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Rebecca J Bird, George S. Baillie, Stephen J Yarwood. Interaction with receptor for activated C kinase 1 (RACK1) sensitises PDE4D5 towards hydrolysis of cyclic AMP and activation by protein kinase C. *Biochemical Journal*, 2010, 432 (1), pp.207-216. 10.1042/BJ20101010 . hal-00529107

HAL Id: hal-00529107

<https://hal.science/hal-00529107>

Submitted on 25 Oct 2010

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Interaction with Receptor for Activated C Kinase 1 (RACK1) Sensitises PDE4D5 towards Hydrolysis of Cyclic AMP and Activation by Protein Kinase C

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Running head: Activation of PDE4D5 by PKC Requires Interactions with RACK1

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Key words: Cyclic AMP; RACK1; Phosphodiesterases; Protein kinase C; Intracellular Targeting

The abbreviations used are: PDE(s), cyclic nucleotide phosphodiesterase(s), PDE4(s), cAMP-specific phosphodiesterase(s); RACK1, receptor for activated C kinase; PKC, protein kinase C; PMA, phorbol 2-myristate 3-acetate; rolipram, 4-[3-(cyclopentoxyl)-4-methoxyphenyl]-2-pyrrolidinone; WD-repeat, a protein sequence motif containing repetitive sequences bounded by tryptophan and aspartic acid residues; VSV, vesicular stomatitis virus; SDS, sodium dodecyl sulphate; PAGE, polyacrylamide gel electrophoresis.

Accepted Manuscript

Synopsis

We have identified the Protein Kinase C (PKC) anchoring protein, Recceptor for Activated Cinase 1 (RACK1), as a specific binding partner for the cyclic AMP-specific phosphodiesterase, PDE4D5, suggesting a potential site for cross-talk between the PKC- and cyclic AMP-signalling pathways. Here we find that elevation of intracellular cyclic AMP with the β 2-adrenoceptor agonist, isoproterenol, led to activation of PDE4 enzymes in the particulate and soluble fractions of HEK293 cells, whereas activation of PDE4D5 by 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 did demonstrate that RACK1 alters the conformation of particulate-associated PDE4D5 so that it more readily interacts with its substrate, cyclic AMP, and with rolipram, a PDE4 inhibitor that specifically targets the enzymes active site. Interaction with RACK1 was also essential for PKC-dependent, and ERK-independent, phosphorylation (on Ser 126) 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 HEK293 cells in response to elevations in intracellular cyclic AMP.

Introduction

Cyclic AMP is a ubiquitous second messenger that controls pleiotropic cellular actions through the direct activation of its effectors, proteins kinase A (PKA) and exchange protein activated by cyclic AMP (EPAC) proteins [1, 2]. Cyclic AMP phosphodiesterases (PDEs) are crucial components of the cyclic AMP signalling system and represent the only means of reducing cyclic AMP levels in cells through hydrolysis to 5'AMP [3]. Of the 11 known classes of cyclic nucleotide PDEs, 8 are able to hydrolyse cyclic AMP [4, 5]. Of these the PDE4, cyclic AMP-specific class is widely expressed in a variety of tissues and accounts for the majority of cyclic AMP hydrolysis activity in cells [6]. PDE4s are encoded by 4 genes that, through complex alternate splicing of cognate mRNAs, give rise to around twenty 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]. For example, the phosphodiesterase PDE4D5 has been found to interact with the ubiquitously expressed, WD-repeat signalling scaffold protein, receptor for activated protein kinase C 1 (RACK1) [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 Protein Kinase C (PKC) isoforms, dynamin-1, Ras-GAP, 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 sub-cellular 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 cyclic AMP 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 this blades of the RACK1 β -propeller form a groove to which the N-terminus of PDE4D5 binds [8]. An 88 amino acid sub-domain, termed the RACK1 interaction domain (RAID1), 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 while 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; for example migration of breast cancer cells in response to IGF-1 is thought to be regulated by a RACK1-directed protein complex involving the IGF-1 receptor, integrin receptors and focal adhesion kinase (FAK) [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 regards 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, 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, like cyclic AMP-activatable PKC α [13], is largely unknown and may reveal key areas of novel signalling regulation and crosstalk. The aims of the current investigation therefore are to determine the consequences of interaction with RACK1 on the regulation of PDE4D5.

Materials and Methods

Materials

GF 109203X, Phorbol 12-myristate 13-acetate, Ro31-7549, Rolipram, and Isoproterenol were purchased from Merck Biosciences. Antibodies to GAPDH, PKC α , RACK1 (IgM clone) and VSV 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

HEK293 cells were cultured at 37°C in 5% (v/v) CO₂ in Dulbecco's Modified Eagle's Medium (DMEM; Sigma-Aldrich) containing 10% (v/v) foetal bovine serum (Sigma-Aldrich), 2mM L-glutamine and 2% (w/v) Penicillin/Streptomycin.

Transfection of Cells

Cells were transfected at approximately 50% confluence with a N-[1-(2,3-Dioleoyloxy)propyl]-N,N,N-trimethylammoniummethylsulfate (DOTAP)/DNA mixture, prepared by diluting 7.5 μ g plasmid DNA 1:10 (v/v) in DMEM then mixing with DOTAP diluted in DMEM according to the manufacturers instructions. The mixture was then incubated at room temperature for 30 minutes before being added to cells in fresh culture medium. Cells were then incubated overnight at 37°C in 5% (v/v) CO₂ 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 (10mM Tris-HCl, pH 7.5, 0.1mM EDTA, containing protease inhibitor cocktail (Boehringer)) and then lysed by seven strokes of a 26½ G needle fixed to a 1ml disposable syringe. Unbroken cells and nuclei were pelleted in a bench top centrifuge at 1000 x g_{max} for 5 minutes at 4°C. Supernatants were then transferred to chilled Beckman ultracentrifuge tubes and then centrifuged in a bench top ultrafuge at 100,000 x g_{max} for 30 minutes 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 again at 100,000 x g_{max} for a further 30 minutes. 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 an OD600 of 0.6 and then induced with 1.0 mM isopropyl-b-D-thiogalactopyranoside (Roche) and grown a further 4 h at 30°C. Bacteria were then harvested by centrifugation at 4000 x g_{max} for 10 min at 4°C and the bacterial pellets frozen at -80°C overnight. To purify recombinant GST-PDE4D5 pellets were resuspended in 10 ml of ice-cold resuspension buffer, 50 mM Tris-HCl, pH 8.0, 100 mM NaCl, 1mM EDTA, 10mM β -mercaptoethanol and Complete protease inhibitor mixture (Boehringer), and sonicated on a maximal setting for 4 x 30 sec on ice. Triton X-100 was then added to a final concentration of 0.02% (v/v), and cell debris was removed by centrifugation at 10000x g_{max} for 10 min at 4°C. The cleared lysate was then incubated with 1/10th volume of pre-equilibrated glutathione-Sepharose beads (Pharmacia) for 1 hour end-over-end at 4°C. The beads were then collected by centrifugation at 2000 x g_{max} 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 (10mM Tris-HCl, pH 7.5, 0.1mM EDTA, containing protease inhibitor cocktail (Boehringer)). Following this 0.1% (v/v) Triton X-100 was added to each sample and incubated on ice for 15 minutes. At this stage 50 μl per sample was retained and boiled in Laemli electrophoresis buffer [14] as input controls. Samples were pre-cleared by adding 5 μg of mouse IgM and 50 μl anti-mouse IgM agarose (Sigma) to each sample. Samples were then incubated on an orbital shaker for 30 minutes at 4°C . Beads were then pelleted and the supernatant transferred to fresh eppendorfs. Duplicates of each sample were then treated with either 5 μg of anti-RACK1 antibody (IgM clone) or 5 μg mouse IgM and incubated on a wheel in cold room for 30 min. 50 μl of anti-mouse IgM-agarose was then added to each eppendorf and samples incubated at 4°C for a further 30 minutes. The agarose beads were then pelleted for 30 seconds at $13000 \times g_{\text{max}}$ and washed 3 times with 500 μl lysis buffer. Beads were then boiled in electrophoresis buffer and separated by SDS-PAGE along with the retained input controls.

