A slowly ADP-ribosylated pertussis-toxin-sensitive GTP-binding regulatory protein is required for vasopressin-stimulated Ca^{2+} inflow in hepatocytes

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The roles of heterotrimeric GTP-binding regulatory proteins (G-proteins) and inositol polyphosphates in the mechanism by which vasopressin stimulates Ca^{2+} inflow in hepatocytes were investigated by using single cells loaded with fura2 by microinjection. Vasopressin-stimulated Ca^{2+} inflow was mimicked by microinjection of guanosine 5′-[γ-thio]triphosphate (GTP[S]) or guanosine 5′-[βγ-imido]triphosphate to the cells, but not adenosine 5′-[γ-thio]triphosphate (ATP[S]) or guanosine 5′-[β-thio]diphosphate (GDP[S]). Extracellular Gd^{3+} (5 μM) inhibited both vasopressin- and GTP[S]-stimulated Ca^{2+} inflow. GDP[S], but not GMP, administered to hepatocytes by microinjection, completely inhibited vasopressin-stimulated Ca^{2+} inflow and partially inhibited vasopressin-induced release of Ca^{2+} from intracellular stores. The microinjection of pertussis toxin had no effect either on the release of Ca^{2+} from intracellular stores or on Ca^{2+} inflow induced by vasopressin, but completely inhibited changes in these processes induced by epidermal growth factor (EGF). Hepatocytes isolated from rats treated with pertussis toxin for 24 h exhibited no vasopressin- or GTP[S]-stimulated Ca^{2+} inflow, whereas the vasopressin-stimulated release of Ca^{2+} from intracellular stores was similar to that observed for control cells. Heparin or ATP[S] inhibited, or delayed the onset of, both vasopressin-induced release of Ca^{2+} from intracellular stores and vasopressin-stimulated Ca^{2+} inflow. Vasopressin-induced oscillations in intracellular [Ca^{2+}] were observed in some heparin-treated cells. It is concluded that the stimulation by vasopressin of Ca^{2+} inflow to hepatocytes requires inositol 1,4,5-trisphosphate ($InsP_3$) and, by implication, the pertussis-toxin-insensitive G-protein required for the activation of phospholipase C_β [Taylor, Chae, Rhee and Exton (1991) Nature (London) **350**, 516–518], and another G-protein which is slowly ADP-ribosylated by pertussis toxin and acts between $InsP_3$ and the putative plasma-membrane Ca^{2+} channel. EGF-stimulated Ca^{2+} inflow involves at least one G-protein which is rapidly ADP-ribosylated and is most likely required for $InsP_3$ formation.

INTRODUCTION

Extracellular signals which use free Ca^{2+} (Ca^{2+}_i) as an intracellular messenger in hepatocytes stimulate the inflow of Ca^{2+} to the cytoplasmic space across the plasma membrane (Barritt et al., 1981; Mauger et al., 1984; Reinhart et al., 1984), as well as the release of Ca^{2+} from a portion of the endoplasmic reticulum (Shears and Kirk, 1984). The latter is mediated by inositol 1,4,5-trisphosphate ($InsP_3$) (Joseph et al., 1984; Burgess et al., 1984). These extracellular signals, which include vasopressin, adrenaline, angiotensin II and epidermal growth factor (EGF), appear to open a putative Ca^{2+} channel in the plasma membrane (Mauger et al., 1984; Crofts and Barritt, 1990; Kass et al., 1990; Butta et al., 1993; Striggow and Bohnensack, 1993). This latter process has been called a receptor-operated Ca^{2+} channel, a receptor-mediated Ca^{2+} entry or a receptor-activated Ca^{2+}-inflow system (RACIS) (reviewed in Barritt and Hughes, 1991). In comparison with knowledge of several other Ca^{2+} channels, including voltage-operated Ca^{2+} channels, the $InsP_3$ receptor, the cyclic GMP-gated cation channel and the ryanodine-sensitive Ca^{2+}-efflux channel (Furuichi et al., 1989; Catterall, 1991; Jan and Jan, 1992; Strong and Gutman, 1993), relatively little is known about the nature and mechanism of activation of the RACIS in hepatocytes and RACISs present in other cell types.

Three main ideas have been developed to explain the process by which agonists activate the hepatocyte RACIS. These are (i) the interaction of $InsP_3$ (Hansen et al., 1991) or inositol 1,3,4,5-tetrakisphosphate (Irvine, 1990; but see Hansen et al., 1991) with a putative RACIS channel protein, (ii) opening of the channel as a consequence of the $InsP_3$-induced release of Ca^{2+} from the endoplasmic reticulum (Putney, 1990; Hansen et al., 1991; Glennon et al., 1992), and (iii) direct interaction of a heterotrimeric GTP-binding regulatory protein (G-protein) with the putative channel protein (Hughes and Barritt, 1987, 1989; Hughes et al., 1987). These proposed mechanisms may not be mutually exclusive. Although each of these proposals is consistent with some of the available experimental data, there is at present insufficient evidence to eliminate conclusively any of these proposed mechanisms.

Some evidence which indicates that agonist-induced increases in $[Ca^{2+}]_i$ and protein kinase C are not involved in activation of the hepatocyte RACIS by vasopressin has been previously obtained (Hughes and Barritt, 1987; Kass et al., 1990). The results of other experiments have suggested firstly that at least

Abbreviations used: $[Ca^{2+}]_i$, concentration of intracellular free Ca^{2+}; $[Ca^{2+}]_o$, concentration of extracellular Ca^{2+}; $InsP_3$, inositol 1,4,5-trisphosphate; EGF, epidermal growth factor; ATP[S], adenosine 5′-[γ-thio]triphosphate; GTP[S], guanosine 5′-[γ-thio]triphosphate; GDP[S], guanosine 5′-[β-thio]diphosphate; G-protein, heterotrimeric GTP-binding regulatory protein; G_{PTS}, a slowly ADP-ribosylated pertussis-toxin-sensitive G-protein; G_{PTR}, a rapidly ADP-ribosylated pertussis-toxin-sensitive G-protein; G_{INS}, a G-protein which is insensitive to pertussis toxin; RACIS, receptor-activated Ca^{2+}-inflow system.

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one G-protein is involved in the mechanism(s) by which vasopressin and EGF stimulate Ca^{2+} inflow (Hughes and Barritt, 1987; Hughes et al., 1987; Kass et al., 1990; Hansen et al., 1991; Butta et al., 1993) and secondly that this or another essential G-protein is sensitive to pertussis toxin (Hughes et al., 1987; Butta et al., 1993). Thus it was shown that the stimulation by agonists of Ca^{2+} inflow can be mimicked by AlF_4^- and guanosine 5'-[γ-thio]triphosphate (GTP[S]) (Hughes and Barritt, 1987, 1989). Moreover, pre-treatment of hepatocytes with pertussis toxin (injected into rats 24 h before preparation of the hepatocytes) blocked the stimulation of Ca^{2+} inflow by vasopressin and EGF, but did not block vasopressin-induced release of Ca^{2+} from intracellular stores or the vasopressin-induced increase in inositol trisphosphates (Hughes et al., 1987) (but see Kass et al., 1990).

