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# Inhibitors of protein kinase C prolong the falling phase of each free-calcium transient in a hormone-stimulated hepatocyte

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Many cells generate oscillations in cytoplasmic free $Ca^{2+}$ concentration ('free Ca') when stimulated with Ca-mobilizing hormones. The frequency of repetitive free-Ca transients in a rat hepatocyte is a function of hormone concentration and can be depressed by phorbol esters. We show here that the protein kinase C (PKC) inhibitors staurosporine and sphingosine can reverse the effects of phorbol dibutyrate on the frequency of free-Ca transients induced by phenylephrine or vasopressin. An important feature of the hepatocyte free-Ca oscillator is that the transient's time course, particularly the rate of fall of free Ca from peak to resting, depends on the species of agonist, and is measurably different for phenylephrine, vasopressin, angiotensin II or ATP. We show here that the rate of fall of free Ca in transients induced by phenylephrine or vasopressin is markedly decreased after treatment of the cells with a PKC inhibitor. A receptor-controlled oscillator model is discussed, in which PKC provides negative feedback during the falling phase of free-Ca transients.

## INTRODUCTION

Calcium-mobilizing hormones induce oscillations of cytoplasmic free $Ca^{2+}$ concentration ('free Ca') in many cells. How hormonal activation of the phosphatidylinositol signalling pathway leads to free-Ca oscillations is not yet known, but two main hypotheses have been proposed [1]. One model proposes that the cytoplasmic level of $InsP_3$ is held relatively stable and that a cytoplasmic mechanism, such as cyclical release and re-uptake of calcium by intracellular compartments, generates the free-Ca oscillation. Evidence for such a mechanism comes from micro-injection of $InsP_3$ or its non-metabolizable phosphorothioate analogues into *Xenopus* oocytes [2,3] or acinar cells [4]. On the other hand, we have proposed that the hormone-induced frequency-modulated repetitive free-Ca transients in rat hepatocytes [5,6] arise from repetitive transient generation of $InsP_3$ as a result of transient activation of phosphoinositidase C (PIC) [1,6–8]. Our model had to explain the key observation that, in the same individual hepatocyte, the time course of a free-Ca transient depends on the agonist species [1,6]. Thus transients induced by phenylephrine or ADP have a much faster fall from peak free Ca back to resting (~4 s) than transients induced by vasopressin (~10 s), angiotensin II (~14 s) or ATP (up to 40 s; [9]). Such observations are difficult to explain by a cytoplasmic oscillator remote from the plasmalemma, since receptor-specific information appears to be retained on a second-by-second basis. We therefore proposed that the rising phase of the transient is due to sudden switch-on of PIC and that the subsequent fall of free Ca results from negative feedback from protein kinase C (PKC) acting to phosphorylate, and hence inactivate, the various receptors and/or their specific G-proteins. Differences in the falling phase of free-Ca transients are postulated to arise from different dynamics in PKC-mediated inactivation of the different receptors and/or G-proteins [1,6–8]. Since we envisage the rising phase of each transient being caused by a sudden activation of PIC, it follows that PKC activity would also be transient, following the sudden increase in diacylglycerol (DG) production, along with $InsP_3$, and the rise in free Ca. In an initial attempt to test this concept of dynamic PIC activity, we found that the phorbol esters phorbol 12-myristate 13-acetate (PMA) or phorbol 12,13-dibutyrate (PDB) merely depressed the frequency of the free-Ca transients, having little effect on each transient's time course [10]. Since transient frequency is normally a function of agonist concentration, the effect of phorbol esters could have arisen from a sustained PKC-mediated phorphorylation of a proportion of the receptors, thereby decreasing receptor affinity for agonist, and hence free-Ca transient frequency. The failure of phorbol esters to affect transient time course, we argued [10], results from their failure to mimic the putatively transient time course of PKC activation by DG and free Ca. During a transient, the rise in free Ca and the putative sudden rise in DG could lead to a sudden, but intense, activation of PKC which cannot be mimicked by exogenously added phorbol esters, since sufficiently high phorbol ester doses would first prevent spike initiation by phosphorylating all receptors to a low-affinity state. In the experiments reported here, we aimed to allow the cell to generate the activators of PKC (DG and free Ca) as normal, and to investigate the effects on free transients of inhibiting PKC with either staurosporine [11] or sphingosine [12]. Both PKC inhibitors induce a prolongation of the falling phase of free Ca in each transient, lending support to the concept of each free-Ca transient being generated by a receptor-controlled oscillator [1,6–8] in which transient activation of PIC is curtailed by negative feedback from PKC.

