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Evidence from studies with hepatocyte suspensions that store-operated Ca^{2+} inflow requires a pertussis toxin-sensitive trimeric G-protein

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The role of heterotrimeric GTP-binding proteins in the process of store-operated Ca^{2+} inflow in hepatocytes was investigated by testing the ability of pertussis toxin to inhibit thapsigargin- and 2,5-di-tert-butylhydroquinone (DBHQ)-induced bivalent cation inflow. Hepatocytes isolated from rats treated with pertussis toxin for 24 h exhibited markedly inhibited rates of both Ca^{2+} and Mn^{2+} inflow when these were stimulated by vasopressin, angiotension II, epidermal growth factor, thapsigargin and DBHQ. Pertussis toxin had little effect on the basal intracellular free Ca^{2+} concentration ($[Ca^{2+}]_i$), basal rates of Ca^{2+} and Mn^{2+} inflow, the abilities of vasopressin, angiotensin II, thapsigargin and DBHQ to induce the release of Ca^{2+} from intracellular stores, and the maximum value of $[Ca^{2+}]_i$ reached following agonist-induced release of Ca^{2+} from intracellular stores. It is concluded that store-operated Ca^{2+} inflow in hepatocytes employs a slowly ADP-ribosylated trimeric GTP-binding protein and is the physiological mechanism, or one of the physiological mechanisms, by which vasopressin and angiotensin stimulate plasma membrane Ca^{2+} inflow in this cell type.

INTRODUCTION

In common with most other non-excitable cells hepatocytes possess receptor-operated Ca^{2+} channels in the plasma membrane. These channels are activated by a variety of agonists including vasopressin, angiotensin II, adrenalin and epidermal growth factor (EGF) (reviewed in Barritt and Hughes, 1991). The putative Ca^{2+} channels appear to be non-selective for bivalent cations since there is evidence that they admit Mn^{2+}, some other bivalent cations (reviewed in Barritt and Hughes, 1991) and Na^+ (Lidofsky et al., 1993). Agonist-stimulated bivalent cation inflow is mimicked by the incubation of hepatocytes with thapsigargin or 2,5-di-tert-butylhydroquinone (DBHQ) (Kass et al., 1989, 1990; Hansen et al., 1991; Llopis et al., 1992; Glennon et al., 1992; Strazzari and Hughes, 1993). These agents induce the release of Ca^{2+} from intracellular Ca^{2+} stores without increasing the concentration of inositol 1,4,5-trisphosphate ($InsP_3$) (Moore et al., 1987; Takemura et al., 1989). Agonist-stimulated bivalent cation inflow in hepatocytes also exhibits requirements for a prior increase in $InsP_3$ (Hansen et al., 1991; Berven et al., 1994) and a slowly ADP-ribosylated pertussis toxin-sensitive trimeric GTP-binding protein (trimeric G-protein) (Hughes et al., 1987; Butta et al., 1993; Berven et al., 1994). The 'capacitative' or 'store-operated' Ca^{2+} inflow hypothesis (Putney, 1986) is currently considered to be the best hypothesis for the mechanism of activation of the hepatocyte receptor-operated Ca^{2+} channels as it is consistent with all the available data (Glennon et al., 1992). This hypothesis states that the emptying of $InsP_3$-sensitive Ca^{2+} stores (induced by the agonist) activates the putative Ca^{2+} channels. However, little is known about the mechanism of this activation.

In previous experiments in which the role of a pertussis toxin-sensitive trimeric G-protein in agonist-stimulated bivalent cation inflow was investigated, the cation monitored was Ca^{2+} (Hughes et al., 1987; Butta et al., 1993; Berven et al., 1994). So far, no evidence for the requirement for a pertussis toxin-sensitive trimeric G-protein in agonist-stimulated Mn^{2+} inflow has been reported. Moreover, Kass et al. (1990) have reported that the treatment of hepatocytes with pertussis toxin for up to 4.5 h did not inhibit vasopressin-stimulated Mn^{2+} inflow.

The aims of the present experiments were, first, to determine whether agonist-stimulated Mn^{2+} inflow is blocked by pertussis toxin and, secondly, to determine whether bivalent cation inflow stimulated by thapsigargin and DBHQ requires a pertussis toxin-sensitive trimeric G-protein. The results indicate that a pertussis toxin-sensitive trimeric G-protein is required for store-operated Ca^{2+} inflow initiated by these agents. Moreover, the results provide further evidence that store-operated Ca^{2+} inflow is part of the physiological mechanism of action of vasopressin, angiotensin II and some other agonists in increasing the intracellular free Ca^{2+} concentration ($[Ca^{2+}]_i$) in hepatocytes.

