Evidence for two Ca^{2+} -mobilizing purinoceptors on rat hepatocytes

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Aequorin measurements of cytosolic free Ca^{2+} in single rat hepatocytes show that ADP and ATP, thought to act through the same P_{2Y} purinoceptor, elicited very different responses in the majority of cells tested. ADP invariably induced transients of short duration (approx. 9 s), whereas ATP induced either similar transients or transients with a much longer duration (approx. 49 s). We explain this variability in terms of two separate purinoceptors on rat hepatocytes, one of which responds to either ATP or ADP to generate free- Ca^{2+} transients of short duration, and the other responds to ATP only, with transients of longer duration.

INTRODUCTION

Oscillations in cytosolic free Ca^{2+} concentration ($[\text{Ca}^{2+}]_{\text{free}}$) have been demonstrated in many cell types in response to agonists acting through the phosphoinositide signalling pathway (Woods *et al.*, 1986; Berridge *et al.*, 1988; Rink & Jacob, 1989; Rooney *et al.*, 1989). In the hepatocyte, agonist concentration modulates the transients' frequency, but has no effect on the time course of individual transients. We have previously shown that the time course of the $[\text{Ca}^{2+}]_{\text{free}}$ transients generated by an aequorin-injected hepatocyte are characteristic of the stimulating agonist, so that transients of very different time course can be recorded from the same individual cell; phenylephrine induces spikes of short duration (approx. 7 s), whereas vasopressin and angiotensin evoke longer spikes (approx. 10 s and 15 s respectively; Woods *et al.*, 1987). The different time courses result from differences in the rate of fall of $[\text{Ca}^{2+}]_{\text{free}}$ from its peak (Woods *et al.*, 1987; Berridge *et al.*, 1988). $[\text{Ca}^{2+}]_{\text{free}}$ transients with very different time courses have been recorded in hepatocytes in response to ATP and ADP (Cobbold *et al.*, 1988a). Since these purinergic agonists are thought to act through the same P_{2Y} purinoceptor to elevate intracellular $[\text{Ca}^{2+}]_{\text{free}}$ (Burnstock & Kennedy, 1985; Gordon, 1986; Keppens & De Wulf, 1986), we were interested in determining the reason for this difference. Vasopressin and oxytocin, acting at the same V_1 receptor on the hepatocyte, elicit spikes of the same time course, despite oxytocin having a 500-fold lower affinity for this receptor (Cobbold *et al.*, 1990). Therefore the distinct ATP/ADP responses do not appear to be explicable in terms of affinity differences for the P_{2Y} purinoceptor. The ATP/ADP responses have been re-examined in greater detail here, and evidence is presented for the existence of two subtypes of Ca^{2+} -mobilizing purinoceptor on the rat hepatocyte.

MATERIALS AND METHODS

Hepatocytes were isolated from fed male Wistar-strain rats and prepared for microinjection with aequorin as described previously (Woods *et al.*, 1987). Microdialysis of aequorin was performed as described previously (Cobbold *et al.*, 1988b). The experimental medium was Williams Medium E (Flow Laboratories) to which ADP or ATP (Sigma) was added. Collection and computer analysis of the data were performed as described previously to allow a comparison of transient parameters generated in response to the different agonists (Woods *et al.*, 1986).

RESULTS

Single aequorin-injected hepatocytes responded to extracellular ADP and ATP with series of repetitive transients in $[\text{Ca}^{2+}]_{\text{free}}$, the frequency of which depended on agonist concentration. In response to ADP, the transients were consistent in duration and waveform with a fast falling phase (Fig. 1). The overall duration was short, namely 9.3 ± 2.2 s (mean $\pm$ s.d.; range 5.8–14.2; $n = 14$ cells; no. of transients = 109). The response to ATP was, however, more complex, and three different classes of cells were distinguishable.

In group 1, 28 % of cells responded to ATP with transients similar to those induced by ADP (Fig. 2a), with a duration of 9.1 ± 2.7 s (mean $\pm$ s.d.; range 5.2–20; $n = 14$ cells; no. of transients = 147).

In group 2, 28 % of cells responded to ATP with significantly longer transients (Fig. 2b); duration 49.3 ± 20.7 s (mean $\pm$ s.d.; range 14.6–177.8; $n = 14$ cells; number of transients = 139).

