Oscillations in cytosolic free Ca^{2+} induced by ADP and ATP in single rat hepatocytes display differential sensitivity to application of phorbol ester

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We have previously described differences in the oscillatory responses of cytosolic free Ca^{2+} concentration ($[Ca^{2+}]_i$) in hepatocytes to ADP and ATP, which we have interpreted as evidence that these two nucleotides are acting at distinct receptors. We show here that ADP- and ATP-induced oscillations are differentially sensitive to application of the phorbol ester 4β-phorbol 12,13-dibutyrate (PDB). ADP-induced $[Ca^{2+}]_i$ oscillations are abolished by low concentrations of PDB (5–10 nM), whereas ATP-induced oscillations of long duration are refractory to PDB, even at greatly elevated concentrations (100 nM). The data illustrate a further difference in the actions of ADP and ATP, strengthening the argument that these agonists are not acting at the same receptor on rat hepatocytes.

INTRODUCTION

Rat hepatocytes express receptors for a range of agonists, including ADP and ATP, linked to the hydrolysis of PtdIns(4,5)P_2 by phospholipase C [1–4]. The Ins(1,4,5)P_3 formed in this reaction mobilizes Ca^{2+} from internal stores [5,6]. Single aequorin-injected rat hepatocytes, in common with many other cell types, generate oscillations in cytosolic free Ca^{2+} concentration ($[Ca^{2+}]_i$) when stimulated with these agonists [7,8]. The duration of the $[Ca^{2+}]_i$ oscillations has been shown to be dependent on the receptor species being activated, so that oscillations of very different duration can be induced in the same individual hepatocyte by different agonists [9]. Activation of a given receptor species evokes oscillations of consistent duration within the same cell, and also between cells [9]. Increasing the agonist concentration increases the frequency of oscillations without altering the duration of individual oscillations [7].

In addition to Ins(1,4,5)P_3, phospholipase C-catalysed hydrolysis of PtdIns(4,5)P_2 yields diacylglycerol, which activates protein kinase C [10]. The activation of protein kinase C by endogenously produced diacylglycerol can be mimicked using the tumour-promoting phorbol esters [10]. A negative-feedback role has been attributed to protein kinase C in many cell types, including hepatocytes, in which its activation by phorbol esters inhibits Ins(1,4,5)P_3 production and therefore the $[Ca^{2+}]_i$ rise [11–13]. Bird et al. [14] proposed that the sinusoidal oscillations observed in mouse lacrimal acinar cells result from oscillating negative feedback by protein kinase C on Ins(1,4,5)P_3 production. These sinusoidal oscillations differ from the frequency-modulated constant-amplitude $[Ca^{2+}]_i$ oscillations observed in hepatocytes [7,9,15]. However, negative feedback by protein kinase C was invoked in the model proposed by Woods et al. [9] for frequency-modulated oscillations, following the observation that activation of protein kinase C by phorbol esters decreased the frequency of, or completely inhibited, $[Ca^{2+}]_i$ oscillations in hepatocytes [16]. Furthermore, the oscillations induced by vasopressin and phenylephrine displayed differential sensitivity to phorbol esters, suggesting that the site of protein kinase C regulation is either the receptors themselves or agonist-specific G-proteins [16].

In hepatocytes, the receptor believed to mediate the effects of both ADP and ATP has been characterized as belonging to the P_{2y}-subclass of purinoceptor [17]. Activation of a single receptor species would be predicted to yield oscillations of consistent duration in response to either nucleotide. However, ADP and ATP induce $[Ca^{2+}]_i$ oscillations with very different characteristics. As summarized below, we have identified differences in the duration of oscillations induced by ADP and ATP [18], in the effects of raised intracellular cyclic AMP levels [19], and in the response to co-addition of the methylated derivative of ATP, adenosine 5′-[$\alpha\beta$-methylene]triphosphate (pp[CH_2]pA) [20]. Thus the durations of $[Ca^{2+}]_i$ oscillations induced by ADP and ATP are different in the majority of hepatocytes [18]. ADP consistently produces oscillations of short duration (approx. 9 s). In contrast, ATP can elicit three different types of response in hepatocytes. The first group of hepatocytes respond to ATP with oscillations of short duration, indistinguishable from those induced by ADP. The second group produce oscillations of much longer duration (up to 2 min), whereas in the final group, ATP induces oscillations of variable duration within a single response. ADP- and ATP-induced oscillations are modulated differently by raising intracellular cyclic AMP levels [19]. Thus the frequency and amplitude of ADP-induced oscillations are enhanced when cyclic AMP levels are raised. In contrast, oscillations resulting from ATP stimulation of hepatocytes display an increase in the duration of individual oscillations, with peak $[Ca^{2+}]_i$ and frequency unaffected [19]. Furthermore, oscillations induced by ATP, but not those induced by ADP, are potentiated by the addition of the methylated derivative of ATP, pp[CH_2]pA [20]. Interestingly, the oscillations of short duration induced by ATP are modulated differently by co-application of pp[CH_2]pA [20] and by elevated cyclic AMP concentration [19], compared with the oscillations of short duration induced by ADP, despite being indistinguishable in terms of duration [18]. These three differences in the oscillatory responses of hepatocytes to ADP and ATP are inconsistent with their effects being mediated by a single receptor.

