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Modulation of cytosolic-[Ca^{2+}] oscillations in hepatocytes results from cross-talk among second messengers

The synergism between the α_1-adrenergic response, glucagon and cyclic AMP, and their antagonism by insulin and diacylglycerol manifest themselves in the control of the cytosolic-[Ca^{2+}] oscillations

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Hepatocytes respond to stimulation by glycogenolytic agonists acting via phosphoinositide (PI) breakdown through oscillations of the free cytosolic concentration of Ca^{2+} ($[Ca^{2+}]_{cyt.}$). Since the second-messenger repertoire of hepatocytes includes many other factors besides Ca^{2+}, we investigated to what degree the regulation of $[Ca^{2+}]_{cyt.}$ oscillations is integrated into these other signalling systems. $[Ca^{2+}]_{cyt.}$ was recorded in single rat hepatocytes by using the Ca^{2+}-indicator fura-2. Parallel stimulation with phenylephrine (an α_1-adrenergic agonist of PI breakdown) and glucagon resulted in a synergistic stimulation of $[Ca^{2+}]_{cyt.}$ oscillations. Direct activation of the cyclic-AMP-dependent pathway with several stimuli (forskolin, 8-bromo cyclic AMP, 8-CPT cyclic AMP) mimicked the response to glucagon. In contrast, $[Ca^{2+}]_{cyt.}$ oscillations induced by various combinations of these agonists could be antagonized by the glycogenic hormone insulin. As one of the options in the insulin-signalling network, we tested a diacylglycerol activator of protein kinase C, DiC_8. It also acted as an inhibitor of $[Ca^{2+}]_{cyt.}$ oscillations. We investigated how these observations could be reconciled with our previously introduced model of $[Ca^{2+}]_{cyt.}$ oscillations in hepatocytes [Somogyi and Stucki (1991) J. Biol. Chem. 266, 11068–11077]. First of all, the effect of calmodulin inhibitors (calmidazolium and CGS 9343 B), acting at the core of our model on the feedback of Ca^{2+} on Ins(1,4,5)P_3-induced Ca^{2+} release, was not altered by the new modulators. In addition, all agonists and antagonists could be used interchangeably in combination and introduced no significant change in the oscillatory pattern or spike shape. Since the response was solely limited to frequency modulation, over- or under-stimulation of the oscillatory system, there is no need to create a new oscillator or to introduce further reaction steps into the core of the model. We conclude that the regulation of $[Ca^{2+}]_{cyt.}$ via the explored second-messenger pathways can be embedded into the oscillatory system as modulation of rate constants already present in this model.

INTRODUCTION

It has become apparent for a number of different cell types that the response to stimuli activating PI breakdown takes the shape of repetitive Ca^{2+} spikes [1–3]. These observations are made possible by measuring $[Ca^{2+}]_{cyt.}$ in individual cells by using fluorescent probes [4]. Nevertheless, $[Ca^{2+}]_{cyt.}$ is only one of many parameters involved in intracellular signal processing. The hepatocyte system is an established focus for the interaction of hormones and second messengers. For glycogenolytic stimuli, the $[Ca^{2+}]_{cyt.}$-elevating response of one type acting via PI breakdown (e.g. α_1-adrenergic agonists, vasopressin) is augmented by another type which elevates $[cAMP]_{cyt.}$ (e.g. glucagon, β-adrenergic agonists, forskolin, cAMP analogues) [5–7]. This synergism reflects the common metabolic goal of these agonists [5]. While pitting glycogenolytic agonists against the glycogenic insulin, insulin can decrease cAMP formed during glucagon stimulation [8] and $[Ca^{2+}]_{cyt.}$ increased by α_1-adrenergic stimulation [9,10] but not by vasopressin [9]. In the regulation of a step following second-messenger formation, cAMP dominates by suppressing insulin-induced glucokinase gene expression [11]. In contrast, the inhibition of phosphoenolpyruvate carboxykinase gene transcription by insulin cannot be reversed by cAMP or glucocorticoids [12]. The complex nature of the insulin signal-transduction system [13,14] may underlie the intricacies of the competition between insulin and its metabolic antagonists.

Considering that $>90\%$ of hepatocytes challenged by Ins(1,4,5)P_3-generating hormones respond with oscillations in $[Ca^{2+}]_{cyt.}$ [15–17], one may ask whether this consistency also applies to the response of these oscillations to other signalling factors. Beyond the sequential additive exposure to an InsP_3-generating hormone and cAMP [18], we will examine the effect of the physiological agonist glucagon. Furthermore, does insulin act as an inhibitor of oscillations? If so, is this dependent on the stimulus employed? Is cAMP-dependent protein kinase (PKA) involved in this network? What is the role of diacylglycerol, produced with InsP_3 or upon insulin exposure [13,14]? Does it parallel the response to phorbol esters, another class of protein kinase C (PKC) activator [19]?

We have previously introduced a model of $[Ca^{2+}]_{cyt.}$ oscillations in hepatocytes [17] based on one InsP_3-sensitive intracellular Ca^{2+} pool and positive feedback of Ca^{2+} on its own release [20], mediated by calmodulin [17,21]. The type of network created by the interaction of several signalling components allows certain predictions on the outcome of the oscillatory pattern within this model. Exposure to additional signalling factors may (1) abolish oscillations, (2) create complex patterns by introducing further oscillators, or (3) result in a simple change of frequency, effecting

Abbreviations used: 8-bromo-cAMP, 8-bromoadenosine 3′:5′-cyclic monophosphate; 8-CPT-cAMP, 8-(4-chlorophenylthio)-adenosine 3′:5′-cyclic monophosphate; $[Ca^{2+}]_{cyt.}$, free cytosolic Ca^{2+} concentration; cAMP, adenosine 3′:5′-cyclic monophosphate; $[cAMP]_{cyt.}$, cytosolic concentration of cAMP; DiC_8, 1,2-dioctanoyl-*sn*-glycerol; PI, phosphoinositide; PKA, cAMP-dependent protein kinase; PKC, protein kinase C; R_p-cAMPS, adenosine 3′:5′-cyclic monophosphorothioate, R_p-diastereomer.

no qualitative changes in spike form and regularity. The first two cases would require substantial modification of our model, since they would correspond to, respectively, (1) the oscillator being destroyed, or (2) the introduction of additional, essentially independent, oscillators. The third case could simply be described by modulatory functions on the existing rate laws, i.e. the core model [17]. This would predict that the activators and inhibitors of the oscillations could be used interchangeably in combination. In > 150 experiments we have tested different combinations of activators and inhibitors to help answer these questions on the experimental level.

MATERIALS AND METHODS

Preparation, fura-2 loading and $[Ca^{2+}]_{cyt.}$ imaging of hepatocytes

The preparation, freezing, thawing, plating and imaging protocol was as described previously [17] with minor modifications. After thawing and washing, hepatocytes were incubated for 2 h in F12 culture medium supplemented with 1% BSA, 1.8 mM-$CaCl_2$ and 20 mM-glucose (total concentrations) to enable attachment. The solution was changed to F12 without $NaHCO_3$ (containing 1% BSA, 1.8 mM-$CaCl_2$, 20 mM-glucose and 10 mM-Hepes). The cells were then stored at 4 °C. Immediately before an experiment, cells were loaded for 20 min with 2 μM fura-2 AM in the same buffer at 37 °C. BSA and fura-2 AM were omitted from this buffer during the experiments. All solutions were adjusted to pH 7.4 with NaOH, except for insulin wash-out, for which the pH was adjusted to 6.0 with HCl. Flattened, generally double-nucleated, cells of the brightest fura-2 fluorescence were selected for the experiments.

Materials

BSA, calmidazolium (R 24571), EGTA, forskolin, 8-CPT-cAMP, glucagon and phenylephrine were purchased from Sigma (St. Louis, MO, U.S.A.), collagenase H, F12 culture medium and fura-2 AM from Boehringer (Mannheim, Germany), Hepes, insulin and 8-bromo-cAMP from Fluka (Buchs, Switzerland); collagen R was from Serva (Heidelberg, Germany), R_p-cAMPS from BIOLOG (Bremen, Germany), DiC_8 from Molecular Probes (Eugene, OR, U.S.A.), and fetal-calf serum from Seromed/Biochrom (Berlin, Germany). CGS 9343 B was generously given by Ciba–Geigy, Basel, Switzerland.