The aim of the present experiments was to resolve further the function of G-proteins in the mechanism by which agonists stimulate Ca^{2+} inflow in hepatocytes. The approach has been to microinject activators or inhibitors of G-proteins into cells and to determine their effect on agonist-stimulated Ca^{2+} inflow. This approach overcomes some of the difficulties inherent in the uptake of compounds such as GTP[S] by intact hepatocytes (Hughes and Barritt, 1989). Ca^{2+} inflow was measured by using fura2 introduced into the cytoplasmic space of hepatocytes by microinjection, in order to avoid potential difficulties associated with the uptake of the acetoxymethyl ester of fura2 (Glennon et al., 1992). The present results indicate that vasopressin-stimulated Ca^{2+} inflow requires a slowly ADP-ribosylated pertussis-toxin-sensitive G-protein as well as the pertussis-toxin-insensitive G-protein which activates phospholipase C_{β_1} (Taylor et al., 1991). EGF-stimulated Ca^{2+} inflow requires a rapidly ADP-ribosylated pertussis-toxin-sensitive G-protein.

EXPERIMENTAL

Isolation and incubation of hepatocytes

Hepatocytes were isolated from fed male hooded Wistar rats (200–250 g) by collagenase digestion by using the method of Berry and Friend (1969) as described previously (Barritt et al., 1981). Cell viability was determined by using Trypan Blue (Berry et al., 1991); over 90% of the cells employed in all experiments excluded Trypan Blue. The experiments were conducted in 35 mm plastic Petri dishes in which the centre of the dish was replaced by a glass coverslip (Polverino et al., 1991). The coverslip portion of each dish was coated with Type I collagen as described by Berry et al. (1991). The procedure for attachment of cells to the collagen-coated coverslips was adapted from that described by Hansen et al. (1991). Freshly isolated hepatocytes were suspended at 2×10^5 cells/ml in Dulbecco's Modified Eagle's Medium (DMEM) (Gibco–BRL, Glen Waverley, Victoria, Australia) containing 44 mM $NaHCO_3$, 1 mM sodium pyruvate and 2 g/l BSA, and were then allowed to attach to the collagen-coated coverslips by incubating 3 ml of the cell suspension in each dish at 37 °C. After 30 min the medium was changed to fresh DMEM, and unattached cells were removed. The attached cells were incubated at 37 °C for a further 1 h before the microinjection of fura2–dextran.

Introduction of fura2–dextran and other agents to hepatocytes by microinjection

Hepatocytes attached to a collagen-coated coverslip were transferred to CO_2-Independent Medium (Gibco–BRL), loaded with fura2–dextran by pressure injection, and maintained at 30 °C on the microscope stage during fluorescence measurements. The micropipette (pulled by using a Kopf vertical pipette puller; David Kopf Instruments, Tujunga, CA, U.S.A.) was filled with a solution of 10 mM fura2–dextran in 125 mM KCl. The pipette tip was introduced to the cell by using a Nikon Narashige micromanipulator. Fura2–dextran was injected into the cytoplasmic space by the application of a pulse of 62 kPa (9 lb/in^2) for 20–200 ms using a Picospritzer (General Valve Corp., Fairfield, NJ, U.S.A.). The volume delivered was less than 1% of the total cell volume. After injection, the cells were incubated for 20 min in order to allow the membrane to re-seal before the measurement of fluorescence and addition of agonists. GTP[S] and other nucleotides were introduced by co-injection with fura2–dextran. Heparin (15000 Da) or low-molecular-mass heparin (4000–6000 Da) (4–20 mM in the pipette tip) was co-injected with fura2–dextran, and the cell was incubated for 20–40 min before the measurement of fluorescence. In all experiments, injected cells were transferred to modified Hanks' medium, which consisted of 137 mM NaCl, 5.4 mM KCl, 0.8 mM $MgSO_4$, 0.8 mM Na_2HPO_4, 0.4 mM KH_2PO_4, 4.2 mM $NaHCO_3$, 20 mM Hepes, pH 7.4, and 5.5 mM glucose (Incubation Medium) (Hansen et al., 1991) just before the fluorescence measurements. For experiments performed in the presence of extracellular Ca^{2+}, 1.3 mM $CaCl_2$ was added to the Incubation Medium as indicated.

Agonists and Ca^{2+} were added to the extracellular medium either by microperfusion or by direct addition to the whole dish. For microperfusion, a micropipette filled with agonist or Ca^{2+} (as indicated in legends to Figures) was positioned by using a micromanipulator approx. 10 μm from the surface of the cell under investigation. The solution was delivered near the cell by a pulse of 34.5 kPa (5 lb/in^2) for 30–60 s by using a Picospritzer. Since the agonist or Ca^{2+} mixed with extracellular medium during microperfusion, the concentrations of agonists and Ca^{2+} employed in these experiments were approx. 3 times that required to obtain similar effects by adding the agonist or Ca^{2+} to the entire contents of the dish. Direct addition of an agonist was achieved by adding 1 ml of Incubation Medium containing the agonist or Ca^{2+} at 3 times the final concentration required to 2 ml of Incubation Medium (in the incubation dish). The contents of the dish were mixed carefully by drawing medium into a pipette and then expelling the medium back to the dish.

Measurement of $[Ca^{2+}]_i$

The fluorescence of single hepatocytes was measured by using an inverted TMD-EF fluorescence microscope (Nikon Corp., Tokyo, Japan) and the UMANS computer system as described by Polverino et al. (1991). Excitation was at 345 and 385 nm. Neutral density filters were incorporated in the excitation light-path in order to decrease photobleaching. Changes in fura2 fluorescence are presented as the ratio of signals obtained at the 345 and 385 nm excitation wavelengths.

Treatment of hepatocytes with pertussis toxin

In experiments in which pertussis toxin (Sigma–Aldrich, Castle Hill, N.S.W., Australia) was injected into single hepatocytes, the toxin (1.4–4.5 mg/ml in the micropipette tip) was co-injected with fura2–dextran, and the cell was incubated for 30–60 min before addition of agonists and measurement of fluorescence. In some experiments pertussis toxin was 'activated' by the method of Cantiello et al. (1989). However, results obtained with 'activated' pertussis toxin were similar to those obtained with non-activated toxin. In experiments in which hepatocytes were isolated from pertussis-toxin-treated rats, non-activated pertussis

toxin was administered to rats (25 μg/100 g body wt.) by intraperitoneal injection (Lynch et al., 1986) 24 h before the preparation of hepatocytes.

Materials

GTP[S], other nucleotides, 2,3-diphospho-D-glycerate and EGF were obtained from Boehringer Mannheim, Castle Hill, N.S.W., Australia; heparin (15000 Da), vasopressin and pertussis toxin were from Sigma–Aldrich; low-molecular-mass heparin (4000–6000 Da) was from Calbiochem–Novabiochem, Alexandria, N.S.W., Australia; fura2–dextran was from Molecular Probes, Eugene, OR, U.S.A.; and $GdCl_3$ from Aldrich Chemical Co., Castle Hill, N.S.W., Australia. All other reagents were of the highest grade commercially available.