EXPERIMENTAL

Materials

Thapsigargin, DBHQ and pertussis toxin were obtained from Sigma–Aldrich (Castle Hill, N.S.W., Australia). All other chemicals were of the highest grade commerically available and were obtained from the sources described previously (Fernando and Barritt, 1994; Berven et al., 1994). Stock solutions of thapsigargin and DBHQ were prepared in dimethyl sulphoxide. The final concentration of this solvent in the cell incubation medium was less than 0.5% (v/v).

Measurement of Ca^{2+} inflow and release of Ca^{2+} from intracellular stores

The isolation and incubation of hepatocytes, loading of hepatocytes with Quin 2 and the measurement of agonist-stimulated Ca^{2+} inflow in hepatocytes loaded with Quin 2 were performed as described previously (Crofts and Barritt, 1989; Fernando and Barritt, 1994). The calibration of plots of fluorescence as a function of time and conversion of units of fluorescence into values of $[Ca^{2+}]_i$ were performed by the method of Tsien et al.

Abbreviations used: $[Ca^{2+}]_i$, intracellular free Ca^{2+} concentration; DBHQ, 2,5-di-tert-butylhydroquinone; $InsP_3$, inositol 1,4,5-trisphosphate; G-protein, GTP-binding regulatory protein; $Ca^{2+}{}_o$, extracellular Ca^{2+}; EGF, epidermal growth factor.

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Table 1 Effects of pertussis toxin treatment on agonist-stimulated Mn^{2+} and Ca^{2+} inflow and agonist-induced release of Ca^{2+} from intracellular stores

Rates of Mn^{2+} and Ca^{2+} inflow and amounts of Ca^{2+} released from intracellular stores were measured as described in the Experimental section. The values are the means $\pm$ S.E.M. for the number of experiments indicated in parentheses, conducted with three different cell preparations. For untreated cells, the degrees of significance for a comparison of rates of Mn^{2+} or Ca^{2+} inflow in the presence of agonist with the corresponding rate in the absence of agonist are $^{*}P \leqslant 0.01$; and $^{**}P \leqslant 0.001$. The degrees of significance for a comparison of rates of bivalent cation inflow for untreated cells with the corresponding rate for pertussis toxin-treated cells incubated in the presence of vasopressin, angiotensin II and EGF are $P \leqslant 0.001$, $P \leqslant 0.01$ and $P \leqslant 0.00001$ respectively, for Mn^{2+} inflow, and $P \leqslant 0.05$, $P \leqslant 0.001$ and $P \leqslant 0.005$ respectively for Ca^{2+} inflow. The degree of significance for a comparison of the amount of Ca^{2+} released by EGF from untreated cells with pertussis toxin-treated cells was $P \leqslant 0.001$.

	Rate of Mn^{2+} inflow (decrease in fluorescense units/s)		Rates of Ca^{2+} inflow (increase in $[Ca^{2+}]_i$, mM/s)		Amount of Ca^{2+} released from intracellular stores (increase in $[Ca^{2+}]_i$, nM)	
Agonist	Untreated cells	Pertussis toxin-treated cells	Untreated cells	Pertussis toxin-treated cells	Untreated cells	Pertussis toxin-treated cells
None	0.08 ± 0.01 (4)	0.07 ± 0.004 (4)	0.13 ± 0.01 (7)	0.15 ± 0.02 (7)	–	–
Vasopressin (50 nM)	0.15 ± 0.01 (5)*	0.08 ± 0.01 (5)	0.91 ± 0.05 (5)**	0.11 ± 0.01 (5)	41 ± 2 (5)	38 ± 2 (6)
Angiotensin II (5 nM)	0.17 ± 0.01 (5)**	0.11 ± 0.01 (5)	0.93 ± 0.11 (5)**	0.19 ± 0.01 (5)	48 ± 6 (6)	38 ± 6 (5)
EGF (100 nM)	0.12 ± 0.003 (4)**	0.08 ± 0.001 (5)	0.51 ± 0.11 (4)**	0.10 ± 0.03 (5)	38 ± 6 (5)	8 ± 2 (6)

(1982) as described previously (Crofts and Barritt, 1989; Fernando and Barritt, 1994). Rates of Ca^{2+} inflow are expressed as the rate of increase in $[Ca^{2+}]_i$ (nM/s) observed following the addition of extracellular Ca^{2+} ($Ca^{2+}{}_o$) to cells incubated in the absence of added $Ca^{2+}{}_o$. The amounts of Ca^{2+} released from intracellular stores in response to an agonist were estimated from the maximal increase in $[Ca^{2+}]_i$ (nM) observed after the addition of an agonist to cells incubated in the absence of added $Ca^{2+}{}_o$. Rates of agonist-induced Ca^{2+} release from intracellular stores were determined using the same procedure as that employed for estimation of rates of Ca^{2+} inflow.

