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Involvement of calyculin A inhibitable protein phosphatases in the cyclic AMP signal transduction pathway of mouse corticotroph tumour (AtT20) cells

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1 The role of non-calcineurin protein phosphatases in the cyclic AMP signal transduction pathway was examined in mouse pituitary corticotroph tumour (AtT20) cells.

2 Blockers of protein phosphatases, calyculin A and okadaic acid, were applied in AtT20 cells depleted of rapidly mobilizable pools of intracellular calcium and activated by various cyclic AMP generating agonists. Inhibitors of cyclic nucleotide phosphodiesterases were present throughout. The accumulation of cyclic AMP was monitored by radioimmunoassay, phosphodiesterase activity in cell homogenates was measured by radiometric assay.

3 Neither calyculin A nor okadaic acid altered basal cyclic AMP levels but cyclic AMP formation induced by 41 amino acid residue corticotrophin releasing-factor (CRF) was strongly inhibited (up to 80%). 1-Norokadaone was inactive. Similar data were also obtained when isoprenaline or pituitary adenylate cyclase activating peptide$_{1-38}$ were used as agonists.

4 Pertussis toxin did not modify the inhibition of CRF-induced cyclic AMP production by calyculin A.

5 Pretreatment with calyculin A completely prevented the stimulation of cyclic AMP formation by cholera toxin even in the presence of 0.5 mM isobutylmethylxanthine (IBMX) and 0.1 mM rolipram. Cholera toxin mediated ADP-ribosylation of the 45K and 52K molecular weight $G_{s\alpha}$ isoforms in membranes from calyculin A-pretreated cells was enhanced to 150–200% when compared with controls.

6 Cholera toxin-induced cyclic AMP was reduced by calyculin A within 10 min when calyculin A was applied after a 90 min pretreatment with cholera toxin. Under these conditions the effect of calyculin A could be blocked by the combination of 0.5 mM IBMX and 0.1 mM rolipram, but not by 0.5 mM IBMX alone.

7 Phosphodiesterase activity in AtT20 cell homogenates showed a significant, 2.7 fold increase after treatment with calyculin A. In control cells phosphodiesterase activity was blocked by 80% in the presence of IBMX (0.5 mM), or IBMX plus rolipram (0.1 mM). In calyculin A-treated cells phosphodiesterase activity was also strongly inhibited by IBMX, but because of the stimulating effect of calyculin A, the activity remaining was still 55% of that found in control homogenates. This activity was reduced to 5% of control by using IBMX and rolipram in combination. Assay of phosphodiesterase in Ca^{2+} free conditions showed that calyculin A markedly increases the activity of rolipram sensitive (type 4) phosphodiesterase.

8 Taken together, blockers of protein phosphatases (PPases) impaired signal transduction through Gs-mediated pathways and activated cyclic AMP degrading phosphodiesterase(s), indicating that PPases 1 and/or 2A are essential for agonist-mediated regulation of cyclic AMP levels in AtT20 cells, and are thus important in maintaining the secretory phenotype of the cells.

Keywords: Protein phosphatase; calyculin A; okadaic acid; G proteins; corticotrophin releasing factor; pituitary tumour; phosphodiesterase; rolipram; cyclic AMP; FK506

Introduction

Protein phosphatases (PPase) are essential elements of cellular control (Cohen, 1989). However, knowledge about their role in the generation of the cyclic AMP response to agonists is largely limited to the control of cell surface receptor recycling (Sibley *et al*., 1987; Pitcher *et al*., 1995). It is generally accepted that in addition to receptors, G proteins (Houslay, 1994), as well as adenylyl cyclases (Jacobowitz & Iyengar, 1994; Kawabe *et al*., 1994) are substrates for protein kinases. Hence the state of phosphorylation of these signal-transducing proteins is likely to be influenced by protein phosphatases. Previous work in S49 lymphoma cells (Clark *et al*., 1993) and AtT20 corticotrope tumour cells (Koch & Lutz-Bucher, 1994) has indicated a role for PPases in receptor-stimulated cyclic AMP production, which was apparently distinct from the control of cell-surface receptors.

One of the most prominent PPases, PPase 1, is under the control of inhibitor-1 and DARPP-32 (Klee & Cohen, 1988). These are closely related regulatory phosphoproteins which in the phosphorylated form inhibit PPase 1. The inhibition is relieved upon dephosphorylation of the regulatory protein by the Ca^{2+}/calmodulin-activated protein phosphatase calcineurin (Klee & Cohen, 1988). The latter mechanism provides for a functionally co-ordinated PPase (calcineurin-PPase 1) cascade opposing the actions of protein kinases (Cohen, 1989). Calcineurin-PPase 1 cascades are thought to operate in a number of functionally important systems, such as regulation of glycogenolysis in skeletal muscle (Klee & Cohen, 1988) ion transport in kidney tubule cells (Aperia *et al*., 1992) and hippocampal long-term depression (Mulkey *et al*., 1994).

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Previous work (Antoni *et al*., 1995) in mouse pituitary corticotrope tumour (AtT20) cells has shown that calcineurin inhibits the formation of cyclic AMP induced by the polypeptide hormone 41 amino acid residue corticotrophin releasing-factor (CRF). The present study investigated whether the control of adenosine 3′:5′ cyclic monophosphate (cyclic AMP) accumulation by calcineurin involves the operation of a calcineurin-PPase 1 cascade in this system. The data showed that inhibitors of PPase 1/2A abolish the regulation of cyclic AMP levels by Ca^{2+}/calcineurin. This is nominally consistent with the operation of a PPase cascade. However, there was also a marked reduction of agonist-induced cyclic AMP responses. Furthermore, PPase blockers were also effective in Ca^{2+} depleted cells, where calcineurin is inactive, clearly demonstrating multiple sites of action of PPase 1/2A in the cyclic AMP signal transduction cascade. Overall, the results suggest that PPase 1/2A operate in a functionally co-ordinated manner to maintain the responsiveness of AtT20 cells to receptors coupling to Gs.

A preliminary account of this work has been published (Antaraki & Antoni, 1994).

Methods

Cells

Mouse pituitary corticotroph tumour (AtT20 D16:16) cells (passage 18–35) were maintained as previously described (Woods *et al*., 1992).

Measurement of cyclic AMP formation

The protocol for experiments with forskolin and agonists of G protein-coupled receptors has been described previously (Antoni *et al*., 1995). Briefly, cells in 24-well tissue culture plates were washed free of serum and incubated at 37°C in Hank's balanced salt solution without Ca^{2+} and Mg^{2+} (HBSS, Life Technologies, Paisley, U.K.), supplemented with 25 mM HEPES pH 7.4, 2 mM $CaCl_2$, 1 mM $MgSO_4$ and bovine serum albumin (0.25% w/v).

