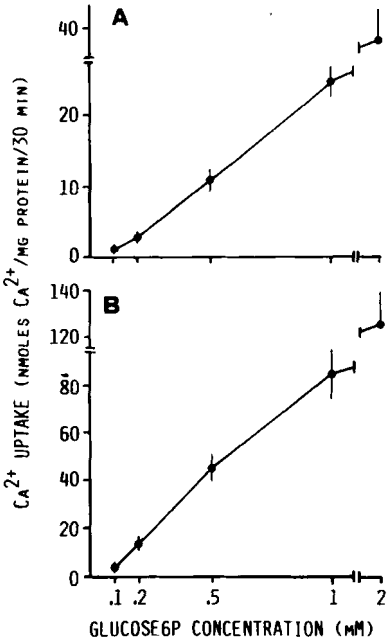


FIG. 3. **Glucose-6-P stimulation of MgATP-dependent $Ca^{2+}$ uptake by rough (*A*) and smooth (*B*) microsomes as a function of glucose-6-P concentration.** The two microsomal fractions were incubated for glucose-6-P-stimulated MgATP-dependent $Ca^{2+}$ uptake as reported in legend to Fig. 2. Glucose-6-P was present in the incubation mixture at the indicated concentrations. Net glucose-6-P-stimulated $Ca^{2+}$ uptake values were obtained by subtracting the values determined in the absence of glucose-6-P (in the presence of MgATP only) from those determined in presence of the different concentrations of glucose-6-P. $Ca^{2+}$ uptake values at 30 min of incubation in the absence of glucose-6-P were 5.2 ± 0.71 and 8.03 ± 0.61 nmol of $Ca^{2+}$/mg of protein for rough and smooth microsomes, respectively. Data shown represent means ± S.E. of three experiments.

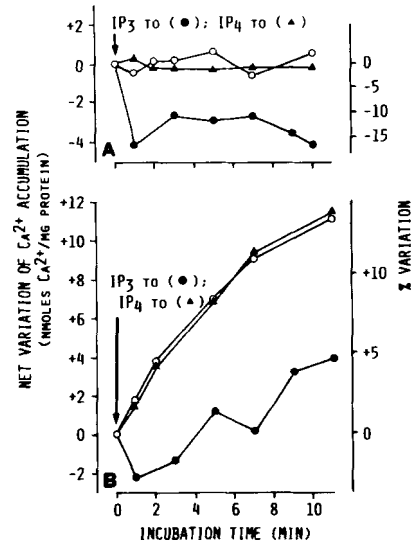


FIG. 4. **$Ca^{2+}$-releasing effect of $IP_3$ and lack of such an effect of $IP_4$ in rough (*A*) and smooth (*B*) microsomes which had previously accumulated $Ca^{2+}$ in the presence of MgATP and glucose-6-P.** The two microsomal fractions were incubated for glucose-6-P-stimulated MgATP-dependent $Ca^{2+}$ uptake at 37 °C as reported in legend to Fig. 2, except that 5 mM $NaN_3$ was employed as mitochondrial inhibitor instead of ruthenium red. At 30 min of incubation aliquots of the incubation mixture were transferred into tubes containing either $IP_3$ (●) or $IP_4$ (▲) in a small volume of 0.15 M KCl (5 μM, final concentration) or 0.15 M KCl alone (○) and further incubated at 37 °C. The mean values for accumulated $Ca^{2+}$ at 30 min of incubation (referred to as zero time in the figure) were 25.70 and 83.22 nmol of $Ca^{2+}$/mg of protein in rough and smooth microsomes, respectively. Values shown (mean of two different experiments) represent the differences in $Ca^{2+}$ accumulation (expressed as nmol of $Ca^{2+}$/mg of protein) at each indicated time with respect to $Ca^{2+}$ accumulated by microsomes by the time of $IP_3$ or $IP_4$ additions. Percent variations of $Ca^{2+}$ accumulation, calculated assuming as 100% the zero time mean value of $Ca^{2+}$ accumulation by microsomes (see above), are also indicated on the right scale of the figure.

tion in rough and in smooth microsomes at concentrations (0.1–0.5 mM) which have been reported to occur in resting as well as in stimulated hepatocytes (31–34).