RESULTS

Synergism between phenylephrine, glucagon and cAMP

Although $[Ca^{2+}]_{cyt.}$ oscillations are typically observed in hepatocytes exposed to hormones acting via PI breakdown [15–17,22], glucagon and other agents tied to the cAMP messenger system could conceivably induce or stimulate these oscillations. Fig. 1 demonstrates that glucagon, applied in the pM range, could induce oscillations after sub-threshold stimulation with the α_1-adrenergic agonist phenylephrine. In Fig. 1(*a*), 20 pM-glucagon caused a spike, whereas oscillations ensued after raising glucagon to 40 pM. At 60 pM-glucagon the system made the transition to the state which we refer to as overstimulation [17], which is characterized by a pattern of low-amplitude oscillations at an elevated steady-state $[Ca^{2+}]_{cyt.}$; a concentration of glucagon as low as 4 pM could also be sufficient in causing oscillations (Fig. 1*b*) (the remaining part of the experiment is considered below).

To establish the correlation of glucagon-stimulated oscillations to the cAMP messenger system, we tested forskolin (increasing $[cAMP]_{cyt.}$ by activating adenylate cyclase [23]) and membrane-permeant cAMP analogues as to whether they could mimic the glucagon results. Fig. 2(*a*) shows an experiment in which $[Ca^{2+}]_{cyt.}$ oscillations, induced by 500 nM-phenylephrine, were further stimulated by 100 nM-forskolin (the remaining part of the experiment is considered below). Here forskolin led to a frequency increase, followed by overstimulation. Are cAMP-elevating stimuli only effective if $[Ca^{2+}]_{cyt.}$ oscillations have been induced previously by phenylephrine? We applied cAMP-elevating modulators in the absence of oscillations, but 5–10 min after sub-threshold stimulation by 100 nM-phenylephrine, analogous to the experiments shown in Fig. 1. Addition of 1 μM-forskolin led to a response of the overstimulated type in Fig. 3 (the remaining part of the experiment is considered below). Similar results were obtained with the membrane-permeant cAMP analogue 8-bromo-cAMP, which was titrated to 10 μM until it elicited oscillations in Fig. 4 (the remaining part of the experiment is considered below). All experiments dealing with glucagon and cAMP analogues are summarized in Table 1.

Could glucagon and other cAMP-dependent stimuli initiate $[Ca^{2+}]_{cyt.}$ oscillations independent of an additional PI-breakdown-stimulating agonist? We exposed hepatocytes to a wide range of concentrations of glucagon or 8-bromo-cAMP, at or above those previously determined to be effective. After each addition we waited for ~ 10 min before elevating the concentration again. Phenylephrine was added at the end of each experiment, to which a response was observed in every case.

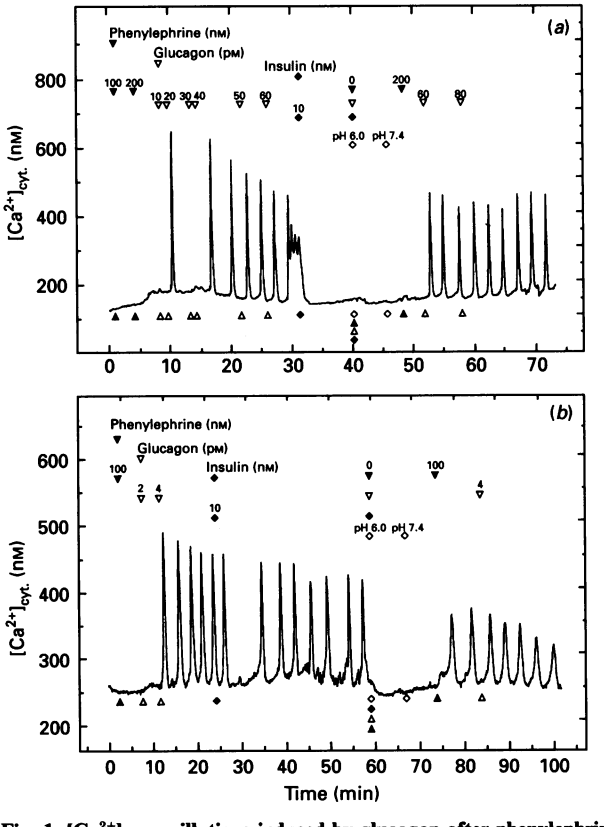

Fig. 1. $[Ca^{2+}]_{cyt.}$ oscillations induced by glucagon after phenylephrine pre-stimulation: response to insulin and insulin wash-out

Additions of phenylephrine, glucagon and insulin are denoted by ▼, ▽ and ◆ respectively. Wash-out of all substances is marked by ◇. The pH changes from pH 7.4 to pH 6.0 and back to pH 7.4, necessary for insulin wash-out, are shown above the ◇ symbols. Phenylephrine and glucagon were added after complete wash-out as marked. Two experiments using two different cells are depicted. Whereas insulin caused complete inhibition of the oscillations after overstimulation by glucagon in (*a*), it only led to a decrease in frequency of glucagon-induced oscillations in (*b*). Also, the sensitivity to hormone after insulin wash-out was decreased in (*a*) and increased in (*b*).

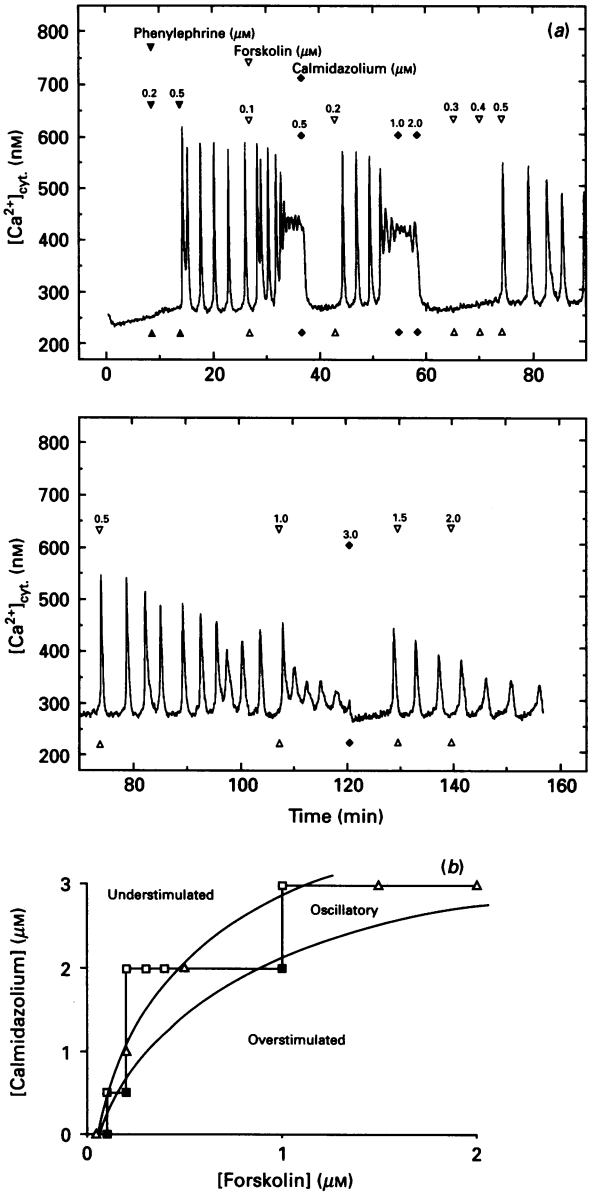

Fig. 2. (*a*) Modulation of phenylephrine-induced $[Ca^{2+}]_{cyt.}$ oscillations by forskolin and calmidazolium, and (*b*) hypothetical boundary of stability in the plane of forskolin and calmidazolium concentrations

In (*a*) the symbols mark additions of concentration changes of the agents used. Generally, final concentrations are noted above each symbol in the units shown in parentheses for each substance. For additional clarity, two sets of symbols corresponding to the same time points were placed at the top and bottom of the graph. Owing to the length of this experiment, it is shown in two overlapping segments. Additions of phenylephrine (α_1-adrenergic agonist), forskolin (agent stimulating cAMP production) and calmidazolium (calmodulin inhibitor) are marked by ▲, △ and ◆ respectively. Three cycles of overstimulation by forskolin and antagonism by calmidazolium are shown. (*b*) The region within the two concave boundary lines represents the unstable oscillatory state, whereas the areas above and below correspond to the understimulated and overstimulated stable states: □, △ and ■ respectively correspond to locations in the understimulated, oscillatory and overstimulated regions. Increases in forskolin and calmidazolium should roughly represent increases in α and a [17] respectively.

