Adenosine 5′-[αβ-methylene]triphosphate potentiates the oscillatory cytosolic Ca^{2+} responses of hepatocytes to ATP, but not to ADP

C. Jane DIXON,* Peter H. COBBOLD and Anne K. GREEN
Department of Human Anatomy and Cell Biology, University of Liverpool, P.O. Box 147, Liverpool L69 3BX, U.K.

Single rat hepatocytes microinjected with aequorin generate oscillations in cytosolic free Ca^{2+} concentration ($[Ca^{2+}]_i$) when stimulated with agonists acting through the phosphoinositide signalling pathway. The duration of these transients has been shown to be characteristic of the stimulating agonist, so that transients of very different duration can be induced in the same individual hepatocyte by different agonists. In a previous study we have shown that ADP and ATP, which are believed to act through a single P_{2y}-purinoceptor species, elicit very different $[Ca^{2+}]_i$ responses in most of the hepatocytes. We have interpreted this as evidence for two Ca^{2+}-mobilizing purinoceptors. The methylated derivative of ATP, adenosine 5′-[αβ-methylene]-triphosphate (pp[CH_2]pA), is only a weak P_{2y}-purinoceptor agonist. When 100 μM pp[CH_2]pA was supplied to aequorin-injected hepatocytes, there was no effect on $[Ca^{2+}]_i$. However, 25 μM pp[CH_2]pA co-supplied with ATP causes a potentiation of the $[Ca^{2+}]_i$ response in most of the hepatocytes. The effect was specific for ATP-induced transients; $[Ca^{2+}]_i$ transients induced by other agonists, and importantly by ADP, were not affected by addition of pp[CH_2]pA. This further illustrates differences in the actions of ADP and ATP, strengthening the argument for separate receptors for these nucleotides.

INTRODUCTION

Oscillations in cytosolic free Ca^{2+} ($[Ca^{2+}]_i$) induced by agonists acting through the phosphoinositide signalling pathway have been demonstrated in many cell types including rat hepatocytes (Berridge, 1990; Cobbold et al., 1991). Single rat hepatocytes microinjected with the Ca^{2+}-sensitive photoprotein aequorin exhibit trains of repetitive $[Ca^{2+}]_i$ transients, each rising from a resting value of approx. 200 nM to a peak value of approx. 600 nM. The hepatocyte expresses receptors for a range of Ca^{2+}-mobilizing agonists, including ADP and ATP (Charest et al., 1985). The primary isolated rat hepatocyte therefore provides a useful system for the study of the phenomenon of ADP- and ATP-induced $[Ca^{2+}]_i$ oscillations. The duration of individual $[Ca^{2+}]_i$ transients has been previously shown to be dependent on the receptor species being activated; transients with different durations could be recorded from the same cell responding to agonists such as phenylephrine, vasopressin and angiotensin II (Woods et al., 1987). The variability results from differences in the rate of fall of $[Ca^{2+}]_i$ from its peak (Woods et al., 1987). ADP and ATP are believed to act to mobilize Ca^{2+} in rat hepatocytes through a single class of P_2-purinoceptor characterized as belonging to the P_{2y} subclass, as defined by the classification of Burnstock and Kennedy (1985). It is therefore interesting that, in a previous study, ADP and ATP were found to produce transients with very different durations in the majority of hepatocytes (Dixon et al., 1990). ADP consistently produced transients of short duration (approx 9 s). The response to ATP was more complex, and three different classes of cells 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 much longer duration (approx. 49 s); in the final group of cells (44%) ATP induced a variable response, with transients of different durations during a single response. We have explained these responses in terms of two receptors and postulate that one receptor, termed P_{2ys}, mediates transients of short duration and is activated by both ADP and ATP. It is proposed that the second receptor type, designated P_{2yl}, responds exclusively to ATP and results in the generation of transients of long duration. The generation of transients of mixed duration is postulated to result from stimulation of both P_{2ys}- and P_{2yl}-purinoceptor subtypes.

A further difference in the oscillatory $[Ca^{2+}]_i$ responses of single hepatocytes to ADP and ATP is described here. The slowly hydrolysable ATP analogue adenosine 5′-[αβ-methylene]ATP (pp[CH_2]pA) is ineffective in eliciting a $[Ca^{2+}]_i$ response when administered alone to single aequorin-injected hepatocytes. However, in combination with ATP, but not ADP, this analogue causes a potentiation of the $[Ca^{2+}]_i$ response in most hepatocytes.

