Elevated intracellular cyclic AMP exerts different modulatory effects on cytosolic free Ca^{2+} oscillations induced by ADP and ATP in single rat hepatocytes

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Single aequorin-injected hepatocytes respond to agonists acting via the phosphoinositide signalling pathway by the generation of oscillations in cytosolic free Ca^{2+} concentration ($[Ca^{2+}]_{free}$). The duration of $[Ca^{2+}]_{free}$ transients is characteristic of the stimulating agonist. We have previously reported that ADP and ATP, which are believed to act through a single P_{2y}-purinoceptor species, induce very different oscillatory $[Ca^{2+}]_{free}$ responses in the majority of hepatocytes. We have interpreted these data as evidence for two separate Ca^{2+}-mobilizing purinoceptors for these nucleotides. We show here that the elevation of intracellular cyclic AMP concentration, by the co-application of either dibutyryl cyclic AMP or 7β-desacetyl-7β-[γ-(*N*-methylpiperazino)butyryl]-forskolin (L858051), exerts different modulatory effects on $[Ca^{2+}]_{free}$ oscillations induced by ADP and ATP in single rat hepatocytes. Elevated intracellular cyclic AMP levels enhance the frequency and peak $[Ca^{2+}]_{free}$ of transients induced by ADP. In contrast, the elevation of intracellular cyclic AMP levels in hepatocytes producing $[Ca^{2+}]_{free}$ oscillations in response to ATP stimulates either an increase in the duration of transients or a sustained rise in $[Ca^{2+}]_{free}$. The data illustrate a further difference between the oscillatory $[Ca^{2+}]_{free}$ responses of hepatocytes to ADP and ATP, thus further arguing against ADP and ATP acting via a single purinoceptor species.

INTRODUCTION

Oscillations in cytosolic free Ca^{2+} concentration ($[Ca^{2+}]_{free}$) have been demonstrated in many cell types, including rat hepatocytes, in response to agonists acting via the phosphoinositide signalling pathway (Berridge, 1990; Cobbold et al., 1991). Single rat hepatocytes, microinjected with the Ca^{2+}-sensitive photoprotein aequorin, exhibit trains of repetitive $[Ca^{2+}]_{free}$ transients whose frequency is modulated by agonist concentration (Woods et al., 1986). The duration of individual $[Ca^{2+}]_{free}$ transients is dependent on the stimulating agonist; the variability results from differences in the rate of fall of $[Ca^{2+}]_{free}$ from its peak (Woods et al., 1987). Thus the duration of the $[Ca^{2+}]_{free}$ transients generated is characteristic of the receptor species being activated. ADP and ATP are believed to act through a single class of P_2-purinoceptor characterized as belonging to the P_{2y} sub-class (Keppens and De Wulf, 1986), as defined by the classification of Burnstock and Kennedy (1985), to stimulate the hydrolysis of phosphatidylinositol 4,5-bisphosphate and subsequent mobilization of Ca^{2+} in rat hepatocytes (Charest et al., 1985). However, aequorin measurements of $[Ca^{2+}]_{free}$ in single rat hepatocytes have shown that ADP and ATP elicit $[Ca^{2+}]_{free}$ transients of very different duration. ADP invariably induces transients of short duration (approx 9 s), whereas the response to ATP is more complex. Three different classes of hepatocytes were distinguishable: 28% of cells responded with transients of short duration indistinguishable from those induced by ADP; a further 28% of cells produced transients of a much longer duration; in the third group of cells (44%) ATP induced a variable response, with a mixture of short and long transients within a single response (Dixon et al., 1990). We have interpreted these data as evidence that rat hepatocytes possess two Ca^{2+}-mobilizing purinoceptors, termed P_{2ys} and P_{2yl}. Thus, it is postulated that one receptor, P_{2ys}, is activated by both ADP and ATP to mediate $[Ca^{2+}]_{free}$ transients of short duration, whereas a second receptor subtype, P_{2yl}, is activated exclusively by ATP to mediate $[Ca^{2+}]_{free}$ transients of long duration. The generation of a variable response, as seen in the third group of cells, is postulated to result from stimulation of both P_{2ys} and P_{2yl} receptor subtypes (Dixon et al., 1990). We have recently described a further difference between the actions of ADP and ATP on rat hepatocytes whereby co-application of adenosine 5′-[$\alpha\beta$-methylene]triphosphate (pp[CH_2]pA) potentiates the oscillatory $[Ca^{2+}]_{free}$ responses of single hepatocytes to ATP but not to ADP (Dixon et al., 1993).

The present work describes the effects of elevated cyclic AMP on $[Ca^{2+}]_{free}$ oscillations induced by ADP and ATP. A number of workers have shown that interactions exist between the Ca^{2+}/phosphoinositide- and the cyclic AMP-mediated signalling pathways. In hepatocyte populations, elevated cyclic AMP has been shown to potentiate several actions of Ca^{2+}-mobilizing agonists, including increases in $[Ca^{2+}]_{free}$(Morgan et al., 1984), inositol lipid turnover (Pittner and Fain, 1989, 1990) and Ca^{2+} influx (Mauger et al., 1985; Poggioli et al., 1986; Bygrave et al., 1993). It has been shown that the increase in $[Ca^{2+}]_{free}$ induced by ADP and ATP in isolated rat hepatocytes can be potentiated by glucagon (Charest et al., 1985). Measurements of $[Ca^{2+}]_{free}$ in single hepatocytes have shown that elevated intracellular cyclic AMP levels modulate $[Ca^{2+}]_{free}$ oscillations induced by agonists acting via the phosphoinositide signalling pathway (Schöfl et al., 1991; Somogyi et al., 1992). Furthermore, elevated intracellular cyclic AMP levels exert different modulatory effects on $[Ca^{2+}]_{free}$ oscillations depending on the Ca^{2+}-mobilizing agonist. Elevated cyclic AMP increases the frequency and peak $[Ca^{2+}]_{free}$ of oscillations induced by the α_1-adrenergic agonist phenylephrine. In contrast, elevated cyclic AMP has no effect on peak $[Ca^{2+}]_{free}$ of vasopressin-induced $[Ca^{2+}]_{free}$ transients, but markedly pro-