## MATERIALS AND METHODS

Hepatocytes were isolated from fed male Wistar rats by collagenase perfusion as described previously [6]. Single hepatocytes were micro-injected with aequorin as described previously [5,6,10]. Staurosporine (Calbiochem) and PDB (Sigma) were dissolved in dimethyl sulphoxide, and sphingosine (Sigma) was dissolved in ethanol, and stored in small aliquots at −70 °C, to avoid freeze–thawing. Fresh solutions were prepared daily, in Williams Medium E (Flow Laboratories) gassed with

Abbreviations used: free Ca, cytoplasmic concentration of free $Ca^{2+}$; PIC, phosphoinositidase C; PKC, protein kinase C; DG, diacylglycerol; PMA, phorbol 12-myristate 13-acetate; PDB, phorbol 12,13-dibutyrate.

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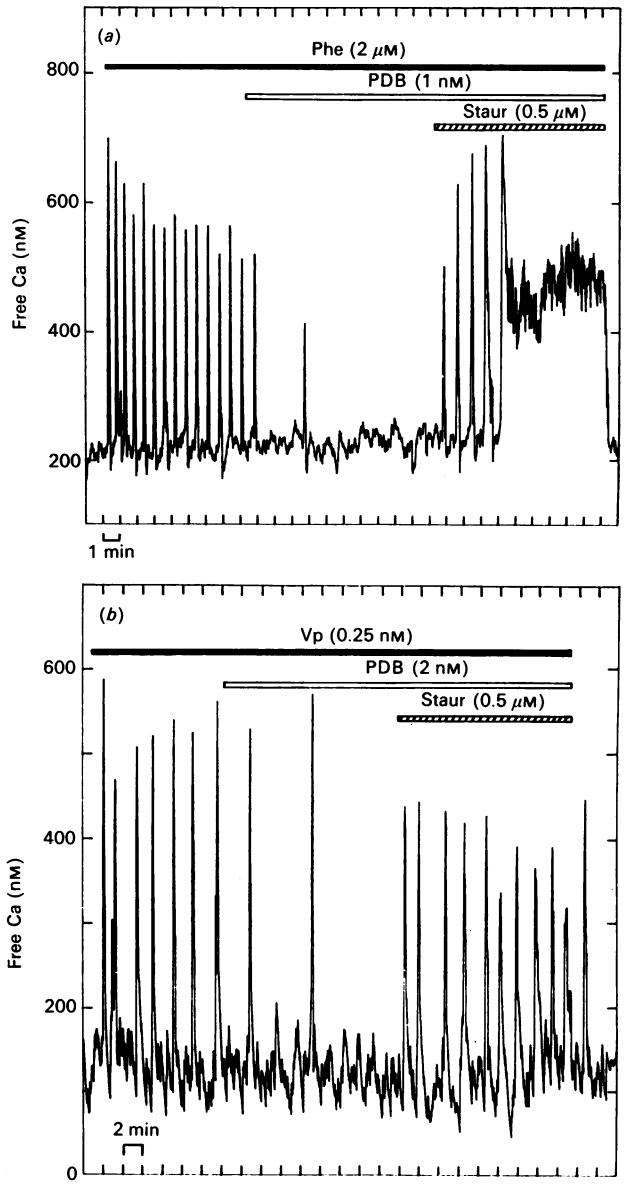


**Fig. 1. Aequorin signals calibrated as free Ca concentration (nM), obtained from single rat hepatocytes microinjected with aequorin and superfused with (*a*) 2 μM-phenylephrine (Phe) and 1 nM-PDB or (*b*) 0.25 nM-[Arg$^8$]vasopressin (Vp) and 2 nM-PDB**

Staurosporine (500 nM) was superfused at the times indicated. The time constant for resting concentration of free Ca was 10 s, and for transients 1 s. For details see the text.

air/$CO_2$ (19:1); temperature 36–37 °C. The aequorin data were collected and analysed as described previously [6]. Statistical significance was calculated by Student's *t* test.