We have proposed the existence of three functionally distinct purinoceptors which mediate the oscillatory responses recorded in aequorin-injected hepatocytes to ADP and ATP [19]. One is activated solely by ADP, which we have termed the ADP receptor, and mediates oscillations of short duration. The two

Abbreviations used: $[Ca^{2+}]_i$, cytosolic free calcium concentration; PDB, 4β-phorbol 12,13-dibutyrate; PMA, 4β-phorbol 12β-myristate 13α-acetate; pp[CH_2]pA, adenosine 5′-[$\alpha\beta$-methylene]triphosphate.

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remaining subtypes proposed are responsive to ATP, but not to ADP. We propose that one of these ATP receptors, designated ATP_S, is responsible for oscillations of short duration, and the other for oscillations of long duration, designated ATP_L. The $[Ca^{2+}]_i$ oscillations of variable duration are postulated to arise from activation of both ATP_S and ATP_L subtypes.

Here, ADP- and ATP-induced oscillations are shown to be differentially sensitive to application of the phorbol ester 4β-phorbol 12,13-dibutyrate (PDB). The $[Ca^{2+}]_i$ oscillations of long duration induced by ATP are unusually insensitive to PDB even at concentrations which completely inhibit oscillations induced by ADP, phenylephrine and vasopressin.

MATERIALS AND METHODS

Single hepatocytes were isolated from fed male Wistar-strain rats (200–250 g) by collagenase perfusion. Preparation of cells, microinjection with the photoprotein aequorin, and collection of data were as described previously [21]. The experimental medium was Williams Medium E (Gibco), to which ADP and ATP (Sigma Chemical Co.) were added. PDB, and the inactive phorbol ester 4α-phorbol 12,13-dibutyrate (LC Laboratories), were dissolved in dimethyl sulphoxide to give 10 mM stocks. Portions were added to Williams Medium E to give the required concentrations. Data were plotted by using exponential smoothing with time constants as indicated.

RESULTS

Single aequorin-injected hepatocytes responded to extracellular ADP and ATP in the concentration range 1–10 μM, with series of repetitive oscillations in $[Ca^{2+}]_i$, as previously observed [18].

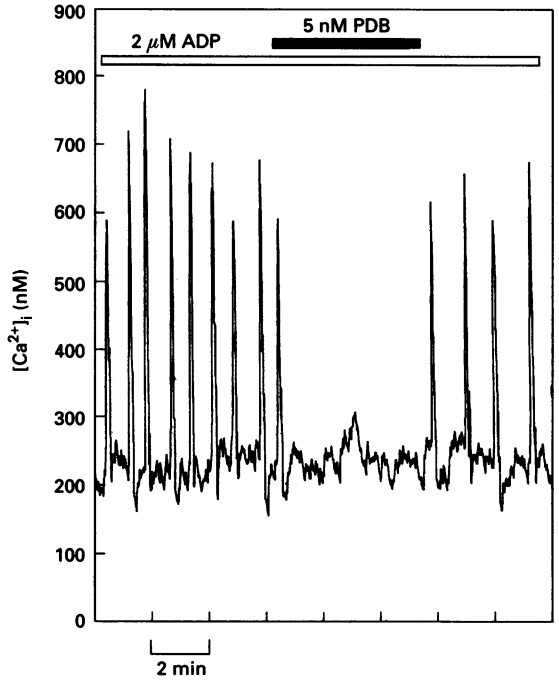

Figure 1 ADP-induced $[Ca^{2+}]_i$ oscillations are inhibited by low concentrations of PDB

A single aequorin-injected hepatocyte responded to 2 μM ADP with $[Ca^{2+}]_i$ oscillations which were promptly abolished upon application of 5 nM PDB. The inhibitory effect was reversed upon removal of PDB. Time constants: for resting $[Ca^{2+}]_i$, 12 s; for oscillations, 1 s.