*$IP_3$-induced Release of $Ca^{2+}$ from Rough and Smooth Microsomes*—The $Ca^{2+}$-releasing effect of $IP_3$ on microsomal fractions, which had been previously loaded with $Ca^{2+}$ in the presence of MgATP and glucose-6-P, is shown in Fig. 4. Upon addition of 5 μM $IP_3$ the $Ca^{2+}$ contents of both rough (Fig. 4*A*) and smooth microsomes (Fig. 4*B*) rapidly (1 min) decrease as compared to their controls (*i.e.* samples to which $IP_3$ has not been added), indicating a rapid $Ca^{2+}$ release from microsomal vesicles. Higher concentrations of $IP_3$ do not result in higher $Ca^{2+}$ releases (data not shown).

Since it has been shown that stimulated cells (45–47) including the hepatocyte (48), besides $IP_3$, also accumulate $IP_4$, it was investigated whether $IP_4$ releases $Ca^{2+}$ from liver microsomal vesicles. As can be seen in Fig. 4, $IP_4$ (5 μM) does not release $Ca^{2+}$ from either rough (Fig. 4*A*) or smooth microsomes (Fig. 4*B*). Lack of $Ca^{2+}$ mobilization by $IP_4$ from a nonmitochondrial pool (likely endoplasmic reticulum) in permeabilized hepatocytes (48) as well as in nonhepatic cells (49) also has been reported. Thus, effects other than $Ca^{2+}$ mobilization from endoplasmic reticulum might be elicited by $IP_4$ in the liver cell. An activity of $IP_4$ in controlling $Ca^{2+}$ flux at the plasma membrane level has been recently suggested in the sea urchin egg (50).

As can be seen in Fig. 5, in both the microsomal fractions which previously accumulated $Ca^{2+}$ in the presence of MgATP only (*i.e.* in the absence of glucose-6-P), a $Ca^{2+}$ release elicited by $IP_3$ is also present, although such a release is minimal in smooth microsomes.

$IP_3$ releases variable portions (about 7–20%) of the $Ca^{2+}$ accumulated, depending on the experimental system (Figs. 4 and 5). $Ca^{2+}$ loading of smooth vesicles under glucose-6-P stimulation results in an increase of net $Ca^{2+}$ release (6–9 *versus* 0.5 nmol of $Ca^{2+}$/mg of protein, compare Fig. 4*B* with Fig. 5*B*), while the percentage of $Ca^{2+}$ released is similar (7–10% *versus* 7%). Similarly, $Ca^{2+}$ loading of rough microsomes in the present of glucose-6-P results in an increase of net $Ca^{2+}$ release (3–4 *versus* 1–1.5 nmol of $Ca^{2+}$/mg of protein, compare Fig. 4*A* with Fig. 4*B*), even if the percentage of released $Ca^{2+}$ is somewhat decreased (15% *versus* 20%). Previous results obtained with permeabilized hepatocytes (17, 25, 51) also showed that $IP_3$ can release a part not exceeding 20–25% of the accumulated $Ca^{2+}$.

The $IP_3$-induced release of $Ca^{2+}$ from liver microsomes was further studied by adding $IP_3$ to the microsomal preparations at the beginning of the glucose-6-P-stimulated $Ca^{2+}$ uptake.

As can be seen in Fig. 6, in the presence of 5 μM $IP_3$ rough (Fig. 6*A*) and smooth microsomes (Fig. 6*B*) actively accumulate $Ca^{2+}$ under glucose-6-P stimulation, although at a lower rate as compared to their controls (*i.e.* samples not containing $IP_3$). At the latest time of incubation (30 min), the decreases in $Ca^{2+}$ accumulation (18 and 11% in rough and smooth microsomes, respectively; Fig. 4) essentially correspond to the decreases in $Ca^{2+}$ content observed when $IP_3$ was added to microsomes previously loaded with $Ca^{2+}$ (see Fig. 3).