Measurement of Mn^{2+} inflow

Rates of Mn^{2+} inflow were estimated from the rate of quenching of the fluorescence of intracellular Quin 2 by Mn^{2+} (Hallam and Rink, 1985) as described previously (Crofts and Barritt, 1990; Fernando and Barritt, 1994). Plots of fluorescence as a function of time were linear for the first 2 min after the addition of Mn^{2+} and were used to estimate rates of Mn^{2+} inflow. These are expressed as decreases in fluorescence units/s. For experiments in which Gd^{3+} was present, Mn^{2+} was added to hepatocytes loaded with Quin 2 and the rate of quenching by Mn^{2+} of Quin 2 fluorescence measured for 2 min (0–2 min after the addition of Mn^{2+}) then Gd^{3+} added and the rate of quenching measured for a further period of 2 min (2–4 min after Mn^{2+} addition). The slope of the plot of fluorescence as a function of time for the 2 min following the addition of Gd^{3+} was taken as the rate of quenching by Mn^{2+} of Quin 2 fluorescence in the presence of Gd^{3+}. The rate of fluorescence quenching by Mn^{2+} in the absence of Gd^{3+} (i.e. the control rate) was obtained from the same trace by measuring the slope of the plot of fluorescence as a function of time for the 0–2 min period following the addition of Mn^{2+}. [Since, in the absence of Gd^{3+}, plots of fluorescence as a function of time exhibited a decrease (11 ± 1 %; mean $\pm$ S.E.M., $n = 5$) in slope in the period 2–4 min after Mn^{2+} addition compared with the slope in the 0–2 min period after Mn^{2+} addition, a correction for this decrease was made when estimating control rates of Mn^{2+} inflow].

Treatment of rats with pertussis toxin

Pertussis toxin (25 μg/100 g of body weight) was administered to rats by intraperitoneal injection (Lynch et al., 1986) 24 h before the isolation of hepatocytes, as described previously (Hughes et al., 1987; Berven et al., 1994).

Presentation of results

All results are expressed as the mean $\pm$ S.E.M. of the number of experiments indicated in parentheses. Degrees of significance were determined using Student's *t*-test for unpaired samples. Values of $P \leqslant 0.05$ were considered to be significant. The traces shown in the Figures were obtained as follows. The data files for each individual plot [values of fluorescence (units) at 1 s intervals] obtained under a given set of experimental conditions were transferred from the TD file of the Perkin–Elmer LS 50 spectrofluorimeter FLDM programme to a WK 1 file in Lotus 1-2-3 format, then imported to Microsoft Excel. For the measurement of $[Ca^{2+}]_i$ (see Figure 3), fluorescence units were first converted into values of $[Ca^{2+}]_i$. After alignment of the times of reagent addition (thapsigargin, Mn^{2+}, Gd^{3+} or Ca^{2+}), the means and S.E.M.s of the fluorescence or $[Ca^{2+}]_i$ values were determined for each time point (i.e. every second). The traces shown in the Figures are the mean values with error bars ($\pm$S.E.M.) at 20–50 s intervals.

RESULTS

In hepatocytes isolated from rats treated with pertussis toxin for 24 h, the ability of vasopressin, angiotensin II and EGF to stimulate Mn^{2+} inflow was completely inhibited when compared with the effects of these agonists on untreated hepatocytes (Table 1). Pertussis toxin had no effect on the rate of Mn^{2+} inflow in the absence of agonist (basal Mn^{2+} inflow) (Table 1). The toxin also blocked the stimulation of Ca^{2+} inflow by each agonist without altering the basal rate of Ca^{2+} inflow (Table 1). Treatment with pertussis toxin did not substantially affect the ability of vasopressin and angiotensin II to induce the release of Ca^{2+} from intracellular stores but, as shown by others (Johnson et al., 1986; Yang et al., 1991; Liang and Garrison, 1991, 1992; Berven et al., 1994), did inhibit the release of Ca^{2+} induced by EGF (Table 1).