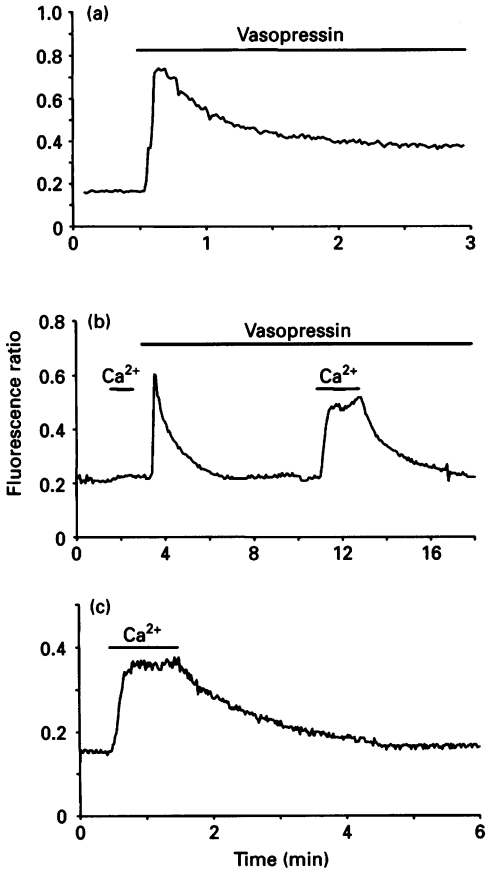

Figure 1 Stimulation of Ca^{2+} inflow by vasopressin and GTP[S]

Hepatocytes were loaded with fura2–dextran by microinjection, and fluorescence was measured as described in the Experimental section. (**a**) Hepatocytes were incubated in the presence of 1.3 mM $Ca^{2+}{}_o$. Vasopressin (40 nM) was added to the incubation dish at the beginning of the period indicated by the horizontal bar. The trace shown is one of similar traces obtained for each of 15/16 cells tested. (**b**) Hepatocytes were incubated initially in the absence of added $Ca^{2+}{}_o$. During the periods indicated by the horizontal bars, Ca^{2+} (5.2 mM) was applied to the extracellular medium adjacent to the cell by microperfusion. Vasopressin (40 nM) was added to the incubation dish at the beginning of the period indicated by the horizontal bar. The trace shown is one of similar traces obtained for each of 16/20 cells tested. (**c**) Hepatocytes were loaded with fura2–dextran and GTP[S] (16 mM in pipette tip) by microinjection and incubated initially in the absence of added $Ca^{2+}{}_o$. During the period indicated by the horizontal bar, Ca^{2+} (5.2 mM in the micropipette tip) was applied by microperfusion to the extracellular medium adjacent to the cell. The trace shown is one of similar traces obtained for each of 20/20 cells subjected to this regime.

RESULTS

Actions of guanine nucleotides

In hepatocytes loaded with fura2–dextran and incubated in the presence of 1.3 mM extracellular Ca^{2+} ($Ca^{2+}{}_o$), vasopressin induced a rapid increase in $[Ca^{2+}]_i$ which subsequently decreased to a plateau above baseline (Figure 1a) (cf. Monck et al., 1988; Rooney et al., 1989; but see Kawanishi et al., 1989). In the absence of added $Ca^{2+}{}_o$ and vasopressin, the microperfusion of $Ca^{2+}{}_o$ near the extracellular surface of hepatocytes caused a negligible or no increase in $[Ca^{2+}]_i$ (Figure 1b). Addition of vasopressin to the medium caused a transient increase in $[Ca^{2+}]_i$, after which $[Ca^{2+}]_i$ returned to the basal value (Figure 1b). A second microperfusion of $Ca^{2+}{}_o$ caused a large increase in $[Ca^{2+}]_i$ (Figure 1b). The results of these two sets of experiments indicate that vasopressin stimulates Ca^{2+} inflow across the plasma membrane of single hepatocytes (c.f. Zhang et al., 1991; Glennon et al., 1992; and Hansen et al., 1991).

In cells pre-loaded with fura2–dextran and GTP[S] and incubated in the absence of $Ca^{2+}{}_o$, an increase in $[Ca^{2+}]_i$ was observed after the microperfusion of $Ca^{2+}{}_o$ (Figure 1c), indicating that GTP[S] also stimulates Ca^{2+} inflow (cf. Hansen et al., 1991). An increase in $[Ca^{2+}]_i$ similar to that shown in Figure 1(c) was also observed after the addition of $Ca^{2+}{}_o$ to cells loaded with guanosine 5′-[$\beta\gamma$-imido]triphosphate instead of GTP[S], but not when adenosine 5′-[γ-thio]triphosphate (ATP[S]), cyclic GMP or guanosine 5′-[β-thio]diphosphate (GDP[S]) was employed (results not shown). The actions of both vasopressin and GTP[S] in stimulating Ca^{2+} inflow were blocked by Gd^{3+}, which has been shown to inhibit some RACIS (Popp and Gogelein, 1992; Bear and Li, 1991) (Figures 2a–d). Gd^{3+} did not affect vasopressin-stimulated release of Ca^{2+} from intracellular stores (Figure 2a, cf. Figure 2b).

The results described above indicate that activation of a G-protein can lead to the stimulation of Ca^{2+} inflow in single hepatocytes. To test whether a G-protein is required in coupling between receptors and the RACIS, the ability of GDP[S] to inhibit vasopressin-stimulated Ca^{2+} inflow was tested. In hepatocytes loaded with GDP[S] by microinjection, no stimulation by vasopressin of Ca^{2+} inflow was observed (Figure 3a). Under the conditions of the experiment shown in Figure 3, GDP[S] did not inhibit the ability of vasopressin to release Ca^{2+} from intracellular stores. However, in most cells tested GDP[S] did alter the shape of the transient increase in $[Ca^{2+}]_i$ induced by vasopressin through introduction of a shoulder after the main transient (Figure 3a; cf. Figure 1b). Higher concentrations of GDP[S] (90 mM in the pipette tip) were also tested, but had no effect on vasopressin-induced release of Ca^{2+} from intracellular stores, while inhibiting vasopressin-stimulated Ca^{2+} inflow.

The effects of GDP[S] were also studied at a much lower concentration of vasopressin (1 nM) (cf. 40 nM employed in the experiments shown in Figure 3). This concentration of hormone caused the release of Ca^{2+} from intracellular stores, and Ca^{2+} inflow, with a magnitude similar to that observed at 40 nM vasopressin (4/4 cells; results not shown). At 1 nM vasopressin, GDP[S] caused a 4–6 min delay in the transient increase in $[Ca^{2+}]_i$ induced by vasopressin, and completely inhibited vasopressin-stimulated Ca^{2+} inflow (results not shown). In these cells the magnitude of the transient increase in $[Ca^{2+}]_i$ induced by vasopressin was similar to that observed in control cells (1 nM vasopressin, absence of GDP[S]).

