Biochem. J. (1991) 273, 799–802 (Printed in Great Britain)

Frequency and amplitude enhancement of calcium transients by cyclic AMP in hepatocytes

Christof SCHÖFL,*† Antonio SANCHEZ-BUENO, Georg BRABANT,* Peter H. COBBOLD and K. S. Roy CUTHBERTSON

Department of Human Anatomy and Cell Biology, University of Liverpool, P.O. Box 147, Liverpool L69 3BX, U.K.

Interactions between signalling pathways such as the cyclic AMP and the Ca^{2+}/phosphatidylinositol pathway may occur and be of major relevance in the regulation of cell function. We demonstrate here that cyclic-AMP-dependent mechanisms cause a marked increase in frequency and peak free Ca^{2+} of α_1-receptor-induced Ca^{2+} transients in single hepatocytes and lower the threshold for α_1-receptor agonists. Adrenaline at low physiological concentrations generates α_1-receptor-induced Ca^{2+} transients, which requires activation of the β_2-receptor signalling pathway. We conclude that an interaction between the α_1-receptor signalling pathway and cyclic-AMP-dependent mechanisms activated by β_2-receptor occupation is crucial to elicit a complete adrenergic response to adrenaline at physiological concentrations in rat hepatocytes.

INTRODUCTION

In many cell types two major systems to transduce signals across the cell membrane, the cyclic AMP and the Ca^{2+}/phosphatidylinositol pathway, co-exist [1]. Cells under physiological conditions are exposed to a variety of hormones activating different signalling pathways at the same time. Interactions between these pathways are therefore of major importance in the regulation of cell function. In the liver the release of glucose is stimulated by both cyclic AMP and Ca^{2+} [2]. Catecholamines such as adrenaline and noradrenaline act on two different adrenoceptors, the α_1-receptor and the β_2-receptor, to stimulate glucose release from the liver [2]. The α_1-receptor activates the phosphatidylinositol pathway, thereby causing repetitive Ca^{2+} transients in single liver cells [3], whereas the β_2-receptor stimulates cyclic AMP formation. Adrenaline, and to a lesser degree noradrenaline, activate both adrenoceptor types. Interactions between the signalling pathways may occur and may be important in the adrenergic regulation of liver glucose production. Results from previous reports using cell populations suggest a synergistic effect of cyclic-AMP-mediated mechanisms on hepatic Ca^{2+} mobilization [4–7].

MATERIALS AND METHODS

Single hepatocytes were isolated and injected with the photoprotein aequorin as previously [3,8], and the free-Ca^{2+} changes were recorded by photomultiplier measurements of aequorin luminescence. The experimental medium was Williams' Medium E (Flow Laboratories) gassed with CO_2/air (1:19) at 37 °C. All substances used were added to this medium. Adrenaline, phenylephrine, phentolamine, propranolol and dibutyryl cyclic AMP were from Sigma, Poole, Dorset, U.K., and forskolin was from Calbiochem, Nottingham, U.K.

RESULTS

As shown in Figs. 1 and 2, the α_1-agonist phenylephrine induced repetitive Ca^{2+} transients, as reported previously [3,8]. Dibutyryl cyclic AMP, a cell-permeant analogue of cyclic AMP, at concentrations of 1–100 μM increased frequency and peak free Ca^{2+} of the transients generated by phenylephrine in 9 out of 9 cells (Fig. 1). The concentrations of dibutyryl cyclic AMP required to induce a maximum response varied from cell to cell, and like all lipophilic substances dibutyryl cyclic AMP could be expected to accumulate in single cells during the perfusion. Therefore no dose–response curve was obtained. The frequency of the free-Ca^{2+} oscillations induced by concentrations of phenylephrine that were just above threshold were increased by dibutyryl cyclic AMP (1–100 μM) to the maximum stable frequency induced by α_1-adrenergic stimulation (approx. 2 min^{-1}). Typically 3–4-fold increases in frequency were obtained. Dibutyryl cyclic AMP increased the average peak free calcium by up to 50% (e.g. from 780 to 1220 nM; $P = 0.0003$). Subtle (approx. 40%) but statistically highly significant ($P < 0.00001$) increases in spike length (defined here as residence time of Ca^{2+} above 300 nM) were induced by dibutyryl cyclic AMP [e.g. from 5.0 ± 0.6 s ($n = 29$) to 7.2 ± 1.6 s ($n = 17$)]. The combined effect of increased transient amplitude and duration was a 2–3-fold increase in aequorin counts per spike, which indicates the likely scale of the relative increase per transient in the activation of native Ca^{2+}-effector proteins resulting from maximal activation of protein kinase A. Dibutyryl cyclic AMP (100 μM) given alone had no effect on the cytosolic free Ca^{2+} (results not shown).

