Evidence that a pertussis-toxin-sensitive substrate is involved in the stimulation by epidermal growth factor and vasopressin of plasma-membrane Ca^{2+} inflow in hepatocytes

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1. In hepatocytes, epidermal growth factor (EGF) (a) increased the rate of $^{45}\text{Ca}^{2+}$ exchange in cells incubated at 1.3 mM extracellular Ca^{2+} , (b) increased the activity of glycogen phosphorylase *a* and the intracellular free Ca^{2+} concentration (measured with quin2) in a process dependent on the concentration of extracellular Ca^{2+} , and (c) enhanced the increase in glycogen phosphorylase activity which follows the addition of Ca^{2+} to cells previously incubated in the absence of Ca^{2+} . It is concluded that EGF stimulates plasma-membrane Ca^{2+} inflow. 2. The effects of the combination of EGF and vasopressin on the rate of $^{45}\text{Ca}^{2+}$ exchange and on the rate of increase in glycogen phosphorylase activity were the same as those of vasopressin alone. 3. The amount of $^{45}\text{Ca}^{2+}$ released by EGF from internal stores was about 30 % of that released by vasopressin. No detectable increase in [^3H]inositol mono-, bis- or tris-phosphate was observed after the addition of EGF to cells labelled with *myo*-[^3H]inositol. 4. In hepatocytes isolated from rats treated with pertussis toxin, the effects of EGF and vasopressin on phosphorylase activity (measured at 1.3 mM- Ca^{2+}) and on the rate of Ca^{2+} inflow (measured with quin2) were markedly decreased compared with those in normal cells. 5. Treatment with pertussis toxin did not impair the ability of vasopressin to release Ca^{2+} from internal stores, but decreased vasopressin-stimulated [^3H]inositol polyphosphate formation by 50 %. 6. It is concluded that the mechanism(s) by which vasopressin and EGF stimulate plasma-membrane Ca^{2+} -inflow transporters in hepatocytes involves a GTP-binding regulatory protein sensitive to pertussis toxin, and does not require an increase in the concentration of inositol trisphosphate comparable with that which induces the release of Ca^{2+} from the endoplasmic reticulum.

INTRODUCTION

Vasopressin and a number of other agonists stimulate Ca^{2+} inflow across the liver cell plasma membrane (Barritt *et al.*, 1981; Mauger *et al.*, 1984; Reinhart *et al.*, 1984) and release Ca^{2+} from intracellular stores (Blackmore *et al.*, 1979; Reinhart *et al.*, 1982; Shears & Kirk, 1984). Although some progress has been made in elucidating the mechanism of the latter process (Joseph *et al.*, 1984; Burgess *et al.*, 1984), little is known about the mechanism(s) which link receptors to the plasma-membrane Ca^{2+} -inflow transporters.

The stimulation of plasma-membrane Ca^{2+} inflow does not appear to require increases in the concentration of free cytoplasmic Ca^{2+} ($[\text{Ca}^{2+}]_i$) or activation of protein kinase C (Hughes & Barritt, 1987). It has been proposed that inositol 1,4,5-trisphosphate [$\text{Ins}(1,4,5)\text{P}_3$] stimulates plasma-membrane Ca^{2+} inflow in cells which possess receptor-activated plasma-membrane Ca^{2+} -inflow transporters (Slack *et al.*, 1986; Putney, 1986). More recently, some evidence which suggests that inositol 1,3,4,5-tetrakisphosphate (InsP_4) may stimulate Ca^{2+} inflow in hepatocytes (Hansen *et al.*, 1986) and egg cells (Irvine & Moor, 1986) has been reported. In a number of cell types, including hepatocytes, epidermal growth factor