When GMP was used in place of GDP[S], vasopressin-induced release of Ca^{2+} from intracellular stores was not inhibited, although, as observed with GDP[S], in some cells GMP altered the shape of the vasopressin-induced transient increase in $[Ca^{2+}]_i$

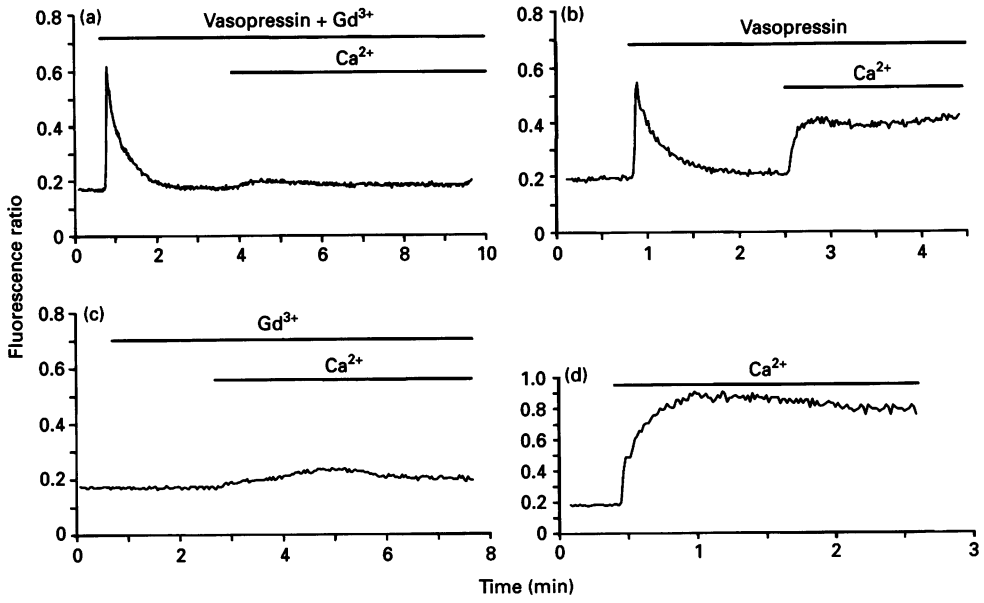

Figure 2 Inhibition by Gd^{3+} of Ca^{2+} inflow stimulated by vasopressin (a, b) and GTP[S] (c, d)

Hepatocytes were loaded with fura2–dextran and GTP[S], and fluorescence was measured as described in the Experimental section. (**a, b**) Cells were loaded with fura2–dextran only and incubated initially in the absence of added Ca^{2+}_o. At the beginning of the horizontal bars, vasopressin (40 nM) plus Gd^{3+} (5 μM), vasopressin (40 nM) or Ca^{2+} (1.3 mM) were added to the incubation dish as indicated. (**c, d**) Cells were loaded with fura2–dextran and GTP[S] (16 mM in the pipette tip) and incubated initially in the absence of added Ca^{2+}_o. At the beginning of the horizontal bars, Gd^{3+} (5 μM) or Ca^{2+} (1.3 mM) was added to the incubation dish as indicated. The numbers of cells tested were: (**a**) vasopressin plus Gd^{3+}, 10 (8 showed no inflow, as in the trace shown, and 2 gave some inflow); (**b**) vasopressin alone, 9 (9 gave results similar to the trace shown); (**c**) GTP[S] plus Gd^{3+}, 8 (8 gave results similar to the trace shown); and (**d**) GTP[S] alone, 8 (7 cells gave results similar to the trace shown).

(Figure 3b). In cells loaded with GMP, vasopressin-stimulated Ca^{2+} inflow was not affected in 6/9 cells tested, and was partially inhibited in 3/9 cells (Figure 3b).

Actions of EGF and pertussis toxin

In single hepatocytes incubated in the presence of 1.3 mM Ca^{2+}_o, EGF caused an increase in $[Ca^{2+}]_i$ (Figure 4a), in agreement with the results of others (Yang et al., 1991). However, as described in the legend of Figure 4(a), the response to EGF was variable. In cells incubated in the absence of added Ca^{2+}_o, EGF induced a transient increase in $[Ca^{2+}]_i$ (Figure 4b), which was somewhat variable in magnitude and duration in different cells. The subsequent addition of Ca^{2+}_o induced an increase in $[Ca^{2+}]_i$ (Figure 4b). These results indicate that, as observed in bulk suspensions of hepatocytes (Hughes et al., 1987), EGF stimulates Ca^{2+} inflow, thus confirming the observations of Yang et al. (1991) with single cells. The stimulation by EGF of Ca^{2+} inflow was not as great as that induced by vasopressin (Figure 4b, cf. Figure 1b).

The ability of EGF to increase $[Ca^{2+}]_i$ in hepatocytes incubated in the presence of 1.3 mM Ca^{2+}_o was completely inhibited by the microinjection of pertussis toxin to hepatocytes 30–60 min before the measurement of changes in $[Ca^{2+}]_i$ (Figure 4c). This regime for the administration of pertussis toxin did not affect either vasopressin-stimulated release of Ca^{2+} from intracellular stores or vasopressin-stimulated Ca^{2+} inflow, as judged from experiments conducted in the presence of added Ca^{2+}_o (Figure 4c, cf. Figure 1a) or in the initial absence of Ca^{2+}_o (results not shown).

In contrast with the results obtained when pertussis toxin was microinjected into cells, hepatocytes isolated from rats 24 h after the animals had received an intraperitoneal injection of the toxin exhibited no vasopressin-induced Ca^{2+} inflow when this was assessed either by addition of Ca^{2+}_o to hepatocytes incubated in the absence of added Ca^{2+}_o (Figure 5a) or by addition of vasopressin in the presence of Ca^{2+}_o (Figure 5b). In the latter experiment, $[Ca^{2+}]_i$ returned to the basal value after the transient increase induced by vasopressin. Treatment with the toxin for 24 h also completely inhibited GTP[S]-stimulated Ca^{2+} inflow (Figure 5c) and the EGF-stimulated release of Ca^{2+} from intracellular stores and EGF-stimulated Ca^{2+} inflow (Figure 5b, cf. Figure 4b) (cf. Yang et al., 1991, 1993; Liang and Garrison, 1992).

Role of $InsP_3$

To investigate the role of $InsP_3$ in the mechanism by which vasopressin stimulates Ca^{2+} inflow, experiments were conducted with ATP[S] (a slowly hydrolysable analogue of ATP), 2,3-diphospho-D-glycerate and heparin. These compounds inhibit the binding of $InsP_3$ to its receptor in the endoplasmic reticulum (Ghosh et al., 1988; Guillemette et al., 1988; Willcocks and Nahorski, 1988; Nunn and Taylor, 1990). The microinjection of 2,3-diphospho-D-glycerate at the highest concentrations attainable (60 mM in the microinjection pipette) had no effect on vasopressin-stimulated Ca^{2+} inflow (results not shown). However, ATP[S] inhibited vasopressin-stimulated Ca^{2+} inflow and inhibited or delayed the onset of vasopressin-stimulated release of Ca^{2+} from intracellular stores (Figure 6a, cf. Figure 6d).