Thirty to forty five minutes later the medium was replaced with fresh medium containing inhibitors of PPases and cyclic nucleotide phosphodiesterases (PDE). Importantly, in all experiments except for those shown in Figure 1b, a Ca^{2+}-depleting medium was applied at this stage (HBSS, supplemented with 25 mM HEPES pH 7.4, 2 mM NaEGTA, 1 mM $MgSO_4$, 5 μM A23187, 5 μM nimodipine and bovine serum albumin (0.25% w/v)), and unless otherwise indicated, this medium was used in the rest of the experiment. These conditions were necessary in order to eliminate Ca^{2+}-dependent regulation of cyclic AMP production (Antoni *et al*., 1995). In cases where, after preincubation in Ca^{2+}-depleting conditions, $CaCl_2$ was introduced with the CRF-stimulus to reach 2 mM free extracellular $CaCl_2$, the pH of the added CRF containing medium was buffered as required to maintain pH 7.4 during the test stimulation. Because of the intensive degradation of cyclic AMP by cyclic nucleotide phosphodiesterase in AtT20 cells (Woods *et al*., 1992; Koch & Lutz-Bucher, 1995) 0.5 mM isobutylmethylxanthine (IBMX) was always added to the cells at this stage. In some experiments rolipram, a highly selective blocker of type 4 PDE (Beavo & Reifsnyder, 1990) (courtesy of Schering, A.G., Berlin, F.R.G.), was also included with IBMX as indicated in the figure legends.

The cells were incubated for a further 30 min at 37°C, then cooled to 24°C for 5 min, after which time inducers of cyclic AMP formation were added at 24°C for 10 min (except for time-course studies). This protocol was used because pilot studies had shown calyculin A to be fully effective within 5 min at 37°C, while having no appreciable effect within 30 min at 24°C. Test incubations were carried out at 24°C, because the effects of calcium ions and calcineurin inhibitors were greater under these conditions (Antoni *et al*., 1995). Note, however, that as cholera toxin action required incubations at 37°C, all experiments with cholera toxin and the analysis of the time-course of calyculin A action in cells pre-activated by CRF (Figures 4 and 6) were carried out at 37°C.

The 10 min test incubation with agonists was terminated by adding HCl to reach a final concentration of 0.1 mM, and cyclic AMP was quantified by direct radioimmunoassay (Brooker *et al*., 1979). Under the conditions used 90% of the immunoreactive cyclic AMP was intracellular.

Experiments with cholera toxin

Where intact cells were used, all incubations were carried out at 37°C, in order to ensure that ADP-ribosylation proceeded optimally. All other procedures for cyclic AMP determination were the same.

ADP-ribosylation was carried out in cell membranes as previously described (Longbottom & van Heyningen, 1989). Briefly, approximately 2.5×10^7 cells preincubated for 30 min in Ca^{2+} depleting medium with or without 50 nM calyculin A, were homogenized in 20 mM Tris/HCl (pH 7.4), 1 mM EDTA, 250 mM sucrose, 0.1 mM phenyl methyl sulphonyl fluoride (PMSF) and 2 mM benzamidine. Homogenization was carried out by 10 strokes of a glass-Teflon homogenizer and the membrane fraction was separated from the crude homogenate by sequential centrifugations at 300, 2,000, 9,800 and 35,000 x*g*, respectively. Protein content was measured by the Coomassie blue method (Bradford, 1976). The ADP ribosylation assay mixture (final volume 0.1 ml) consisted of 20 μg ml^{-1} pre-activated cholera toxin, 5 μM [adenylate-^{32}P]-NAD$^+$ (25–35 Ci mmol^{-1}), 200 mM K_2HPO_4/NaH_2PO_4 (pH 7.5), 10 mM $MgCl_2$, 2 mM EDTA, 5 mM ATP, 0.5 mM GTP, 10 mM creatine phosphate, 50 u ml^{-1} creatine phosphokinase, 10 mM thymidine and 10 mM isoniazide. The reaction was initiated by the addition of 50 μg membrane protein. The assay mixture was incubated at 30°C for 30 min (maximal incorporation of radioactivity under these conditions) and the reaction was stopped by the addition of 1 ml of 'ice-cold' 10 mM MOPS/0.13 M NaCl (pH 7.5). Subsequently, the mixture was centrifuged at 9,000 x*g*, the pellet solubilized in 60 μl of 0.1% SDS, 50 mM Tris/HCl pH 7.8, 1 mM dithiothreitol (DTT) and 10 μl aliquots of this mixture were applied to a 12% polyacrylamide gel (Pharmacia, Milton Keynes U.K.) and electrophoresed under denaturing conditions. The gel was stained with Coomassie blue, dried and autoradiographed on X-ray film or in Molecular Dynamics phosphorimager cassettes, after which the optical densities of the radioactive bands were quantified with the Imagequant software as supplied by the manufacturer.

Measurement of cyclic nucleotide phosphodiesterase (PDE) activity

Crude homogenates of AtT20 cells were prepared in Teflon/glass homogenizers in homogenizing buffer (20 mM Tris/HCl (pH 8), 1 mM EDTA, 0.2 mM EGTA, 50 mM NaF, 10 mM sodium pyrophosphate, 50 mM benzamidine, 2 mM PMSF, 0.5 μg ml^{-1} leupeptin, 0.7 μg ml^{-1} pepstatin, 4 μg ml^{-1} aprotinin and 10 μg ml^{-1} soybean trypsin inhibitor) 50 μl/10^6 cells, in order to minimize proteolytic cleavage and dephosphorylation (Sette *et al*., 1994) of PDE. The radiometric assay of PDE activity was by column chromatography (Manganiello & Vaughan, 1973; Kono, 1988) or a co-precipitation/filtration assay (Schilling *et al*., 1994) which gave comparable results. The PDE assay reaction (250 μl) contained 100 mM TES-HCl pH 7.5, 5 mM $MgSO_4$, 1 or 10 μM substrate cyclic AMP, [^{3}H]-cyclic AMP (NET-275, NEN, Dupont) in tracer amounts, and 50 μl of homogenate. In some experiments, 0.5 mM EGTA was also included to reduce Ca^{2+}-dependent PDE activity to a minimum.

In experiments with PDE inhibitors and calyculin A, the requisite compounds were added to intact cells in order to reproduce the conditions of cyclic AMP experiments as closely as possible. PDE blockers were also added at the requisite concentrations into the PDE assay. The PDE reaction was terminated when it was linear both with respect to time and the amount of homogenate protein added, (5–10 μg or 80–100 μg/assay point, at 1 or 10 μM substrate cyclic AMP, respectively), so that quantitative comparisons between treatment groups could be made.

Reagents

Unless otherwise specified, all reagents were from Sigma Ltd (Poole, Dorset, U.K.). Sources of other materials were as follows: phorbol 12,13 dibutyrate ester (PdBu), calyculin A and okadaic acid, LC-Labs Boston (dissolved at 5 mM in ethanol and stored at -40°C); rat corticotrophin releasing-factor$_{1-41}$ (CRF) Bachem U.K. (Saffron Walden, Essex, U.K.); pituitary adenylate cyclase activating peptide$_{1-38}$ (PACAP) Peninsula Laboratories, (St Helen, Merseyside, U.K.), A23187 (Calbiochem-Novabiochem, Nottingham, U.K.); nimodipine Semat Ltd (St Albans, Herts, U.K.); pertussis toxin (List Laboratories). FK506 (tacrolimus), courtesy of Fujisawa GMBH, München.