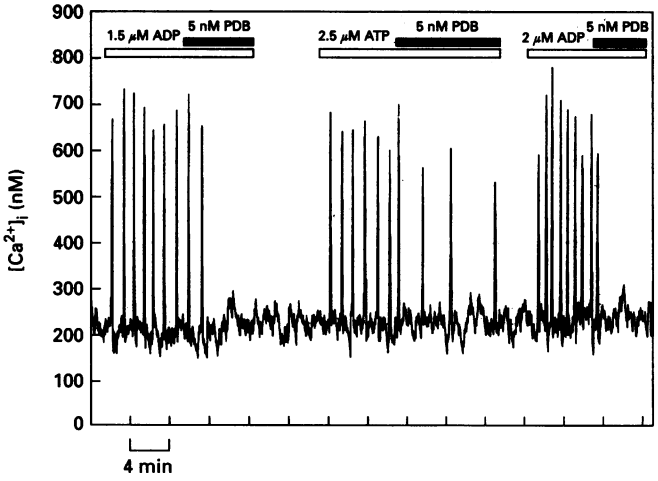

Figure 2 ADP- and short ATP-induced $[Ca^{2+}]_i$ oscillations display subtle differences in sensitivity to PDB

$[Ca^{2+}]_i$ oscillations induced by 1.5 μM ADP were promptly abolished by application of 5 nM PDB. Subsequent $[Ca^{2+}]_i$ oscillations of short duration induced by ATP displayed a decrease in frequency, but were not abolished, by application of 5 nM PDB. The hepatocyte was then re-stimulated with ADP, and the oscillations were again abolished by 5 nM PDB. Time constants: for resting $[Ca^{2+}]_i$, 12 s; for oscillations, 1 s.

Consistent with the inhibition of phenylephrine- and vasopressin-induced oscillations by phorbol esters [16], 18/19 hepatocytes displayed a decreased frequency or abolition of the short-duration (approx. 9 s) ADP-induced oscillations by addition of 5 nM PDB. The oscillations were abolished in 10/19 hepatocytes and displayed a large decrease in frequency in 8/19 cells; only 1/19 cells was unaffected by application of 5 nM PDB. Figure 1 shows a typical result where application of 5 nM PDB promptly abolished the $[Ca^{2+}]_i$ oscillations induced by ADP. When the 8 cells which continued to oscillate in the presence of 5 nM PDB were exposed to 10 nM PDB, only 2 cells continued to produce oscillations, although at a much decreased frequency. The inhibitory effect of these low concentrations of PDB, applied for short periods, was promptly reversed, although the frequency of oscillations upon removal of PDB was often slightly decreased (see Figure 1). This reversibility is consistent with earlier observations of the effect of PDB on phenylephrine- and vasopressin-induced oscillations, and contrasts with the action of the more hydrophobic phorbol ester, 4β-phorbol 12β-myristate 13α-acetate (PMA), which was not reversible [16].

Oscillations of short duration induced by ATP are indistinguishable in terms of duration from those induced by ADP [18]. Although similar low concentrations of PDB (5–10 nM) produced a decrease in frequency or abolition of short ATP-induced oscillations in 3/3 cells, these oscillations displayed a subtle difference in sensitivity to the application of PDB compared with ADP-induced oscillations in the same cell. Thus the ADP-induced oscillations depicted in Figure 2 were abolished by 5 nM PDB, which caused only a decrease in frequency of short ATP-induced oscillations. This was not a result of decreased sensitivity of the cell to repeated applications of PDB, as, subsequently, ADP-induced oscillations in the same hepatocyte were again abolished by 5 nM PDB (Figure 2).

The effect of PDB on hepatocytes producing $[Ca^{2+}]_i$ oscillations of long duration in response to ATP was more surprising. Of 9 cells in which ATP evoked oscillations of long duration, exposure to 5 nM PDB had no effect on the frequency of oscillations in 7

