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Interaction with receptor for activated C-kinase 1 (RACK1) sensitizes the phosphodiesterase PDE4D5 towards hydrolysis of cAMP and activation by protein kinase C

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We have previously identified the PKC (protein kinase C)-anchoring protein RACK1 (receptor for activated C-kinase 1), as a specific binding partner for the cAMP-specific phosphodiesterase PDE4D5, suggesting a potential site for cross-talk between the PKC and cAMP signalling pathways. In the present study we found that elevation of intracellular cAMP, with the β_2 -adrenoceptor agonist isoproterenol (isoprenaline), led to activation of PDE4 enzymes in the particulate and soluble fractions of HEK (human embryonic kidney)-293 cells. In contrast activation of PDE4D5, with isoproterenol and the PKC activator PMA, was restricted to the particulate fraction, where it interacts with RACK1; however, RACK1 is dispensable for anchoring PDE4D5 to the particulate fraction. Kinetic studies demonstrated that RACK1 alters the conformation of particulate-associated PDE4D5 so that it more readily interacts with its substrate cAMP

and with rolipram, a PDE4 inhibitor that specifically targets the active site of the enzyme. Interaction with RACK1 was also essential for PKC-dependent and ERK (extracellular-signal-regulated kinase)-independent phosphorylation (on Ser¹²⁶), and activation of PDE4D5 in response to PMA and isoproterenol, both of which trigger the recruitment of PKC α to RACK1. Together these results reveal novel signalling cross-talk, whereby RACK1 mediates PKC-dependent activation of PDE4D5 in the particulate fraction of HEK-293 cells in response to elevations in intracellular cAMP.

Key words: cAMP, intracellular targeting, phosphodiesterase (PDE), protein kinase C (PKC), receptor for activated C-kinase 1 (RACK1).

INTRODUCTION

cAMP is a ubiquitous second messenger that controls pleiotropic cellular actions through the direct activation of its effectors, PKA (protein kinase A) and EPAC (exchange protein activated by cAMP) [1,2]. cAMP PDEs (phosphodiesterases) are crucial components of the cAMP signalling system and represent the only means of decreasing cAMP levels in cells, through hydrolysis to 5'-AMP [3]. Of the 11 known classes of cyclic nucleotide PDEs, eight are able to hydrolyse cAMP [4,5]. Of these the PDE4 cAMP-specific class is widely expressed in a variety of tissues and accounts for the majority of cAMP hydrolysis activity in cells [6]. PDE4s are encoded by four genes that, through complex alternative splicing of cognate mRNAs, give rise to approx. 20 PDE subgroups, termed A–D [6]. Each PDE4 subgroup has a unique C-terminus and alternative splicing at the N-terminus results in a unique N-terminal region for each of the PDE4 classes, which is thought to influence stability, enzyme activity and intracellular targeting [6,7]. For example, the PDE4D group is distinguishable by unique N-terminal regions that have been found to readily interact with other cellular proteins [6]; phosphodiesterase PDE4D5 has been found to interact with the ubiquitously expressed WD-repeat signalling scaffold protein RACK1 (receptor for activated C-kinase 1) [8,9], although to date the precise function of this signalling complex is largely unknown. RACK1 has been found to act as both anchor and shuttle for a

number of cellular proteins, including conventional PKC (protein kinase C) isoforms, dynamin-1, Ras-GAP (GTPase-activating protein), Src and several integrin receptors, as well as a variety of viral proteins [8]. It has been suggested that RACK1 recruits these proteins to their appropriate subcellular locations thereby integrating their actions into intracellular signalling pathways [8]. This raises the question as to whether RACK1 affects the intracellular targeting and activity of PDE4D5 in a similar manner.

The interaction between PDE4D5 and RACK1 is highly specific since, to date, RACK1 has not been shown to bind to any other members of the cAMP-specific PDE4 family, nor does PDE4D5 bind to other WD-repeat proteins [9]. RACK1 and PDE4D5 are believed to interact in a manner similar to the heterotrimeric G-protein subunits, G β and G γ [8] in that blades of the RACK1 β -propeller form a groove to which the N-terminus of PDE4D5 binds [8]. An 88-amino-acid subdomain, termed the RAID1 (RACK1-interaction domain), located in the N-terminal region of PDE4D5 was identified as an essential requirement for RACK1 binding [10]. In addition, a low-affinity RACK1-binding site has also been located in the PDE4D5 catalytic domain [11]. Further studies have shown that although PDE4D5 appears to be only able to bind one protein at a time [11], it is likely that RACK1 has the facility to bind multiple proteins simultaneously, e.g. migration of breast cancer cells in response to IGF (insulin-like growth factor)-1 is thought to be regulated by a RACK1-directed protein complex involving the IGF-1

Abbreviations used: β_2 -AR, β_2 -adrenoceptor; CREB, cAMP-response-element-binding protein; DMEM, Dulbecco's modified Eagle's medium; DOTAP, dioleoyltrimethylammonium propane; ECL, enhanced chemiluminescence; ERK, extracellular-signal-regulated kinase; GFX, GF109203X; GST, glutathione transferase; HEK, human embryonic kidney; HRP, horseradish peroxidase; IGF, insulin-like growth factor; MEK, MAPK (mitogen-activated protein kinase)/ERK kinase; PDE, phosphodiesterase; PKA, protein kinase A; PKC, protein kinase C; PP2A, protein phosphatase 2A; RACK1, receptor for activated C-kinase 1; TBST, Tris-buffered saline containing 0.1% Tween 20; VSV, vesicular stomatitis virus.

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receptor, integrin receptors and FAK (focal adhesion kinase) [12]. This ability of RACK1 raises questions as to whether additional proteins binding to RACK1 can influence the function and specificity of the complex with regard to the intracellular targeting, conformation and activity of RACK1-bound PDE4D5. In support of this it has been shown that interaction with RACK1 *in vitro* increases the sensitivity of PDE4D5 to the PDE4-selective inhibitor rolipram {4-[3-(cyclopentoxyl)-4-methoxyphenyl]-2-pyrrolidinone}, which suggests that RACK1 may have some influence on the conformation of bound PDE4D5 [9]. Moreover, the influence of additional RACK1-binding partners on the status of PDE4D5, such as conventional PKC isoforms, e.g. cAMP-activatable PKC α [13], is largely unknown and may reveal key areas of novel signalling regulation and cross-talk. The aims of the present study are therefore to determine the consequences of interaction with RACK1 on the regulation of PDE4D5.

MATERIALS AND METHODS

Materials

GFX (GF109203X), PMA, Ro31-7549, rolipram and isoproterenol (isoprenaline) were purchased from Merck Biosciences. Antibodies against GAPDH (glyceraldehyde-3-phosphate dehydrogenase), PKC α , RACK1 (IgM clone) and the VSV (vesicular stomatitis virus) epitope were purchased from Ambion, Cell Signalling Technology, BD Transduction Laboratories and Sigma–Aldrich respectively. Human wild-type PDE4D5 and PDE4D5-L33D cDNAs [11], both with a C-terminal VSV tag were generously provided by Professor Miles D. Houslay (University of Glasgow, Scotland, U.K.).

Cell culture

HEK (human embryonic kidney)-293 cells were cultured at 37°C under a 5% (v/v) CO₂ atmosphere in DMEM (Dulbecco's modified Eagle's medium; Sigma–Aldrich) containing 10% (v/v) fetal bovine serum (Sigma–Aldrich), 2 mM L-glutamine and 2% (w/v) penicillin/streptomycin.

Transfection of cells

Cells were transfected at approx. 50% confluence with a DOTAP (dioleoyltrimethylammonium propane) methyl sulfate/DNA mixture, prepared by diluting 7.5 μ g of plasmid DNA 1:10 (v/v) in DMEM then mixing with DOTAP methyl sulfate diluted in DMEM according to the manufacturer's instructions. The mixture was then incubated at room temperature (18°C) for 30 min before being added to cells in fresh culture medium. Cells were then incubated overnight at 37°C under a 5% (v/v) CO₂ atmosphere before being used in experiments.

High-speed cell fractionation

To obtain membrane pellet and soluble fractions, cells were treated with pharmacological agents, harvested into lysis buffer [10 mM Tris/HCl, pH 7.5, 0.1 mM EDTA and protease inhibitor cocktail (Boehringer)] and then lysed by seven strokes of a 26.5 gauge needle fixed to a 1-ml disposable syringe. Unbroken cells and nuclei were pelleted in a bench-top centrifuge at 1000 g for 5 min at 4°C. Supernatants were then transferred into chilled Beckman ultracentrifuge tubes and then centrifuged in a bench-top ultracentrifuge at 75 000 rev./min in a TLA-110 rotor for 30 min at 4°C. The supernatant fraction was retained and stored at –80°C

for future use. The pellet fraction was resuspended in 500 μ l of lysis buffer and centrifuged as above for a further 30 min. The resulting supernatant was discarded and the membrane fraction resuspended in lysis buffer and stored at –80°C for future use.

