Permissive role of cAMP in the oscillatory Ca^{2+} response to inositol 1,4,5-trisphosphate in rat hepatocytes

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Rat hepatocytes respond to α -adrenergic stimulation by intracellular production of *myo*-inositol 1,4,5-trisphosphate (IP_3) which stimulates the periodic release and reuptake of intracellular store (IS) Ca^{2+} . The generation of these Ca^{2+} oscillations was investigated by simultaneously monitoring Ca^{2+} changes in the cytosol and IS by combined fluorescence microscopy and whole-cell patch clamp. Intracellular IP_3 perfusion (1–50 μM in the pipette) produced three types of Ca^{2+} response: understimulation, oscillations and overstimulation, i.e. with Ca^{2+} levels not returning to baseline. In a total of 57 experiments, only three displayed oscillations during continuous IP_3 infusion, in a narrow range of IP_3 concentration centred around 5–8 μM in the pipette.

In oscillating cells, cytosolic Ca^{2+} spikes were synchronized with transient Ca^{2+} depletions of the IS, consistent with a direct exchange of Ca^{2+} between the two compartments. Application of 8-Br-cAMP to cells infused with IP_3 increased the probability of eliciting Ca^{2+} oscillations by a factor of 4–5 for IP_3 concentrations in the range 1–10 μM , whereas IP_3 concentrations above 10 μM always resulted in overstimulation. IP_3 photorelease experiments and measurements of IS Ca^{2+} content indicated that 8-Br-cAMP enhanced the affinity of the IP_3 receptor and increased the pool of releasable Ca^{2+} . We propose that cAMP has a permissive role in the generation of IP_3 -induced Ca^{2+} oscillations by extending the window of IP_3 concentrations able to elicit oscillations.

INTRODUCTION

Myo-inositol 1,4,5-trisphosphate (IP_3) generated after activation of the phosphoinositide pathway is involved in the mechanism of agonist-induced Ca^{2+} oscillations in several non-excitable cell types. In hepatocytes, hormones using this pathway include α -adrenergic hormones, vasopressin, angiotensin II and adenosine nucleotides [1].

Based on the growing experimental data, the most recent models [2–4] assign the intracellular Ca^{2+} stores (IS) as the major source of Ca^{2+} for the oscillatory process in rat hepatocytes. The binding of the agonist to its plasma-membrane receptor activates the phosphoinositide pathway, which results in the production of IP_3 and the activation of the IP_3 receptor/ Ca^{2+} channel of the IS. The IP_3 receptor/ Ca^{2+} channel then triggers the rapid release of Ca^{2+} into the cytosol, which lasts until feedback mechanisms close or inactivate the channel, allowing the cell to restore both cytosolic and IS Ca^{2+} to their resting value. Ca^{2+} spiking repeats itself in a periodical way at a stable frequency, which is mainly determined by the concentration of the agonist in the extracellular medium [1]. Above a certain agonist concentration, cytosolic Ca^{2+} no longer oscillates but remains chronically increased, a type of response that is referred to as overstimulation [5].

Studies using whole-cell patch-clamp techniques have demonstrated that continuous G-protein activation [6] or continuous IP_3 infusion [7,8] are sufficient ingredients for the induction of Ca^{2+} oscillations in pancreatic acinar cells in whole-cell patch-clamp experiments. Non-metabolized analogues of IP_3 have also been found to elicit Ca^{2+} oscillations in guinea-pig hepatocytes [9] as well as in pancreatic acinar cells [8], which argues against pulsatile IP_3 production as the basis of Ca^{2+} oscillations.

The present study investigates the role of cAMP in the generation of IP_3 -induced Ca^{2+} oscillations in rat hepatocytes, in a situation where both cytosolic and IS Ca^{2+} levels were monitored. The results presented in this study suggest that cAMP, probably through its activation of protein kinase A (PKA), is a

permissive or possibly necessary factor for the generation of Ca^{2+} oscillations in rat hepatocytes.