In untreated cells, Mn^{2+} inflow was stimulated by thapsigargin, as previously shown by Glennon et al. (1992) (Figures 1a, cf. 1b, and Table 2). In cells from pertussis toxin-treated rats the ability of thapsigargin to stimulate Mn^{2+} inflow was completely inhibited (see Figure 1c, cf. Figures 1a and 1d, and Table 2). Likewise,

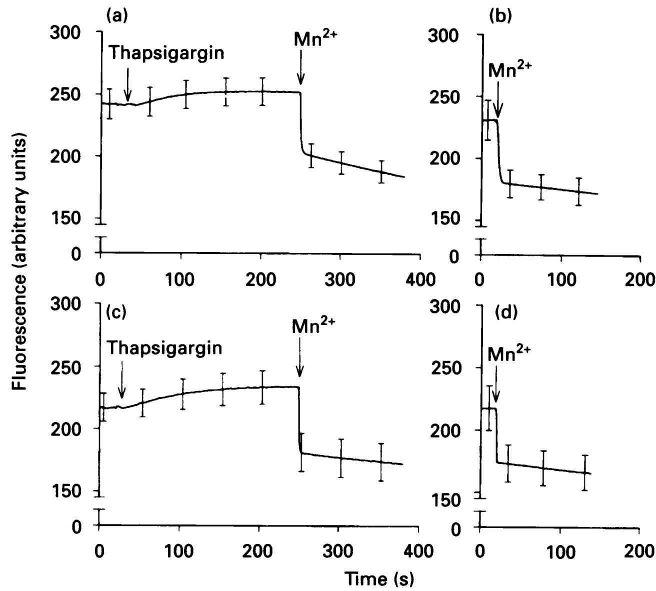

Figure 1 Inhibition by pertussis toxin of thapsigargin-stimulated Mn^{2+} inflow

Plots of fluorescence as a function of time for Quin 2-loaded hepatocytes from untreated (normal) (**a, b**) and pertussis toxin-treated (**c, d**) rats. The hepatocytes were incubated in the presence (**a, c**) and absence (basal Mn^{2+} inflow) (**b, d**) of thapsigargin. Additions of thapsigargin (100 nM) and Mn^{2+} (100 μM) were made at the times indicated by the arrows. The treatment of rats with pertussis toxin, the loading of hepatocytes with Quin 2, and the measurement of fluorescence were conducted as described in the Experimental section. The very rapid decrease in fluorescence observed immediately after the addition of Mn^{2+} is due to the quenching by Mn^{2+} of extracellular Quin 2 (Crofts and Barritt, 1990). Each plot is composed of the mean values obtained after combination of the results (combined as described in the Experimental section) obtained from 20 (seven cell preparations) or 10 (three cell preparations) experiments conducted with untreated and toxin-treated hepatocytes respectively, in the presence of thapsigargin (**a** and **c**), and 15 (five cell preparations) and 11 (five cell preparations) experiments conducted with untreated and toxin-treated hepatocytes respectively, in the absence of thapsigargin (**b** and **d**). The error bars represent the S.E.M. Note that below 150 fluorescence units the ordinate scale is condensed.

DBHQ-stimulated Mn^{2+} inflow was substantially inhibited in pertussis toxin-treated hepatocytes (Table 2). Pertussis toxin did not inhibit the basal rate of Mn^{2+} inflow (Figures 1d, cf. 1b, and Table 2). The degree of inhibition by pertussis toxin of thapsigargin- and DBHQ-stimulated Mn^{2+} inflow was similar to

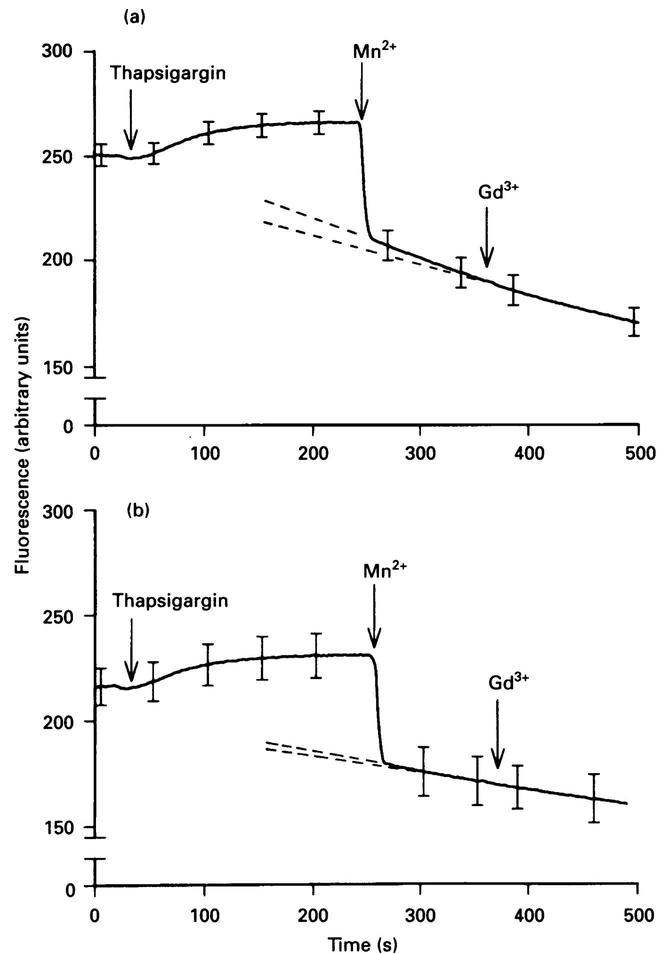

Figure 2 Inhibition of thapsigargin-stimulated Mn^{2+} inflow in untreated (a) and in pertussis toxin-treated (b) hepatocytes by Gd^{3+}