Abbreviations used: pp[CH_2]pA, adenosine 5′-[$\alpha\beta$-methylene]triphosphate; $[Ca^{2+}]_{free}$, cytosolic concentration of free Ca^{2+}; L858051, 7β-desacetyl-7β-γ-(*N*-methylpiperazino)butyryl]forskolin.

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longs the falling phase of each $[Ca^{2+}]_{free}$ transient (Sanchez-Bueno et al., 1993).

We show here that elevated cyclic AMP concentration elicits markedly different modulatory effects on $[Ca^{2+}]_{free}$ oscillations induced by ADP and ATP. The present data highlight another difference between the oscillatory $[Ca^{2+}]_{free}$ responses of rat hepatocytes to ADP and ATP, thus further arguing against a single receptor species for these two agonists.

MATERIALS AND METHODS

Hepatocytes were isolated from fed 150–250 g male Wistar rats by collagenase digestion and prepared for microinjection with aequorin as described previously (Woods et al., 1987). Collagenase was from Boehringer. Microdialysis of aequorin, microinjection and data acquisition and analysis have been described previously (Cobbold and Lee, 1991). The experimental medium was Williams medium E (Gibco) gassed with CO_2/air (1:19) at 37 °C. ADP, ATP, dibutyryl cyclic AMP and 7β-desacetyl-7β-[γ-(*N*-methylpiperazino)butyryl]forskolin (L858051) were added to this medium. ADP, ATP and dibutyryl cyclic AMP were from Sigma. L858051 (Calbiochem) is a chemically modified forskolin with an enhanced stability and water solubility, whose adenylate cyclase-stimulating activity and binding capacity are approximately equal to those of forskolin (Laurenza et al., 1987).

RESULTS

Single aequorin-injected hepatocytes responded to extracellular ADP and ATP in the range 1–5 μM by the generation of oscillations in $[Ca^{2+}]_{free}$ similar in duration to those previously reported (Dixon et al., 1990). Elevation of intracellular cyclic AMP levels by the co-application of the cell-permeant analogue of cyclic AMP, dibutyryl cyclic AMP (1–10 μM), stimulated an increase in the frequency and peak $[Ca^{2+}]_{free}$ of ADP-induced transients (14/16 cells), as shown in Figure 1(a). In the remaining 2 cells, the co-application of dibutyryl cyclic AMP evoked a conversion from discrete transients to a sustained rise in $[Ca^{2+}]_{free}$. Elevation of intracellular cyclic AMP levels by direct activation of adenylate cyclase by co-application of L858051 (2–10 μM; 3/3 cells) stimulated a similar increase in the frequency and peak $[Ca^{2+}]_{free}$ of ADP-induced transients, as illustrated in Figure 1(b).

In the majority of hepatocytes, the experimental elevation of intracellular cyclic AMP levels also potentiated $[Ca^{2+}]_{free}$ oscillations induced by ATP. However, the modulatory effects of elevated cyclic AMP concentration on the profile of ATP-induced $[Ca^{2+}]_{free}$ transients differed greatly from those observed on ADP-induced transients. Since, as outlined in the Introduction, considerable variation exists in the hepatocyte oscillatory $[Ca^{2+}]_{free}$ response to ATP, it is necessary to consider the observed modulatory effects of elevated cyclic AMP in relation to the profile of $[Ca^{2+}]_{free}$ transients induced by ATP before the elevation of cyclic AMP:

(i) Elevation of intracellular cyclic AMP levels in single aequorin-injected hepatocytes responding to ATP with $[Ca^{2+}]_{free}$ oscillations of consistent short duration, by the co-application of L858051 (2–10 μM; $n = 8$), stimulated a marked increase in the duration of each transient. A typical result is shown in Figure 2(a). As shown in Figure 2(b), a similar prolongation of short ATP-induced transients was observed when dibutyryl cyclic AMP was used to elevate intracellular cyclic AMP concentration (1–5 μM; $n = 4$). In addition, the co-application of 5 μM dibutyryl cyclic AMP to two hepatocytes generating short ATP-induced transients led to a sustained rise in $[Ca^{2+}]_{free}$, as shown in Figure 2(c).

(ii) Elevation of intracellular cyclic AMP levels in those hepatocytes in which ATP alone induces $[Ca^{2+}]_{free}$ transients of long duration evoked a more varied response. In the majority of hepatocytes in this group, elevated cyclic AMP levels potentiated the $[Ca^{2+}]_{free}$ response. Of a total 21 hepatocytes responding to ATP alone by the generation of long $[Ca^{2+}]_{free}$ transients, 5 cells continued to produce discrete transients in the presence of elevated intracellular cyclic AMP (by either 1–10 μM dibutyryl cyclic AMP or 2–10 μM L858051), but the duration of each transient was much longer than those evoked by ATP alone (Figure 3a). In a further 9 cells in this group, elevation of intracellular cyclic AMP concentration, by co-application of either L858051 (10 μM) or dibutyryl cyclic AMP (1–10 μM), led to a sustained rise in $[Ca^{2+}]_{free}$, as illustrated in Figure 3(b). Three