Fig. 1. $[\text{Ca}^{2+}]_{\text{free}}$ transients induced in an aequorin-injected hepatocyte by ADP

A single cell was microinjected with aequorin and superfused with Williams Medium E containing $2 \mu\text{M}$ -ADP. Time constants: for transients, 0.4 s; for resting Ca^{2+} levels, 5 s. This result was typical of ten out of ten cells examined.

Abbreviations used: ATP[S], adenosine 5'-[γ -thio]triphosphate; G-protein, guanine-nucleotide-binding protein.

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Fig. 2. Variability in the $[Ca^{2+}]_{free}$ response of rat hepatocytes to ATP

(a) $[Ca^{2+}]_{free}$ transients of short duration induced in a single cell by $3 \mu M$ -ATP. Time constants: for transients, 0.4 s; for resting levels, 5 s. This result was typical of 14 out of 50 cells. (b) $[Ca^{2+}]_{free}$ transients evoked by $1.5 \mu M$ -ATP in a single hepatocyte, showing prolonged falling phases. This result was typical of 14 out of 50 cells. Time constants were 0.2 s and 8 s. (c) $[Ca^{2+}]_{free}$ response to $2 \mu M$ -ATP in a single hepatocyte, showing the variability of spike duration and the broad spectrum of waveforms; the result is typical of 22 out of 50 cells. Time constants were 1 s and 8 s.

In group 3, 44 % of cells responded to ATP with a mixture of 'ADP-like' transients and transients of longer duration during a single response (Fig. 2c); 18.3 ± 9.1 s (mean $\pm$ S.D.; range 5.8–47.4; $n = 22$ cells; number of transients = 205).

Fig. 3 shows the frequency distributions of spike lengths for each of these three groups. It is clear that the distributions for groups 1 (short transients) and 3 (long transients) are very well differentiated, with minimal overlap (t test; $P < 0.0001$). In Fig. 4, the frequency distributions of transient lengths for group 1 (short transients) and ADP are displayed; there was no significant difference between the groups ($P = 0.56$). A third distribution was obtained by extracting transients from group 3 (variable transients) of less than 15 s duration, the minimum histogram break-point greater than all ADP transient durations; the mean duration of these transients (11.0 s) is slightly, but significantly, greater than the means of both the ADP and group-1 transients

Fig. 3. Normalized frequency distributions of transient lengths for ATP

The distribution of transient lengths from each of the three groups of ATP response is shown (see the text). There is minimal overlap between the distributions of transient durations from group 1 (----, short transients) and group 2 (—, long transients). The distribution for group 3 (—; variable transients) appears to combine the group-1 (short transients) distribution with a reduced version of the group-2 (long transients) distribution and is explained in terms of interaction between two separate receptors responsible for transients of short and long duration.

(9.3 and 9.1 s respectively; $P < 0.0001$). The mean duration of transients of longer than 15 s from group 3 (variable transients) is significantly shorter than that of group 2 (long transients) (25.7 and 49.3 s respectively; $P < 0.0001$).

The possibility that influx of extracellular Ca^{2+} could account for the prolonged ATP response through a receptor-operated Ca^{2+} channel was explored in experiments in which the extracellular Ca^{2+} levels were reduced to $100 \mu M$ or below. Fig. 5 shows the result of such an experiment where extracellular Ca^{2+} was removed. In all experiments a decrease in transient frequency was observed (as expected from analogous experiments with phenylephrine and vasopressin; Woods *et al.*, 1990). No shortening of ATP-induced transients occurred, even over a period of 20 min in low- Ca^{2+} medium ($100 \mu M$). In Ca^{2+} -free medium (+0.5 mM-EGTA), three to five transients were produced before the response was abolished. Again, these transients did not show a truncated falling phase. In three cells low- Ca^{2+} medium caused a prolonging of transients induced by ATP, an effect which persisted when the cell was re-stimulated with ATP in normal medium (results not shown).

Fig. 4. Normalized frequency distributions of transient lengths for ADP, group-1 ATP (short transients) and transients of less than 15 s duration from group 3 (variable transients)

The distributions of ADP (—) and group-1 ATP transient lengths (----) are similar, whereas the short transients from group 3 (—; variable transients) have a slightly longer mean duration.