MATERIALS AND METHODS

Hepatocytes were isolated from fed male Wistar-strain rats by collagenase digestion and prepared for microinjection with the Ca^{2+}-sensitive photoprotein aequorin as described previously (Cobbold and Lee, 1991). Experimental medium was Williams Medium E (Gibco), to which ADP, ATP and pp[CH_2]pA were added. All agonists were purchased from Sigma Chemical Co. Data acquisition and analysis was performed as described previously (Cobbold and Lee, 1991).

RESULTS

Single aequorin-injected hepatocytes responded to extracellular ADP and ATP in the range 1–5 μM, with a series of repetitive transients in $[Ca^{2+}]_i$, the frequency of which is dependent on agonist concentration. When applied to hepatocytes at concentrations up to 100 μM, pp[CH_2]pA alone had no effect on $[Ca^{2+}]_i$ and did not alter the subsequent cellular response to ADP ($n = 3$; Figure 1). However, when ADP was replaced by ATP, the

Abbreviations used: pp[CH_2]pA, adenosine 5′-[αβ-methylene]triphosphate; $[Ca^{2+}]_i$, cytosolic free Ca^{2+} concentration.
* To whom correspondence and reprint requests should be addressed.

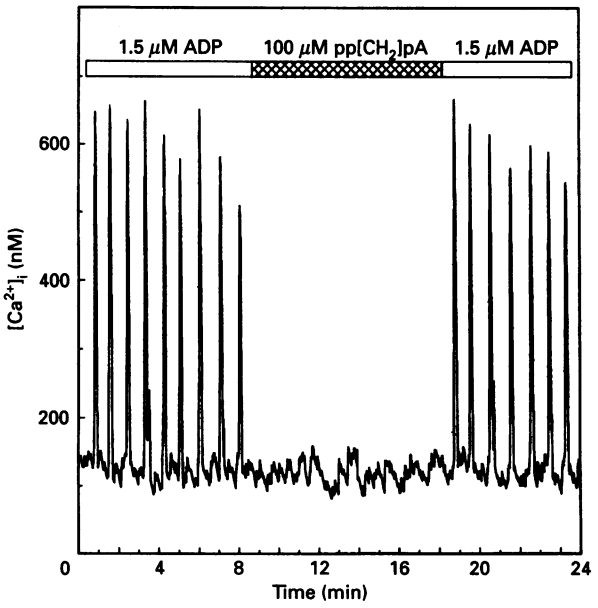

Figure 1 pp[CH_2]pA does not induce $[Ca^{2+}]_i$ transients in aequorin-injected hepatocytes

A single hepatocyte was microinjected with aequorin and superfused with Williams Medium E containing 1.5 μM ADP, producing transients of short duration. Application of 100 μM pp[CH_2]pA did not elicit a response. Time constants: for resting $[Ca^{2+}]_i$, 12 s; for transients, 1 s.

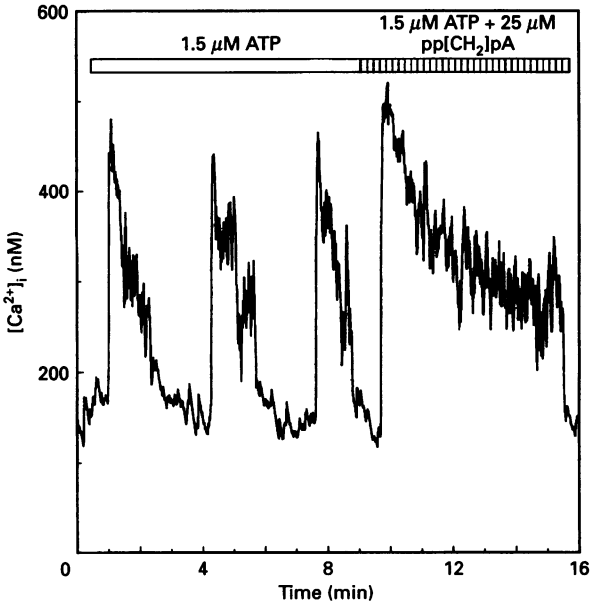

Figure 2 Conversion of ATP-induced $[Ca^{2+}]_i$ transients into a sustained rise of $[Ca^{2+}]_i$ through addition of pp[CH_2]pA