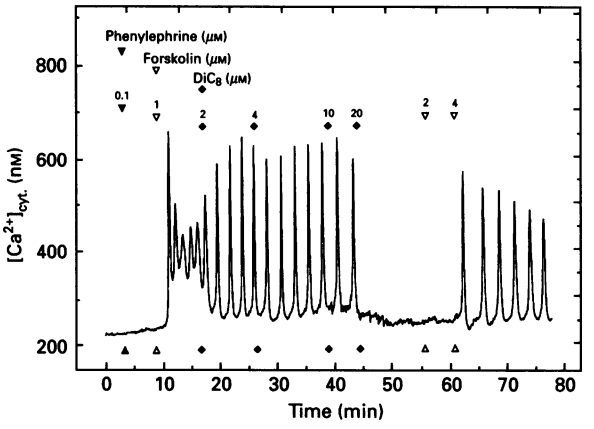

Fig. 3. $[Ca^{2+}]_{cyt.}$ oscillations induced by forskolin after phenylephrine pre-stimulation: response to DiC_8

Additions of phenylephrine, forskolin and DiC_8 (an activator of PKC) are marked by ▲, △ and ◆ respectively. DiC_8 was able to counteract overstimulation by forskolin and then to inhibit the oscillations altogether, in a manner reversible by further forskolin addition.

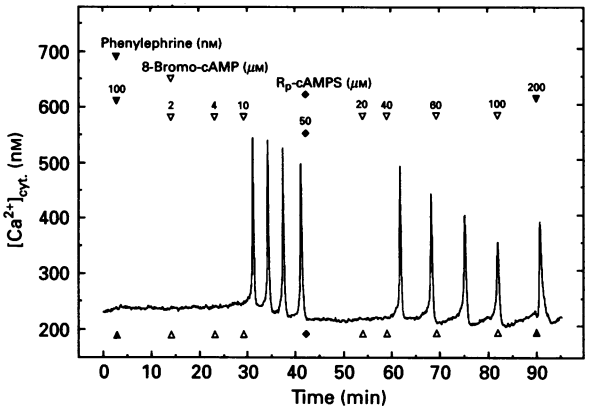

Fig. 4. $[Ca^{2+}]_{cyt.}$ oscillations induced by 8-bromo-cAMP after phenylephrine pre-stimulation: response to R_p-cAMPS

Additions of phenylephrine, 8-bromo-cAMP (a membrane-permeant cAMP analogue) and R_p-cAMPS (an inhibitor of PKA) are marked by ▲, △ and ◆ respectively. The inhibition of the oscillations by R_p-cAMPS was reversed by increasing 8-bromo-cAMP.

Although glucagon could induce a Ca^{2+} response in spherical cells, as mentioned in a previous report [24], the flattened hepatocytes exclusively examined in the present work behaved differently. Glucagon in the range 10 pM–100 nM (four experiments), and even at 100 μM (two experiments), had no effect on $[Ca^{2+}]_{cyt.}$. Similarly, 2–200 μM-8-bromo-cAMP (eight experiments) did not cause a change in $[Ca^{2+}]_{cyt.}$.

Role of PKA in $[Ca^{2+}]_{cyt.}$ oscillations

Can the stimulatory effects of cAMP be reversed by the membrane-permeant PKA inhibitor, R_p-cAMPS [25]? Oscillations induced by 8-bromo-cAMP in combination with phenylephrine were suppressed by 50 μM-R_p-cAMPS, in a fashion reversible by further addition of 8-bromo-cAMP (Fig. 4). The possibility may be considered that some level of PKA activity is necessary for $[Ca^{2+}]_{cyt.}$ oscillations to occur in the first place. We have therefore tested whether R_p-cAMPS can inhibit oscillations elicited by phenylephrine alone. In Fig. 5(*a*), addition of 50 μM-R_p-cAMPS suppressed $[Ca^{2+}]_{cyt.}$ oscillations stimulated by 40 nM phenylephrine, which could be reversed by further phenylephrine addition (the remaining part of the experiment is considered below). This inhibitory effect was not always evident, as shown in Fig. 5(*b*). Here R_p-cAMPS (50 μM) inhibited oscillations

Table 1. Overview of the combinations of activators and inhibitors used in the modulation of $[Ca^{2+}]_{cyt.}$ oscillations

The numbers of experiments documenting the stimulatory or inhibitory action of each combination is shown. For the experiments in which forskolin, 8-bromo-cAMP, 8-CPT-cAMP or glucagon were used, phenylephrine was applied in concentrations under the threshold to induce oscillations by itself (40–100 nM). If phenylephrine application alone resulted in oscillations, and the respective positive modulator caused further stimulation, the number of experiments is marked by an asterisk. All of the cells tested responded to the respective stimulator and inhibitor, except for insulin and R_p-cAMPS, for which the total number of experiments is shown in parentheses. The results obtained with insulin are detailed in Table 2. A total of 136 experiments is included in the Table. The concentration range for each agent refers to the minimal concentrations used to elicit effects. These concentrations may have been raised even higher in some experiments in which agonists were titrated against antagonists, and vice versa. The sum of inhibitory experiments may exceed the number of stimulatory experiments in a row, because more than one antagonist was occasionally tested in the same experiment.

		Inhibition by antagonist			
Agonist	Stimulatory action	CGS 9343 B (0.2–20 μM) or calmidazolium (1–3 μM)	DiC_8 (1–10 μM)	Insulin (10 nM)	R_p-cAMPS (50 μM)
Phenylephrine (0.04–4 μM)	42	12	8	17 (20)	5 (8)
Forskolin (0.02–4 μM)	4	–	2	1 (2)	
	17*	2*	3*	0 (1)*	–
8-Bromo-cAMP (1–10 μM)	10	3	–	1 (3)	1 (1)
	4*	3*			
8-CPT-cAMP (10–100 nM)	13	5	1	3 (4)	–
	20*	5*	1*	1 (1)*	
Glucagon (1–100 pM)	15	2	–	4 (4)	–
	11*			2 (2)*	

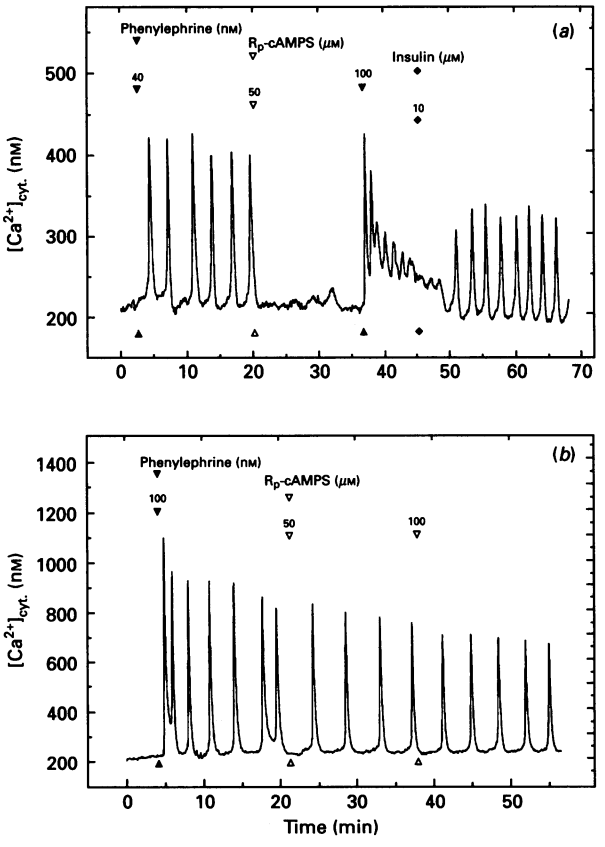

Fig. 5. (*a*) Inhibition of phenylephrine-induced $[Ca^{2+}]_{cyt.}$ oscillations by R_p-cAMPS: response to insulin, and (*b*) effect of R_p-cAMPS on phenylephrine-induced $[Ca^{2+}]_{cyt.}$ oscillations

(*a*) Additions of phenylephrine, R_p-cAMPS and insulin are marked by ▲, △ and ◆ respectively. Insulin is able to inhibit the oscillations in the presence of R_p-cAMPS. (*b*) Additions of phenylephrine and R_p-cAMPS are marked by ▲ and △ respectively. R_p-cAMPS does not have a clear inhibitory effect at two concentrations tested.

induced by 100 nM-phenylephrine only slightly, if at all. This apparent lack of response was not due to an insufficient amount of antagonist, since no additional effect was seen at 100 μM-R_p-cAMPS. The results concerning R_p-cAMPS (summarized in Table 1) indicate that PKA activity may have been involved in, but was not mandatory for, $[Ca^{2+}]_{cyt.}$ oscillations.