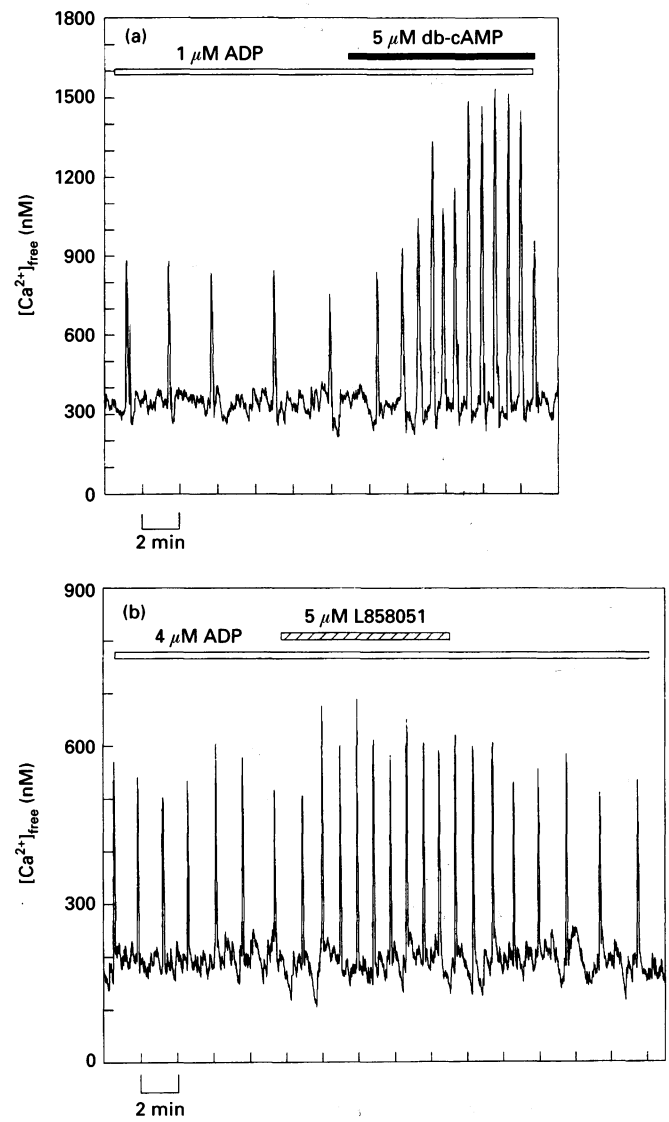

Figure 1 Elevation of intracellular cyclic AMP levels enhances the frequency and peak $[Ca^{2+}]_{free}$ of $[Ca^{2+}]_{free}$ oscillations induced by extracellular ADP

Single aequorin-injected hepatocytes responding to ADP by the generation of $[Ca^{2+}]_{free}$ transients were co-supplied with (**a**) 5 μM dibutyryl cyclic AMP (db-cAMP) or (**b**) 5 μM L858051 for the periods indicated. Time constants: for resting $[Ca^{2+}]_{free}$, 10 s; for transients, 1 s.

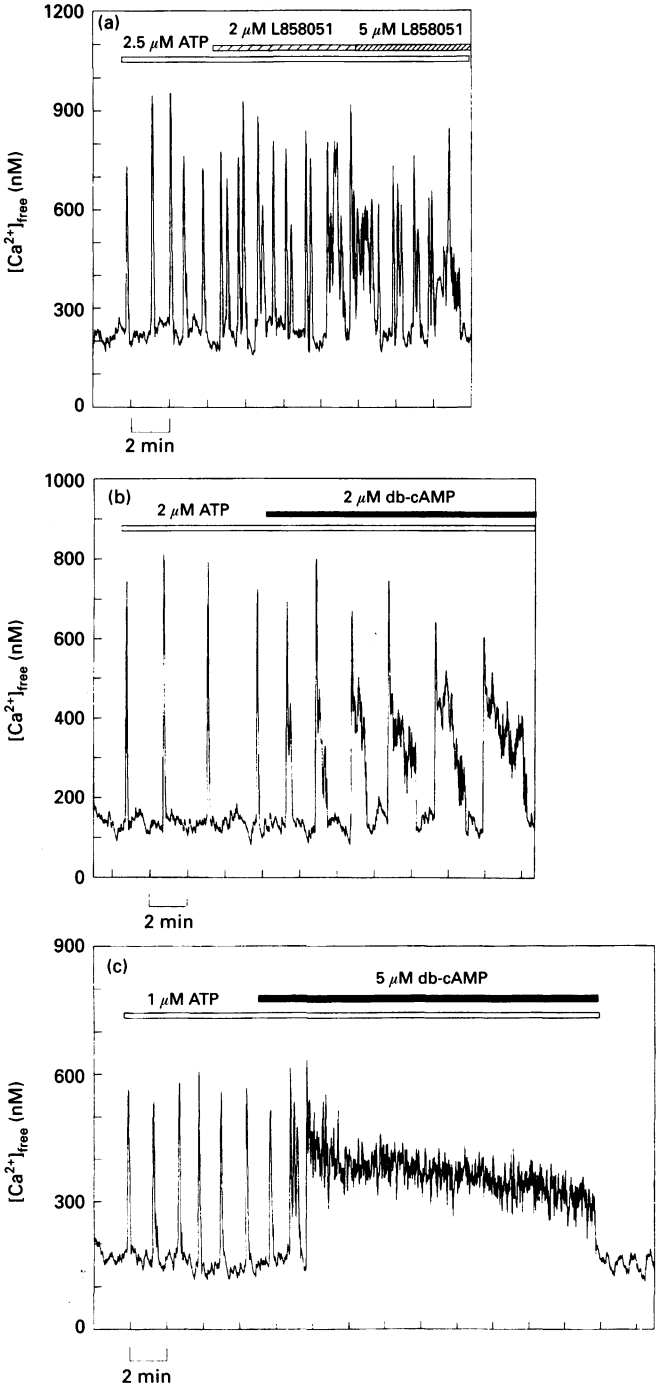

Figure 2 Elevation of intracellular cyclic AMP levels modulates short ATP-induced $[Ca^{2+}]_{free}$ oscillations via either an increase in the duration of individual transients or a sustained rise in $[Ca^{2+}]_{free}$