MATERIALS AND METHODS

Rat hepatocytes were isolated from male Wistar rats and attached to collagen-coated glass coverslips as described previously [10].

The setup used for fluorescence microscopy and flash photolysis was as described in detail previously [4,10]. Simultaneous measurements of Ca^{2+} in the cytosol and the IS of hepatocytes were carried out as previously described [11]. Briefly, hepatocytes were loaded for 30 min at 37 °C with 4–8 μM mag-fura 2 acetoxymethyl ester in Ham F12/Hepes with 1% BSA and placed in a dye-free solution for an additional 30 min. This procedure resulted in substantial localization of the dye in subcellular compartments. Whole-cell patch of dye-loaded cells was performed using a pipette containing anionic fluo 3 (see composition below). Mag-fura 2 and fluo 3 were selected for their K_D for Ca^{2+} (53 μM and 316 nM respectively) and their compatible spectral characteristics. During the first few minutes of intracellular perfusion, cytosolic mag-fura 2 was washed out while fluo 3 simultaneously filled the cytosolic compartment. At equilibrium, i.e. about 10 min after establishment of whole-cell configuration, fluo 3 was located in the cytosol, and mag-fura 2 was mainly associated with intracellular compartments, which have been functionally associated with the IS [11]. When relevant, the inhibitor of the endoplasmic-reticulum Ca^{2+} -ATPase, thapsigargin (1 μM), was added towards the end of the experiment to discharge Ca^{2+} completely from the IS and define the minimum mag-fura 2 ratio. Permeabilization of the plasma and IS membranes was achieved using ionomycin (4 μM) and provided the maximal ratio of saturating Ca^{2+} concentration. IS Ca^{2+} concentration was calculated as described previously [11] and yielded a mean value of $370 \pm 87 \mu\text{M}$ in this series of experiments ($n = 9$).

Exchange of the pipette solution during the experiment [12] was achieved using a tapered fused silica capillary positioned

Abbreviations used: IP_3 , *myo*-inositol 1,4,5-trisphosphate; 3-F- IP_3 , 3-deoxy-3-fluoro-D-*myo*-inositol 1,4,5-trisphosphate; IS, intracellular Ca^{2+} stores; PKA, protein kinase A.

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about 100 μm away from the pipette tip and used in conjunction with a precision pump (Adams & List, New York, NY, U.S.A.). In experiments in which only cytosolic Ca^{2+} was monitored, anionic fura 2 (100 μM) was included in the pipette solution and a 340 nm/380 nm excitation ratio was used.

Solutions and materials

The bath solution was Ham F12 culture medium containing 10 mM Hepes and 1.8 mM CaCl_2 and adjusted to pH 7.4, or a similar solution containing (mM): 145 NaCl, 5.6 KCl, 0.8 MgSO_4 , 1.8 CaCl_2 , 10 Hepes, 10 glucose. The pipette solution for the patch-clamp experiment contained (mM): 138 potassium glutamate, 8 NaCl, 5 MgCl_2 , 1 EGTA, 0.5 CaCl_2 , 10 Hepes, 1 ATP, 1 GTP, 2 glutathione, 0.02–0.03 fluo 3 or 0.1 fura 2, and was adjusted to pH 7.2. The pipette solution used for micro-injection contained (mM): 138 potassium glutamate, 8 NaCl, 5 MgCl_2 , 1 EGTA, 0.5 CaCl_2 , 10 Hepes, 2 fluo 3, 0.8 or 1.5 *myo*-inositol 1,4,5-trisphosphate $\text{P}^{4(5)}$ -1-(2-nitrophenyl)ethyl ester (caged IP_3). Experiments were performed at room temperature.