Purification of recombinant PDE4D5

Bacteria expressing pGEX-5X-3 containing a cDNA for wild-type PDE4D5 were grown to a D_{600} of 0.6 and then induced with 1.0 mM isopropyl β -D-thiogalactopyranoside (Roche) and grown for a further 4 h at 30°C. Bacteria were then harvested by centrifugation at 4000 g for 10 min at 4°C and the bacterial pellets frozen at –80°C overnight. To purify recombinant GST (glutathione transferase)–PDE4D5, pellets were resuspended in 10 ml of ice-cold resuspension buffer (50 mM Tris/HCl, pH 8.0, 100 mM NaCl, 1 mM EDTA, 10 mM 2-mercaptoethanol and complete protease inhibitor mixture) and sonicated on a maximal setting for 4 \times 30 s on ice. Triton X-100 was then added to a final concentration of 0.02%, and cell debris was removed by centrifugation at 10 000 g for 10 min at 4°C. The cleared lysate was then incubated with a 1/10 volume of pre-equilibrated glutathione–Sepharose beads (Pharmacia) for 1 h with end-over-end turning at 4°C. The beads were then collected by centrifugation at 2000 g for 1 min and washed three times with resuspension buffer. The fusion proteins were eluted by the addition of 10 mM glutathione in 50 mM Tris/HCl, pH 8.0. The fusion proteins were dialysed three times against 50 mM Tris/HCl, pH 8.0, 100 mM NaCl and 5% (v/v) glycerol, then stored at –80°C until required.

Co-immunoprecipitation experiments

Equal amounts of cell pellet (crude membrane) or supernatant (cytosol) protein were prepared in equal amounts of lysis buffer. Following this 0.1% Triton X-100 was added to each sample which were then incubated on ice for 15 min. At this stage a 50 μ l aliquot of each sample was retained and boiled in Laemmli electrophoresis buffer [14] as input controls. Samples were pre-cleared by adding 5 μ g of mouse IgM and 50 μ l of anti-(mouse IgM) antibody–agarose (Sigma–Aldrich) to each sample. Samples were then incubated on an orbital shaker for 30 min at 4°C. Beads were then pelleted (13 000 g for 0.5 min) and the supernatant transferred to fresh Eppendorf tubes. Duplicates of each sample were then treated with either 5 μ g of anti-RACK1 antibody (IgM clone) or 5 μ g of mouse IgM and incubated on a wheel in a cold room for 30 min. A total of 50 μ l of anti-(mouse IgM) antibody–agarose was then added to each Eppendorf tube and the samples were incubated at 4°C for a further 30 min. The agarose beads were then pelleted for 30 s at 13 000 g and washed three times with 500 μ l of lysis buffer. Beads were then boiled in electrophoresis buffer and separated by SDS/PAGE (12% gels) along with the retained input controls.

Western immunoblotting

Cells were scraped directly into Laemmli electrophoresis buffer [14] and separated by SDS/PAGE (12% gels) before being transferred on to nitrocellulose membranes. Membranes were either blocked in 5% (w/v) dried milk powder in TBST (Tris-buffered saline containing 0.1% Tween 20) or 5% (w/v) BSA in TBST for phospho-specific antibodies. Membranes were then incubated overnight with the appropriate primary antibody at 4°C. Primary antibody was then removed and membranes were

washed three times with TBST and were then incubated for 1 h at room temperature with HRP (horseradish peroxidase)-conjugated secondary antibody. Blots were developed using ECL (enhanced chemiluminescence; GE Healthcare) according to the manufacturer's instructions.

Peptide array experiments

RACK1 peptide arrays were produced by automatic SPOT synthesis [15] and were synthesized on continuous cellulose membrane supports on Whatman 50 cellulose membranes using Fmoc (fluoren-9-ylmethoxycarbonyl)-chemistry with an AutoSpot-Robot ASS 222 (Intavis Bioanalytical Instruments AG) [15,16]. The interaction of spotted peptides with GST–PDE4D5 fusion protein was determined by overlaying the cellulose membranes with 10 $\mu\text{g/ml}$ recombinant protein. Bound recombinant GST–PDE4D5 was detected with specific rabbit antisera and secondary anti-rabbit HRP-coupled antibody (1:2500 dilution; Dianova), and was visualized by ECL (as above).

PDE enzyme assay

PDE activity in samples was assayed in cell extracts as described previously [4], using a 0.2 μM cAMP substrate solution containing 5 μCi [^3H]cAMP per ml prepared in assay buffer (40 mM Tris/HCl, pH 7.4, 5 mM MgCl_2 and 3.75 mM 2-mercaptoethanol). Reactions were incubated for 10 min at 30°C and then stopped by boiling for 2 min. Tubes were then transferred into watery ice and left to cool completely. Snake venom solution (25 μl of 1 mg/ml) from *Crotalus atrox* was then added and the tubes were incubated for 10 min at 30°C. The tubes were then returned to ice and 400 μl of pre-prepared Dowex[®]/water/ethanol (1:1:1) slurry was added to each tube. The tubes were left on ice for 15 min, with occasional vortex-mixing, and then centrifuged at 13000 g for 2 min at 4°C in a refrigerated bench-top centrifuge. A total of 150 μl of supernatant from the reaction tubes was added to scintillation tubes containing 4 ml of scintillant, with total counts being obtained by the addition of 50 μl of cAMP substrate solution and then assayed in a scintillation counter. Initial rates were taken from linear time courses of activity in all experiments. The K_m and V_{max} determinations were performed at substrate concentrations between 1 μM and 40 μM cAMP, defined experimentally and confirmed by fitting to the hyperbolic form of the Michaelis–Menten equation using GraphPad InStat 3. Dose-dependent inhibition by rolipram was determined in the presence of 0.2 μM cAMP with rolipram at concentrations between 0.01 nM and 1 mM.

Densitometry and statistical analysis

Non-saturating exposures from multiple experiments were quantified densitometrically using ImageJ software. Densitometric values for PDE4, the VSV tag and PKC α levels were normalized to the expression of RACK1 protein levels in the same sample. Statistical significance was determined using a two-tailed Student's *t* test or one-way ANOVA, where appropriate.

RESULTS

The effect of RACK1 on PDE4D5 subcellular localization

We have identified RACK1 protein as a cellular binding partner for PDE4D5 previously [9]; however, the functional importance of this interaction has yet to be determined. In particular the role of RACK1 in the intracellular targeting of PDE4D5 has yet to be investigated. To address this,

Table 1 K_m , V_{max} and IC_{50} values for PDE4D5-WT and PDE4D5-L33D expressed in HEK-293 cells

Significant differences relative to pellet-associated PDE4D5-WT (*, ** and ***) or PDE4D5-L33D (#, ## and ###) are indicated ($P < 0.05$, $P < 0.01$ and $P < 0.001$ respectively).

Transfection	Fraction	K_m (μM)	V_{max} (pmol/min per mg)	IC_{50} Rolipram (μM)
PDE4D5-WT	Pellet	6.3 ± 0.9	0.32 ± 0.07	0.10 ± 0.01
PDE4D5-WT	Soluble	$16.7 \pm 1.3^{**}$	$9.71 \pm 2.09^{***\#}$	$0.08 \pm 0.02^{###}$
PDE4D5-L33D	Pellet	$20.0 \pm 0.9^{***}$	0.87 ± 0.09	$10 \pm 0.90^{***}$
PDE4D5-L33D	Soluble	$10.0 \pm 1.5^{\#}$	$7.14 \pm 1.62^{*\#}$	$0.09 \pm 0.02^{###}$

HEK-293 cells were transfected with VSV-tagged cDNAs encoding either wild-type PDE4D5 (PDE4D5-WT) or VSV-tagged PDE4D5 where the leucine residue at position 33 in the unique N-terminus had been mutated to an aspartate residue (PDE4D5-L33D). The L33D mutation in PDE4D5 has been shown previously to prevent interaction with RACK1 [17] and immunoprecipitation experiments confirmed that this was the case in HEK-293 cells (Supplementary Figure S1 at <http://www.BiochemJ.org/bj/432/bj4320207add.htm>). Moreover, immunoblotting and cAMP PDE assays of fractions demonstrated that the relative distributions of PDE4D5-WT and PDE4D5-L33D protein and activity were approximately equal between the two fractions (Figure 1), whereas both PDE4D5-WT and PDE4D5-L33D immunoreactivity was enriched in the cytosolic fraction, with lower levels of expression in the particulate fractions (Figure 1). Together these results suggest that association with RACK1 does not play a significant role in determining the subcellular distribution of PDE4D5 in HEK-293 cells.