Single aequorin-injected hepatocytes responded to ATP with transients of short duration. Intracellular cyclic AMP concentration was elevated by the co-application, for the periods indicated, of (**a**) 2 μM and 5 μM L858051, (**b**) 2 μM dibutyryl cyclic AMP (db-cAMP) or (**c**) 5 μM dibutyryl cyclic AMP (db-cAMP). Time constants: for resting $[Ca^{2+}]_{free}$, 10 s; for transients, 1 s.

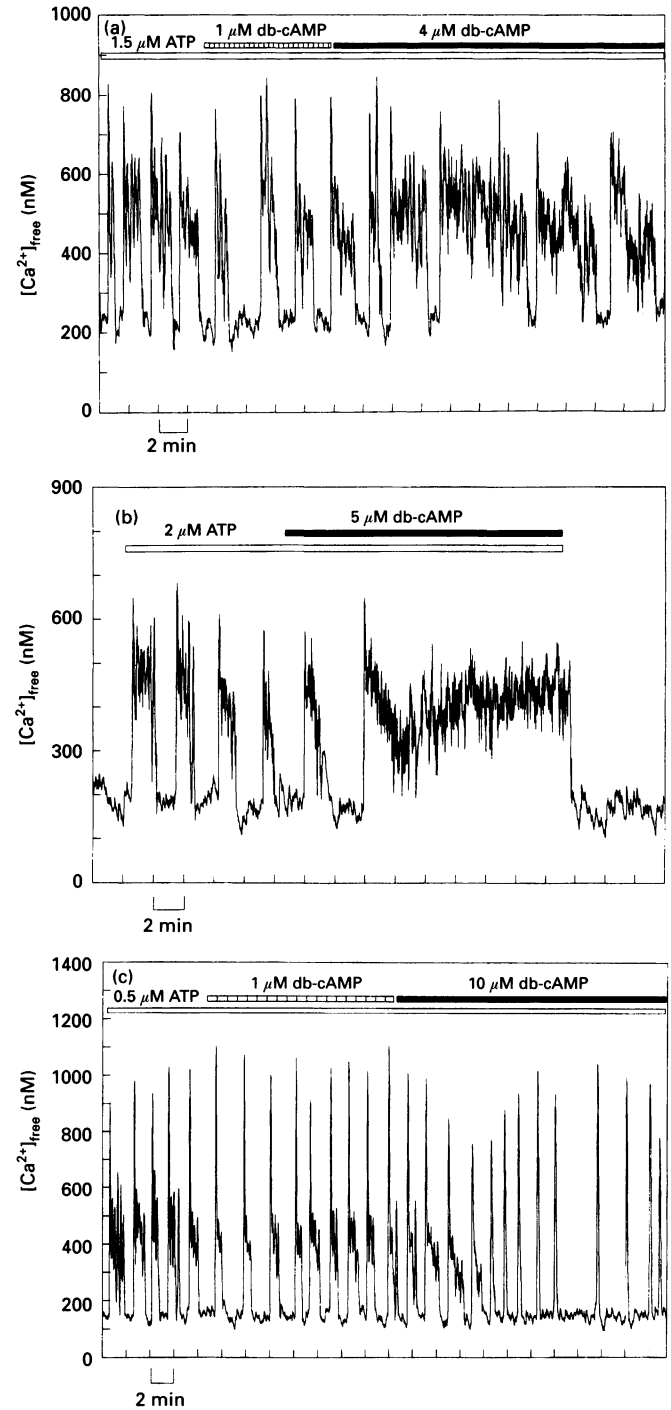

Figure 3 Effects of elevated intracellular cyclic AMP levels on long ATP-induced $[Ca^{2+}]_{free}$ oscillations

Single aequorin-injected hepatocytes responded to ATP with $[Ca^{2+}]_{free}$ transients of long duration. Intracellular cyclic AMP concentration was elevated by the co-application of dibutyryl cyclic AMP (db-cAMP) at the concentrations indicated and for the periods indicated. The elevation of intracellular cyclic AMP levels stimulated (**a**) an increase in the duration of individual transients, or (**b**) a sustained rise in $[Ca^{2+}]_{free}$, or (**c**) a marked truncation of the falling phase of individual transients. Time constants: for resting $[Ca^{2+}]_{free}$, 10 s; for transients, 1 s.

hepatocytes were found in which the co-application of L858051 had no effect on long ATP $[Ca^{2+}]_{free}$ transients. The remaining 4 hepatocytes generating long $[Ca^{2+}]_{free}$ transients in the presence of ATP responded in a curious manner to the addition of dibutyryl cyclic AMP. In all 4 cells, 1 μM dibutyryl cyclic AMP appeared to have no effect on the oscillatory $[Ca^{2+}]_{free}$ response,