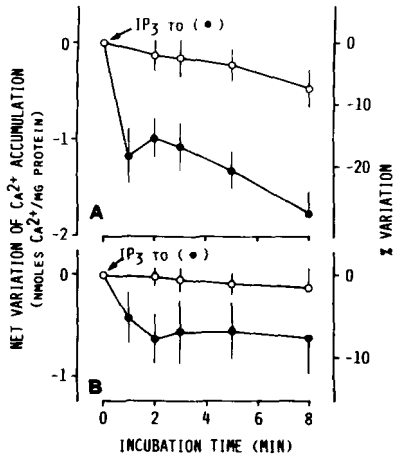


FIG. 5. **$Ca^{2+}$-releasing effect of $IP_3$ in rough (*A*) and smooth (*B*) microsomes which had previously accumulated $Ca^{2+}$ in the presence of MgATP.** The two microsomal fractions were incubated for MgATP-dependent $Ca^{2+}$ uptake (in the absence of glucose-6-P) at 37 °C as reported in the legend to Fig. 2, except that 5 mM $NaN_3$ was employed as a mitochondrial inhibitor instead of ruthenium red. At 8 min of incubation, aliquots of the incubation mixture were transferred into tubes containing $IP_3$ (●) in a small volume of 0.15 M KCl (5 μM, final concentration) or 0.15 M KCl alone (○) and further incubated at 37 °C. The mean ± S.E. values for accumulated $Ca^{2+}$ at 8 min of incubation (referred to as zero time in the figure) were 6.10 ± 0.22 and 8.20 ± 0.65 nmol of $Ca^{2+}$/mg of protein in rough and smooth microsomes, respectively. Values shown (means ± S.E. of six or five different experiments for rough or smooth microsomes, respectively) represent the differences in $Ca^{2+}$ accumulation (expressed in nmol of $Ca^{2+}$/mg of protein) at each indicated time with respect to $Ca^{2+}$ accumulated by microsomes by the time of $IP_3$ addition. Percent variations of $Ca^{2+}$ accumulation calculated assuming as 100% the zero time mean value of $Ca^{2+}$ accumulation by microsomes (see above) are also indicated on the right scale of the figure.

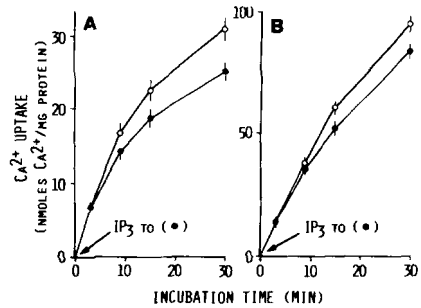


FIG. 6. **Inhibitory effect of $IP_3$ on the glucose-6-P-stimulated MgATP-dependent $Ca^{2+}$ uptake by rough (*A*) and smooth (*B*) microsomes.** The two microsomal fractions were incubated for glucose-6-P-stimulated MgATP-dependent $Ca^{2+}$ uptake at 37 °C as reported in the legend to Fig. 2 except that 5 mM $NaN_3$ was employed as mitochondrial inhibitor instead of ruthenium red in the presence (●) or in the absence (○) of 5 μM $IP_3$. At the indicated times, aliquots of the incubation mixture were drawn to measure the $^{45}Ca^{2+}$ taken up by microsomes, and $Ca^{2+}$ uptake was calculated as reported under "Experimental Procedures." The amount of $Ca^{2+}$ nonspecifically bound to microsomes (*i.e.* in the absence of ATP in the incubation mixture) was in all experimental conditions (rough and smooth microsomes, in the presence and in the absence of $IP_3$) lower than 0.9 nmol of $Ca^{2+}$/mg of protein. Data shown represent means ± S.E. of four experiments.