Data analysis

Student's t test and analysis of variance were used where appropriate and are indicated in the figure legends.

Results

Actions of okadaic acid and calyculin A on receptor-induced cyclic AMP formation

Pilot studies showed that in cells treated with IBMX, both calyculin A (Figure 1a) and okadaic acid (not shown) inhibited CRF-induced cyclic AMP formation irrespective of the calcium status of the cells. Notably, in the presence of maximally effective concentrations of calyculin A, the enhancement of cyclic AMP formation by Ca^{2+}-depletion was no longer apparent. Furthermore, the enhancement of the cyclic AMP response by the calcineurin blocker FK506 (Antoni *et al*., 1995) could not be observed in the presence of calyculin A (Figure 1b). On the one hand, these data are compatible with the PPase cascade hypothesis, i.e. the serial coupling of calcineurin and PPase1, although the marked reduction of the cyclic AMP response complicates the interpretation of the data. On the other hand, the effects observed in Ca^{2+}-depleted cells clearly indicate that type 1/2A PPases are involved in the control of cyclic AMP accumulation independently of Ca^{2+}calcineurin. In order to examine this problem further, the subsequent studies were carried out in Ca^{2+}-depleted cells.

Under these conditions inhibition of CRF-induced cyclic AMP accumulation by 100 nM calyculin A was evident at all time-points examined after the addition of CRF (Figure 2a). Unless otherwise indicated, all reactions were terminated at 10 min. The reduction of cyclic AMP accumulation by 100 nM calyculin A was observed at all concentrations of CRF that caused a significant enhancement of cyclic AMP production (Figure 2b).

The dose-response curves for okadaic acid and calyculin A revealed that okadaic acid was approximately 80 fold less potent than calyculin A in reducing CRF-induced cyclic AMP formation, moreover 1-norokadaone, an okadaic acid analogue that is not bound by PPases (Nishiwaki *et al*., 1990), had no effect on cyclic AMP formation (Figure 2c).

Similar data were obtained with the β-adrenoceptor agonist isoprenaline, that activates a β_2 adrenoceptor (Heisler *et al*., 1983) in AtT20 cells, and PACAP (Figure 2d).

Lack of involvement of pertussis toxin sensitive G-proteins

The half-effective concentration and the maximal effect of calyculin A with respect to CRF-induced cyclic AMP formation was not altered by preincubation of the cells with pertussis toxin (1 μg ml^{-1} 18 h) (Figure 3a).

The same PTX treatment markedly attenuated the inhibitory effect of 10 nM somatostatin (Antoni *et al*., 1995), a peptide hormone known to reduce cyclic AMP formation through G_i proteins in AtT20 cells (Reisine, 1985).

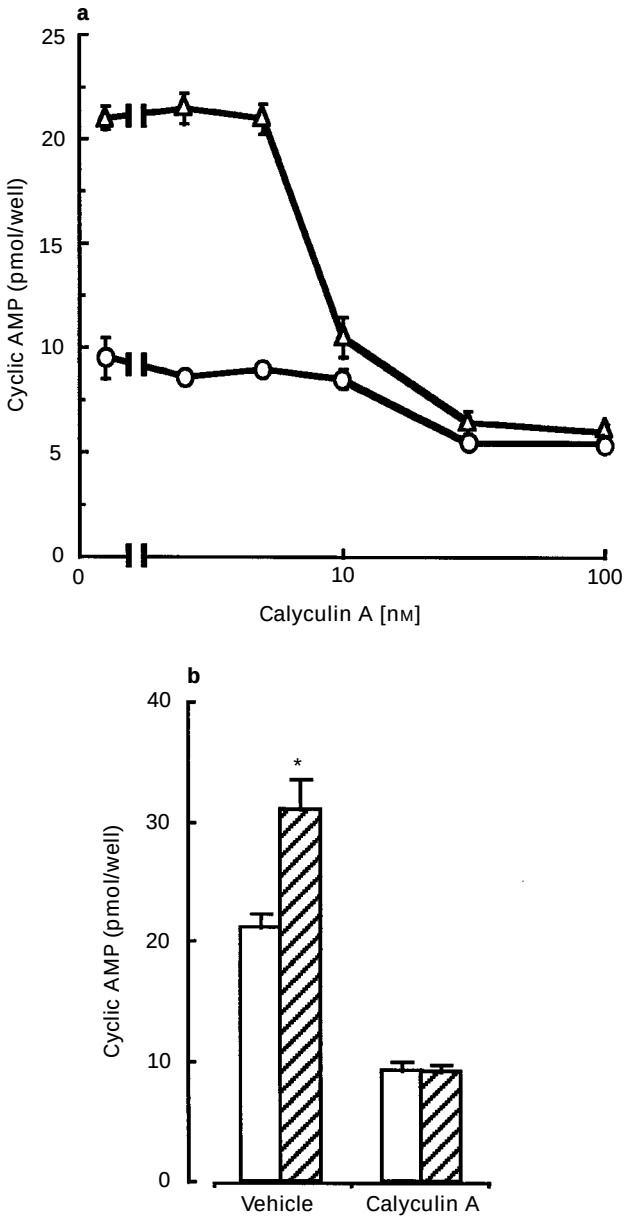

Figure 1 (a) Inhibition of cyclic AMP accumulation in response to 10 nM CRF by 50 nM calyculin A in (△) Ca^{2+}-depleted cells and (○) Ca^{2+}-depleted cells repleted with Ca^{2+} at the time of addition of CRF (see Methods); 0.5 mM IBMX and calyculin A were added 30 min before CRF. Basal levels of cyclic AMP were 0.65 ± 0.002 and 0.54 ± 0.02 pmol/well (mean$\pm$s.e.mean) in the Ca^{2+}-depleted and Ca^{2+}-repleted groups, respectively. (b) Blockade of calcineurin modulation of 10 nM CRF-induced cyclic AMP by calyculin A in AtT20 cells incubated in medium containing 2 mM $CaCl_2$ (without ionophore) throughout the course of the experiment. All groups of cells were preincubated with 0.5 mM IBMX, and the indicated combinations of vehicle (0.4% ethanol), 50 nM calyculin A and 2 μM FK506 for 30 min; open columns, control; hatched columns, FK506. Basal cyclic AMP level was 1.6 ± 0.09 pmol/well. Data are means$\pm$s.e.mean; $n=4$/group. *$P<0.05$ vs vehicle, one-way analysis of variance followed by Newman-Keuls test.

Cholera toxin induced cyclic AMP synthesis was blocked by calyculin A

Pretreatment with 50 nM calyculin A completely blocked the formation of cyclic AMP induced by cholera toxin despite the presence of IBMX (not shown and (Koch & Lutz-Bucher, 1994)) or IBMX as well as rolipram (Figure 4).

