Evidence that guanosine 5'-[γ -thio]triphosphate stimulates plasma membrane Ca^{2+} inflow when introduced into hepatocytes

Bernard P. HUGHES and Gregory J. BARRITT

Department of Medical Biochemistry, Flinders University School of Medicine, Bedford Park, South Australia 5042, Australia

1. Slowly hydrolysable analogues of GTP were introduced into hepatocytes by incubating the cells in the absence of Mg^{2+} and in the presence of ATP^{4-} . Experiments using guanosine 5'-[γ - ^{35}S]thio]triphosphate (GTP[^{35}S]) indicated that about 50 % of the GTP[S] loaded into the cells was subsequently hydrolysed. 2. In cells loaded with GTP[S] and incubated in the absence of added extracellular Ca^{2+} (Ca^{2+}_o), the rate of activation of glycogen phosphorylase observed after addition of 1.3 mM- Ca^{2+}_o was 250 % greater than the rate observed in unloaded cells. Smaller effects (130 %) were observed in cells loaded with either guanylyl-5'-yl imidodiphosphate or guanosine 5'-[β -thio]diphosphate (GDP[S]). Cells loaded with adenosine 5'-[γ -thio]triphosphate showed no increase in glycogen phosphorylase activity on addition of Ca^{2+}_o . 3. The effect of a submaximal concentration of GTP[S] on the Ca^{2+} -induced activation of glycogen phosphorylase was additive with that of a half-maximally effective concentration of vasopressin. GTP[S] did not increase the effect of a maximally effective concentration of the hormone. 4. Cells loaded with GTP[S] exhibited an increased initial rate of $^{45}\text{Ca}^{2+}$ exchange measured at 1.3 mM- Ca^{2+}_o . 5. GTP[S] did not affect the amount of $^{45}\text{Ca}^{2+}$ exchanged by cells incubated at 0.1 mM- Ca^{2+}_o or the ability of vasopressin to release $^{45}\text{Ca}^{2+}$ from these cells. 6. It is concluded that the introduction of slowly hydrolysable analogues of GTP to the liver cell cytoplasmic space stimulates the inflow of Ca^{2+} across the plasma membrane through a channel similar to that activated by vasopressin.

INTRODUCTION

The increase in the concentration of free Ca^{2+} in the liver cell cytoplasm which is caused by vasopressin and a number of other agonists results from the release of Ca^{2+} from the endoplasmic reticulum (Shears & Kirk, 1984) and enhanced inflow of Ca^{2+} across the plasma membrane (Barritt *et al.*, 1981; Mauger *et al.*, 1984; Reinhart *et al.*, 1984). It has been shown that the former process is mediated by inositol 1,4,5-trisphosphate (Joseph *et al.*, 1984; Burgess *et al.*, 1984). However, the mechanism by which agonists stimulate plasma-membrane Ca^{2+} inflow is not well understood.

Previous studies, which employed NaF (Hughes & Barritt, 1987) and pertussis toxin (Hughes *et al.*, 1987), have provided evidence that a GTP-binding regulatory protein is involved in the process by which agonists stimulate plasma-membrane Ca^{2+} inflow in the liver cell. Increases in the concentration of free cytoplasmic Ca^{2+} , cyclic AMP (Hughes & Barritt, 1987), inositol trisphosphate (Hughes *et al.*, 1987) or in the activity of protein kinase C (Hughes & Barritt, 1987) are not likely to be involved. The aim of the present work was to further investigate the role of GTP-binding proteins in this process by investigating the effects of guanosine 5'-[γ -thio]triphosphate (GTP[S]), a slowly hydrolysable analogue of GTP, on plasma-membrane Ca^{2+} inflow. GTP[S] and other slowly hydrolysable analogues of GTP, such as guanylyl-5'-yl imidodiphosphate (p[NH]ppG), bind to GTP-binding proteins and lead to a prolonged activation of these proteins (reviewed by Gilman, 1987).

Since the liver cell plasma membrane is not readily permeable to GTP and its analogues, these molecules were initially introduced to the cells by incubation of cells in the absence of Mg^{2+} and in the presence of ATP^{4-} . Experiments with a number of other types of cells have shown that ATP^{4-} causes the plasma membrane to become permeable to small molecules (Cockcroft & Gomperts, 1980; Gomperts, 1983; Ratan *et al.*, 1986; Mustelin *et al.*, 1986; Steinberg *et al.*, 1987). Cells loaded with GTP[S] exhibited increased rates of plasma-membrane Ca^{2+} inflow, as judged by increases in: (i) the rate of activation of glycogen phosphorylase which follows the addition of Ca^{2+} to cells previously incubated in the absence of extracellular Ca^{2+} (Ca^{2+}_o) and (ii) the initial rate of $^{45}\text{Ca}^{2+}$ exchange.

EXPERIMENTAL

Introduction of GTP[S] and related compounds into liver cells

The isolation of hepatocytes from fed male rats, incubation of the cells as stirred suspensions in cylindrical chambers and the assessment of cell viability by Trypan Blue exclusion were performed as described previously (Barritt *et al.*, 1981). Except where indicated otherwise, the incubation medium contained 117 mM-NaCl, 4.6 mM-KCl, 1.2 mM- KH_2PO_4 , 1.2 mM- MgSO_4 , 24 mM- NaHCO_3 , 20 mM-Tes/NaOH, pH 7.4 (Medium A), and hepatocytes [2×10^6 cells (30 mg wet wt.)/ml].