To test whether intracellularly formed cyclic AMP had the same effect, the action of forskolin, which directly activates adenylate cyclase, on phenylephrine-induced Ca^{2+} transients was examined. As shown in Figs. 2(*a*) and 2(*b*), forskolin, like dibutyryl cyclic AMP, increased frequency and peak free Ca^{2+} of the transients. This was observed at concentrations of 1 and 10 μM. These concentrations are known to be sufficient to stimulate adenylate cyclase. This increase in frequency and peak free Ca^{2+} was slowly reversible over several minutes after forskolin had been washed off (Fig. 2*b*). Forskolin (1 μM) could increase transient frequency approx. 2-fold to near maximum (approx. 1.5 min^{-1}). Forskolin induced relative increases in transient amplitude (10–20%), duration (15–40%) and total aequorin counts (50%), which were somewhat less than those induced by dibutyryl cyclic AMP. Where this could be resolved from the data, the rate of free-Ca^{2+} rise during the transient upstroke (estimated by linear regression) was also increased by

* Present address: Abteilung für Klinische Endokrinologie, Medizinische Hochschule Hannover, Postfach 61 01 80, 3000 Hannover 61, Federal Republic of Germany.
† To whom correspondence and reprint requests should be addressed, at the Hannover address.

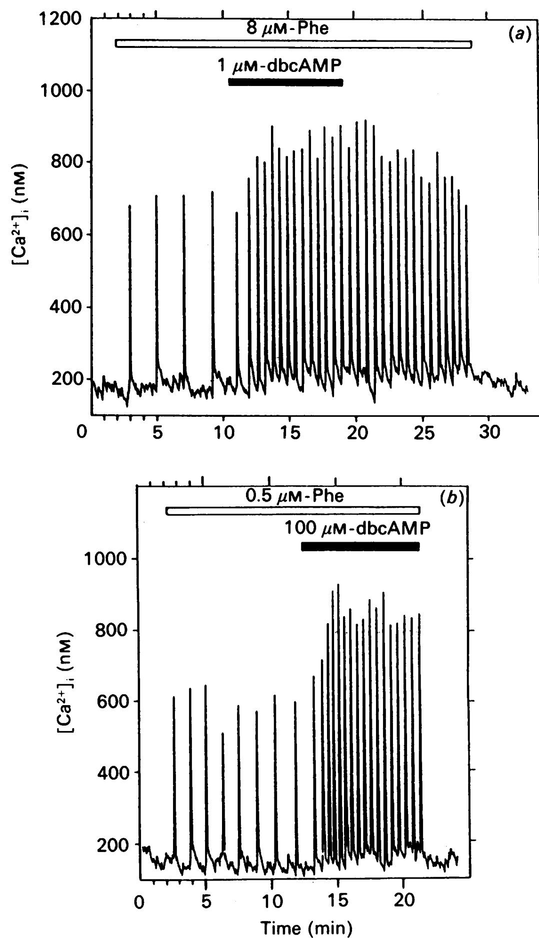

Fig. 1. Aequorin signals from single rat hepatocytes calibrated as free Ca^{2+} (nM) exposed to phenylephrine (Phe) and dibutyryl cyclic AMP (dbcAMP)

Dibutyryl cyclic AMP at 1 μM (*a*) and 100 μM (*b*) increased frequency and peak height of phenylephrine-induced Ca^{2+} transients. Representative tracings are shown of 3 (*a*) or 5 (*b*) independent experiments. Duration of exposure to phenylephrine or dibutyryl cyclic AMP is indicated by the horizontal bars. Time constants for the plot were 10 s for resting levels and 1 s for the transients.

1 μM-forskolin [e.g. from 379 ± 70 nM/s ($n = 14$) to 540 ± 87 nM/s ($n = 12$); $P = 0.00003$]. Forskolin (1–50 μM) given alone had no effect on cytosolic free Ca^{2+} (results not shown). This contrasts with some previous reports where dibutyryl cyclic AMP, forskolin, or agonists which stimulate cyclic AMP formation, caused a moderate increase in cytosolic Ca^{2+} in suspensions of liver cells [9–11].

