Agonist-specific behaviour of the intracellular Ca^{2+} response in rat hepatocytes

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A variety of agonists stimulate in hepatocytes a response that takes the shape of repetitive cytosolic free Ca^{2+} transients called Ca^{2+} oscillations. The shape of spikes and the pattern of oscillations in a given cell differ depending on the agonist of the phosphoinositide pathway that is applied. In this study, the response of individual rat hepatocytes to maximal stimulation by arginine vasopressin (AVP), phenylephrine and ADP was investigated by fluorescence microscopy and flash photolysis. Hepatocytes loaded with Ca^{2+} -sensitive probes were stimulated with a first agonist to evoke a maximal response, and then a second agonist was added. When phenylephrine or ADP was used as the first agonist, AVP applied subsequently could elicit an additional response, which did not happen when AVP was first applied and phenylephrine or ADP was applied later. Cells microinjected

with caged *myo*-inositol 1,4,5-trisphosphate (IP_3) were challenged with the different agonists and, when a maximal response was obtained, photorelease of IP_3 was triggered. Cells maximally stimulated with AVP did not respond to IP_3 photorelease, whereas those stimulated with phenylephrine or ADP responded with a fast Ca^{2+} spike above the elevated steady-state level, which was followed by an undershoot. In contrast, with all three agonists, IP_3 photorelease triggered at the top of an oscillatory Ca^{2+} transient was able to mobilize additional Ca^{2+} . These experiments indicate that the differential response of cells to agonists is found not only during Ca^{2+} oscillations but also during maximal agonist stimulation and that potency and efficacy differences exist among agonists.

INTRODUCTION

Hepatocytes as well many other types of cells have been shown to respond to a variety of hormonal stimuli by altering their Ca^{2+} concentration in a periodical way called Ca^{2+} oscillations ([1,2]; recently reviewed in [3]). At high concentrations of agonist, Ca^{2+} levels no longer oscillate but remain chronically elevated. In hepatocytes, these hormones include α -adrenergic hormones, vasopressin, angiotensin II and adenosine nucleotides (ATP, ADP, AMP) among others. Theoretical models have been proposed to explain the mechanism of oscillation generation [4,5]. The latest models generally involve the participation of intracellular Ca^{2+} stores as a major source of Ca^{2+} for the oscillatory behaviour. The proposed cascade of events that leads to Ca^{2+} spiking can be summarized by the following sequence: binding of the agonist to its receptor, activation of the associated G-proteins, followed by activation of phospholipase C which produces *myo*-inositol 1,4,5-trisphosphate (IP_3). Binding of IP_3 to the IP_3 receptor/ Ca^{2+} channel located in the membrane of intracellular Ca^{2+} stores stimulates the rapid release of Ca^{2+} into the cytosol. This release persists until feedback mechanisms close the channel allowing the cell to restore both cytosolic and intracellular-store Ca^{2+} to their respective resting levels. At constant agonist concentration, periodical Ca^{2+} spiking is routinely observed with a frequency that is mainly determined by the agonist concentration, whereas the amplitude remains fairly constant regardless of agonist concentration.

Although several agonists are known to induce such an oscillatory behaviour in hepatocytes [6], the shape of the Ca^{2+} transients and the pattern of oscillations can be substantially

different, suggesting that the idealized cascade of events described above is, in addition, modulated by other agonist-specific factors. For instance, we have recently shown [7] that the frequency of oscillations generated with the α -adrenergic agonist phenylephrine is very sensitive to intracellular pH changes, whereas oscillations obtained with other agonists such as vasopressin and ADP are independent of these changes.

In the present study we investigated the maximal response of hepatocytes to a set of different agonists, and showed by both combinations of agonists and photorelease of IP_3 that the extent of Ca^{2+} mobilization is different depending on the agonist.