Interestingly, $IP_3$ does not reduce the initial rate of $Ca^{2+}$ uptake (see Fig. 6, *A* and *B*; 3 min of incubation: rough microsomes, 6.3 ± 0.7 and 6.4 ± 0.6 nmol of $Ca^{2+}$/mg of protein in the absence and in the presence of $IP_3$, respectively; smooth microsomes, 13.9 ± 1 and 14.3 ± 1.3 nmol of $Ca^{2+}$/mg

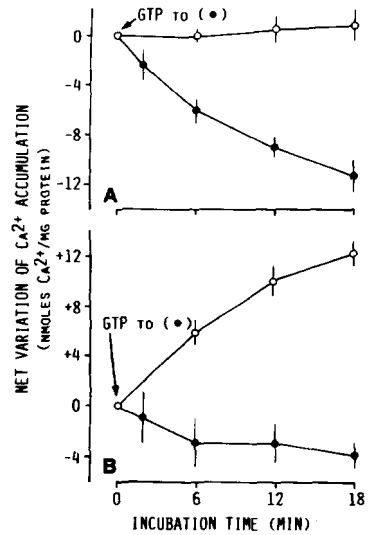


FIG. 7. **$Ca^{2+}$-releasing effect of GTP in rough (*A*) and smooth (*B*) microsomes which had previously accumulated $Ca^{2+}$ in the presence of MgATP and glucose-6-P.** The two microsomal fractions were incubated for glucose-6-P-stimulated MgATP-dependent $Ca^{2+}$ uptake at 37 °C as reported in the legend to Fig. 2, except that 5 mM $NaN_3$ was employed as mitochondrial inhibitor instead of ruthenium red; 3% (w/v) PEG was also present in the incubation mixture. At 30 min of incubation aliquots of the incubation mixture were transferred into tubes containing GTP (●) in a small volume of 0.15 M KCl (10 μM, final concentration) or 0.15 M KCl alone (○) and further incubated at 37 °C. The mean ± S.E. values for accumulated $Ca^{2+}$ at 30 min of incubation (referred to as zero time in the figure) were 32.2 ± 2.6 and 90.0 ± 3.1 nmol of $Ca^{2+}$/mg of protein in rough and smooth microsomes, respectively. Values shown (means ± S.E. of three different experiments) represent the differences in $Ca^{2+}$ accumulation (expressed in nmol of $Ca^{2+}$/mg of protein) at each indicated time with respect to $Ca^{2+}$ accumulated by microsomes by the time of GTP addition.

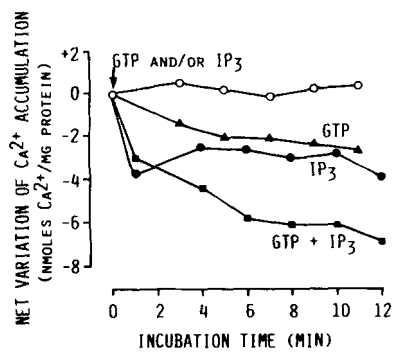


FIG. 8. **$Ca^{2+}$-releasing effects of GTP and/or $IP_3$ in rough microsomes which had previously accumulated $Ca^{2+}$ in the presence of MgATP and glucose-6-P.** The rough microsomal fraction was incubated for glucose-6-P-stimulated MgATP-dependent $Ca^{2+}$ uptake at 37 °C as reported in the legend to Fig. 2 except that 5 mM $NaN_3$ was employed as mitochondrial inhibitor instead of ruthenium red. At 30 min of incubation aliquots of the incubation mixture were transferred into tubes containing either GTP (▲) or $IP_3$ (●) or both GTP and $IP_3$ (■) in a small volume of 0.15 M KCl or 0.15 M KCl alone (○) and further incubated at 37 °C. The final concentrations of GTP and $IP_3$ were 10 and 5 μM, respectively. The value for accumulated $Ca^{2+}$ by microsomes at 30 min of incubation (referred to as zero time in the figure) was 29.81 nmol of $Ca^{2+}$/mg of protein. Values shown (means of two different experiments) represent the differences in $Ca^{2+}$ accumulation (expressed as nmol of $Ca^{2+}$/mg of protein) at each indicated time with respect to $Ca^{2+}$ accumulated by microsomes by the time of GTP and/or $IP_3$ additions.