In view of the complete block of the effect of cholera toxin by preincubation with calyculin A, the effects of calyculin A pretreatment on ADP-ribosylation were examined. In membranes prepared from calyculin A-pretreated cells (Figure 5), there was a clear increase (50% and 100% expressed in arbitrary optical density units in experiment 1 and 2, respectively) of [^{32}P]-ADP ribose incorporation into two major radiolabelled bands corresponding to the two commonly occurring 45K and 52K molecular weight splice variants (Robishaw *et al*., 1986) of $G_{s\alpha}$. No differential changes in the intensity of radiolabelling of these bands were discernible.

In order to circumvent the apparent blockade of the activation of G_s by calyculin A, the cells were activated by cholera toxin for 90 min, after which PDE blockers and calyculin A were added in sequence. Under these conditions (Figure 6a) when only 0.5 mM IBMX was present, a marked inhibition of cyclic AMP accumulation developed within 10 min after the addition of 50 nM calyculin A. However, 0.5 mM IBMX and 0.1 mM rolipram in combination blocked the effect of calyculin A at all time points, except at 30 min when a small (20%), but statistically significant ($P<0.05$, one-way ANOVA followed by Newman Keuls test) inhibition developed.

As IBMX and rolipram in combination also markedly (close to 150%) enhanced the cyclic AMP response to cholera toxin when compared with IBMX alone, the response to 10 nM CRF was re-examined. This was also increased upon the addition of 0.1 mM rolipram and 0.5 mM IBMX, but only by 20% when compared with the response observed in the presence of 0.5 mM IBMX alone (not shown). However, the profound inhibition by calyculin A either as preincubation (not shown), or when given 1.5 min after the addition of CRF (Figure 6b) prevailed even in the presence of 0.1 mM rolipram and 0.5 mM IBMX.

Effect of calyculin A on phosphodiesterase activity

Analysis of the time-course of calyculin A action on cholera toxin-induced cyclic AMP formation (see Figure 6a) indicated that calyculin A may enhance phosphodiesterase activity in

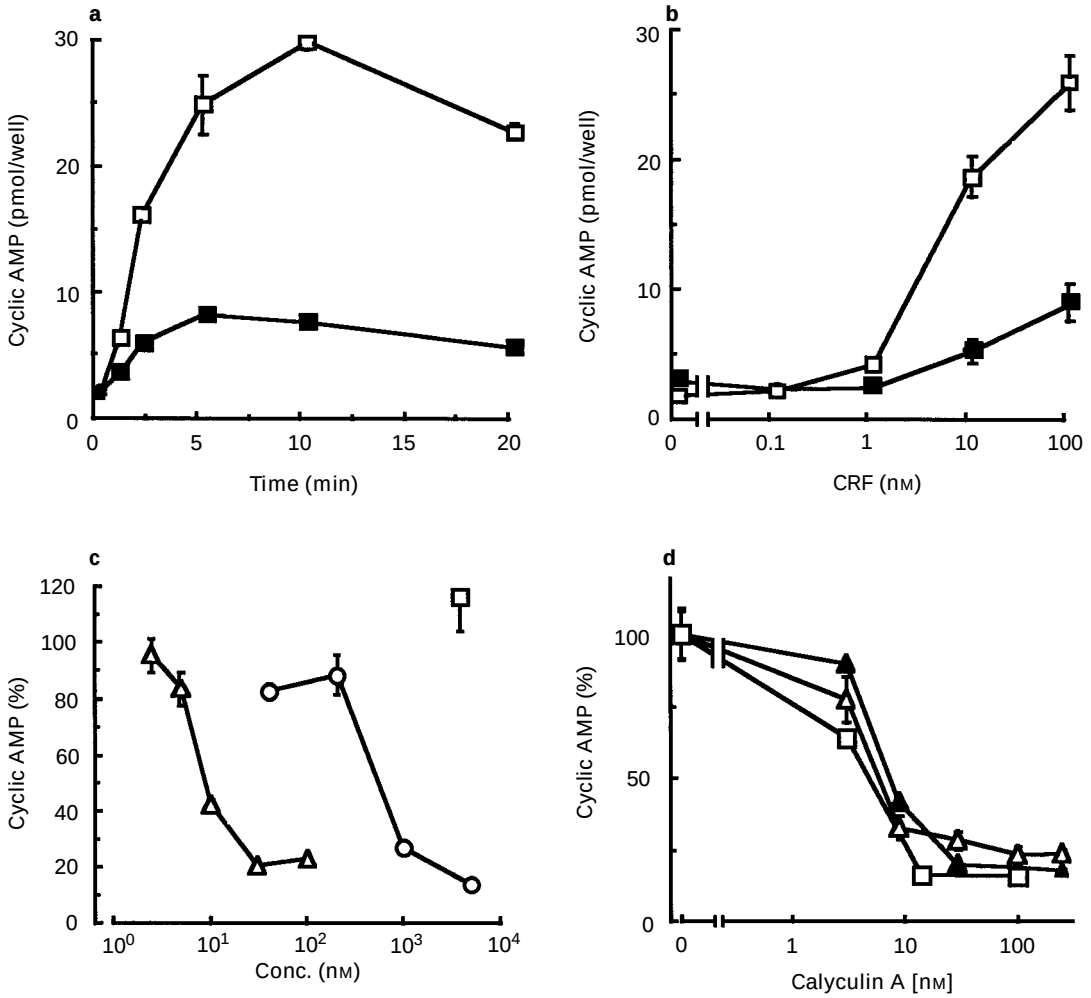

Figure 2 Inhibition of CRF-induced cyclic AMP production by protein phosphatase (PPase) blockers in Ca^{2+}-depleted AtT20 cells in the presence of 0.5 mM IBMX. PPase blockers and IBMX were applied 30 min before the addition of CRF. Data are means and vertical lines show s.e.mean; $n=4-6$/group, and representative of at least two identical experiments. (a) Time-course of cyclic AMP formation induced by 10 nM CRF in cells treated with (□) vehicle or (■) 100 nM calyculin A. (b) Effect of (■) 100 nM calyculin A and (□) vehicle on cyclic AMP production in response to various concentrations of CRF, the reaction was terminated 10 min after the addition of CRF. (c) Specificity of PPAse blocker action on cyclic AMP production evoked by 10 nM CRF. Data are expressed as percentage of the cyclic AMP response to 10 nM CRF. Basal levels of cyclic AMP were 0.79 ± 0.05 pmol/well, CRF-induced cyclic AMP taken as 100% was 11.3 ± 0.4 pmol/well. Effects (△) calyculin A, (○) okadaic acid and (□) 1-nor-okadone are shown. (d) Concentration-dependent effect of calyculin A on cyclic AMP accumulation induced by (▲) 10 nM CRF, (△) PACAP or (□) isoprenaline. All stimuli caused comparable increases (10–20 fold basal) of total cyclic AMP.