Sensitivity to calmodulin inhibitors

To investigate whether stimulation by cAMP-dependent modulators is tied to the same oscillatory mechanism as proposed for phenylephrine, we examined the effect of calmodulin inhibitors, which act at the core of our model [17]. The experiment in Fig. 2(*a*) demonstrates that overstimulation by 0.1 μM-forskolin during oscillations induced by 0.5 μM-phenylephrine could be reversed by addition of 0.5 μM of the calmodulin antagonist calmidazolium [26]. Raising forskolin to 0.2 μM then led to gradual overstimulation, which was again antagonized by 2 μM-calmidazolium. Oscillations were restored by 0.5 μM-forskolin and overstimulated by 1 μM. Finally, 3 μM-calmidazolium caused a decrease in frequency, which was reversed by 1.5 μM-forskolin.

Analogous to previously published results [17], the additions of agonist and inhibitor can be mapped as an empirical stability graph on the agonist/inhibitor plane (Fig. 2*b*). Three areas have been defined, corresponding to the unstable (sustained oscillatory), and the stable (non-oscillatory), overstimulated and understimulated states. Additions of modulators (exact concentrations as used in Fig. 2*a*) can move the system from one state to another across these boundaries of stability. Note that the boundaries are both concave, in contrast with those previously fitted for phenylephrine [17]. This could be explained by different implicit functions of phenylephrine and cAMP governing α, the rate constant affecting the open probability of the $InsP_3$-sensitive channel. The antagonism between calmidazolium, desensitizing Ca^{2+} feedback on Ca^{2+} release, and forskolin, sensitizing Ca^{2+} release to a presumably constant $InsP_3$, can be interpreted by our

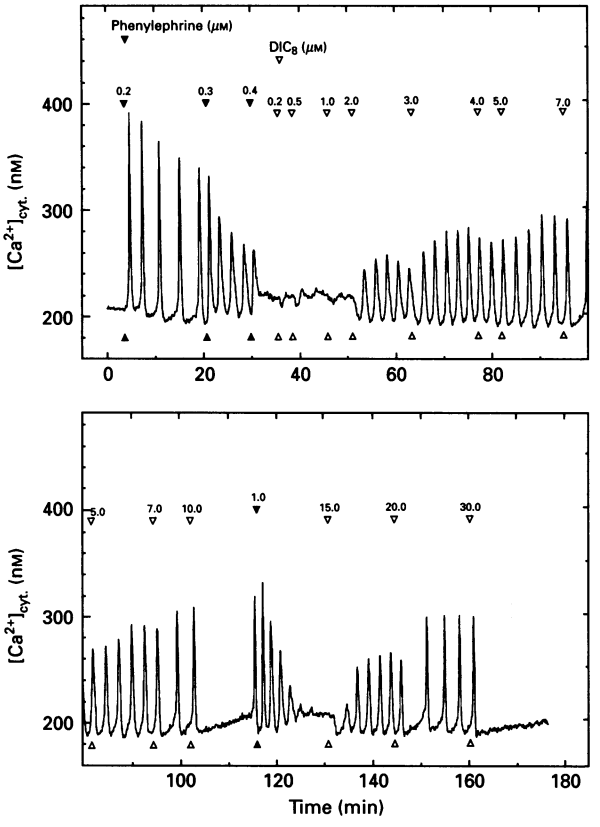

Fig. 6. Inhibition of phenylephrine-induced $[Ca^{2+}]_{cyt.}$ oscillations by DiC_8

Additions of phenylephrine and DiC_8 are marked by ▲ and △ respectively. The experiment is shown in two overlapping segments, owing to its length of almost 3 h. Two cycles of stimulation and inhibition of the oscillations are shown.

model as a modulation of the affinity and rate constant, a and α respectively [17]. Inhibition of $[Ca^{2+}]_{cyt.}$ oscillations by the calmodulin inhibitors calmidazolium and CGS 9343 B [27] was observed in experiments using various combinations of phenylephrine (also at sub-threshold levels) and cAMP-dependent modulators (see Table 1).

Role of PKC

PKC, activated by diacylglycerols formed during PI breakdown, is part of the same signalling network which leads to $[Ca^{2+}]_{cyt.}$ oscillations. To see whether PKC directly interacts with the oscillatory mechanism, we tested a synthetic diacylglycerol, DiC_8, which stimulates PKC like its cellular counterparts [28]. In Fig. 6, oscillations induced by 0.2 μM-phenylephrine were overstimulated by raising the agonist concentration to 0.4 μM. Titration with DiC_8 was started at 0.2 μM until overstimulation was antagonized and the oscillations resumed at 2 μM-DiC_8. Further addition of DiC_8 up to 10 μM led to a progressive amplitude increase and frequency decrease, consistent with the predictions of our model [17]. This was readily reversible by raising the phenylephrine concentration to 1.0 μM, again leading to overstimulation. The following increase of DiC_8 to 15 μM guided the system to the sustained oscillatory state. At 20 μM-DiC_8 the amplitude increased and the frequency decreased, whereas complete inhibition resulted at 30 μM-DiC_8. DiC_8 is also an effective inhibitor if the acute stimulant of the oscillations is forskolin and not phenylephrine. Overstimulation caused by forskolin after pre-stimulation with sub-threshold phenylephrine concentrations could be reversed by 2 μM-DiC_8 (Fig. 3). Further increases in DiC_8 resulted in a complete inhibition, which was overcome by raising forskolin to 4 μM. The inhibitory action of DiC_8 appears not to depend on the type of stimulation protocol used to cause $[Ca^{2+}]_{cyt.}$ oscillations (summarized in Table 1).

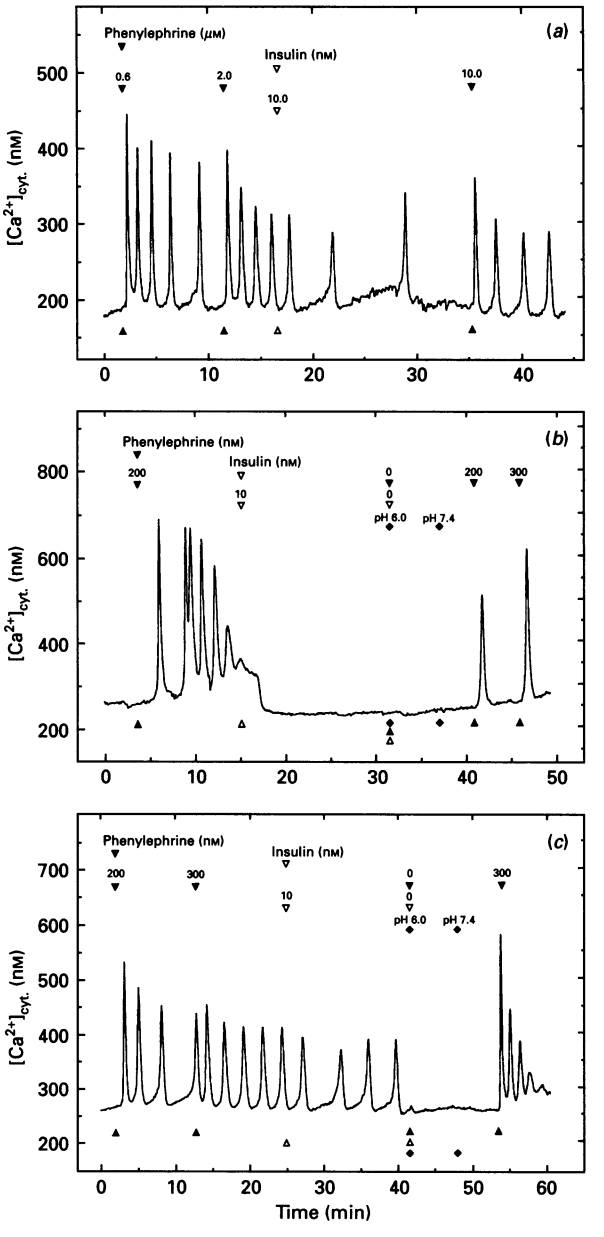

Fig. 7. Inhibition of phenylephrine-induced $[Ca^{2+}]_{cyt.}$ oscillations by insulin

