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A role for a pertussis toxin-sensitive trimeric G-protein in store-operated Ca^{2+} inflow in hepatocytes

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Abstract

The mechanism of store-operated Ca^{2+} inflow in hepatocytes was investigated using fluo-3 and fura-2 to monitor changes in the concentration of intracellular free Ca^{2+} in single cells, and 1-(α-glycerophosphoryl)-*myo*-inositol 4,5-diphosphate, $P^{4(5)}$-1-(2-nitrophenyl)ethyl ester ('caged' $GPIP_2$) and 'caged' guanosine 5′-[γthio]triphosphate (GTPγS) (introduced into the cytoplasmic space by microinjection), thapsigargin and 2,5-di-*tert*-butylhydroquinone (DBHQ) to stimulate Ca^{2+} inflow. Photolysis of 'caged' $GPIP_2$ or 'caged' GTPγS stimulated Ca^{2+} inflow. The abilities of $GPIP_2$, thapsigargin and DBHQ to stimulate Ca^{2+} inflow were inhibited by the pre-treatment of hepatocytes with pertussis toxin in vivo for 36 h. Thapsigargin-stimulated Ca^{2+} inflow was also inhibited by guanosine 5′-[β-thio]diphosphate (GDPβS) (introduced by microinjection). It is concluded that, in hepatocytes, store-operated Ca^{2+} inflow induced by the actions of either inositol 1,4,5-trisphosphate, thapsigargin or DBHQ requires a pertussis toxin-sensitive trimeric G-protein.

Key words: Ca^{2+} inflow; Hepatocyte; Trimeric G-protein; Pertussis toxin; Inositol trisphosphate

1. Introduction

Receptor-activated Ca^{2+} inflow across the plasma membrane of hepatocytes and other animal cells plays an essential role in the processes by which extracellular signals induce physiological responses [1–4]. The stimulation of Ca^{2+} inflow by an agonist-receptor complex appears to be a consequence of the depletion of Ca^{2+} present in inositol 1,4,5-trisphosphate ($InsP_3$)-sensitive stores [5–9] such that an increase in $InsP_3$ is a prerequisite for the stimulation of Ca^{2+} inflow. Thus, in hepatocytes and many types of animal cells the depletion of Ca^{2+} in intracellular stores, induced by agonists, thapsigargin and some other agents, has been shown to mimic the action of extracellular signals in stimulating Ca^{2+} inflow [8–11]. It has not yet been established conclusively whether this store-operated [12] Ca^{2+} inflow is part of the physiological actions of extracellular signals in stimulating Ca^{2+} inflow. Moreover, the molecular events in store-operated Ca^{2+} inflow have not been elucidated, although they may involve a mobile intracellular messenger [13–16].

It has previously been shown that, in hepatocytes, GTPγS (introduced by a cell permeation technique or by microinjection) mimics the ability of vasopressin to stimulate Ca^{2+} inflow [17,18]. Moreover, the treatment of hepatocytes with pertussis toxin in vivo for 24–72 h inhibits vasopressin-and GTPγS-stimulated Ca^{2+} inflow but not the release of Ca^{2+} from intracellular stores induced by these agents [18–20]. Inhibition of vasopressin-stimulated Ca^{2+} inflow was not observed when hepatocytes were exposed to pertussis toxin for 1 h following microinjection of the toxin to the cells [18]. These results have led to the conclusion that the actions of a slowly-ADP-ribosylated trimeric GTP-binding protein (G-protein) are required in the mechanism by which vasopressin stimulates Ca^{2+} inflow [18–20] (but see [21]).