The treatment of rats with pertussis toxin, the loading of hepatocytes with Quin 2, and the measurement of fluorescence were conducted as described in the Experimental section. Addition of thapsigargin (100 nm), Mn^{2+} (100 μM) and Gd^{3+} (20 μM) were made at the times indicated by the arrows. Each plot is composed of the mean values obtained after combination of the results (combined as described in the Experimental section) obtained from 13 experiments (three to four cell preparations). The error bars represent the S.E.M. The broken lines are extrapolations of the slopes of the lines obtained before and after Gd^{3+} addition. Note that below 150 fluorescence units the ordinate scale is condensed.

Table 2 Effects of pertussis toxin treatment on Mn^{2+} and Ca^{2+} inflow induced by thapsigargin and DBHQ

Rates of Mn^{2+} and Ca^{2+} inflow, and amounts of Ca^{2+} released from intracellular stores were estimated as described in the Experimental section. The values are the means ± S.E.M. for the number of experiments shown in parentheses conducted using between two and seven different cell preparations. The degrees of significance for a comparison of rates of Mn^{2+} or Ca^{2+} inflow obtained in the presence of an agonist with the rate observed in the absence of agonist are $^*P \leqslant 0.05$; $^{**}P \leqslant 0.01$; and $^{***}P \leqslant 0.00001$. The degrees of significance for a comparison of untreated with pertussis toxin-treated cells incubated in the presence of vasopressin, 20 nM thapsigargin, 100 nM thapsigargin or 25 μM DBHQ are $P \leqslant 0.001$, $P \leqslant 0.00001$, $P \leqslant 0.000001$ and $P \leqslant 0.01$ respectively, for Mn^{2+} inflow and $P \leqslant 0.00001$ and $P \leqslant 0.0001$ for Ca^{2+} inflow in the presence of vasopressin and 100 nM thapsigargin respectively.

	Rate of Mn^{2+} inflow (decrease in fluorescence units/s)		Rate of Ca^{2+} inflow (increase in $[Ca^{2+}]_i$, nM/s)	
Agonist	Untreated cells	Pertussis toxin-treated cells	Untreated cells	Pertussis toxin-treated cells
None	0.062 ± 0.005 (15)	0.066 ± 0.01 (11)	0.16 ± 0.02 (6)	0.15 ± 0.01 (6)
Vasopressin (50 nM)	0.14 ± 0.009 (13)***	0.082 ± 0.006 (15)	0.80 ± 0.04 (11)***	0.26 ± 0.04 (6)*
Thapsigargin (20 nM)	0.12 ± 0.012 (7)***	0.062 ± 0.005 (7)	0.82 ± 0.21 (6)**	0.24 ± 0.09 (4)
Thapsigargin (100 nM)	0.145 ± 0.007 (20)***	0.069 ± 0.005 (10)	0.81 ± 0.08 (9)***	0.28 ± 0.03 (6)*
DBHQ (25 μM)	0.13 ± 0.008 (10)***	0.094 ± 0.006 (8)	Not determined	Not determined

Table 3 Effects of pertussis toxin treatment on the degree of inhibition of vasopressin- and thapsigargin-stimulated Mn^{2+} inflow caused by Gd^{3+}

Rates of Mn^{2+} inflow were measured in the absence and presence of 20 μM Gd^{3+} as described in the Experimental section and in Figure 2. The values are the means ± S.E.M. for the number of experiments shown in parentheses conducted using between three and nine different cell preparations. The degrees of significance for comparison of the rate of Mn^{2+} inflow in the presence of Gd^{3+} with that observed in the absence of the lanthanide are: $^{*}P \leqslant 0.0001$ and $^{**}P \leqslant 0.00001$.