The method employed for the loading of hepatocytes with analogues of GTP was based on methods used for

Abbreviations used: ATP[S], adenosine 5'-[γ -thio]triphosphate; GTP[S], guanosine 5'-[γ -thio]triphosphate; GDP[S], guanosine 5'-[β -thio]diphosphate; p[NH]ppG, guanylyl-5'-yl imidodiphosphate; $[\text{Ca}^{2+}]_o$, concn. of extracellular Ca^{2+} .

other cell types (Gomperts, 1983; Ratan *et al.*, 1986; Mustelin *et al.*, 1986). Cells (500–600 mg wet wt.) were washed twice in 15 ml of Medium A modified by the inclusion of 0.5 mM- Na_2EDTA and the omission of MgSO_4 (Medium B), and resuspended in 15 ml of Medium B. Samples (6 ml) of this suspension, containing 35–40 mg wet wt./ml of cells, were added to 25 ml plastic screw-top pots. Na_2ATP (3 μM) and the agent to be loaded were added. Control cells were incubated in an identical manner, except that water was added in place of the agent under test. The pots were shaken at 37 °C at 90 oscillations/min under O_2/CO_2 (19:1). After 5 min, 1.8 mM- MgSO_4 was added, and the incubation continued for a further 2 min. The cells were harvested by centrifugation at 50 *g* for 1 min. The resulting pellet was suspended in 2 ml of Medium C [137 mM- NaCl , 5.4 mM- KCl , 0.8 mM- MgSO_4 , 0.8 mM- Na_2HPO_4 , 0.4 mM- KH_2PO_4 and 25 mM- NaHCO_3 , containing 0.1% (w/v) Phenol Red, pH 7.4]. This suspension was mixed in a 10 ml plastic centrifuge tube with a solution composed of 2 ml of Medium C and 4 ml of a mixture of 9 vol. of Percoll and 1 vol. of Medium C in which the components were present at 10 times their normal concentrations (Berry *et al.*, 1983). The tube was centrifuged at 1100 *g* for 10 min in a bench centrifuge (MSE Super Minor). At the conclusion of this centrifugation, the viable cells (which excluded Trypan Blue) formed a pellet. Damaged cells (which admitted Trypan Blue) formed a layer at the top of the tube. The supernatant and damaged cells were removed by aspiration, the sides of the tube were wiped with tissue, and the pellet was resuspended in 6 ml of Medium A. The cells were centrifuged at 50 *g* for 1 min, resuspended in 6 ml of Medium A and used immediately.

At the conclusion of this procedure, the yield of cells (on a wet-wt. basis) from the initial suspension was $66 \pm 3\%$ ($n = 14$). The viability of the cells was $91 \pm 1\%$ ($n = 10$). Cells which had undergone the loading procedure but which had not been subjected to the Percoll-gradient step were $80 \pm 1\%$ ($n = 12$) viable. In the experiments reported, all cells, except for those used in the experiments described in Fig. 1(b), were subjected to the Percoll-gradient-centrifugation step.

Measurement of plasma-membrane Ca^{2+} inflow

Rates of plasma-membrane Ca^{2+} inflow were estimated from measurements of (a) the initial rate of $^{45}\text{Ca}^{2+}$ exchange (Barritt *et al.*, 1981) and (b) the increase in glycogen phosphorylase *a* activity which follows the addition of Ca^{2+} to cells previously incubated in the absence of added Ca^{2+} (Cooper *et al.*, 1985; Hughes *et al.*, 1986a,b). Glycogen phosphorylase *a* activity was assayed by using [^{14}C]glucose 1-phosphate (Hutson *et al.*, 1976). A unit of enzyme activity is 1 μmol of [^{14}C]glucose 1-phosphate incorporated into glycogen/min per g wet wt. of cells.

Estimates of the amounts of [^{14}C]sucrose or GTP^{35}S associated with the cells were made after separation of the cells from the medium through a layer of silicone oil (Barritt *et al.*, 1981). To estimate the amount of ^{35}S that was still bonded to the terminal phosphate atom of $\text{GTP}[\text{S}]$, trichloroacetic acid extracts of the cell suspensions were prepared (Charest *et al.*, 1985b) and fractionated by anion-exchange chromatography with AG 1-X8 resin (formate form) as described by Krishnan & Krishna (1976). The columns were eluted successively with 5×2 ml fractions each of water, 2 M-formic acid, 4 M-

formic acid, 8 M-formic acid/0.02 M-ammonium formate, 8 M-formic acid/0.2 M-ammonium formate, 8 M-formic acid/1 M-ammonium formate, 8 M-formic acid/2 M-ammonium formate and 8 M-formic acid/3 M-ammonium formate. The uptake of Lucifer Yellow was assessed by observation of the cells with an Ortholux Microscope equipped for cell fluorescence measurements (filter block D, band-pass 355–425 nm; Ernst Leitz, Wetzlar, Germany). The preparation of hepatocytes labelled with [^3H]inositol and measurement of mono-, bis- and tris-phosphates labelled with [^3H]inositol were performed as described previously (Hughes & Barritt, 1987).