The aims of the present experiments were two-fold. Firstly to conduct a more direct test of the abilities of intracellular $InsP_3$ and GTPγS to stimulate Ca^{2+} inflow by determining whether Ca^{2+} inflow can be stimulated by the photolysis of 1-(α-glycerophosphoryl)-*myo*-inositol 4,5-diphosphate, $P^{4(5)}$-1-(2-nitrophenyl)ethyl ester ('caged' $GPIP_2$) or 'caged' guanosine 5′-[γ-thio]triphosphate (GTPγS). Secondly to determine whether a pertussis toxin-sensitive G-protein is required when Ca^{2+} inflow is stimulated by the depletion of Ca^{2+} in intracellular stores, induced by $InsP_3$, thapsigargin or 2,5-di-*tert*-butylhydroquinone (DBHQ) (i.e. required for store-operated Ca^{2+} inflow). We show here, using single hepatocytes and the technique of microinjection, that intracellular $InsP_3$ and GTPγS stimulate Ca^{2+} inflow. Moreover, the results indicate that a trimeric G-protein, sensitive to pertussis toxin, is required for store-operated Ca^{2+} inflow when this is initiated by $InsP_3$, thapsigargin or DBHQ. The results provide further evidence which indicates that the process of store-operated Ca^{2+} inflow is a component of the physiological pathway by which agonists stimulate Ca^{2+} inflow in hepatocytes, and point

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Abbreviations: $InsP_3$, inositol 1,4,5-trisphosphate; 'caged' $GPIP_2$, 1-(α-glycerophosphoryl)-*myo*-inositol 4,5-diphosphate, $P^{4(5)}$-1-(2-nitrophenyl)ethyl ester; GTPγS, guanosine 5′-[γthio]triphosphate; GDPβS, guanosine 5′-[β-thio]diphosphate; ADPβS, adenosine 5′-[β-thio]-diphosphate; G-protein, GTP-binding protein; Ca^{2+}_o, extracellular Ca^{2+}; DBHQ, 2,5-di-*tert*-butylhydroquinone.

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to a central function for a trimeric G-protein in this process.

2. Materials and methods

2.1. Materials

Fluo-3, fura-2 and 'caged' GTPγS were obtained from Molecular Probes, Eugene, OR, USA; 'caged' $GPIP_2$ and adenosine 5'-[β-thio]diphosphate from Calbiochem-Novabiochem, Alexandria, NSW, Australia; and thapsigargin, DBHQ and pertussis toxin from Sigma-Aldrich, Castle Hill, NSW, Australia. All other chemicals were obtained from the sources described previously [18].

2.2. Methods

Hepatocytes were isolated, attached to coverslips coated with collagen, and fluo-3 (10 mM in the pipette tip), fura-2 (10 mM in the pipette tip), 'caged' $GPIP_2$ (24 mM in the pipette tip), 'caged' GTPγS (30 mM in the pipette tip), GDPβS (90 mM in the pipette tip), ADPβS (90 mM in the pipette tip) and GMP (90 mM in the pipette tip) were introduced to the cytoplasmic space by microinjection, as described previously [18]. In order to estimate the degree of dilution of nucleotides and other agents injected into hepatocytes, the fluorescence of cells loaded with fluo-3 by microinjection and incubated at saturating $[Ca^{2+}]_i$ was compared with the fluorescence of microdroplets (with a similar volume to that of hepatocytes) formed by the microinjection of Ca^{2+}-saturated fluo-3 into silicon oil. The dilution factor for the microinjection of agents to hepatocytes was found to be 50–100 fold. Thus the concentrations of GDPβS and 'caged' GTPγS in the cytoplasmic space of hepatocytes were estimated to be 0.9–1.8 mM and 0.3–0.5 mM, respectively.

Pertussis toxin (25 μg/100 g body weight) was administered to rats by intraperitoneal injection 36 h before isolation of the hepatocytes. Ca^{2+} inflow to single hepatocytes was monitored by measuring the increase in fluorescence of intracellular fluo-3 or fura-2 following the addition of extracellular Ca^{2+} (Ca^{2+}_o) to hepatocytes incubated in the absence of added Ca^{2+}_o, as described previously [18]. Mn^{2+} inflow was measured in cells loaded with fura-2 by microinjection, using a modification of the methods described previously [22].