Western Immunoblotting

Cells were scraped directly into Laemli electrophoresis buffer [14] and separated by SDS-PAGE electrophoresis on 12% resolving gels before being transferred to nitrocellulose membranes. Membranes were blocked in 5% (w/v) milk powder in Tris-buffered saline containing 0.1% (v/v) Tween 20 (TBST), or 5% (w/v) bovine serum albumin (BSA) in TBST for phospho-specific antibodies. Membranes were then incubated overnight in appropriate primary antibody at 4°C . Primary antibody was then removed and membranes washed three times with TBST and then incubated for 1 hour at room temperature with horse radish peroxidase (HRP)-conjugated secondary antibody. Blots were developed using Enhanced Chemiluminescence (ECL) (GE Healthcare UK) according to 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-chemistry with an AutoSpot-Robot ASS 222 (Intavis Bioanalytical Instruments AG, Köln, Germany) [15, 16]. The interaction of spotted peptides with GST-PDE4D5 fusion protein was determined by overlaying the cellulose membranes with 10 $\mu\text{g}/\text{ml}$ of recombinant protein. Bound recombinant GST-PDE4D5 was detected with specific rabbit antisera and detection performed with secondary anti-rabbit horseradish peroxidase coupled antibody (1:2,500 dilution; Dianova) and visualisation by ECL, as in the *Western Immunoblotting* protocol.

Phosphodiesterase Enzyme Assay

Phosphodiesterase (PDE) activity in samples was assayed in cell extracts as previously described [4], using a 0.2 μM cyclic AMP substrate solution containing 5 μCi ^3H -cyclic AMP per ml prepared in assay buffer (40mM Tris-HCl, 5mM MgCl_2 , 3.75mM β -mercaptoethanol).

Reactions were incubated for 10min at 30°C and then stopped by boiling for 2 minutes. Tubes were transferred to watery ice and left to cool completely. 25µl of snake venom solution (1mg/ml) from *Crotalus atrox* was then added and the tubes incubated for 10min at 30°C. The tubes were then returned to ice and 400µl of previously prepared Dowex/water/ethanol (1:1:1) slurry was added to each tube. The tubes were left on ice for 15 minutes, with occasional vortexing, and then centrifuged at 13000 x g_{max} for 2 minutes at 4°C in a refrigerated bench top centrifuge. 150µl of supernatant from the reaction tubes was added to scintillation tubes containing 4ml scintillant, with total counts being obtained by the addition of 50µl cyclic AMP substrate solution, added to 4ml of scintillant and then assayed in a scintillation counter. Initial rates were taken from linear time courses of activity in all experiments. K_m and V_{max} determinations were done over a range of substrate concentrations between 1µM and 40µM cyclic AMP, 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 0.2µM cyclic AMP over a range of rolipram concentrations between 0.01nM and 1mM.

Densitometry and Statistical Analysis

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

Results

The Effect of RACK1 on PDE4D5 Subcellular Localisation We have previously identified RACK1 protein as a cellular binding partner for PDE4D5 [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, HEK293 cells were transfected with VSV-tagged cDNAs encoding either wild type PDE4D5 (PDE4D5-WT) or VSV-tagged PDE4D5 where the leucine at position 33 in the unique N-terminus had been mutated to aspartate (PDE4D5-L33D). The PDE4D5-L33D mutation in PDE4D5 has previously been shown to prevent interaction with RACK1 [17] and immunoprecipitation experiments confirmed that this was the case in HEK293 cells (Supplementary Figure 2). Moreover, immunoblotting and cyclic AMP PDE assays of fractions demonstrated that the relative distributions of PDE4D5-WT and PDE4D5-L33D protein and activity, was approximately equal between the two fractions (Figure 1) whereas both PDE4D5-WT and PDE4D5-L33D immunoreactivity is enriched in the cytosolic fraction with lower levels of expression in the particulate fractions (Figure 1). Together this data suggests that association with RACK1 does not play a significant role in determining the sub-cellular distribution of PDE4D5 in HEK293 cells.

The 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 cyclic AMP (Michaelis-Menten constant or K_m) by varying the amount of substrate (ranging between $1\mu\text{M}$ and $40\mu\text{M}$). Lineweaver-Burke plots of results demonstrated that Michaelis/Menten kinetics was being followed at each of these substrate concentrations (Supplementary Figure 1). V_{\max} values determined for pellet fractions of both PDE4D5-WT and PDE4D5-L33D were low, at 0.32 ± 0.07 and 0.87 ± 0.09 pmol/min/mg respectively, while the supernatant fractions displayed higher rates of catalytic activity, with PDE4D5-WT possessing a V_{\max} of 9.71 ± 2.09 pmol/min/mg, greater than the PDE4D5-L33D V_{\max} value of 7.14 ± 1.62 pmol/min/mg (Table 1). The higher rate of catalysis observed in the supernatant fractions of PDE4D5-WT and PDE4D5-L33D transfected HEK293 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 cyclic AMP. The pellet fraction of PDE4D5-WT, however, shows 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 cyclic AMP when compared to 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 high affinity of PDE4D5 for cyclic AMP in the membrane fraction of cells. Next we determined the effect of RACK1 binding on susceptibility of PDE4D5 to a specific enzyme inhibitor was investigated by measuring the dose response of PDE4D5-WT and PDE4D5-L33D to the PDE4 inhibitor, rolipram (0.01nM to 1mM). The responses of PDE4D5-WT and PDE4D5-L33D to inhibition by rolipram were very similar; whereas the pellet fraction graph shows 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 HEK293 cells. Together these results (summarised in Table 1) suggest that PDE4D5 binding to RACK1 is necessary to enable high affinity binding of PDE4D5 to its substrate, cyclic AMP, and for effective inhibition by rolipram in the particulate fraction of HEK293 cells.

To test this idea further we carried out competition experiments to attempt to disrupt association of PDE4D5-WT with RACK1 in the particulate fraction of HEK293 cells with RACK1-derived peptides ($500\mu\text{M}$). Pellet fractions were incubated with 0.2 μM rolipram (Figure 3a); a concentration of rolipram induced an approximate 40% inhibition of PDE4D5

in this fraction (Figure 3a). Rolipram inhibition was effectively ablated by RACK1-derived 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 to by amino acids 35-48 and 99-108. To test this assumption peptide array experiments were carried out using a range of immobilised peptides, that 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 immobilised 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 a conformational changes in PDE4D5 that are characterised by high affinity interaction with rolipram.

PMA Promotes PDE Activation in the Particulate, but not the Soluble Fraction, of HEK293 cells Results so far suggest that RACK1 can sensitise PDE4D5 towards interaction with cyclic AMP and may therefore play a role in regulating PDE4D5 activity *per se*. We therefore sought to determine the role of the RACK1-interaction partner, protein kinase C (PKC), on the activation of PDE4 isoforms in HEK293 cells. Cells were treated for 15 minutes in the presence or absence of the β 2-Adrenoreceptor (β 2-AR) agonist isoproterenol (10 μ M) [18] or the PKC-activator, phorbol-12myristate-13-acetate (PMA, 10 μ M) [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 *Materials and Methods*. Analysis of 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 rolipram (10 μ M), in the assay (Figure 4). Analysis of the supernatant fraction also demonstrated that isoproterenol stimulates cell PDE4 activity, with an approximate 1.6 fold increase when compared to 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 HEK293 cells. These results suggest that an increase in cyclic AMP levels following isoproterenol stimulation causes a significant increase in PDE4 activity in soluble and particulate fractions of HEK293 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 HEK293 Cells

The effects of PDE4D5 binding to RACK1 on the cellular localisation and activity of PDE4D5 has yet to be determined. We therefore sought to determine whether translocation of PDE4D5/RACK1, as previously reported for the PDE4D5/ β -arrestin signalling complex [20], contributed to the increases in particulate PDE activity following isoproterenol stimulation described in Figure 4. HEK293 cells were stimulated between 0 and 60 min with or without 10 μ M isoproterenol and then fractionated into particulate (pellet or P2) and cytosolic (supernatant or S/N) fractions. Immunoblotting fractions with antibodies to PDE4D, which recognises all PDE4D isoforms, and RACK1 (Figure 5a) demonstrated that RACK1 immunoreactivity is clearly enriched in the pellet fraction whereas PDE4D3 and PDE4D5 isoforms [4] are 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 cyclic AMP response element binding protein (CREB), which is phosphorylated in response to increased cyclic AMP levels in a wide variety of cell types [21] (Figure 5b). These results indicate that isoproterenol is able to stimulate elevations in intracellular cyclic AMP levels in HEK293 cells; however the RACK1/PDE4D5 does not appear to undergo activation-coupled translocation in response to elevated levels of intracellular cyclic AMP 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 HEK293 cells (Figure 4). RACK1 was immunoprecipitated from the pellet and soluble fractions of HEK293 cells that had been stimulated with isoproterenol (10 μ M) for 15 minutes and PDE4D5 activity associated with the immunoprecipitates determined by PDE assay (Supplementary Figure 3). Results demonstrated that isoproterenol stimulation provoked a significant 1.6 fold increase in PDE4D5 activity associated with RACK1 immunoprecipitates from solubilised particulate fractions, but not from soluble fractions (Supplementary Figure 3). This indicates that association with RACK1 causes a conformational change in PDE4D5 associated with particulate fractions that enables PDE4D5 activation by the cyclic AMP signalling cascade.