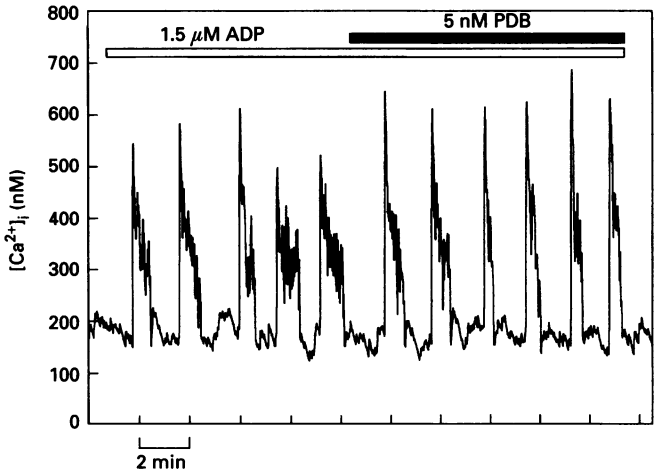

Figure 3 Application of 5 nM PDB has no effect on the frequency of $[Ca^{2+}]_i$ oscillations of long duration induced by ATP

$[Ca^{2+}]_i$ oscillations of long duration were recorded from a single cell stimulated with 1.5 μM ATP. Addition of 5nM PDB for the period indicated did not affect the frequency of oscillations: however, a slight decrease in the duration of individual oscillations was recorded. Time constants: for resting $[Ca^{2+}]_i$, 12 s; for oscillations, 1 s.

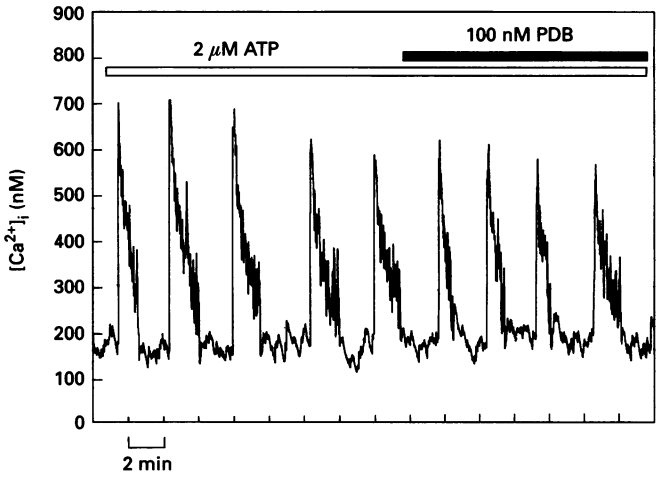

Figure 4 $[Ca^{2+}]_i$ oscillations of long duration induced by ATP are not inhibited by the addition of 100 nM PDB

A single aequorin-injected hepatocyte responded to 2 μM ATP by generating oscillations of long duration. Application of 100 nM PDB for the period indicated did not affect the frequency of these oscillations, although a small decrease in the duration of individual oscillations was recorded. Time constants: for resting $[Ca^{2+}]_i$, 12 s; for oscillations, 1 s.

hepatocytes, although there was a small decrease in the duration of individual oscillations in 5 of these 7 hepatocytes (Figure 3). In the remaining 2/9 hepatocytes, 5 nM PDB caused a decrease in frequency of oscillations in one cell, and abolished the oscillations in the final cell. The majority of hepatocytes were, in addition, resistant to much higher concentrations of PDB; $[Ca^{2+}]_i$ oscillations continued, at unaltered frequency, in 8/11 cells after addition of 100 nM PDB (Figure 4); in the remaining 3 hepatocytes oscillations were abolished. In 5 of the 8 cells which continued to oscillate in the presence of 100 nM PDB, there was

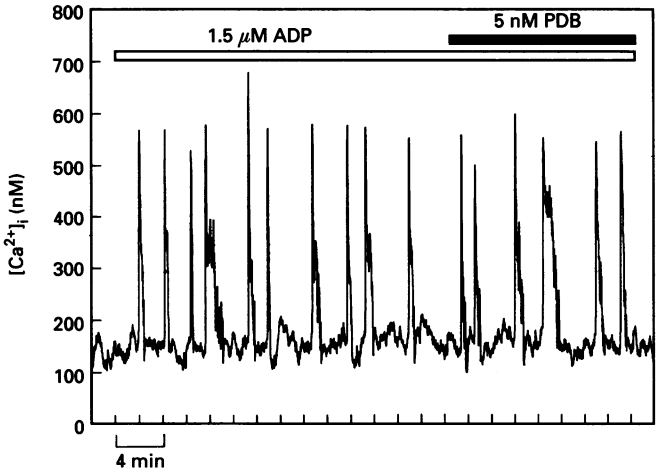

Figure 5 $[Ca^{2+}]_i$ oscillations of variable duration induced by ATP are not affected by the addition of 5 nM PDB

A single aequorin-injected hepatocyte responded to 1.5 μM ATP by the generation of oscillations of variable duration. Application of 5 nM PDB for the period indicated had no effect on these oscillations. Time constants: for resting $[Ca^{2+}]_i$, 12 s; for oscillations, 1 s.

a small decrease in the duration of individual oscillations (see Figure 4).