Fluorescence was measured using an inverted TMD-EF fluorescence microscope, a photometer (Nikon Corporation, Tokyo, Japan) and the UMANS filter changing and data recording system, as described previously [18]. The excitation light source was a mercury lamp. Conditions for the excitation of fura-2 and the measurement of fura-2 fluorescence were as described previously [18]. For experiments involving 'caged' compounds, fluo-3 was employed for the measurement of intracellular free Ca^{2+} concentrations ($[Ca^{2+}]_i$) [23]. Excitation of fluo-3 was at 490 nm and a Nikon barrier filter B (wavelength 520 nm) was employed to isolate emitted light. Photolysis of 'caged' compounds was achieved by exposing the cell to the full spectrum of light from the mercury lamp for 3–5 s while the shutter to the photometer was closed.

3. Results

3.1. Activation of Ca^{2+} inflow by photolysis of 'caged' $GPIP_2$ and 'caged' GTPγS

To investigate whether $InsP_3$ can activate Ca^{2+} inflow across the plasma membrane of hepatocytes, 'caged' $GPIP_2$, which yields a slowly-hydrolysable form of $InsP_3$ upon photolysis [24], was introduced to hepatocytes by microinjection together with fluo-3. Fig. 1a (thick solid line) shows that, in a single hepatocyte incubated initially in the absence of added Ca^{2+}_o, the photolysis of 'caged' $GPIP_2$ induced a rapid transient increase in fluorescence due to the $InsP_3$-induced release of Ca^{2+} from intracellular stores [24]. Addition of Ca^{2+}_o induced a further increase in fluorescence resulting from Ca^{2+} inflow across the plasma membrane [11,18]. No substantial increase in fluorescence was observed following the addition of Ca^{2+}_o to hepatocytes treated with 'caged' $GPIP_2$ but not exposed to UV light (Fig. 1a, broken line). In cells loaded only with fluo-3, exposure to UV light caused a small increase in fluorescence which, in most cases, occurred 1–2 min after UV exposure (Fig. 1a, thin solid line). These cells showed no significant increase in fluorescence following the addition of Ca^{2+}_o.

The ability of $GPIP_2$ to stimulate divalent cation inflow was confirmed using Mn^{2+} [21,22]. Thus in cells loaded with 'caged' $GPIP_2$ and fluo-3, photolysis led to a stimulation of Mn^{2+} inflow, assessed by the quenching of fluo-3 (results not shown).

In cells loaded with 'caged' GTPγS, photolysis of this 'caged' compound also stimulated Ca^{2+} inflow (Fig. 1b, solid line). In control cells loaded with 'caged' GTPγS but not exposed to UV light little Ca^{2+} inflow was observed following the addition of Ca^{2+}_o (Fig. 1b, broken line).

3.2. Effects of pertussis toxin and GDPβS on $GPIP_2$-, thapsigargin- and DBHQ-stimulated Ca^{2+} inflow

The ability of 'caged' $GPIP_2$ to stimulate Ca^{2+} inflow was almost completely inhibited in hepatocytes isolated from rats treated with pertussis toxin (Fig. 2a). The toxin had no observable effect on the amount of Ca^{2+} released from intracellular stores. Treatment with pertussis toxin also blocked thapsigargin-stimulated Ca^{2+} inflow to hepatocytes without altering the ability of thapsigargin to release Ca^{2+} from intracellular stores (Fig. 2b). A similar inhibition by pertussis toxin was observed when DBHQ (25 μM in the extracellular medium) was used in place of thapsigargin (9 out of 9 cells tested, results not shown). These observations suggest that a pertussis toxin-sensitive trimeric G-protein is required in the process by which thapsigargin and DBHQ stimulate Ca^{2+} inflow. This idea was tested further using guanosine 5'-[β-thio]diphosphate (GDPβS), an inhibitor of the action of trimeric G-proteins [25]. Introduction of GDPβS to the cytoplasmic space of hepatocytes by microinjection inhibited by more than 50% thapsigargin-stimulated Ca^{2+} inflow but had no observable effect on the ability of thapsigargin to release Ca^{2+} from intracellular stores (Fig. 3). When GMP was employed in place of GDPβS there was no inhibition of thapsigargin-stimulated Ca^{2+} inflow (Fig. 3). Attempts were made to use ADPβS in place of GDPβS. However, the results suggested that ADPβS inhibits Ca^{2+} efflux from the cytoplasmic space which precluded the use of ADPβS as a control.