Except where indicated otherwise, the results are means $\pm$ S.E.M. for the numbers of experiments indicated. Degrees of significance were determined by using Student's *t* test for paired samples. Values of $P < 0.05$ were considered to be not significant.

Materials

The tetralithium salts of $\text{GTP}[\text{S}]$, adenosine 5'-[γ -thio]-triphosphate ($\text{ATP}[\text{S}]$) and p[NH]ppG and the trilithium salt of guanosine 5'-[β -thio]diphosphate ($\text{GDP}[\text{S}]$) were purchased from Boehringer Mannheim Australia Pty., North Ryde, N.S.W., Australia. $\text{GTP}[\text{S}]$ and $\text{GDP}[\text{S}]$ were also purchased from Sigma Chemical Co., St. Louis, MO, U.S.A., as was Lucifer Yellow CH (dilithium salt). GTP^{35}S was purchased from Du Pont, Melbourne, Victoria, Australia. All other chemicals were of the highest grade available and were obtained from the sources described previously (Barritt *et al.*, 1981; Hughes *et al.*, 1986b; Hughes & Barritt, 1987).

RESULTS

Uptake of analogues of GTP by hepatocytes

GTP^{35}S was taken up by hepatocytes incubated in the absence of Mg^{2+} and in the presence of ATP^{4-} (Table 1). When cells loaded with GTP^{35}S were subsequently incubated at 37 °C for 5, 10 or 15 min, the amounts of radioactivity associated with the cells were 77 ± 10 , 65 ± 2 and $49 \pm 2\%$ (mean $\pm$ range, $n = 2$) respectively of those observed immediately after the conclusion of the Percoll-gradient step.

To estimate the maximum amount of ^{35}S that was still bonded to the terminal phosphate atom of $\text{GTP}[\text{S}]$, cells loaded with GTP^{35}S were extracted with trichloroacetic acid, the acid was removed with diethyl ether, and the extracts were subjected to anion-exchange chromatography. In extracts obtained from cells immediately after the loading procedure, $31 \pm 6\%$ (mean $\pm$ range, $n = 2$) of the total radioactivity was eluted from the column at 8 M-formic acid/1 M-ammonium formate (Fig. 1a). This coincided with standard $\text{GTP}[\text{S}]$ (Fig. 1b). For cells incubated for a further 15 min at 37 °C this percentage was $34 \pm 3\%$ (Fig. 1a).

Experiments were performed to test whether the absence of Mg^{2+} and the presence of ATP^{4-} were responsible for the uptake of GTP^{35}S by hepatocytes. Cells incubated in either the absence or the presence of Mg^{2+} took up the same amount of GTP^{35}S as did those incubated in the absence of Mg^{2+} and in the presence of ATP^{4-} (Table 1). This result was checked by using [^{14}C]sucrose. The uptake of [^{14}C]sucrose was not significantly increased by either the omission of Mg^{2+} or the inclusion of ATP^{4-} in the absence of Mg^{2+} (Table 1).

Fig. 1. Anion-exchange chromatography of $\text{GTP}^{[35\text{S}]}$

(a) Cells were loaded with $380 \mu\text{M}$ - $\text{GTP}^{[35\text{S}]}$ containing $10 \mu\text{Ci}$ of $\text{GTP}^{[35\text{S}]}$. A 3 ml sample was added to 0.6 ml of 80% (w/v) trichloroacetic acid either immediately ($\bullet$) or after incubation for 15 min at 37°C ($\blacktriangle$). The samples were treated and fractionated as described in the Experimental section. The data are representative of two experiments which gave similar results. The amount of radioactivity applied to each column was 27500 ± 1600 d.p.m. (mean $\pm$ S.E.M., $n = 4$). The recovery of the applied radioactivity was $94 \pm 2\%$ ($n = 4$). (b) A sample of $10 \mu\text{M}$ - $\text{GTP}^{[35\text{S}]}$ containing $2.5 \mu\text{Ci}$ of $\text{GTP}^{[35\text{S}]}$ was diluted to a volume of 5 ml with water and applied to the column. The column was eluted as described in the Experimental section.

In order to assess whether (a) the same amount of extracellular molecules are taken up by each cell present in the incubation and (b) the molecules that are taken up are evenly distributed within a given cell, experiments were performed with the fluorescent molecule Lucifer Yellow (M_r 457). About 75% of the cells loaded with Lucifer Yellow showed a detectable increase in fluorescence relative to cells that had been incubated in the absence of the dye (Table 1). Cells that took up the dye showed fluorescence which was evenly distributed throughout the whole cell (results not shown).