A single aequorin-injected hepatocyte responded to 1.5 μM ATP with transients of long duration. When the cell was additionally supplied with 25 μM pp[CH_2]pA at the time indicated, the transients were converted into a sustained rise in $[Ca^{2+}]_i$. Time constants: for resting $[Ca^{2+}]_i$, 12 s; for transients, 1 s.

first transient evoked by ATP after removal of pp[CH_2]pA was prolonged ($n = 3$; results not shown). At this point the cell may be exposed to a combination of ATP and pp[CH_2]pA in the perfusion chamber before wash-off of pp[CH_2]pA was complete. This prompted an investigation into the effect of applying a lower dose of pp[CH_2]pA in combination with ATP. When 25 μM pp[CH_2]pA was co-supplied to hepatocytes producing transients in response to ATP, the $[Ca^{2+}]_i$ response was enhanced in 10/16 cells. In 4 of these 10 cells addition of pp[CH_2]pA led to a sustained rise in $[Ca^{2+}]_i$, as illustrated in Figure 2. In the remaining 6 cells in which pp[CH_2]pA led to a potentiation of the $[Ca^{2+}]_i$ response, the result was the production of discrete transients in $[Ca^{2+}]_i$ with much longer time-course than those evoked by ATP alone (Figure 3). Table 1 relates the effect of pp[CH_2]pA to the shape of the initial transients induced by ATP. In contrast with the potentiation of the ATP-induced $[Ca^{2+}]_i$ changes, when cells

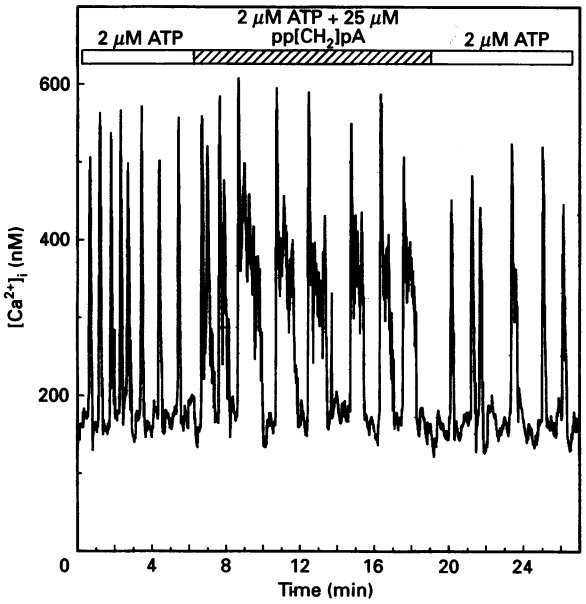

Figure 3 Prolongation of individual ATP-induced $[Ca^{2+}]_i$ transients by addition of pp[CH_2]pA

Transients of short duration were recorded from a single cell stimulated with 2 μM ATP. Addition of 25 μM pp[CH_2]pA at the time indicated led to a reversible prolongation of the transients. Time constants: for resting $[Ca^{2+}]_i$, 12 s; for transients, 1 s.

Table 1 Effect of pp[CH_2]pA on $[Ca^{2+}]_i$ transients induced by ATP

Three different classes of hepatocyte can be distinguished, based on the duration of transients induced by ATP. The effect of addition of pp[CH_2]pA is related here to the duration of the initial transients induced by ATP. The values given are numbers of individual aequorin-injected hepatocytes.

	Duration of ATP-induced $[Ca^{2+}]_i$ transients		
Effect of pp[CH_2]pA addition	Short	Variable	Long
Prolongation of transients	5	1	0
Sustained rise in $[Ca^{2+}]_i$	1	0	3
No effect	0	5	1