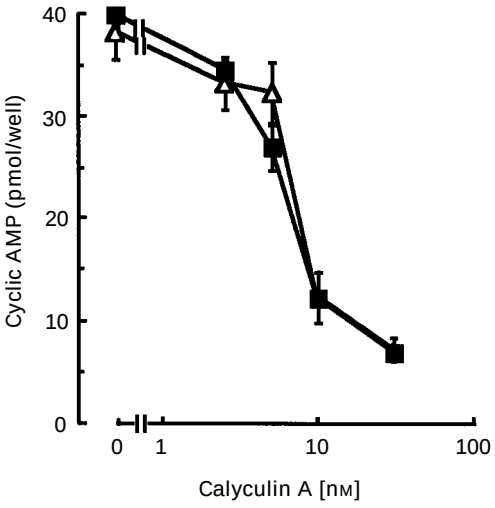

Figure 3 Effect of various concentrations of calyculin A on cyclic AMP production induced by 10 nM CRF in calcium-depleted AtT20 cells after 18 h of treatment with (■) vehicle or (△) 1 μg ml^{-1} pertussis toxin. Calyculin A was applied 30 min before stimulation by CRF. IBMX (0.5 mM) was present during the addition of calyculin A and CRF. Data are means and vertical lines show s.e.mean; $n = 4$, representative of two experiments.

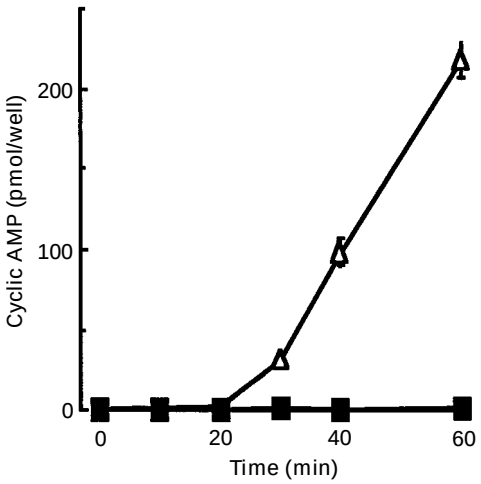

Figure 4 Interaction of calyculin A with cholera toxin in calcium depleted AtT20 cells. Levels of cyclic AMP in AtT20 cells pretreated with 0.5 mM IBMX and 0.1 mM rolipram plus vehicle (△) or 50 nM calyculin A (■) for 30 min. Cholera toxin (125 ng ml^{-1}) was added at 0 min. Means ± s.e.mean (vertical lines) $n = 4$/group, representative of two experiments.

AtT20 cells. Measurement of PDE activity in Ca^{2+}-depleted cells revealed a clear stimulation of cyclic AMP hydrolysis upon 30 min of pretreatment with 50 nM calyculin A (Figure 7a and b). Treatment with PDE blockers by use of the same protocol as in the experiments for cyclic AMP accumulation and also including the blockers in the PDE assay (10 μM substrate) showed (Figure 7b) that IBMX inhibited PDE activity up to 80%. However, because of the marked enhancement of PDE activity in calyculin A treated cells, the activity remaining in the presence of IBMX was 55% of that found in control cells. Combination of rolipram with IBMX reduced this activity to 5% of control.

The time-course of the onset of the effect of calyculin A on PDE activity was assayed under Ca^{2+} free conditions at 1 μM substrate thus favouring detection of PDE4. A significant increase of activity was evident within 5 min of addition to the cells (Figure 7c) and was completely blocked by addition of 10 μM rolipram in the PDE assay (Figure 7c). Addition of 30 nM calyculin A into the PDE assay did not mimic this effect,

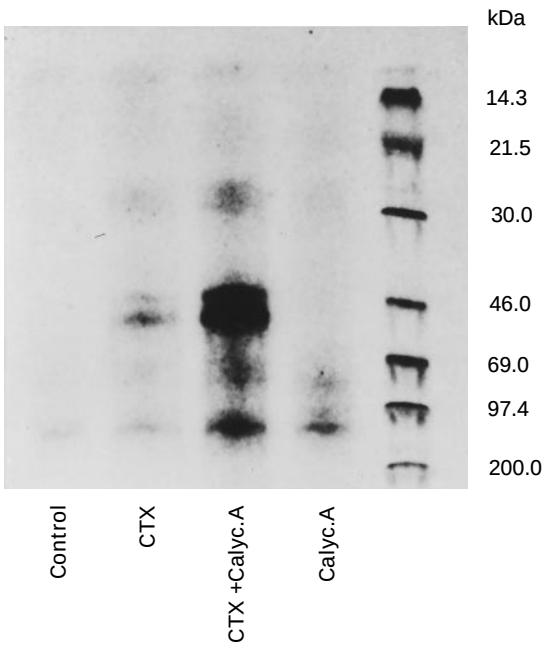

Figure 5 Effect of pretreatment with calyculin A on cholera toxin induced ADP-ribosylation in AtT20 cell membranes. Cells were incubated with 0.4% (v/v) ethanol or 50 nM calyculin A in Ca^{2+}-depleting medium for 30 min and membranes were reacted with cholera toxin (CTX) in ADP-ribosylation buffer or ADP-ribosylation buffer alone for 30 min, after which the proteins were resolved by gel electrophoresis under denaturing conditions. Autoradiography was carried out with X-ray film as well as phosphorimager cassettes. Data shown are representative of two identical experiments. Control: vehicle treated cells, membranes in ADP ribosylation buffer only, CTX: vehicle treated cells, membranes treated with CTX; CTX + Calyc. A: cells treated with calyculin A membranes reacted with CTX and ADP ribosylation buffer; Calyc.A: cells treated with calyculin A, membranes in ADP ribosylation buffer only. Quantification of the optical densities by image analysis indicated a 2 fold increase of incorporation into the two bands migrating approximately at 45K and 52K molecular weight. The high molecular weight band at 97K may be a dimer of the lower molecular weight forms.

furthermore, assays in the presence of 100 μM Ca^{2+}, 100 nM calmodulin and 10 μM rolipram showed no increase of PDE activity after treatment with calyculin A (results not shown). Collectively, these data indicate selective activation of a type 4 PDE by calyculin A.

Effects of calyculin A are not mimicked by activation of protein kinase C

In view of the possible activation of protein kinase C by calyculin A (Gopalakrishna *et al*., 1992) the effects of phorbol dibutyrate ester (PdBu) on CRF-induced cyclic AMP accumulation were also examined. In calcium-depleted cells pretreated with 0.5 mM IBMX, 10 nM PdBu produced a significant ($P < 0.0001$, two-way ANOVA) 30% inhibition of CRF-evoked cyclic AMP, which was also apparent in the presence of a maximally effective concentration (50 nM) of calyculin A (Figure 8). The actions of PdBu and calyculin A on CRF-induced cyclic AMP were not additive, PdBu produced a smaller decrement in the presence of calyculin A ($P < 0.01$ for interaction between PdBu and calyculin A in a linear ANOVA model). Finally, treatment of Ca^{2+}-depleted cells with 10 nM PdBu for 30 min failed to alter PDE activity (not shown).