Effects of GTP analogues on the Ca^{2+} activation of glycogen phosphorylase

After addition of 1.4 mM - Ca^{2+} , cells loaded with $\text{GTP}^{[35\text{S}]}$ exhibited a faster and greater increase in glycogen phosphorylase activity compared with control cells (Fig. 2). The increase was more substantial when the loading procedure was conducted in presence of ATP^{4-} (Fig. 2). Since $\text{GTP}^{[35\text{S}]}$ was present as the Li^+ salt, the presence of 1 mM - LiCl (in place of $\text{GTP}^{[35\text{S}]}$) during the loading

Table 1. Uptake of small molecules by hepatocytes

Cells were incubated in either Medium A (containing 1.2 mM - Mg^{2+}) or Medium B (Mg^{2+} omitted, EDTA included) or Medium B which also contained $3 \mu\text{M}$ - Na_2ATP in the presence of either 0.4 mM - ^{14}C -sucrose ($0.5 \mu\text{Ci/mol}$), 0.4 mM - $\text{GTP}^{[35\text{S}]}$ ($3 \mu\text{Ci/mol}$) or 0.5 mM -Lucifer Yellow. After 5 min, 1.8 mM - MgSO_4 was added to each incubation, and the cells were harvested by centrifugation, resuspended and subjected to centrifugation through a Percoll gradient as described in the Experimental section. The data are the results of experiments conducted with three or four preparations of hepatocytes. In the case of Lucifer Yellow, the number of cells examined in each experiment was 310 ± 45 ($n = 4$).

Modification to incubation conditions	Amount of molecule taken in 5 min incubation		
	$\text{GTP}^{[35\text{S}]}$ (pmol/mg wet wt.)	^{14}C -Sucrose (pmol/mg wet wt.)	Lucifer Yellow (no. of fluorescent cells, % of total)
0 mM - Mg^{2+} + $3 \mu\text{M}$ - ATP	2.2 ± 0.4	14.6 ± 1.8	74 ± 3
0 mM - Mg^{2+}	2.0 ± 0.2	11.1 ± 0.4	65 ± 5
1.2 mM - Mg^{2+}	2.7 ± 0.4	8.7 ± 1.4	70 ± 2

Fig. 2. Effect of loading conditions on the effect of $\text{GTP}^{[35\text{S}]}$ on the activation by Ca^{2+} of glycogen phosphorylase in cells previously incubated in the absence of Ca^{2+}

Cells loaded with $190 \mu\text{M}$ - $\text{GTP}^{[35\text{S}]}$ ($\bullet$, $\blacktriangle$) or controls ($\circ$, $\triangle$) were incubated in the absence of Ca^{2+} for 5 min before the addition of 1.4 mM - Ca^{2+} as indicated by the arrow. The loading conditions were Medium A (containing 1.2 mM - Mg^{2+}) ($\triangle$, $\blacktriangle$) or Medium B (Mg^{2+} omitted, EDTA included) which also contained $3 \mu\text{M}$ - Na_2ATP ($\circ$, $\bullet$). The data are means $\pm$ S.E.M. for experiments conducted with 5–11 preparations of hepatocytes.

Fig. 3. Effect of GTP[S] on the activation by Ca^{2+} of glycogen phosphorylase in cells previously incubated in the absence of Ca^{2+}

(a) Cells loaded with a nucleotide analogue ($\bullet$, $\blacktriangle$, $\blacksquare$) or controls ($\circ$, $\square$) were incubated in the absence of Ca^{2+} for 5 min before the addition of 1.4 mM-Ca^{2+} as indicated by the arrow. The conditions are ($\circ$) control, ($\square$) 10 nM -vasopressin added at $t = 0 \text{ min}$, ($\bullet$) $190 \mu\text{M}$ GTP[S], ($\blacktriangle$) $380 \mu\text{M}$ -GTP[S] and ($\blacksquare$) $380 \mu\text{M}$ -ATP[S]. The data are means $\pm$ S.E.M. for experiments conducted with 3–11 preparations of hepatocytes. (b) Cells loaded with GTP[S] ($\bullet$, $\blacktriangle$, $\blacksquare$) or controls ($\circ$, $\triangle$, $\square$) were incubated in the absence of Ca^{2+} for 5 min before the addition of 1.4 mM-Ca^{2+} as indicated by the arrow. The conditions are ($\circ$) control, ($\triangle$) 20 pM -vasopressin added at $t = 0 \text{ min}$, ($\square$) 10 nM -vasopressin added at $t = 0 \text{ min}$, ($\bullet$) $190 \mu\text{M}$ -GTP[S], ($\blacktriangle$) $190 \mu\text{M}$ -GTP[S] and 20 pM -vasopressin added at $t = 0 \text{ min}$ and ($\blacksquare$) $190 \mu\text{M}$ -GTP[S] and 10 nM -vasopressin added at $t = 0 \text{ min}$. The data are means $\pm$ S.E.M. for experiments conducted with 3–6 preparations of hepatocytes. The errors bars have been omitted for the first three time points for clarity. (c) Cells loaded with $380 \mu\text{M}$ -GTP[S] were incubated in the absence of Ca^{2+} for 20 min before the addition of 1.4 mM-Ca^{2+} as indicated by the arrow. Either 0.5% (v/v) dimethyl sulphoxide ($\bullet$) or $200 \mu\text{M}$ -verapamil ($\blacktriangle$) dissolved in dimethyl sulphoxide was added at $t = 0 \text{ min}$. The data are means $\pm$ S.E.M. for experiments conducted with four preparations of hepatocytes. (d) Cells loaded with 1 mM-p[NH]ppG ($\bullet$), 1 mM-GDP[S] ($\blacktriangle$) or controls ($\circ$) were incubated in the absence of Ca^{2+} for 5 min before the addition of 1.4 mM-Ca^{2+} as indicated by the arrow. The data are means $\pm$ S.E.M. for experiments conducted with 3–5 preparations of hepatocytes.

procedure was tested. This had no effect on the activation of glycogen phosphorylase by subsequent Ca^{2+} addition (results not shown). A concentration of $380 \mu\text{M}$ -GTP[S] had an effect which was approximately twice that of $190 \mu\text{M}$ -GTP[S] and was similar to that of vasopressin (Fig. 3a). The effect of $190 \mu\text{M}$ -GTP[S] was approximately additive with that of a half-maximally effective concentration (20 pM) of vasopressin, but did not increase the effect of a maximally effective concentration of vasopressin (Fig. 3b).