Given that RACK1 is a scaffold protein for activated conventional PKC isoforms [22], and that cyclic AMP 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. Firstly we found that stimulation of cells with the PKC activator, PMA (10 μ M), provoked similar levels of PDE4D5 activation as isoproterenol in the particulate fraction, but not soluble fraction, of HEK293 cells (Figure 4). HEK293 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). PKC α was clearly found to translocate to the particulate fraction in response to PMA stimulation (Supplementary Figure 4, indicating enzyme activation as previously reported [23], where it was found to associate with RACK1 as determined by immunoprecipitation (Figure 6a). Surprisingly, isoproterenol stimulation also provoked an increase in PKC α 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 PKC α in the particulate fraction of HEK293 cells. This idea is supported by experiments showing that isoproterenol stimulation is sufficient to provoke phosphorylation of a large number of intracellular proteins in HEK293 cells in a PKC-dependent manner (Supplementary Figure 5). Moreover, we also found that stimulation of HEK293 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, GF109203X (GFX) (Figure 6b). Given that phosphorylation of Ser126 has been shown to be vital for PDE4D5 activation in response to elevations in intracellular cyclic AMP [24], the cyclic AMP and PKC-dependent phosphorylation of this site provides a potential mechanism for the activation of RACK1-bound PDE4D5 in HEK293 cells.

The identification of a stable interaction between RACK1 and PKC α and the subsequent phosphorylation of Ser126 of PDE4D5 leads to the question as to whether the interaction between PKC α and RACK1 has any affect on RACK1-bound PDE4D5 activity in the particulate fraction of HEK293 cells. Our results from PDE assays carried out on RACK1 immunoprecipitates suggest that this might be the case (Supplementary Figure 3). 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 agent requires PKC. In contrast, co-incubation with H-89 only inhibited 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 (Figure 7b). Therefore, to demonstrate that it is PKC activation *per se*, and not the action of the downstream PKC-effector pathway, the Raf-ERK cascade [25], that is responsible for the activation of PDE4D5 in the particulate fraction of HEK293 cells, cells were treated with general PKC inhibitors, and the inhibitor of the Raf substrate, MEK [25], and PDE activity levels determined (Figure 7c). HEK293 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 minutes, then stimulated for 15 minutes 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 HEK293 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 minutes, before being harvested, fractionated and the PDE4D5 activity in fractions assayed by PDE assay. 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 non-stimulated PDE4D5-WT pellet fraction (Figure 8a). In contrast, the pellet fraction from PMA-stimulated PDE4D5-L33D cells shows a PDE activity fold change of around only 1.5 (Figure 8a). These results show that interaction with RACK1 in the particulate fraction of HEK293 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-permeable peptide inhibitors of the interaction between PDE4D5 and RACK1, compared with an additional peptide that disrupts interaction between PDE4D5 and β -arrestin [11, 26]. We found that disruption of PDE4D5/RACK1 interaction caused a significant reduction in PDE activity in PDE4D5-specific immunoprecipitates from isoproterenol-stimulated HEK293 cells, whereas disruption of PDE4D5/ β -arrestin interaction had little effect on isoproterenol-stimulated PDE4D5 activity (Figure 8b).

Discussion

To fully appreciate the pleiotropic actions of cyclic AMP in cells the role of specific cyclic AMP 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 characterisation of protein interactions with the cyclic AMP-specific PDE, 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 cyclic AMP in the vicinity of the β 2-AR [20, 28]. In the current investigation we found that isoproterenol stimulation was sufficient to elevate cyclic AMP and activate PDE4 activity in general in HEK293 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 β -arrestin/PDE4D5 translocation seems to occur in cells that express β 2-AR to a very high level, for example in cardiomyocytes [30] or HEK293 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 HEK293 cells, with no overexpression of the β 2-AR (Dr George Baillie, unpublished data). 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 as to what are the structural influences that 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) that sensitises PDE4D5 towards cyclic AMP, 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 [32], but has a number of side effects, including an emetic effect [33]. The implication that RACK1 binding to PDE4D5 lowers the effective concentration 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.

This work also highlights the potential for direct phosphorylation and activation RACK1-bound of PDE4D5 by PKC α , however there have been no previous published reports demonstrating that PDE4D5 is a *bona fide* 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 in the current study. The second involves inactivation of the RACK1-binding partner [34, 35], protein phosphatase 2A (PP2A), by PKC, leading to an increase in the phosphorylation of stimulatory Ser-126 on PDE4D5 [36]. In the current study we did find that both isoproterenol and PMA provoked a rapid PKC-dependent phosphorylation of RACK1-associated PDE4D5 on Ser 126 (Figure 5b), however the PP2A inhibitor, okadaic acid, has 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 5). It therefore remains to be determined therefore whether PP2A is in fact a PKC-controlled regulator of RACK1-associated PDE4D5.

Altogether, the results from the present study demonstrate that the complex formed between PDE4D5 and RACK1 provides a novel point of crosstalk between the cyclic AMP and PKC signalling pathways, providing potential point of negative feedback in the cyclic AMP signalling cascade. This potential has come to light given recent findings that increased cyclic

AMP levels have been shown to activate PKC [13], which we confirm in the current study (Figure 6a and Supplementary Figure 5). Therefore if PKC activation increases PDE activity as we demonstrate in the current paper, this has the potential to reduce cellular cyclic AMP levels and, in turn, limit PKC activation.

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Funding

This work was funded by a studentship from the Biotechnology and Biological Sciences Research Council awarded to R.J.B. and a British Heart Foundation Project Grant (Grant number PG/10/026/28303) awarded to S.J.Y.

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Figure Legends

Figure 1 Distribution PDE4D5-WT and PDE4D5-L33D Activity and Immunoreactivity between Soluble and Particulate Fractions of HEK293 Cells Pellet (P2) and cytosol (S/N) fractions from HEK293 cells transfected with VSV-tagged, PDE4D5 wild type (PDE4D5-WT) or VSV-tagged, PDE4D5 L33D (PDE4D5-L33D), non-RACK1 binding mutant DNA, were immunoblotted with anti-RACK1 and anti-VSV specific antibodies. Densitometric values were obtained from non-saturating immunoblots from 3 separate experiments and plotted on the *upper* graph, to show the relative distributions of PDE4D5-WT and PDE4D5-L33D between fractions, and the *centre* graph, to show the relative distribution of RACK1. PDE assays were also carried out on fractions from transfected cells and these are displayed in the *lower* graph.

Figure 2 Dose-response of PDE4D5-WT and PDE4D5-L33D to Inhibition by Rolipram Particulate (P2) or soluble (S/N) fractions from HEK293 cells transfected with cDNAs encoding either PDE4D5-WT or PDE4D5-L33D and then cells were treated with various concentrations of the PDE4 inhibitor, rolipram, ranging from 0 to 1mM, then assayed for PDE activity. PDE activities obtained from three separate experiments were then plotted as means \pm SEM. PDE activities, in the absence of inhibitor, for PDE4D5-WT or PDE4D5-L33D were 60 \pm 10 pmol/min/mg, in the pellet fraction, and 550 \pm 50 pmol/min/mg in the soluble fraction, respectively.