## RESULTS

Neither staurosporine nor sphingosine has absolute specificity for inhibiting PKC [12,13], so we sought first to show that PKC is an important target for both inhibitors by demonstrating their ability to reverse the effects of phorbol esters on decreasing the frequency of agonist-induced free-Ca transients [10]. Fig. 1 shows that free-Ca transients induced by either phenylephrine (Fig. 1*a*) or vasopressin (Fig. 1*b*) could be decreased in frequency or blocked completely with nanomolar concentrations of PDB, and that this blockade could be promptly overcome, at least in part, by 500 nM-staurosporine in the superfusate. Fig. 2 shows a

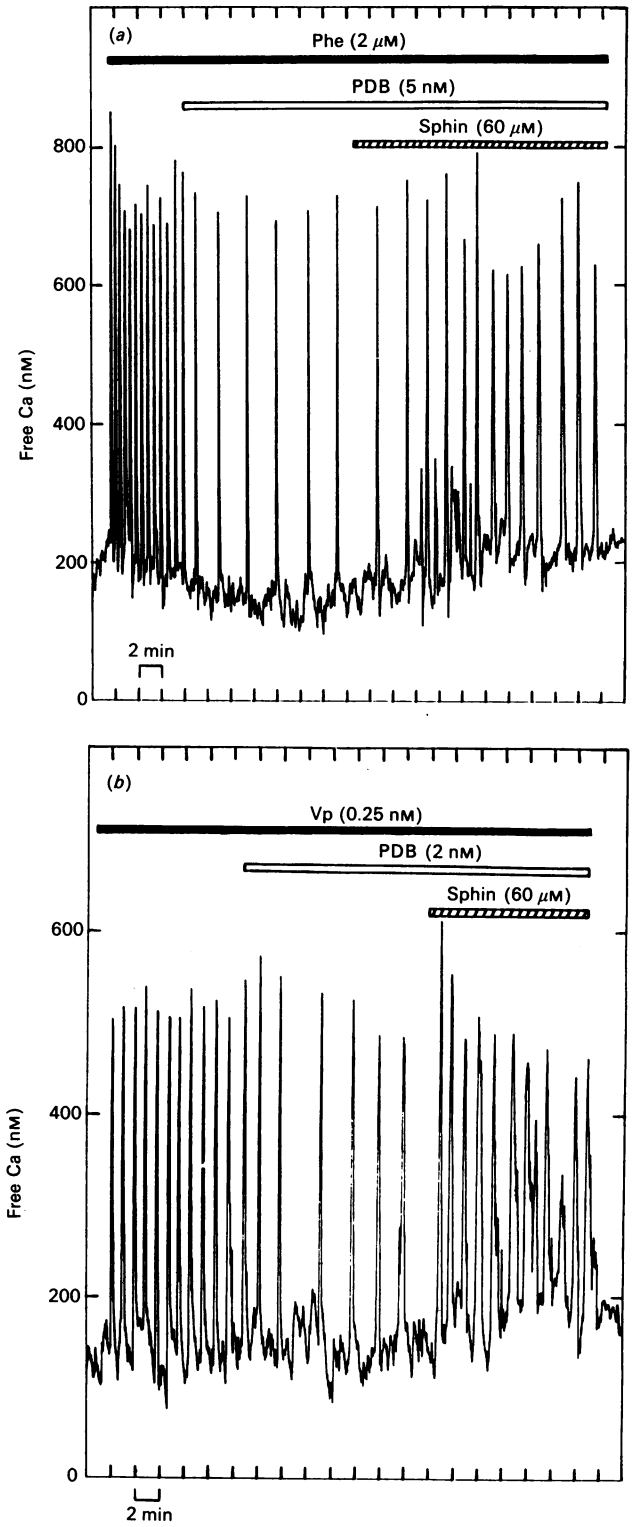


**Fig. 2. Sphingosine-promoted reversal of PDB-induced blockade of free-Ca transients in single aequorin-injected hepatocytes exposed to phenylephrine (*a*) or vasopressin (*b*)**

The time constants, abbreviations, and other details were as in Fig. 1.

similar protocol, but with sphingosine (60 μM) as PKC inhibitor. These data establish that PKC is an important target for inhibition by staurosporine and sphingosine.