Fura 2, mag-fura 2 acetoxymethyl ester and fluo-3 were from Molecular Probes (Eugene, OR, U.S.A.). ATP, Ham F12 medium, BSA, thapsigargin and ionomycin were from Sigma (St. Louis, MO, U.S.A.). IP_3 hexapotassium was from LC Laboratories/Alexis Corp. (Läufelfingen, Switzerland). Caged IP_3 and 3-deoxy-3-fluoro-D-*myo*-inositol 1,4,5-trisphosphate (3-F- IP_3) were from Calbiochem (La Jolla, CA, U.S.A.). Rp-cAMPs were from Biomol/Anawa Trading (Wangen, Switzerland). The other chemicals were from Fluka (Buchs, Switzerland).

Expression of results

Data are presented as means $\pm$ S.E.M. When appropriate, paired Student's *t* test was performed to assess the statistical significance of differences.

RESULTS

IP_3 infusion and Ca^{2+} oscillations

According to many models of Ca^{2+} oscillation in non-excitable cells, IP_3 and its intracellular receptor are the central elements for the generation of Ca^{2+} oscillations. To examine whether IP_3 alone could trigger oscillations in rat hepatocytes, we designed experiments in which IP_3 was delivered to cells. Because the half-life of IP_3 is short [13] as the result of extensive metabolism, steady IP_3 stimulation was achieved by continuous delivery. Thus, in addition to the whole-cell patch-clamp technique outlined previously for monitoring both cytosolic and IS Ca^{2+} changes [11], perfusion of the pipette was implemented to allow replacement of the control intracellular solution with a second one containing IP_3 .

Figure 1 depicts such an experiment in which internal perfusion of IP_3 elicited cytosolic Ca^{2+} oscillations as well as downward deflections of Ca^{2+} concentration in the IS. These periodic changes in IS Ca^{2+} concentration occurred in phase with cytosolic oscillations and were consistent with a cycling of Ca^{2+} between the IS and cytosol. This oscillatory behaviour was found to be qualitatively identical with that observed previously using the α -adrenergic agonist phenylephrine instead of IP_3 [11]. One can assume that IP_3 is able to generate Ca^{2+} oscillations of the same type as agonists of the phosphoinositide pathway and is therefore a sufficient ingredient for the generation of Ca^{2+} oscillations in rat hepatocytes.

Application of IP_3 did not systematically result in Ca^{2+} oscillations. In fact, the oscillatory response was observed in only a very few cells, regardless of the concentration of IP_3 present in

Figure 1 Ca^{2+} oscillations triggered by IP_3 infusion

Hepatocytes were exposed to variable amounts of IP_3 by internal perfusion from a patch pipette. In the experiment presented, 5 μM IP_3 was applied as indicated by the bar inside the graph. Cytosolic Ca^{2+} was monitored using fluo 3 at 480 nm excitation (—), and Ca^{2+} in the IS was measured with mag-fura 2 using the 340 nm/380 nm excitation ratio (.....).

the patch pipette. We initially expected that a range of IP_3 concentration would be determined that would consistently produce Ca^{2+} oscillations. However, we found that cells would either not respond to IP_3 application or would exhibit overstimulation, in a quasi all-or-none manner. In order to examine this phenomenon more closely, we performed a systematic analysis of the type of response for a wide range of IP_3 concentrations. Experiments were sorted according to three types of response: understimulated, where no Ca^{2+} response was elicited after IP_3 infusion; overstimulated, where Ca^{2+} release was maximally stimulated and Ca^{2+} concentration remained at an elevated steady-state; oscillatory, where more than one Ca^{2+} transient was observed. The result of this analysis is depicted in Figure 2. As could be expected, the number of understimulated cells decreased as the IP_3 concentration was raised, and the percentage of overstimulated cells increased proportionally. The oscillatory response was observed with an overall probability of about 5% ($n = 57$) and occurred only at pipette IP_3 concentrations centred around 5–8 μM .

