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Effect of perturbing diacylglycerol metabolism on cytosolic free Ca^{2+} oscillations induced in single hepatocytes

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Single rat hepatocytes microinjected with aequorin show free Ca oscillations when stimulated with Ca^{2+}-mobilizing hormones. We show here that an inhibitor of diacylglycerol kinase (R 59 022) and an analogue of native diacylglycerol (diC_8) inhibit free Ca oscillations induced by phenylephrine and vasopressin. These results agree with a negative feedback effect of protein kinase C on free Ca oscillations.

Ca^{2+} oscillation; Diacylglycerol; R 59 022; diC_8; Single hepatocyte

1. INTRODUCTION

Diacylglycerol (DG), an intermediate in the biosynthesis and degradation of glycerolipids in eukaryotic cells, has been demonstrated to play an important role as an intracellular second messenger, in particular in activating protein kinase C (PKC) [1]. DG may be formed by several pathways, the best characterized of which is the hydrolysis of phosphatidyl inositol 4,5-bisphosphate to DG and inositol 1,4,5-trisphosphate catalysed by a specific phospholipase C [2]. DG can also be formed by breakdown of phosphatidyl choline, by another phospholipase C [3]. The degradation of a phosphatidyl inositol-glycan could be a third source of DG [4]. Rapid removal of DG is required to terminate the response to agonists, and to reset the system for subsequent signalling events. DG attenuation may occur by two pathways. DG can be phosphorylated by DG-kinase to phosphatidic acid: this is the predominant route [5]. Alternatively, DG may be degraded by DG-lipase [6].

Ca^{2+}-mobilizing agonists, which are mediated by inositol 1,4,5-trisphosphate, induce free Ca^{2+} oscillations (spikes) in single hepatocytes [7]. While the frequency of oscillations depends on agonist dose, the time–course of an individual spike does not change with agonist concentration for a given agonist [8]. The frequency of spiking is depressed by phorbol ester activation of PKC [9], while inhibitors of PKC prolong the falling phase of each spike [10]. Here we show the effect on free Ca oscillations of perturbing DG metabolism using a DG-kinase inhibitor, R 59 022, which has been shown to raise DG levels in platelets [11] and hepatocytes [12], and a DG analogue, diC_8.

2. MATERIALS AND METHODS

Hepatocytes were isolated from fed, 150–250 g, male Wistar rats by collagenase perfusion as described previously [13]. Microinjection of single hepatocytes with aequorin, and data acquisition, have been previously described [14]. The experimental medium was Williams' Medium E (Flow Laboratories) gassed with CO_2/air (1:19) at 37°C. All substances were added to this medium. The DG-kinase inhibitor, R 59 022, was from Janssen Pharmaceutical; and diC_8, phenylephrine and vasopressin were from Sigma. Statistical significance was calculated by Student's *t*-test.

3. RESULTS

Fig. 1 shows the effect of R 59 022 on phenylephrine-induced spikes; R 59 022 (0.1 μM) reversibly reduced the frequency, and peak free Ca, of phenylephrine-induced spikes (Fig. 1a; representative of 7 hepatocytes exposed to concentrations of R 59 022 between 2–500 nM). These effects of R 59 022 are detailed in Table I where data from spikes from 4 hepatocytes whose free Ca oscillations showed similar frequency and peak free Ca in response to phenylephrine (1 μM for 3 hepatocytes and 0.6 μM for 1 hepatocyte). The period between spikes showed a ca. 2.5-fold increase, while peak free Ca shows a small but significant ($P < 0.01$) decrease. Neither the duration of the spikes nor the time constant of the falling phase of the spikes are affected (Table I).

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Abbreviations: free Ca, cytosolic concentration of free Ca^{2+}; DG, *sn*-1,2-diacylglycerol; diC_8, *sn*-1,2-dioctanoylglycerol.