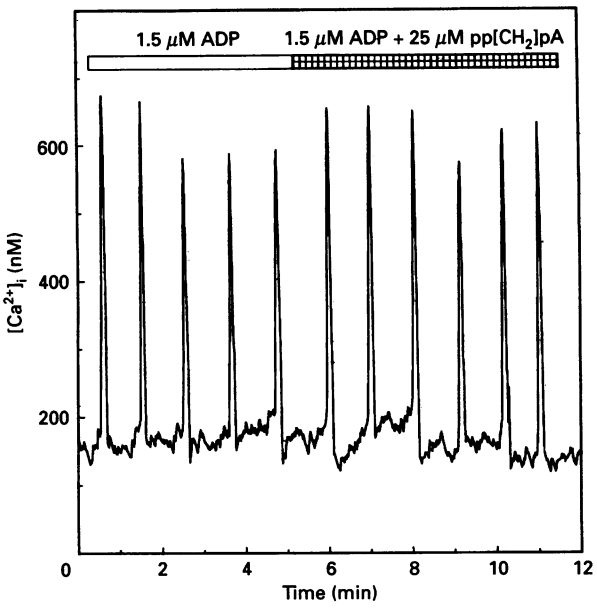

Figure 4 $[Ca^{2+}]_i$ transients induced by ADP are not altered by addition of pp[CH_2]pA

A single aequorin-injected hepatocyte was superfused with ADP and transients of short duration were recorded. pp[CH_2]pA was added at the time indicated. Time constants: for resting $[Ca^{2+}]_i$, 12 s; for transients, 1 s.

responding to ADP were exposed to pp[CH_2]pA, none of the cells showed any modulation of the $[Ca^{2+}]_i$ response ($n = 8$; Figure 4). Transients induced in hepatocytes by vasopressin ($n = 2$) or phenylephrine ($n = 4$) were similarly unaffected by co-addition of 25 μM pp[CH_2]pA (results not shown).

DISCUSSION

Addition of pp[CH_2]pA to hepatocytes already displaying $[Ca^{2+}]_i$ transients in response to ATP leads to a potentiation of the $[Ca^{2+}]_i$ response in most of the cells as described above (Figures 2 and 3). This effect cannot simply be explained by an increased agonist concentration, as pp[CH_2]pA itself at much higher concentrations is ineffective in eliciting a $[Ca^{2+}]_i$ response in hepatocytes (Figure 1). Moreover, in 5/16 cells, individual transients were converted from short to long duration by addition of pp[CH_2]pA (Figure 3), which is inconsistent with an increased agonist concentration acting at a single receptor site, which does not cause changes in transient shape, but only in frequency (Woods et al., 1986).

The methylated derivatives of ATP are the most potent agonists at the P_{2x} subclass of purinoceptor according to the classification of Burnstock and Kennedy (1985). Agonist interaction at P_{2x}-purinoceptors has been shown in a number of cell types to activate plasmalemmal Ca^{2+} channels (Kennedy, 1990). One possible explanation for the enhanced $[Ca^{2+}]_i$ response of hepatocytes co-stimulated with ATP and pp[CH_2]pA is that it reflects an influx of extracellular Ca^{2+} through such channels, although hepatocytes are not believed to possess P_{2x}-purinoceptors (Keppens and De Wulf, 1986). Okajima et al. (1987) have proposed that two subtypes of P_2-purinoceptor mediate the response of hepatocytes to purine nucleotides. One subtype is coupled to phospholipase C (termed P_{2p}) and the other mediates inhibition of cyclic AMP generation via a G_i-protein (termed P_{2i}). pp[CH_2]pA was found to be effective only at the P_{2i} subtype, which is consistent with the lack of effect of this analogue on $[Ca^{2+}]_i$. There is evidence for a role for G_i in agonist-stimulated Ca^{2+} influx in hepatocytes (Barritt and Hughes, 1991). It is therefore possible that increased activation of G_i resulting from the addition of the methylated ATP analogue prolongs the cellular $[Ca^{2+}]_i$ response to ATP. An inhibition by pp[CH_2]pA of the Ca^{2+} pump which restores $[Ca^{2+}]_i$ to resting levels between transients could also explain the observed prolongation of ATP-induced $[Ca^{2+}]_i$ transients. It must, however, be emphasized that pp[CH_2]pA specifically potentiates the $[Ca^{2+}]_i$ response in hepatocytes to ATP; $[Ca^{2+}]_i$ transients evoked by other agonists, and importantly by ADP, were unaffected by the co-addition of pp[CH_2]pA. It is therefore unlikely that any of the above mechanisms underlie the effect of pp[CH_2]pA, as a similar potentiation of the $[Ca^{2+}]_i$ response would be expected irrespective of agonist species.