Additions of phenylephrine and insulin are denoted by ▲ and △ respectively. (*a*) Reversibility by agonist. The decrease in frequency by insulin was reversed by an increase in the phenylephrine concentration. (*b*) and (*c*) Responsiveness after insulin wash-out. Removal of all substances is marked by ◆ at the pH shown above the symbols. Phenylephrine was re-introduced as marked. Experiments (*b*) and (*c*) correspond to two different cells. Insulin antagonized overstimulation by phenylephrine completely and decreased the sensitivity to hormone after wash-out in *b*. In contrast, only a decrease in frequency was observed in (*c*), followed by sensitization to hormone after wash-out.

Inhibition by insulin

Is the antagonism between glycogenolytic agonists and insulin also reflected in the control of $[Ca^{2+}]_{cyt.}$ oscillations? In the experiments below we have applied insulin at the optimal concentration (10 nM) able to elicit maximal physiological effects

Table 2. Responsiveness of $[Ca^{2+}]_{cyt.}$ oscillations to insulin in three concentration ranges

Insulin was tested in the concentrations shown in the column headings. The sub-columns headed by '−', '0' and '+' list the number of experiments in which insulin inhibited, had no effect on, or stimulated $[Ca^{2+}]_{cyt.}$ oscillations, respectively. A total of 62 experiments is included in the Table. This is smaller than the sum of all numbers shown in the Table, since more than one concentration of insulin has been tested in some experiments. The '−' sub-column at 10 nM-insulin is the same as the 'Insulin' column in Table 1. See Table 1 legend for further details.

	Insulin								
	2 pM– < 10 nM			10 nM			> 10 nM–2 μM		
Agonist	−	0	+	−	0	+	−	0	+
Phenylephrine (0.2–4 μM)	3	–	3	17	2	1	17	3	8
Forskolin (0.02–4 μM)	–	–	–	1	1 1*	–	1	1	–
8-Bromo-cAMP (1–10 μM)	–	–	–	1	2	–	–	–	–
8-CPT-cAMP (10–100 nM)	1	–	–	3 1*	1	–	1	1	2
Glucagon (1–100 pM)	1	–	1	4 2*	–	–	1 1*	–	–

[8,11,29]. During oscillations induced by 2.0 μM-phenylephrine, addition of 10 nM-insulin clearly resulted in a lowering of the frequency of oscillations in Fig. 7(*a*). This was counteracted by raising phenylephrine to 10 μM. The experiments depicted in Figs. 7(*b*) and 7(*c*) determined whether insulin has longer-term effects on $[Ca^{2+}]_{cyt.}$ oscillations, not reversible by wash-out. In Fig. 7(*b*), 10 nM-insulin antagonized overstimulation induced by 200 nM-phenylephrine. A complete inhibition of the oscillations was observed over a time range > 10 min. Insulin and phenylephrine were washed out at pH 6.0 to remove receptor-bound insulin [11,12,30], and the pH was then restored to pH 7.4. After exposure to insulin, phenylephrine at 200 nM and 300 nM only caused low-frequency spiking after wash-out, suggesting the cell has lost some of its sensitivity to phenylephrine. In contrast, Fig. 7(*c*) shows an experiment in which insulin alone led to a decrease in frequency of sustained oscillations induced by 300 nM-phenylephrine in a time interval of > 10 min. This cell was more responsive to phenylephrine after complete wash-out, since 300 nM agonist then caused overstimulation. Insulin may have minor, longer-term, modulatory effects on $[Ca^{2+}]_{cyt.}$ oscillations which are not clearly predictable.

Is the inhibition by insulin dependent on the agonist that produced the oscillations? In Fig. 1(*a*), insulin (10 nM) antagonized overstimulation by a combination of 60 pM-glucagon and 200 nM-phenylephrine. This caused complete inhibition of the oscillations for ~ 8 min. After wash-out as described above, oscillations could again be induced by a combination of 200 nM-phenylephrine and 60 pM-glucagon. Since this combination led to overstimulation before insulin treatment, the cell had apparently lost some of its hormone-sensitivity. In another experiment (Fig. 1*b*), application of 10 nM-insulin after oscillations had been induced by 4 pM-glucagon (sub-threshold pre-stimulation with 100 nM-phenylephrine) resulted in a short pause in spiking. This was followed by sustained oscillations, albeit at a slightly lower frequency than before. The hormone-sensitivity of this cell had apparently increased slightly, as 100 nM-phenylephrine alone was sufficient to stimulate oscillations after wash-out of all agents. Insulin could also counteract the stimulatory effect of forskolin, 8-bromo-cAMP and 8-CPT-cAMP (summarized in Tables 1 and 2).

We have tested insulin in only one concentration above, as opposed to titrating it over a wide range, because its effects can vary at much lower or higher concentrations than 10 nM. This

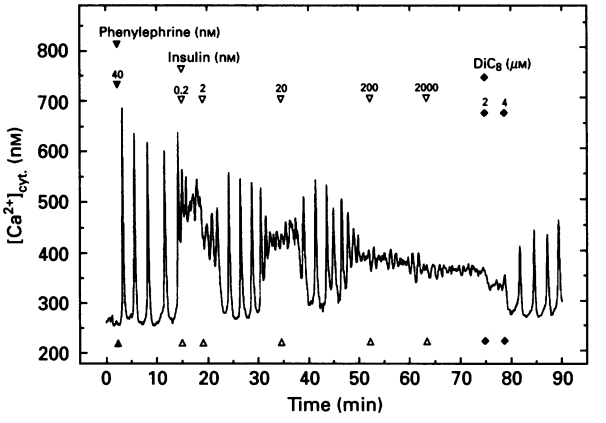

Fig. 8. Modulation of phenylephrine-induced $[Ca^{2+}]_{cyt.}$ oscillations by insulin and DiC_8

Additions of phenylephrine, insulin and DiC_8 are marked by ▲, △ and ◆ respectively. The experiment shows transient 'anomalous' stimulatory and inhibitory responses to insulin in a wide range of concentrations. DiC_8 acts as an effective inhibitor even after a maximal dose of insulin.

'anomalous' response is exemplified by the experiment shown in Fig. 8. After inducing oscillations with 40 nM-phenylephrine, addition of 0.2 nM-insulin led to overstimulation, which could only be transiently reversed by raising insulin to 2 nM. This temporary inhibition could be repeated by increasing the insulin concentration to 20 nM. Additional steps to 200 nM- and then to 2 μM-insulin had no further noticeable effect on overstimulation. The experiments dealing with three concentration ranges of insulin, below, above and exactly at 10 nM, are pooled in Table 2. Although 10 nM-insulin inhibited $[Ca^{2+}]_{cyt.}$ oscillations in a clear majority of the cells, some experiments also showed stimulatory effects of insulin in the higher and lower concentration ranges.