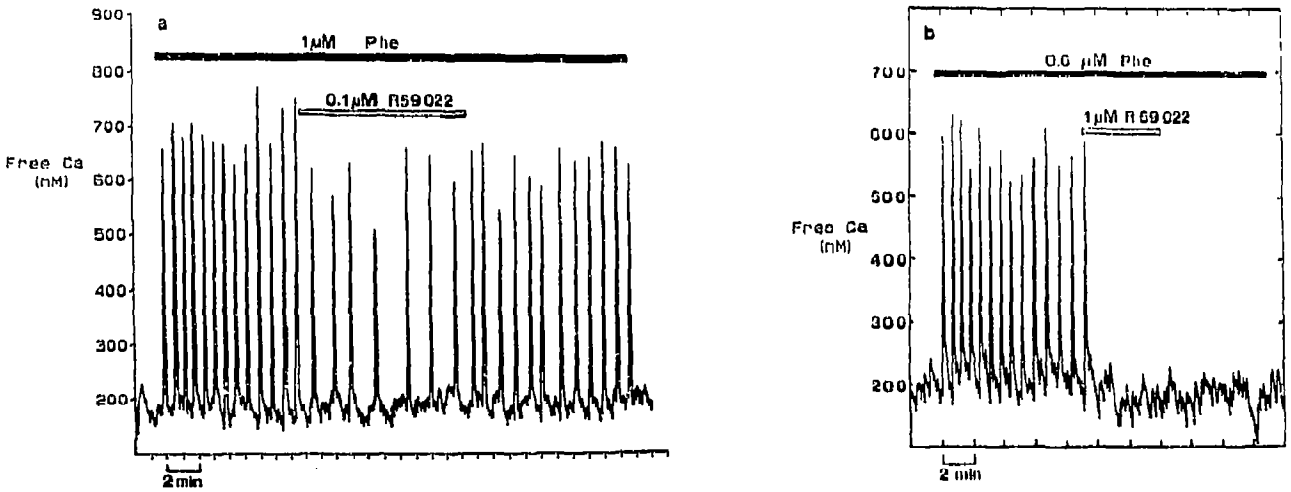

Fig. 1. Two different hepatocytes were microinjected with aequorin and superfused at the times indicated with (a) 0.1 μM R 59 022 and 1 μM phenylephrine (Phe), and (b) 1 μM R 59 022 and 0.6 μM phenylephrine (Phe). The time constant for the resting concentration of free Ca was 10 s, and for oscillations 1 s.

Higher concentrations of R 59 022 (1 μM) blocked the spikes induced by phenylephrine, usually irreversibly (Fig. 1b; representative of 7 hepatocytes at concentrations of R 59 022 between 0.05–15 μM. In only one of these 7 hepatocytes was the effect of R 59 022 reversible).

High concentrations of R 59 022 (10 μM), sufficient to completely abolish phenylephrine-induced spikes, either reduce the frequency of free Ca oscillations induced by 0.5 nM vasopressin (Fig. 2, representative of 4 hepatocytes), or completely abolished spiking (3 hepatocytes; in two of them these effects were irreversible, data not shown). The minimum concentration of the DG-kinase inhibitor, R 59 022, needed to affect oscillations induced by vasopressin was ca. 1 μM, compared with 0.1 μM for phenylephrine responses.

Another way to increase DG levels in the cell is by adding DG analogues such as diC_8, which is permeant to the cell membrane [15]. Fig. 3a shows the effect of diC_8 on free Ca oscillations induced by 1 μM phenylephrine. At 10 μM diC_8 the frequency of the oscillations is slightly reduced, but an increase in the diC_8 concentration to 15 μM reversibly blocked the free Ca oscillations (Fig. 3a). diC_8 at concentrations of 10–25 μM either reduced the frequency of the oscillations induced by phenylephrine without any effect on other spikes characteristics (5 out of 11 hepatocytes), or completely blocked the oscillations (6 out of 11 hepatocytes). In only two of these 6 hepatocytes was the blockade irreversible (data not shown); in the other 4 the oscillations recovered, but with a lower frequency and a slight reduction in peak free Ca (Fig. 3a). Fig. 3b shows that 20 μM diC_8 reduced the frequency and peak free Ca of the oscillations induced by 0.25 nM vasopressin (7 hepatocytes). In 2 of these 7 hepatocytes the effect on frequency was not reversible, while the effect on peak free