		Rate of Mn^{2+} inflow (decrease in fluorescence units/s)	
Agonist	Hepatocytes	No inhibitor	Gd^{3+} present
None	Untreated	0.084 ± 0.006 (10)	0.087 ± 0.005 (10)
	Pertussis toxin-treated	0.067 ± 0.009 (8)	0.071 ± 0.01 (8)
Thapsigargin (100 nM)	Untreated	0.174 ± 0.006 (13)	0.134 ± 0.005 (13)**
	Pertussis toxin-treated	0.081 ± 0.011 (13)	0.076 ± 0.011 (13)
Vasopressin (50 nM)	Untreated	0.137 ± 0.006 (11)	0.104 ± 0.004 (11)*
	Pertussis toxin-treated	0.092 ± 0.007 (23)	0.087 ± 0.006 (24)

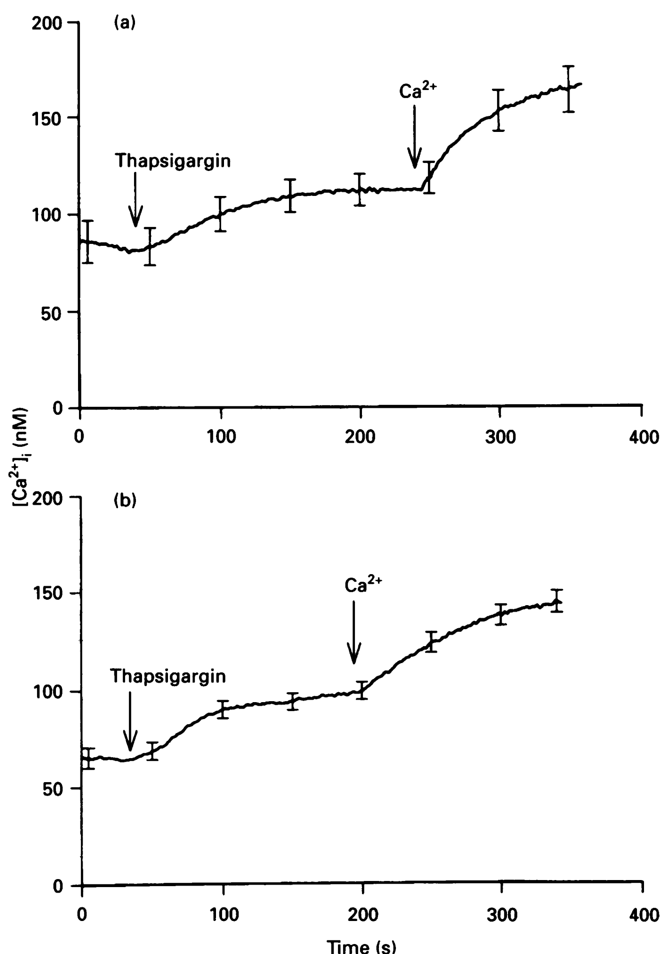

Figure 3 Inhibition by pertussis toxin of thapsigargin-stimulated Ca^{2+} inflow

Plots of $[Ca^{2+}]_i$ as a function of time for Quin 2-loaded hepatocytes from untreated (normal) (**a**) and pertussis toxin-treated (**b**) rats. Additions of thapsigargin (100 nM) and Ca^{2+} (1.3 mM) were made at the times indicated by the arrows. Treatment of rats with pertussis toxin, the loading of hepatocytes with Quin 2, and the measurement of fluorescence were conducted as described in the Experimental section. Each plot is composed of the mean values obtained after combination of the results (combined as described in the Experimental section) obtained from five (two cell preparations) or six (two cell preparations) experiments conducted with untreated and toxin-treated hepatocytes respectively. The error bars represent the S.E.M.

the inhibition of vasopressin-stimulated Mn^{2+} inflow (Table 2). A further test of the inhibition of agonist-stimulated Mn^{2+} inflow by pretreatment with pertussis toxin was made using Gd^{3+}. It has previously been shown that this trivalent cation inhibits agonist-stimulated Ca^{2+} and Mn^{2+} inflow in hepatocyts but does not affect basal (absence of agonist) bivalent cation inflow (Fernando and Barritt, 1994). In untreated hepatocytes, Gd^{3+} inhibited the rate of quenching by Mn^{2+} of the fluorescence of intracellular Quin 2 in the presence of thapsigargin or vasopressin by 23% and 24% respectively (Figure 2a and Table 3), whereas the inhibition by Gd^{3+} observed in hepatocytes treated with pertussis toxin was 6% and 5% for thapsigargin and vasopressin respectively (Figure 2b and Table 3). Gd^{3+} did not inhibit basal rates of Mn^{2+} inflow in untreated or in pertussis toxin-treated hepatocytes (Table 3).

Treatment with pertussis toxin also caused substantial inhibition of thapsigargin-stimulated Ca^{2+} inflow (Figure 3b, cf. Figure 3a and Table 2). As shown in Table 4, the toxin had little effect on basal $[Ca^{2+}]_i$, the maximum value of $[Ca^{2+}]_i$ attained after addition of vasopressin or thapsigargin or the amount of Ca^{2+} released by these agonists and DBHQ. The toxin caused no significant change in the rate of agonist-induced release of Ca^{2+} from intracellular stores (increase in $[Ca^{2+}]_i$) (Table 4 and Figure 3) although in the case of thapsigargin this parameter was more difficult to measure as there was some variation in the time elapsed between addition of thapsigargin and the increase in $[Ca^{2+}]_i$.