Figure 3 RACK1-derived Peptides corresponding to the RACK1/PDE4D5 Interaction Interface Block the Inhibition of Particulate PDE4D5 by Rolipram

- Particulate fractions were prepared from HEK293 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 *Experimental Procedures*. RACK1-derived peptides (corresponding to amino acids 10-19, 35-48, 59-68, 99-108 and 272-283 or RACK1 as indicated) were also included in PDE assays. Results are presented as percentage inhibition of PDE4D5 activity and significant differences relative to samples treated with rolipram, but no peptide or rolipram plus RACK1 peptide (59-68) are indicated (*, $p < 0.05$ and #, $p < 0.05$ respectively). PDE activities, in the absence of inhibitor, for PDE4D5-WT or PDE4D5-L33D were 60 \pm 10 pmol/min/mg, in the pellet fraction, and 550 \pm 50 pmol/min/mg in the soluble fraction, respectively.
- RACK1-derived peptide array overlay experiments were carried out using purified, recombinant GST-PDE4D5-WT as described in *Experimental Procedures*. Two arrays of five overlapping peptides were prepared corresponding to the region of RACK1 that encompasses RACK1 peptides 35-48 and 99-108, respectively.

Figure 4 Enhanced PDE4 Activity Following Stimulation of HEK293 Cells with Isoproterenol or PMA HEK293 cells were stimulated with 10 μ M isoproterenol or 10 μ M PMA for 15 minutes and then harvested and fractionated into high speed pellet and supernatant fractions then assayed for PDE hydrolytic activity in the presence or absence of the type 4 PDE inhibitor, rolipram (10 μ M), as described in *Experimental Procedures*. Values obtained from 3 separate experiments were normalised and plotted as means \pm SEM for each cellular fraction. Significant differences relative to rolipram treated samples are indicated by *, $p < 0.05$.

Figure 5 RACK1 and PDE4D5 do not Undergo Activation-coupled Translocation following Isoproterenol Stimulation of HEK293 Cells

- Pellet and supernatant fractions from HEK293 cells stimulated with 10 μ M isoproterenol at the indicated time points were Immunoblotted with anti-PDE4D and anti-RACK1 specific antibodies. RACK1 was enriched in the pellet fraction, while PDE4D5 and PDE4D3 (upper and lower bands of the doublet, of 105 and 95 kDa

respectively, shown on the blot) are enriched in the supernatant fraction. Immunoblots are representative of 3 separate experiments.

- b) HEK293 cells were stimulated with 10 μ M isoproterenol for the indicated times between 0 and 60 minutes and then immunoblotted with anti-CREB and anti-phosphoCREB (Ser133) specific antibodies. Results are representative of 3 separate immunoblots.

Figure 6 PMA or Isoproterenol Stimulation Promotes the Association of PKC α with RACK1 and Promotes PKC-dependent Phosphorylation of PDE4D5 on Ser 126 in the Particulate Fraction of HEK293 Cells.

- a) Soluble (S/N) or particulate (pellet) fractions from HEK293 cells were stimulated with diluent (DMSO), isoproterenol or PMA for 15 minutes and then immunoprecipitated with either mouse IgM (negative control) or anti-RACK1 (IgM clone) antibody and then immunoblotted with anti-RACK1 and anti-PKC α antibodies. Densitometric values from the RACK1-immunoprecipitated samples were plotted as means \pm SEM (n=3) in the *lower panel* and represent 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) HEK293 cells were stimulated with diluent (DMSO), isoproterenol or PMA for 15 minutes, 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 to pan-PDE4D, RACK1 or phospho-specific PDE4D, which recognises phosphorylated Ser126 of PDE4D5 (upper band) and PDE4D3 (lower band), respectively.

Figure 7 Activation of RACK1-associated PDE4D5 by PMA or Isoproterenol Requires PKC and not ERK

- a) HEK293 cells were stimulated for 15 minutes 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 *Experimental Procedures*. Results represent an experiment carried out on three separate occasions and are presented as means \pm SEM. Significant differences relative to cells stimulated with isoproterenol or PMA alone are indicated (***, p<0.001 and ####, p<0.001, respectively).
- 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 pretreated for 30 min with either diluent (DMSO) or inhibitors U0126, Ro-31-7549 or GFX and then stimulated with or without PMA for 15 minutes. Lysates were then immunoblotted with total ERK and 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 Uo126, Ro-31-7549 or GFX prior to 15 minutes PMA stimulation. Cells were then fractionated and PDE4D5 activity associated with the particulate fraction of cells was assessed by PDE assay. Results are expressed as percentage of maximum (PMA-treated cells) and are means \pm SEM for 3 separate experiments. Significant differences relative to PMA-treated cells are indicated, **, p<0.01.

Figure 8 Association with RACK1 is Required for PDE4D5 to be Activated by PMA in the Particulate Fraction of HEK293 Cells

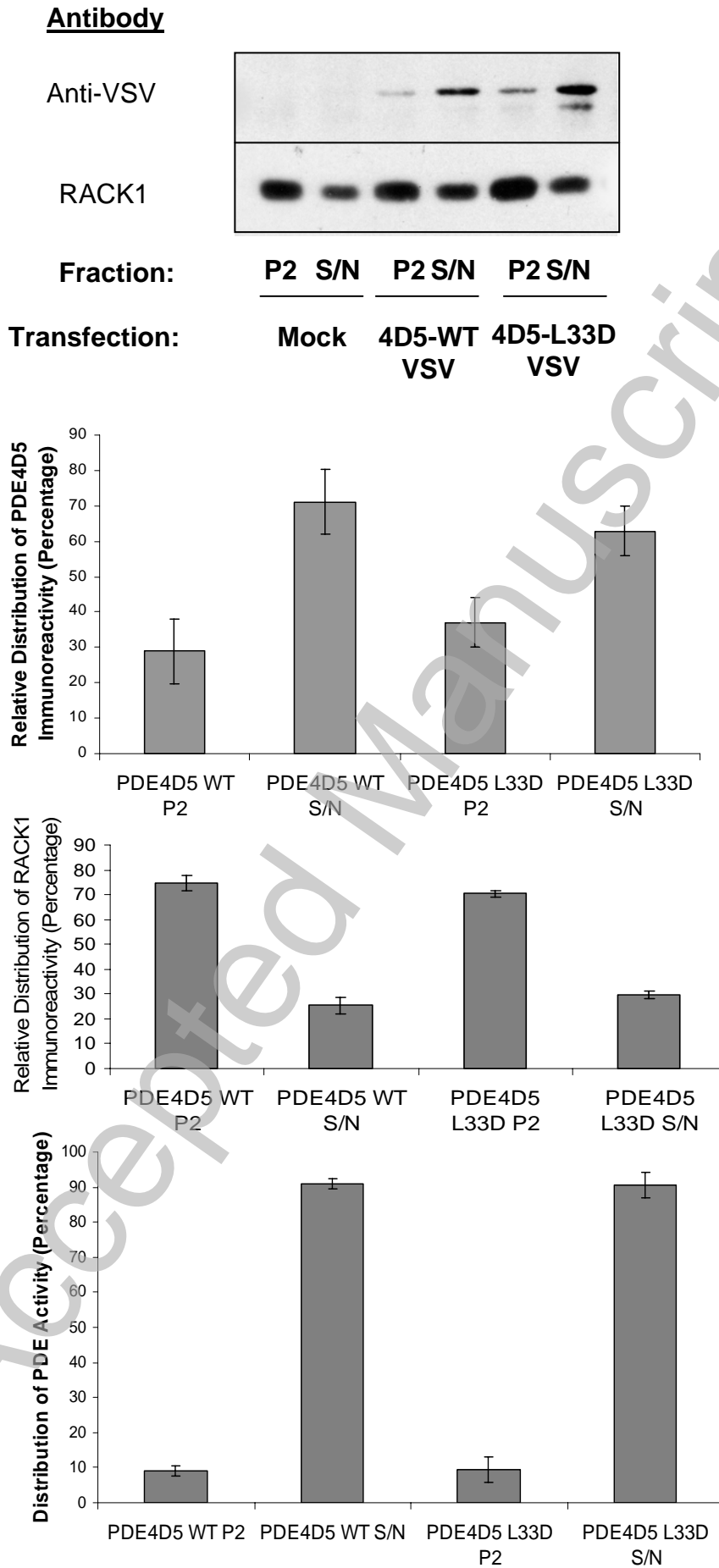
- a) Particulate (pellet) and soluble (S/N) fractions from HEK293 cells transfected with either PDE4D5-WT or PDE4D5-L33D and then stimulated with either diluent (DMSO) or PMA for 15 minutes were assayed for PDE activity. Significant differences relative to control are indicated, ** = $p < 0.01$ ($n=3$). PDE activities for PDE4D5-WT or PDE4D5-L33D prior to treatment were 60 ± 10 pmol/min/mg, in the pellet fraction, and 550 ± 50 pmol/min/mg in the soluble fraction, respectively.
- b) HEK293 cells were stimulated with diluent or $10 \mu\text{M}$ isoproterenol in the presence or absence of cell-permeable, 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 specific for PDE4D5. Immunoprecipitates were then assayed for PDE activity as described in *Experimental Procedures*. Results are means \pm SEM ($n=3$) and significant differences relative to unstimulated cells or cells stimulated with isoproterenol alone are indicated (**, $p < 0.01$, ***, $p < 0.001$ or #, $p < 0.05$, respectively).