4. Discussion

Upon photolysis, 'caged' $GPIP_2$ liberates $GPIP_2$, a slowly-hydrolysable analogue of $InsP_3$ [24]. Thus the ob-

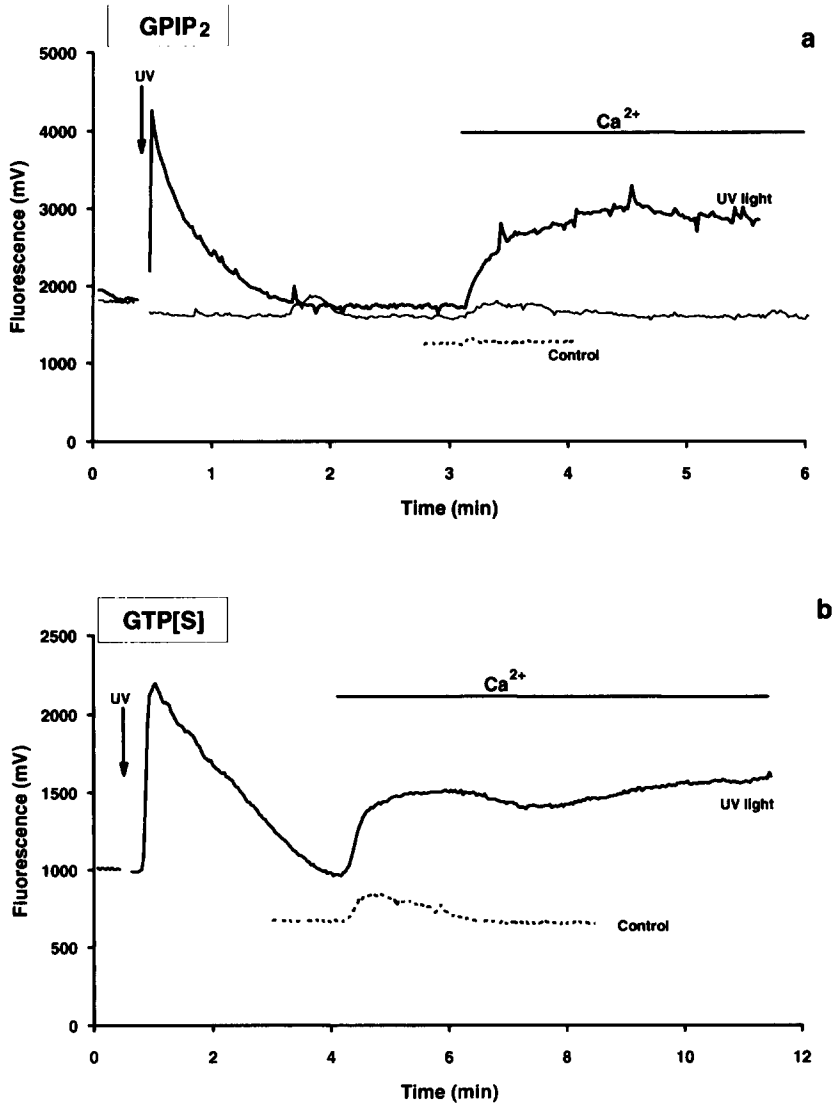

Fig. 1. The stimulation of Ca^{2+} inflow by $GPIP_2$ and GTPγS. The thick solid line is a representative trace obtained from a single hepatocyte loaded with fluo-3 and 'caged' $GPIP_2$ (a) or 'caged' GTPγS (GTP[S]) (b), incubated initially in the absence of added Ca^{2+}_o then exposed to UV light (arrow) and 1.3 mM Ca^{2+}_o (added at the beginning of the period indicated by the horizontal bar). The results shown are those obtained for 15 of 22 cells tested and 8 of 14 cells tested for (a) and (b), respectively. The broken lines in (a) and (b) show control experiments in which the cells were not exposed to UV light to photolyse the 'caged' compound before the addition of Ca^{2+}_o. For each of (a) and (b), 4 cells were tested in the control experiment. Each gave a trace similar to the one shown. The thin solid line in (a) represents an experiment in which cells injected with fluo-3 (in the absence of $GPIP_2$ or GTPγS) were exposed to UV and then to Ca^{2+}_o. Traces similar to the thin solid line were obtained with each of eight cells tested.