DISCUSSION

The present results indicate, first, that the treatment of hepatocytes with pertussis toxin for 24 h *in vivo* inhibits (i) angiotensin II-stimulated bivalent cation inflow as well as vasopressin- and EGF-stimulated bivalent cation inflow, (ii) vasopressin-, angiotensin II- and EGF-stimulated Mn^{2+} inflow as well as Ca^{2+} inflow induced by these agonists, and (iii) thapsigargin- and DBHQ-stimulated bivalent cation (Ca^{2+} or Mn^{2+}) inflow. The conclusion that pertussis toxin inhibits agonist-stimulated Mn^{2+} inflow is also consistent with the observation that Gd^{3+} causes a substantial inhibition of vasopressin- and thapsigargin-stimulated Mn^{2+} inflow in untreated but not in pertussis toxin-treated hepatocytes, coupled with the observation that neither treatment with the toxin nor addition of Gd^{3+} inhibits the basal rate of Mn^{2+} inflow.

Treatment with pertussis toxin had little effect on the basal rates of Ca^{2+} and Mn^{2+} inflow, basal values of $[Ca^{2+}]_i$, the

Table 4 Intracellular free Ca^{2+} ($[Ca^{2+}]_i$), the amount of Ca^{2+} released from intracellular stores by thapsigargin, vasopressin and DBHQ, and the rate of agonist-induced release of Ca^{2+} in untreated and in pertussis toxin-treated hepatocytes

Values of $[Ca^{2+}]_i$, amounts of Ca^{2+} released from intracellular stores, and rates of release of Ca^{2+} from intracellular stores were estimated as described in the Experimental section. Unless indicated, the concentrations of thapsigargin, vasopressin and DBHQ were 100 nM, 50 nM and 25 μM respectively. The values are the means ± S.E.M. of the numbers of experiments shown in parentheses conducted using between two and seven different cell preparations. For each parameter, comparison of the value for untreated cells with that for pertussis toxin-treated cells using Student's *t*-test for unpaired samples gave values of $P > 0.05$ (i.e. the differences are not significant).

	$[Ca^{2+}]_i$ nM)			Amount of Ca^{2+} released from intracellular stores (increase in $[Ca^{2+}]_i$, nM)				Rate of release of Ca^{2+} from intracellular stores (increase in $[Ca^{2+}]_i$, nM/s)		
Cells	Before agonist (basal)	After thapsigargin addition	After vasopressin addition	Thapsigargin (20 nM)	Thapsigargin (100 nM)	Vasopressin	DBHQ	Thapsigargin (20 nM)	Thapsigargin (100 nM)	Vasopressin
Untreated	95 ± 9 (15)	136 ± 14 (10)	146 ± 8 (9)	48 ± 6 (15)	51 ± 6 (15)	48 ± 2 (26)	48 ± 2 (10)	0.4 ± 0.1 (5)	0.8 ± 0.2 (8)	5.1 ± 0.7 (9)
Pertussis toxin-treated	76 ± 7 (22)	117 ± 11 (13)	126 ± 7 (13)	54 ± 8 (15)	54 ± 6 (16)	41 ± 2 (24)	51 ± 2 (8)	0.3 ± 0.02 (5)	0.7 ± 0.1 (10)	5.3 ± 0.8 (10)

maximum value of $[Ca^{2+}]_i$ observed following agonist addition, and the rate of increase in $[Ca^{2+}]_i$ after agonist addition (rate of release of Ca^{2+} from intracellular stores). Moreover, control experiments performed previously have not detected changes in other hormone-induced responses, which might be attributed to general cell damage, in rats treated with pertussis toxin (Hughes et al., 1987; Butta et al., 1993). Therefore it is concluded that the inhibition of agonist-stimulated bivalent cation inflow is a specific action of pertussis toxin and is unlikely to be the result of general damage to the hepatocytes induced by the toxin.

Thapsigargin and DBHQ artificially initiate the process of store-operated Ca^{2+} inflow, whereas it is proposed that the physiological initiator of this process is InsP_3 (reviewed by Putney, 1990). Thus it may be concluded from the present observations that store-operated bivalent cation inflow initiated by InsP_3 also requires a pertussis toxin-sensitive protein. Moreover, since bivalent cation inflow stimulated by vasopressin or angiotensin II has been shown to require a pertussis toxin-sensitive protein (Hughes et al., 1987; Butta et al., 1993; Berven et al., 1994; present results), the idea that store-operated bivalent cation inflow is the physiological mechanism by which these agonists stimulate Ca^{2+}inflow is consistent with the present data. (The possibility that store-operated Ca^{2+} inflow shares a common pertussis toxin-sensitive step with agonist-stimulated inflow, but is not part of the physiological mechanism of action of agonists, is considered unlikely.)