Tables

Table 1 K_m , V_{max} and IC_{50} values for PDE4D5-WT and PDE4D5-L33D Expressed in HEK293 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).

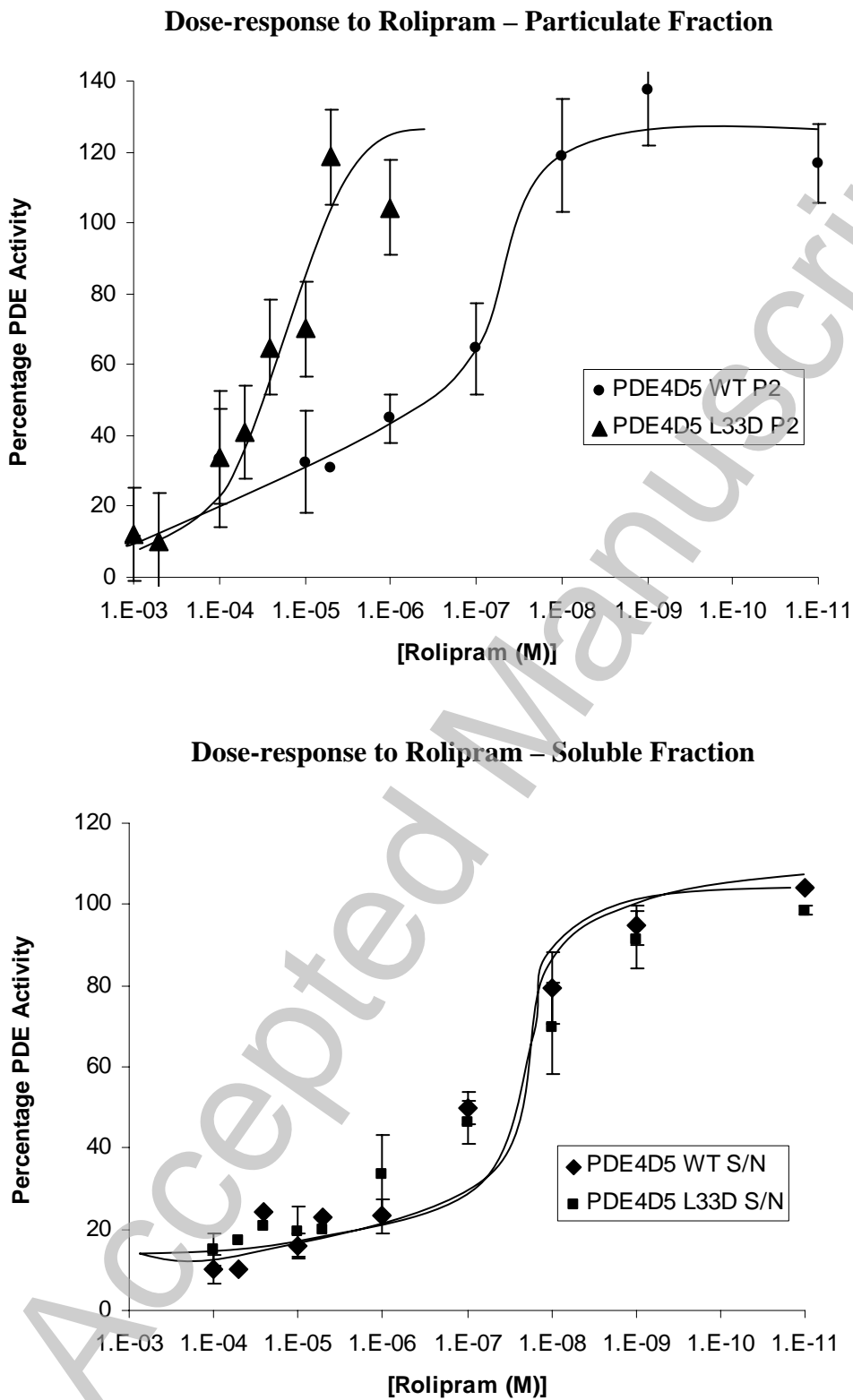
Transfection	Fraction	K_m (μM)	V_{max} (pmol/min/mg)	IC_{50} Rolipram (μM)
PDE 4D5- WT	Pellet	6.3 ± 0.9	0.32 ± 0.07	0.10 ± 0.01
PDE 4D5- WT	S/N	$16.7 \pm 1.3^{**}$	$9.71 \pm 2.09^{**\#}$	$0.08 \pm 0.02^{###}$
PDE 4D5- L33D	Pellet	$20.0 \pm 0.9^{***}$	0.87 ± 0.09	$10 \pm 0.90^{***}$
PDE 4D5- L33D	S/N	$10.0 \pm 1.5^{\#}$	$7.14 \pm 1.62^{*\#}$	$0.09 \pm 0.02^{###}$

Figure 1



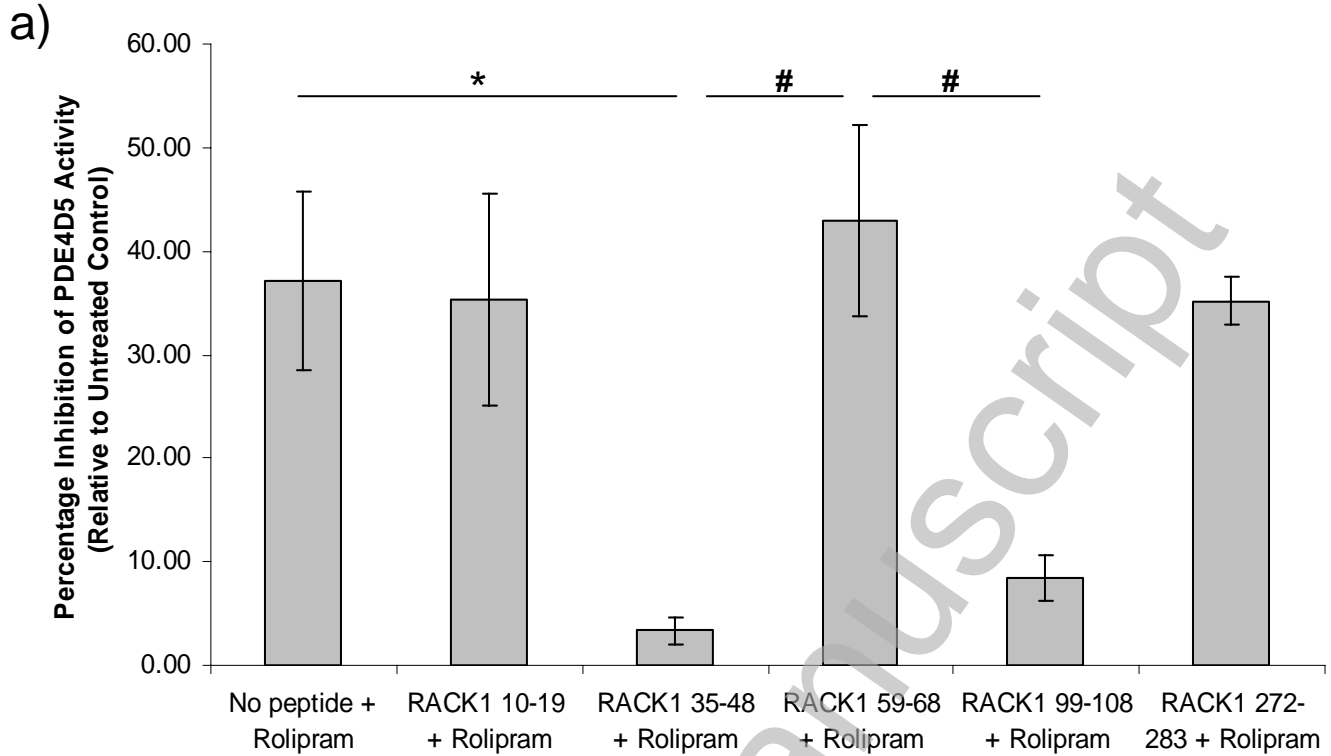
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Figure 2