servation that the photolysis of 'caged' $GPIP_2$ stimulates Ca^{2+} inflow provides further evidence for the idea [11,18] that an increase in $InsP_3$ is a necessary step in the mechanism by which vasopressin and other G-protein-coupled receptors stimulate plasma membrane Ca^{2+} inflow in hepatocytes. While the results of previous experiments have suggested a role for $InsP_3$ in this process [11,18], the use of a 'caged' analogue of $InsP_3$ has allowed a more direct test of the proposal.

As in the case of the generation of $GPIP_2$ from 'caged' $GPIP_2$, the results obtained with 'caged' GTPγS provide more direct evidence that intracellular GTPγS can stimulate Ca^{2+} inflow. The photolysis of 'caged' GTPγS, which generates a slowly-hydrolysable analogue of GTP, would be expected to activate all trimeric G-proteins in the hepatocyte [26]. These include $G_{q/11}$, the activator of phospholipase Cβ. This enzyme catalyses the formation of $InsP_3$ when this is induced by vasopressin and other G-protein-coupled receptors [27]. In the present experiments, the ability of GTPγS to stimulate Ca^{2+} inflow is likely to be due to the generation of $InsP_3$, through the activation of phospholipase Cβ, and also to the activation of any other trimeric G-proteins which may lie on the pathway between the receptor and the putative plasma membrane Ca^{2+} channel.

The results obtained with pertussis toxin indicate that

the process by which $InsP_3$, thapsigargin and DBHQ induce plasma membrane Ca^{2+} inflow (i.e. store-operated Ca^{2+} inflow) requires a pertussis toxin-sensitive protein. The most likely candidate for this protein is a trimeric G-protein [26]. Previous experiments have shown that the inability of vasopressin to stimulate Ca^{2+} inflow in hepatocytes treated with pertussis toxin in vivo is unlikely to be due to general cell damage induced by the toxin [18–20]. Further evidence for the involvement of a trimeric G-protein in the actions of $InsP_3$ and thapsigargin comes from the results obtained with GDPβS and 'caged' GTPγS. GDPβS inhibits the abilities of vasopressin [18] and thapsigargin (present results) to stimulate Ca^{2+} inflow, whereas GTPγS activates Ca^{2+} inflow (in a pertussis toxin-sensitive manner) (present results and [18]). Since one of the characteristics of trimeric G proteins is activation by GTPγS and inhibition by GDPβS [25,26], the idea that a trimeric G protein is involved in store-operated Ca^{2+} inflow is consistent with these results.

Since G_{i2} and G_{i3} are the only known pertussis toxin-sensitive trimeric G proteins in hepatocytes [28,29], it is likely that one of these proteins is the trimeric G-protein involved in store-operated Ca^{2+} inflow. The requirement to treat hepatocytes with pertussis toxin for 36 h in order to inhibit $InsP_3$-, thapsigargin- and DBHQ-stimulated