Fig. 5. Effect of chelating extracellular Ca^{2+} on the $[\text{Ca}^{2+}]_{\text{free}}$ transients induced by ATP

A single aequorin-injected hepatocyte was superfused with $3 \mu\text{M}$ -ATP in Williams Medium E (1.8 mM - Ca^{2+}) and nominally Ca^{2+} -free Williams Medium E with 0.5 mM -EGTA added, at the times indicated. Time constants: for transients, 0.8 s ; for resting Ca^{2+} levels, 8 s .

Adenosine 5'-[γ -thio]triphosphate (ATP[S]), a non-hydrolysable ATP analogue, was employed to test whether the ecto-ATPase activity described by Lin & Russell (1988) was responsible for the differences in the ADP/ATP transients. If hydrolysis of the terminal phosphate by this enzyme accounted for the prolonged Ca^{2+} response to ATP in some cells, then ATP[S] should produce ADP-like transients. However, in every cell tested ($n = 6$) the transients induced by ATP[S] were indistinguishable from those generated in response to ATP (results not shown).

A single cell was found which produced transients in response to $1.5 \mu\text{M}$ -ATP, but which failed to respond to ADP at concentrations up to $80 \mu\text{M}$ (Fig. 6).

Fig. 6. $[\text{Ca}^{2+}]_{\text{free}}$ transients induced in an aequorin-injected hepatocyte by ATP but which showed no response to ADP

A single hepatocyte responded to ATP with prolonged and oscillatory $[\text{Ca}^{2+}]_{\text{free}}$ transients, but did not respond to ADP at concentrations up to $80 \mu\text{M}$. Activity of the ADP solution was confirmed on another cell from the same population. Time constants: for transients, 0.8 s ; for resting Ca^{2+} levels, 8 s .

DISCUSSION

A previous study by Cobbold *et al.* (1988a) reported that the shape of $[\text{Ca}^{2+}]_{\text{free}}$ transients generated in a single cell by ADP and ATP are very different. ADP was found to increase transients of a much shorter duration than those induced by ATP. Here the ATP/ADP responses have been examined further. Consistent with the previous study, ADP invariably elicited transients of short duration. The response to extracellular ATP, however, was found to be more complex than previously reported. About 44% of hepatocytes responded with transients that included, in the same individual cell, a wide range in duration and a broad spectrum of waveforms. The variability was observed in the falling phase, which ranged from being brief and non-oscillatory to prolonged with superimposed secondary oscillations. These secondary oscillations have been recorded in fertilized mouse eggs (Cuthbertson & Cobbold, 1985). The remaining hepatocytes displayed transients with one or other of these two extremes of waveform only. It is notable that ATP is the only agonist found to induce a variable response; other agonists always elicited characteristic and consistent transient shapes (Wood *et al.*, 1987). If ATP and ADP are acting at the same receptor, one possible explanation for the different responses is that the agonists bind with very different affinities, and hence kinetics. However, vasopressin analogues with a 1000-fold range in affinity for the same V_1 receptor elicited transients with no obvious differences (Cobbold *et al.*, 1990a).

Benham & Tsien (1987) described a receptor-operated Ca^{2+} channel in smooth muscle activated by $1 \mu\text{M}$ -ATP, but only weakly affected by ADP at a 100-fold higher concentration. However, an influx of extracellular Ca^{2+} does not appear to be a contributory factor in the prolonged ATP response here, since chelating extracellular Ca^{2+} decreased the transient frequency, but did not cause shortening of the transients (Fig. 5).

It is unlikely that conversion of ATP into ADP by ectonucleotidases is responsible for the transients of short duration seen in 28% of hepatocytes examined. ATP was continually superfused over the cell surface, avoiding the possibility of a local accumulation of ADP. In addition, hydrolysis of ATP to ADP in the superfusate was not significant, since short transients were also recorded from cells superfused with medium containing ATP in a regenerating system [5 units of creatine

phosphokinase/ml + 5 mM-phosphocreatine (Sigma)]. Lin & Russell (1988) suggested that the previously purified ($\text{Ca}^{2+} + \text{Mg}^{2+}$)-dependent ATPase (Lin & Fain, 1984) is an ecto-ATPase and that its function may be to regulate the effect of extracellular ADP and ATP. If differential activation of this enzyme by ADP and ATP is responsible for the differences in the Ca^{2+} responses to these agonists, then ATP analogues not hydrolysed by the ecto-ATPase should produce transients more like those induced by ADP. However, the transients induced by ATP[S] were identical with those induced by ATP, arguing against a critical role for this enzyme in controlling transient duration. Consistent with the observation by Okajima *et al.* (1987) that methylated derivatives of ATP are effective through an inhibition of cyclic AMP accumulation rather than through effects on inositol lipid metabolism, no free- Ca^{2+} transient behaviour was recorded from cells stimulated with either adenosine 5'-[$\alpha\beta$ -methylene]triphosphate or adenosine 5'-[$\beta\gamma$ -methylene]triphosphate (both ATP analogues), at concentrations up to 60 μM (results not shown).