(EGF) has been shown to increase $[\text{Ca}^{2+}]_i$ without a detectable increase in inositol polyphosphates (Moolenaar *et al.*, 1986; Johnson *et al.*, 1986; Bosch *et al.*, 1986; but see Sawyer & Cohen, 1981). These observations suggested to us that EGF could be used as a tool in further investigation of the role of inositol polyphosphates in the process by which plasma-membrane Ca^{2+} -inflow transporters are activated. Indirect evidence which indicates that a GTP-binding regulatory protein plays a role in the stimulation of plasma-membrane Ca^{2+} inflow in hepatocytes has been previously obtained by using NaF and AlCl_3 to activate GTP-binding regulatory proteins in intact cells (Hughes & Barritt, 1987). In some cell types, the stimulation by agonists of plasma-membrane Ca^{2+} inflow is inhibited by pertussis toxin (Murayama & Ui, 1985; Kojima *et al.*, 1986; Holz *et al.*, 1986; Hescheler *et al.*, 1987), which ribosylates and blocks the action of the inhibitory GTP-binding regulatory protein linked to adenylate cyclase (Bokoch & Gilman, 1984; Nakamura & Ui, 1985). In hepatocytes, the action of pertussis toxin on agonist-induced plasma-membrane Ca^{2+} inflow has not been reported, although this toxin does not inhibit agonist-stimulated phosphoinositide hydrolysis (Lynch *et al.*, 1986; Johnson *et al.*, 1986).

Abbreviations used: InsP , *myo*-inositol monophosphate; InsP_2 , *myo*-inositol bisphosphate; InsP_3 , *myo*-inositol trisphosphate; InsP_4 , *myo*-inositol 1,3,4,5-tetrakisphosphate; EGF, epidermal growth factor; quin2, 2-[[2-bis(carboxymethyl)amino-5-methylphenoxy]methyl]-6-methoxy-8-bis(carboxymethyl)aminoquinoline; $[\text{Ca}^{2+}]_o$, concentration of extracellular Ca^{2+} ; $[\text{Ca}^{2+}]_i$, concentration of free cytoplasmic Ca^{2+} .

The aims of the present experiments were to determine whether EGF stimulates plasma-membrane Ca^{2+} inflow in hepatocytes, to evaluate the role of inositol polyphosphates in this action of EGF, and to determine whether a pertussis-toxin-sensitive GTP-binding protein is required for the stimulation by agonists of Ca^{2+} inflow. The ability of an agonist to stimulate plasma-membrane Ca^{2+} inflow was assessed by (i) measurement of rates of $^{45}\text{Ca}^{2+}$ exchange (Barritt *et al.*, 1981), (ii) the ability of the agonist to activate glycogen phosphorylase *a* in the presence of extracellular Ca^{2+} (Ca^{2+}_o) after Ca^{2+} had been released from intracellular stores (DeWitt & Putney, 1983; Binet *et al.*, 1985; Charest *et al.*, 1985; Joseph *et al.*, 1985; Hughes *et al.*, 1986a), and (iii) the activation of phosphorylase *a* by Ca^{2+}_o in cells previously incubated in the presence of an agonist but in the absence of added Ca^{2+}_o (Cooper *et al.*, 1985; Hughes *et al.*, 1986a,b). The results indicate that EGF does stimulate plasma-membrane Ca^{2+} inflow in hepatocytes under conditions in which the growth factor causes a small release of Ca^{2+} from intracellular stores, but no detectable increase in inositol polyphosphates. Moreover, it is shown that the stimulation by vasopressin and EGF of plasma-membrane Ca^{2+} inflow requires a pertussis-toxin-sensitive GTP-binding regulatory protein.

EXPERIMENTAL

Isolation and incubation of hepatocytes

The isolation of hepatocytes from fed male hooded Wistar rats, incubation of cells, and measurement of $^{45}\text{Ca}^{2+}$ exchange were performed as described previously (Barritt *et al.*, 1981; Hughes *et al.*, 1986b). The incubation medium contained 117 mM-NaCl, 4.7 mM-KCl, 1.2 mM- KH_2PO_4 , 1.2 mM- MgSO_4 , 24 mM- NaHCO_3 , 20 mM-Tes/NaOH, 1.3 mM- CaCl_2 (except where indicated otherwise) and hepatocytes [2×10^6 cells (30 mg wet wt.)/ml]. Additions of vasopressin and EGF were made in 154 mM-NaCl/0.1% (w/v) bovine serum albumin.

Glycogen phosphorylase *a* activity was measured by using [^{14}C]glucose 1-phosphate (Hutson *et al.*, 1976), as described previously (Hughes *et al.*, 1986b). A unit of enzyme activity is μmol of [^{14}C]glucose 1-phosphate incorporated into glycogen/min per g wet wt. of cells. Total phosphorylase activity (the sum of activity in forms *a* and *b*) was determined by measuring enzyme activity in the presence of 3 mM-AMP and 80 mM-glucose 1-phosphate, but in the absence of caffeine (Tan & Nuttall, 1975). Measurement of the amount of cyclic AMP in HClO_4 extracts of cell incubation mixtures were conducted as described previously (Whiting & Barritt, 1982). The concentration of hEGF (recombinant human EGF) in the incubation medium was measured by homologous radioimmunoassay (fluid phase) as described previously (Read *et al.*, 1986).