A more detailed examination of Figs. 1 and 2 reveals that both PKC inhibitors have effects on the time course of the free-Ca

transients. These effects develop rather later after exposure to inhibitor than their immediate effect on resumption of spiking. Thus in Fig. 1(*a*) the fourth transient generated after ~3 min in staurosporine is detectably prolonged compared with the control spikes. The sustained 'plateau' of free Ca during the falling phase of the fifth transient is most notable, since phenylephrine, even at millimolar concentrations, in normal cells never induces a sustained rise, but always repetitive transients with a period of ~20 s [5,6]. The free-Ca plateau is not due to any ill-defined toxic effect on the cell, since removal of the agonist promptly restored free Ca to normal (Fig. 1*a*). A plateau of free Ca was recorded in five staurosporine-treated hepatocytes exposed to phenylephrine concentrations in the range 2–10 μM. At 0.5 μM or 1 μM-phenylephrine a plateau was not seen, but the spikes became progressively lengthened in their falling phase (three cells). Similar observations of a plateau have been obtained in cells that had not been exposed to PDB (see Fig. 3*b*, below). Interestingly, a plateau of free Ca was not recorded in four out of five cells stimulated with vasopressin at concentrations from 0.1 to 0.8 nM (Fig. 1*b*); a small prolongation of the falling phase was observed, while peak free Ca remained unaltered. In the fifth cell peak free Ca was slightly decreased (by 200 nM). The fifth cell did, however, show a plateau of free Ca similar to that induced by high phenylephrine concentrations. Rather more obvious effects of staurosporine on the shape of spikes induced by vasopressin have been recorded in the absence of PDB treatment (see Fig. 4, below).

Fig. 2 shows that sphingosine is also able to reverse the effects of PDB on transient frequency. Detailed examination of the effects of sphingosine on spike time course reveals a lengthening of transients induced by phenylephrine (Fig. 2*a*) or by vasopressin (Fig. 2*b*). In five cells exposed to sphingosine and to phenylephrine at concentrations of 0.6–5 μM a plateau of free Ca, such as is seen with staurosporine (above), was not found. In five cells pretreated with sphingosine (60 μM, 10 min) phenylephrine induced about four spikes, all with prolonged falling phases, and then spiking ceased, whereas pretreatment with staurosporine (Fig. 3, below) did not lead to cessation of the free-Ca responses to phenylephrine.

Since Figs. 1 and 2 are complicated by the presence of phorbol esters and the time-dependence of the effects of the PKC inhibitors, especially staurosporine, we resorted to pretreatment of the hepatocyte with the inhibitor alone. Fig. 3 shows that pretreatment with staurosporine (500 nM, 10 min) has an effect which persists when phenylephrine was re-applied in the absence of staurosporine. As described above, whether phenylephrine continues to induce spikes (as in Fig. 3*a*) or induces a plateau of free Ca (Fig. 3*b*) depends on the concentration of phenylephrine. At 0.5 μM-phenylephrine (Fig. 3*a*) an obvious prolongation of the falling phase of the transient was recorded (four out of four cells). A decrease in peak height does not usually occur (three out of four cells); Fig. 3(*a*) is from the fourth cell, when a small peak fall is seen. In all cells (four out of four) a decrease in the frequency of the transients occurred, although the period for which free Ca is close to resting levels (e.g. 300 nM or below) is somewhat shorter than in the control sequence of spikes. So the decrease in frequency appears to be mostly a reflection of the increase in the length of the phase of falling free Ca. When a higher concentration of phenylephrine [2 μM (one cell); 5 μM (two cells); 50 μM (two cells)] is applied to a staurosporine-treated cell a sustained plateau of free Ca is seen (Fig. 3*b*), which appears to result from an enormous prolongation of the falling phase of the first spike. As discussed above (Fig. 1*a*), this is a most unusual response for a cell to make to phenylephrine, which is notable for its ability to induce repetitive transients even at very high concentrations (e.g. 1 mM; [5,6]), whereas vasopressin, even at concentrations of only ~10 nM, produce a sustained rise in free Ca that only returns to resting after ~5 min.