Effect of interaction with RACK1 on the conformation of PDE4D5

Experiments were then carried out to determine the maximum rate of catalysis (V_{max}) of PDE4D5-WT or PDE4D5-L33D, and the affinity of PDE4D5-WT or PDE4D5-L33D for cAMP (K_m) by using various concentrations of substrate (between 1 μM and 40 μM). Lineweaver–Burk plots of the results demonstrated that Michaelis–Menten kinetics were being followed at each of these substrate concentrations (Supplementary Figure S2 at <http://www.BiochemJ.org/bj/432/bj4320207add.htm>). The V_{max} values determined for the pellet fractions of both PDE4D5-WT and PDE4D5-L33D were low, at 0.32 ± 0.07 and 0.87 ± 0.09 pmol/min per mg respectively, whereas the supernatant fractions displayed higher rates of catalytic activity, with PDE4D5-WT possessing a V_{max} of 9.71 ± 2.09 pmol/min per mg, greater than the PDE4D5-L33D V_{max} value of 7.14 ± 1.62 pmol/min per mg (Table 1). The higher rate of catalysis observed in the supernatant fractions of PDE4D5-WT- and PDE4D5-L33D-transfected HEK-293 cells agreed with the relatively low K_m values of 16.7 ± 1.3 and 10.0 ± 1.5 μM respectively, indicating a fairly high affinity for cAMP. The pellet fraction of PDE4D5-WT, however, showed a comparatively lower K_m value of 6.3 ± 0.9 μM , indicating a higher affinity for its substrate (Table 1). Conversely, the non-RACK1-binding PDE4D5-L33D mutant pellet fraction has a K_m value of 20.0 ± 0.9 μM , demonstrating a loss of affinity for cAMP when compared with the other PDE4D5 forms in the other fractions. The significant difference in K_m values indicates that the binding of RACK1 to PDE4D5 in particulate fractions is essential for maintaining the high affinity of PDE4D5 for cAMP in the membrane fraction of cells. Next, we determined the effect of RACK1 binding on the susceptibility of PDE4D5 to a specific enzyme inhibitor by measuring the dose-response

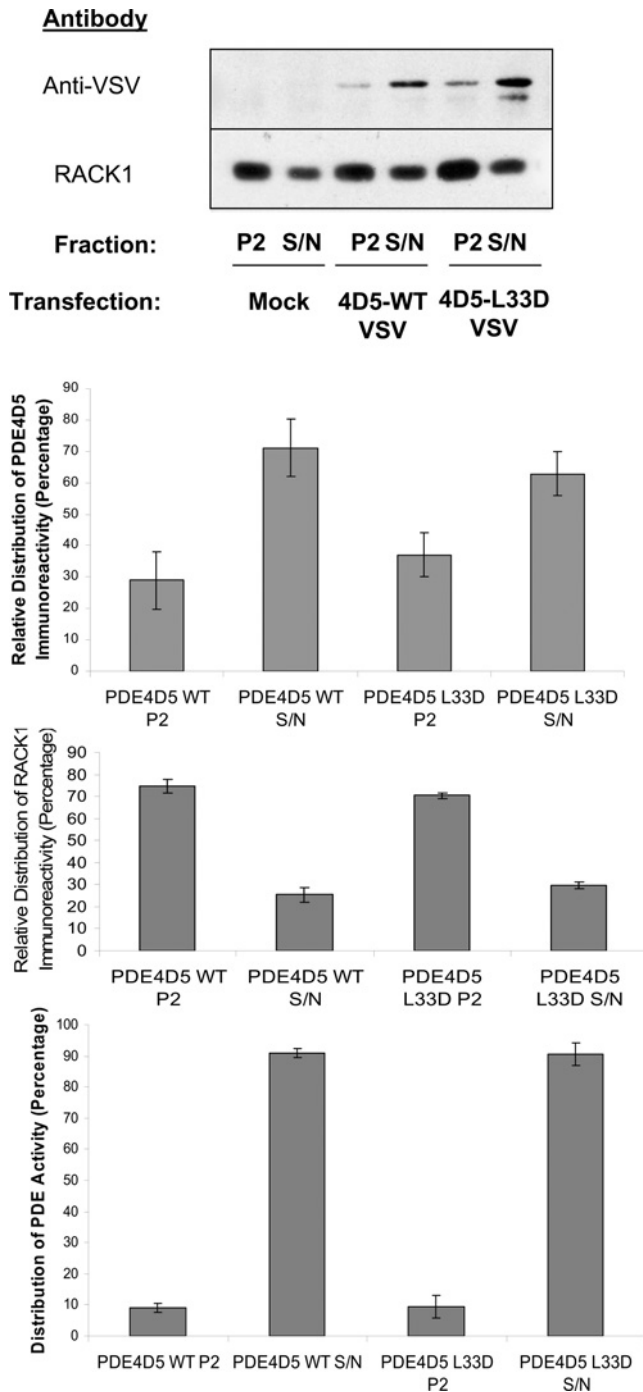


Figure 1 Distribution of PDE4D5-WT and PDE4D5-L33D activity and immunoreactivity between soluble and particulate fractions of HEK-293 cells

Pellet (P2) and cytosol (S/N) fractions from HEK-293 cells transfected with cDNAs encoding VSV-tagged wild-type PDE4D5 (PDE4D5-WT) or VSV-tagged PDE4D5-L33D (PDE4D5-L33D), the non-RACK1-binding mutant, were immunoblotted with anti-RACK1 and anti-VSV antibodies. Densitometric values were obtained from non-saturating immunoblots from three separate experiments and plotted on the top histogram, to show the relative distributions of PDE4D5-WT and PDE4D5-L33D between fractions. The middle histogram shows the relative distribution of RACK1. PDE assays were also carried out on fractions from transfected cells and these are displayed in the bottom histogram.

of PDE4D5-WT and PDE4D5-L33D to the PDE4 inhibitor rolipram (at concentrations between 0.01 nM and 1 mM). The responses of the soluble fraction PDE4D5-WT and

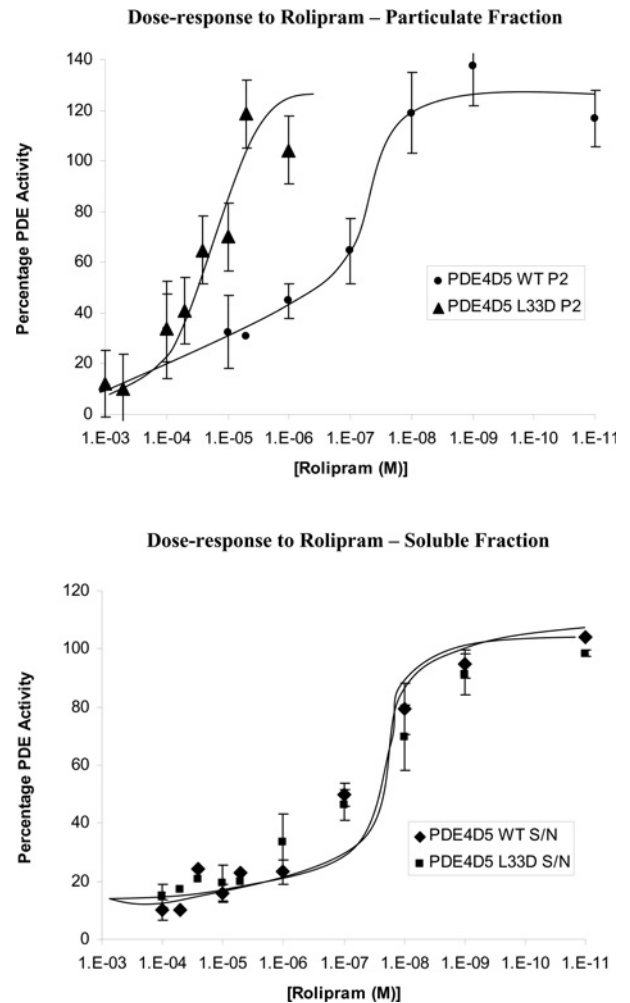


Figure 2 Dose-response curves of PDE4D5-WT and PDE4D5-L33D to inhibition by rolipram

Particulate (P2) or soluble (S/N) fractions, prepared from HEK-293 cells transfected with cDNAs encoding either PDE4D5-WT or PDE4D5-L33D, were treated with various concentrations of the PDE4 inhibitor rolipram, ranging from 0 to 1 mM, and then assayed for PDE activity. PDE activities obtained from three separate experiments were then plotted as means \pm S.E.M. ($1.E-03 = 1.0 \times 10^{-3}$ etc.) PDE activities, in the absence of inhibitor, for PDE4D5-WT or PDE4D5-L33D were 60 ± 10 pmol/min per mg, in the pellet fraction, and 550 ± 50 pmol/min per mg, in the soluble fraction, respectively.

PDE4D5-L33D to inhibition by rolipram were very similar; whereas the pellet fraction graph showed a clear difference in the dose-responses of PDE4D5-WT and PDE4D5-L33D, with PDE4D5-L33D being far less susceptible to inhibition by rolipram (Figure 2). These results suggest that interaction with RACK1 is important for maintaining the conformation of PDE4D5 in the particulate fraction of HEK-293 cells. Together these results (summarized in Table 1) suggest that PDE4D5 binding to RACK1 is necessary to enable high-affinity binding of PDE4D5 to its substrate cAMP, and for effective inhibition by rolipram in the particulate fraction of HEK-293 cells.