MATERIALS AND METHODS

Preparation of hepatocytes

Isolated rat hepatocytes were prepared as described previously [8]. Briefly, the liver of male Wistar rats was perfused with an oxygenated Ca^{2+} -free EDTA solution. Mechanical dissociation of liver tissue followed by two sequential centrifugations allowed the isolation of single hepatocytes with a population viability of more than 80%. Cells were then placed in fetal bovine serum supplemented with 10–15% DMSO and frozen in liquid nitrogen for up to 10 days. A new batch of frozen cells was thawed every day. Cells were washed and seeded on collagen-coated glass coverslips and incubated at 37 °C in 5% CO_2 /95% air for 2 h in Ham's F12/bicarbonate medium containing 2% BSA. Unattached cells were resuspended and washed out, and hepatocytes attached to coverslips were kept at 4 °C in Ham's F12/Hepes with 1% BSA for a whole day.

Abbreviations used: IP_3 , *myo*-inositol 1,4,5-trisphosphate; caged IP_3 , *myo*-inositol 1,4,5-trisphosphate $P^{(4(5))}$ -1-(2-nitrophenyl)ethyl ester; AVP, [Arg⁸]vasopressin; AM, acetoxymethyl ester.

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Fluorescence microscopy

For Ca^{2+} measurements in the cytosol alone, cells were incubated at 37 °C in the presence of 3–5 μM fura 2 acetoxymethyl ester (fura 2 AM) for 20 min and then in the absence of dye for another 10 min. When relevant, the cytosolic Ca^{2+} signal was calibrated at the end of the experiment as previously described [9,10]. Simultaneous measurements of Ca^{2+} in the cytosol and in the intracellular Ca^{2+} stores were performed as previously described in detail [11]. Briefly, the low-affinity Ca^{2+} dye mag fura 2 acetoxymethyl ester (mag fura 2 AM; 4–8 μM) was loaded at 37 °C for 30 min and cells were then placed in a dye-free solution for an additional 30 min. This dye-loading procedure ensured adequate accumulation of mag fura 2 in thapsigargin-sensitive subcellular compartments. A whole-cell patch clamp was then performed using a pipette containing fluo 3. After an equilibration period of about 10–15 min, during which fluo 3 diffused from the pipette into the cytosol and the cytosolic mag fura 2 was washed out into the pipette, fluo 3 and mag fura 2 signals were associated with cytosol and intracellular stores respectively.

The fluorescence microscopy set up consisted of a video camera with image intensifier (Videoscope Int., Washington D.C., U.S.A.) attached to an inverted microscope (Nikon, Tokyo, Japan) equipped with a 100 $\times$ 1.3 N.A. objective lens (Nikon). The video image was digitized by an eight-bit image processor (Leutron, Glattburg, Switzerland) that allowed the selection of regions of interest and intensity measurement inside single cells. A fluorescence filter wheel (Sutter Instruments, Novato, CA U.S.A.) was used to illuminate the specimen with 480 nm (fluo 3) or 340 nm and 380 nm light (fura 2, mag fura 2). The filter cube contained a 510 nm dichroic mirror and 520 nm long-pass emission filter.

Microinjection and flash photolysis

Hepatocytes attached to coverslips were injected using micropipettes bevelled to approx. 50 M Ω (BV-10 Microelectrode Beveler; Sutter Instruments), and back-filled with an injection solution containing 2 mM fluo 3 and 1.5 mM *myo*-inositol, 1,4,5-trisphosphate $\text{P}^{(4,5)}$ -1-(2-nitrophenyl)ethyl ester (caged IP_3), using the Eppendorf 5246 (Eppendorf, Hamburg, Germany) injection system. The Ca^{2+} probe fluo 3 was chosen because its visible excitation at 480 nm does not cause unwanted photolysis of caged IP_3 .

The final concentration of caged IP_3 in the injected cells was estimated to be in the order of 20–25 μM . After injection, the cells were allowed to recover for 20–30 min before the start of the experiments. The coverslips with injected cells were then transferred to the fluorescence microscope described above.