Although it is widely accepted that ADP and ATP act through the same receptor, designated ' P_{2Y} ' by Burnstock & Kennedy (1985), one explanation for the variability in the ATP response reported here is that two functionally distinct subtypes of the P_{2Y} purinoceptor exist on the hepatocyte-cell surface. One subtype may be activated exclusively by ATP, producing long oscillatory $[\text{Ca}^{2+}]_{\text{free}}$ transients (see, e.g., Fig. 2b); we designate these ' P_{2YL} '. A second subtype may be activated by ADP to produce transients of short duration, designated ' P_{2YS} '. The P_{2YS} receptor could also be stimulated by ATP, but activation always leads to transients of short duration (see, e.g., Fig. 2a). The ADP transients and short ATP transients (group 1) are then envisaged to arise from stimulation of a single receptor species. Under this dual-receptor scheme the response of a cell to ATP may depend on the proportion of receptors of each subset expressed on the cell surface. Other than in the total absence of receptors of both subsets, all cells will respond to ATP. We propose that the cells responding to ATP with transients of short duration lack receptors of the P_{2YL} subtype. A subpopulation of cells may be expected which possess only receptors of the P_{2YL} subtype; such cells would respond to ATP with prolonged transients and not respond to ADP. This interpretation is supported by the result from a single cell that was unresponsive to ADP up to 80 μM , but which generated prolonged transients with 1.5 μM ATP (Fig. 6). Fluorescence imaging of fields of cells may confirm the existence of such a subpopulation. The response of cells to ATP which included transients of variable duration (group 3) is postulated to result from stimulation of both P_{2YL} and P_{2YS} receptor subtypes. However, the mean duration of transients lasting under 15 s from this group is significantly greater than that of transients postulated to arise from P_{2YS} stimulation alone (ADP and group-1 ATP transients). In addition, the transients of longer than 15 s from this variable group are shorter than the group-2 transients postulated to arise from stimulation of the P_{2YL} subtype. These differences may be explicable in terms of an interaction between the two receptors, so that when both are activated there is a modification of the transients associated with each subtype alone. Decreasing extracellular Ca^{2+} caused prolonging of ATP-induced transients in three cells; this may reflect a change in the proportion of receptors being activated under these conditions. Other groups have reported ATP to be ineffective in causing changes in $[\text{Ca}^{2+}]_{\text{free}}$ in systems where ATP mobilizes intracellular Ca^{2+} stores (Gonzalez *et al.*, 1989a; Kuroki *et al.*, 1989). These findings could be interpreted as evidence for separate receptors for ATP and ADP. Recently, Gonzalez *et al.* (1989b) presented evidence for two separate ATP receptors responsible for

mediating cell permeabilization and Ca^{2+} mobilization in Swiss 3T6 fibroblasts.

We interpret the observed dependence of transient shape on agonist species to indicate that the oscillator lies close to the receptors, implying that the activity of phosphoinositidase C oscillates. The distinct falling phases of the $[\text{Ca}^{2+}]_{\text{free}}$ transients might arise from a differential response of receptor-related G (guanine-nucleotide-binding)-protein subtypes to the negative feedback element of the oscillator, putatively provided by protein kinase C (Woods *et al.*, 1987; Cobbold *et al.*, 1988c) or a stimulation of GTPase activity by activated phosphoinositidase C (Cuthbertson, 1989). In this hypothesis the difference in the $[\text{Ca}^{2+}]_{\text{free}}$ responses resulting from activation of the two P_{2Y} purinoceptor subtypes proposed here is due to distinct kinetics of inactivation of the G-proteins associated with them.

We suggest that the hepatocyte P_{2Y} purinoceptor may encompass two receptor subtypes. The ' P_{2YL} ' subtype induces $[\text{Ca}^{2+}]_{\text{free}}$ transients with a long falling phase and is responsive solely to ATP. The ' P_{2YS} ' subtype responds to either ATP or ADP to generate Ca^{2+} transients of short falling phase.

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