Hepatocytes producing $[Ca^{2+}]_i$ oscillations of variable duration in response to ATP were similarly resistant to exposure to PDB. Of 9 cells exposed to 2–5 nM PDB, 2 showed a decrease in frequency of oscillations, but 7 were unaffected (Figure 5). When exposed to 10 nM PDB, 7/7 hepatocytes responding to ATP with $[Ca^{2+}]_i$ oscillations of mixed duration continued to produce oscillations, although 3 displayed a decrease in frequency. Application of 25 nM PDB abolished the $[Ca^{2+}]_i$ oscillations in 2/5 cells, but had no effect on either the duration or the frequency of oscillations in 3/5 hepatocytes. Although 25 nM PDB would inhibit oscillations of short duration induced by ATP, when these oscillations formed part of the variable response to ATP they were resistant to this concentration of PDB.

The negative control compound 4α-phorbol 12,13-dibutyrate had no effect on ADP- or ATP-induced $[Ca^{2+}]_i$ oscillations at concentrations up to 100 nM (results not shown).

DISCUSSION

Application of phorbol esters has previously been shown to attenuate or abolish the ATP-induced Ca^{2+} response in many cell types [22–29], including populations of hepatocytes [2]. However, most of these studies did not compare the effect of phorbol esters on ADP- and ATP-mediated Ca^{2+} responses. We have shown here that ADP- and ATP-induced $[Ca^{2+}]_i$ oscillations are differentially sensitive to application of phorbol esters; ADP-induced oscillations are inhibited by low concentrations of PDB (5–10 nM), whereas, remarkably, ATP-induced oscillations of long duration are resistant to PDB, even at greatly elevated concentrations (100 nM), although a slight decrease in the duration of oscillations was often recorded (see Figure 4). Significantly, results from two studies which did compare the effects of phorbol esters on both ADP- and ATP-induced responses are consistent with the differential sensitivity reported

here. Thus Keppens et al. [30] reported that, in populations of hepatocytes, application of PMA only slightly decreased the ability of ATP to stimulate glycogenolysis through Ca^{2+}-dependent activation of phosphorylase. This was in contrast with the glycogenolytic effect of the ADP analogue adenosine 5′-[β-thio]diphosphate, which was abolished by this treatment [30]. Similarly, Purkiss et al. [31] reported that the phorbol-ester-induced attenuation of ADP-mediated Ins(1,4,5)P_3 accumulation in bovine aortic endothelial cells was far greater than attenuation of the ATP-mediated response.

$[Ca^{2+}]_i$ oscillations induced by ADP, and those of short duration induced by ATP, display subtle differences in sensitivity to PDB. Similar differences have previously been noted in the sensitivity of vasopressin- and phenylephrine-induced $[Ca^{2+}]_i$ oscillations in single aequorin-injected rat hepatocytes to application of phorbol esters [16]. Thus, concentrations of PDB sufficient to abolish phenylephrine-induced oscillations resulted only in a decrease in frequency of vasopressin-induced oscillations. One explanation for these agonist-specific differences is that feedback by protein kinase C acts at the receptors. Phorbol esters are known to lead to a decrease in receptor affinity for ligands, which has been attributed to protein kinase C-mediated phosphorylation [12,32–35]. Some receptors may be more readily phosphorylated than others, undergoing a greater decrease in affinity, which could explain the agonist-specific differences in sensitivity to phorbol esters. The differential sensitivity of ADP- and ATP-induced oscillations would then further argue that these two nucleotides do not act at a single receptor. The postulated ADP receptor is the most sensitive to application of phorbol esters. The response mediated by the proposed ATP_S receptor subtype is also susceptible to inhibition by PDB, although higher concentrations are required than to inhibit ADP-induced oscillations (Figure 2). This is consistent with the proposal that ADP-induced oscillations and ATP-induced oscillations of short duration, although indistinguishable in terms of duration, are mediated by distinct receptors: the ADP receptor and the ATP_S receptor [19].