Discussion

Previous work has indicated a prominent role of PPases in cellular responsiveness through the dephosphorylation of G-protein coupled membrane receptors (Sibley *et al*., 1987;

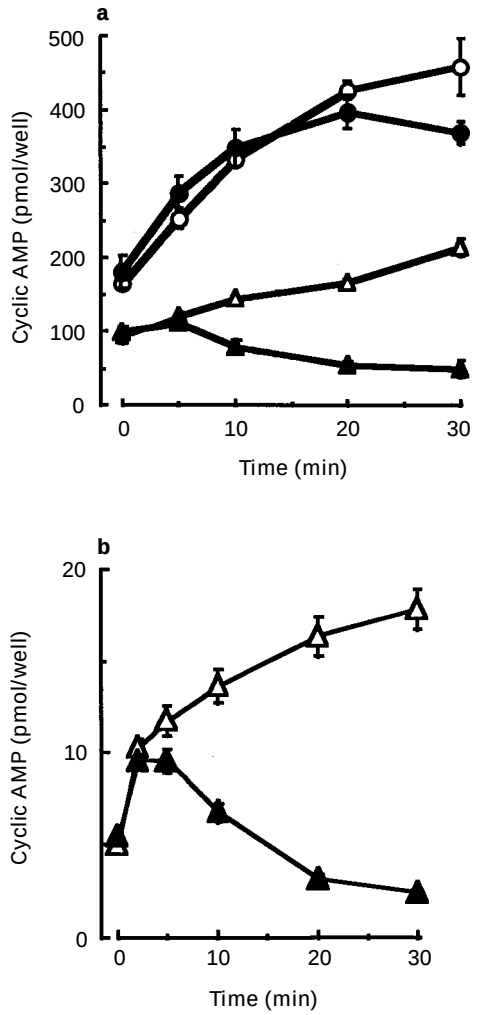

Figure 6 (a) Levels of cyclic AMP in AtT20 cells pretreated for 90 min with (125 ng ml^{-1}) cholera toxin; 0.5 mM IBMX (△, ▲) or 0.5 mM IBMX and 0.1 mM rolipram (○, ●) were added 5 min before vehicle (△, ○) or 50 nM calyculin A (▲, ●) was introduced at 0 min. Data are representative of two identical experiments. (b) Levels of cyclic AMP in AtT20 cells pretreated with 0.5 mM IBMX and 0.1 mM rolipram for 30 min; 10 nM CRF was added 1.5 min before the addition of vehicle (△) or 50 nM calyculin A (▲) at 0 min.

Pitcher *et al*., 1995). While it is possible that some of the effects observed in the present study are due to the depletion of cell surface receptor pools required for a sustained response to agonist stimulation, the data clearly show effects of PPase blockers downstream of membrane receptors. The reduction of agonist as well as cholera toxin-stimulated cyclic AMP synthesis indicates impairment of the activation of adenylyl cyclase by G_s. Moreover, a marked stimulation of cyclic AMP phosphodiesterase activity was demonstrated which was functionally relevant with respect to cyclic AMP accumulation.

Specificity of PPase inhibitors

The relative potencies of calyculin A and okadaic acid to inhibit receptor-induced cyclic AMP formation respectively, conform with the pharmacology of these compounds to inhibit PPase1 activity (Cohen *et al*., 1990). Similarly, a previous study (Koch & Lutz-Bucher, 1994) found a 100 fold difference in the potencies of calyculin A and okadaic acid to block CRF- and PACAP-induced cyclic AMP production in AtT20 cells. A close structural analogue of okadaic acid, 1-norokadaone, that does not bind to protein phosphatases (Nishiwaki *et al*., 1990) was inactive in our system. The cyclic AMP response to forskolin, a direct activator of adenylyl cyclase, is unchanged

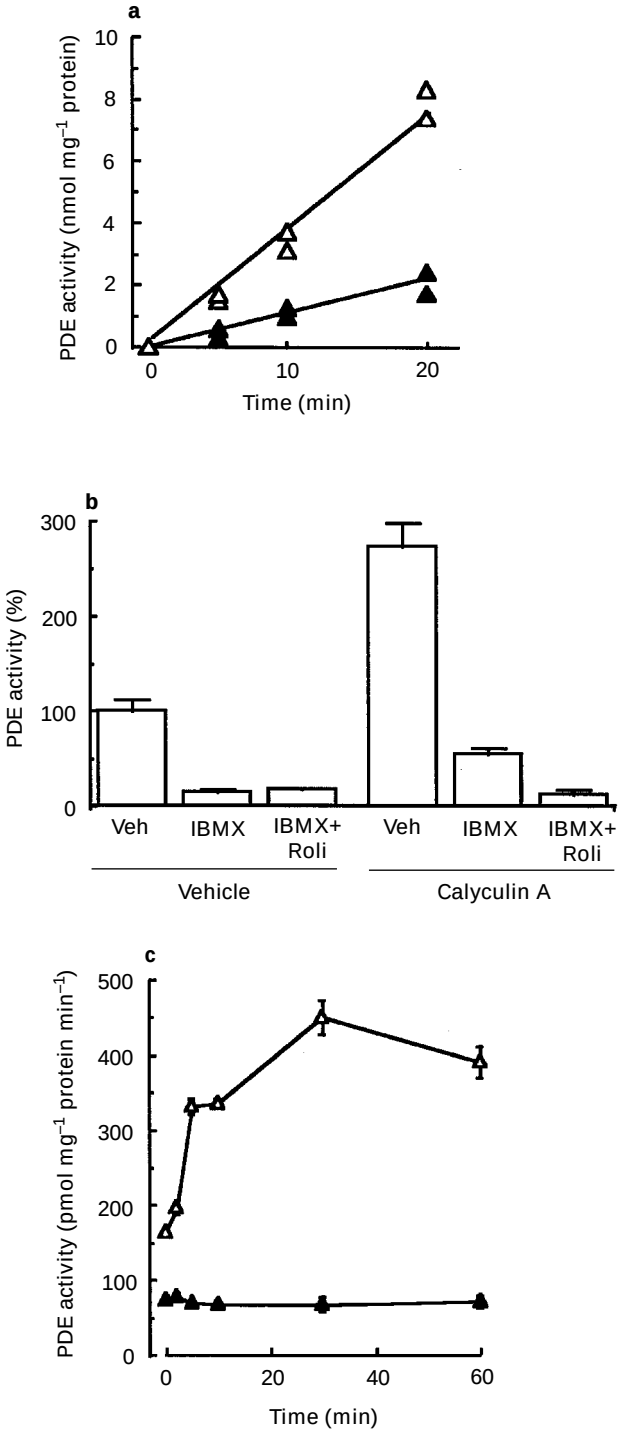

Figure 7 Effect of calyculin A and phosphodiesterase (PDE) blockers on cyclic AMP phosphodiesterase activity in calcium-depleted AtT20 cells. (a) Time-course of PDE activity in homogenates prepared from (▲) vehicle (Veh) or (△) 50 nM calyculin A-treated AtT20 cells with 10 μM cyclic AMP as substrate. Individual points from a representative experiment. (b) Cyclic AMP PDE activity in homogenates prepared from AtT20 cells pretreated with vehicle or 50 nM calyculin A plus 0.5 mM IBMX or 0.5 mM IBMX and 0.1 mM rolipram (Roli) PDE blockers were also added to the assay reaction mixture (substrate 10 μM cyclic AMP), the reactions were terminated at 10 min. Data are means ± s.e.mean, $n = 4$/group, pooled from 3 separate experiments, the basal activity taken as 100% ranged between 60 and 100 pmol mg^{-1} protein min^{-1} in these experiments. (c) Cyclic AMP PDE activity of homogenates prepared from AtT20 cells pretreated with 50 nM calyculin A for the indicated time intervals. The PDE activity was assayed in the presence of (△) 0.1% (v/v) DMSO (Veh) or (▲) 10 μM rolipram the assay buffer contained 0.5 mM EGTA and 1 μM cyclic AMP substrate.