Which messenger system is the main target of insulin antagonism of $[Ca^{2+}]_{cyt.}$ oscillations? Since PKC activation is one of the proposed elements of the insulin-signalling system, we examined whether the restricted inhibition by insulin could be linked to a lack of available PKC. We tested the PKC activator DiC_8 in experiments in which an increase in insulin concentration

could not lead to a further inhibition. In Fig. 8, addition of DiC_8 (2 μM) was still able to lower the steady-state $[Ca^{2+}]_{cyt.}$, then reinstate the oscillations at 4 μM-DiC_8, while insulin was already titrated to a concentration not eliciting any further response (2 μM). In a total of nine experiments, application of DiC_8 was still effective while insulin addition could not counteract stimulators of the oscillations. This suggests that insulin only has a limited capacity to activate PKC. As an alternative, the modulation of $[Ca^{2+}]_{cyt.}$ oscillations by insulin may be due to a decrease in cAMP and PKA activity. If this were the sole action of insulin, insulin should be without effect if PKA activity was eliminated before insulin application, which is not what we observed. In Fig. 5(*a*), insulin could clearly antagonize overstimulation by 100 nM-phenylephrine after addition of 50 μM of the PKA inhibitor R_p-cAMPS.

DISCUSSION

We have shown that $[Ca^{2+}]_{cyt.}$ oscillations can (*a*) be stimulated by glucagon and other agents acting via the cAMP-dependent messenger pathway, (*b*) still occur in the presence of an inhibitor of PKA, (*c*) be inhibited by an activator of PKC and by calmodulin inhibitors, and (*d*) be negatively modulated by insulin. Moreover, they are generally sensitive to the listed inhibitors, independently of the stimulation protocol, and show no obvious alteration of the oscillatory pattern. None of these modulators principally acts by irreversibly eliminating $[Ca^{2+}]_{cyt.}$ oscillations. Instead, appropriate doses of these substances can be used interchangeably to adjust the frequency of the oscillations in a reversible and competitive fashion. These results therefore do not necessitate principal changes to our model of $[Ca^{2+}]_{cyt.}$ oscillations in hepatocytes [17]. All of these observations can theoretically be explained by the modulation of rate constants already contained in the model. As a first approximation (Fig. 9), these modulators may influence the agonist-dependent formation of $InsP_3$ or the coupling of $InsP_3$ to the rate constant α, which together with Ca^{2+} feedback controls Ca^{2+} release from intracellular stores.

Role and mechanism of modulation of $[Ca^{2+}]_{cyt.}$ oscillations by glucagon and cAMP

We have shown that glucagon consistently exerted a positive modulatory influence on $[Ca^{2+}]_{cyt.}$ oscillations. Furthermore, it was also able to trigger oscillations in response to sub-threshold concentrations of phenylephrine. Is this response due to activation of the cAMP or the $InsP_3$ signalling pathways, as glucagon has also been associated with the production of $InsP_3$ in hepatocytes [31]? In some cells, sustained oscillations of $[Ca^{2+}]_{cyt.}$ were observed with glucagon as the sole stimulus [24], interpretable as an elevation of $InsP_3$. But this was limited to spherical cells, and was never observed in the flattened ones exclusively used in the present work. We have employed glucagon alone in a range of concentrations both lower (2 pM) and higher (> 1 μM) than those shown to generate $InsP_3$ [31], without observing a Ca^{2+} response. If $InsP_3$ was produced after glucagon stimulation, either owing to activation of another receptor type [31] or indirectly via cAMP [32], it was not sufficient in causing any measurable change in $[Ca^{2+}]_{cyt.}$. Glucagon-induced Ca^{2+} responses in the absence of other agonists may also be related to the condition of the cells; it could be speculated that spherical or freshly isolated cells either have higher resting $InsP_3$ levels, essentially mimicking the action of sub-threshold pre-stimulation with a PI-breakdown-linked agent, or may express additional, distinct, signalling mechanisms. Though potentially significant for hepatocytes *in situ*, glucagon-mediated elevation of $InsP_3$ does not need to be invoked to explain the results described here.

Stimuli directly activating the cAMP signalling system showed the same type of positive modulation as glucagon, consistent with the interpretation that the effects of glucagon on $[Ca^{2+}]_{cyt.}$ oscillations are mainly mediated by cAMP. Forskolin and the cAMP analogues 8-bromo-cAMP and 8-CPT-cAMP (see Table 1) increased the frequency of phenylephrine-induced $[Ca^{2+}]_{cyt.}$ oscillations or induced overstimulation, or triggered oscillations after sub-threshold prestimulation with phenylephrine. Comparable results have been shown for forskolin and dibutyryl cAMP [18]. In addition, we demonstrated that the PKA inhibitor R_p-cAMPS [24], which antagonizes cAMP-induced protein phosphorylation in hepatocytes [33], could suppress oscillations induced by the α_1-adrenergic agonist phenylephrine, with or without additional stimulation of the cAMP pathway. This suggests that the action of basal or elevated cAMP with respect to $[Ca^{2+}]_{cyt.}$ oscillations involves PKA-dependent phosphorylation. Since this inhibitory effect was not always clearly detectable in the absence of a cAMP-linked stimulus, it appears that the cAMP messenger pathway is not mandatory for, but serves an important modulatory function in, the generation of $[Ca^{2+}]_{cyt.}$ oscillations.

Which mechanism may be responsible for the enhancement of the PI-breakdown-dependent Ca^{2+} response by cAMP? A key study using intact and permeabilized hepatocytes has demonstrated that the target of cAMP lies after the generation of $InsP_3$, in the coupling of $InsP_3$ to Ca^{2+} release from an intracellular store [34]. It has been shown that β-adrenergic stimulation does not affect PI metabolism, while potentiating the Ca^{2+} response to PI-breakdown-linked stimulation. Circumventing the hormone-receptor signal-transduction pathway, the catalytic subunit of PKA or 8-bromo-cAMP increases the Ca^{2+} release induced by $InsP_3$ in permeabilized cells. Treatment with PKA leads to a shift of the dose/response curve for $InsP_3$-induced Ca^{2+} release, resulting in a 5-fold lowering of the EC_{50} for $InsP_3$. This effect could be eliminated by the wiptide inhibitor of PKA, analogous to the antagonism of cAMP-dependent stimulation by R_p-cAMPS observed here. A cAMP-dependent phosphorylation, possibly of the $InsP_3$ receptor, may underlie the synergism between PI breakdown and cAMP-linked agonists in the control of $[Ca^{2+}]_{cyt.}$ [34]. This corresponds to a positive modulation of the rate constant α in our model [17] by sensitizing the response of α to $InsP_3$ (Fig. 9). Such a model of cAMP action may be restricted to this particular preparation; in cerebellar microsomes, phosphorylation of the $InsP_3$ receptor by PKA is actually correlated with a decrease in $InsP_3$-induced Ca^{2+} release [35]. On the other hand, this paradox could be reconciled if the positive modulation by cAMP in hepatocytes is due to the phosphorylation of a transducing element and not of the $InsP_3$ receptor itself, the former sensitizing effect being dominant over the inhibition directly on the receptor level.

Additional interactions may be involved in the modulation of $[Ca^{2+}]_{cyt.}$ by cAMP. The binding of noradrenaline to hepatocytes is increased after treatment of hepatocytes with forskolin, dibutyryl cAMP or glucagon [5]. Since these authors did not distinguish between α_1 and α_2 receptors, the latter not acting via PI breakdown, the link to increased $InsP_3$ production has not yet been positively established. An inconsistency still exists regarding the effects of cAMP in the absence of phospholipase C-linked agonists. Though a cAMP-stimulated increase in $InsP_3$ [36] and $[Ca^{2+}]_{cyt.}$ [32,36], independent of a PI-breakdown-associated agonist, has been reported, this finding could not be corroborated by other investigations [18,34]. At any rate, if this mechanism were operative, it is apparently without significant effect on the Ca^{2+} responses examined here; like glucagon, direct cAMP agonists did not independently evoke a Ca^{2+} response under the prevailing experimental conditions.