Heparin induced several different types of behaviour. Thus, in the absence of added Ca^{2+}_o heparin (4–20 mM in the pipette tip) had no effect, completely inhibited (results not shown), or delayed (1–2 min) (Figure 6b, cf. Figure 6d) the vasopressin-induced

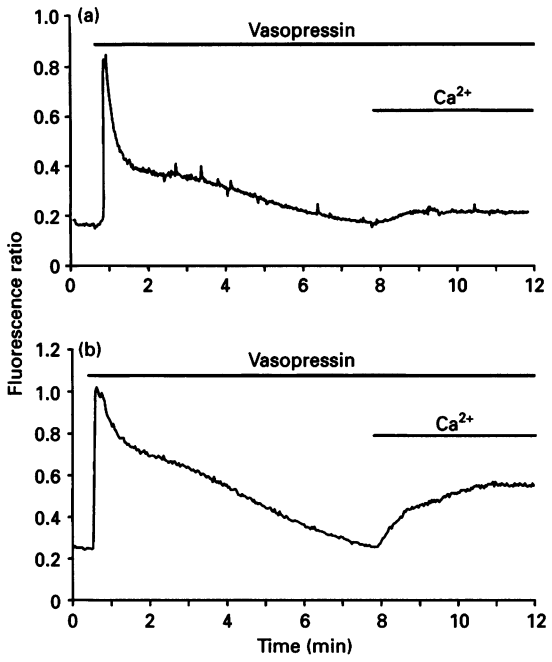

Figure 3 Inhibition of vasopressin-stimulated Ca^{2+} inflow by GDP[S]

Cells were loaded with fura2–dextran and GDP[S] (30 mM in the pipette tip) (**a**) or fura2–dextran and GMP (30 mM in the pipette tip) (**b**), incubated initially in the absence of added Ca^{2+}_o, and fluorescence was monitored as described in the Experimental section. At the beginning of the horizontal bars, vasopressin (40 mM) and $CaCl_2$ (1.3 mM) were added to the incubation dish as indicated. The results shown in (**a**) (GDP[S]) represent those obtained with one of 20 cells tested; 18 of the cells tested showed no substantial increase in $[Ca^{2+}]_i$ after addition of Ca^{2+}_o, and 2 cells showed some increase in $[Ca^{2+}]_i$ after addition of Ca^{2+}_o. All 20 cells showed a vasopressin-stimulated increase in $[Ca^{2+}]_i$ similar to that shown. The results shown in (**b**) represent those obtained with one of 9 cells tested. All 9 cells tested showed a vasopressin-stimulated increase in $[Ca^{2+}]_i$ similar in magnitude to that observed in control cells (no GMP). In 3/9 cells a transient increase in $[Ca^{2+}]_i$ with an unusual shape (as shown in this trace) was observed after addition of vasopressin. After addition of Ca^{2+}_o, 6/9 cells showed an increase in $[Ca^{2+}]_i$ similar to that observed in control cells (no GMP) and 3/9 cells exhibited a somewhat diminished increase in $[Ca^{2+}]_i$ (as shown in this trace).

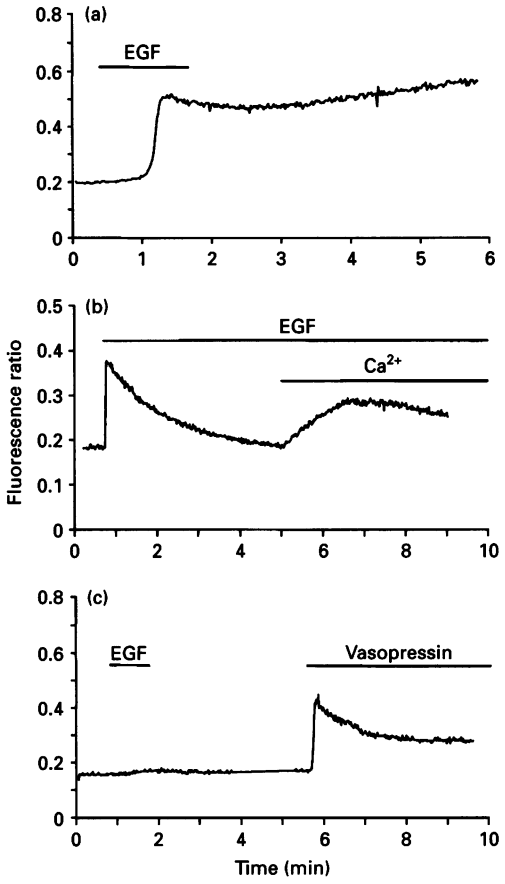

Figure 4 Effects of EGF and vasopressin on $[Ca^{2+}]_i$ in the absence and presence of pertussis toxin introduced to the cells by microinjection

The loading of the cells with fura2–dextran, incubations and measurement of fluorescence were conducted as described in the Experimental section. (**a**) The increase in $[Ca^{2+}]_i$ induced by EGF in the presence of 1.3 mM Ca^{2+}_o. EGF (800 nM in the pipette tip) was introduced by microperfusion to the medium adjacent to the cell as indicated by the horizontal bar. The results shown represent those obtained with one of 33 cells tested; 26 of these 33 cells gave a response to EGF. However, this response was variable. Of the 26 cells which responded to EGF, 12 exhibited a sustained increase in $[Ca^{2+}]_i$, whereas 14 gave a transient increase in $[Ca^{2+}]_i$. Some cells (8 of all those which responded to EGF) exhibited a delay of 30–60 s in the onset of the sustained or transient increase in $[Ca^{2+}]_i$ (cf. Yang et al., 1991). (**b**) The effect of EGF on $[Ca^{2+}]_i$ in cells initially incubated in the absence of added Ca^{2+}_o. At the beginning of the horizontal bars, EGF (400 nM) or $CaCl_2$ (1.3 mM) was added to the incubation dish as indicated. The trace shown represents the results obtained for 5 out of 6 cells tested. (**c**) Inhibition by pertussis toxin of the action of EGF measured in the presence of 1.3 mM Ca^{2+}_o. The microinjection of pertussis toxin was performed as described in the Experimental section. EGF (800 nM in the pipette tip) and vasopressin (60 nM in the pipette tip) were introduced by microperfusion to the medium adjacent to the cell for the periods indicated by the horizontal bars. The trace shown represents the results obtained for 14 out of 16 cells tested.

increase in $[Ca^{2+}]_i$ and the increase in $[Ca^{2+}]_i$ induced by the addition of Ca^{2+}_o to cells previously incubated in the absence of Ca^{2+}_o and presence of vasopressin. Some cells, in which heparin induced a delay in vasopressin-stimulated Ca^{2+} inflow, exhibited oscillations in $[Ca^{2+}]_i$ after addition of Ca^{2+}_o (Figure 6c). In all cells in which heparin either blocked or caused a delay in onset of vasopressin-induced increase in $[Ca^{2+}]_i$, heparin also either blocked or caused a delay in onset of the increase in $[Ca^{2+}]_i$ which followed addition of $[Ca^{2+}]_o$. In cells not exposed to heparin, delays in the onset of vasopressin-stimulated Ca^{2+} release from intracellular stores and Ca^{2+} inflow were never observed. The use of low-molecular-mass (4000–6000 Da) heparin or longer incubation times gave the same pattern of results (i.e. inhibition, a delay in onset, or oscillations) as was obtained for cells treated with higher-molecular-mass heparin. [Low-molecular-mass heparin has been shown to cause greater inhibition of $InsP_3$-induced Ca^{2+} release in smooth-muscle cells compared with higher-molecular-mass (15000 Da) heparin (Chopra et al., 1989).]

In 75% of those heparin-treated cells which showed a delay in response after the addition of vasopressin, a change in the shape of the transient increase in $[Ca^{2+}]_i$ induced by vasopressin was also noticed (Figure 6b, cf. Figure 6d). This shape change, characterized by the appearance of a shoulder after the main peak, was similar to the shape of the transients observed in the presence of GDP[S] and GMP (Figures 3a and 3b). In cells injected with heparin in which there was no vasopressin-stimulated Ca^{2+} release, subsequent addition of thapsigargin (2 μM) caused a transient increase in $[Ca^{2+}]_i$ (results not shown). This result indicates that the absence of vasopressin-induced release of Ca^{2+} from intracellular stores in heparin-treated cells is not due to the depletion of Ca^{2+} in these stores.