Alternatively, the agonist-specific differences in sensitivity to phorbol esters may indicate that protein kinase C regulation acts at agonist-specific G-proteins, if these exist. Ashkenazi et al. [36] provided evidence that functionally distinct G-proteins can selectively couple different muscarinic receptors to PtdIns(4,5)P_2 hydrolysis. In addition, Dasso and Taylor [37] have suggested that different receptor species may interact with different pools of G-proteins. If coupling of agonist-specific G-proteins to phospholipase C was differentially affected by protein kinase C-mediated phosphorylation, this could account for the differences in sensitivity of oscillations induced by individual agonists to phorbol esters.

The resistance of ATP-induced oscillations of long duration is remarkable, and contrasts with the subtle differences in sensitivity of phenylephrine-, vasopressin-, ADP- and short-duration ATP-induced oscillations to phorbol esters. ATP-induced oscillations of long duration are refractory to PDB concentrations at least 10 times higher than those required to abolish phenylephrine- or ADP-induced oscillations. These data conflict with reports from many cell types, including a study of populations of rat hepatocytes [2], in which application of phorbol esters has been shown to abolish the ATP-induced accumulation of Ins(1,4,5)P_3 or the rise in $[Ca^{2+}]_i$ [25,26]. However, the abolition of the $[Ca^{2+}]_i$ rise in hepatocyte populations was achieved with a high concentration (1 μM) of the more hydrophobic phorbol ester, PMA. More usually, application of phorbol esters attenuated, rather than abolished, the ATP-induced response [22–24,28–30]. Assuming that the distribution of the three subtypes of purinoceptor proposed above (see the Introduction) is not limited to hepatocytes, this may reflect an inhibition of the response mediated by the ATP_S receptor, with the ATP_L response remaining unaffected. The continuation of the ATP-induced oscillations of long duration in the presence of high phorbol ester concentrations indicates that protein kinase C does not play a central role in the generation of these oscillations. The subtle effect of PDB on the length of some long ATP oscillations (Figures 3 and 4), however, suggests that protein kinase C may have a minor, modulatory, role. The duration of $[Ca^{2+}]_i$ oscillations induced by phenylephrine (approx. 7 s) and vasopressin (approx. 10 s) can be greatly increased by inhibiting protein kinase C with staurosporine or sphingosine, which prolong the falling phase of these oscillations [38]. The elongated oscillations, evoked in the absence of feedback inhibition by protein kinase C, resemble the ATP-induced oscillations of long duration, strengthening the proposal that these oscillations are not regulated by protein kinase C. Although ATP-induced activation of protein kinase C has been demonstrated in some cell types [27], ATP induces mobilization of intracellular Ca^{2+} stores in murine 3T6 and 3T3 fibroblasts, without activation of protein kinase C [39,40], which may indicate that this response is not subject to feedback inhibition by protein kinase C. The values for the rate of rise and peak $[Ca^{2+}]_i$ of oscillations of long duration induced by ATP are indistinguishable from those of oscillations induced by other agonists acting through the phosphoinositide-signalling pathway [41], indicating that a common mechanism is likely to be involved. The present data therefore have implications for our understanding of the oscillator mechanism, and argue against a role for negative feedback by protein kinase C in determining the rate of rise or peak $[Ca^{2+}]_i$ of oscillations. The resistance of ATP-induced oscillations of long duration to PDB may reflect the involvement of an additional element in the oscillator which confers PDB resistance upon the ATP_L-mediated response. As ATP is known to act at P_{2x} receptors, which have been characterized as cation-selective channels permeable to Ca^{2+} [42], it is possible that this additional element is ATP-stimulated Ca^{2+} entry. However, the distribution of P_{2x} receptors appears to be limited to excitable cells [42,43], and they are believed to be absent from rat hepatocytes [44]. Indeed, the mRNA encoding a recently cloned P_{2x} receptor was not detected in rat liver [45]. Moreover, decreasing extracellular Ca^{2+} decreases the frequency of ATP-induced oscillations, without any effect on the duration of individual oscillations [18]. It is therefore unlikely that ATP-activated Ca^{2+} influx is a critical factor in the mechanism underlying hepatocyte ATP-induced oscillations of long duration, or that it accounts for the resistance that these oscillations display to phorbol esters.

The data presented here reveal that ATP oscillations of long duration are, uniquely, resistant to phorbol esters. This has important implications for our understanding of the oscillator and the role of protein kinase C in the underlying mechanism. In addition, the differential sensitivity in the $[Ca^{2+}]_i$ oscillatory responses of hepatocytes to ADP and ATP furthers the argument that these nucleotides are acting at functionally distinct receptors.

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