Table I

Effect of 0.1 μM R 59 022 on the characteristics of the free Ca oscillations induced by phenylephrine

Treatment	n	Peak free Ca (nM)	Period between oscillations (s)	Length (s)	Falling time constant (s)
Phenylephrine	60	728 ± 12	37.54 ± 1.79	5.1 ± 0.52	3.06 ± 0.1
Phenylephrine + R 59 022	25	653 ± 21*	86.45 ± 23.34**	4.99 ± 0.54	3.91 ± 0.98

The results show the means ± S.E.M. for a number (n) of oscillations. *$P < 0.01$; **$P < 0.001$.

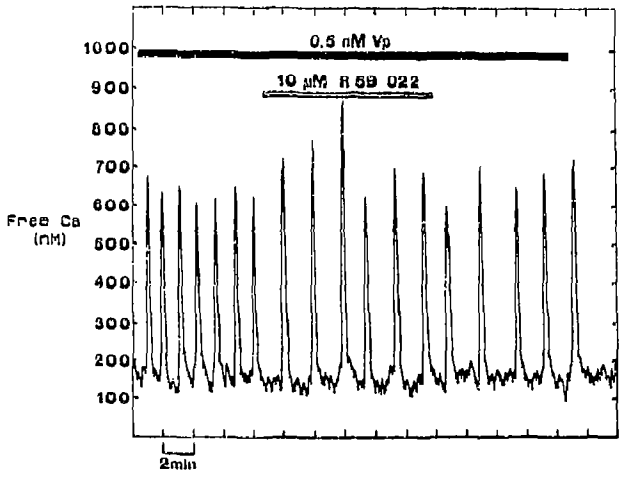

Fig. 2. Effect of 10 μM R 59 022 on free Ca oscillations induced by 0.5 nM vasopressin (Vp). The time constants and other details were as in Fig. 1.

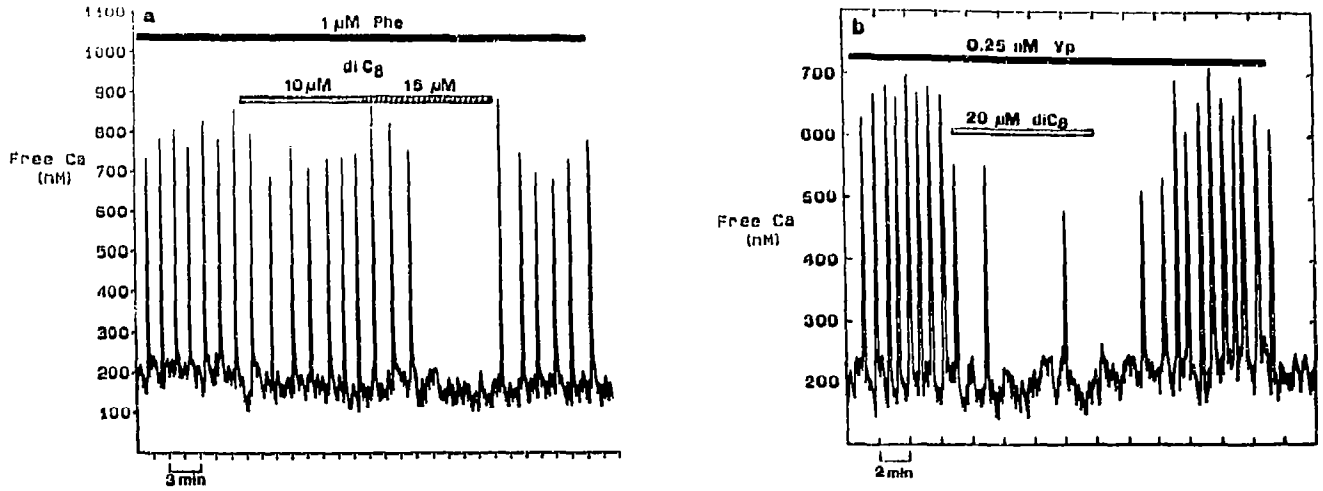

Fig. 3. Effect of (a) 10 and 15 μM diC_8 on free Ca oscillations induced by 1 μM phenylephrine (Phe), and (b) 20 μM diC_8 on free Ca oscillations induced by 0.25 nM vasopressin (Vp). The time constants and other details were as in Fig. 1.