The preparation of hepatocytes labelled with *myo*- ^3H inositol, and separation and measurement of InsP_1 , InsP_2 and InsP_3 labelled with ^3H inositol were performed as described previously (Hughes & Barritt, 1987).

Determination of $[\text{Ca}^{2+}]_i$ and rates of Ca^{2+} inflow in quin2-loaded cells

The loading of hepatocytes with quin2 and calculation of $[\text{Ca}^{2+}]_i$ were conducted by the methods of Tsien *et al.* (1982) as described previously (Hughes *et al.*, 1986b).

Rates of Ca^{2+} inflow across the plasma membrane were estimated by measuring the rate of increase in the intracellular concentration of Ca^{2+} bound to quin2 ($[\text{Caquin2}]$). This was obtained from the slope of the trace of fluorescence (*F*) as a function of time after addition of 1.3 mM- CaCl_2 to hepatocytes incubated for 5 min in the presence of 13 nM-vasopressin and in the absence of added Ca^{2+} . Experiments were conducted in the presence of vasopressin in order to inhibit uptake of Ca^{2+} by the endoplasmic reticulum (Hughes *et al.*, 1986b). $[\text{Caquin2}]$ was calculated by using the following equation, which is a re-arrangement of the equations described by Tsien *et al.* (1982):

$$[\text{Caquin2}] = a[\text{quin2}]/(a+1)$$

where $a = (F - F_{\min.})/(F_{\max.} - F)$ and $[\text{quin2}]$ represents the intracellular concentration of quin2. Correction was made for the fluorescence of unloaded cells, and the contribution of external quin2 was estimated by the method of Rink & Pozzan (1985). In order to measure the intracellular concentration of quin2, the cells were lysed with 0.1% Triton X-100, and the amount of quin2 released was estimated from a standard curve constructed by adding quin2 free acid to a suspension of lysed unloaded hepatocytes. The volume of the cell cytoplasm was calculated by using the relationship that 1 mg wet wt. of cells is equivalent to cytoplasmic space of $0.5 \mu\text{l}$ (Krebs *et al.*, 1974). The apparent rate of Ca^{2+} influx was expressed as nmol of Ca^{2+} /min per mg wet wt. of cells.

Treatment of rats with pertussis toxin

Non-activated pertussis toxin (50 μg) was administered to rats (body wt. 210–230 g) by intraperitoneal injection (Lynch *et al.*, 1986) 24 h before the preparation of hepatocytes. The effectiveness of the treatment was monitored by assessing the ability of angiotensin II to attenuate the stimulation by glucagon of cyclic AMP formation (Pobiner *et al.*, 1985; Lynch *et al.*, 1986). The increase in cyclic AMP concentrations produced by glucagon (10 nM) in cells from toxin-treated rats was 40% of the increase observed in control cells (results not shown). Inhibition of the glucagon response by angiotensin II (100 nM) was 46% ($n = 2$) in cells from control rats and $26 \pm 3\%$ ($n = 3$) in cells from pertussis-toxin-treated rats. These results indicate that about 90% of the form of the GTP-binding regulatory protein sensitive to inhibition by pertussis toxin was ribosylated (Pobiner *et al.*, 1985).

Materials

Pertussis toxin (islet-activating protein) was purchased from List Biological Laboratories, Campbell, CA, U.S.A. hEGF, synthesized in yeast by using recombinant techniques (Urdea *et al.*, 1983; Brake *et al.*, 1984) was generously provided by Chiron Corp., Emeryville, CA, U.S.A. [^{14}C]Glucose 1-phosphate, *myo*- ^3H inositol, the acetoxymethyl ester of quin2 and ACS II scintillant were purchased from Amersham (Australia), Surrey Hills, N.S.W., Australia. Quin2 free acid was purchased from Lancaster Synthesis, Morecambe, Lancs., U.K. Verapamil was kindly provided by Knoll A.G., Ludwigshafen, Germany. All other chemicals were of the highest grade available and were obtained from the sources described previously (Whiting & Barritt, 1982; Hughes *et al.*, 1984).