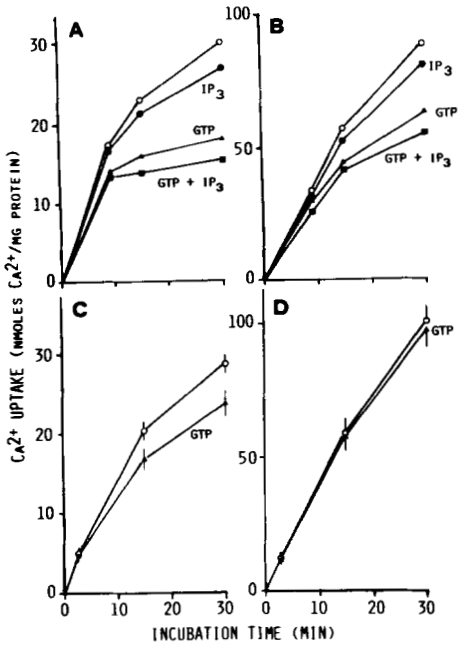


FIG. 9. **Inhibitory effect of GTP and/or $IP_3$ on glucose-6-P-stimulated MgATP-dependent $Ca^{2+}$ uptake by rough and smooth microsomes in the presence or in the absence of PEG.** *A*, *C*: rough microsomes, with or without 3% (w/v) PEG, respectively. *B*, *D*: smooth microsomes, with or without 3% (w/v) PEG, respectively. The two microsomal fractions were incubated for glucose-6-P-stimulated MgATP-dependent $Ca^{2+}$ uptake at 37 °C as reported in legend to Fig. 2, except that 5 mM $NaN_3$ was employed as mitochondrial inhibitor instead of ruthenium red. Either 10 μM GTP (▲) or 5 μM $IP_3$ (●) or 10 μM GTP plus 5 μM $IP_3$ (■) were present in the incubation mixtures; ○, $Ca^{2+}$ uptake in the absence of GTP and/or $IP_3$. At the indicated times, aliquots of the incubation mixture were drawn to measure the $^{45}Ca^{2+}$ taken up by microsomes, and $Ca^{2+}$ uptake was calculated as reported under "Experimental Procedures." The amount of $Ca^{2+}$ nonspecifically bound to microsomes (*i.e.* in the absence of ATP in the incubation mixture) was in all experimental conditions lower than 0.9 μmol of $Ca^{2+}$/mg of protein. *A*, *B*: a typical experiment out of three is shown. *C*, *D*: data are the means ± S.E. of three experiments.

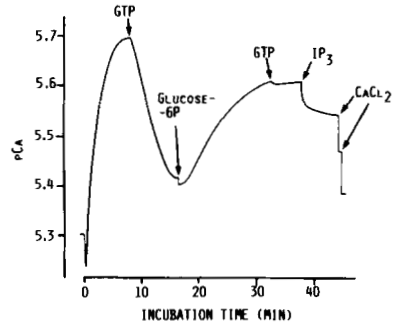


FIG. 10. **Effects of subsequent additions of GTP, glucose-6-P, and $IP_3$ on $Ca^{2+}$ uptake by rough microsomes.** $Ca^{2+}$ uptake by rough microsomes was measured by monitoring the free $Ca^{2+}$ concentration in the incubation system for MgATP-dependent $Ca^{2+}$ uptake with a $Ca^{2+}$ electrode (see "Experimental Procedures" for details). Rough microsomes were incubated in a thermostated (37 °C) Plexiglas vessel in which a $Ca^{2+}$ electrode and a reference electrode (Radiometer K4040) were immersed as described by Affolter and Sigel (42). The incubation system was composed as follows: 1 mg/ml of microsomal protein, 100 mM KCl, 20 mM NaCl, 5 mM $MgCl_2$, 1 mM $KH_2PO_4$, 10 mM MOPS, pH 7.2, 5 mM $NaN_3$ 3% (w/v) PEG, and 2 mM ATP plus an ATP-regenerating system (creatine phosphate, 10 mM and creatine phosphokinase, 10 mM units/ml). The volume of the incubation mixture was 1 ml. The incubation was started by the addition of the microsomal suspension (50 μl). Where indicated in the figure, additions of GTP (final concentration 10 μM), glucose-6-P (final concentration 0.5 mM), or $IP_3$ (final concentration 5 μM) in a small volume of 0.15 M KCl (2–5 μl) were made. At the end of the incubation, two subsequent additions of standard $CaCl_2$ (2 nmol each) were also made. The $Ca^{2+}$ electrode was calibrated before and after the incubation with $Ca^{2+}$ buffers as reported by others (43). The amount of total $Ca^{2+}$ present in the incubation medium before the addition of the microsomal fraction was 13.3 nmol/ml, as measured by atomic absorption spectroscopy (16). A typical experiment out of three is shown in the figure.