This unexpectedly low rate of occurrence of oscillations led us to consider the possibility that cells stimulated with agonists produce, in addition to IP_3 , some cofactor that makes the oscillatory behaviour more likely to occur. Along with IP_3 , phospholipase C generates diacylglycerol, a known activator of protein kinase C. We tested whether protein kinase C activators, which have a negative regulatory effect on Ca^{2+} oscillations [14,15], would bring the cells from the overstimulated to the oscillatory state. PMA (100 nM), di-octanoyl glycerol (20 μM) and oleoyl-2-acetyl-*sn*-glycerol (30 μM) had no influence on the behaviour of cells ($n = 7$; results not shown). Another alternative could have been that, under these experimental conditions, the fast metabolism of IP_3 rendered the oscillatory response unlikely. To investigate this possibility, experiments were conducted using a non-metabolized analogue of IP_3 , namely 3-F- IP_3 , which was reported to activate the IP_3 receptor with similar potency to natural IP_3 [16,17]. 3-F- IP_3 was internally perfused at concentrations ranging from 5 to 50 μM ($n = 41$), and the cytosolic Ca^{2+} response was monitored. Cells responded in a very similar way to 3-F- IP_3 as to native IP_3 : 3-F- IP_3 concentrations below 25 μM resulted in understimulation in 80% of the cells tested ($n = 31$) and concentrations of 25 μM and above yielded an

Figure 2 Distribution of Ca^{2+} response type as a function of pipette IP_3 concentration

Experiments performed with IP_3 concentrations in the pipette ranging from 1 to 50 μM were pooled and the number of observations for each group is given in the graph. The ordinate represents the percentage of cells that exhibited understimulation (□), oscillation (■) or overstimulation (▨) for each range of IP_3 concentration.

overstimulatory Ca^{2+} response in 80 % of the cases ($n = 10$). No clearly defined Ca^{2+} oscillations were obtained after 3-F- IP_3 infusion. Thus, infusion of both the non-metabolized analogue 3-F- IP_3 and native IP_3 resulted in an all-or-none Ca^{2+} response in the majority of cells.

Effects of cAMP on the response to IP_3 infusion

Another modulator of Ca^{2+} oscillations in rat hepatocytes is cAMP acting via stimulation of PKA. It has been shown that cAMP enhances the response of hepatocytes to agonists of the phosphoinositide pathway [15,18]. We tested whether stimulation of PKA using 8-Br-cAMP, a cell-permeable and phosphodiesterase-resistant analogue of cAMP, had any influence on the response to IP_3 infusion.

In a first series of experiments, the cells were internally perfused with 4–10 μM IP_3 , the range of concentrations at which Ca^{2+} oscillations are most likely to be observed (Figure 2). If no Ca^{2+} response was elicited (understimulation), 8-Br-cAMP was applied at concentrations ranging from 10 to 40 μM . Figure 3 shows such an experiment, in which Ca^{2+} oscillations started appearing after 8-Br-cAMP application and proceeded in a sustained manner. Cytosolic Ca^{2+} oscillations were accompanied by corresponding periodic depletion in the IS, as was observed after IP_3 stimulation in the absence of 8-Br-cAMP and after agonist stimulation [11], indicating that Ca^{2+} oscillations elicited by these three different types of stimulation probably shared a common mechanism.

Using this protocol, the rate of occurrence of oscillations increased considerably, by a factor of about 5, compared with cells stimulated by IP_3 in the same concentration range (4–10 μM) but in the absence of 8-Br-cAMP. In the control group (IP_3 alone), three of 28 cells responded with Ca^{2+} oscillations, while seven of 13 cells subsequently treated with 8-Br-cAMP displayed

Figure 3 Ca^{2+} oscillations triggered by internal perfusion of IP_3 after application of 8-Br-cAMP

Hepatocytes were exposed to variable amounts of IP_3 by internal perfusion from a patch pipette, and, if no response was elicited, 8-Br-cAMP (10–40 μM) was added to the bath. In the experiment shown, 10 μM IP_3 was internally perfused, followed by addition to the bath of 10 and 20 μM 8-Br-cAMP, as indicated inside the graph. Cytosolic and IS Ca^{2+} were monitored as in Figure 1.

oscillations. The same effect of 8-Br-cAMP was observed with 3-F- IP_3 ($n = 3$).