In hepatocytes, the major known targets for ADP-ribosylation by pertussis toxin are the trimeric G-proteins G_{i2} and G_{i3} (Bushfield et al., 1990; Pobiner et al., 1991). Thus it is concluded that the target of pertussis toxin detected in the present experiments is G_{i2} or G_{i3}. However, the possibility that another type of protein has been altered by the action of the toxin (e.g. McCarthy and Bicknell, 1992) cannot be completely eliminated. The results indicate that activation of the hepatocyte receptor-operated Ca^{2+} channel by a G-protein-linked receptor requires the action of $G_{i2/3}$ at a step which lies downstream from InsP_3-induced release of Ca^{2+} from intracellular stores. This requirement is in addition to $G_{q/11}$, which is required in the activation of phospholipase Cβ and the generation of InsP_3 (Taylor et al., 1991).

Orrenius and his colleagues have investigated the ability of a variety of agonists to stimulate Mn^{2+} inflow in hepatocytes, and have tested the ability of pertussis toxin to inhibit vasopressin-stimulated Mn^{2+} inflow (Kass et al., 1990; Llopis et al., 1992). The results reported here differ from those obtained by these workers in two ways. These authors did not observe stimulation of Mn^{2+} inflow by either thapsigargin or DBHQ (Llopis et al., 1992) nor an inhibition by pertussis toxin of vasopressin-induced Mn^{2+} inflow (Kass et al., 1990). The difference in the observed effects of agonists, thapsigargin and DBHQ on Mn^{2+} inflow may be due to differences in the methods employed to measure Mn^{2+} present in the cytoplasmic space of hepatocytes. The present results were obtained using hepatocytes loaded with the acetoxy methyl ester of Quin 2 (Crofts and Barritt, 1989, 1990) because in this laboratory it had not been possible to find conditions for the successful loading of Fura 2 to the cytoplasmic space of hepatocytes in suspension using the acetoxy methyl ester of Fura 2 (J. N. Crofts and G. J. Barritt, unpublished work). Moreover, Glennon et al. (1992) have provided evidence which indicates that results obtained for the measurement of Mn^{2+} inflow in hepatocytes loaded with the acetoxy methyl ester of Fura 2 may reflect the fact that, under these conditions, Fura 2 is partly located in intracellular organelles. They found that when the introduction of Fura 2 to intracellular organelles was minimized by the microinjection of Fura 2 free acid into hepatocytes, a stimulation of Mn^{2+} inflow by thapsigargin was observed.

With respect to the effect of pertussis toxin, differences between the results reported by Kass et al. (1990) and the present results may reflect differences in the time of exposure of the hepatocytes to the toxin (24 h in the present experiments compared with 4.5 h in the experiments of Kass et al.). It has been shown, using cultured cells, that about 10 h is required to fully ADP-ribosylate pertussis toxin-sensitive trimeric G-proteins in hepatocytes (Yang et al., 1993) [cf. (Lynch et al., 1986) and the slow ADP-ribosylation of trimeric G-proteins also observed in some other cell types (Neylon et al., 1992; Blitzer et al., 1993)].

There are some similarities between hepatocytes, in which it appears that a pertussis toxin-sensitive trimeric G-protein is required for store-operated bivalent cation inflow, and the mechanism of agonist-stimulated Ca^{2+} inflow in some other cell types which have also been shown to involve a pertussis toxin-sensitive trimeric G-protein (Komori et al., 1992; Krautwurst et al., 1992; Gollasch et al., 1993). Moreover, Jaconi et al. (1993) have interpreted the results of their recent experiments with granulocytes in terms of the requirement for a trimeric G-protein in the process of store-operated Ca^{2+} inflow. Evidence that a low-molecular-mass (monomeric) G-protein is required for store-

operated bivalent cation inflow in mast cells (Fasolato et al., 1993) and lacrimal acinar cells (Bird and Putney, 1993) has recently been reported. Since, to our knowledge, there are no reports which indicate that monomeric G-proteins are ADP-ribosylated, it is unlikely that the target of pertussis toxin detected in the present studies is a monomeric G-protein. However, the present experiments do not exclude the possibility that both a trimeric and a monomeric G-protein are required in the process of store-operated Ca^{2+} inflow in hepatocytes.

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