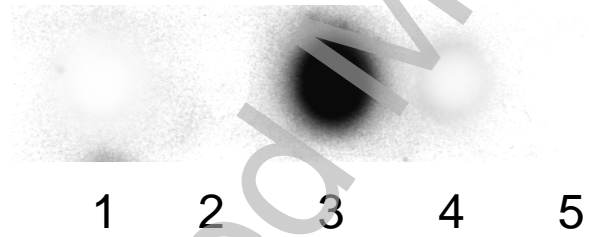


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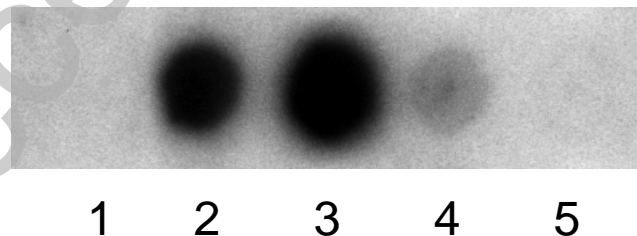
Figure 3



b)



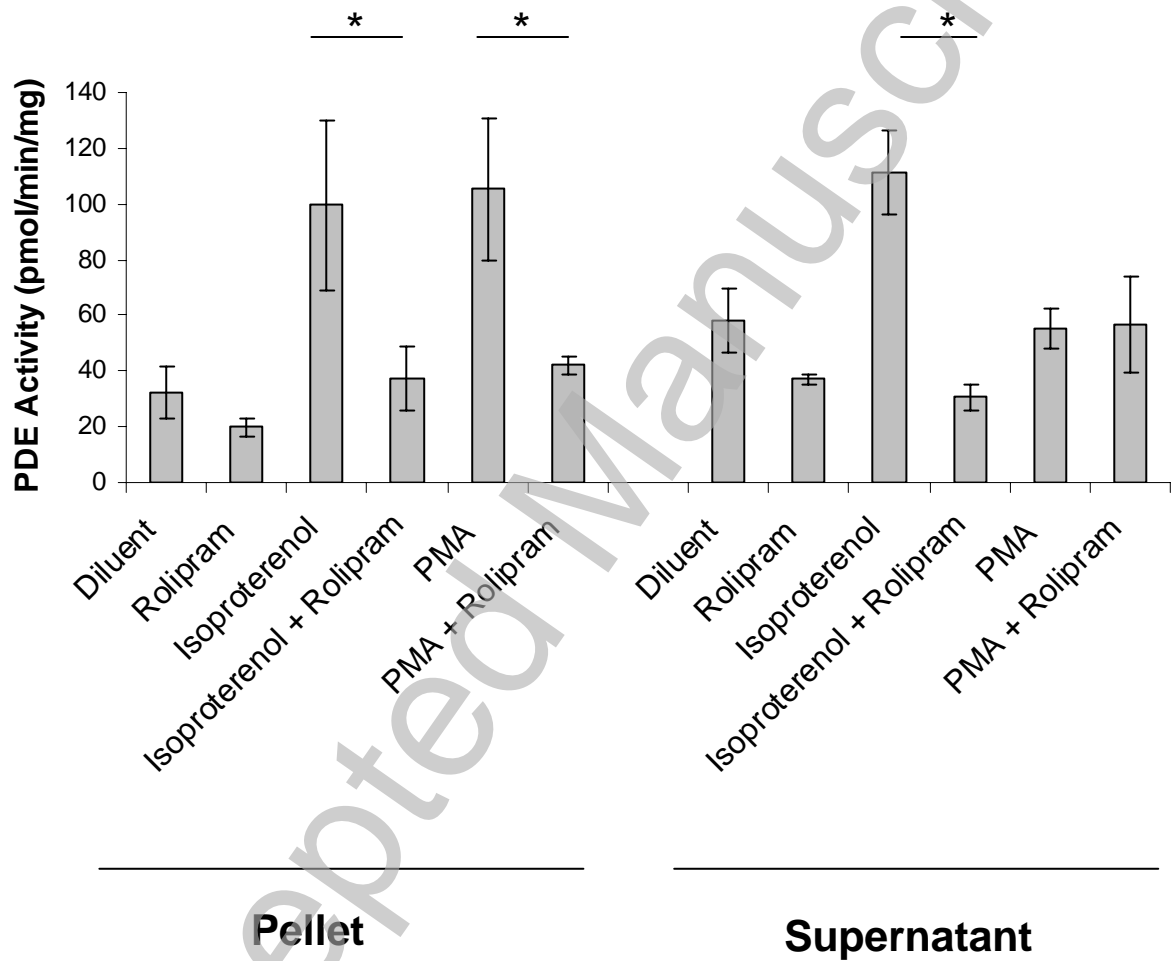
Peptide 1	IATTPQFPDM ILSASRDKTI	(hRACK1 21-40)
Peptide 2	QFPDM ILSASRDKTI IMWKL	(hRACK1 26-45)
Peptide 3	LSASRDKTIIMWKLTRDET	(hRACK1 31-50)
Peptide 4	DKTI IMWKLTRDET NYGIP	(hRACK1 36-55)
Peptide 5	MWKLTRDETNYGIPQRALR	(hRACK1 41-60)



Peptide 1	LRLWDLTTGTTTRRFVGH	(hRACK1 86-105)
Peptide 2	LTTGTTTRRFVGHTKDVLS	(hRACK1 91-110)
Peptide 3	TTRRFVGHTKDVLSVAFSS	(hRACK1 96-115)
Peptide 4	VGHTKDVLSVAFSSDNRQI	(hRACK1101-120)
Peptide 5	DVLSVAFSSDNRQIVSGSR	(hRACK1106-125)

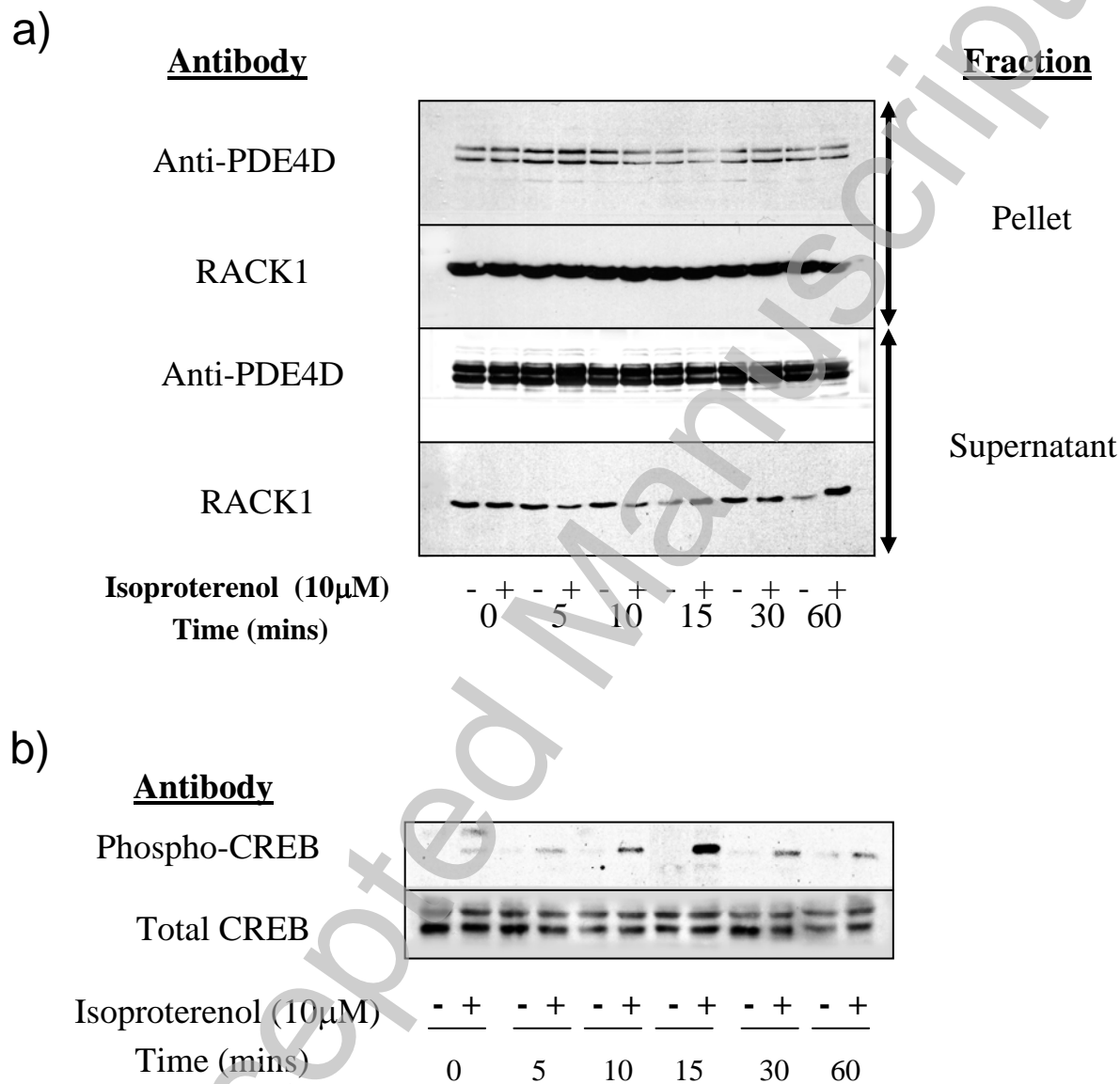
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Figure 4