Flashes of UV light of 1 ms duration were produced using a Xenon-flash-lamp system (XF-10; Hi-Tech Science, Salisbury, Wilts., U.K.) equipped with a UG-11 filter to select a band of UV light (300–390 nm) [12]. A liquid light guide with quartz focusing lens was used to bring the light to the microscope stage and produce a 2–3 mm² spot on the cells.

The energy delivered during each flash was adjusted by changing the charge voltage and/or by changing the combination of capacitors to a maximum calculated output energy of 331 J. The timing of flash-lamp triggering and image acquisition was controlled by computer. A cellular Ca^{2+} response could be observed after up to a dozen flashes, indicating that only a small fraction of the total pool of caged IP_3 injected underwent photolysis during one flash. Therefore repetitive photorelease could be routinely achieved in a given cell injected with caged IP_3 .

Solutions and materials

Bath solution was Ham's F12 culture medium containing 10 mM Hepes and 1.8 mM CaCl_2 and set to pH 7.4, or a similar solution containing 145 mM NaCl, 5.6 mM KCl, 0.8 mM MgSO_4 , 1.8 mM CaCl_2 , 10 mM Hepes, 10 mM glucose. The pipette solution of microinjection and whole-cell patch clamp contained (mM) 138 potassium glutamate, 8 NaCl, 5 MgCl_2 , 1 EGTA, 0.5 CaCl_2 and 10 Hepes, and was adjusted to pH 7.2. Patch-clamp pipette solution contained in addition (mM): 1 ATP, 1 GTP, 2 GSH and 0.02 fluo-3.

Caged IP_3 was from Calbiochem (La Jolla, CA, U.S.A.). Fura 2 AM, mag fura 2 AM and fluo 3 (K^+ salt) were from Molecular Probes (Eugene, OR, U.S.A.). ADP, Ham's F12 medium, BSA, thapsigargin and ionomycin were from Sigma (St. Louis, MO, U.S.A.). All other chemicals were from Fluka (Buchs, Switzerland).

Expression of data

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

RESULTS

Additive response of agonists

Hepatocytes stimulated with an appropriate dose of agonists such as phenylephrine respond with oscillations of the cytosolic free Ca^{2+} concentration. If the dose of agonist is increased in a stepwise fashion, the frequency of oscillations increases but the amplitude of Ca^{2+} spiking is not significantly altered. This acceleration of the frequency persists to a point where the cytosolic Ca^{2+} response is characterized by an elevated Ca^{2+} baseline sometimes accompanied by low-amplitude oscillations. This state of stimulation will be referred to as overstimulated [13] in the present study, as opposed to the oscillatory and the understimulated states, the latter being observed with a dose of agonist too low to elicit any Ca^{2+} response. In the overstimulated state, cytosolic Ca^{2+} levels remain elevated, and it has been implied [13] that this state corresponds to maximal stimulation of the IP_3 receptor. As a consequence, one could expect that the intracellular Ca^{2+} stores are depleted during overstimulation.

Three representative agonists were selected that act on distinct classes of receptors but which all use the phosphoinositide pathway leading to the intracellular production of IP_3 and to a Ca^{2+} response: the α_1 -adrenergic agonist phenylephrine, ADP acting on P_{2y} purinoceptors [14] and arginine vasopressin (AVP) acting on V_1 -vasopressin receptors [15]. Cytosolic Ca^{2+} was measured using fura 2 loaded via its acetoxymethyl ester derivative, and, as depicted in Figures 1 and 2, a combination of these agonists was then applied in the following sequence: the cells were stimulated with the first agonist using a concentration sufficient to reach an overstimulated cytosolic Ca^{2+} response (2–4 μM phenylephrine, 2–20 μM ADP, 1–20 nM AVP). The dose was then increased to at least double the initial amount to ensure that the response was maximal, after which a second agonist was applied at a concentration that would usually cause overstimulation when applied alone. Figure 1(A) presents an experiment where phenylephrine was used as the first agonist to obtain overstimulation. When AVP was subsequently added, further and abrupt Ca^{2+} mobilization was observed. By contrast, when AVP was applied as the first agonist, phenylephrine produced no additional effect on the Ca^{2+} concentration (Figure 1B). Corresponding experiments using combinations of ADP and AVP are presented in Figure 2.