RESULTS

Effects of EGF on hepatocytes from normal rats

EGF increased the rate of $^{45}\text{Ca}^{2+}$ exchange in cells incubated at 1.3 mM-Ca^{2+}_o for 15 min before the addition of $^{45}\text{Ca}^{2+}$ (Fig. 1). The effect of EGF alone was comparable with that of vasopressin and with that caused by EGF and vasopressin together (Fig. 1).

The increase in glycogen phosphorylase activity caused by the addition of EGF to cells incubated at 1.3 mM-Ca^{2+}_o (Johnson *et al.*, 1986; Bosch *et al.*, 1986) was substantially decreased in the absence of added Ca^{2+}_o (Fig. 2). At 1.3 mM-Ca^{2+}_o , the concentration of EGF required to cause half-maximal increase in phosphorylase (measured 1 min after addition of EGF) was 2 nM, and maximum increase was observed at 5 nM-EGF (results not shown). Other experiments (results not shown) showed that the concentration of EGF in the extracellular medium did not decrease during the incubation with hepatocytes.

In the presence of EGF, the addition of Ca^{2+} to cells previously incubated in the absence of added Ca^{2+}_o caused an increase in glycogen phosphorylase activity (Fig. 3a). This was almost completely inhibited by a high concentration of verapamil (Fig. 3a, inset). The rate of increase in glycogen phosphorylase observed within the first 20 s after the addition of Ca^{2+} to cells pre-treated for 1 min with EGF (measured in the absence of verapamil) was about 30 % of that observed in cells pre-treated with

vasopressin (Fig. 3b). The rate of increase in cells pre-treated with EGF and vasopressin together was comparable with that in cells pre-treated with vasopressin alone (Fig. 3b). The rate of increase in phosphorylase observed when Ca^{2+} was added 5 min after EGF [when phosphorylase activity had returned to the basal value (cf. Fig. 2)] was the same as that observed after Ca^{2+} addition 1 min after EGF (results not shown).

The results obtained for the increase in glycogen phosphorylase activity (Fig. 2) suggest that the effect of EGF on $[\text{Ca}^{2+}]_i$ is dependent on the presence of extracellular Ca^{2+} . This proposal was tested directly in hepatocytes loaded with quin2. The increase in $[\text{Ca}^{2+}]_i$ induced by the addition of EGF to cells incubated at 1.3 mM-Ca^{2+}_o (Johnson *et al.*, 1986; Bosch *et al.*, 1986) was considerably less than that induced by vasopressin (Fig. 4a) and was substantially decreased in the absence of added Ca^{2+}_o (Fig. 4b). The addition of Ca^{2+} to cells incubated in the absence of added Ca^{2+}_o and in the presence of vasopressin increased $[\text{Ca}^{2+}]_i$ to a greater extent than did addition of Ca^{2+} in the absence of an agonist (Fig. 4b). However, the increase in $[\text{Ca}^{2+}]_i$ induced by the addition of Ca^{2+} to cells incubated in the presence of EGF was similar to that observed in cells incubated in the absence of an agonist (Fig. 4b).

EGF released $^{45}\text{Ca}^{2+}$ from hepatocytes incubated in the presence of $0.1 \text{ mM-}^{45}\text{Ca}^{2+}_o$ (Fig. 5a). However, the effect was only about 30 % of that induced by vasopressin (Fig. 5a). In cells labelled with $[^3\text{H}]$ inositol, EGF caused

Fig. 1. Effects of EGF and vasopressin on $^{45}\text{Ca}^{2+}$ exchange

Cells were incubated for 15 min in the presence of 1.3 mM-Ca^{2+} before the addition of 17 nM-EGF (■), $13 \text{ nM-vasopressin}$ (●), 17 nM-EGF plus $13 \text{ nM-vasopressin}$ (▲) or vehicle (○). After a further 2 min, a tracer amount of $^{45}\text{Ca}^{2+}$ was added and the amounts of $^{45}\text{Ca}^{2+}$ associated with the cells were measured as described in the Experimental section. The data are the means $\pm$ S.E.M. of experiments conducted with three cell preparations.

Fig. 2. Effect of EGF on glycogen phosphorylase activity

Cells were incubated either in the presence of 1.3 mM-Ca^{2+} (●) or with no added Ca^{2+} (○) for 20 min before the addition of EGF (17 nM) (indicated by the arrow). At the times indicated, samples were removed for measurement of the activity of glycogen phosphorylase α , as described in the Experimental section. The data are the means $\pm$ S.E.M. of experiments conducted with four or five cell preparations.