To test this idea further we carried out competition experiments to attempt to disrupt the association of PDE4D5-WT with RACK1 in the particulate fraction of HEK-293 cells using RACK1-derived peptides (500 μ M). Pellet fractions were incubated with 0.2 μ M rolipram (Figure 3a; a concentration of rolipram induced an approx. 40% inhibition of PDE4D5 in this fraction). Rolipram inhibition was effectively ablated by RACK1-derived

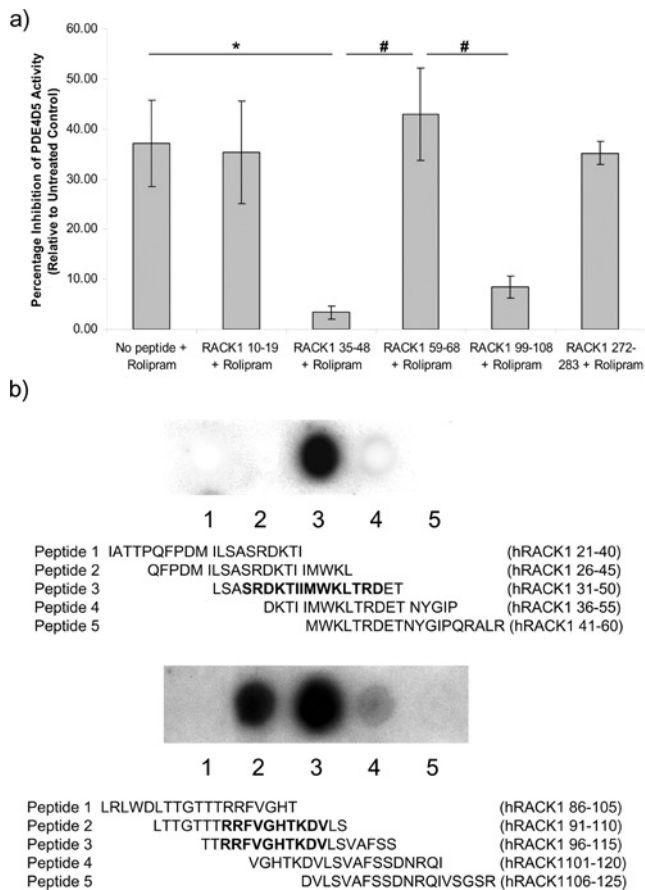


Figure 3 RACK1-derived peptides corresponding to the RACK1–PDE4D5 interaction interface block the inhibition of particulate PDE4D5 by rolipram

(a) Particulate fractions were prepared from HEK-293 cells that had been transfected with PDE4D5-WT and PDE assays were carried out in the presence or absence of 0.1 μ M rolipram as described in the Materials and methods section. RACK1-derived peptides (corresponding to amino acids 10–19, 35–48, 59–68, 99–108 and 272–283 of RACK1 as indicated) were also included in PDE assays. Results are presented as the percentage inhibition of PDE4D5 activity and significant differences relative to samples treated with rolipram without peptide ($*P < 0.05$), or with rolipram plus RACK1 peptide (59–68) ($\#P < 0.05$), are indicated. PDE activities, in the absence of inhibitor, for PDE4D5-WT or PDE4D5-L33D were 60 ± 10 pmol/min per mg, in the pellet fraction, and 550 ± 50 pmol/min per mg, in the soluble fraction. (b) RACK1-derived peptide array overlay experiments were carried out using purified, recombinant GST–PDE4D5-WT as described in the Materials and methods section. Two arrays of five overlapping peptides were prepared corresponding to the region of RACK1 that encompasses RACK1 peptides 35–48 and 99–108 respectively.

peptides corresponding to amino acids 35–48 and 99–108 of RACK1 (Figure 3a). Peptides corresponding to amino acids 10–19, 59–68 and 272–283 of RACK1 had little effect on the ability of 0.2 μ M rolipram to inhibit PDE4D5 activity in this fraction. These results suggest that part of the RACK1 interaction interface with PDE4D5 is contributed by amino acids 35–48 and 99–108. To test this assumption peptide array experiments were carried out using a range of immobilized peptides, which span the region of RACK1 corresponding to amino acids 21–60 and 86–125, overlaid with purified recombinant PDE4D5-WT (Figure 3b). PDE4D5-WT was found to interact most strongly with immobilized peptides corresponding to amino acids 35–48 and 99–108 of RACK1 (Figure 3b). Together these results support the idea that binding of PDE4D5 to amino acids 35–48 and 99–108 of particulate RACK1 is sufficient to evoke conformational changes in PDE4D5 that is characterized by high-affinity interaction with rolipram.

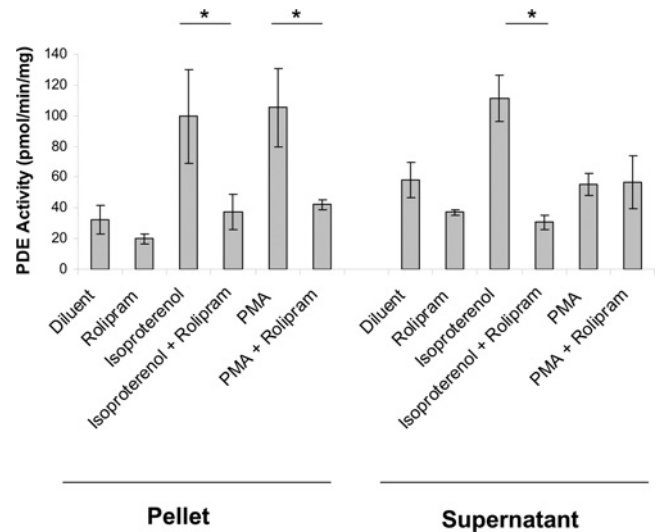


Figure 4 Enhanced PDE4 activity following stimulation of HEK-293 cells with isoproterenol or PMA

HEK-293 cells were stimulated with 10 μ M isoproterenol or 10 μ M PMA for 15 min, and then harvested and fractionated into high-speed pellet and supernatant fractions, and assayed for PDE hydrolytic activity in the presence or absence of the PDE4 inhibitor rolipram (10 μ M) as described in the Materials and methods section. Results obtained from three separate experiments were normalized and plotted as means \pm S.E.M. for each cellular fraction. Significant differences relative to rolipram-treated samples are indicated ($*P < 0.05$).

PMA promotes PDE activation in the particulate, but not the soluble fraction, of HEK-293 cells

Results so far suggest that RACK1 can sensitize PDE4D5 towards interaction with cAMP and therefore may play a role in regulating PDE4D5 activity. We therefore sought to determine the role of the RACK1-interaction partner, PKC, on the activation of PDE4 isoforms in HEK-293 cells. Cells were treated for 15 min in the presence or absence of the β_2 -AR (β_2 -adrenoreceptor) agonist 10 μ M isoproterenol [18] or the PKC-activator PMA [19], fractionated into high-speed pellet and cytosolic fractions, and assessed for PDE activity in the presence or absence of the PDE4-specific inhibitor rolipram, as described in the Materials and methods section. Analysis of the total PDE activity in the pellet fraction showed a significant increase in PDE activity following isoproterenol or PMA stimulation, which was completely ablated by inclusion of 10 μ M rolipram in the assay (Figure 4). Analysis of the supernatant fraction also demonstrated that isoproterenol stimulated cell PDE4 activity, with an approx. 1.6-fold increase when compared with non-stimulated cells (Figure 4). However, in contrast with what was observed in the pellet fraction, PMA failed to induce activation of PDE4 enzymes in the supernatant fraction of HEK-293 cells. These results suggest that an increase in cAMP levels following isoproterenol stimulation causes a significant increase in PDE4 activity in soluble and particulate fractions of HEK-293 cells; however, the action of PKC isoforms on PDE4 activity is limited to the pellet fraction in these cells.

RACK1 and PDE4D5 do not undergo activation-coupled translocation in HEK-293 cells

The effects of PDE4D5 binding to RACK1 on the cellular localization and activity of PDE4D5 has yet to be determined. We therefore sought to determine whether translocation of PDE4D5–RACK1, as reported previously for the PDE4D5– β -arrestin signalling complex [20], contributed to the increases in particulate PDE activity following isoproterenol stimulation

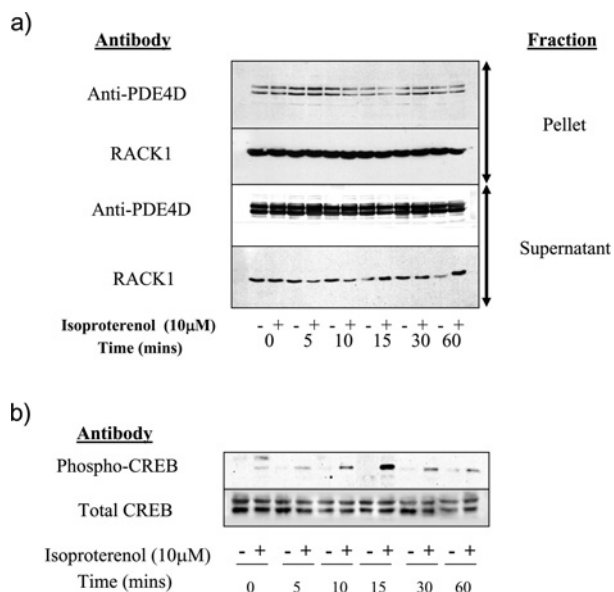


Figure 5 RACK1 and PDE4D5 do not undergo activation-coupled translocation following isoproterenol stimulation of HEK-293 cells

(a) Pellet and supernatant fractions from HEK-293 cells stimulated with 10 μM isoproterenol at the indicated time points were immunoblotted with anti-PDE4D and anti-RACK1 antibodies. RACK1 was enriched in the pellet fraction, whereas PDE4D5 and PDE4D3 (upper and lower bands, 105 and 95 kDa respectively, of the doublet shown on the blot) are enriched in the supernatant fraction. The immunoblots are representative of three separate experiments. (b) HEK-293 cells were stimulated with 10 μM isoproterenol for the indicated times (between 0 and 60 min) and then immunoblotted with anti-CREB and anti-phospho-CREB (Ser¹³³) antibodies. Results are representative of three separate immunoblots.