(Koch & Lutz-Bucher, 1994) or even enhanced (A. Antaraki, & F.A. Antoni, unpublished data), upon treatment with PPase blockers, which argues against an impairment of adenylyl cyclase catalytic activity or a generalized toxic damage to cellular metabolism. Finally, activation of protein kinase C with phorbol ester did not mimic the effects of calyculin A. It is therefore reasonable to suggest that the effects observed here are due mainly to blockade of PPase activity, primarily types 1 and 2A, and not to non-specific interferences with other mechanisms of cellular control.

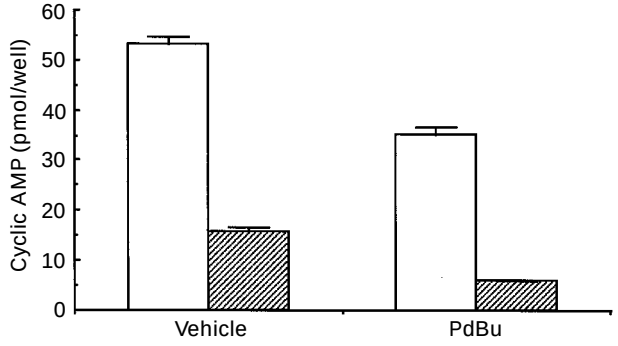

Figure 8 Effect of 10 nM phorbol 12, 13 dibutyrate ester (PdBu) on cyclic AMP accumulation induced by 10 nM CRF in Ca^{2+}-depleted AtT20 cells in the presence (hatched columns) or absence (open columns) of a maximally effective (50 nM) concentration of calyculin A. Cells were preincubated for 30 min with 0.5 mM IBMX and the indicated combinations of vehicle (0.4% ethanol), calyculin A and PdBu. Data are means ± s.e.mean, $n = 4$/group, representative of two experiments. Two-way ANOVA $P < 0.0001$ for the effects of calyculin A and PdBu, there was also a significant interaction $P < 0.01$ between the two treatments.

Blockade of modulation of cyclic AMP by Ca^{2+} in the presence of calyculin A

Calcium ions exert a powerful and functionally relevant negative feedback effect on cyclic AMP formation in AtT20 cells (Shipston *et al*., 1994; Antoni *et al*., 1995) which is interrupted by blockers of calcineurin such as FK506. In the present study calyculin A blocked the effects of calcium depletion and repletion previously (Antoni *et al*., 1995) shown to enhance and inhibit the CRF-induced cyclic AMP response, respectively. Accordingly, the effect of FK506 was also abolished. Nominally, these data are consonant with the calcineurin-PPase1 cascade hypothesis (Figure 9). However, calyculin A caused a strong net inhibition of the cyclic AMP response to activation by G_s-coupled receptors, whereas blockers of calcineurin enhanced it. Furthermore, the suppression of the cyclic AMP response by calyculin A was also observed in Ca^{2+}-depleted cells. Thus, while these findings favour that calcineurin operates through PPase1 in AtT20 cells as proposed previously for other systems (Klee & Cohen, 1988), it is clear that further sites of PPase action are involved that are readily demonstrable in cells depleted of rapidly mobilizable Ca^{2+}.

Effects of PPase blockade on G_s mediated responses

Overall, the consequence of PPase inhibition was a marked reduction of the rate of receptor- as well as cholera toxin-stimulated cyclic AMP formation. Pertussis toxin sensitive G-proteins have no apparent role in this effect of PPase blockers.

Surprisingly, despite the complete blockade of cholera toxin-induced cyclic AMP formation by calyculin A, the ADP-ribosylation of $G_{s\alpha}$ by cholera toxin was enhanced. As recent work suggests that $G_{s\alpha}$ in the heterotrimeric complex with β and γ subunits is a much better substrate for cholera toxin than the dissociated α-subunit (Toyoshige *et al*., 1994), these data