Fig. 3. Effects of EGF and vasopressin on the activation by Ca^{2+} of glycogen phosphorylase in hepatocytes previously incubated in the absence of Ca^{2+} .

(a) Cells were incubated in the absence of added Ca^{2+} for 20 min before the addition of saline (○) or 17 nM-EGF (●). At 21 min, 1.3 mM- CaCl_2 was added (indicated by the arrow). The results are the means of two incubations conducted with each of two cell preparations. (a) (inset): cells were incubated in the absence of added Ca^{2+} before the addition of 0.5% (v/v) dimethyl sulphoxide (○) or 200 μM-verapamil (dissolved in dimethyl sulphoxide) (●) at 15 min, 17 nM-EGF at 29 min and 1.3 mM CaCl_2 at 30 min (indicated by the arrow). The results are the means $\pm$ S.E.M. of experiments conducted with three cell preparations. (b) Cells were incubated for 15 min in the absence of added Ca^{2+} before the addition of vasopressin or vehicle. After a further 14 min, EGF or vehicle was added. Samples were then taken for the measurement of glycogen phosphorylase activity (enzyme activity at 0 s on the graph) and 1.3 mM- CaCl_2 was added (indicated by the arrow). Further samples for the determination of glycogen phosphorylase activity were removed at the times indicated. The symbols are: ■, EGF; ●, vasopressin; ▲, vasopressin + EGF. The results are the means of experiments conducted with two cell preparations.

Fig. 4. Effects of EGF and vasopressin on $[\text{Ca}^{2+}]_i$ in hepatocytes loaded with quin2

(a) Cells loaded with quin2 were incubated for approx. 4 min in the presence of 1.3 mM- Ca^{2+} before the addition of 17 nM-EGF (■) or vasopressin (●). (b) Cells loaded with quin2 were incubated for approx. 4 min in the absence of added Ca^{2+} before the addition of 13 nM-vasopressin (●), 17 nM-EGF (■) or vehicle (○) (indicated by the first arrow). After 4 min, 1.3 mM- CaCl_2 was added (second arrow). Values of $[\text{Ca}^{2+}]_i$ were calculated as described in the Experimental section. The data are the means $\pm$ S.E.M. for experiments conducted with three to six preparations of hepatocytes.

no detectable increase in [^3H]inositol polyphosphates (Table 1) (cf. Bosch *et al.*, 1986).

Effects of EGF on cells from pertussis-toxin-treated rats

In hepatocytes treated with pertussis toxin, the total activity of glycogen phosphorylase (the sum of that present in forms *a* and *b*) was 51% (mean of two determinations) of that present in untreated cells (results not shown). Moreover, in cells incubated in the absence of an agonist, the activity of glycogen phosphorylase *a* in toxin-treated cells (Fig. 6) was lower than that present in normal cells (Fig. 2).

For cells incubated at 1.3 mM-Ca^{2+}_o , the increase in glycogen phosphorylase activity induced by vasopressin

and EGF in toxin-treated cells was substantially less than the increases induced by the agonists in normal cells (Fig. 6a). When added to toxin-treated cells, glucagon and ionophore A23187 caused a prolonged and a transient increase, respectively, in glycogen phosphorylase (Fig. 6b). These changes were similar to those induced by glucagon and A23187 in untreated cells (results not shown). The transient nature of the increases in glycogen phosphorylase activity induced by the agonists in toxin-treated cells is consistent with the proposal that plasma-membrane Ca^{2+} inflow is inhibited. This proposal was further tested by determining the effect of Ca^{2+} addition to cells previously treated with the agonists in the absence of added Ca^{2+}_o . The ability of EGF and

Fig. 5. Effects of EGF and vasopressin on $^{45}\text{Ca}^{2+}$ release

Cells from either normal (a) or pertussis-toxin-treated (b) rats were incubated in the presence of $0.1 \text{ mM-}^{45}\text{Ca}^{2+}$. After 30 min (indicated by the arrow), either 17 nM-EGF (■) or $13 \text{ nM-vasopressin}$ (●) was added. The amounts of $^{45}\text{Ca}^{2+}$ associated with the cells were determined as described in the Experimental section. The data are the means $\pm$ S.E.M. for experiments conducted with either three (normal cells) or four (pertussis-toxin-treated) preparations of hepatocytes.