(Figure 4). HEK-293 cells were stimulated for between 0 and 60 min with or without 10 μM isoproterenol and then fractionated into particulate (pellet or P2) and cytosolic (soluble or S/N) fractions. Immunoblotting fractions with antibodies against PDE4D (recognizing all PDE4D isoforms) and RACK1 (Figure 5a) demonstrated that RACK1 immunoreactivity was clearly enriched in the pellet fraction, whereas PDE4D3 and PDE4D5 isoforms [4] were enriched in the supernatant fraction of cells (Figure 5a). Stimulation with isoproterenol seemed to have little effect on the distribution of PDE4D3, PDE4D5 or RACK1 between pellet and supernatant fractions at any of the time points tested (Figure 5a); however, isoproterenol could stimulate the phosphorylation of the PKA substrate CREB (cAMP-response-element-binding protein) (Figure 5b), which is phosphorylated in response to increased cAMP levels in a wide variety of cell types [21]. These results indicate that isoproterenol is able to stimulate elevations in intracellular cAMP levels in HEK-293 cells; however, RACK1–PDE4D5 does not appear to undergo activation-coupled translocation in response to elevated levels of intracellular cAMP in this cell type.

Effect of interaction with RACK1 on the activation of PDE4D5 by isoproterenol and PKC isoforms

We next sought to investigate whether interaction with RACK1 has an effect on the ability of particulate PDE4D5 to be activated by isoproterenol, which is a potent regulator of PDE4 activity in HEK-293 cells (Figure 4). RACK1 was immunoprecipitated from the pellet and soluble fractions of HEK-293 cells that had been stimulated with 10 μM isoproterenol for 15 min and PDE4D5 activity associated with the immunoprecipitates was determined by PDE assay (Supplementary Figure

S3 at <http://www.BiochemJ.org/bj/432/bj4320207add.htm>). The results demonstrated that isoproterenol stimulation provoked a significant 1.6-fold increase in PDE4D5 activity associated with RACK1 immunoprecipitates from solubilized particulate fractions, but not from soluble fractions (Supplementary Figure S3). This indicates that association with RACK1 causes a conformational change in the PDE4D5 associated with the particulate fractions, which enables PDE4D5 activation by the cAMP signalling cascade.

Given that RACK1 is a scaffold protein for activated conventional PKC isoforms [22], and that cAMP elevation has recently been linked to the activation of PKC isoforms in a number of different cell types [13], we next investigated whether conventional PKC isoforms could be involved in mediating the actions of isoproterenol on RACK1-bound PDE4D5. First, we found that stimulation of cells with the PKC activator PMA (10 μM) provoked similar levels of PDE4D5 activation to isoproterenol in the particulate fraction, but not the soluble fraction, of HEK-293 cells (Figure 4). HEK-293 cells were next stimulated with either isoproterenol or PMA and then cells were harvested and fractionated into particulate and soluble fractions, which were then immunoprecipitated with anti-RACK1 antibodies (Figure 6). PKCα was clearly found to translocate to the particulate fraction in response to PMA stimulation (Supplementary Figure S4 at <http://www.BiochemJ.org/bj/432/bj4320207add.htm>) indicating enzyme activation, as reported previously [23], where it was also found to associate with RACK1 as determined by immunoprecipitation (Figure 6a). Surprisingly, isoproterenol stimulation also provoked an increase in PKCα immunoreactivity in pellet fractions and in RACK1 immunoprecipitates from pellet fractions (Figure 6a), although the degree of translocation elicited by isoproterenol was far less than that produced by PMA. These results demonstrate that isoproterenol stimulation provokes a significant activation of RACK1-associated PKCα in the particulate fraction of HEK-293 cells. This idea is supported by experiments showing that isoproterenol stimulation is sufficient to provoke phosphorylation of a large number of intracellular proteins in HEK-293 cells in a PKC-dependent manner (Supplementary Figure S5 at <http://www.BiochemJ.org/bj/432/bj4320207add.htm>). Moreover, we also found that stimulation of HEK-293 cells with either isoproterenol or PMA provoked a robust phosphorylation of Ser¹²⁶ of RACK1-associated PDE4D5, which was blocked by co-incubation with the general PKC inhibitor GFX (Figure 6b). Given that phosphorylation of Ser¹²⁶ has been shown to be vital for PDE4D5 activation in response to elevations in intracellular cAMP [24], the cAMP- and PKC-dependent phosphorylation of this site provides a potential mechanism for the activation of RACK1-bound PDE4D5 in HEK-293 cells.

The identification of a stable interaction between RACK1 and PKCα, and the subsequent phosphorylation of Ser¹²⁶ of PDE4D5 leads to the question as to whether the interaction between PKCα and RACK1 has any effect on RACK1-bound PDE4D5 activity in the particulate fraction of HEK-293 cells. Our results from PDE assays carried out on RACK1 immunoprecipitates suggest that this might be the case (Supplementary Figure S3). To test this further we examined the PDE activity associated with RACK1 immunoprecipitated from cells that had been stimulated with either isoproterenol or PMA, in the presence or absence of the PKA inhibitor H-89 or the PKC inhibitor GFX (Figure 7a). Both isoproterenol and PMA were found to provoke a robust activation of RACK1-associated PDE activity, which was completely ablated by co-incubation with GFX (Figure 7a), indicating that the stimulation of PDE activity by both agents requires PKC. In contrast, co-incubation with H-89 only inhibited the

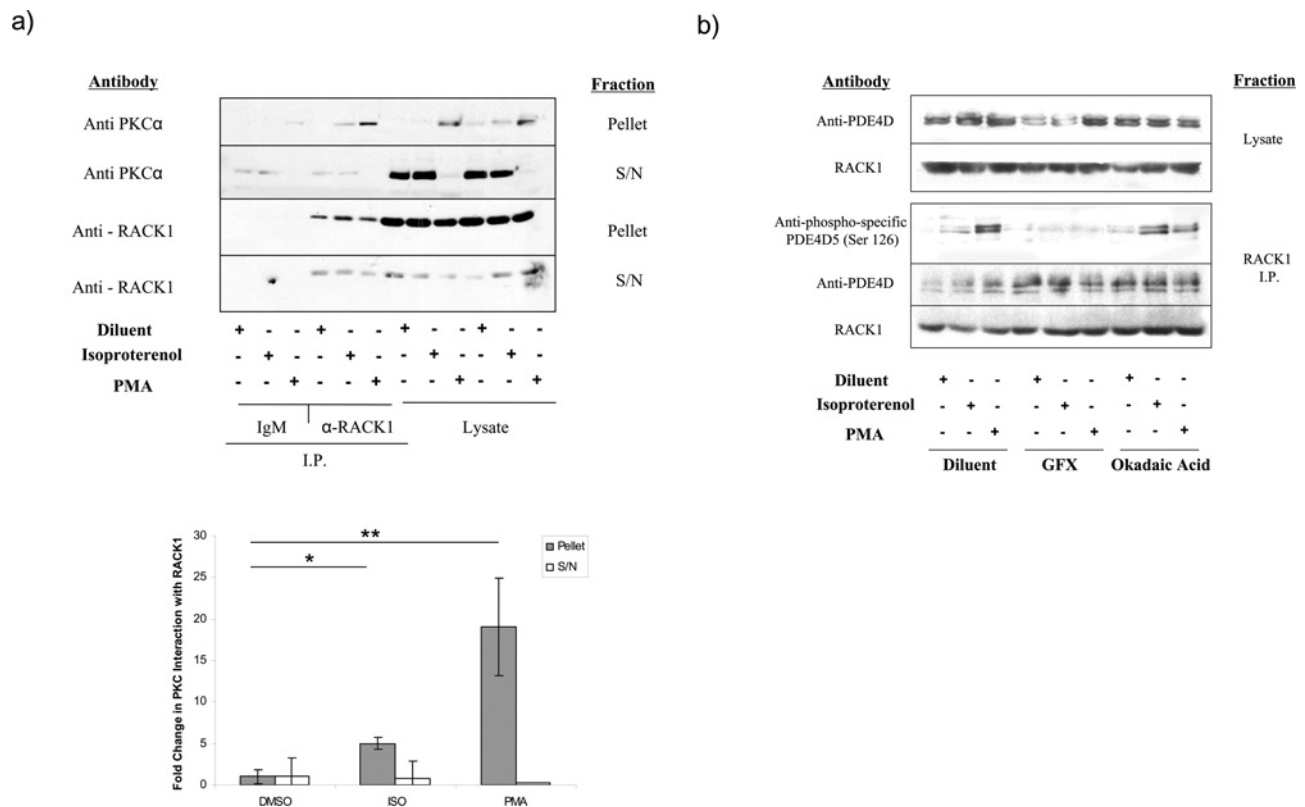


Figure 6 PMA or isoproterenol stimulation promotes the association of PKC α with RACK1 and promotes PKC-dependent phosphorylation of PDE4D5 on Ser¹²⁶ in the particulate fraction of HEK-293 cells