Ca was always reversible (data not shown). In a further 3 hepatocytes oscillations induced by vasopressin were reversibly blocked by 20–30 μM diC_8 (data not shown). The solvents for diC_8 was ethanol, and for R 59 022 was DMSO; and they had no effect on phenylephrine or vasopressin-induced spiking, or alone, at concentrations added to the experimental medium: up to 0.25% (V/V) for ethanol, and up to 0.025% (V/V) for DMSO.

4. DISCUSSION

It has been shown that the DG-kinase inhibitor, R 59 022, increases [^{3}H]DG and decreases [^{32}P]phosphatidic acid levels in hepatocytes [12]. Thus, we can assume that R 59 022 inhibits DG-kinase in hepatocytes. The different sensitivities to R 59 022 of the free Ca oscillations induced by phenylephrine or vasopressin are comparable to those found in hepatocytes populations for vasopressin and adrenaline on glycogen phosphorylase activity [12]. Different effects of R 59 022 have been reported on two DG-kinase isoenzymes [16]. It is possible that the differential sensitivity to R 59 022 of the free Ca responses to phenylephrine or vasopressin are caused by these agonists coupling to different DG-kinase isoenzymes. A further consideration is an unsubstantiated suggestion that R 59 022 acts as a weak α_1-adrenergic antagonist [11]. However, since R 59 022 enhances rather than diminishes the increase in [^{3}H]DG induced by adrenaline in hepatocytes [12], it is unlikely that the actions of R 59 022 are predominantly due to the displacement of the α_1-agonist from its receptor.

The DG analogue, diC_8, has effects on free Ca oscillations that resemble those of R 59 022, as has been found in other cell types [17]. However, in hepatocytes, R 59 022 is more effective on a molar basis than exogenously added synthetic DG. Also, free Ca oscillations induced by vasopressin are less sensitive to diC_8 than those induced by phenylephrine, as has been shown for phorbol esters [9].

The DG-kinase inhibitor, R 59 022 [11,12], and diC_8 both increase the level of endogenous DG, and will elicit an activation of PKC. The decrease in peak free Ca and in the frequency of the oscillations induced by both R 59 022 and diC_8 supports the prediction that an enhancement in PKC activity would diminish peak free Ca [18,19]. However, an increase in R 59 022 or diC_8 concentration led to a blockade of the oscillations before a substantial reduction in peak free Ca was observed. Thus we are not yet confident that these data provide convincing support for our 'receptor controlled model' of the hepatocyte calcium oscillator [18,19], in which transient generation of DG would activate PKC transiently. The reduction of free Ca oscillation frequency by R 59 022 or diC_8 could be explained by continuous phosphorylation of the hormone receptors by continuous activation of PKC [9,20]. Indeed, PKC inhibitors tend to raise the frequency of the free Ca oscillations when frequency is depressed by phorbol esters [10]. A differential susceptibility of the α_1-adrenergic and vasopressin receptors to negative feedback from PKC is our favoured explanation for the greater sensitivity of the phenylephrine-induced spiking to R 59 022 and diC_8 shown here.

In summary, we show here that an increase in the level of endogenous DG, by inhibition of DG-kinase with R 59 022, or by adding DG exogenously as diC_8, has similar inhibitory effects on free Ca oscillations to those produced by phorbol ester activators of PKC [9,10]. The data agree with the concept of negative feedback being provided by PKC in the hepatocyte free Ca oscillator and, in particular, implicate endogenously generated DG in the mechanism.

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