Fig. 6. Effects of EGF and vasopressin on glycogen phosphorylase activity in hepatocytes from pertussis-toxin-treated rats

(a) Hepatocytes isolated from either normal rats (□, ▲) or from rats treated with pertussis toxin (●, ■) were incubated in the presence of 1.3 mM-Ca^{2+} . After 20 min either 17 nM-EGF (□, ■) or $13 \text{ nM-vasopressin}$ (●, ▲) was added (indicated by the arrow). At the times indicated, samples were removed for the determination of glycogen phosphorylase activity as described in the Experimental section. The data are the means $\pm$ S.E.M. for experiments conducted with either 5–11 (normal cells) or three (pertussis-toxin-treated hepatocytes) preparations of hepatocytes. (The data for EGF treatment of cells from normal rats are the same data as shown in Fig. 2 for the 1.3 mM-Ca^{2+} condition). (b) Hepatocytes isolated from rats treated with pertussis toxin were incubated in the presence of 1.3 mM-Ca^{2+} . After 20 min, 17 nM-EGF (■), $13 \text{ nM-vasopressin}$ (●), $10 \mu\text{M-A23187}$ (▲) or 200 nM-glucagon (△) was added. The data are the means of experiments conducted with two cell preparations.

vasopressin to enhance the increase in phosphorylase caused by the subsequent addition of Ca^{2+} to cells pre-incubated in the absence of added Ca^{2+} , was almost completely abolished by pre-treatment with pertussis toxin (Fig. 7).

Treatment of hepatocytes with pertussis toxin also decreased the rate of plasma-membrane Ca^{2+} inflow, estimated by measuring the rate of increase in Ca^{2+} bound to intracellular quin2 observed after the addition of 1.3 mM-Ca^{2+} to cells incubated in the presence of vasopressin. The rates of Ca^{2+} inflow were 0.12 ± 0.02 (3) and 0.18 ± 0.01 (5) nmol/min per mg wet wt. for toxin-treated cells and untreated cells respectively ($P < 0.02$ for a comparison of unpaired samples). The value of $[\text{Ca}^{2+}]_i$ measured in cells incubated in the absence of both added Ca^{2+} and agonists, and the maximum value of $[\text{Ca}^{2+}]_i$ measured after the addition of vasopressin, were the same for toxin-treated and untreated cells (results not shown).

In toxin-treated cells incubated at $0.1 \text{ mM-}^{45}\text{Ca}^{2+}$, the amount of $^{45}\text{Ca}^{2+}$ released by vasopressin (Fig. 5b) was similar to that released by the hormone in untreated cells (Fig. 5a). EGF released little $^{45}\text{Ca}^{2+}$ in toxin-treated cells (Fig. 5b). The increases in $[\text{H}^3]\text{inositol polyphosphates}$ induced by vasopressin in toxin-treated cells labelled with $[\text{H}^3]\text{inositol}$ were about 50% of those induced by the hormone in untreated cells (Table 1).

DISCUSSION

Several observations provide evidence which indicates that EGF stimulates Ca^{2+} inflow across the liver cell plasma membrane. These are (i) the stimulation by EGF of $^{45}\text{Ca}^{2+}$ exchange (cf. Barritt *et al.*, 1981), (ii) the requirement for Ca^{2+}_o for the increases in glycogen phosphorylase *a* and $[\text{Ca}^{2+}]_i$ induced by EGF, (iii) the Ca^{2+} -induced increase in the activity of glycogen phosphorylase *a* in cells previously incubated in the absence of Ca^{2+}_o and in the presence of EGF (cf. Hughes

Fig. 7. Effect of treatment with pertussis toxin on the actions of EGF and vasopressin on Ca^{2+} -induced increases in glycogen phosphorylase in cells previously incubated in the absence of added Ca^{2+} .

Cells from either normal ($\blacktriangle$) or pertussis-toxin-treated rats ($\circ$, $\bullet$, $\blacksquare$) were incubated in the absence of added Ca^{2+} . After 25 min, $13 \text{ nM-vasopressin}$ ($\bullet$, $\blacktriangle$) or vehicle ($\circ$) was added, followed by addition of 17 nM-EGF ($\blacksquare$) after 29 min and 1.3 mM-Ca^{2+} (indicated by the arrow) after 30 min. The 1 min time of exposure of cells to EGF before Ca^{2+} addition was chosen because of the transient nature of the effects of EGF on glycogen phosphorylase *a* activity (Figs. 2 and 3a). The data are the means $\pm$ S.E.M. for experiments conducted with either ten (normal cells) or three (pertussis-toxin-treated) preparations of hepatocytes.