We then investigated whether this effect of 8-Br-cAMP applied only in the range of IP_3 concentrations tested (4–10 μM), or whether it was present regardless of the IP_3 concentration. Cells were incubated with 8-Br-cAMP (10 μM) for at least 3 min before internal IP_3 perfusion. For these experiments, only cytosolic Ca^{2+} was monitored using fura 2 in the patch pipette. The distribution of the type of Ca^{2+} response in the presence of 8-Br-cAMP was constructed as above and is presented in Figure 4. A

Figure 4 Distribution of Ca^{2+} response type as a function of pipette IP_3 concentration in the presence of 8-Br-cAMP

Cells were incubated for at least 3 min in the presence of 10 μM 8-Br-cAMP before internal perfusion of IP_3 . The percentage of cells exhibiting understimulation (□), oscillations (■) or overstimulation (▨) is represented for IP_3 concentrations as for Figure 2. The number of experiments for each group is given in the graph.

Figure 5 Effect of 8-Br-cAMP on supramaximal IP₃ stimulation

(A) Hepatocytes were exposed to 50 μM IP₃ by internal perfusion from a patch pipette. Once a steady-state Ca²⁺ level in the IS was reached, 8-Br-cAMP (20–50 μM) was added to the bath. Towards the end of the experiments, thapsigargin (TG; 1 μM) and ionomycin (Iono; 4 μM) were added to first completely deplete and then saturate respectively the IS with Ca²⁺. Cytosolic Ca²⁺ was monitored using fluo 3 at 480 nm excitation (—), and Ca²⁺ in IS was measured with mag-fura 2 using the 340 nm/380 nm excitation ratio (· · · ·). The graph is representative of 10 experiments. (B) The relative amount of Ca²⁺ released after 8-Br-cAMP application, expressed as percentage of total Ca²⁺ capacity of the IS, is plotted against the relative amount of Ca²⁺ remaining in the IS after IP₃ internal perfusion. The total capacity of the IS (i.e. 100%) was calculated from the difference between the IS Ca²⁺ concentration before IP₃ infusion (full stores) and after thapsigargin treatment (empty stores). The dotted line was obtained by linear regression, which yielded a correlation coefficient of 0.941.

first observation revealed that, in the range of IP₃ concentration (4–10 μM) used in the first 8-Br-cAMP protocol, the increase in the occurrence of oscillations was similar, about 4-fold higher than in control experiments. This enhancing effect of 8-Br-cAMP on Ca²⁺ oscillations was not only present in the narrow window of IP₃ concentration found with IP₃ alone (Figure 2), but yielded a constant probability of eliciting oscillations of about 40% from 1 to 10 μM IP₃. At higher IP₃ concentrations, however, only overstimulation was observed, as was the case in the absence of 8-Br-cAMP. Thus 8-Br-cAMP not only increased the chances of eliciting oscillations with IP₃ in its optimal range of concentration

but in addition extended the range of IP₃ concentration able to evoke oscillations.

Mechanisms of cAMP action

We then attempted to provide explanations for the observed effects of cAMP. The IP₃ receptor is an appropriate target of modulation for cAMP, and the next series of experiments was designed to examine whether cAMP was directly altering the properties of the IP₃ receptor.

The first hypothesis considered was that cAMP acts by preventing the system from falling almost systematically in overstimulation when enough IP₃ was present to elicit a response. This would allow the IS to maintain higher levels of Ca²⁺ during stimulation or to become replenished more easily. This hypothesis was tested using the following experimental protocol: cells were overstimulated with 50 μM IP₃ and the Ca²⁺ content of the stores was monitored. 8-Br-cAMP (20–50 μM) was then applied. In 10 experiments, IS partially discharged by overstimulation of IP₃ did not recover after 8-Br-cAMP addition (Figure 5A), but, on the contrary, released even more Ca²⁺. This hypothesis was therefore discarded.