Since dibutyryl cyclic AMP and forskolin clearly potentiated the α_1-adrenoceptor-induced Ca^{2+} response, a possible influence of cyclic-AMP-mediated mechanisms on the threshold for phenylephrine-induced Ca^{2+} transients was investigated. When cells were perfused with sub-threshold concentrations of phenylephrine (0.1–0.5 μM), confirmed by the absence of Ca^{2+} transients for a 10 min perfusion period, the addition of forskolin (10 μM) sensitized the α_1-receptor pathway sufficiently to initiate the production of Ca^{2+} transients (Fig. 2*c*). This was reversible when forskolin was removed from the perfusion medium (Fig. 2*c*).

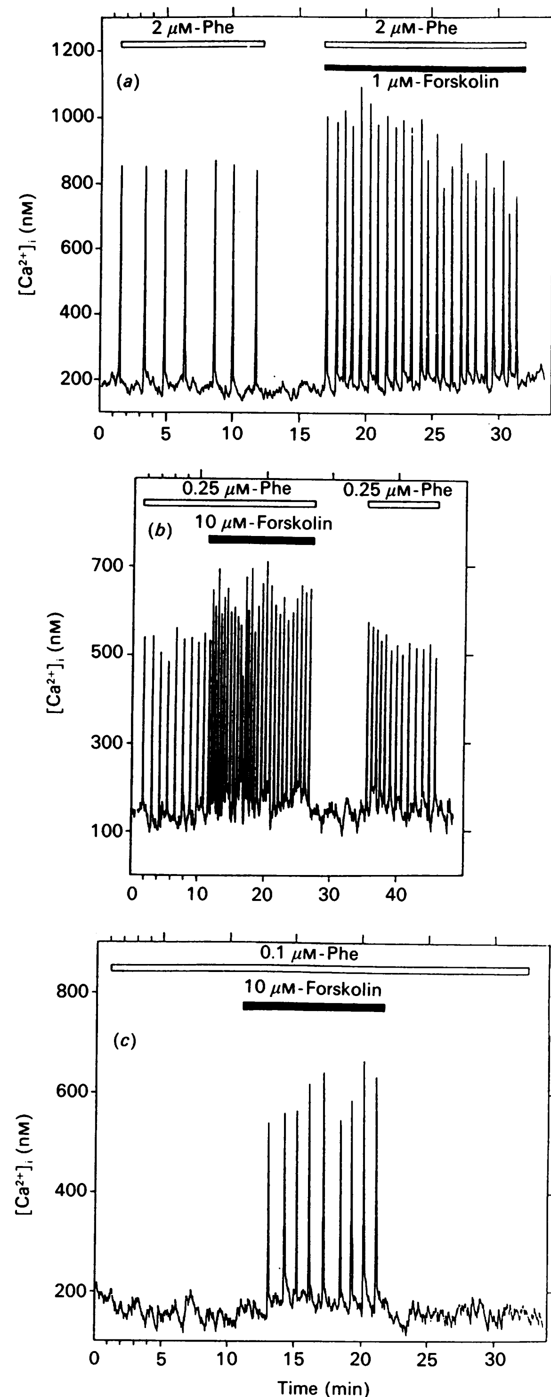

Fig. 2. Aequorin signals plotted as free Ca^{2+} (nM) from single hepatocytes exposed to phenylephrine (Phe) and various concentrations of forskolin

Time constants were as for Fig. 1. (*a*, *b*) Forskolin caused an increase in frequency and in the peak height of each phenylephrine-induced Ca^{2+} transient. This was reversible, as shown in (*b*). (*c*) A sub-threshold concentration of phenylephrine did not induce any Ca^{2+} spikes. In the presence of forskolin, however, the same concentration was sufficient to elicit Ca^{2+} transients. This was reversed by removing forskolin from the perfusion medium. Representative tracings are shown from 3 (*a*), 6 (*b*) and 6 (*c*) independent experiments.