DISCUSSION

The key results presented in this paper can be summarized as follows. (*a*) Analogues of GTP which are known to activate G-proteins (reviewed by Gilman, 1987) stimulated plasma-mem-

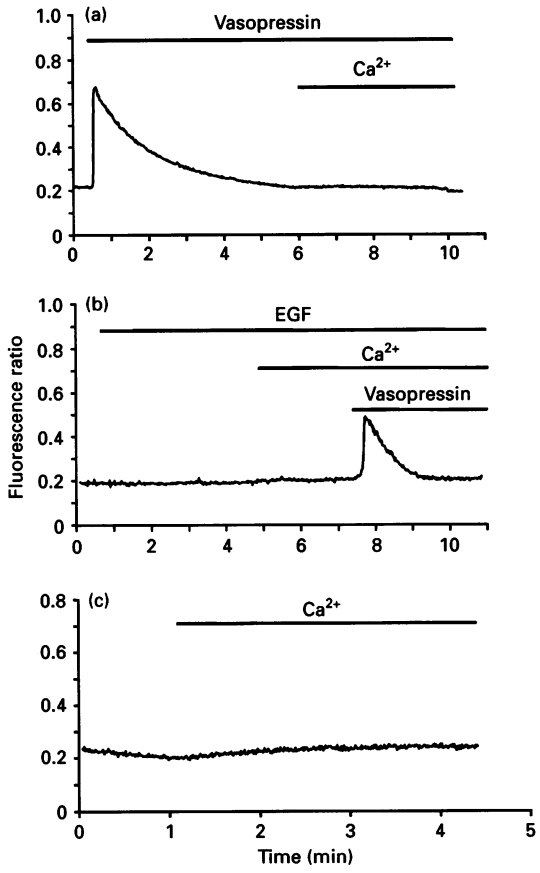

Figure 5 Effect of 24 h treatment with pertussis toxin in *vivo* on vasopressin-induced increases in $[Ca^{2+}]_i$ (a), EGF- and vasopressin-induced increases in $[Ca^{2+}]_i$ (b), and GTP[S]-induced increases in $[Ca^{2+}]_i$ (c)

The administration of pertussis toxin to rats, loading of cells with fura2–dextran and GTP[S], incubation of the cells in the absence of added Ca^{2+}_o and measurement of fluorescence was conducted as described in the Experimental section. The results were obtained from two separate cell preparations. At the beginning of the horizontal bars, EGF (400 nM), vasopressin (40 nM) or Ca^{2+} (1.3 mM $CaCl_2$) was added to the incubation dish as indicated. The results shown in (**a**) represent those obtained with one of 12 cells tested. Of these, all 12 showed a vasopressin-stimulated increase in $[Ca^{2+}]_i$ similar to that shown. After addition of Ca^{2+}_o, 8/12 cells gave traces similar to the one shown and 4/12 showed a vasopressin-stimulated Ca^{2+} inflow similar to that observed for control cells. The results shown in (**b**) represent those obtained with 8 cells in which EGF was tested. In 4 of these, vasopressin was also added. Of the 8 cells, 7 showed no response to EGF (as shown) and one showed EGF- and Ca^{2+}-stimulated increases in $[Ca^{2+}]_i$ (reflecting both release of Ca^{2+} from intracellular stores and enhanced Ca^{2+} inflow). In the 4 cells where vasopressin was also added, all 4 gave a trace similar to that shown. The results shown in (**c**) represent those obtained with 10 cells in which GTP[S] (16 mM in the micropipette tip) was co-injected with fura2–dextran. Of these cells, 7/10 showed no response to the addition of Ca^{2+}_o (as shown), 2/10 cells exhibited a delayed increase in $[Ca^{2+}]_i$ and one cell showed a response similar to that observed in control cells.

brane Ca^{2+} inflow. (*b*) Gd^{3+}, which has been shown to inhibit some RACIS (Popp and Gogelein, 1992; Bear and Li, 1991), inhibited both vasopressin- and GTP[S]-stimulated Ca^{2+} inflow. (*c*) GDP[S], an inhibitor of G-protein action (Eckstein et al., 1979), inhibited vasopressin-stimulated Ca^{2+} inflow, but did not readily affect the vasopressin-stimulated release of Ca^{2+} from intracellular stores. (*d*) Treatment of hepatocytes with pertussis toxin *in vivo* for 24 h, but not *in vitro* for 30–60 min, inhibited vasopressin- and GTP[S]-stimulated Ca^{2+} inflow, but not vasopressin-stimulated release of Ca^{2+} from intracellular stores. (*e*) Heparin and ATP[S], which each inhibit the binding of $InsP_3$ to its receptor, inhibited both vasopressin-induced Ca^{2+} inflow and the release of Ca^{2+} from intracellular stores.

From these results the following conclusions can be drawn. (i) An increase in the concentration of $InsP_3$ is required for the activation by vasopressin of plasma-membrane Ca^{2+} inflow. (ii) Two different G-proteins are necessary for the activation by vasopressin of plasma-membrane Ca^{2+} inflow. These are a pertussis-toxin-insensitive G-protein (G_{INS}) which couples vasopressin receptors to phospholipase $C_{\beta 1}$ (Taylor et al., 1991) and a slowly ADP-ribosylated G-protein (G_{PTS}). (iii) G_{PTS} is required in the process by which $InsP_3$ activates Ca^{2+} inflow (i.e. its acts 'downstream' from $InsP_3$). G_{INS} is most likely G_q or G_{11}, which are not ADP-ribosylated by pertussis toxin (Lynch et al., 1986; Taylor et al., 1991; reviewed by Rhee and Choi, 1992).

The conclusion that an increase in $InsP_3$ is a necessary step in the process by which vasopressin stimulates Ca^{2+} inflow is based on the observation that the inhibition or delay in onset of vasopressin-induced plasma-membrane Ca^{2+} inflow caused by heparin or ATP[S] was always associated with an inhibition or delay in onset in vasopressin-induced release of Ca^{2+} from intracellular stores. A similar conclusion that $InsP_3$ is required for agonist-stimulated Ca^{2+} inflow in hepatocytes has been reached by Hansen et al. (1991), who used a different experimental approach. Evidence for this requirement for $InsP_3$ in agonist-stimulated Ca^{2+} inflow has also been obtained for some other cell types (Davis, 1992; McDonald et al., 1993). $InsP_3$ may stimulate Ca^{2+} inflow by acting directly on a plasma-membrane Ca^{2+} channel (Khan et al., 1992a,b) or via the endoplasmic reticulum, as proposed in the capacitative model of Putney (1990). Another possibility, which cannot at present be excluded, is that the requirement for $InsP_3$ reflects a requirement for inositol 1,3,4,5-trisphosphate, which interacts with the plasma-membrane Ca^{2+} channel (Irvine, 1990). The failure of cyclic GMP, introduced into hepatocytes by microinjection, to stimulate Ca^{2+} inflow indicates that cyclic GMP is not involved in activation of the hepatocyte RACIS [cf. the role of the cyclic nucleotide in retinal cells (reviewed by Yau and Baylor, 1989) and its proposed role in pancreatic acinar cells (Pandol and Schoeffield-Payne, 1990)].