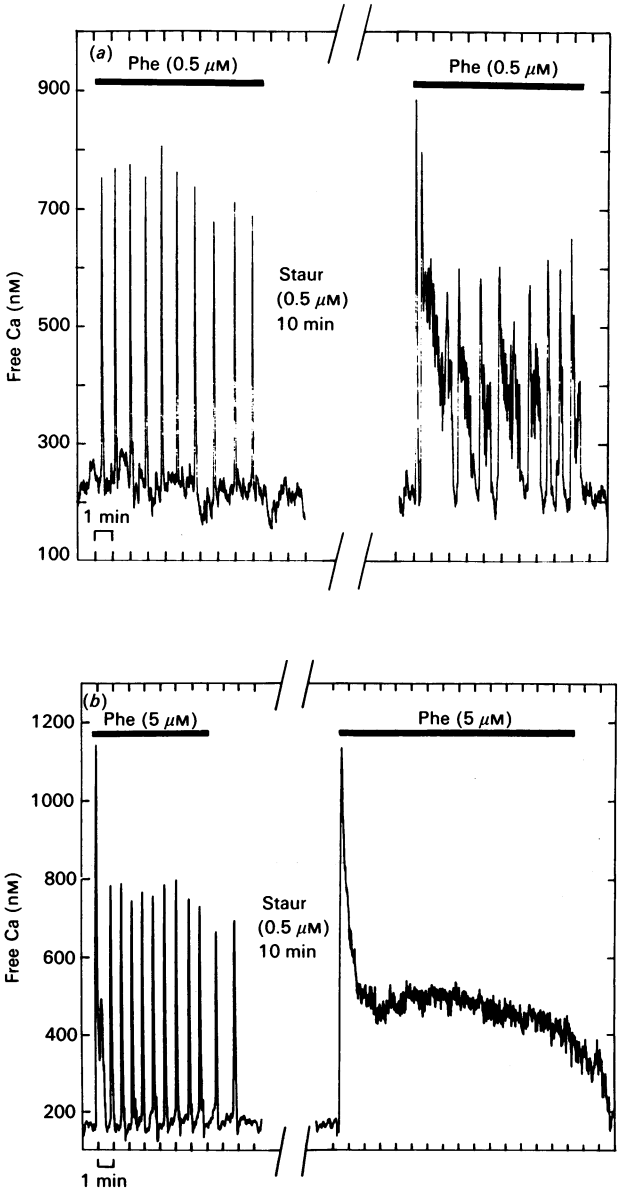


**Fig. 3. Pretreatment of hepatocytes with staurosporine (500 nM, 10 min) induces prolongation of the falling phase of free Ca in phenylephrine (0.5 μM)-induced transients (*a*), or a sustained plateau of free Ca in a cell exposed to a higher (5 μM) phenylephrine concentration (*b*)**

The time constants and other details were as in Fig. 1.

Close examination of Fig. 3(*a*) reveals an initial spike on the leading edge of the first transient after staurosporine treatments. At higher time resolution (not shown) this initial spike is seen to rise from resting free Ca to ~900 nM and fall back to resting in a total of 9 s, whereupon free Ca rose, after ~15 s at resting levels, rapidly back to 800 nM. These initial spikes were seen in three out of four cells subjected to this protocol, the exception being the cell shown in Fig. 3(*b*).

Prolongation of the falling phase of free Ca is also seen in staurosporine-pretreated hepatocytes when vasopressin is the agonist (Fig. 4; Table 1), at a range of vasopressin concentrations [0.1 nM (three cells); 0.4 nM (one cell); 0.8 nM (one cell)]. Peak free Ca remained similar (four out of five cells) or slightly

**Table 1. Effect of treatment with staurosporine (500 nM, 10 min) on the characteristics of transients induced by 1 nM-vasopressin**

Results are means ± S.D. for the number ($n$) of transients in an individual cell. *$P < 0.01$.

| | $n$ | Smoothed peak free Ca (nM) | Length of the transient (s) | Falling time constant (s) |
|---|---|---|---|---|
| Control | 11 | 601 ± 46 | 12.51 ± 3.15 | 10.00 ± 2.70 |
| After staurosporine | 9 | 610 ± 107 | 19.69 ± 3.87* | 16.99 ± 6.49* |