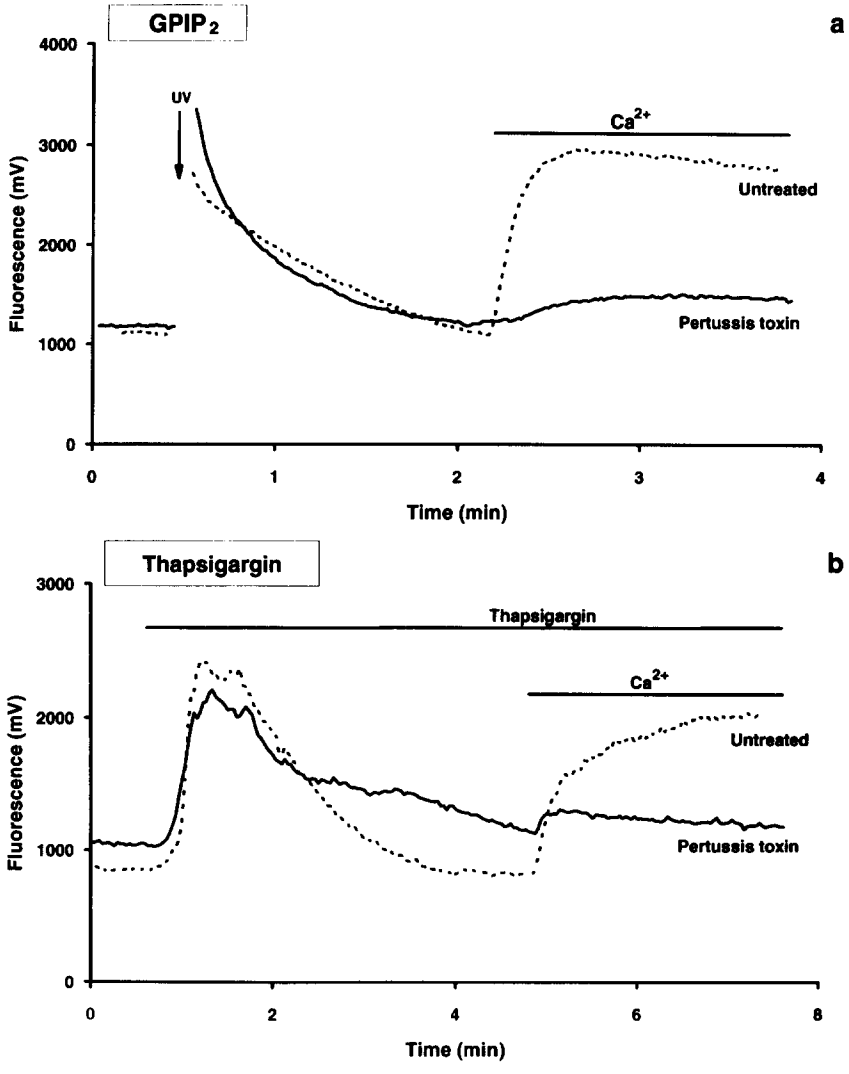

Fig. 2. The inhibition by pertussis toxin of $GPIP_2$-stimulated and thapsigargin-stimulated Ca^{2+} inflow. (a) The solid line is a representative trace obtained from a single hepatocyte from a rat treated with pertussis toxin, loaded with fluo-3 and 'caged' $GPIP_2$ by microinjection, incubated initially in the absence of added Ca^{2+}_o, exposed to UV light (arrow) and 1.3 mM Ca^{2+}_o (added at the beginning of the period indicated by the solid bar). The broken line represents a control experiment showing that $GPIP_2$ stimulates Ca^{2+} inflow in untreated (normal) hepatocytes. (b) The solid line is a representative trace obtained from a single hepatocyte from a rat treated with pertussis toxin, loaded with fluo-3, incubated in the absence of added Ca^{2+}_o and exposed to thapsigargin (10 μM in the extracellular medium) and 1.3 mM Ca^{2+}_o, added at the beginning of the periods indicated by the horizontal bars. The broken line represents a control experiment which shows that Ca^{2+} inflow was stimulated by thapsigargin in an untreated (normal) hepatocyte. The results shown are those obtained for 15 of 20 cells tested and 8 of 11 cells tested for (a) and (b), respectively.