The requirement for two different G-proteins in the pathway for the stimulation of Ca^{2+} inflow by vasopressin in hepatocytes is based on the observations that (*a*) $InsP_3$, and hence G_{INS} (most likely G_q or G_{11}, as discussed above), is required for vasopressin-stimulated Ca^{2+} inflow, (*b*) pertussis toxin, which does not inhibit the transient increase in $[Ca^{2+}]_i$ induced by vasopressin, blocks vasopressin-stimulated Ca^{2+} inflow, and (*c*) GDP[S] blocks vasopressin-stimulated Ca^{2+} inflow, but does not readily inhibit vasopressin-induced release of Ca^{2+} from intracellular stores. This last result indicates not only that a second G-protein acts 'downstream' from G_{INS}, but also that the properties of this second G-protein differ from those of G_{INS}. The physiological function for a slowly ADP-ribosylated G-protein, G_{PTS}, proposed on the basis of the present studies, is consistent with previous observations of the rates at which hepatic G-proteins are ADP-ribosylated by pertussis toxin (Lynch et al., 1986). Moreover, Yang et al. (1993) have recently shown that in cultured hepatocytes treated with pertussis toxin a substantial proportion of the pertussis-toxin-sensitive G-protein is not ADP-ribosylated within 6 h. A period of at least 10 h exposure to the toxin was required in order to achieve complete ribosylation.

The conclusion that G_{PTS} is required in the process by which $InsP_3$ activates Ca^{2+} inflow is based on two observations. Firstly, treatment with pertussis toxin for 24 h did not substantially inhibit vasopressin-stimulated $InsP_3$ production (as assessed by the lack of an effect of the toxin on the ability of vasopressin to release Ca^{2+} from intracellular stores), but did inhibit vasopressin-

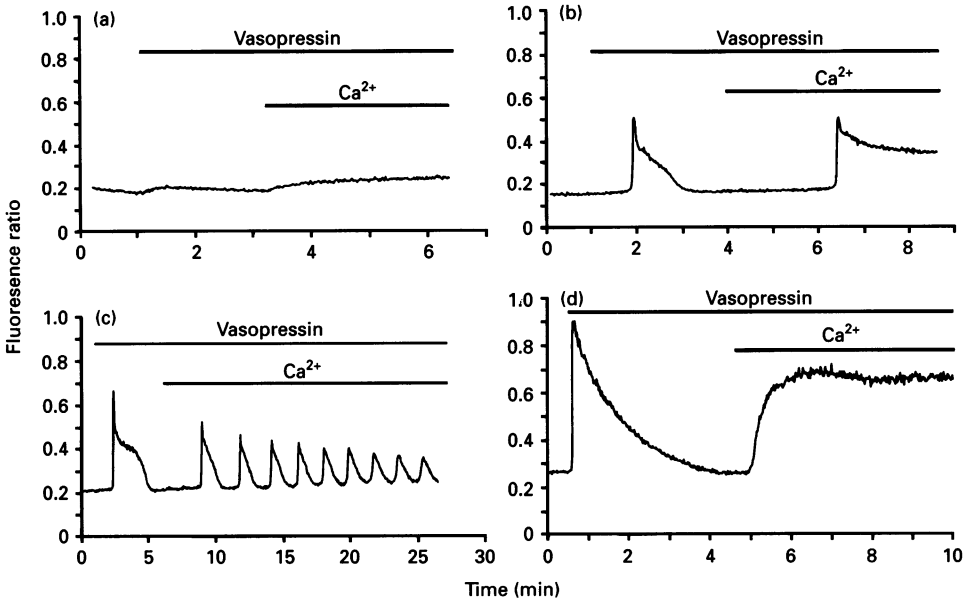

Figure 6 Effect of ATP[S] (a) and heparin (b, c) on vasopressin- and Ca^{2+}_o-induced increases in $[Ca^{2+}]_i$

The microinjection of ATP[S] and fura2–dextran (**a**) or heparin and fura2–dextran (**b, c**) or fura2–dextran alone (**d**), incubation of cells and measurement of fluorescence were performed as described in the Experimental section. ATP[S] (90 mM in the pipette tip) or heparin was co-injected with fura2–dextran. Vasopressin (40 nM) or Ca^{2+} (1.3 mM $CaCl_2$) was added to the incubation dish at the beginning of the periods indicated by the bars. The results shown in (**a**) represent those obtained with one of a total of 6 cells tested. In 5/6 of these cells ATP[S] inhibited vasopressin-stimulated Ca^{2+} inflow. In 3/6 cells ATP[S] completely inhibited vasopressin-stimulated release of Ca^{2+} from intracellular stores (as shown in trace **a**) whereas in the other 3/6 cells ATP[S] caused a delay in onset of the vasopressin-induced release of Ca^{2+} from intracellular stores (results not shown). The results shown in (**b**) represent those obtained with 8 out of a total of 20 cells treated with heparin (4–20 mM in the pipette tip). In response to vasopressin, 6 of the 20 cells tested responded normally (i.e. the same as control cells), 8/20 cells exhibited a lag (1–2 min) in response to vasopressin (as shown in trace **b**) and 6/20 cells showed no stimulation of Ca^{2+} release from intracellular stores. The subsequent addition of Ca^{2+}_o (1.3 mM) to cells previously injected with heparin and exposed to vasopressin (but not Ca^{2+}_o) showed the following responses: 3 of the 20 cells tested showed no change in vasopressin-stimulated inflow compared with control cells, 11/20 cells showed a delay (1–2 min) in the increase in $[Ca^{2+}]_i$ (shown in trace **b**), and 6/20 cells showed no vasopressin-stimulated Ca^{2+} inflow. Of the 8 cells showing a delay in onset of vasopressin- and Ca^{2+}-induced changes, 4 cells also showed oscillations in $[Ca^{2+}]_i$ (shown in trace **c**). Trace (**d**) (control) was obtained with one of 12 cells which each gave similar traces.

stimulated Ca^{2+} inflow. Secondly, GDP[S] did not substantially inhibit vasopressin-stimulated $InsP_3$ production (assessed by Ca^{2+} release), but did block vasopressin-stimulated Ca^{2+} inflow. In both these instances vasopressin-stimulated Ca^{2+} inflow was inhibited in the presence of an apparently normal increase in $InsP_3$.

The idea that G_{PTS} is required in the process by which $InsP_3$ stimulates Ca^{2+} inflow is considered to be the simplest explanation for the present data. However, it is possible that the role of G_{PTS} is to couple the vasopressin receptor to an isoenzyme of phospholipase C which differs from the $\beta 1$ form [which is activated by G_{INS} (G_q or G_{11}) (Taylor et al., 1991; Rhee and Choi, 1992)]. For some other cell types there is evidence which indicates that the $\beta\gamma$ subunits of G-proteins can activate some forms of phospholipase C, such as the $\beta 2$ isoenzyme, in a pertussis-toxin-sensitive pathway (Moriarty et al., 1989; Blank et al., 1992; Boyer et al., 1992; Camps et al., 1992; Katz et al., 1992; Park et al., 1993). This could lead to the formation of a pool of $InsP_3$ which selectively activates the RACIS. This possibility cannot at present be excluded.

