Oscillations of cytosolic free calcium concentration in the presence of intracellular antibodies to phosphatidylinositol 4,5-bisphosphate in voltage-clamped guinea-pig hepatocytes

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In liver cells, the stimulation of α_1 -adrenoceptors by noradrenaline induces the production of $\text{Ins}(1,4,5)\text{P}_3$ through the degradation of membrane polyphosphoinositides [$\text{PtdIns}(4,5)\text{P}_2$]. InsP_3 evokes in turn the release of Ca^{2+} from internal stores. Our results show that the internal perfusion of single guinea-pig hepatocytes with monoclonal anti- PtdInsP_2 antibody blocks the rise in cytosolic free Ca^{2+} concn. ($[\text{Ca}^{2+}]_i$) evoked by noradrenaline, an InsP_3 -dependent agonist, but not by the monohydroxylated bile acid tauroolithocholate 3-sulphate, which is known to permeabilize the endoplasmic reticulum. In these conditions, the bile acid elicited either fast or slow fluctuations of $[\text{Ca}^{2+}]_i$, independently of any InsP_3 production. The responses to the bile acid were also observed in the absence of external Ca^{2+} . The presence of intracellular anti- PtdInsP_2 antibody does not affect the response to a photolytic release of InsP_3 ($1.5 \mu\text{M}$ final concn.) from a caged precursor.

INTRODUCTION

Stimulation of plasma-membrane α_1 -adrenoceptors by noradrenaline leads to a large and sustained rise in cytosolic free Ca^{2+} concn. ($[\text{Ca}^{2+}]_i$) in mammalian hepatocytes [1–4]. It has been shown that monohydroxylated bile acids are able to reproduce these effects in rat and guinea-pig liver cells [5–7]. It is now clear that $\text{Ins}(1,4,5)\text{P}_3$ generated through the degradation of plasma-membrane $\text{PtdIns}(4,5)\text{P}_2$ mediates the release of Ca^{2+} from the internal stores. However, it was interesting to investigate whether we could induce a mobilization of the internal Ca^{2+} in the absence of InsP_3 production. This was made possible by blocking the degradation of membrane PtdInsP_2 . A monoclonal antibody, kt3g, raised against PtdInsP_2 has been produced in BALB/c mice [8,9]. It has been successfully used to block mitogenesis in response to platelet-derived growth factor (PDGF) and bombesin in 3T3 cells [8] and the PDGF-induced rise in Ca^{2+} in rat vascular smooth-muscle cells [10]. We have investigated the effects of the anti- PtdInsP_2 antibody on the noradrenaline-induced rise in the apamin-sensitive Ca^{2+} -dependent K^+ conductance in voltage-clamped guinea-pig hepatocytes, which has been shown to be a good monitor of the variations of $[\text{Ca}^{2+}]_i$ near the plasma membrane [3,11]. Our results show first, that intracellular perfusion of the anti- PtdInsP_2 antibody is able to block the noradrenaline-evoked response, and second, that, in these conditions, an application of the bile acid can induce either fast or slow fluctuations of $[\text{Ca}^{2+}]_i$.

EXPERIMENTAL

Materials

Collagenase was obtained from Worthington Biochemical Corp. All other reagents were of the highest purity commercially available and were obtained from Sigma or Boehringer Mannheim.

Methods

All the procedures utilized in this study described here,

including cell preparation, cell incubation and whole-cell recording techniques were as described in [7]. Experiments were done in Cl^- -free conditions, with gluconate as substitute. The external solution contained (mM): sodium gluconate, 145; potassium gluconate, 5.6; CaSO_4 , 5; MgSO_4 , 1.2; NaH_2PO_4 , 0.4; Hepes, 8 (pH 7.3). In Ca^{2+} -free experiments, the external medium contained (mM): sodium gluconate, 149; potassium gluconate, 5.6; MgSO_4 , 3; NaH_2PO_4 , 0.8; EGTA, 0.04; Hepes, 8 (pH 7.3). Patch pipettes contained (mM): potassium gluconate, 153; Na_2ATP , 3; MgSO_4 , 3; Na_2GTP , 0.3; Hepes, 8 (pH 7.3). The dish was continuously perfused, and hormones or drugs were added to the external medium. All solutions were sterilized by passage through $0.22 \mu\text{m}$ -pore Millipore filters.

Photolysis was produced by the same procedure as in [11]. Caged InsP_3 [the 1-(nitrophenyl)ethyl ester of InsP_3 , esterified on position 4] was introduced into the cell via the patch pipette, allowing 2–3 min for equilibration. Photolysis was produced by a 1 ms pulse from a xenon arc flash-lamp [12] focused to a spot of about $2 \text{ mm} \times 3 \text{ mm}$ around the cell. The energy output of the lamp was set to produce approx. 10% conversion of caged InsP_3 into InsP_3 .