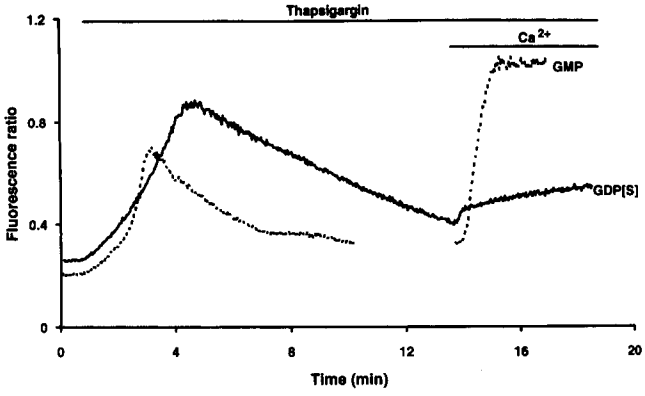

Fig. 3. The inhibition by GDPβS of thapsigargin-stimulated Ca^{2+} inflow. The solid line is a representative trace obtained from a single untreated (normal) hepatocyte loaded with fura-2 and GDPβS (GDP[S]), incubated in the absence of added Ca^{2+}_o and treated with thapsigargin (10 μM in the extracellular medium) and 1.3 mM Ca^{2+}_o, added at the beginning of the periods indicated by the horizontal bars. The broken line represents a control experiment in which a single untreated (normal) hepatocyte was loaded with fura-2 and GMP, incubated in the absence of added Ca^{2+}_o then exposed to thapsigargin and Ca^{2+}_o. The results shown are those for 9 of 12 cells tested and 4 of 5 cells tested for hepatocytes loaded with GDPβS and GMP, respectively.

Ca^{2+} inflow is consistent with the previous observation that, in hepatocytes, $G_{i\alpha}$ subunits are ADP-ribosylated slowly [30].

Recently, evidence for the involvement of monomeric (low molecular weight) G-proteins, of the type which mediate membrane fusion, in store-operated Ca^{2+} inflow in a mast cell line [15] and in lacrimal acinar cells [31] has been reported. (These G-proteins are inhibited by GTPγS [15,31] but, in the case of mast cells, not by GDPβS [15].) The requirement for a monomeric G-protein in store-operated Ca^{2+} inflow in hepatocytes is not consistent with the present results (activation of Ca^{2+} inflow by GTPγS and inhibition of inflow by GDPβS). Moreover, it is not known whether the stimulation of Ca^{2+} inflow in mast cells and lacrimal acinar cells is sensitive to pertussis toxin. Nevertheless, the possibility that a monomeric G-protein does work in conjunction with $G_{i2/3}$ in activating plasma membrane Ca^{2+} inflow in the hepatocyte, but this action of a monomeric G-protein was not detected in the present experiments, cannot be excluded.

It is presently considered that the sequence of events which leads from a G-protein-coupled receptor to activation of the putative Ca^{2+} channel in the plasma membrane of hepatocytes is as follows. (i) The agonist binds to the receptor and activates phospholipase Cβ through the action of $G_{q/11}$. (ii) This, in turn, increases $InsP_3$ which induces the release of Ca^{2+} from a region of the endoplasmic reticulum. (iii) Partial depletion of Ca^{2+} in the endoplasmic reticulum causes, through an unknown mechanism, opening of the putative Ca^{2+} channel. The observation that pertussis toxin inhibits thapsigargin-, DBHQ- and $InsP_3$-induced Ca^{2+} inflow (present results) as well as agonist-stimulated Ca^{2+} inflow [18–20] provides further evidence that this process of store-operated Ca^{2+} inflow is the physiological mechanism by which G-protein-coupled receptors stimulate Ca^{2+} inflow in hepatocytes. It may be concluded from the present results that the putative pertussis toxin-sensitive trimeric G protein $G_{i2/3}$ lies between the release of Ca^{2+} from the endoplasmic reticulum and activation of the putative Ca^{2+} channel. (Since pertussis toxin did not affect the ability of $InsP_3$, thapsigargin or DBHQ to release Ca^{2+} from the endoplasmic reticulum, it is unlikely that $G_{i2/3}$ is required in this process.)

$G_{i2/3}$ may be involved in the actions of recently-described putative intracellular messengers which have been proposed to link intracellular Ca^{2+} stores to the putative plasma membrane Ca^{2+} channel [13–15] and/or may allow association of the endoplasmic reticulum Ca^{2+} store with the plasma membrane [32]. The idea that a pertussis toxin-sensitive trimeric G-protein is a component of the mechanism of store-operated Ca^{2+} inflow in hepatocytes is consistent with mechanisms proposed for some other cell types in which a requirement for a pertussis toxin-sensitive trimeric G-protein in agonist-stimulated Ca^{2+} inflow has been reported [33,34], and the recent observation which implicates a trimeric G protein in store-operated Ca^{2+} inflow in granulocytes [35].

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