Table 1. Effects of EGF and vasopressin on the formation of $[\text{H}^3]\text{inositol polyphosphates}$ in cells from normal and pertussis-toxin-treated rats

Hepatocytes labelled with $[\text{H}^3]\text{inositol}$ were incubated for 5 min before the addition of EGF, vasopressin or vehicle (control). After 1 or 10 min (as indicated), trichloroacetic acid extracts were prepared and ^3H -labelled inositol polyphosphates separated as described in the Experimental section. For each inositol polyphosphate, the amount of radioactivity present in cells treated with vasopressin is expressed as a percentage of that present in the corresponding control incubation. The results are the means $\pm$ S.E.M. for the numbers of experiments shown. The data obtained for untreated cells exposed to vasopressin for 10 min are from Hughes & Barritt (1987). The degrees of significance for a comparison of the amount of radioactivity in inositol polyphosphates in cells from normal rats versus cells from pertussis-toxin-treated rats are $*P < 0.05$, $**P < 0.025$ and $***P < 0.01$ (Student's *t* test for unpaired samples). The degrees of significance for a comparison of values obtained in the presence of vasopressin with the corresponding controls are $\dagger P < 0.05$, $\ddagger P < 0.025$, $\ddagger\ddagger P < 0.005$ and $\ddagger\ddagger\ddagger P < 0.0005$ (Student's *t* test for paired samples).

Cells	Agonist	Time of exposure to agonist (min)	Radioactivity in inositol polyphosphates (% of corresponding control)		
			InsP	InsP ₂	InsP ₃
Untreated	Vasopressin (20 nM)	1	110 $\pm$ 10 (3)	200 $\pm$ 20 (3) $\dagger\dagger$	470 $\pm$ 100 (3) $\dagger\dagger$
	EGF (17 nM)	1	96 $\pm$ 4 (3)	100 $\pm$ 10 (3)	120 $\pm$ 30 (3)
	Vasopressin (20 nM)	10	150 $\pm$ 7 (6) $\dagger\dagger\dagger$	290 $\pm$ 30 (6) $\dagger\dagger\dagger$	1103 $\pm$ 200 (6) $\dagger\dagger$
Pertussis-toxin-treated	Vasopressin (20 nM)	10	120 $\pm$ 5 (3) $***\dagger$	170 $\pm$ 20 (3) $**\dagger$	500 $\pm$ 70 (3) $\dagger\dagger$

et al., 1986b), and (iv) the inhibition of this Ca^{2+} -induced increase in phosphorylase by verapamil (cf. Hughes *et al.*, 1986a). The observation that the effects of EGF plus vasopressin on (a) $^{45}\text{Ca}^{2+}$ exchange and (b) the initial rate of increase in Ca^{2+} -induced phosphorylase activity were no greater than those of vasopressin alone on these parameters suggests that vasopressin and EGF activate the same Ca^{2+} -inflow transporter. However, other explanations for these results, including inactivation of the EGF receptor by protein kinase C (Johnson *et al.*, 1986), cannot be eliminated.

The effects of EGF on $[\text{Ca}^{2+}]_i$, monitored by the fluorescence of quin2, were less clearly defined and not the same as those monitored by measurement of the activity of glycogen phosphorylase *a*. These differences may be due to the small magnitude of the changes in $[\text{Ca}^{2+}]_i$ induced by EGF, the greater sensitivity of phosphorylase *a* to small changes in $[\text{Ca}^{2+}]_i$, and to the buffering by quin2 of changes in $[\text{Ca}^{2+}]_i$ (Kojima & Ogata, 1986; Woods *et al.*, 1987). It is considered unlikely that the transient nature of the response of phosphorylase *a* to EGF is caused by degradation or internalization of the growth factor. No decrease in the concentration of extracellular EGF was observed during cell incubations. Moreover, it has been shown that longer incubation times are required in order to decrease the number of EGF receptors (Ballard & Gunn, 1984).