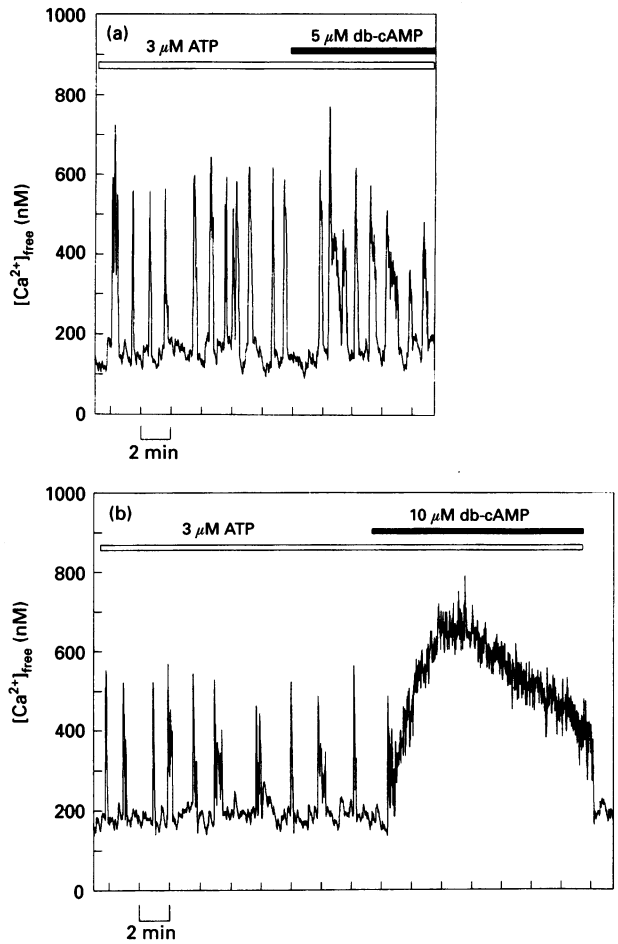

Figure 4 Effects of elevated intracellular cyclic AMP concentration on the oscillatory $[Ca^{2+}]_{free}$ of hepatocytes producing $[Ca^{2+}]_{free}$ transients of variable duration in response to extracellular ATP

Single hepatocytes, microinjected with aequorin, responded to ATP by the generation of $[Ca^{2+}]_{free}$ transients of variable duration. The elevation of intracellular cyclic AMP concentration by the co-application of either L858051 or dibutyryl cyclic AMP (db-cAMP), for the periods indicated, stimulated either (**a**) an increase in the duration of individual transients, or (**b**) a sustained rise in $[Ca^{2+}]_{free}$. Time constants: for resting $[Ca^{2+}]_{free}$, 10 s; for transients, 1 s.

but the subsequent addition of 10 μM dibutyryl cyclic AMP effected a marked truncation of the falling phase of the $[Ca^{2+}]_{free}$ transients. A typical response of this type is illustrated in Figure 3(c).

(iii) In hepatocytes responding to ATP alone by the generation of $[Ca^{2+}]_{free}$ transients of variable duration, the subsequent elevation of intracellular cyclic AMP concentration, by addition of either dibutyryl cyclic AMP (1–10 μM) or L858051 (5–10 μM), stimulated an increase in the duration of transients (10 cells; Figure 4a) or a sustained rise in $[Ca^{2+}]_{free}$ (7 cells; Figure 4b).

The data described above are summarized in Table 1, which relates the effects of the elevation of intracellular cyclic AMP concentration by the addition of either dibutyryl cyclic AMP or L858051 to the shape of the initial transients induced by ATP.

In agreement with the observations of Schofl et al. (1991) and Sanchez-Bueno et al. (1993), neither dibutyryl cyclic AMP nor L858051 alone induced any changes in $[Ca^{2+}]_{free}$ (results not shown).

Table 1 Effects of elevated cyclic AMP levels on $[Ca^{2+}]_{free}$ transients induced by ATP

The effect of elevation of intracellular cyclic AMP concentration by the co-application of either dibutyryl cyclic AMP or L858051 is related to the duration of the individual transients induced by ATP. The values given are the numbers of individual hepatocytes.

Effect of elevated intracellular cyclic AMP	Duration of ATP-induced $[Ca^{2+}]_{free}$ transients		
	Short	Long	Variable
Prolongation of transients			
(i) by addition of dibutyryl cyclic AMP	4	3	7
(ii) by addition of L858051	8	2	3
Sustained rise in $[Ca^{2+}]_{free}$			
(i) by addition of dibutyryl cyclic AMP	2	7	7
(ii) by addition of L858051	0	2	0
No effect			
(i) by addition of dibutyryl cyclic AMP	0	0	0
(ii) by addition of L858051	0	3	0
Truncation of transients			
(i) by addition of dibutyryl cyclic AMP	0	4	0
(ii) by addition of L858051	0	0	0

DISCUSSION

We have shown here that elevated intracellular cyclic AMP levels potentiate $[Ca^{2+}]_{free}$ oscillations induced by extracellular ADP and ATP in single aequorin-injected rat hepatocytes. As outlined in the Introduction, aequorin measurements of $[Ca^{2+}]_{free}$ in single rat hepatocytes have shown that ADP and ATP, thought to act through the same P_{2y}-purinoceptor, elicit $[Ca^{2+}]_{free}$ transients of very different durations in the majority of cells. Briefly, ADP invariably induces transients of short duration, whereas ATP induces similar short transients, transients of a longer duration, or a mixture of short and long transients within a single response (Dixon et al., 1990). The present study illustrates a further difference between the oscillatory $[Ca^{2+}]_{free}$ responses of hepatocytes to ADP and ATP; $[Ca^{2+}]_{free}$ transients induced by extracellular ADP are modulated differently by elevated intracellular cyclic AMP concentration compared with those induced by ATP. Elevation of intracellular cyclic AMP levels potentiates $[Ca^{2+}]_{free}$ oscillations induced by ADP by enhancing the frequency and peak $[Ca^{2+}]_{free}$ of transients. In contrast, elevated cyclic AMP levels in hepatocytes producing $[Ca^{2+}]_{free}$ oscillations in response to ATP stimulate either an increase in the duration of each transient or a sustained rise in $[Ca^{2+}]_{free}$ in the majority of hepatocytes.