The effect of GTP[S] was not decreased when the time of incubation of cells before the addition of Ca^{2+} was increased from 5 to 20 min (results not shown). The

increase induced by GTP[S] was inhibited by $200 \mu\text{M}$ -verapamil (Fig. 3c). However, as shown previously (Hughes *et al.*, 1986a), addition of verapamil alone activated glycogen phosphorylase. At a concentration of $380 \mu\text{M}$, ATP[S] had no effect on the Ca^{2+} -induced activation of glycogen phosphorylase (Fig. 3a). Both p[NH]ppG and, surprisingly, GDP[S] increased the effect of Ca^{2+} on phosphorylase relative to control (Fig. 3d).

Experiments were performed to test whether the observed effects of GTP[S] on the activation of glycogen phosphorylase by the addition of Ca^{2+} were due to effects of GTP[S] at a receptor on the plasma membrane (Okajima *et al.*, 1987). After addition of 1.4 mM-Ca^{2+} ,

Fig. 4. Effect of nucleotide analogues on glycogen phosphorylase activity in untreated cells

(a) Cells were incubated in the absence of added Ca^{2+} . After 20 min, 0.9% NaCl (○), 190 μM -GTP[S] (●), 15 μM -GTP[S] (▲), 7.5 μM -GTP[S] (■), 0.75 μM -GTP[S] (◆) or 13 nM-vasopressin (□) was added. At 30 min (indicated by the arrow) 1.4 mM- Ca^{2+} was added. The data are means of two experiments or are means $\pm$ S.E.M. for experiments conducted with 3–5 preparations of hepatocytes. (b) Cells were incubated in the presence of 1.3 mM- Ca^{2+} . After 20 min (indicated by the arrow) 0.9% NaCl (○), 190 μM -GTP[S] (●), 190 μM -ATP[S] (■), 1 mM-GDP[S] (▲) or 13 nM-vasopressin (□) was added. The data are means of two experiments or are means $\pm$ S.E.M. for experiments conducted with three preparations of hepatocytes.

untreated cells incubated in the presence of 190 μM -GTP[S] exhibited a rate of activation of glycogen phosphorylase similar to that observed in the presence of vasopressin (Fig. 4a). The effect of GTP[S] on the rate of increase of glycogen phosphorylase activity was concentration-dependent, with a concentration of 0.75 μM having little effect compared with control (Fig. 4a).

When added to untreated cells incubated in the presence of 1.3 mM- Ca^{2+} , GTP[S] caused an activation of glycogen phosphorylase similar in time of onset and magnitude to that observed with vasopressin, except that the increase was transient, with the enzyme activity returning to basal values within 10 min with 190 μM -GTP[S] (Fig. 4b) or 20 min with 380 μM -GTP[S] (results not shown). ATP[S] caused an increase in glycogen phosphorylase activity similar to that induced by vasopressin. The effect of ATP[S] was maintained for at least 10 min (Fig. 4b). This result is consistent with the known effects of ATP on glycogen phosphorylase activity and inositol trisphosphate production (Charest *et al.*, 1985a; Okajima *et al.*, 1987).

Effect of GTP[S] on $^{45}\text{Ca}^{2+}$ exchange and release

In cells which had undergone the loading procedure in the absence of GTP[S], vasopressin caused a stimulation

of the initial rate of $^{45}\text{Ca}^{2+}$ exchange (Fig. 5a) similar to that which has previously been observed in untreated cells (Hughes *et al.*, 1986a). Cells loaded with GTP[S] exhibited an increase of about 25% in the initial rate of $^{45}\text{Ca}^{2+}$ exchange compared with control cells (Fig. 5b).

The effect of the loading of cells with GTP[S] on inositol 1,4,5-trisphosphate concentrations was tested indirectly by measuring the amount of $^{45}\text{Ca}^{2+}$ exchanged at low $[\text{Ca}^{2+}]_o$, and the size of the vasopressin-releasable $^{45}\text{Ca}^{2+}$ pool. The data in Fig. 6 show that loading of cells with GTP[S] did not alter the amount of $^{45}\text{Ca}^{2+}$ exchanged or the size of the vasopressin-releasable $^{45}\text{Ca}^{2+}$ pool in hepatocytes incubated in the presence of 0.1 mM- $^{45}\text{Ca}^{2+}$.

Experiments were performed to determine the effect of the loading of cells with GTP[S] on the amounts of ^3H -labelled polyphosphates. Cells were loaded with GTP[S], put through the Percoll-gradient step and then incubated for 10 min in the absence of Li^+ . The amounts of ^3H -labelled inositol mono-, bis- and tris-phosphates (expressed as percentages of values obtained for cells treated in the same manner but with the loading procedure conducted in the absence of GTP[S]) were 94, 97 and 94% respectively (means of two experiments). When cells treated in the absence of GTP[S] were incubated for 5 min with vasopressin, the amounts of ^3H -labelled inositol mono-, bis- and tris-phosphates (expressed as

Fig. 5. Effect of GTP[S] on $^{45}\text{Ca}^{2+}$ exchange at 1.4 mM-Ca^{2+} .