Characterization and purification of anti- PtdInsP_2 antibody

The mouse monoclonal anti- PtdInsP_2 antibody designated as clone kt3g was obtained by immunization of BALB/c mice with PtdInsP_2 from bovine spinal cords. The characterization and the specificity of this antibody were previously described in detail [8]. Briefly, the antibody kt3g, identified as IgG_2b class, was found to bind to PtdInsP_2 and to have no specific affinity for other phospholipids and for InsP_3 . The IgG_2b fraction was further purified from ascites by using a Sepharose–Protein A column as described in [13]. The purity of IgG_2b fraction was tested by SDS/PAGE and its ability to recognize PtdInsP_2 after purification was assayed by dot-blot experiments. Pure IgG_2b was finally stored at -30°C in phosphate-buffered saline (40 mg/ml) and used at a concentration of 50–100 $\mu\text{g}/\text{ml}$ inside the patch pipette for the patch-clamp experiments.

Abbreviations used: $[\text{Ca}^{2+}]_i$, cytosolic free Ca^{2+} concn.; PDGF, platelet-derived growth factor; TLC-S, tauroolithocholate 3-sulphate.

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RESULTS

Guinea-pig liver cells possess a Ca^{2+} -dependent K^+ conductance that has been characterized in detail [3,11,14] and provides a measure of hormone-mediated $[\text{Ca}^{2+}]_i$ rises in the immediate vicinity of the plasma membrane. The response to noradrenaline is characterized by a variable delay, a fast rise in K^+ conductance followed by one of two main patterns of responses: a plateau phase which shows fast fluctuations of $[\text{Ca}^{2+}]_i$ or regular spikes of $[\text{Ca}^{2+}]_i$ separated by variable intervals. The involvement of PtdInsP_2 in the sequence which follows the α_1 -adrenoceptors stimulation was tested by the intracellular perfusion of anti- PtdInsP_2 antibody. This antibody (50–100 $\mu\text{g}/\text{ml}$ inside the patch pipette) diffused into the cell via the patch pipette. Fig. 1 shows the effects of the internal perfusion of antibody kt3g on the noradrenaline-induced response. On this cell, we were able to record a response to noradrenaline in the first 5 min after going to whole-cell recording configuration. After 10 min, corresponding to the time taken for the antibodies to reach the effective concentration, the response to noradrenaline was totally blocked.

The inhibitory effect of the intracellular perfusion of guinea-pig hepatocytes with the anti- PtdInsP_2 antibody on the noradrenaline-evoked responses was observed in 60 % of the 59 cells tested. In those cells sensitive to the antibody, half of them had their noradrenaline responses blocked within the first 4 min,

Fig. 1. Effect of internal perfusion of the same guinea-pig liver cell by the anti- PtdInsP_2 antibody on the activation of the Ca^{2+} -dependent K^+ conductance evoked by the InsP_3 -dependent agonist (noradrenaline; NOR) and by the monohydroxylated bile acid (TLC-S)

The patch pipette contained 100 μg of anti- PtdInsP_2 antibody/ml which diffused into the cell. The time elapsed since the beginning of the internal perfusion after going to whole-cell recording conditions is indicated above each trace. The effectors (NOR, 1 μM ; TLC-S, 200 μM) were applied to the cell as indicated by the horizontal bars. These four traces show that the noradrenaline-induced rise in K^+ conductance was inhibited after 14 min of internal perfusion of the antibodies, whereas the response to TLC-S in the same cell remained unaffected after 17 min of the same treatment. Further application of TLC-S gave a similar rise in conductance (result not shown). Membrane potential, 0 mV.

Fig. 2. Different patterns of responses (a and b) and absence of effect of external Ca^{2+} -free solutions (c) on the TLC-S-evoked activation of Ca^{2+} -dependent K^+ conductance in the presence of 100 μg of anti- PtdInsP_2 antibody/ml

The responses to 1 μM -noradrenaline (NOR) applied as indicated by the horizontal bars were blocked by internal perfusion of the antibodies (time shown inside each trace). The application of TLC-S (100 μM) evoked a response with a plateau phase showing fast oscillations of $[\text{Ca}^{2+}]_i$ on the first cell (a) and repetitive spikes of $[\text{Ca}^{2+}]_i$ from a resting baseline level on a second cell (b). The third trace (c) shows the lack of effect of a superfusion with a Ca^{2+} -free medium on the response evoked by 200 μM -TLC-S. Membrane potentials were held at 0 mV. Scale bars: vertical, 100 pA; horizontal, 60 s.