ADP and ATP are believed to act through a single receptor species on the rat hepatocyte, which has been characterized as a P_{2y}-purinoceptor (Keppens and De Wulf, 1986). We have previously shown that in most cells ADP and ATP produce transients of very different time course, which we have interpreted as evidence for two separate receptors (Dixon et al., 1990). According to this proposal, transients of short duration induced by either ADP or ATP are mediated by a subtype of the P_{2y}-purinoceptor, the P_{2ys} subtype, distinct from that which mediates transients of long duration, the P_{2yl} subtype. It is possible that the application of pp[CH_2]pA selectively sensitizes the P_{2yl} purinoceptor. The effect then of administering pp[CH_2]pA coincidentally with ATP would be to potentiate the response mediated by the P_{2yl}-purinoceptor, mimicking an increase in agonist concentration specifically at this receptor. However, 5 of the 6 cells in which individual transients were prolonged by addition of pp[CH_2]pA belonged to the class producing transients of short duration in response to ATP alone (Table 1). To accommodate these findings, such cells are envisaged as expressing receptors of both subtypes, although the P_{2ys} subtype predominates. With the addition of pp[CH_2]pA, the $[Ca^{2+}]_i$ response will be determined by the sensitized P_{2yl} subtypes, so that transients of short duration, produced in response to ATP alone, will be replaced by transients of long duration. Increasing the concentration of pp[CH_2]pA may convert these long transients into a sustained rise in $[Ca^{2+}]_i$. In hepatocytes producing transients of long duration in response to ATP, the concentration range for transient production is very narrow. Above the upper limit, transients are replaced by a sustained rise in $[Ca^{2+}]_i$. If pp[CH_2]pA mimics an increase in agonist concentration through sensitization of the P_{2yl} receptor, conversion of individual transients of long duration into a sustained rise in $[Ca^{2+}]_i$ would be the expected outcome. This was observed in 3 of the 4 cells producing transients of long duration in response to ATP alone. The remaining cell showed no effect. It is curious that cells producing transients of variable duration in response to ATP were generally unaffected by the addition of pp[CH_2]pA.

The lack of effect of pp[CH_2]pA on ADP-induced transients may, alternatively, indicate the involvement of an ADP-specific receptor class distinct from that mediating transients of short duration in response to ATP.

The mechanism by which pp[CH_2]pA acts to modify the $[Ca^{2+}]_i$ response induced only by ATP and not by other agonists such as ADP, vasopressin and phenylephrine is unclear. Nonetheless, the data presented here reveal a further difference between ADP and ATP, supporting the idea of separate Ca^{2+}-mobilizing receptors for these nucleotides on hepatocytes. Importantly for

this interpretation, several systems have been documented in which ADP is ineffective in causing changes in $[Ca^{2+}]_i$, but in which ATP mobilizes intracellular Ca^{2+} stores (Dubyak and De Young, 1985; Cockcroft and Stutchfield, 1989; Gonzalez et al., 1989a,b; Kuroki et al., 1989; Brown et al., 1991; Dulon et al., 1991). The sub-classification of P_2-purinoceptors is based upon rank order potency studies on smooth-muscle preparations (Burnstock and Kennedy, 1985). Anomalous potency series have, however, been reported for smooth-muscle preparations (Burnstock and Warland, 1987; Tawada et al., 1987; Wiklund and Gustafsson, 1988; Bailey and Hourani, 1990). Among non-excitable cells the situation is equally confusing, with many cell types displaying anomalous series, prompting investigators to propose the involvement of novel purinoceptor subtypes (Fine et al., 1989; Gonzalez et al., 1989b; Allsup and Boarder, 1990; Davidson et al., 1990; Murphy and Tiffany, 1990; Merritt and Moores, 1991; Martin, 1992). Indeed, Keppens and De Wulf (1991) have proposed that hepatocytes possess additional P_2-purinoceptors distinct from those of the P_{2y} subclass, based on differences in the actions of ATP and 2-methylthio-ATP. The variability in reported potency series may in some part result from activation of separate receptors for ADP and ATP. Platelets express receptors which are responsive solely to ADP (P_{2t}-purinoceptors; Gordon, 1986). In addition to mediating inhibition of adenylate cyclase, P_{2t}-purinoceptors increase $[Ca^{2+}]_i$ by evoking Ca^{2+} release from intracellular stores and influx of extracellular Ca^{2+} (Sage et al., 1990). This receptor may therefore be related to that proposed here to mediate the effects of ADP in hepatocytes.