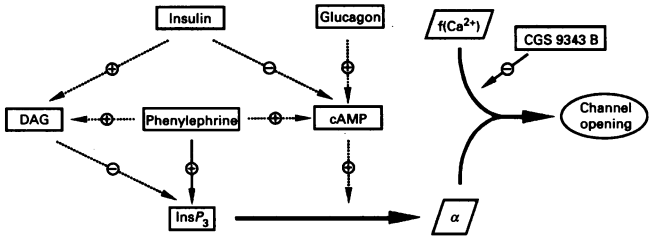

Fig. 9. Cross-talk between second messengers in the control of $[Ca^{2+}]_{cyt.}$ oscillations

The scheme depicts interactions of the signalling pathways investigated here, as documented by other studies (see the Discussion section). The positive and negative arrows resemble stimulatory or inhibitory influences respectively. Broken arrows resemble 'modulatory' effects, not mandatory for oscillations to occur as such. Continuous arrows refer to steps involved in the 'core' of our model, as described in detail previously [17]. The central oscillatory mechanism or 'core' is based on Ca^{2+} release from an intracellular store through an $InsP_3$- and Ca^{2+}/calmodulin-regulated channel (wide arrows). $InsP_3$ increases the rate constant α, whereas Ca^{2+} exerts its positive co-operative feedback through $f(Ca^{2+})$ {f(y) previously [17]}, the target of the calmodulin antagonist CGS 9343 B. The product of α and $f(Ca^{2+})$ then controls channel opening. In this scheme, the modulatory steps simply affect either $InsP_3$ formation or the coupling of $InsP_3$ to α. Abbreviation: DAG, diacylglycerol.

We have argued above that the modulatory effects of cAMP on $[Ca^{2+}]_{cyt.}$ oscillations fit into the general framework of our previously proposed model at a simple interpretative level [17]. An essential feature of this model is the dual control of Ca^{2+} release (see Fig. 9) from an intracellular store by $InsP_3$ and positive co-operative feedback of Ca^{2+}. This is in accord with Ca^{2+} facilitating its own release at low concentrations (0.1–3 μM), but acting as an inhibitor in a higher range (3–100 μM) [20]. Invariable inhibition of $[Ca^{2+}]_{cyt.}$ oscillations by several chemically unrelated calmodulin antagonists [17], demonstrated to inhibit $InsP_3$-induced Ca^{2+} release from permeabilized cells [21], suggests that a calmodulin-related element is involved in operating the $InsP_3$-sensitive Ca^{2+} channel in hepatocytes. To test whether cAMP induces $[Ca^{2+}]_{cyt.}$ oscillations within this framework, we examined the ability of previously employed calmodulin inhibitors in suppressing the oscillations. Calmidazolium and CGS 9343 B (discussed in [17]) effectively antagonized cAMP modulation of $[Ca^{2+}]_{cyt.}$ oscillations in all cases (Table 1). In addition, phenylephrine could generally reverse the action of these calmodulin inhibitors applied in concentrations > 10 times higher than those necessary for initial inhibition [17]. Cyclic-AMP-linked agonists could only antagonize calmodulin inhibitors employed in lower concentrations (results not shown). These results on the antagonism between cAMP and calmodulin inhibitors underline the modulatory nature of cAMP stimulation and their compliance with our model of $[Ca^{2+}]_{cyt.}$ oscillations.

Modulation of $[Ca^{2+}]_{cyt.}$ oscillations by PKC

It may seem paradoxical that PKC activation by diacylglycerol, generated together with $InsP_3$ during PI breakdown, inhibits $[Ca^{2+}]_{cyt.}$ oscillations. A simple explanation for this would be that diacylglycerol exerts a negative feedback on PI breakdown, as evidenced on two levels in hepatocytes. In the case of the α_1-adrenergic response, inhibition by PKC is located in part on the receptor itself [37]. Concerning the PKC antagonism of vasopressin, no negative effect is observed at the receptor level [37]. This second target of PKC appears to lie between receptor stimulation, G-protein and phospholipase C activation. Activators of PKC inhibit (1) guanosine 5'-[γ-thio]triphosphate-stimulated PI breakdown in astrocytoma cells [38] and polymorphonuclear leucocytes [39], and (2) guanosine 5'-[γ-thio]-triphosphate-induced current response in lacrimal cells [40]. Since the α_1-adrenergic agonist phenylephrine was always present, at least for pre-stimulation, modulation of agonist binding may partially explain the inhibitory effects of the synthetic diacyglycerol, DiC_8, on the $[Ca^{2+}]_{cyt.}$ oscillations observed here. But, since vasopressin-induced oscillations could be antagonized by PKC activators as well [19], a step between the receptor and phospholipase C may also be sensitive to PKC. Both possibilities are compatible with our scheme (Fig. 9). It has been proposed that periodic negative feedback of PKC on $InsP_3$ production is fundamental to the generation of $[Ca^{2+}]_{cyt.}$ oscillations [41]. This assumption is not supported by the observation of $[Ca^{2+}]_{cyt.}$ oscillations in the absence of PKC activity in fibroblasts [42]. As an alternative, the significance of negative feedback by PKC on $InsP_3$ formation may lie in extending the range of hormone concentrations wherein oscillations occur.

Insulin and $[Ca^{2+}]_{cyt.}$ oscillations

So far, only the interactions between glycogenolytic stimuli have been investigated in the generation of $[Ca^{2+}]_{cyt.}$ oscillations. Is the physiologically antagonistic role of insulin as a glycogenic hormone reflected in negative modulation of $[Ca^{2+}]_{cyt.}$ oscillations? The evidence presented above shows that insulin can counteract stimulation of $[Ca^{2+}]_{cyt.}$ oscillations by phenylephrine, glucagon and various cAMP-liked agonists. Unlike the other inhibitors employed, i.e. calmodulin and PKC activators, insulin was not always able to suppress $[Ca^{2+}]_{cyt.}$ oscillations completely and could not be repeatedly titrated against stimulatory agents. In addition, at concentrations other than 10 nM (the most effective concentration for physiological responses [8,11,29]), insulin occasionally led to an 'anomalous' positive modulation of $[Ca^{2+}]_{cyt.}$ oscillations (see Table 2). Since the insulin signalling system is a composite of various second-messenger pathways [13,14], several outcomes with respect to a particular parameter as $[Ca^{2+}]_{cyt.}$ may be possible, depending on the relative contribution of each component.

Could the action of insulin be due to modulation of the level of cAMP? Thomas & Williamson [43] have suggested 'that insulin antagonism of α_1-adrenergic effects on glycogenolysis in liver is mediated at a step distal to hormone binding to the α_1 receptor and activation of inositol lipid breakdown but prior to intracellular Ca^{2+} mobilization.' A decrease in $[cAMP]_{cyt.}$ might be expected owing to activation of a phosphodiesterase [8], which is presumably regulated by the inositol-glycan second messenger of insulin [44–46]. This second messenger also seems to be associated with an inhibition of adenylate cyclase and PKA [45]. Inhibition of $[Ca^{2+}]_{cyt.}$ oscillations by insulin via decreases in cAMP and PKA activity appears feasible, recalling the positive modulatory role of cAMP and glucagon. Even stimulation by phenylephrine, not primarily associated with activation of adenylate cyclase, may be sensitive to antagonism of the cAMP pathway. This has been demonstrated by using the PKA inhibitor R_p-cAMPS. The limited response of phenylephrine-induced oscillations to R_p-cAMPS and insulin may be due to different basal contributions of cAMP in different cells.

Next to the effects on the level of cAMP, which role should be attributed to diacylglycerol appearing after insulin stimulation via different pathways [47]? The degree of diacylglycerol production and activation of PKC by insulin is comparable with those of the PI-breakdown-linked hormones vasopressin and angiotensin II in hepatocytes [48]. Since DiC_8 and other PKC activators [19] are effective inhibitors of $[Ca^{2+}]_{cyt.}$ oscillations, the diacylglycerol pathway could account in part for the negative

modulation by insulin. We explored the possibility that insulin's limited capacity as an antagonist may be due to maximal PKC activation. Although insulin had reached its maximal inhibitory effect, application of DiC_8 still led to further inhibition. Therefore, maximal stimulation of PKC had apparently not yet occurred. Nonetheless, the two established pathways for insulin action, i.e. cAMP antagonism and diacylglycerol formation, could complement each other in the inhibition of $[Ca^{2+}]_{cyt.}$ oscillations.