The physiological catecholamine adrenaline caused Ca^{2+} transients similar to those with phenylephrine, but was more potent (Fig. 3). The frequency of the transients was dependent on the concentration of adrenaline. The threshold concentrations for adrenaline-induced Ca^{2+} transients were 5–10 nM. Higher adrenaline concentrations (50–100 nM) produced transients every 20–30 s. These concentrations are in the physiological range

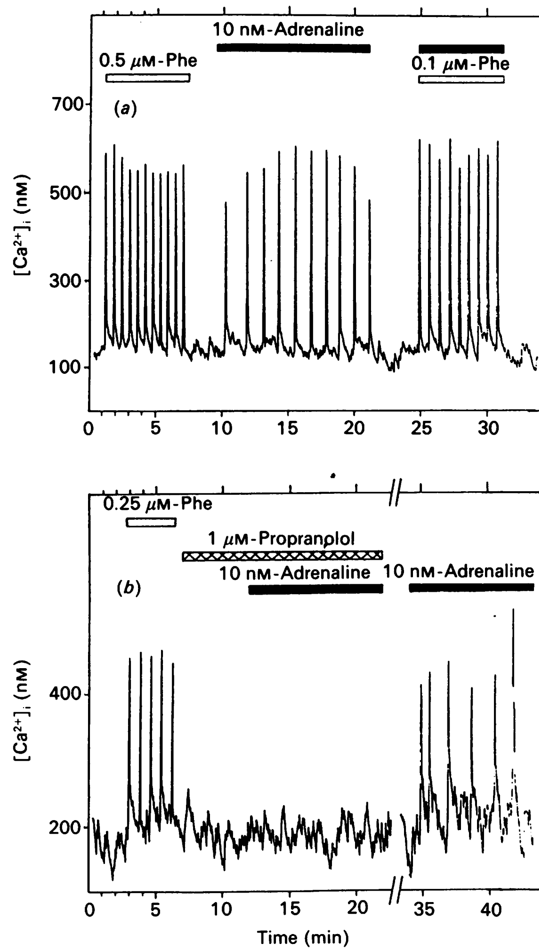

Fig. 3. Aequorin signals from single rat hepatocytes plotted as free Ca^{2+} (nM) exposed to phenylephrine and adrenaline in presence or absence of propranolol

(*a*) Adrenaline at a physiological concentration (10 nM) caused Ca^{2+} transients which were identical with phenylephrine (Phe)-induced ones. The addition of a sub-threshold concentration of phenylephrine (0.1 μM) to adrenaline (10 nM) further increased the frequency of the Ca^{2+} transients. (*b*) β-receptor blockade with propranolol (1 μM) prevented adrenaline from having any effect on cytosolic Ca^{2+}. After propranolol had been washed off for several minutes, a low concentration of adrenaline was sufficient to cause Ca^{2+} transients. Representative tracings of 8 (*a*) or 7 (*b*) independent experiments are shown (time constants were as in Figs. 1 and 2).

occurring under stress conditions, when glucose release from the liver is stimulated by catecholamines such as adrenaline [12]. The transient durations were indistinguishable from those of phenylephrine-induced Ca^{2+} transients. Ca^{2+} transients caused by adrenaline were blocked by the α-adrenoceptor blocker phentolamine, indicating that adrenaline induced Ca^{2+} transients by activating the α_1-adrenoceptor pathway in hepatocytes (results not shown). Furthermore, sub-threshold concentrations of phenylephrine (0.1 μM) were effective at speeding up the adrenaline-induced Ca^{2+} transients (Fig. 3*a*). Since we had demonstrated that cyclic-AMP-mediated mechanisms potentiated and sensitized the α_1-adrenoceptor pathway, we used propranolol, a β-receptor blocker, to investigate the potential role of the β_2-receptor in the activation of the α_1-receptor pathway by adrenaline at low agonist concentrations. As shown in Fig. 3(*b*), in the presence of propranolol (1 μM) a low concentration of adrenaline did not cause any Ca^{2+} transients. In contrast, high concentrations of adrenaline (100–500 nM) were still effective in producing free-Ca^{2+} oscillations under these conditions (results not shown). After propranolol had been washed off, the same low concentration of adrenaline induced Ca^{2+} transients (Fig. 3*b*). This indicates that adrenaline at a low physiologically occurring concentration (10 nM) only activates the α_1-receptor pathway when the β_2-receptor pathway is stimulated simultaneously. The results above suggest that this involves a cyclic-AMP-dependent mechanism.