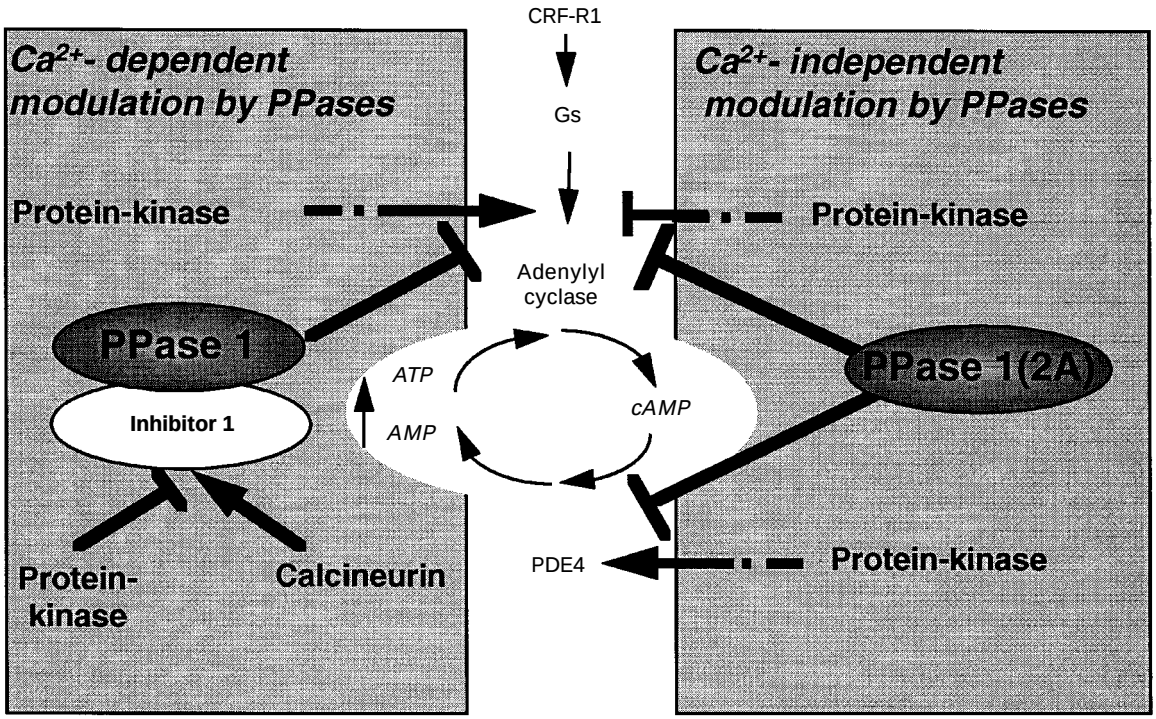

Figure 9 Schematic summary of multiple sites of PPase modulation of the cyclic AMP (cAMP) signal transduction cascade in AtT20 cells. Arrows and T-s represent stimulation and inhibition, respectively. The broken effector lines indicate that multiple enzymatic steps may be involved. Ca^{2+}-dependent modulation by PPases: the operation of a calcineurin-PPase1 cascade is suggested by the loss of Ca^{2+}/calcineurin modulation of agonist-induced cyclic AMP synthesis by calyculin A. The respective protein kinase(s) that appear to be facilitatory to G_S-cyclase coupling remain to be identified. The site of PPase1 action is not known. Ca^{2+}-independent modulation by PPases: a calyculin A-sensitive mechanism is required for efficient coupling of G_S and adenylyl cyclase implying the existence of inhibitory phosphorylation. The site(s) of this modulation and the protein kinases are not known. Calyculin A enhanced the activity of a rolipram-sensitive PDE, indicating constitutive stimulant phosphorylation of a PDE 4 isotype.

could be interpreted to suggest that the probability of G_s subunit dissociation, which is a prerequisite for the activation of adenylyl cyclase (Gilman, 1987), is reduced by calyculin A. This hypothesis is also compatible with the observation that after irreversible activation of $G_{s\alpha}$ by cholera toxin, the principal mediator of calyculin A action appeared to be a rolipram/IBMX sensitive PDE, whereas this was clearly not the case when calyculin A was applied before cholera toxin. Blockade of PDE with rolipram/IBMX also failed to prevent the inhibition of CRF-induced activation of adenylyl cyclase by calyculin A, where cycling of $G_{s\alpha}$ from the activated (dissociated) to the deactivated (heterotrimeric) state is likely to be essential for the stimulating effect of the agonist (Gilman, 1987). In addition, other workers have shown a drastic reduction of the cyclic AMP response to CRF in membranes of AtT20 cells pretreated with calyculin A (Koch & Lutz-Bucher, 1994). Thus calyculin A interferes with the activation of adenylyl cyclase by G_s. This implies that in AtT20 cells constitutive phosphorylation/dephosphorylation reactions regulate the coupling of G_s to adenylyl cyclase.

Phosphorylation of $G_{s\alpha}$ by a variety of protein kinase cascades, particularly epidermal growth factor-induced kinase cascades, has been observed previously (see Houslay, 1994; Liebman *et al*., 1996 for reviews). One apparent outcome of $G_{s\alpha}$ phosphorylation by epidermal growth factor-induced mechanisms is the inhibition of the activation of adenylyl cyclase by G_s-coupled receptors, which conforms with the findings presented here. Another potential mechanism that could underlie the reduction in the stimulation of adenylyl cyclase by receptor-activated G_s is the phosphorylation of adenylyl cyclase by protein kinases (Premont *et al*., 1992). Adenylyl cyclase type 9 is the predominant cyclase isotype expressed in AtT20 cells (Antoni *et al*., 1995; Paterson *et al*., 1995), which, like other mammalian cyclase isoforms, is potentially phosphorylated by several protein kinases (Antoni, 1997). However, the functional relevance of specified phosphorylation sites in adenylyl cyclases remains to be investigated.

Effects on PDE

Calyculin A substantially (2–3 fold) enhanced cyclic AMP hydrolysis in homogenates from calcium-depleted cells, and this was also evident in cells incubated in normal, calcium-containing medium (A. Antaraki, K.L. Ang & F.A. Antoni, unpublished data) showing that the effects are not due to depletion of intracellular calcium stores.

The aim of the preliminary analysis of AtT20 cell PDE activity presented here was to clarify the results obtained with cholera toxin. AtT20 cells contain both Type 1 and Type 4 PDE activity (Ang & Antoni, 1996) and calyculin A specifically increased the latter. Moreover, at 10 μM of substrate cyclic AMP, IBMX blocked 80% of total PDE activity while rolipram caused a 40% inhibition. As PDE activity rose almost 3 fold in response to calyculin A, PDE activity was substantial, 55% of that in vehicle-treated cells, in the presence of 0.5 mM IBMX. Combination of rolipram and IBMX in calyculin A-treated cells produced close to complete (95%) inhibition of PDE activity. These results can be correlated with the observation that when cyclic AMP formation was stimulated by cholera toxin i.e. irreversibly activated $G_{s\alpha}$ the combination of rolipram and IBMX but not IBMX alone blocked the inhibitory effect of calyculin A on cyclic AMP accumulation. Thus under these conditions the effect of calyculin A on cyclic AMP accumulation was largely due to activation of PDE.

These findings indicate that a large proportion of PDE activity in AtT20 cells is under stimulant control by constitutively active protein kinase(s) and is inhibited by calyculin A sensitive dephosphorylation. Regulation of various PDEs by phosphorylation is an important element of cellular control in several types of cell (Beltman *et al*., 1993). Specifically, other investigators have formed an increase of PDE activity by okadaic acid in adipocytes (Shibata *et al*., 1991), and phosphorylation by protein kinase A influences the activity of Type 4 PDE in a thyroid cell line (Sette *et al*., 1994). Moreover, control of calmodulin activated Type 1 PDEs by phosphorylation has been also shown (Sharma *et al*., 1988). However, the impact of these changes on cyclic AMP signals awaits further analysis.

Functional implications

A diagrammatic summary of the results presented in this paper is shown in Figure 9. Taken together, the present observations suggest that in AtT20 cells multiple phosphorylations by constitutively active protein kinases block cellular responses to cyclic AMP generating agonists. PPases inhibited by calyculin A and okadaic acid (primarily PPase 1 with possible requirement for concomitant blockade PPase 2A) have essential and functionally consonant actions in opposing constitutive phosphorylations and thus maintaining the transduction of extracellular signals operating through heterotrimeric G protein activation. AtT20 cells have been cultured as a relatively well-differentiated secretory cell line for almost three decades (Yasumura, 1968), while normal rat pituitary corticotrophs and human corticotroph adenoma cells generally dedifferentiate in similar culture conditions within a few weeks (Westphal *et al*., 1986; Childs *et al*., 1989). As cyclic AMP may provide the signal for proliferation of a differentiated phenotype of endocrine cell (Dumont *et al*., 1989) the constitutively active protein kinases are likely to promote non-differentiated growth, which is suppressed by PPases. Evidence specifically showing unique patterns of constitutional Ser/Thr phosphorylation and a resultant increase in the activity of pp60 (c-*src*) in AtT20 cells has been obtained (Gould *et al*., 1989). Thus the present findings may help to explain the tumour promoter actions of PPase blockers, i.e. the facilitation of proliferation without differentiation.

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