This series of experiments revealed some new aspects of the mode of action of IP₃. (1) Even supramaximal concentrations of IP₃ did not generally empty the thapsigargin-sensitive Ca²⁺ pools, in some cases, the Ca²⁺ in the IS being only barely altered. (2) As can be seen in Figure 5(A), 8-Br-cAMP enabled the release of a fraction of stored Ca²⁺ that was previously not releasable by supramaximal IP₃ alone. As depicted in Figure 5(B), inspection of the IS content in these experiments revealed that there was a strong correlation between the amount of Ca²⁺ released after 8-Br-cAMP application (expressed as percentage of total IS capacity) and the amount of Ca²⁺ left after IP₃ internal perfusion (percentage of total IS capacity). Furthermore, the fact that the amount of Ca²⁺ released after 8-Br-cAMP treatment tended toward zero when most of the Ca²⁺ had been released by IP₃ alone indicated that the mobilized Ca²⁺ ions originate from a common source. Taken together, these experiments indicated that 8-Br-cAMP increased the size of the pool of Ca²⁺ accessible to IP₃.

Another possible effect of cAMP on the IP₃ receptor could be acceleration of the transition from the closed to the open state, either by enhancing the co-operative effect of cytosolic Ca²⁺ itself on the receptor or by increasing the conductance of the channel. Both should result in a faster release of Ca²⁺ from the IS. To investigate this option, photorelease of microinjected caged IP₃ was performed. Ca²⁺ spikes were triggered by photolysis of caged IP₃ using 1 ms flashes of UV light delivered by a xenon flash lamp in the absence and then in the presence of 8-Br-cAMP (10–20 μM). Figure 6 shows an example of such an experiment in which cells had been injected with 0.8 mM caged IP₃ in the injection pipette (approx. 10 μM intracellular) and low intensity flashes were applied in a control period and then in the presence of 8-Br-cAMP after 3–5 min of incubation. Kinetic characteristics of Ca²⁺ spikes were described in terms of (1) lag time between the photorelease and the onset of the Ca²⁺ response, (2) rate of rise of Ca²⁺ signal calculated as the time to 50% ($t_{50\%}$) and 100% ($t_{100\%}$) of the peak full amplitude, (3) rate of recovery calculated from a single exponential fit of the data. The results of these experiments are summarized in Table 1 and indicate that the only parameter that was influenced by 8-Br-cAMP was the amplitude of Ca²⁺ spiking which increased by a factor of approx. 4. In three experiments of this series, the cAMP antagonist Rp-cAMPs (50 μM) [19] was added after washout of 8-Br-cAMP and was

Figure 6 Effect of 8-Br-cAMP on Ca^{2+} spikes induced by IP_3 photorelease

Hepatocytes were microinjected with the photolabile inactive precursor of IP_3 (caged IP_3) and the Ca^{2+} indicator fluo 3. Flashes of UV light (1 ms) were applied at the time indicated by the arrow. The response to a control flash (curve a) is compared with the response obtained after a 3–5 min incubation in the presence of 10 μM (curve b) and 20 μM (curve c) 8-Br-cAMP on the same cell. For clarity, the three curves have been shifted in time to make the flashes coincide. No other scaling was used in the plots. The kinetic analysis of this series of experiments is summarized in Table 1.

Table 1 Effects of 8-Br-cAMP on the kinetics of Ca^{2+} spikes induced by photorelease of IP_3

Kinetic parameters of Ca^{2+} transients obtained from 16 separate experiments are presented for caged IP_3 -injected rat hepatocytes. The lag time is defined as the time elapsed between the 1 ms flash and the appearance of the Ca^{2+} response. The parameters $t_{50\%}$ and $t_{100\%}$ are defined as the time needed to reach 50% and 100% respectively of the maximal Ca^{2+} peak amplitude from the baseline. Amplitude changes are expressed as percentage of the fluo 3 intensity changes measured in the gray scale. The rate of recovery was computed from single exponential kinetics fitted to the curve. Paired Student's *t* test was performed to assess the significance of differences of the 16 experiments: **P* < 0.0001.