DISCUSSION

It is now widely accepted that frequency-modulated repetitive Ca^{2+} transients rather than amplitude-modulated sustained increases in cytosolic Ca^{2+} constitute the intracellular signal activated by many Ca^{2+}-mobilizing hormones [13]. Previous reports from cell populations suggested a synergistic interaction between cyclic-AMP-dependent mechanisms and Ca^{2+}-mobilizing hormones in hepatocytes [4–7,14,15]. Here we demonstrate for the first time that cyclic-AMP-dependent mechanisms potentiate the intracellular Ca^{2+} signal in response to an α_1-adrenoceptor agonist by increasing both the frequency of Ca^{2+} transients and the peak free Ca^{2+} of each transient in individual hepatocytes. There are several points of interaction which could possibly lead to an increase in frequency and peak free Ca^{2+} of the transients. We suggest that phosphorylation of the α_1-adrenoceptor by cyclic-AMP-dependent protein kinase A leads to a higher affinity or efficiency of the receptor, and hence faster pulses at sub-maximal agonist concentrations and a lower threshold for agonists to generate Ca^{2+} transients. Likewise, cyclic-AMP-dependent phosphorylation of the Ins(1,4,5)P_3 receptor could increase its affinity or efficiency, leading with the same amount of Ins(1,4,5)P_3 generated during each transient to an increase in the amplitude of the Ca^{2+} transients. Indeed, both, α_1-adrenoceptors [16] and Ins(1,4,5)P_3 receptors [17] possess sites for phosphorylation by cyclic-AMP-dependent protein kinase A. It has been shown that binding of α_1-agonists to plasma membranes of liver cells is increased by cyclic-AMP-dependent mechanisms, suggesting that this is a major site of interaction between the two signalling pathways [4]. Phosphorylation of the Ins(1,4,5)P_3 receptor has been shown to inhibit, rather than potentiate, Ins-(1,4,5)P_3-induced Ca^{2+} release in the cerebellum [18]. However, organization, control and distribution of Ins(1,4,5)P_3 receptors may well be different in other cell types. Whether cyclic-AMP-dependent phosphorylation of Ins(1,4,5)P_3 receptors potentiates Ins(1,4,5)P_3-induced Ca^{2+} mobilization in hepatocytes remains to be shown. Such potentiation might be by a change in threshold or kinetics as well as by a change in affinity or activity.

According to the 'two-pool' model [13,19,20], cytosolic free-Ca^{2+} oscillations are generated by an Ins(1,4,5)P_3-independent Ca^{2+} pool 'overloaded' by release of free Ca^{2+} from the Ins(1,4,5)P_3-dependent pool. Therefore, an increase in Ins(1,4,5)P_3 formation, or a potentiation of its action by phosphorylation of its receptor or the associated Ca^{2+} channel, would result in an increase in the frequency of Ca^{2+} transients indistinguishable from that induced by an increase in agonist concentration. However, changes in the peak height and the kinetics of each transient induced by cyclic-AMP-dependent mechanisms could only be explained by changes in characteristics of the putative Ins(1,4,5)P_3-independent pool.

According to models for the generation of Ca^{2+} transients published by this group [8,13,21], phosphorylation, and thereby increasing the activity of G-proteins (guanine-nucleotide-binding proteins) and phospholipase C, by protein kinase A could also increase the frequency, amplitude and duration of free-Ca^{2+} transients, for example by decreasing the rate of G-protein inactivation, by increasing the interaction between G-proteins

and phospholipase C, or by increasing the activity of phospholipase C itself. In experiments with liver cells, using AlF_3 to stimulate G-proteins directly, cyclic-AMP-dependent mechanisms have been reported to potentiate AlF_3-induced Ca^{2+} mobilization as well as Ins(1,4,5)P_3 formation [22]. This points to a site of interaction proximal to the hydrolysis of phosphatidylinositol 4,5-bisphosphate at the level of G-proteins or phospholipase C. However, there is some controversy as to whether in response to agonist stimulation the formation of Ins(1,4,5)P_3, or just cytosolic Ca^{2+}, is potentiated by cyclic-AMP-dependent mechanisms [4–6,14,15,22]. Establishing the sites of cyclic AMP action will provide further insight in the mechanism involved in the generation of Ca^{2+} transients.