(a) Soluble (S/N) or particulate (pellet) fractions from HEK-293 cells were stimulated with diluent (DMSO), isoproterenol or PMA for 15 min, and then immunoprecipitated with either mouse IgM (negative control) or anti-RACK1 antibody (IgM clone), and immunoblotted with anti-RACK1 and anti-PKC α antibodies. Densitometric values from the RACK1-immunoprecipitated samples were plotted as means \pm S.E.M. for three separate experiments and the results are shown in the lower panel as the fold change in PKC α interaction with RACK1 in each fraction. Significant increases in PKC α immunoreactivity are indicated (* P < 0.05 and ** P < 0.01). (b) HEK-293 cells were stimulated with diluent (DMSO), isoproterenol or PMA for 15 min in the presence or absence of the general PKC inhibitor, GFX (10 μ M), or the PP2A inhibitor okadaic acid (10 μ M). RACK1 immunoprecipitates were then immunoblotted with specific antisera against pan-PDE4D, RACK1 and phospho-PDE4D, which recognizes phosphorylated Ser¹²⁶ of PDE4D5 (upper band) and PDE4D3 (lower band).

isoproterenol-stimulated PDE activity (Figure 7a). This indicates that part of the action of isoproterenol on RACK1-associated PDE activity requires PKA, as well as PKC, as previously reported [24]. We also found that our RACK1 immunoprecipitates from PMA-stimulated cells contained significant amounts of activated ERK (extracellular-signal-regulated kinase) (Figure 7b). Therefore to demonstrate that it is PKC activation in itself, and not the action of the downstream PKC-effector pathway, i.e. the Raf/ERK cascade [25], that is responsible for the activation of PDE4D5 in the particulate fraction of HEK-293 cells, cells were treated with general PKC inhibitors and the inhibitor of the Raf substrate MEK [MAPK (mitogen-activated protein kinase)/ERK kinase] [25], and PDE activity levels were determined (Figure 7c). HEK-293 cells were pre-treated with U0126, a specific inhibitor of MEK1 and 2, a known downstream effector of PKC signalling [25], or with the PKC specific inhibitors Ro-31-7549 or GFX for 30 min, then stimulated for 15 min with or without PMA. Immunoblots of cell lysates demonstrated a robust activation of the MEK substrate ERK following PMA stimulation, which is completely ablated following U0126, Ro-31-7549 or GFX treatment (Figure 7c). In contrast, treatment with U0126 failed to block particulate PDE4D5 activation following PMA stimulation, whereas treatment of PMA-stimulated cells with Ro-31-7549 and GFX caused a significant reduction in PDE activity (Figure 7d).

These results show that activation of PDE4D5 by PMA-stimulated PKC isoforms is through a direct effect of PKC on PDE4D5 and not through activation of the downstream effector MEK.

Finally, to test whether interaction with RACK1 is required for activation of particulate PDE4D5 by PKC, the effect of PMA was tested on HEK-293 cells transfected with PDE4D5-WT or the PDE4D5-L33D RACK1-binding mutant. Transfected cells were stimulated in the presence or absence of PMA for 15 min, before being harvested, fractionated and the PDE4D5 activity in fractions assayed by PDE assay. The results demonstrated an almost 3.5-fold increase in PDE4D5 activity in the pellet fraction from PDE4D5-WT-transfected cells stimulated with PMA, in comparison with the non-stimulated PDE4D5-WT pellet fraction (Figure 8a). In contrast, the pellet fraction from PMA-stimulated PDE4D5-L33D cells shows a PDE activity change of only approx. 1.5-fold (Figure 8a). These results show that interaction with RACK1 in the particulate fraction of HEK-293 cells is required for PKC to be able to efficiently activate PDE4D5. To confirm that interaction with RACK1 is required for effective activation of RACK1-associated PDE4D5, we took advantage of cell-permeant peptide inhibitors of the interaction between PDE4D5 and RACK1, compared with an additional peptide that disrupts the interaction between PDE4D5 and β -arrestin [11,26]. We found that disruption of the PDE4D5-RACK1 interaction

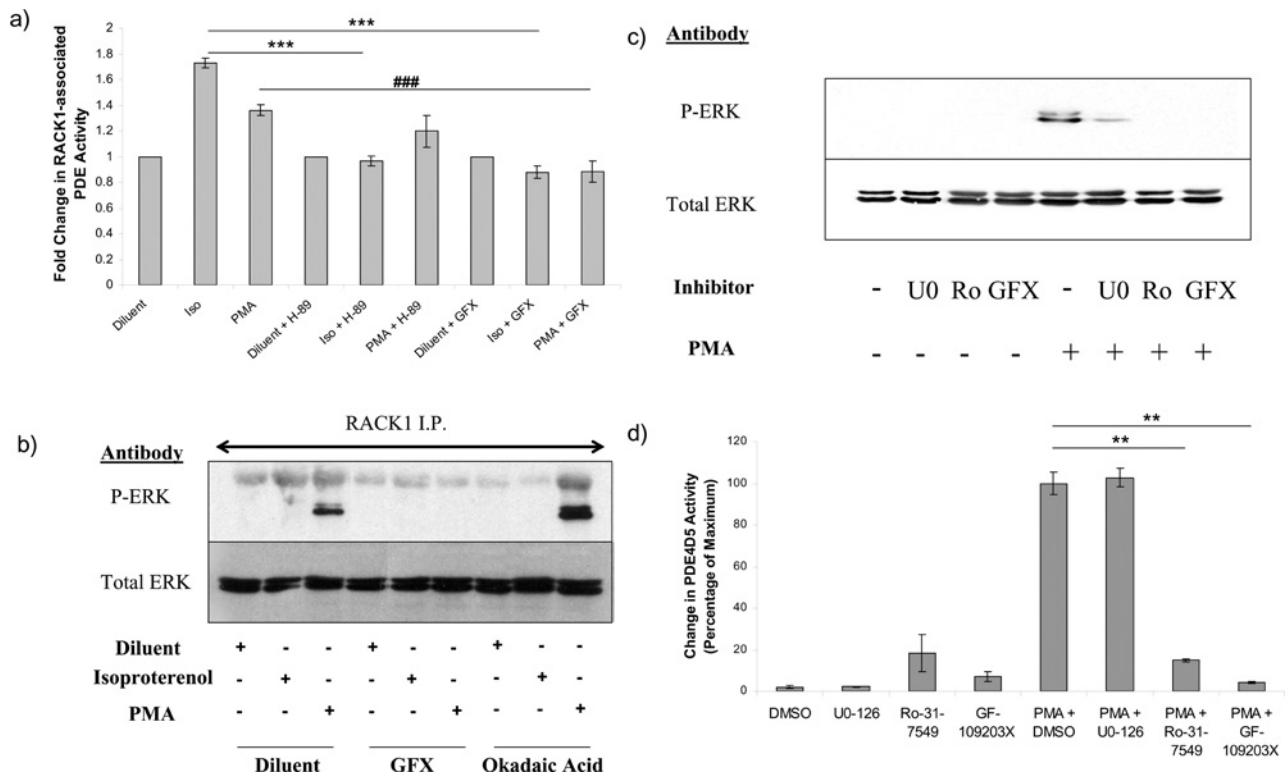


Figure 7 Activation of RACK1-associated PDE4D5 by PMA or isoproterenol requires PKC, but not ERK

(a) HEK-293 cells were stimulated for 15 min with either 10 μ M PMA or 10 μ M isoproterenol, in the presence or absence of the PKA inhibitor H-89 (10 μ M) or GFX (10 μ M). RACK1 was then immunoprecipitated from cells and associated PDE activities were determined as described in the Materials and methods section. Results represent means \pm S.E.M. for three separate experiments. Significant differences relative to cells stimulated with isoproterenol or PMA alone are indicated ($***P < 0.001$ and $###P < 0.001$). (b) RACK1 immunoprecipitates from cells stimulated with either 10 μ M PMA or 10 μ M isoproterenol, in the presence or absence of GFX (10 μ M) or okadaic acid (10 μ M), were immunoblotted with anti-phospho-ERK or anti-total-ERK antibodies. Results are a representative immunoblot from an experiment carried out on three separate occasions with similar results. (c) HEK293 cells were transfected with cDNA encoding PDE4D5-WT and then pre-treated for 30 min with either diluent (DMSO) or inhibitor (U0126, Ro-31-7549 or GFX) and then stimulated with or without PMA for 15 min. Lysates were then immunoblotted with anti-total-ERK and anti-phospho-ERK antibodies. Results represent a single experiment carried out on three separate occasions with similar results. (d) PDE4D5-WT-transfected cells were pre-treated with U0126, Ro-31-7549 or GFX prior to 15 min of PMA stimulation. Cells were then fractionated and PDE4D5 activity associated with the particulate fraction of cells was assessed with the PDE assay. Results are expressed as a percentage of the maximum (PMA-treated cells) and are means \pm S.E.M. for three separate experiments. Significant differences relative to PMA-treated cells are indicated ($**P < 0.01$).

caused a significant reduction in PDE activity in PDE4D5-specific immunoprecipitates from isoproterenol-stimulated HEK-293 cells, whereas disruption of the PDE4D5– β -arrestin interaction had little effect on isoproterenol-stimulated PDE4D5 activity (Figure 8b).