The specificity of the effect of pp[CH_2]pA to prolong transients induced only by ATP provides yet another example of agonist-specificity in the hepatocyte $[Ca^{2+}]_i$ oscillatory mechanism (Cobbold et al. 1991). This ATP analogue may prove to be a useful tool for probing the separate identities of the putative receptors responsible for mediating the effects of ADP and ATP.

We are grateful for funding from the Wellcome Trust.

Received 17 February 1993/30 March 1993; accepted 1 April 1993

REFERENCES

Allsup, D. J. and Boarder, M. R. (1990) Mol. Pharmacol. **38**, 84–91
Bailey, S. J., and Hourani, S. M. O. (1990) Br. J. Pharmacol. **100**, 753–756
Barritt, G. J. and Hughes, B. P. (1991) Cell. Signalling **3**, 283–292
Berridge, M. J. (1990) J. Biol. Chem. **265**, 9583–9586
Brown, H. A., Lazarowski, E. R., Boucher, R. C. and Harden, T. K. (1991) Mol. Pharmacol. **40**, 648–655
Burnstock, G. and Kennedy, C. (1985) Gen. Pharmacol. **16**, 433–440
Burnstock, G. and Warland, J. J. L. (1987) Br. J. Pharmacol. **90**, 383–391
Charest, R., Blackmore, P. F. and Exton, J. H. (1985) J. Biol. Chem. **260**, 15789–15794
Cobbold, P. H. and Lee, J. A. C. (1991) in Techniques in Calcium Research (McCormack, J. G. and Cobbold, P. H., eds.), pp. 55–81, I. R. L. Press, Oxford
Cobbold, P. H., Sanchez-Bueno, A. and Dixon, C. J. (1991) Cell Calcium **12**, 87–95
Cockcroft, S. and Stutchfield, J. (1989) Biochem. J. **263**, 715–723
Davidson, J. S., Wakefield, I. K., Sohnius, U., Van der Merwe, P. A. and Millar, R. P. (1990) Endocrinology (Baltimore) **126**, 80–87
Dixon, C. J., Woods, N. M., Cuthbertson, K. S. R. and Cobbold, P. H. (1990) Biochem. J. **269**, 499–502
Dubyak, G. R. and De Young, M. B. (1985) J. Biol. Chem. **260**, 10653–10661
Dulon, D., Mollard P. and Aran, J. M. (1991) Neuro Report **2**, 69–72
Fine J., Cole, P. and Davidson, J. S. (1989) Biochem. J. **263**, 371–376
Gonzalez, F. A., Rozengurt, E. and Heppel, L. A. (1989a) Proc. Natl. Acad. Sci. U.S.A. **86**, 4530–4534
Gonzalez, F. A., Alfonzo, G. A., Toro, J. R. and Heppel, L. A. (1989b) J. Cell. Physiol. **141**, 606–617
Gordon, J. L. (1986) Biochem. J. **233**, 309–319
Kennedy, C. (1990) Arch. Int. Pharmacodyn. **303**, 30–50
Keppens, S. and De Wulf, H. (1986) Biochem. J. **240**, 367–371
Keppens, S. and De Wulf, H. (1991) Br. J. Pharmacol. **104**, 301–304
Kuroki, M., Takeshige, K. and Minakami, S. (1989) Biochim. Biophys. Acta **1012**, 103–106
Martin, S. C. (1992) J. Membr. Biol. **125**, 243–253
Merritt, J. E. and Moores, K. E. (1991) Cell. Signalling **3**, 243–249
Murphy, P. M. and Tiffany, H. L. (1990) J. Biol. Chem. **265**, 11615–11621
Okajima, F., Tokumitsu, Y., Kondo, Y. and Ui, M. (1987) J. Biol. Chem. **262**, 13483–13490
Sage, S. O., Reast, R. and Rink, T. J. (1990) Biochem. J. **265**, 675–680
Tawada, Y., Furukawa, K.-I. and Shigekawa, M. (1987) J. Biochem. (Tokyo) **102**, 1499–1509
Wiklund, N. P. and Gustafsson, L. E. (1988) Eur. J. Pharmacol. **148**, 361–370
Woods, N. M., Cuthbertson, K. S. R. and Cobbold, P. H. (1986) Nature (London) **319**, 600–602
Woods, N. M., Cuthbertson, K. S. R. and Cobbold, P. H. (1987) Cell Calcium **8**, 79–100