Figure 1 Combination of phenylephrine and AVP during overstimulation

Hepatocytes were stimulated with a dose of a first agonist sufficient to produce an overstimulated Ca^{2+} response. A higher dose of this agonist was subsequently applied to ensure that maximal stimulation was achieved. A second agonist was then added to the first at a dose also sufficient to produce a full response. (A) AVP given on top of phenylephrine; (B) reverse order of agonist application: phenylephrine given on top of AVP stimulation. Both experiments are representative of a series of agonist combinations summarized in Table 1.

The cellular responses to combinations of the three agonists are summarized in Table 1. As can be seen, AVP had the capacity to produce a further Ca^{2+} increase on cells overstimulated with either phenylephrine or ADP. However, when AVP was used as the first agonist, neither phenylephrine nor ADP added later produced any further Ca^{2+} increase. Combinations of ADP and phenylephrine resulted in an additive response, although ADP appeared to induce a somewhat more potent effect on the Ca^{2+} release than phenylephrine. From these experiments, the efficacy of Ca^{2+} release of these agonists given at a saturating concentration can be tentatively sorted as follows: $\text{AVP} > \text{ADP} \geq \text{phenylephrine}$.

The next experiments were designed to test whether the efficacy

Figure 2 Combination of ADP and AVP during overstimulation

The same experimental protocol as in Figure 1 was used but ADP and AVP were the agonists. The experiments presented are representative of a series of agonist combinations summarized in Table 1.

of agonists manifested itself also when these agonists were individually applied on cells at saturating concentrations. Single cells were stimulated with phenylephrine to reach overstimulation, after which the agonist was washed out and the cells allowed to recover for 5–10 min. A second agonist (AVP or ADP) was then applied. The amplitude of the Ca^{2+} concentration from the baseline to the overstimulated plateau was measured and compared between two agonists on the same cell. The same protocol was applied to compare ADP and AVP, as well as ADP and phenylephrine. In this series of experiments, AVP produced a higher overstimulated Ca^{2+} concentration than phenylephrine ($147 \pm 10\%$; $n = 8$; $P < 0.01$) or ADP ($153 \pm 19\%$; $n = 8$; $P < 0.05$). Ca^{2+} mobilization by ADP was higher than that obtained with phenylephrine ($122 \pm 8\%$; $n = 7$; $P < 0.05$), but the difference was not as pronounced as with AVP. These results

Table 1 Effects of agonist combination during overstimulation experiments

Hepatocytes were challenged with a first agonist (left column) at a dose yielding an overstimulated Ca^{2+} response. After an overstimulated plateau was reached a higher dose of the first agonist was applied to ensure that maximal stimulation was achieved. A second agonist was then given. The number of independent experiments on single cells is given in parentheses.

First agonist	Second agonist...	Effect		
		Phenylephrine	ADP	AVP
Phenylephrine	—	—	Further mobilization ($n = 4$)	Further mobilization ($n = 5$)
ADP	Mixed response* ($n = 3/5$)	—	—	Further mobilization ($n = 3$)
AVP	No further mobilization ($n = 3$)	No further mobilization ($n = 3$)	—	—

* Further Ca^{2+} mobilization was observed in three of five experiments.

Figure 3 Amplitude of oscillatory peaks induced by different agonists on the same cell

Single-cell Ca^{2+} measurements were performed during sequential stimulation with phenylephrine (4.4 μM), AVP (1.4 nM) and ADP (3 μM), applied at doses eliciting an oscillatory response. Respective agonists were applied only during the period indicated by the horizontal bars.

essentially confirmed the sequence of efficacy deduced from the combination of agonists during overstimulation (see above).