DISCUSSION

To fully appreciate the pleiotropic actions of cAMP in cells the role of specific cAMP PDEs, their interaction with cellular proteins and the effects of this interaction on PDE activity must be determined. Progress is starting to be made in this direction, particularly in the characterization of protein interactions with the cAMP-specific PDE4D5 and the scaffold protein β -arrestin [27–29]. This complex is recruited from the cytosol to the plasma membrane, in response to activation of the β 2-AR, to specifically hydrolyse cAMP in the vicinity of the β 2-AR [20,28]. In the present study we found that isoproterenol stimulation was sufficient to elevate cAMP and activate PDE4 activity in general in HEK-293 cells (Figure 4), and PDE4D5 specifically associated with RACK1 in the particulate fraction of cells (Figure 7a); however, we did not see any translocation of the RACK1–PDE4D5 complex (Figure 5a). This is perhaps not

surprising since the β -arrestin–PDE4D5 translocation seems to occur in cells that express β 2-AR to a very high level, e.g. in cardiomyocytes [30] or HEK-293 cells stably transfected to express high levels of receptor [20,30], and may reflect the ability of β -arrestin to develop high-affinity interactions with this receptor type. Indeed, little translocation of β -arrestin–PDE4D5 is seen in wild-type HEK-293 cells, with no overexpression of the β 2-AR (G. Baillie, unpublished work). The β 2-AR may therefore present an effective recruitment site for β -arrestin–PDE4D5 signalling complexes, but not for RACK1–PDE4D5 complexes. It therefore remains to be determined what structural influences direct the recruitment of RACK1–PDE4D5 complexes to the particulate fraction of cells.

We found that RACK1 binding exerted a conformational change in PDE4D5 in the particulate fraction of cells (Table 1), which sensitizes PDE4D5 towards cAMP, perhaps priming PDE4D5 for activation in this fraction. Rolipram also interacts with the active-site of PDE4D5 [31] and dose–response experiments revealed that particulate PDE4D5 was more susceptible to inhibition by rolipram if bound to RACK1 (Figures 2 and 3). Rolipram is a drug that has the potential to treat a variety of diseases, ranging from depression to Alzheimer's disease [32], but has a number of side effects, including an emetic effect [33]. The implication that RACK1 binding to PDE4D5 lowers the effective concentration

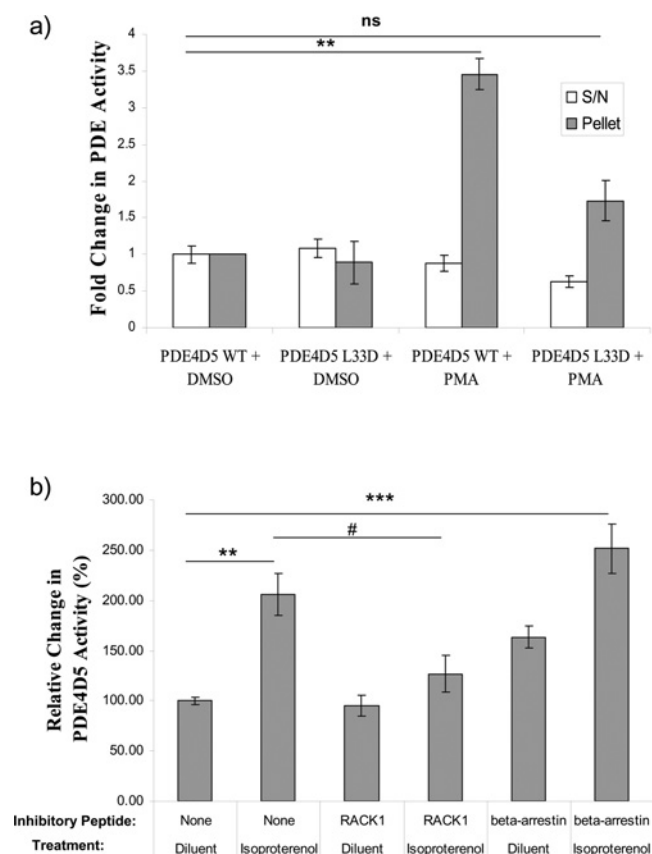


Figure 8 Association with RACK1 is required for PDE4D5 to be activated by PMA in the particulate fraction of HEK-293 cells

(a) Particulate (pellet) and soluble (S/N) fractions from HEK-293 cells transfected with either PDE4D5-WT or PDE4D5-L33D and then stimulated with either diluent (DMSO) or PMA for 15 min were assayed for PDE activity. Significant differences relative to control are indicated; $**P < 0.01$ ($n = 3$). PDE activities for PDE4D5-WT or PDE4D5-L33D prior to treatment were 60 ± 10 pmol/min per mg, in the pellet fraction, and 550 ± 50 pmol/min per mg, in the soluble fraction. (b) HEK-293 cells were stimulated with diluent or $10 \mu\text{M}$ isoproterenol in the presence or absence of cell-permeant inhibitory peptides that disrupt the interaction between PDE4D5 and RACK1, or PDE4D5 and β -arrestin, as indicated. Cell extracts were prepared and immunoprecipitated with an antibody against PDE4D5. Immunoprecipitates were then assayed for PDE activity. Results are means \pm S.E.M. for three separate experiments and significant differences relative to unstimulated cells or cells stimulated with isoproterenol alone are indicated ($**P < 0.01$; $***P < 0.001$; $\#P < 0.05$).

of rolipram, provides the possibility of RACK1-derived peptides (such as amino acids 35–48 and 99–108 in Figure 3), or synthetic analogues, being taken therapeutically to potentially lessen these side effects.

The present study also highlights the potential for direct phosphorylation and activation of RACK1-bound PDE4D5 by PKC α ; however, there have been no previous published reports demonstrating that PDE4D5 is a PKC substrate. Two mechanisms have been suggested by which PMA treatment leads to PDE4D5 activation. The first involves activation of an ERK-controlled autocrine loop [24]; however, this mechanism is unlikely to account for the rapid ERK-independent activation of RACK1-associated PDE4D5 seen in the present study. The second mechanism involves inactivation of the RACK1-binding partner [34,35] PP2A (protein phosphatase 2A) by PKC, leading to an increase in the phosphorylation of stimulatory Ser¹²⁶ on PDE4D5 [36]. In the present study we did find that both isoproterenol and PMA provoked a rapid PKC-dependent phosphorylation

of RACK1-associated PDE4D5 on Ser¹²⁶ (Figure 5b); however, the PP2A inhibitor okadaic acid had little effect on Ser¹²⁶ phosphorylation (Figure 6b), although it did enhance intracellular phosphorylation of PKC substrates in response to both of these agents (Supplementary Figure S5). It therefore remains to be determined whether PP2A is in fact a PKC-controlled regulator of RACK1-associated PDE4D5.

Altogether, the results of the present study demonstrate that the complex formed between PDE4D5 and RACK1 provides a novel point of cross-talk between the cAMP and PKC signalling pathways, providing a potential point of negative feedback in the cAMP signalling cascade. This potential has come to light given recent findings that increased cAMP levels activate PKC [13], which we confirm in the present study (Figure 6a and Supplementary Figure S5). Therefore if PKC activation increases PDE activity, as we demonstrate in the present study, this has the potential to reduce cellular cAMP levels and, in turn, limit PKC activation.

AUTHOR CONTRIBUTION

Rebecca Bird performed out all the experiments apart from the peptide array, which was performed by George Baillie. The study concept was devised by Stephen Yarwood, who also guided the day-to-day research and wrote the manuscript.

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SUPPLEMENTARY ONLINE DATA

Interaction with receptor for activated C-kinase 1 (RACK 1) sensitizes the phosphodiesterase PDE4D5 towards hydrolysis of cAMP and activation by protein kinase C

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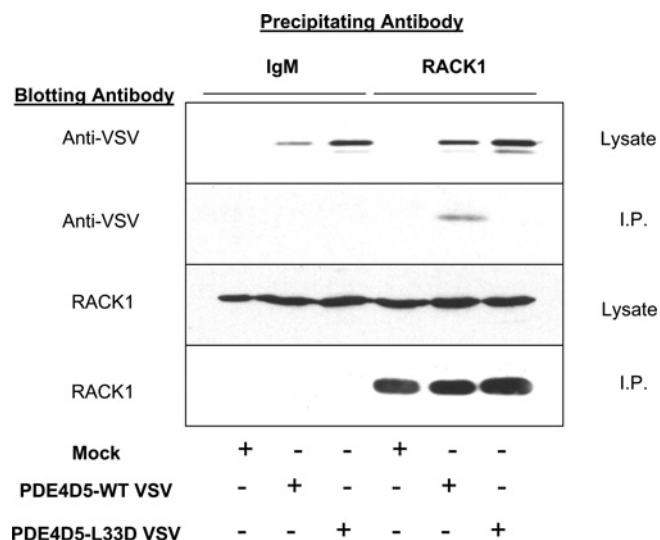


Figure S1 Wild-type PDE4D5, but not PDE4D5-L33D, associates with RACK1 in HEK-293 cells

Confluent HEK-293 cells were transiently transfected with VSV-tagged PDE4D5 wild-type (PDE4D5-WT) or VSV-tagged PDE4D5-L33D mutant (PDE4D5-L33D). Whole-cell lysates were then prepared and immunoprecipitated (I.P.) with control (IgM) antibody or anti-RACK1 antibody (IgM clone). Immunoprecipitates were then immunoblotted with anti-VSV antibodies, to detect PDE4D5 forms, or anti-RACK1 antibody. Results are representative of an experiment carried out on three separate occasions.