On hepatocytes there are at least two adrenoceptor subtypes present coupled to the cyclic AMP or Ca^{2+}-signalling pathway. Both receptors stimulate the release of glucose. In this study we demonstrated that at low physiological concentrations adrenaline generated α_1-receptor-induced Ca^{2+} transients in individual hepatocytes. This required the activation of the cyclic-AMP-forming β_2-receptor pathway, indicating that an interaction between the cyclic AMP and the Ca^{2+}-signalling pathway is crucial for the adrenergic regulation of hepatocyte function under physiological conditions at a single-cell level. The underlying mechanisms appear to be cyclic-AMP-mediated, and may involve the same sites of interaction as discussed above. Furthermore, the present results clearly demonstrate that the two signalling pathways in single hepatocytes which are responsible for the stimulation of glucose release from the liver in response to adrenergic stimulation synergize not only in the activation of phosphorylase *b* kinase [2] but also in the transmembrane Ca^{2+} signal. Whether similar synergistic interactions between two signalling pathways are required for the regulation of cell functions at physiological agonist concentrations in other cell types where different adrenoceptor subtypes are present and synergize in their action [2] or in other hormonal systems remains to be shown.

We thank The Wellcome Trust, the Royal Society and the Basque Government for funding. C. S. is grateful for the support of Professor R.-D. Hesch and Professor A. von zur Mühlen of Abteilung für Klinische Endokrinologie, Medizinische Hochschule Hannover, Germany.

REFERENCES

1. Nishizuka, Y. (1986) Science **233**, 305–312
2. Exton, J. H. (1985) Am. J. Physiol. **248**, E633–E647
3. Woods, N. M., Cuthbertson, K. S. R. & Cobbold, P. H. (1986) Nature (London) **319**, 600–602
4. Morgan, N. G., Charest, R., Blackmore, P. F. & Exton, J. H. (1984) Proc. Natl. Acad. Sci. U.S.A. **81**, 4208–4212
5. Poggioli, J., Mauger, J.-P. & Claret, M. (1986) Biochem. J. **235**, 663–669
6. Altin, J. G. & Bygrave, F. L. (1986) Biochem. J. **238**, 653–661
7. Cocks, T. M., Jenkinson, D. H. & Koller, K. (1984) Br. J. Pharmacol. **83**, 281–291
8. Woods, N. M., Cuthbertson, K. S. R. & Cobbold, P. H. (1987) Cell Calcium **9**, 79–100
9. Charest, R., Blackmore, P. F., Berthon, B. & Exton, J. H. (1983) J. Biol. Chem. **258**, 8769–8773
10. Combettes, L., Berthon, B., Binet, A. & Claret, M. (1986) Biochem. J. **237**, 675–683
11. Sistare, F. D., Picking, R. A. & Haynes, R. C., Jr. (1985) J. Biol. Chem. **260**, 12744–12747
12. Clutter, W. E., Bier, D. M., Shah, S. D. & Cryer, P. E. (1980) J. Clin. Invest. **66**, 94–101
13. Berridge, M. J., Cobbold, P. H. & Cuthbertson, K. S. R. (1988) Philos. Trans. R. Soc. London B **320**, 325–343
14. Burgess, G. M., Dooley, R. K., McKinney, J. S., Nanberg, E. & Putney, J. W., Jr. (1986) Mol. Pharmacol. **30**, 315–320
15. Pittner, R. A. & Fain, J. N. (1989) Biochem. J. **257**, 455–460
16. Bouvier, M., Leeb-Lundberg, L. M. F., Benovic, J. L., Caron, M. G. & Lefkowitz, R. J. (1987) J. Biol. Chem. **262**, 3106–3113
17. Furuichi, T., Yoshikawa, S., Miyakawi, A., Wada, K., Maeda, N. & Mikoshiba, K. (1989) Nature (London) **342**, 32–38
18. Supattapone, S., Danoff, S. K., Theibert, A., Joseph, S. K., Steiner, I. & Snyder, S. H. (1988) Proc. Natl. Acad. Sci. U.S.A. **85**, 8747–8750
19. Berridge, M. J. (1990) J. Biol. Chem. **265**, 9583–9586
20. Goldbeter, A., Dupont, G. & Berridge, M. J. (1990) Proc. Natl. Acad. Sci. U.S.A. **87**, 1461–1465
21. Cuthbertson, K. S. R. (1989) in Cell to Cell Signalling: From Experimental to Theoretical Models (Goldbeter, A., ed.), pp. 435–447, Academic Press, London
22. Blackmore, P. F. & Exton, J. H. (1986) J. Biol. Chem. **261**, 11056–11063

Received 17 October 1990/15 November 1990; accepted 4 December 1990