The question as to whether the differential agonist efficacy had a consequence during sustained Ca^{2+} oscillations was next addressed. We examined whether the amplitude of spiking during sustained oscillations was different for these agonists on a given cell, as was observed during overstimulation. Figure 3 shows cytosolic Ca^{2+} oscillations in one hepatocyte sequentially stimulated with phenylephrine, AVP and ADP. Three separate experiments varying the order of agonist application revealed no systematic difference in amplitude. The same observation was made in another study [6] in which the agonists tested were ATP, phenylephrine, angiotensin II and vasopressin. Therefore, whereas the amount of Ca^{2+} released during oscillations is similar for different agonists, this is not so for overstimulation.

It is noteworthy that, in our hands, the oscillatory response seems to be more easily elicited in phenylephrine- than AVP-

Figure 4 IP_3 photorelease during AVP overstimulation

A control flash of UV light was applied to hepatocytes injected with fluo 3 and caged IP_3 first in the absence of agonist in order to show that the cells were responsive to caged IP_3 . The cells were then stimulated with a concentration of AVP producing an overstimulated response. A second flash was then applied to release IP_3 . This graph is an example from a series of six experiments that all gave the same result.

stimulated cells. In most of the cells tested, the range of AVP concentrations that induce Ca^{2+} oscillations is rather narrow, the cells quickly going from under- to over-stimulation. Conversely, on rare occasions, phenylephrine was not able to reach overstimulation, no matter what concentrations were used.

Photorelease of IP_3 during overstimulation

Another consequence of the additive effect observed during overstimulation is that the capacity to release Ca^{2+} does not seem to be exhausted during phenylephrine- or ADP-induced overstimulation, whereas it does after AVP overstimulation. In the next series of experiments, flash photolysis was used to investigate whether the more complete Ca^{2+} release observed during overstimulation by AVP was due to a direct effect of AVP on the responsiveness of the intracellular stores or was rather the result of higher IP_3 production.

Single hepatocytes were co-injected with the Ca^{2+} indicator fluo 3 and caged IP_3 , an analogue of IP_3 that remains inactive until it becomes activated by intense UV illumination [16]. Cells were then stimulated with the different agonists using a dose sufficient to reach overstimulation. When a stable overstimulation was obtained, caged IP_3 was photolysed by 1 ms flashes of UV light.

Figure 4 shows such an experiment in which an hepatocyte was stimulated with AVP. In six separate experiments, no further release of Ca^{2+} by IP_3 photorelease was observed when the cell was under AVP stimulation. This lack of response was also observed previously by another group using similar experimental conditions [17]. However, when the same experimental protocol was used with cells overstimulated with either phenylephrine (Figure 5) or ADP (Figure 6), photorelease of IP_3 induced a Ca^{2+} spike that rose above the elevated steady-state level. Interestingly, after this Ca^{2+} spike an undershoot and a damped quasi-sinusoidal return to the steady-state level was consistently observed in phenylephrine-stimulated cells. ADP-stimulated cells exhibited the same undershoot (Figure 6) although generally not of the same amplitude. Thus the perturbation induced by the release of IP_3 and the brief elevation of Ca^{2+} concentration above the elevated steady-state level appeared to bring the cell transiently into a quasi-oscillatory state.

Figure 5 IP_3 photorelease during phenylephrine overstimulation

The same general protocol as in Figure 4 was used. Photorelease of IP_3 was performed on cells overstimulated with phenylephrine. In the experiment presented two flashes were applied during the overstimulated phase, and in addition, a last flash was applied at the end after agonist washout to show that the cell was still responsive. The result is a representative experiment of five.

Figure 6 IP_3 photorelease during ADP overstimulation

The same general protocol as in Figures 4 and 5 were used. This graph is a typical example of five experiments.