Cells loaded with $380 \mu\text{M}$ -GTP[S] (▲) or controls (O, ●) were incubated in the absence of added Ca^{2+} for 5 min in the presence of either vehicle (O, ▲) or 13 nM -vasopressin (●). CaCl_2 (1.4 mM) was then added. After a further 2 min $^{45}\text{Ca}^{2+}$ tracer was added ($t = 0 \text{ s}$), and the amount of $^{45}\text{Ca}^{2+}$ associated with the cells was determined at the indicated times. The data are means $\pm$ S.E.M. for experiments conducted with four preparations of hepatocytes.

percentages of values obtained in the absence of vasopressin) were 104, 115 and 129 % respectively (means of two experiments).

DISCUSSION

After the Percoll-gradient step the viability of hepatocytes loaded with GTP[S], as judged by Trypan Blue exclusion, was over 90 %. This observation, coupled with the finding that about 75 % of the cells took up Lucifer Yellow, provides evidence that the uptake of [^{14}C]sucrose and GTP[^{35}S] by hepatocytes is not due to uptake by damaged cells. The observation that not all cells accumulate Lucifer Yellow is similar to that reported for the uptake of fura 2 free acid by ATP-permeabilized kidney epithelial cells (Ratan *et al.*, 1986).

Considerable uptake of GTP[S] occurred in the presence of Mg^{2+} and the absence of ATP^{4-} . However, the observation that the Ca^{2+} -induced activation of glycogen phosphorylase is more substantial when cells are loaded with GTP[S] in the presence of ATP^{4-} , compared with cells loaded in the absence of ATP^{4-} , indicates that ATP^{4-} enhances the uptake of GTP[S]. The activity of glycogen phosphorylase may be a more sensitive measure of the permeability of cells to GTP[S]. The uptake of molecules in the presence of Mg^{2+} and the absence of ATP^{4-} may be by micropinocytosis (Sasaki *et al.*, 1987). The effect of ATP^{4-} on cell permeability in hepatocytes is much smaller than its effect in a number of other cell types (Gomperts, 1983; Mustelin *et al.*, 1986; Steinberg *et al.*, 1987). This indicates that hepatocytes do

Fig. 6. Effect of GTP[S] on $^{45}\text{Ca}^{2+}$ exchange at 0.1 mM-Ca^{2+} .

Cells loaded with $380 \mu\text{M}$ -GTP[S] (●) or controls (O) were incubated in the presence of 0.1 mM-Ca^{2+} . After 10 min, tracer $^{45}\text{Ca}^{2+}$ was added and the amount of $^{45}\text{Ca}^{2+}$ associated with the cells was determined at the times indicated. Vasopressin was added at $t = 31 \text{ min}$ as indicated by the arrow. The data are means of experiments conducted with two preparations of hepatocytes; the values for each experiment differed by less than 10 %.

not possess the ATP^{4-} -ligated plasma-membrane channel that has been proposed to exist in macrophages (Steinberg *et al.*, 1987).

The amount of GTP[^{35}S] associated with the cells after

the Percoll-gradient step is approx. 3 pmol/mg wet wt. The data presented in Fig. 1 indicate that about 50 % of the $\text{GTP}^{[35\text{S}]}$ is still in the form of GTP[S] at the times when measurements of glycogen phosphorylase activity or $^{45}\text{Ca}^{2+}$ exchange were conducted. Consistent with a continued substantial concentration of GTP[S] in loaded cells is the observation that the effect of GTP[S] on the Ca^{2+} -induced activation of glycogen phosphorylase is observed in cells incubated for both 5 and 20 min before the addition of Ca^{2+} . From the amount of $\text{GTP}^{[35\text{S}]}$ taken up, the proportion remaining as GTP[S] and the relationship that 1 mg wet wt. of cells is equivalent to a volume of the cytoplasmic compartment of $0.5\ \mu\text{l}$ (Krebs *et al.*, 1974), it is calculated that the concentration of $\text{GTP}^{[35\text{S}]}$ is approx. $3\ \mu\text{M}$. This is within the range of concentrations of GTP[S] known to activate purified GTP-binding proteins (Sternweis *et al.*, 1981; Bokoch *et al.*, 1984).