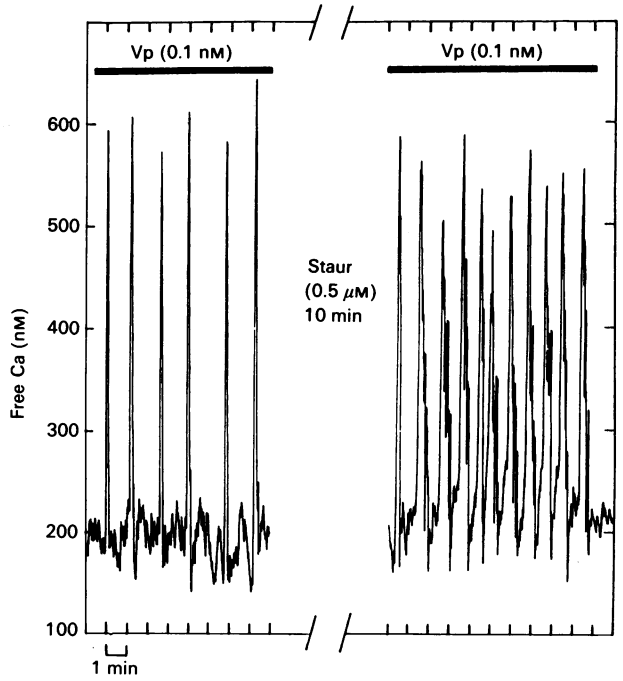


**Fig. 4. Effect of pretreatment with staurosporine (500 nM, 10 min) on hepatocyte free Ca transients induced by 0.1 nM-vasopressin**

The time constants and other details were as in Fig. 1.

decreased (by 200 nM; one cell). Interestingly, in the experiment of Fig. 4, which used a low dose of vasopressin to induce control spikes with a long period (~1.5–2 min), staurosporine treatment resulted in an increase in transient frequency, despite the prolongation of the falling phase of free Ca in each transient. Indeed, the period during which free Ca was at resting levels was several-fold shorter than in the control sequence of spikes. This effect was recorded in three cells; in two cells frequency did not change. We have not detected any fast initial spikes upon re-admitting vasopressin to a staurosporine-treated cell.

More detailed information of the effect of staurosporine on free-Ca transients is given in Table 1. The falling phase and the length of the free-Ca transients induced by vasopressin both increase significantly after treatment of the hepatocyte with staurosporine. However, peak free Ca was not significantly affected. Control experiments showed that dimethyl sulphoxide or ethanol, the solvents for the inhibitors, at concentrations present in these experiments (up to 0.05 % and 0.6 % respectively) had no effect on the free-Ca transients. Neither staurosporine nor sphingosine themselves induced free-Ca transients (results not shown).

## DISCUSSION

Activation of PKC by low concentrations of phorbol ester, including PDB, leads to a decrease in the frequency of hormone-induced repetitive free-Ca transients in a hepatocyte [10]. The prompt effect of staurosporine or sphingosine in reversing the frequency-decreasing action of PDB (Figs. 1 and 2) suggests that these agents do inhibit PKC in the intact hepatocyte. However, neither staurosporine [13] nor sphingosine [12] is specific for PKC, and both could have other cellular effects. Staurosporine inhibits PKC *in vitro* with $K_i = 3$ nM, whereas $K_i = 8$ nM for cyclic AMP-dependent protein kinase. The concentration of staurosporine used here (500 nM) is much higher than the $K_i$ value *in vitro* (but lower than that used to show effects on PtdIns-mediated responses of intact cells [14–16]), possibly because of slow penetration of staurosporine into the cell. On the other hand, in view of the lipophilicity of sphingosine a rapid loading of the cell would be predicted. So we cannot expect the extent of PKC inhibition to be equal for both inhibitors, and they may become effective to different degrees with different time courses. However, it is curious that, although 500 nM-staurosporine and 60 μM-sphingosine are roughly comparable in their ability to reverse the action of PDB promptly, staurosporine, the less permeant inhibitor, quickly leads to more accentuated effects on free Ca (see, e.g., Fig. 1*a*) than does sphingosine (Fig. 2). Furthermore, the plateau of free Ca induced by staurosporine in phenylephrine-stimulated hepatocytes (e.g. Figs. 1*a* and 3*b*) was found in only one out of five cells stimulated with vasopressin (e.g. Fig. 1*b*; [4]). It is conceivable, in view of the evidence that a mechanism for receptor desensitization other than PKC exists for $\alpha_1$-adrenoceptors (but not for vasopressin receptors) in a line of cultured liver-derived cells [17], that staurosporine, but not sphingosine, is also inhibiting this pathway. Indeed, since sphingosine inhibits PKC by interacting with phosphatidylserine stimulation of PKC [18], whereas staurosporine interacts with the catalytic subunit of PKC [19], different side effects are expected to occur when these two non-specific agents are used on intact cells. However, since the $\alpha_1$-adrenoceptor is known to be more susceptible to desensitization by PKC than are vasoactive-peptide receptors (for references, see [12]), we cannot be certain that the plateau is not merely a reflection of differential sensitivity of receptors to PKC inhibition. Another difference between the two inhibitors is that spiking ceases after ~4–6 min exposure to sphingosine (which is why a pre-treatment protocol has not been used with this agent), whereas staurosporine permits sustained spiking. Conceivably a non-specific membrane-perturbing action of sphingosine is involved, as this lipophilic agent accumulates in the cell from an effectively infinite supply in the superfusate. Indeed, since an increase in agonist dose can overcome this sphingosine-induced blockade (results not shown), a PKC-independent mechanism may be involved, as has been suggested in neutrophils [20] and epidermal cacinoma cells [21].