of protein, in the absence and in the presence of $IP_3$, respectively). This indicates that $IP_3$ does not modify the active $Ca^{2+}$ transport into microsomal vesicles, which in turn suggests that $IP_3$ acts by increasing the $Ca^{2+}$ efflux from liver endoplasmic reticulum, as also suggested by other authors in different experimental conditions ((18) see Ref. 24 for a review).

Since glucose-6-P hydrolysis by glucose-6-phosphatase is likely essential for the stimulatory effect of glucose-6-P on microsomal $Ca^{2+}$ uptake (16, 20, 22), the effect of $IP_3$ on glucose-6-phosphatase activity was evaluated in rough and smooth microsomes. It was found that $IP_3$ (up to 20 μM) does not modify glucose-6-P hydrolysis in either fraction (data not shown).

*GTP-induced Release of $Ca^{2+}$ from Rough and Smooth Microsomes*—As mentioned above, previous data have shown that GTP elicits $Ca^{2+}$ release in liver microsomes (29) as well as in nonhepatic cells (30, 52–54). According to different reports, GTP would enhance $IP_3$-mediated $Ca^{2+}$ release from liver microsomal fractions (28, 55) or might act independently from $IP_3$ (29, 30, 53, 54). In addition, it has been reported that the effectiveness of GTP in releasing $Ca^{2+}$ depends on the presence of agents which increase the viscosity of the medium, such as PEG (28–30), polyvinylpyrrolidone (29), or bovine serum albumin (29).

In the present study, the activity of GTP in eliciting $Ca^{2+}$ release was also investigated in liver microsomal systems for MgATP-dependent glucose-6-P-stimulated $Ca^{2+}$ uptake.

The addition of 10 μM GTP to microsomes, which had been previously loaded with $Ca^{2+}$ in the presence of MgATP and glucose-6-P, results in a progressive decrease of the accumulated $Ca^{2+}$ both in rough (Fig. 7*A*) and smooth microsomes (Fig. 7*B*), provided that PEG is present in the incubation system. In the absence of PEG, no effect was observed in smooth microsomes (data not shown). On the other hand, GTP releases $Ca^{2+}$ from rough microsomes even in the absence of PEG (Fig. 8) although the extent of such release is lower than in its presence. The concomitant addition of $IP_3$ results in a higher $Ca^{2+}$ release, which appears to be the sum of the $Ca^{2+}$ releases caused by $IP_3$ and GTP independently (Fig. 8).

The GTP-induced $Ca^{2+}$ release from liver microsomes was further studied by adding GTP to the microsomal preparations at the beginning of the glucose-6-P-stimulated $Ca^{2+}$ uptake. As can be seen in Fig. 9, *A* and *B*, the active glucose-6-P-stimulated $Ca^{2+}$ uptake by both rough and smooth microsomes is strongly decreased in the presence of 10 μM GTP, when PEG is present in the incubation system. In these experimental conditions too, the effects of $IP_3$ and GTP are likely to be independent from each other. In fact (Fig. 9, *A* and *B*), in the presence of $IP_3$ and GTP, $Ca^{2+}$ accumulation by both microsomal fractions is decreased to an extent which is the sum of the decreases caused by $IP_3$ and GTP separately. It should also be mentioned that the presence of PEG in the incubation system essentially does not modify the inhibitory effect of $IP_3$ on $Ca^{2+}$ accumulation (see Fig. 6 for comparison). In the absence of PEG, GTP is moderately effective on rough

microsomes only (Fig. 9C). As shown above for $IP_3$, GTP does not influence the initial rate of $Ca^{2+}$ uptake, which suggests that GTP acts by increasing the $Ca^{2+}$ efflux from vesicles, rather than inhibiting active $Ca^{2+}$ pumping. This had been already suggested by the results obtained by other authors in different experimental systems for microsomal $Ca^{2+}$ uptake (29, 30). As reported above for $IP_3$, GTP (up to 50 $\mu$M) did not modify the glucose-6-P hydrolysis by both rough and smooth microsomes (data not shown).