Experiments using combinations of the agonists (Table 1) as well as those making use of photolysis of caged IP_3 (Figure 4) suggest that, in a maximal or overstimulated state, AVP is able to release more Ca^{2+} from the stores than phenylephrine or ADP. Since this did not appear to be the case during the oscillatory response (Figure 3), photorelease of caged IP_3 was attempted during Ca^{2+} transients of hormone-induced oscillations, more precisely when Ca^{2+} was near its highest level during an oscillatory peak. Figures 7(A)–7(C) show that, with all three agonists tested, namely AVP, phenylephrine and ADP, photorelease of IP_3 was able to evoke a small cytosolic Ca^{2+} spike which reached a slightly higher level than the oscillatory transient.

Intracellular store content during AVP overstimulation

The lack of additional Ca^{2+} response during AVP overstimulation either after addition of a second agonist or after IP_3 photorelease could be the result of complete emptying of the intracellular Ca^{2+} stores. In order to test this hypothesis, Ca^{2+} levels in the cytosol

and in intracellular stores were simultaneously measured using a method developed in previous studies [11]. The whole-cell patch-clamp technique was applied using a pipette containing the Ca^{2+} dye fluo 3 and in which the low-affinity Ca^{2+} -sensitive probe magfura 2 had been preferentially loaded in intracellular stores. Cells were challenged with AVP to obtain an overstimulated response. Figure 8 shows such an experiment in which a first low dose of AVP (2 nM) elicited cytosolic Ca^{2+} oscillations. Concomitantly, the Ca^{2+} signal associated with the intracellular stores displayed periodic decreases that occurred in phase with the cytosolic spikes. Two higher doses of AVP were subsequently able to induce overstimulation, and resulted in a progressive decrease in store Ca^{2+} content. After the level of Ca^{2+} had been stabilized in the stores, thapsigargin, an inhibitor of the endoplasmic reticulum Ca^{2+} -ATPase, was given. This compound has been shown in a number of studies to cause depletion of intracellular Ca^{2+} stores. As can be seen in Figure 8, thapsigargin was able to release an additional amount of Ca^{2+} from the stores which was retrieved in the cytosol. Estimations of the store Ca^{2+} concentration performed as described previously [11] gave a resting Ca^{2+} level of $454 \pm 129 \mu\text{M}$ ($n = 7$) decreasing to $115 \pm 53 \mu\text{M}$ during maximal AVP stimulation. Therefore, although the intracellular stores were markedly depleted, to about 25% of their initial content, they were not completely emptied by AVP.

The same protocol was attempted with phenylephrine and ADP. However, overstimulation was difficult to achieve with these agonists, probably because of the lower responsiveness of cells toward them during whole-cell patch-clamp experiments, as previously noted [11]. Nevertheless, in the cases where overstimulation could be obtained with phenylephrine ($n = 3$) or ADP ($n = 3$), results similar to those with AVP were found, the stores being only partially depleted by application of the agonist at saturating concentrations. Definitive conclusions could not be reached as to whether phenylephrine and ADP caused a more limited store depletion than AVP under these experimental conditions.

DISCUSSION

In this study, the agonist specificity of cellular Ca^{2+} response has been investigated. It was shown that rat hepatocytes, although responding to a variety of agonists of the phosphoinositide signalling pathway by oscillating cytosolic Ca^{2+} concentration, exhibited significant differences in their response to maximal stimulation.

Differences in the Ca^{2+} -mobilizing response to the α -adrenergic agonists, AVP and angiotensin, was recognized early on at the level of perfused liver [18]. At the single-cell level, the agonist-specific type of Ca^{2+} response, sometimes called agonist fingerprinting, was investigated in a few studies (e.g. [14]) that focused mainly on the differences in the shape of the Ca^{2+} and oscillatory patterns produced by different agonists. It was shown for instance that ATP and ADP, two structurally and pharmacologically related molecules that interact with purinoceptors, evoke strikingly different oscillation patterns in hepatocytes [14]. In this case it was concluded that two different subtypes of P_{2y} purinoceptors were activated and resulted in different types of oscillation. Obvious differences were also noted for agonists such as phenylephrine, angiotensin II and vasopressin [6], which led the authors to propose a complex receptor-controlled model of Ca^{2+} oscillation.