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Figure 5

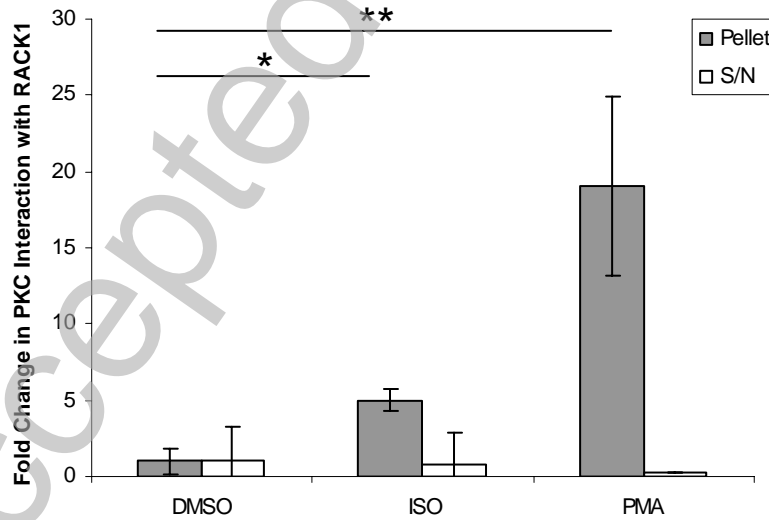
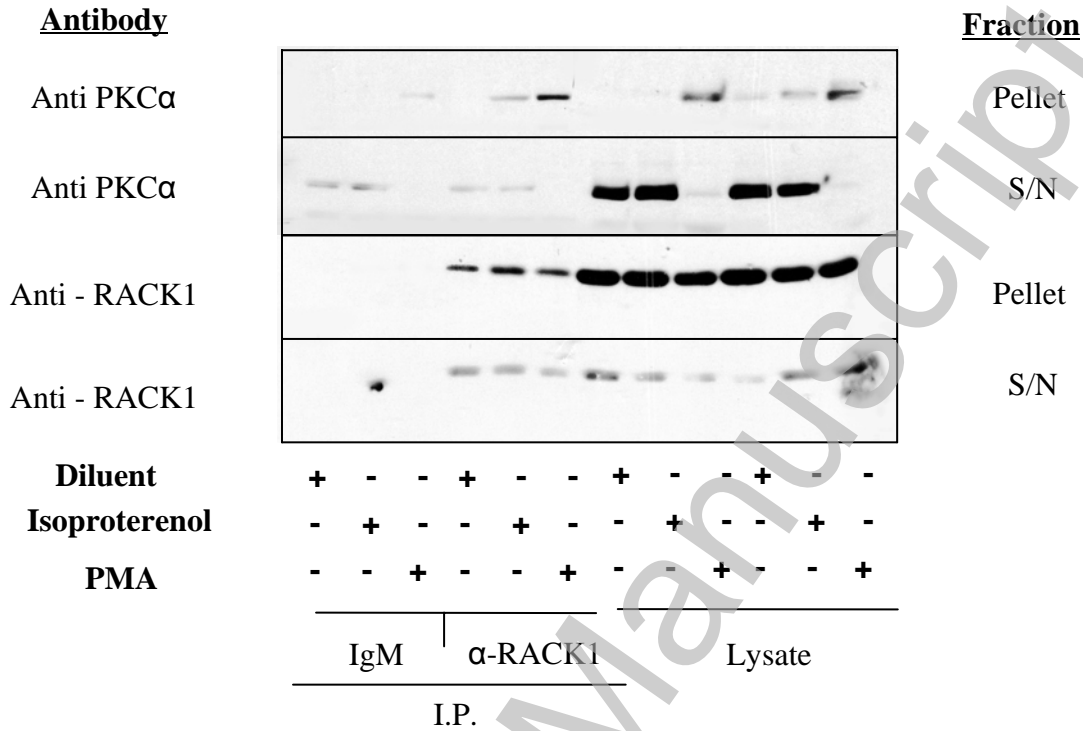


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Figure 6

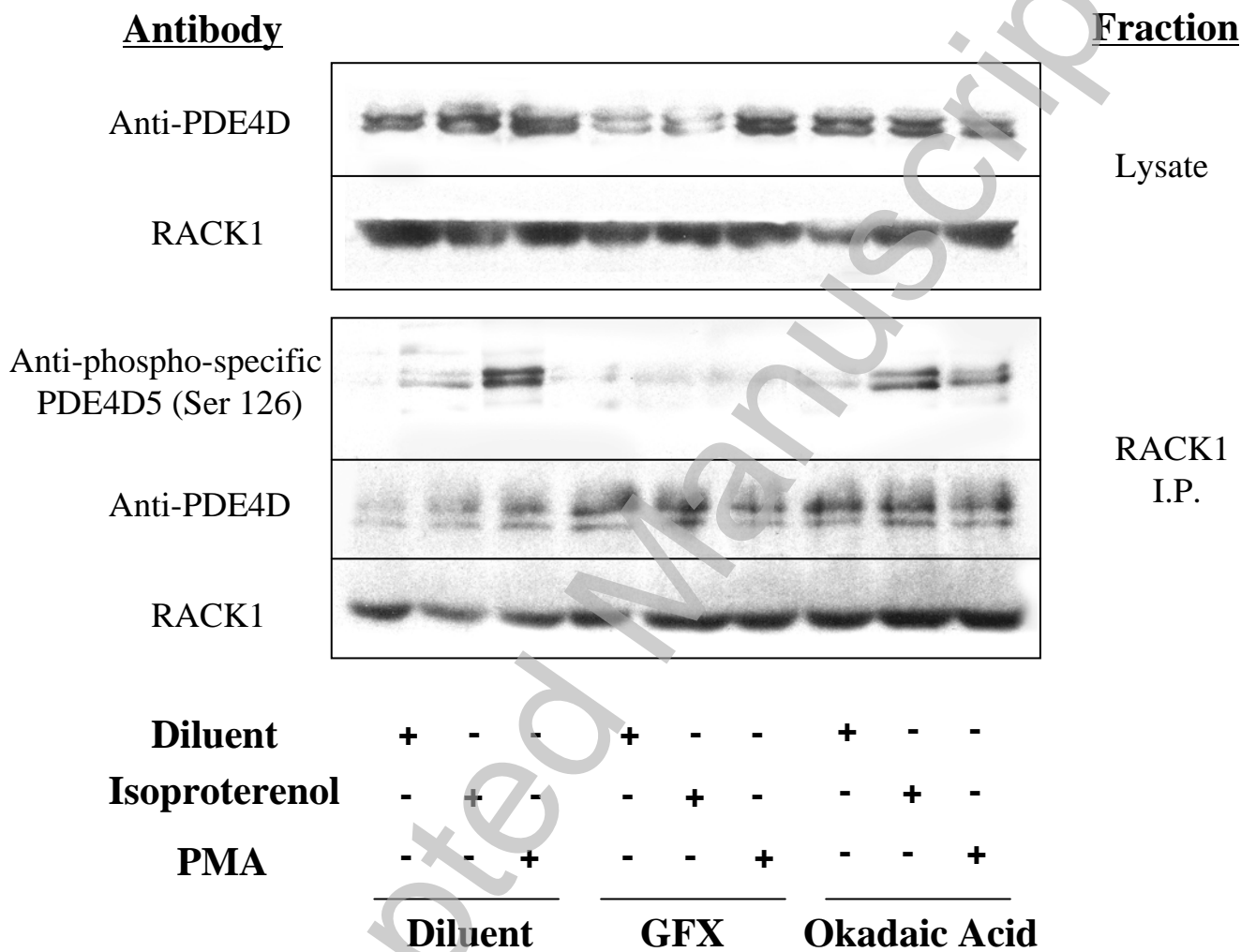
a)