Additional experiments suggest that $IP_3$ and GTP may act on different $Ca^{2+}$ channels and/or on different portions of microsomal membrane. As can be seen in Fig. 10, GTP releases most of $Ca^{2+}$ previously accumulated by rough microsomes in the presence of MgATP only (*i.e.* in the absence of glucose-6-P). A subsequent addition of glucose-6-P results in a substantial reuptake of the released $Ca^{2+}$. A further addition of GTP is ineffective, thus indicating that the maximal $Ca^{2+}$-releasing effect of GTP has already been achieved. On the other hand, the subsequent addition of $IP_3$ still elicits a rapid $Ca^{2+}$ release, whose extent (10–15%) is comparable to that observed when $IP_3$ is added to $Ca^{2+}$-loaded microsomes not previously challenged by GTP and glucose-6-P (data not shown; see also Fig. 5).

## DISCUSSION

The results presented here indicate that $IP_3$ mobilizes $Ca^{2+}$ from purified microsomal fractions isolated from rat liver. This provides further and direct evidence that liver endoplasmic reticulum is the target for $IP_3$ when it causes $Ca^{2+}$ mobilization from intracellular stores. As mentioned above, previous attempts to demonstrate the $Ca^{2+}$-releasing effect of $IP_3$ with isolated subcellular preparations of rat liver were only partially successful (17, 18, 26, 27). This can be explained by the possible influence of several factors such as for instance: (i) loss of $IP_3$-responsive endoplasmic reticulum portions along with heavier organelles during the preparation of microsomal fractions (17); (ii) $IP_3$ degradation by $IP_3$ phosphatase present in the plasma membrane (56), which can contaminate at various extents the microsomal preparations; (iii) influence of $Ca^{2+}$ concentration in the test systems on the $Ca^{2+}$-releasing activity of $IP_3$ (52, 53).

In the experimental conditions used (*i.e.* $Ca^{2+}$ loading of microsomes under glucose-6-P stimulation) $IP_3$ activates $Ca^{2+}$ release in rough as well as in smooth microsomes, thus indicating that the whole liver endoplasmic reticulum can respond to $IP_3$, and, therefore, a loss of selected parts of endoplasmic reticulum responsive to $IP_3$ seems to be unlikely. Also it has been shown that the distribution of binding sites for $IP_3$ parallels the distribution of the endoplasmic reticulum markers in subcellular fractions from rat liver (57), which indicates that $IP_3$ receptors are distributed on the whole endoplasmic reticulum of liver cell. However, since smooth microsomes release a lower percentages of the accumulated $Ca^{2+}$ than rough microsomes (see Figs. 4 and 5), a different sensitiveness of different parts of the endoplasmic reticulum to $IP_3$ can be envisaged.