The molecular basis of these differences is still speculative as are their possible functional implications. It was found that diacylglycerol production was higher during AVP stimulation than during stimulation with angiotensin II or phenylephrine

Figure 7 IP_3 photorelease at the top of oscillatory spikes

Sustained Ca^{2+} oscillations in hepatocytes were obtained by stimulation using appropriate doses of AVP (0.8 nM) (A), phenylephrine (0.4 μM) (B) or ADP (5.6 μM) (C). Flashes of UV light, indicated by the closed triangles, were triggered when the Ca^{2+} level was near its highest in order to release IP_3 .

Figure 8 Simultaneous measurements of Ca^{2+} in the cytosol and intracellular Ca^{2+} stores during AVP-induced overstimulation

Cytosolic Ca^{2+} was monitored with fluo 3 (upper trace, left axis) using 480 nm excitation, and intracellular store Ca^{2+} was measured with mag fura 2 using the excitation ratio 340 nm/380 nm (lower trace, right axis). AVP was added at the different doses indicated, and, towards the end of the experiment, thapsigargin (1 μM) was added to cause complete depletion of stores.

[19]. Diacylglycerol is indeed a possible candidate for involvement since it is a natural activator of protein kinase C, which is known to have an inhibitory effect on the oscillatory Ca^{2+} response of hepatocytes [20,21]. On the other hand, activation of protein kinase C, e.g. by phorbol esters, has been shown to inhibit strongly the Ca^{2+} response of hepatocytes to phenylephrine but to have a much weaker effect on the AVP-induced response [22,23]. This difference in sensitivity to protein kinase C stimulation was confirmed at the level of IP_3 formation [24], which was found to be significantly reduced in phenylephrine-treated cells but not changed in cells stimulated by AVP. These results were obtained using high concentrations of agonists, similar to the situation of overstimulation that we studied, and adds to the disparity of the response of hepatocytes to these hormones. These observations as well as the results of the present study

would be consistent with receptor-specific G-proteins that are differentially regulated by protein kinase C (see below).

Another modulatory factor that could be involved is cAMP through its activation of the protein kinase A. This kinase has opposite effects to those of the protein kinase C in hepatocytes, i.e. it potentiates the cell's response to agonists which translates into an increase in oscillatory frequency which can even lead to overstimulation [21]. It has been shown that, in hepatocytes, not only hormones such as glucagon induce cAMP formation but also α -adrenergic agonists through cross-activation of the α_{1B} -adrenoceptor [25]. Therefore a mixed or differential activation of signalling pathways could account for the variety of Ca^{2+} -response patterns. The different shapes and patterns of Ca^{2+} oscillations that can be found are not explained by most mathematical models of Ca^{2+} oscillation.

The types of response that were observed in the present study are consistent with a difference in the amount of IP_3 that agonists are able to produce. The results of both the experiments with agonist combinations and those involving photorelease of IP_3 during overstimulation would fit with this concept. Indeed, AVP has been shown to cause greater IP_3 production than adrenaline in suspensions of hepatocytes [24,26]. However, the Ca^{2+} response to adrenaline was of the same amplitude and was even slightly faster than the response to AVP [26]. The different IP_3 levels mobilized during overstimulation could result from activation of an agonist-specific set of G-proteins that would activate phospholipase C with different efficacies. It could alternatively be due to activation of different sets of phospholipase C enzymes or to both.