whereas the delay to observe an inhibition ranged from 6 to 17 min in the remaining cells. The delay, and sometimes the absence of effect (24 cells out of 59), can be explained by a limited access of the bulky antibody into the cell, which is especially apparent for high-molecular-mass proteins such as antibodies [15]. Moreover, it should be noted that 95 % of cells not loaded with the antibody are responding to noradrenaline and that these responses continue as long as the duration of the whole-cell recordings (up to 40 min). The slow diffusion process also explains the 10–20 times higher concentration than that used in injected 3T3 cells to block the PDGF-induced mitogenesis [8]. In their experiments 5 $\mu\text{g}/\text{ml}$ was sufficient to block the response induced by a maximal dose of PDGF.

Monohydroxylated bile acids such as tauro lithocholate 3-sulphate (TLC-S) are able to reproduce the effect of noradrenaline on Ca^{2+} mobilization [5–7]. In this experiment, the cell's ability to mobilize Ca^{2+} was tested with TLC-S. The presence of antibody to PtdInsP_2 within the cell had no effect on the TLC-S-evoked rise in the Ca^{2+} -dependent K^+ conductance.

Fig. 3. Lack of effect of the internal perfusion of a single guinea-pig hepatocyte with anti-PtdInsP₂ antibody on the activation of the Ca²⁺-dependent K⁺ conductance by InsP₃ released from a caged precursor

The cell was internally perfused with 50 µg of anti-PtdInsP₂ antibody/ml. The responses evoked by maximal concentrations of noradrenaline (NOR; 5 µM) were blocked after 5 min (time elapsed since the beginning of perfusion is indicated inside each trace), whereas the response to TLC-S (200 µM) remained unaffected even for a longer time. Noradrenaline and TLC-S were applied along the horizontal bars. Photolytic release of InsP₃ (indicated by the vertical arrows) from the caged precursor introduced into the cell via the patch pipette together with the antibody (see under 'Methods') produced a fast and large rise in the K⁺ conductance. Each flash released approx. 1.5 µM-InsP₃ inside the cell. Membrane potential 0 mV.

As explained above, there are two main patterns in the noradrenaline responses. Both of them are reproduced by bile acids [7]. The ability of TLC-S to produce regular spikes of Ca²⁺ is rather striking. Low TLC-S concentrations (100 µM) with no effect on the InsP₃ production [7] are able to mimic the noradrenaline-evoked rises in [Ca²⁺]_i. The effects of the anti-PtdInsP₂ antibody on the shape of the responses induced by TLC-S are shown in Fig. 2. As in Fig. 1, kt3g (100 µg/ml) was introduced into the cell via the patch pipette. In these experiments, the responses to noradrenaline were blocked within the first 4 min after going to whole-cell recording configuration, whereas control cells from the same dish without antibodies responded to noradrenaline on the same day. The application of 100 µM-TLC-S to two different hepatocytes gave rise to the two patterns of responses. In the first (Fig. 2a), we observed a sustained plateau phase with fast fluctuations of [Ca²⁺]_i. In the second (Fig. 2b), regular spikes of Ca²⁺ were recorded. For that cell, the interval between two spikes was approx. 1 min.

It was decided to investigate the effects of Ca²⁺-free external solutions on the TLC-S-evoked rise in [Ca²⁺]_i in the presence of the anti-PtdInsP₂ antibody. When the noradrenaline responses were blocked by the antibodies, cells were superfused with a Ca²⁺-free solution. After approx. 30 s of superfusion to wash out external Ca²⁺, TLC-S was added (Fig. 2c), and elicited the usual large rise in the Ca²⁺-dependent K⁺ conductance. This showed that bile acids could evoke large and fluctuating rises in [Ca²⁺]_i in the absence of external Ca²⁺ and in the absence of any InsP₃ production. This also confirmed previous work on suspensions of rat hepatocytes which showed that the bile-acid-induced rise in [Ca²⁺]_i measured with a fluorescent dye was independent of external Ca²⁺ [5]. The bile acids also have been shown to have no effect on Ca²⁺ permeability of the plasma membrane of rat liver cells [16].

The anti-PtdInsP₂ antibody had no effect on the InsP₃ receptor itself. The response to a photolytic release of InsP₃ from a caged precursor [11,17] introduced into the cell together with 50 µg of the antibody/ml was not affected, whereas in the same cell the noradrenaline-evoked response was totally blocked (Fig. 3).