ATP is the only agonist found to induce a variable oscillatory $[Ca^{2+}]_{free}$ response between cells; other agonists always elicit characteristic and consistent transient shapes (Woods et al., 1987). In addition, ATP is also unique in inducing $[Ca^{2+}]_{free}$ oscillations in some cells in which the falling phase is composed of either one or more discrete secondary oscillations (see Figures 3 and 4). Similar multiple secondary oscillations have been recorded in fertilized mouse oocytes (Cuthbertson and Cobbold, 1985). In some cells the prolongation of ATP-induced transients by elevated intracellular cyclic AMP levels was achieved through an increase in the number of secondary oscillations (Figure 2a).

It might be suggested that the previously described variability in the duration of $[Ca^{2+}]_{free}$ transients induced by ATP alone (Dixon et al., 1990) is simply the result of possible cell-to-cell variations in basal cyclic AMP concentration (Hanoune et al.,

1977). Thus, in those hepatocytes with a relatively high basal cyclic AMP concentration, the oscillatory $[Ca^{2+}]_{free}$ response would be potentiated such that $[Ca^{2+}]_{free}$ transients of longer duration are generated in response to extracellular ATP. However, the duration of vasopressin- and angiotensin II-induced $[Ca^{2+}]_{free}$ transients is also increased by elevated cyclic AMP levels (Sanchez-Bueno et al., 1993; Sanchez-Bueno and Cobbold, 1993). We would thus expect to see, if sufficient cell-to-cell variations in basal cyclic AMP concentration exist, similar variability in the duration of $[Ca^{2+}]_{free}$ transients induced by these agonists. However, in the absence of experimentally elevated cyclic AMP concentration, hepatocytes respond to vasopressin and angiotensin II by the generation of $[Ca^{2+}]_{free}$ transients of consistent duration (Woods et al., 1987). It is therefore unlikely that the variability in the duration of $[Ca^{2+}]_{free}$ transients induced by ATP alone is the result of cell-to-cell variations in basal cyclic AMP concentration.

It might also be proposed that the variability in the oscillatory $[Ca^{2+}]_{free}$ response of hepatocytes to ATP may arise from the stimulation by extracellular ATP, in some hepatocytes, of an accompanying rise in intracellular cyclic AMP concentration, thus resulting in the generation of transients of longer duration. However, ADP and ATP, at concentrations which near-maximally increase $[Ca^{2+}]_{free}$, do not elevate cyclic AMP concentration in hepatocytes (Charest et al., 1985). On the contrary, ATP and ADP, like angiotensin II and vasopressin (Crane et al., 1982; Morgan et al., 1983), have been shown to inhibit the glucagon- (Keppens and De Wulf, 1985) and forskolin- (Okajima et al., 1987), induced rise in cyclic AMP in hepatocytes.

The previously reported variability in the hepatocyte oscillatory $[Ca^{2+}]_{free}$ response to ATP (Dixon et al., 1990) cannot therefore be attributed to differences in intracellular cyclic AMP levels. Indeed, the present data, which illustrate the impact of experimentally elevated cyclic AMP levels on ADP- and ATP-induced $[Ca^{2+}]_{free}$ oscillations, highlight further differences between the oscillatory $[Ca^{2+}]_{free}$ responses of single hepatocytes to ADP and ATP.

Different modulatory effects of elevated intracellular cyclic AMP on $[Ca^{2+}]_{free}$ oscillations, depending on the Ca^{2+}-mobilizing agonist, have been reported previously. Elevated cyclic AMP enhances both the peak $[Ca^{2+}]_{free}$ and the frequency of transients induced by phenylephrine, whereas $[Ca^{2+}]_{free}$ transients induced by vasopressin and angiotensin II are modulated through an increase in the duration of each transient (Sanchez-Bueno et al., 1993; Sanchez-Bueno and Cobbold, 1993). The present data show that the response of ADP-induced $[Ca^{2+}]_{free}$ oscillations to elevated intracellular cyclic AMP resembles that of phenylephrine-induced oscillations, whereas the response of ATP-induced $[Ca^{2+}]_{free}$ oscillations is similar to that of vasopressin- and angiotensin II-induced transients. It thus appears that the potentiating effect of cyclic AMP on the hepatocyte oscillatory $[Ca^{2+}]_{free}$ response depends on the Ca^{2+}-mobilizing agonist, and hence on the receptor species activated.