According to various estimates (31, 33) glucose-6-P is available for a continuous hydrolysis by glycose-6-phosphatase in the liver cell even in resting conditions, since a basal level of glucose-6-P is continuously maintained by the glucose/glucose-6-P "futile" cycle (31–34, 58). Thus, high $Ca^{2+}$ levels could be continuously maintained in endoplasmic reticulum by the concomitant activities of $Ca^{2+}$ pumps and glucose-6-phosphatase. Direct measurements of $Ca^{2+}$ content in rough cysternae, by electron probe x-ray microanalysis, have shown (59) that endoplasmic reticulum is indeed a major intracellular store of $Ca^{2+}$ containing higher $Ca^{2+}$ amounts than the other intracellular compartments including mitochondria. It can, therefore, be envisaged that the glucose-6-P enhancement of $Ca^{2+}$ accumulation into endoplasmic reticulum results in an amplification of the effect of intracellular $Ca^{2+}$-mobilizing mediators like $IP_3$. However, more complex interactions between $IP_3$ and glucose-6-P probably occur in stimulated hepatocytes, *i.e.* when hormones are evoking intracellular increases of $IP_3$ levels ((60) see Refs. 18 and 61 for reviews). This would in fact result soon after in a cytosolic $Ca^{2+}$ increase, which in turn causes (62) or is accompanied by an increase in glucose-6-P via glycogenolysis (4, 63, 64). The increased availability of glucose-6-P could favor $Ca^{2+}$ uptake by the endoplasmic reticulum. This might result either in a lowering cytosolic free $Ca^{2+}$ to resting values or in a further higher release of reticular $Ca^{2+}$ if $IP_3$ is still, or again, available. Since rough appear to be more responsive to $IP_3$ than smooth microsomes, but the latter are much more sensitive to glucose-6-P stimulation of $Ca^{2+}$ accumulation, the possibility exists that different parts of the endoplasmic reticulum play different roles in the regulation of cytosolic $Ca^{2+}$. On the other hand, it also should be considered that, in the stimulated hepatocyte, the elevation of cytosolic $Ca^{2+}$ is likely due only in part (and in the initial phase) to intracellular mobilization (*e.g.* by $IP_3$), being also due to $Ca^{2+}$ entry into the cell (see Ref. 18 for a review). However, work is still needed in order to clarify the interactions between $IP_3$ and glucose-6-P with respect to $Ca^{2+}$ homeostasis in the stimulated hepatocyte.

As far as the effect of GTP on $Ca^{2+}$ accumulation by liver microsomes, GTP itself appears to be able to activate $Ca^{2+}$ release from both rough and smooth microsomes which had previously accumulated $Ca^{2+}$ under glucose-6-P stimulation. The $Ca^{2+}$ release mediated by GTP is less rapid than that mediated by $IP_3$ but progressively increases with time up to levels higher than in the case of $IP_3$. Also, in the present experimental conditions the $Ca^{2+}$-releasing activity of $IP_3$ is not modified by GTP and vice versa. Therefore, the two agents, although producing the same effect (*i.e.* increase in passive efflux of $Ca^{2+}$ ions), appear to act independently from each other. This confirms previous data obtained in different experimental conditions (30, 52–54).

Unlike $IP_3$, the $Ca^{2+}$-releasing activity of GTP on rough and smooth microsomes is highly magnified in the presence of 3% PEG, as previously reported in different experimental conditions (28–30). It has been shown recently (65) that PEG stimulates a high-affinity GTPase in liver microsomes. This strengthens the previous suggestion (55) that the mechanism of GTP in eliciting $Ca^{2+}$ release might consist of a phosphorylation of microsomal proteins as a consequence of GTP hydrolysis. Yet, in our experimental system, rough microsomes appeared to be responsive to GTP action even in the absence of PEG, their $Ca^{2+}$ accumulation being significantly lowered in the presence of GTP alone (Fig. 10). Saponine-permeabilized neuroblastoma cells have been shown to release $Ca^{2+}$ from endoplasmic reticulum upon GTP stimulation in the absence of PEG (52). However, at present it is difficult to give an explanation for a role of GTP in mediating $Ca^{2+}$ release in the liver. According to several estimates (66, 67), in fact, GTP is present in the cytosolic compartment of the hepatocyte at high concentrations, which would continuously stimulate $Ca^{2+}$ efflux from endoplasmic reticulum. Theoretically, in the intact hepatocyte, mechanisms should be operative which counteract and/or modulate the action of GTP (fully expressed *in vitro* in the presence of PEG). One of these mechanisms might well rely on the action of glucose-6-P in favoring $Ca^{2+}$ accumulation into liver endoplasmic reticulum.

Work is in progress in our laboratory in order to further elucidate the possible interactions between GTP and glucose-6-P at the endoplasmic reticulum membrane of the hepatocyte.

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