The observations that cells loaded with GTP[S] exhibit increases in (i) the rate and extent of Ca^{2+} -induced activation of glycogen phosphorylase and (ii) the initial rate of $^{45}\text{Ca}^{2+}$ exchange provide evidence that intracellular GTP[S] stimulates the inflow of Ca^{2+} across the plasma membrane. GTP has been shown to stimulate the release of Ca^{2+} from liver microsomal fractions (Dawson *et al.*, 1987; Benedetti *et al.*, 1988) and the entry of Ca^{2+} into the inositol trisphosphate-sensitive Ca^{2+} pool of neuronal cells, smooth-muscle cells (Mullaney *et al.*, 1987, 1988; Chueh *et al.*, 1987) and permeabilized hepatocytes (Thomas, 1988). These effects appear to require the enzyme-mediated hydrolysis of GTP, in that they are not observed when GTP is replaced by GTP[S] (Gill *et al.*, 1986; Dawson *et al.*, 1987; Mullaney *et al.*, 1987; Thomas, 1988). Consequently, it is unlikely that the effects of GTP[S] on the Ca^{2+} -induced activation of glycogen phosphorylase or the initial rate of $^{45}\text{Ca}^{2+}$ exchange observed in the present study reflect changes in Ca^{2+} handling by the inositol trisphosphate-sensitive Ca^{2+} pool. The observations that the effect of GTP[S] on the Ca^{2+} -induced activation of glycogen phosphorylase is inhibited by verapamil, and is additive to that of a limiting concentration of vasopressin but does not alter the effects of a maximally effective concentration of the hormone, provide evidence that GTP[S] activates the same Ca^{2+} -inflow pathway as that activated by vasopressin.

The possibility that the effect of GTP[S] in loaded cells is due to the action of the nucleotide at a plasma-membrane receptor (Okajima *et al.*, 1987) is considered to be unlikely. Firstly, at $0.75\ \mu\text{M}$ added GTP[S] (30 pmol/mg wet wt.), which is considerably higher than the amount of GTP[S] present in loaded cells (3 pmol/mg wet wt.), GTP[S] has little effect on the rate of Ca^{2+} -induced activation of glycogen phosphorylase. Secondly, ATP[S] stimulates glycogen phosphorylase when added externally (Okajima *et al.*, 1987; the present results), but has no effect when loaded into cells.

In permeabilized hepatocytes (Rubin *et al.*, 1987) and in isolated plasma-membrane preparations (Uhing *et al.*, 1986; Cockcroft & Taylor, 1987) the enzyme phosphatidylinositol 4,5-bisphosphate phosphodiesterase has been shown to be activated after treatment with GTP[S] . It has been previously shown that hormone-stimulated Ca^{2+} release from hepatocytes requires the formation of inositol 1,4,5-trisphosphate (Joseph *et al.*, 1984; Burgess *et al.*, 1984). The results of the $^{45}\text{Ca}^{2+}$ -exchange experi-

ments conducted at $0.1\ \text{mM}$ - $[\text{Ca}^{2+}]_o$, and measurements of ^3H -labelled inositol polyphosphates indicate that, in comparison with control cells, the intracellular concentration of inositol 1,4,5-trisphosphate is not substantially increased during the experimental incubation in cells loaded with GTP[S] . The lack of the observed effect of GTP[S] could be due to the absence of Li^+ from the incubation medium, since others have shown that the increase in the concentration of inositol trisphosphate induced by agonists is transient in the absence of Li^+ , compared with a prolonged increase in the presence of Li^+ (Charest *et al.*, 1985b).

The observation that GDP[S] increased glycogen phosphorylase in a manner similar to that of GTP[S] is puzzling, in view of the results obtained by others for inositol trisphosphate formation in plasma-membrane preparations (Uhing *et al.*, 1986; Cockcroft & Taylor, 1987). Although little is known of the effect of GDP[S] on intact cells, a stimulatory effect of this analogue on inositol trisphosphate production has been reported in a permeabilized platelet system (Oberdisse & Lapetina, 1987). A possible explanation for the observed stimulatory effect of GDP[S] is that it may act to relieve the effects of an inhibitory GTP-binding protein (Oberdisse & Lapetina, 1987).

Taken together with the previous observations that the effects of agonists on plasma-membrane Ca^{2+} inflow are mimicked by NaF (Hughes & Barritt, 1987) and are abolished in cells isolated from pertussis-toxin-treated rats (Hughes *et al.*, 1987), the present results provide further evidence that a GTP-binding regulatory protein is involved in the activation by agonists of Ca^{2+} movement into the liver cell. There may be a direct interaction between the receptor, a component of the GTP-binding protein and the putative Ca^{2+} channel, as proposed for the activation by acetylcholine of K^+ channels (Pfaffinger *et al.*, 1985; Sasaki & Sato, 1987; Yatani *et al.*, 1987).

This work was supported by a grant from the N.H. and M.R.C. of Australia. We are grateful to Nicola Capon, Amanda Auld and Kay Owen for skilled technical assistance, and to Dr. Joel Bornstein, Department of Human Physiology, Flinders University School of Medicine, for advice regarding the use of Lucifer Yellow and fluorescence microscopy.

REFERENCES

- Barritt, G. J., Parker, J. C. & Wadsworth, J. C. (1981) *J. Physiol. (London)* **312**, 29–55
- Benedetti, A., Fulceri, R., Romani, A. & Comporti, M. (1988) *J. Biol. Chem.* **263**, 3466–3473
- Berry, M. N., Farrington, C., Gay, S., Grivell, A. R. & Wallace, P. G. (1983) in *Isolation, Characterization and Use of Hepatocytes* (Harris, R. A. & Cornell, N. W., eds.), pp. 7–10, Elsevier, New York
- Bokoch, G. M., Katada, T., Northup, J. K., Ui, M. & Gilman, A. G. (1984) *J. Biol. Chem.* **259**, 3560–3567
- Burgess, G. M., Godfrey, P. P., McKinney, J. S., Berridge, M. J., Irvine, R. F. & Putney, J. W. (1984) *Nature (London)* **309**, 63–66
- Charest, R., Blackmore, P. F. & Exton, J. H. (1985a) *J. Biol. Chem.* **260**, 15789–15794
- Charest, R., Prpic, V., Exton, J. H. & Blackmore, P. F. (1985b) *Biochem. J.* **227**, 79–90
- Chueh, S.-H., Mullaney, J. M., Ghosh, T. K., Zachary, A. L. & Gill, D. L. (1987) *J. Biol. Chem.* **262**, 13857–13864