Several mechanisms have been proposed whereby cyclic AMP, acting through the activation of protein kinase A, exerts effects on $[Ca^{2+}]_{free}$. Some studies have revealed that treatment of hepatocytes with glucagon or cyclic AMP analogues potentiates agonist-mediated increases in Ins(1,4,5)P_3 levels (Pittner and Fain, 1989, 1990), whereas other workers have found no changes in inositol phosphates or the inositol lipid precursors (Poggioli et al., 1986; Burgess et al., 1991). Since an increase in the concentration of Ca^{2+}-mobilizing agonist will be accompanied by an increase in Ins(1,4,5)P_3 levels, the reported ability of cyclic AMP to potentiate the increase in Ins(1,4,5)P_3 levels would be predicted to mimic the effects on $[Ca^{2+}]_{free}$ oscillations of an increase in agonist concentration. Raising agonist concentration increases the frequency of $[Ca^{2+}]_{free}$ oscillations; the duration is unchanged (Woods et al., 1987). Such an effect is consistent with the observed increase in frequency of ADP-induced transients (and also of phenylephrine-induced transients; Sanchez-Bueno et al., 1993) in the presence of elevated cyclic AMP levels, and also with the conversion of long ATP-induced transients to a sustained rise in $[Ca^{2+}]_{free}$. In hepatocytes producing transients of long duration in response to ATP, the concentration range for transient production is very narrow; above an upper limit, transients are replaced by a sustained rise in $[Ca^{2+}]_{free}$. However, the increased peak $[Ca^{2+}]_{free}$ attained and the increase in the duration of ATP-induced transients of both long and short duration, as observed in the present study (and similarly for vasopressin-induced transients; Sanchez-Bueno et al., 1993), is not consistent with the mimicking of an increase in agonist concentration. Increasing agonist concentration does not alter either the peak $[Ca^{2+}]_{free}$ or the duration of transients (Woods et al., 1987).

Burgess et al. (1991) have shown, in permeabilized guinea-pig hepatocytes, that elevated intracellular cyclic AMP acts, via protein kinase A activation, to potentiate the ability of Ins(1,4,5)P_3 to release Ca^{2+} and to increase the amount of Ca^{2+} available for release by maximal Ins(1,4,5)P_3. Furthermore, in intact rat and guinea-pig hepatocytes in which inositol trisphosphate levels were elevated by microinjection with a subthreshold concentration of Ins(2,4,5)P_3, the subsequent elevation of intracellular cyclic AMP stimulated a rise in $[Ca^{2+}]_{free}$ (Burgess et al., 1991; Bird et al., 1993). These workers have therefore proposed that, since the Ins(1,4,5)P_3 receptor is a major site for cyclic AMP-dependent phosphorylation, a protein kinase A-mediated phosphorylation might increase its sensitivity for Ins(1,4,5)P_3, thus allowing lower concentrations of Ins(1,4,5)P_3 to induce Ca^{2+} release. Indeed a direct action of protein kinase A on the Ins(1,4,5)P_3 receptor to increase its sensitivity to Ins(1,4,5)P_3 for channel opening has recently been confirmed by Hajnóczky et al. (1993). Hajnóczky et al. (1993) also report the ability of protein kinase A to stimulate the ATP-dependent Ca^{2+} pump and to modify the functional distribution of Ca^{2+} stores between Ins(1,4,5)P_3-sensitive and Ins(1,4,5)P_3-insensitive pools.

It is difficult to envisage how any of the mechanisms described above could give rise to the agonist-specific modulatory effects of elevated intracellular cyclic AMP reported here and by Sanchez-Bueno et al. (1993). Such agonist specificity might be more readily explained by interactions with protein kinase A at the level of the relevant phosphoinositide-linked receptors. For example, it could be envisaged that phosphorylation of a receptor may modify its agonist-binding or signal-transducing properties in a manner specific to that particular receptor. Indeed, it has been shown that the binding of α_1-agonists to hepatocyte plasma membranes is increased in cyclic AMP-dependent mechanisms (Morgan et al., 1984). Protein kinase A phosphorylates the α_1-adrenoceptor *in vitro* (Bouvier et al., 1987), and it has also been suggested that the vasopressin V_1-receptor is phosphorylated by protein kinase A (Guillon et al., 1988; Pittner and Fain, 1989). Furthermore, a recently cloned ATP receptor has been shown to have several potential phosphorylation sites (Lustig et al., 1993). However, the demonstration that the $[Ca^{2+}]_{free}$ response induced by fluoroaluminate (which activates G-proteins directly) is also potentiated by cyclic AMP (Blackmore and Exton, 1986) suggests that some of the observed potentiating effects of cyclic AMP may be exerted at a site distal to the receptors.

The stimulation of plasma-membrane Ca^{2+} influx has been shown to be an early action of the elevation of intracellular cyclic AMP in rat hepatocytes (Bygrave et al., 1993). It has been reported that the co-addition of glucagon or dibutyryl cyclic

AMP with vasopressin greatly stimulates Ca^{2+} influx in hepatocytes (Mauger et al., 1985; Poggioli et al., 1986). In perfused rat liver, glucagon stimulates an acceleration of Ca^{2+} influx induced by phenylephrine, vasopressin, angiotensin II (Altin and Bygrave, 1986), ADP and ATP (Altin and Bygrave, 1987). In a recent review of cross-talk between the actions of glucagon and Ca^{2+}-mobilizing agonists in liver (Bygrave and Benedetti, 1993) it was suggested that the different modulatory effects of elevated cyclic AMP on $[Ca^{2+}]_{free}$ oscillations induced by phenylephrine and vasopressin (Sanchez-Bueno et al., 1993) may be related to the observation that, in perfused rat liver, the glucagon-stimulated acceleration of Ca^{2+} influx seen with vasopressin and angiotensin II is much greater than that seen with phenylephrine (Altin and Bygrave, 1986). Unfortunately, most studies on Ca^{2+} influx have used concentrations of vasopressin that are not equivalent to phenylephrine in their effect on the hepatocyte $[Ca^{2+}]_{free}$ oscillations. Usually vasopressin concentrations that induce a sustained $[Ca^{2+}]_{free}$ rise are compared with phenylephrine doses that elicit repetitive $[Ca^{2+}]_{free}$ oscillations. Comparisons of influx data are therefore difficult.