Both staurosporine and sphingosine prolong the phase of falling free Ca in spikes induced by either phenylephrine or vasopressin. We would predict from these single-cell data that there would be an apparent increase in the free-Ca response of a population. Measurements in population of other cells responding to PtdIns-mediated agonists have shown an increased free Ca in the presence of staurosporine (e.g. U-937 cells [14]; platelets [16]) or sphingosine (hepatocytes exposed to ethanol; [22]). Enhanced production of $InsP_3$ has been shown to occur in response to sphingosine treatment in glioma cells stimulated with acetylcholine [23] and mesangial cells responding to angiotensin

II [24]. These data, combined with evidence for the inhibitory effects of PKC activation ([10] and references therein), suggest that PKC is involved in negative-feedback control of the PtdIns pathway and the subsequent increases in free Ca.

How do staurosporine and sphingosine promote, in an agonist-stimulated hepatocyte, free-Ca transients with a prolonged falling phase? We will assume, in view of the ability of these agents to reverse the effects of phorbol esters (Figs. 1 and 2), that PKC is the relevant cellular target for inhibition, having already made our reservations regarding specificity and non-specific actions. We have proposed, in the receptor-mediated oscillator model [1,6–8], that PKC acts to curtail PIC activity in a transient by phosphorylating receptors and/or a receptor-specific G-protein. The different receptors or G-proteins are envisaged as being phosphorylated by PKC to give different dynamics of inactivation, thereby generating the observed agonist-specific rates of decline of free Ca in each spike. The sudden rise in free Ca is envisaged as being the result partly of the sudden activation of PIC by the co-operative behaviour of activated G-proteins, perhaps in combination with receptors [7,8] (together perhaps, with a local sub-plasmalemmal rise in free Ca), and partly by co-operativity of $InsP_3$ at the Ca-release site [8]. In these models it was implicit that, as PIC activity began, both free Ca and DG rose rapidly. This sudden rise in DG, together with the rise in free Ca, activated PKC to such a degree that negative feedback caused PIC activity to reach a peak, thereby causing free Ca to reach a peak [1,6,7]. The data presented here show that inhibition of PKC does not raise peak free Ca. The latest model [8] takes this new evidence into account and proposes that, once the G-protein threshold is reached, a free-Ca transient is generated by activation of a consistent number of PIC molecules, to generate a consistent peak free Ca (i.e. a consistent number of GTP-liganded G-proteins need to accumulate in order to trigger a free-Ca transient, thereby activating a consistent number of PIC molecules). The rationale for our previous experiments with phorbol esters [10] was to test the prediction that enhanced PKC activity should lead to truncated spikes with lowered peak free Ca. Mostly this did not happen, spiking ceasing before any effect on peak height was seen. The cessation of spiking was attributed to the phorbol-ester-activated PKC causing the receptors to be continually phosphorylated and hence less responsive to agonists. Indeed, PKC inhibitors do tend to raise the frequency of the transients when the frequency is depressed by PDB (Figs. 1 and 2). Prolonged exposure to inhibitors, or pre-treatment, leads to transients with prolonged falling phases (Figs. 3 and 4), in agreement with our model in which PKC provides negative feedback during the falling phase [1,6–8]. It is clear that phorbol esters and PKC inhibitors do not possess equal and opposite actions on the hepatocyte transients. We suggest that this is because phorbol esters fail to mimic the transient activation of PKC that, we postulate, occurs during a spike, whereas the experiments using the PKC inhibitors do allow the time course of activation of PKC to be determined by the cell itself. We are as yet unable to explain why PKC inhibitors should lead to a decrease in the period during which free Ca is at, or close to, resting levels between spikes (Figs. 3*a* and 4). It appears that a period during which free Ca is at resting levels is needed before the next spike occurs, since we have never recorded spikes starting during the falling phase of a preceding transient, although some of the records do show marked oscillations within the falling phase (e.g. Figs. 2*b*, 3*a*, 4).