DISCUSSION

In addition to the blockade of the noradrenaline-evoked response by the anti-PtdInsP₂ antibody, it is interesting that it was possible to observe [Ca²⁺]_i fluctuations with TLC-S. We have previously shown that TLC-S was able to mobilize Ca²⁺ from internal stores in the presence of intracellular heparin to block the InsP₃ receptors [7]. However, a recent study has shown that heparin is also able to uncouple the α₁-adrenoceptor from its G-protein in the rat liver plasma-membrane fraction [18]. These authors have suggested that this action of heparin limits its utility as a selective antagonist of the InsP₃ receptor in whole cells. A direct consequence of this observation is that the block of the response to noradrenaline in heparin-loaded liver cells did not necessarily indicate a complete inhibition of the InsP₃ receptors. The possibility remained that, since high doses of TLC-S (200–300 µM) slightly activate InsP₃ production [7], the resulting InsP₃ production would have activated some unaffected InsP₃ receptors to release Ca²⁺ from the intracellular stores. The present results show that TLC-S releases Ca²⁺ from internal stores independently of InsP₃ production. The origin of the Ca²⁺ is intracellular, as shown by the lack of effect of superfusion of the guinea-pig liver cell with a Ca²⁺-free solution. The mechanism by which TLC-S mobilizes Ca²⁺ to create fluctuations of [Ca²⁺]_i similar to those observed with Ca²⁺-mobilizing hormones is still unknown. One way to interpret these results in the light of recent work, showing an increased sensitivity of the InsP₃ receptors to InsP₃ at [Ca²⁺]_i values up to 500 nM [19,20], is to claim that TLC-S produces a primary increase in [Ca²⁺]_i in the vicinity of InsP₃ receptors in a Ca²⁺-induced Ca²⁺-release-like mechanism. This would therefore lead to an amplified rise in [Ca²⁺]_i and to the resulting fast and slow fluctuations in [Ca²⁺]_i by using the same mechanisms as those triggered by noradrenaline. This proposal requires that the resting InsP₃ concentration would be sufficient to trigger the response when the InsP₃ receptor's sensitivity is increased. It has been suggested that the resting InsP₃ concentration in hepatocytes is relatively high [21]. A Ca²⁺-induced sensitization of the InsP₃ receptors would also imply that, in the presence of anti-PtdInsP₂ antibody, the resting InsP₃ concentration stays constant. It is also assumed that the resting InsP₃ value only depends on the degradation of the PtdInsP₂ associated with the plasma-membrane receptors, PtdInsP₂ that are blocked by the antibody. In liver cells, most of the total PtdInsP₂ is located in the plasma membrane and only half of it is sensitive to the hormone-stimulated phospholipase C [22]. However, if the TLC-S effects are linked to a Ca²⁺-dependent activation of the InsP₃ receptors, the origin of the Ca²⁺ which primes the effect remains unclear and cannot be related to an obvious effect of the monohydroxylated bile acids on the Ca²⁺ permeability of plasma membrane or mitochondria [6].

The fact that TLC-S could mobilize Ca²⁺ from a non-mitochondrial InsP₃-insensitive store provides an alternative hypothesis. Preliminary experiments on saponin-treated rat liver cells have shown that TLC mobilizes more Ca²⁺ from the non-mitochondrial pools than does a maximally effective concentration of InsP₃ (L. Combettes, personal communication). Experiments using 2,5-di-(*t*-butyl)-1,4-benzohydroquinone, a blocker of the Ca²⁺-ATPase of the intracellular Ca²⁺ stores, led to similar conclusions [23]. It is therefore likely that the InsP₃-sensitive Ca²⁺ store is only a part of the total non-mitochondrial

Ca²⁺ stores. The presence of multiple Ca²⁺ stores has been suggested in various cells (for review see [24,25]) including liver cells [26]. This heterogeneity of microsomal Ca²⁺ stores has been confirmed by immunocytochemistry in chicken Purkinje neurons [27]. The fact that TLC-S can release more Ca²⁺ than does InsP₃ indicates that the bile acid possibly opens specific channels permeable to Ca²⁺ and produces Ca²⁺ efflux from both InsP₃-sensitive and InsP₃-insensitive non-mitochondrial Ca²⁺ stores. This would then suggest that Ca²⁺ ions could regulate their own release and re-uptake by the Ca²⁺ stores in liver cells.

In conclusion, our results show that [Ca²⁺]_i fluctuations can be produced independently of InsP₃ production, and therefore that they may be linked to an intrinsic property of the intracellular stores to regulate their own Ca²⁺ permeability.

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