Finally, there are other important steps in insulin signalling which may be involved in the modulation of $[Ca^{2+}]_{cyt.}$ oscillations: protein phosphorylation by the insulin-receptor tyrosine kinase and an insulin-activated serine/threonine kinase [13,14]. The mechanisms discussed above cannot explain the 'anomalous' positive modulation and insulin's unpredictable behaviour at non-physiological concentrations; insulin does not generally lead to $InsP_3$ formation and cAMP production [48], the two established stimulators of $[Ca^{2+}]_{cyt.}$ oscillations. Protein tyrosine phosphorylation may offer a link to the 'anomalous' Ca^{2+} response. Though no direct evidence is available for insulin, activation of the epidermal growth factor receptor, a tyrosine kinase, leads to Ca^{2+} elevation in hepatocytes [49] and also to $InsP_3$ production in a hepatocellular carcinoma [50]. On the other hand, EGF is able to mimic some of insulin's ability to decrease cAMP [49], opening the possibility of tyrosine phosphorylation also being involved in the antagonism of cAMP by insulin. Further experiments examining second-messenger formation and protein phosphorylation stimulated by a wider range of insulin and EGF concentrations should help to elucidate some of these interesting possibilities.

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REFERENCES

1. Berridge, M. J., Cobbold, P. H. & Cuthbertson, K. S. R. (1988) Philos. Trans. R. Soc. London B **320**, 325–343
2. Jacob, R. (1990) Biochim. Biophys. Acta **1052**, 427–438
3. Tsien, R. W. & Tsien, R. Y. (1990) Annu. Rev. Cell Biol. **6**, 715–760
4. Tsien, R. W. (1988) Trends Neurosci. **11**, 419–424
5. Morgan, N. G., Charest, R., Blackmore, P. F. & Exton, J. H. (1984) Proc. Natl. Acad. Sci. U.S.A. **81**, 4208–4212
6. Poggioli, J., Mauger, J.-P. & Claret, M. (1986) Biochem. J. **235**, 663–669
7. Combettes, L., Berthon, B., Binet, A. & Claret, M. (1986) Biochem. J. **237**, 675–683
8. Heyworth, C. M., Wallace, A. V. & Houslay, M. D. (1983) Biochem. J. **214**, 99–110
9. Dehaye, J.-P., Hughes, B. P., Blackmore, P. F. & Exton, J. H. (1981) Biochem. J. **194**, 949–956
10. Thomas, A. P., Martin-Requero, A. & Williamson, J. R. (1985) J. Biol. Chem. **260**, 5963–5973
11. Iynedjian, P. B., Jotterand, D., Nouspikel, T., Asfari, M. & Pilot, P.-R. (1989) J. Biol. Chem. **264**, 21824–21829
12. Sasaki, K., Cripe, T. P., Koch, S. R., Andreone, T. L., Peterson, D. D., Beale, E. G. & Granner, D. K. (1984) J. Biol. Chem. **259**, 15242–15251
13. Sale, G. H. (1988) Int. J. Biochem. **9**, 897–908
14. Larner, J. (1988) Diabetes **37**, 262–275
15. Woods, N. M., Cuthbertson, K. S. R. & Cobbold, P. H. (1986) Nature (London) **319**, 600–602
16. Rooney, T. A., Sass, E. J. & Thomas, A. P. (1989) J. Biol. Chem. **264**, 17131–17141
17. Somogyi, R. & Stucki, J. W. (1991) J. Biol. Chem. **266**, 11068–11077
18. Schöfl, C., Sanchez-Buono, A., Brabant, G., Cobbold, P. H. & Cuthbertson, K. S. R. (1991) Biochem. J. **273**, 799–802
19. Woods, N. M., Cuthbertson, K. S. R. & Cobbold, P. H. (1987) Biochem. J. **246**, 619–623
20. Finch, E. A., Turner, T. J. & Goldin, S. M. (1991) Science **252**, 443–446
21. Hill, T. D., Campos-Gonzalez, R., Kindmark, H. & Boynton, A. L. (1988) J. Biol. Chem. **263**, 16479–16484
22. Kawanishi, T., Blank, L. M., Harootunian, A. T., Smith, M. Y. & Tsien, R. Y. (1989) J. Biol. Chem. **264**, 12859–12866
23. Seamon, K. B. & Daly, J. W. (1981) J. Cyclic Nucleotide Res. **7**, 201–224
24. Reber, B. F. X., Somogyi, R. & Stucki, J. W. (1990) Biochim. Biophys. Acta **1018**, 190–193
25. Botelho, L. H. P., Rothermel, J. D., Coombs, R. V. & Jastorff, B. (1988) Methods Enzymol. **159**, 159–172
26. van Belle, H. (1981) Cell Calcium **2**, 483–494
27. Norman, J. A., Ansell, J., Stone, G. A., Wennogle, L. P. & Wasley, J. W. F. (1987) Mol. Pharmacol. **31**, 535–540
28. Ganong, B. G., Loomis, C. R., Hannun, Y. A. & Bell, R. M. (1986) Proc. Natl. Acad. Sci. U.S.A. **83**, 1184–1188
29. Morin, O., Fehlmann, M. & Freychet, P. (1982) Mol. Cell. Endocrinol. **25**, 339–352
30. Tilghman, S. M., Hanson, R. W. & Ballard, F. J. (1976) in Gluconeogenesis—Its Regulation in Mammalian Species (Hanson, R. W. & Mehlman, M., eds.), pp. 47–91, John Wiley and Sons, New York
31. Wakelam, M. J. O., Murphy, G. J., Hruby, V. J. & Houslay, M. D. (1986) Nature (London) **323**, 68–71
32. Staddon, J. M. & Hansford, R. G. (1986) Biochem. J. **238**, 737–743
33. Connelly, P. A., Botelho, L. H. P., Sisk, R. B. & Garrison, J. C. (1987) J. Biol. Chem. **262**, 4324–4332
34. Burgess, G. M., Bird, G. St. J., Obie, J. F. & Putney, J. W., Jr. (1991) J. Biol. Chem. **266**, 4772–4781
35. Supattapone, S., Danoff, S. K., Theibert, A., Joseph, S. K., Steiner, J. & Snyder, S. H. (1988) Proc. Natl. Acad. Sci. U.S.A. **85**, 8747–8750
36. Blackmore, P. F. & Exton, J. H. (1986) J. Biol. Chem. **261**, 11056–11063
37. Lynch, C. J., Charest, R., Bocckino, S. B., Exton, J. H. & Blackmore, P. F. (1985) J. Biol. Chem. **260**, 2844–2851
38. Orellana, S., Solski, P. A. & Brown, J. H. (1987) J. Biol. Chem. **262**, 1638–1643
39. Smith, C. D., Uhing, R. J. & Snyderman, R. (1987) J. Biol. Chem. **262**, 6121–6127
40. Llano, I. & Marty, A. (1987) J. Physiol. (London) **394**, 239–248
41. Sanchez-Bueno, A., Dixon, C. J., Woods, N. M., Cuthbertson, K. S. R. & Cobbold, P. H. (1990) Biochem. J. **268**, 627–632
42. Harootunian, A. T., Kao, J. P. Y., Paranjape, S. & Tsien, R. Y. (1991) Science **251**, 75–78
43. Thomas, A. P. & Williamson, J. R. (1983) J. Biol. Chem. **258**, 1411–1414
44. Saltiel, A. R. & Cuatrecasas, P. (1986) Proc. Natl. Acad. Sci. U.S.A. **83**, 5793–5797
45. Malchoff, C. D., Huang, L., Gillespie, N., Palasi, C. V., Schwartz, C. F. W., Cheng, K., Hewlett, E. L. & Larner, J. (1987) Endocrinology (Baltimore) **120**, 1327–1337
46. Saltiel, A. R. (1987) Endocrinology (Baltimore) **120**, 967–972
47. Farese, R. V. (1988) Am. J. Med. **85** (suppl. 5A), 36–43
48. Cooper, D. R., Hernandez, H., Kuo, J. Y. & Farese, R. V. (1990) Arch. Biochem. Biophys. **276**, 486–494
49. Bosch, F., Bouscarel, B., Slaton, J., Blackmore, P. F. & Exton, J. H. (1986) Biochem. J. **239**, 523–530
50. Gilligan, A., Prentki, M. & Knowles, B. B. (1990) Exp. Cell Res. **187**, 134–142

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