G_{PTS} is most likely to be G_{i2} or G_{i3}, since these are the only known pertussis-toxin-sensitive G-proteins present in hepatocytes (Bushfield et al., 1990; Pobiner et al., 1991). The intracellular location and function of G_{PTS} are at present not known. Most G-proteins are located on the cytoplasmic face of the plasma membrane (reviewed by Gilman, 1987). However, in some cell types the majority of G_{i3} is located in the Golgi apparatus and may be involved in the formation, transport and/or fusion of membrane vesicles (Barr et al., 1991; Wilson et al., 1993). Thus it is conceivable that G_{PTS} plays a role in interactions between the endoplasmic reticulum and plasma membrane (cf. Rossier et al., 1991; Bourguignon et al., 1993). Indeed, recent results from our laboratory have provided evidence which indicates that the ability of thapsigargin to stimulate Ca^{2+} inflow in hepatocytes is blocked by pre-treatment with pertussis toxin or by GDP[S] (L. A. Berven, K. C. Fernando and G. J. Barritt, unpublished work). The results obtained with hepatocytes can be compared with studies reported in vascular smooth-muscle cells. These have revealed a slowly ADP-ribosylated pertussis-toxin-sensitive G-protein, which is proposed to be involved in the action of $InsP_3$ rather than in $InsP_3$ formation (Saida et al., 1988; Neylon et al., 1992).

In the experiments with heparin, a complete inhibition of vasopressin-stimulated release of Ca^{2+} from intracellular stores and vasopressin-stimulated Ca^{2+} inflow was only observed when very high concentrations of heparin were microinjected into hepatocytes. The need for high concentrations may be due to restraints which confine heparin to that region of the cytoplasmic space close to the site of the injection (Parker and Ivorra, 1992) or to the possibility that, in the intact hepatocyte, $InsP_3$ receptors are not readily accessible to heparin (Huang et al., 1991). An interesting feature of the action of heparin is the observation that it induces oscillations of $[Ca^{2+}]_i$ in some cells. These oscillations may be due to an increase in the apparent K_a for $InsP_3$ of the $InsP_3$-sensitive Ca^{2+} channel in the presence of heparin {cf. the observation that agonist-induced oscillations in $[Ca^{2+}]_i$ in hepatocytes are observed at limiting (non-saturating) concentrations of agonist and hence limiting, but not at high,

concentrations of InsP_3 (Woods et al., 1986; Rooney et al., 1989)}.

The observation that GDP[S] was a less effective inhibitor of vasopressin-induced release of Ca^{2+} from the endoplasmic reticulum than of vasopressin-induced Ca^{2+} inflow was unexpected. One explanation for this observation is that G_{INS} and G_{PTS}, the likely targets for inhibition by GDP[S], exhibit different susceptibilities to inhibition by GDP[S]. This would mean that, under the conditions of the present experiments, the concentration of GDP[S] at the site of G_{INS} was not high enough to inhibit this G-protein. An alternative explanation is that GDP[S] causes the same degree of inhibition of G_{INS} and G_{PTS}, but a lower concentration of InsP_3 is required to release Ca^{2+} from intracellular stores than the concentration required to activate the RACIS. Thus Charest et al. (1985) have shown that full release of Ca^{2+} from intracellular stores in hepatocytes is observed when the concentration of inositol trisphosphates is only a small proportion of the maximum achievable value. Differential effects of GDP[S] similar to those reported here have been documented by some other workers. Komori et al. (1992) found that GDP[S] inhibited agonist-stimulated Ca^{2+} inflow, but not the release of Ca^{2+} from intracellular stores in smooth-muscle cells. Moreover, Brock et al. (1988) reported that GDP[S] and GDP, introduced to endothelial cells by the scrape-loading technique, failed to inhibit ATP-stimulated increases in $[Ca^{2+}]_i$ and InsP_3. These observations suggest that the G-proteins which activate phospholipase C in these cell types are also not readily inhibited by GDP[S].

The inability of pertussis toxin microinjected into hepatocytes to inhibit vasopressin-stimulated Ca^{2+} inflow is unlikely to be due to inactivation of the toxin, since, under these conditions, the toxin completely inhibited EGF-induced Ca^{2+} fluxes. Moreover, Kass et al. (1990) have reported that exposure of hepatocytes to toxin in the extracellular medium for periods of 4–6 h also failed to inhibit vasopressin-stimulated bivalent-cation inflow. The time required for the ribosylation of G_{PTS} may indicate that G_{PTS} is not readily accessible to pertussis toxin, or that it is a poor substrate for the toxin [cf. G_0 in *Xenopus laevis* (Blitzer et al., 1993)]. The inhibition of vasopressin-stimulated Ca^{2+} inflow in single hepatocytes obtained from rats treated with pertussis toxin for 24 h is consistent with the observation reported previously that this treatment inhibits vasopressin-stimulated Ca^{2+} inflow measured in suspensions of hepatocytes (Hughes et al., 1987). Butta et al. (1993) have also provided evidence which indicates that 72 h treatment of rats with pertussis toxin inhibits vasopressin-stimulated Ca^{2+} inflow to cells in the perfused liver.

The effects of a 24 h exposure of hepatocytes to pertussis toxin are unlikely to be due to general cell damage, since vasopressin-induced release of Ca^{2+} from intracellular stores and a number of other hepatocyte functions were not affected by this treatment (Hughes et al., 1987; Butta et al., 1993; the present work). Although the possibility cannot be completely excluded, it is also considered unlikely that the target for pertussis toxin in the present studies is a protein which is not a G-protein (cf. McCarthy and Bicknell, 1992). Although the present results clearly show the presence of a rapidly (1–2 h) ADP-ribosylated protein involved in EGF-stimulated Ca^{2+} inflow and a slowly (greater than 2 h) ADP-ribosylated protein involved in vasopressin-stimulated Ca^{2+} inflow, they do not provide precise information on the times taken to give 50% ADP-ribosylation of the two proteins. This is partly because the techniques used to present the toxin to the G-protein were different: microinjection into the cell on the one hand, and administration to the animals (with a requirement for the movement of the toxin across cell membranes) on the other.

The observation that the microinjection of pertussis toxin into hepatocytes inhibits both EGF-stimulated release of Ca^{2+} from intracellular stores and EGF-stimulated Ca^{2+} inflow confirm results reported by other workers (Yang et al., 1991, 1993) and indicate that a pertussis-toxin-sensitive G-protein is also required in the mechanisms by which EGF stimulates Ca^{2+} inflow. This G-protein (G_{PTR}) is ribosylated more rapidly than G_{PTS}, and thus probably differs from G_{PTS}. It is likely that G_{PTR} is required for the activation of phospholipase Cγ by EGF, as proposed by Liang and Garrison (1991) and Yang et al. (1991). The resulting InsP_3 may then stimulate the RACIS by the same mechanism as that employed by vasopressin. Thus G_{PTS} as well as G_{PTR} may be required for the action of EGF in stimulating Ca^{2+} inflow. The present experiments would not have permitted the detection of G_{PTS} in the pathway of EGF-stimulated Ca^{2+} inflow. Another possibility for the role of G_{PTR} in EGF-stimulated Ca^{2+} inflow is that G_{PTR} is required for the activation of phospholipase A_2 (Burch et al., 1986), which in turn catalyses the formation of arachidonic acid. Metabolites of arachidonic acid, such as leukotrienes, have been proposed to activate plasma-membrane Ca^{2+} inflow in at least one cell type (Peppelenbosch et al., 1992).

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