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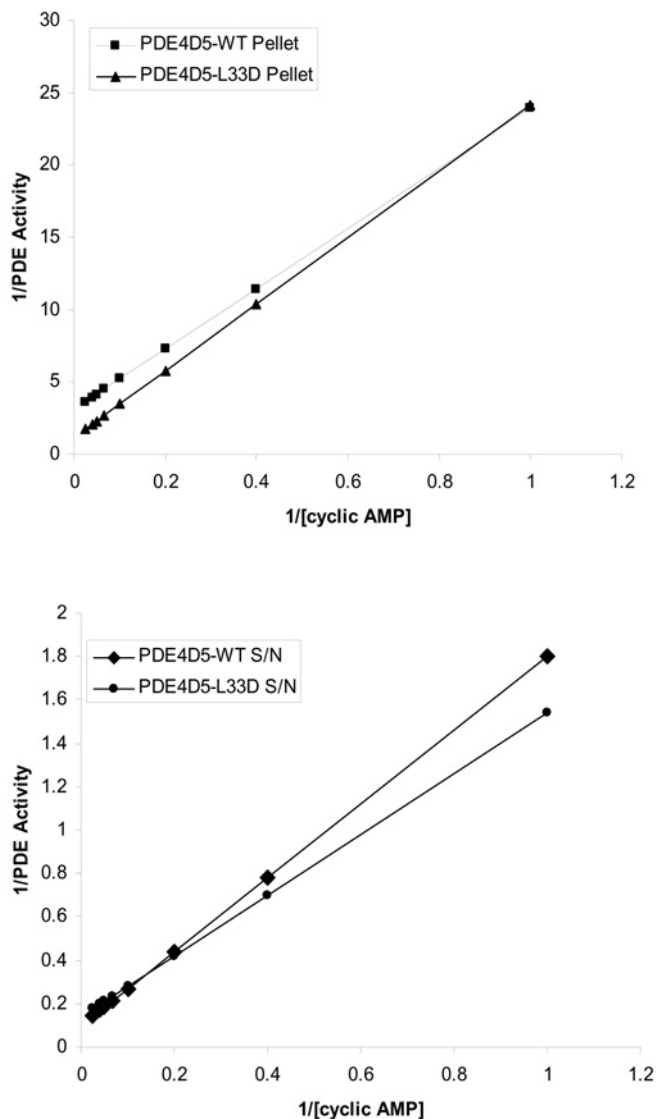


Figure S2 Lineweaver–Burk plots for PDE4D5-WT and PDE4D5-L33D

Particulate (pellet; upper panel) and soluble fractions (S/N; lower panel) from HEK-293 cells were transfected with cDNAs encoding PDE4D5-WT or PDE4D5-L33D and assayed for PDE activity in the presence of cAMP at concentrations between 1 μ M and 40 μ M. Mean PDE activities from three separate experiments were then used to produce Lineweaver–Burk plots for each fraction.

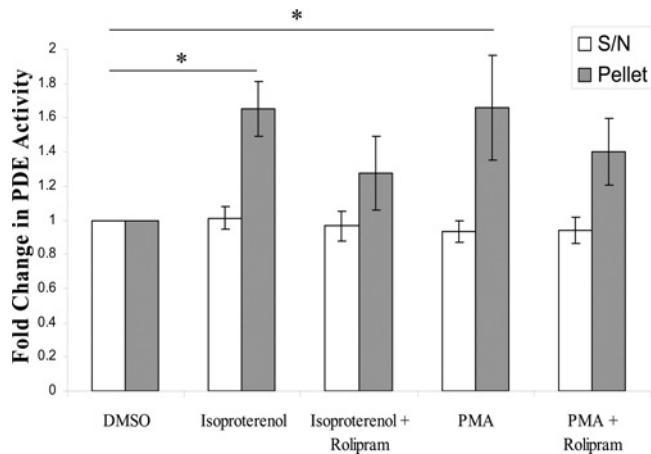


Figure S3 Isoproterenol and PMA stimulate PDE4 activity associated with RACK1 immunoprecipitates from HEK-293 cells

Confluent HEK-293 cells were stimulated with either 10 μ M isoproterenol or 10 μ M PMA and then fractionated into soluble (S/N) and particulate (pellet) fractions, which were then immunoprecipitated with anti-RACK1 antibodies. RACK1 immunoprecipitates were then assayed for associated PDE activity in the presence or absence of rolipram, as indicated. The results are means \pm S.E.M. for three separate experiments and are plotted as the fold change relative to non-stimulated cells (DMSO). Significant differences relative to the control are indicated (* $P < 0.01$).

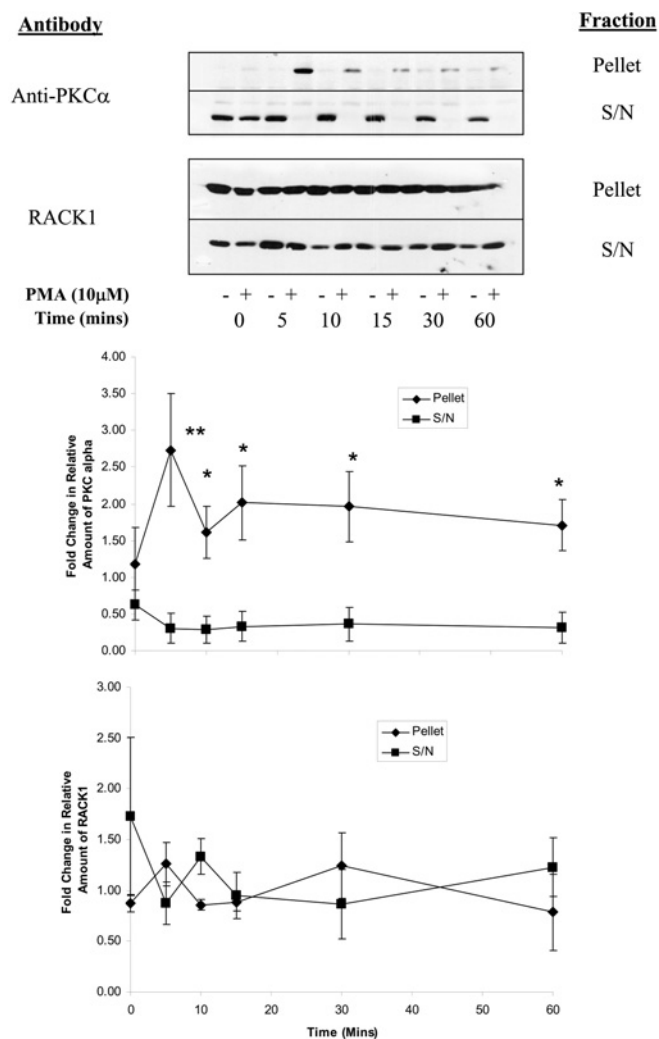


Figure S4 Stimulation of HEK-293 cells with PMA triggers intracellular translocation of PKC α , but not RACK1

HEK-293 cells were stimulated with 10 μ M PMA for between 0 and 60 min, following which cells were fractionated into soluble (S/N) and particulate (pellet) fractions then immunoblotted with anti-RACK1 and anti-PKC α antibodies. The immunoblots show that PKC α translocates from the supernatant to the pellet fraction within 5 min of PMA stimulation. Densitometry was carried out on non-saturated immunoblots from three separate experiments and plotted as means \pm S.E.M. Significant differences relative to non-stimulated control are indicated (* P < 0.05 and ** P < 0.01).

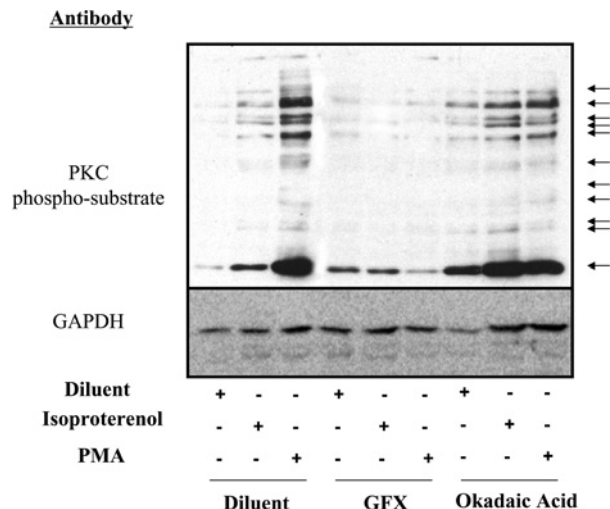


Figure S5 Stimulation of HEK-293 with isoproterenol provokes serine phosphorylation of multiple intracellular proteins in a PKC-dependent manner

HEK-293 cells were stimulated for 15 min with either 10 μ M isoproterenol or 10 μ M PMA in the presence or absence of 10 μ M GFX or 10 μ M okadaic acid, as indicated. Cell lysates were then immunoblotted with an anti-PKC substrate phospho-specific antibody. The arrows on the right-hand side indicate immunoreactive protein species phosphorylated in response to isoproterenol that were both inhibited by GFX and enhanced by okadaic acid.

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