- Cockcroft, S. & Gomperts, B. D. (1980) *Biochem. J.* **188**, 789–798
- Cockcroft, S. & Taylor, J. A. (1987) *Biochem. J.* **241**, 409–414
- Cooper, R. H., Coll, K. E. & Williamson, J. R. (1985) *J. Biol. Chem.* **260**, 3281–3288
- Dawson, A. P., Hills, G. & Comerford, J. G. (1987) *Biochem. J.* **244**, 87–92
- Gill, D. L., Ueda, T., Chueh, S.-H. & Noel, M. W. (1986) *Nature (London)* **320**, 461–464
- Gilman, A. G. (1987) *Annu. Rev. Biochem.* **56**, 615–649
- Gomperts, B. D. (1983) *Nature (London)* **306**, 64–66
- Hughes, B. P. & Barritt, G. J. (1987) *Biochem. J.* **245**, 41–47
- Hughes, B. P., Milton, S. E. & Barritt, G. J. (1986a) *Biochem. Pharmacol.* **35**, 3045–3052
- Hughes, B. P., Milton, S. E., Barritt, G. J. & Auld, A. M. (1986b) *Biochem. J.* **238**, 793–800
- Hughes, B. P., Crofts, J. N., Auld, A. M., Read, L. C. & Barritt, G. J. (1987) *Biochem. J.* **248**, 911–918
- Hutson, N. J., Brumley, F. T., Asimacopoulos, F. D., Harper, S. C. & Exton, J. H. (1976) *J. Biol. Chem.* **251**, 5200–5208
- Joseph, S. K., Thomas, A. P., Williams, R. J., Irvine, R. F. & Williamson, J. R. (1984) *J. Biol. Chem.* **259**, 3077–3081
- Krishnan, N. & Krishna, G. (1976) *Anal. Biochem.* **70**, 18–31
- Krebs, H. A., Cornell, N. W., Lund, P. & Hems, R. (1974) in *Regulation of Hepatic Metabolism* (Lundquist, F. & Tygstrup, N., eds.), pp. 726–750, Munksgaard, Copenhagen
- Mauger, J.-P., Poggioli, J., Guesdon, F. & Claret, M. (1984) *Biochem. J.* **221**, 121–127
- Mullaney, J. M., Chueh, S.-H., Ghosh, T. K. & Gill, D. L. (1987) *J. Biol. Chem.* **262**, 13865–13872
- Mullaney, J. M., Yu, M., Ghosh, T. K. & Gill, D. L. (1988) *Proc. Natl. Acad. Sci. U.S.A.* **85**, 2499–2503
- Mustelin, T., Posso, H. & Andersson, L. C. (1986) *EMBO J.* **5**, 3287–3290
- Oberdisse, E. & Lapetina, E. G. (1987) *Biochem. Biophys. Res. Commun.* **144**, 1188–1196
- Okajima, F., Tokumitsu, Y., Kondo, Y. & Ui, M. (1987) *J. Biol. Chem.* **262**, 13483–13490
- Pfaffinger, P. J., Martin, J. M., Hunter, D. D., Nathanson, N. M. & Hille, B. (1985) *Nature (London)* **317**, 536–538
- Ratan, R. R., Shelanski, M. L. & Maxfield, F. R. (1986) *Proc. Natl. Acad. Sci. U.S.A.* **83**, 5136–5140
- Reinhart, P. H., Taylor, W. M. & Bygrave, F. L. (1984) *Biochem. J.* **220**, 35–42
- Rubin, R., Thomas, A. P. & Hoek, J. B. (1987) *Arch. Biochem. Biophys.* **256**, 29–38
- Sasaki, K. & Sato, M. (1987) *Nature (London)* **325**, 259–262
- Sasaki, A. W., Williams, S. K., Jain, M. & Wagner, R. C. (1987) *J. Cell. Physiol.* **133**, 175–180
- Shears, S. B. & Kirk, C. J. (1984) *Biochem. J.* **220**, 417–421
- Steinberg, T. H., Newman, A. S., Swanson, J. A. & Silverstein, S. C. (1987) *J. Biol. Chem.* **262**, 8884–8888
- Sternweis, P. C., Northup, J. K., Smigel, M. D. & Gilman, A. G. (1981) *J. Biol. Chem.* **256**, 11517–11526
- Thomas, A. P. (1988) *J. Biol. Chem.* **263**, 2704–2711
- Uhing, R. J., Prpic, V., Jiang, H. & Exton, J. H. (1986) *J. Biol. Chem.* **261**, 2140–2146
- Yatani, A., Codina, J., Brown, A. M. & Birnbaumer, L. (1987) *Science* **235**, 207–211

Received 18 April 1988/3 August 1988; accepted 20 September 1988