The effect of EGF on the rate of $^{45}\text{Ca}^{2+}$ exchange, measured at 1.3 mM Ca^{2+}_o , is similar in magnitude to that induced by vasopressin. However, the increase in $[\text{Ca}^{2+}]_i$ induced by EGF is much smaller than that caused by vasopressin. One explanation for these results may be the much smaller or negligible increase in InsP_3 caused by EGF. It has previously been proposed that one of the actions of InsP_3 is to inhibit uptake of Ca^{2+} by the endoplasmic reticulum when plasma-membrane Ca^{2+} inflow is increased, and hence to magnify the resulting increase in $[\text{Ca}^{2+}]_i$ (Hughes *et al.*, 1986b).

Three observations indicate that pertussis toxin blocks the ability of EGF and vasopressin to stimulate plasma-membrane Ca^{2+} inflow in hepatocytes. These are (i) almost complete inhibition by the toxin of the increase in glycogen phosphorylase *a* activity induced by the addition of Ca^{2+} to cells previously incubated in the presence of EGF or vasopressin but in the absence of added Ca^{2+}_o , (ii) inhibition by the toxin of the increase in phosphorylase *a* activity observed at long, as well as short, times after the addition of EGF or vasopressin to cells incubated in the presence of Ca^{2+}_o , and (iii) inhibition by the toxin of the initial rate of increase in $[\text{Ca}^{2+}]_i$ measured in quin2-loaded cells in the presence of vasopressin. Treatment of hepatocytes with pertussis toxin decreased the total amount of glycogen phosphorylase activity (cf. Lynch *et al.*, 1986). However, the observation that the enzyme in these cells responded to glucagon and ionophore A23187 in a manner similar to that in untreated cells indicates that the small transient increases in phosphorylase observed in toxin-treated cells are not due to an impairment in the ability of Ca^{2+} to activate phosphorylase or to activation by the toxin of phosphoprotein phosphatase in toxin-treated cells.

The present results obtained with pertussis toxin, and the observations that this toxin ribosylates and inhibits the action of GTP-binding regulatory proteins (Bokoch & Gilman, 1984; Nakamura & Ui, 1985; Lynch *et al.*, 1986), indicate that a GTP-binding regulatory protein is

involved in the processes by which vasopressin and EGF stimulate receptor-activated Ca^{2+} -inflow transporters. This conclusion is further supported by the results of previous studies with NaF and AlCl_3 (Hughes & Barritt, 1987). The observation that the vasopressin-increased formation of InsP_3 is significantly decreased in toxin-treated cells appears to be inconsistent with the conclusions reached by others that a pertussis-toxin-sensitive GTP-binding regulatory protein is not involved in the link between vasopressin and phosphoinositide-specific phospholipase C in hepatocytes (Lynch *et al.*, 1986; Johnson *et al.*, 1986). The reasons for this are not immediately apparent, but may be due to non-specific effects associated with the administration of the toxin *in vivo* (Lynch *et al.*, 1986). Even though the vasopressin-induced stimulation of InsP_3 production was decreased in toxin-treated cells, there was little effect on the release of $^{45}\text{Ca}^{2+}$ from the cells. These data are consistent with the conclusion that the increase in InsP_3 produced after vasopressin challenge of hepatocytes is in excess of that required for the release of Ca^{2+} from internal stores (Charest *et al.*, 1985; Lynch *et al.*, 1985).

The present results for the effects of EGF on normal hepatocytes indicate that a concentration of this agonist which causes maximum stimulation of plasma-membrane Ca^{2+} inflow causes no detectable increase in $[\text{H}^3]\text{-InsP}_1$, -InsP_2 or -InsP_3 and little release of Ca^{2+} from intracellular stores. As EGF is able to produce some release of Ca^{2+} from intracellular stores, it is likely that a small increase in InsP_3 does occur, but is not detected in the present studies (cf. Bosch *et al.*, 1986). The alternative explanation, that an agent other than InsP_3 is responsible for the EGF-induced release of Ca^{2+} from internal stores, although considered unlikely, cannot be excluded. Although the amounts of InsP_4 were not measured in the present study, results obtained by others indicate that InsP_4 only increases when InsP_3 is increased (Batty *et al.*, 1985; Hansen *et al.*, 1986). These observations indicate that inositol polyphosphates are unlikely to be involved in the mechanism by which EGF stimulates Ca^{2+} inflow in hepatocytes. Alternatively, it can be concluded that an inositol polyphosphate is involved, but that the plasma-membrane Ca^{2+} -inflow transporters are considerably more sensitive to the inositol polyphosphate than is the endoplasmic reticulum to InsP_3 .

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