Thus, although there are many possible sites of interaction between the two signalling pathways, it is difficult to establish which of these are important in the observed receptor-specific modulatory effects of elevated cyclic AMP levels on the hepatocyte oscillatory $[Ca^{2+}]_{free}$ response. It is clear, however, that the markedly different modulatory effects of elevated intracellular cyclic AMP on ADP- and ATP-induced $[Ca^{2+}]_{free}$ oscillations are not consistent with ADP and ATP acting via the same single receptor species. The present observations therefore provide further evidence in support of our previous proposals for the existence of separate receptors for these nucleotides (Dixon et al., 1990, 1993).

It is particularly intriguing that short $[Ca^{2+}]_{free}$ transients induced by ATP (Figure 2) are modulated differently by elevated cyclic AMP concentration compared with those induced by ADP (Figure 1). Short $[Ca^{2+}]_{free}$ transients generated in response to extracellular ATP have previously been shown to be indistinguishable, in terms of duration, from $[Ca^{2+}]_{free}$ transients generated in response to extracellular ADP. It was therefore proposed that the ADP transients and the short ATP transients arise from the stimulation of single receptor species (Dixon et al., 1990). However, in the present study, in all hepatocytes responding to ATP by the generation of short transients, elevation of intracellular cyclic AMP evoked an increase in the duration of each $[Ca^{2+}]_{free}$ transient or a sustained rise in $[Ca^{2+}]_{free}$ (Figure 2) rather than the enhancement of the frequency and peak $[Ca^{2+}]_{free}$ of transients seen in hepatocytes responding to ADP (Figure 1). Consistent with the present observation, we have recently described a difference between the effects of pp[CH_2]pA, a methylated derivative of ATP, on ADP- and ATP-induced $[Ca^{2+}]_{free}$ transients (Dixon et al., 1993). When pp[CH_2]pA was co-supplied to aequorin-injected hepatocytes producing $[Ca^{2+}]_{free}$ oscillations in response to ADP, no modulation of the oscillatory $[Ca^{2+}]_{free}$ response was observed. However, in aequorin-injected hepatocytes responding to ATP by the production of short $[Ca^{2+}]_{free}$ transients, the application of pp[CH_2]pA stimulated a prolongation of the falling phase of each transient.

We have thus illustrated two cases in which ADP-induced $[Ca^{2+}]_{free}$ transients and short ATP-induced $[Ca^{2+}]_{free}$ transients, although indistinguishable in terms of duration, are affected differently by the co-application of a modulatory agonist. Such findings might be interpreted as evidence that short ATP- and ADP-induced $[Ca^{2+}]_{free}$ oscillations are mediated via distinct P_{2y}-purinoceptors rather than via the same subtype of the P_{2y}-purinoceptor, the P_{2ys} subtype, as previously proposed (Dixon et al., 1990). Such an interpretation thus requires the existence of three functionally distinct P_{2y}-purinoceptors on the hepatocyte cell surface. One, we predict, is activated exclusively by ADP to mediate $[Ca^{2+}]_{free}$ transients of short duration which are not affected by the co-application of pp[CH_2]pA. Elevated intracellular cyclic AMP concentration enhances the frequency and peak $[Ca^{2+}]_{free}$ of transients mediated via this receptor, which we term the ADP receptor. A second receptor sub-type, we suggest, is responsive to ATP, but not to ADP, to mediate $[Ca^{2+}]_{free}$ transients of short duration, which we designate 'ATP_S'. In addition, a third receptor sub-type may exist which is responsive solely to ATP to mediate $[Ca^{2+}]_{free}$ transients of long duration; we designate this 'ATP_L'. Transients mediated via these two receptors, ATP_S and ATP_L, are potentiated by the co-application of pp[CH_2]pA and by the elevation of intracellular cyclic AMP concentration, which stimulates either an increase in the duration of individual transients or a sustained rise in $[Ca^{2+}]_{free}$.

In accordance with our proposals for separate receptors for ADP and ATP on hepatocytes, other groups have reported ADP to be ineffective in causing changes in $[Ca^{2+}]_{free}$ in several cell types in which ATP mobilizes intracellular Ca^{2+} stores (Cockcroft and Stutchfield, 1989; Gonzalez et al., 1989a,b; Kuroki et al., 1989; Brown et al., 1991; Dulon et al., 1991; Merten et al., 1993). Platelets express P_{2t}-purinoceptors (Gordon, 1986), which are responsive solely to ADP to mediate both the inhibition of adenylate cyclase and the mobilization of intracellular Ca^{2+} stores (Sage et al., 1990). The distribution of P_{2t}-purinoceptors was previously thought to be restricted to platelets. However, a recent report describes the existence of a receptor with similar properties in K562 leukaemia cells (Murgo and Sistare, 1992). P_{2t}-purinoceptors may therefore have a more widespread distribution than previously envisaged, and may be related to the hepatocyte ADP receptor proposed here.

The cloning and expression of two distinct ATP receptors has recently been reported (Webb et al., 1993; Lustig et al., 1993). One of these was found to be responsive to both ADP and ATP, but the mRNA encoding this receptor was not detected in liver (Webb et al., 1993). Of particular interest is the report of the cloning of an ATP receptor from mouse neuroblastoma cells which is activated by ATP (EC_{50} = 0.7 μM), but by ADP only in the millimolar range, to elicit the mobilization of $[Ca^{2+}]_{free}$. mRNA encoding this receptor has been detected in a number of mouse tissues, including liver. The properties of this receptor thus resemble those of the proposed ATP_S and ATP_L purinoceptor sub-types of rat hepatocytes. Future studies may confirm the existence of a purinoceptor with similar properties in rat liver. In addition, we predict that molecular cloning will reveal the existence of a gene which encodes a purinoceptor that is responsive solely to ADP.

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