In experiments to assess the association of different classes of receptors with G-proteins [27], it was found that activated V_1 -vasopressin receptors prevented α_1 -agonists from associating with G-proteins. In contrast, neither α_1 -agonists nor angiotensin II influenced the interaction of V_1 -vasopressin receptors with their G-proteins, which was described by the authors as asymmetric G-protein scavenging. Our present results obtained at the level of Ca^{2+} signalling are compatible with the findings of that study in that either a tighter or a more developed coupling of vasopressin receptors with G-proteins should result in the different Ca^{2+} mobilization observed under maximal stimulation in the combinations of agonist. Dasso and Taylor [27] proposed that two alternative hypotheses could explain their results: first, a greater affinity of vasopressin receptors for G-proteins which

would prevent activated α_1 -receptors from sequestering G-proteins shared with vasopressin receptors; secondly, vasopressin- and α_1 -receptors share a common pool of G-proteins but vasopressin receptors in addition interact with a second pool of G-proteins not available to the other receptors. They were not able to discriminate between these alternatives using their experimental approach. We believe that our data allow us to distinguish between the two explanations, as our experimental results are best explained by their second hypothesis: in a situation of maximal stimulation of receptors, one may expect that only access to more than one pool of G-proteins would enable vasopressin to mobilize more Ca^{2+} than phenylephrine in combination experiments and prevent photoreleased IP_3 from mobilizing more Ca^{2+} from intracellular stores.

The present study highlights another facet of agonist fingerprinting that does not manifest itself during the oscillatory response but during maximal hormonal stimulation. Indeed, whereas artificially increasing the amount of IP_3 inside cells previously overstimulated with AVP did not result in any additional response, performing the same experiment during oscillations induced by this agonist, even at the highest level of an oscillatory spike, yielded a Ca^{2+} transient that rose higher than the maximal level of the oscillatory transient.

Direct measurements of Ca^{2+} in the intracellular stores indicated that these stores were not completely depleted during AVP overstimulation. As was previously observed during phenylephrine-induced oscillations by direct measurements of Ca^{2+} in intracellular stores [11], our present measurements indicate that the Ca^{2+} stores are not completely depleted during overstimulation triggered by phenylephrine or ADP. We cannot completely exclude the possibility that the residual Ca^{2+} that could not be released by these agonists but that was releasable by thapsigargin, an inhibitor of endoplasmic reticulum Ca^{2+} -ATPase, is the consequence of a heterogeneous population of Ca^{2+} pools, some of them lacking IP_3 receptors or possessing a vastly different sensitivity toward IP_3 . If this were the case, one could argue that AVP indeed empties IP_3 -sensitive pools whereas ADP and phenylephrine only partially discharge them.

Another conclusion that can be drawn is that, even when maximal stimulation of the receptor is imposed during overstimulation with phenylephrine or ADP, the amount of IP_3 endogenously produced is not saturating, since artificially releasing an extra amount of IP_3 during overstimulation evoked a Ca^{2+} spike above the elevated baseline.

The Ca^{2+} spike that arose after photorelease of IP_3 was followed by an undershoot, sometimes pronounced, that returned promptly to the overstimulated elevated steady-state level. The mechanism responsible for this transient undershoot is not certain. However, it has been shown that the IP_3 receptor possesses an intrinsic sensitivity to cytosolic Ca^{2+} concentration [28], a so-called bell-shaped dependency of activation, which is the basis of some of the current models of Ca^{2+} oscillation [29]. A consequence of this property of IP_3 receptors is that the extent of inactivation or desensitization is proportional to peak Ca^{2+} concentration [30]. The undershoot that follows the IP_3 -induced spikes (Figures 5 and 6) is therefore consistent with transient inactivation of the IP_3 receptors caused by the brief increase in Ca^{2+} concentration. This observation supports the notion that

cytosolic Ca^{2+} itself exerts an inhibitory effect on its own release, thus providing the negative feedback element essential for the generation of oscillations.

In summary, it was found that the response of hepatocytes to maximal hormonal stimulation depended on the agonist. The efficacy of Ca^{2+} mobilization in the overstimulated state was higher with AVP than with phenylephrine or ADP. However, during overstimulation with AVP, phenylephrine or ADP, the intracellular stores were not completely depleted. This study points to a different type of agonist fingerprinting not found at the level of the oscillatory patterns but only during maximal stimulation.

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