We continue to think it likely that the cell retains, second by second, receptor-specific information in the mobilization of Ca, presumably via $InsP_3$. The data presented here augment previous observations [6,7] of receptor-specific spike time courses. The phenomenon, in staurosporine-pretreated cells, of a fast transient preceding the slow-falling transients when phenylephrine is the stimulus (Fig. 3*a*), but not when vasopressin is the agonist (Fig. 4), is another example of receptor-specified differences in cellular Ca control. Furthermore, a plateau of free Ca is induced by staurosporine in a phenylephrine-stimulated cell (Fig. 3*b*), but not in vasopressin-stimulated cells (Fig. 4). So we continue to find the concept of the cytoplasmic oscillator, in which free Ca oscillates as a result of shuttling of Ca between $InsP_3$-sensitive and -insensitive stores [3–6], incapable of explaining the various detailed differences in hepatocyte transients. If the oscillator is denied, on a second-by-second basis, receptor-specified information, we would expect all transients to possess the same properties whatever the agonist species. In the hepatocyte this is quite clearly not the case. A similar argument suggests to us that, in the hepatocyte, negative feedback from PKC, through activation of $InsP_3$ kinase [16] or phosphatase [25], cannot be responsible for controlling the falling phase of free Ca unless each receptor type has, somehow, control of separate pools of these $InsP_3$-metabolizing enzymes, within the same individual cell. It is unlikely that PKC activation of these $InsP_3$-metabolizing enzymes is important, in view of the lack of effect of pretreatment with phorbol ester on the activity of both these enzymes in rat hepatocytes [26] and several other cell types [17,27–29]. Likewise, PKC-mediated activation of the plasmalemmal Ca pump [30] would be unlikely to generate receptor-specified time courses to the free-Ca spikes, unless different sub-sets of pumps responded (in the same individual cell) to activation of different receptors. However, there is emerging evidence for sources of DG other than PIC activation, including (in hepatocytes) a phosphatidylcholine-specific phospholipase C and a phospholipase D [31]. It is conceivable that different receptors activate these alternative routes for DG generation, and hence PKC activation, to different degrees, thereby offering a possible explanation for the different rates of fall of free Ca in transients induced by different agonists. Unfortunately for such schemes, one might expect that, if the various phospholipases are continuously active as long as agonist is present, that the steady-state DG level in the plasmalemma would be higher at the higher doses of agonist. The prediction would then be that negative feedback from PKC would then be greater at higher agonist levels, leading to faster rates of fall of free Ca. This does not fit the data; the rate of fall of free Ca is not influenced by agonist dose over the range in which free-Ca transients are observed [6]. In order to explain the dose-invariant time course of the transients, it is necessary to postulate a threshold phenomenon both for Ca mobilization and for activation of PKC. Thus models in which Ca-induced release of Ca accounts for the rise in free Ca suffer from a predicted dose-dependence in the determinants of the rate of fall of free Ca, whether this be by PKC or by inhibition of Ca efflux, as proposed by Thomas and co-workers [32]. The receptor-controlled model [1,6–8] circumvents problems with postulating spatially or functionally distinct zones of Ins(1,4,5)$P_3$-metabolizing enzymes, Ca pumps, or even Ca stores, by proposing that the observed differences in the time course of free-Ca transients result from the dynamics by which negative feedback from PKC, itself activated transiently, curtails the activity of PIC by phosphorylating the different hormone receptors and/or G-proteins at different rates. The data presented here support a role for protein kinase C in determining the rate of fall of free Ca in each transient, in broad agreement with the receptor-controlled model of the hepatocyte Ca oscillator.

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