	Control	8-Br-cAMP (10–20 μM)
Lag time (s)	0.35 $\pm$ 0.03	0.36 $\pm$ 0.03
$t_{50\%}$ (s)	1.62 $\pm$ 0.24	1.55 $\pm$ 0.31
$t_{100\%}$ (s)	4.29 $\pm$ 0.53	4.00 $\pm$ 0.76
Amplitude (%)	100	457 $\pm$ 104*
Rate of recovery (s^{-1})	0.10 $\pm$ 0.03	0.10 $\pm$ 0.02

found to reverse the stimulatory action of 8-Br-cAMP, confirming the specificity of the observed effects.

In another series of experiments ($n = 5$) in which cells had been injected with double the amount of caged IP_3 (1.5 mM in the injection pipette, about 20 μM intracellular) and in which a higher flash intensity had been applied, photorelease of IP_3 in the control conditions resulted in larger Ca^{2+} spikes, and 8-Br-cAMP was not able to alter any kinetic parameter. The fact that 8-Br-cAMP did not alter the amplitude of spiking indicated that the stimulatory effect of 8-Br-cAMP was present only when the amount of IP_3 released was not saturating the IP_3 receptor or emptying the IS.

DISCUSSION

The present study shows that cAMP greatly favours the oscillatory Ca^{2+} response of hepatocytes stimulated by direct application of IP_3 . Although IP_3 is in principle a sufficient and minimal ingredient for the generation of Ca^{2+} oscillations in rat

hepatocytes, its ability to elicit this type of response was found to be limited. Under the experimental conditions used in which the soluble cytosolic components were replaced by a solution of defined composition, Ca^{2+} oscillations rarely occurred after IP_3 addition, the observed responses being either understimulation or overstimulation in an almost all-or-none fashion.

Nevertheless, the present study showed that Ca^{2+} oscillations after IP_3 infusion did occur in rat hepatocytes, as was found with other cell types such as pancreatic acinar cells [7,8] or guinea-pig hepatocytes [9]. Ca^{2+} oscillations induced by IP_3 were found to be based on an exchange of Ca^{2+} between the IS and the cytosol, and were qualitatively indistinguishable from Ca^{2+} oscillations obtained by agonist stimulation [10,11]. Recently, the IS of rat hepatocytes studied in a situation where the cell membrane had been permeabilized also responded to IP_3 application with oscillatory release of Ca^{2+} from the IS [20].

The fact that Ca^{2+} oscillations rarely occurred after IP_3 infusion alone led us to postulate that the oscillatory response would occur only when a perfect match of functional parameters was present in the cell, a condition that hormonal stimulation, as opposed to IP_3 stimulation, would naturally bring about. The response of rat hepatocytes to IP_3 that we observed cannot be attributed to continuous degradation of the IP_3 introduced through the pipette since the non-metabolized analogue 3-F- IP_3 induced the same type of all-or-none response.

Of the possible missing cofactors tested, cAMP was found to significantly increase the rate of occurrence of Ca^{2+} oscillations. This was also noticed in a recent study [20] in which a cAMP analogue was routinely added to improve the responsiveness of the cell preparation. cAMP is produced in rat hepatocytes after activation of β -adrenergic or glucagon receptors and is known for its synergistic effect on hormones of the phosphoinositide pathway [15]. It was also reported to be released to some extent in hepatocytes by α -adrenergic stimulation [21]. Its effects translate into an increase in the frequency of Ca^{2+} oscillations up to overstimulation.

Although cAMP has been shown to enhance the production of IP_3 in the presence of vasopressin [22], it was not the case during phenylephrine stimulation [23,24]. In the context of our present study, the main and most relevant effects of cAMP are probably at the level of the IP_3 receptors, which can undergo phosphorylation by PKA in hepatocytes [25]. Whereas in certain tissues such as brain, cAMP reduces the affinity of the IP_3 receptor for IP_3 [26,27], it increases it in hepatocytes [28,29].

PKA stimulation was associated with an increased affinity of the receptor towards IP_3 for opening the ion channel without altering the number or gating properties of these channels [29]. Our results tend to support this conclusion, since Ca^{2+} spikes induced by photorelease of IP_3 revealed no change in the kinetics of the transients after cAMP stimulation, apart from the increase in amplitude. cAMP would therefore increase the sensitivity of the IP_3 receptor toward IP_3 and yield a stronger receptor stimulation at a given IP_3 concentration. Furthermore, the fact that the rate of recovery was the same in the presence and absence of cAMP allowed us to exclude a significant influence of cAMP on the active transport of Ca^{2+} at the IS or the cell membrane, as well as on the metabolism of IP_3 , as described previously [28].

Our experiments analysing the type of response after IP_3 stimulation showed that cAMP not only increased the probability of the oscillatory response but also broadened the range of IP_3 concentration compatible with oscillations. It is not yet clear by what mechanism an increase in the sensitivity to IP_3 would achieve these effects. They, however, applied only to IP_3 levels that were not already saturating, as evidenced by the lack of

effect of cAMP after photorelease of larger amounts of IP_3 , and by the fact that the oscillatory behaviour did not extend to higher IP_3 concentrations.

The molecular elucidation of the cAMP effects on the IP_3 receptor and of their functional consequences is complicated by the fact that at least three types of receptor have been identified so far [30] which differ in their affinity for IP_3 [31] as well as in their regulation by Ca^{2+} [32] and their ability to undergo phosphorylation by PKA [30]. In liver cells, only IP_3 receptor types I and II have been found, type II being the most abundant [33], and heterotetramers of types I and II are expected to be present [34]. Phosphorylation by PKA of IP_3 receptor type I has been demonstrated in hepatocytes [25]. Since type-II receptors do not contain the consensus phosphorylation sites for PKA [30], one can assume that only type-I IP_3 receptors mediate the effects of cAMP observed in hepatocytes.

Direct measurements of IS Ca^{2+} performed in the present study revealed that the IS were discharged to a variable extent by supramaximal IP_3 stimulation, even though an overstimulatory response was elicited. The same conclusion has been reached in previous studies involving maximal vasopressin, ADP or phenylephrine stimulation [10]. However, in the situation of maximal IP_3 stimulation (overstimulation), 8-Br-cAMP allowed further release of stored Ca^{2+} that was not releasable by IP_3 alone, thus supporting the idea that PKA stimulation increases the pool of releasable Ca^{2+} [28,29]. The correlation found between the amount of Ca^{2+} released after IP_3 plus 8-Br-cAMP and the amount left after IP_3 alone, as well as the fact that they both converge towards zero, indicate that they originate from a common pool. This argues against a 'silent' Ca^{2+} pool that is activated by cAMP.

This incremental emptying of the IS, in a sense, is reminiscent of the so-called quantal Ca^{2+} release [35], but could be explained solely by an increase in receptor affinity toward IP_3 : at lower affinity, the Ca^{2+} efflux from the IS would be moderate and compensated by Ca^{2+} reuptake, resulting in a partial depletion of the IS at steady-state. Increasing the affinity of the IP_3 receptor would suddenly change this steady-state to a more profound depletion. Another alternative is the existence of a multiple population of stores possessing different affinities for IP_3 that would contribute to the Ca^{2+} response. However, as discussed above, our data do not support the presence of distinct pools.

It is probable that, in whole-cell patch-clamp experiments, because of dialysis of low-molecular-mass molecules from the cytosol into the pipette, the resting level of cAMP is lowered considerably and that the basal level of PKA stimulation normally present in intact cells has disappeared. By increasing the affinity of the IP_3 receptors for IP_3 , cAMP would allow a larger number of receptors and/or a larger Ca^{2+} pool to participate in the response, thus allowing a more pronounced Ca^{2+} release. Ca^{2+} spiking would then be favoured by a positive co-operative effect of Ca^{2+} at concentrations of IP_3 below saturation. In conclusion, we propose that cAMP is a permissive

or maybe mandatory factor in the generation of Ca^{2+} oscillations in rat hepatocytes.

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