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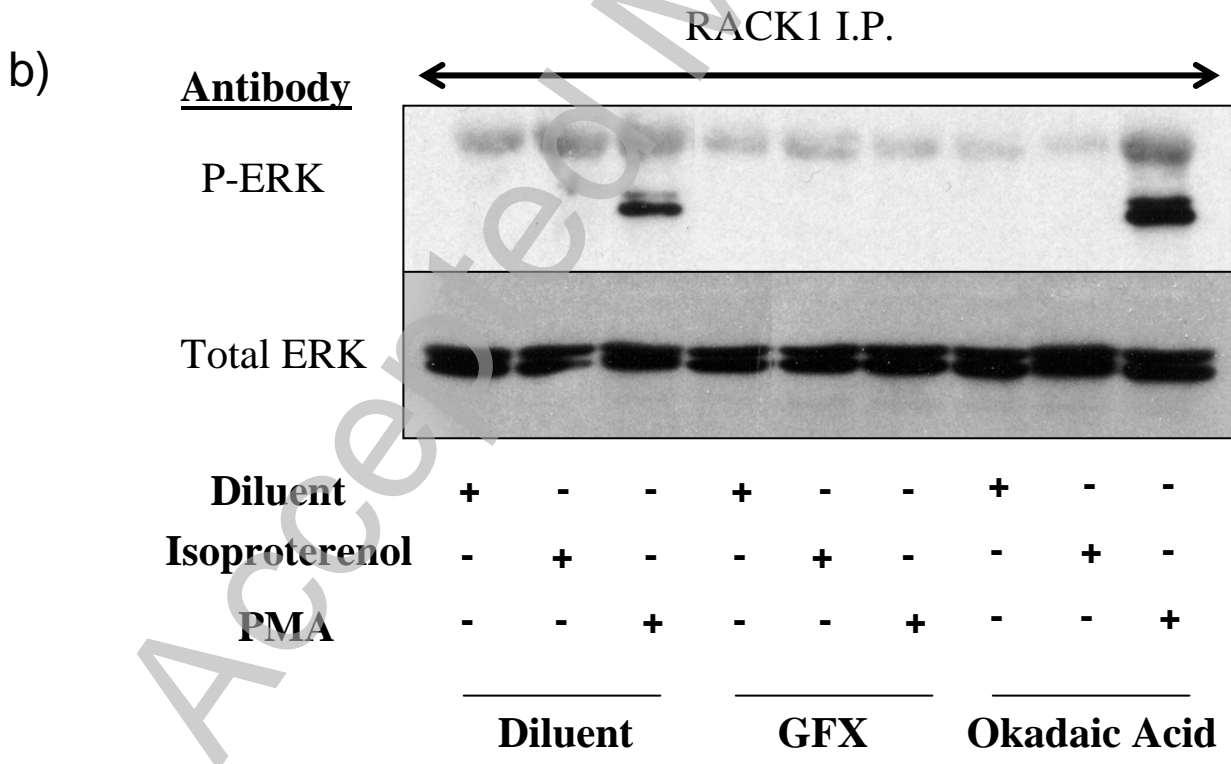
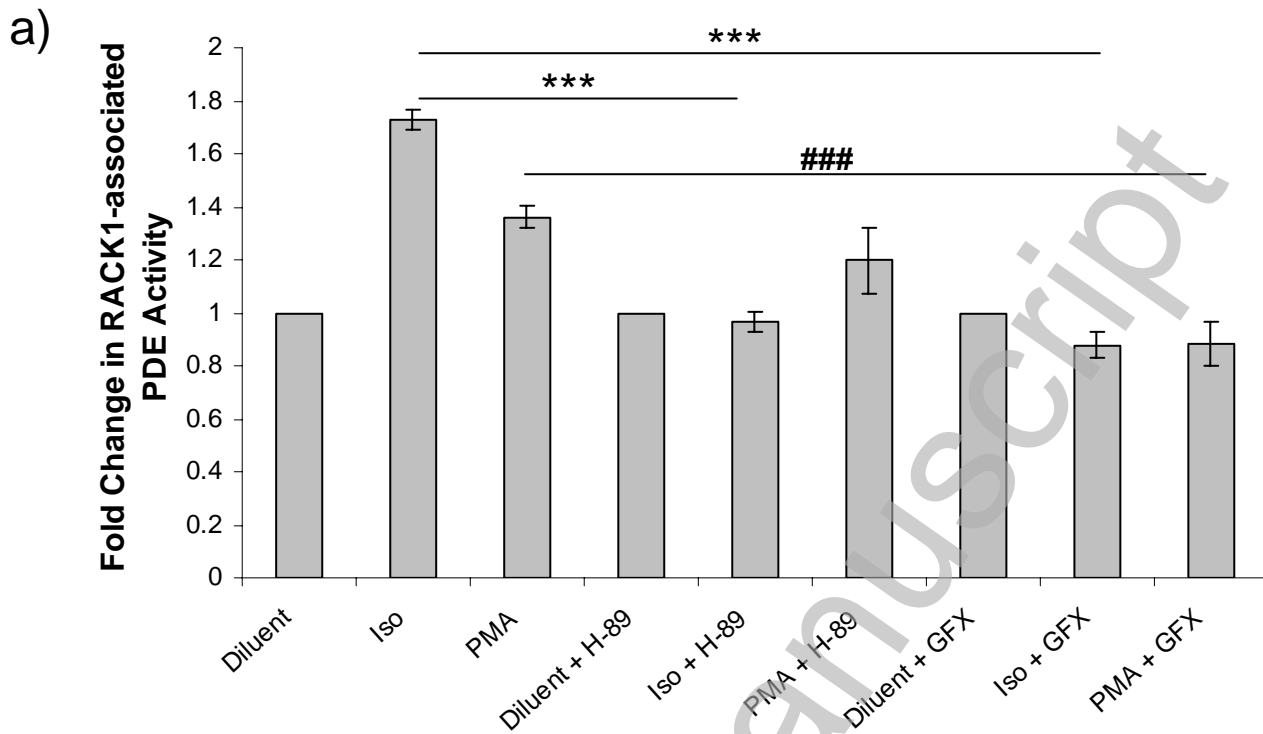
Figure 6

b)



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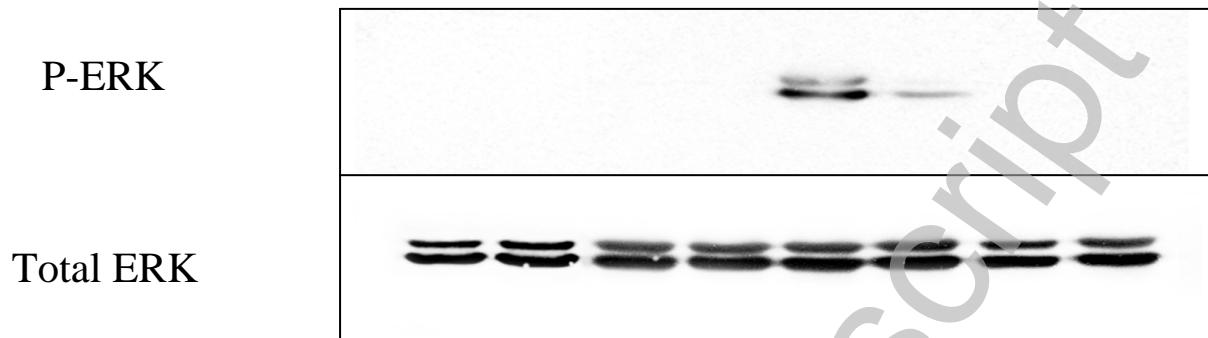
Figure 7



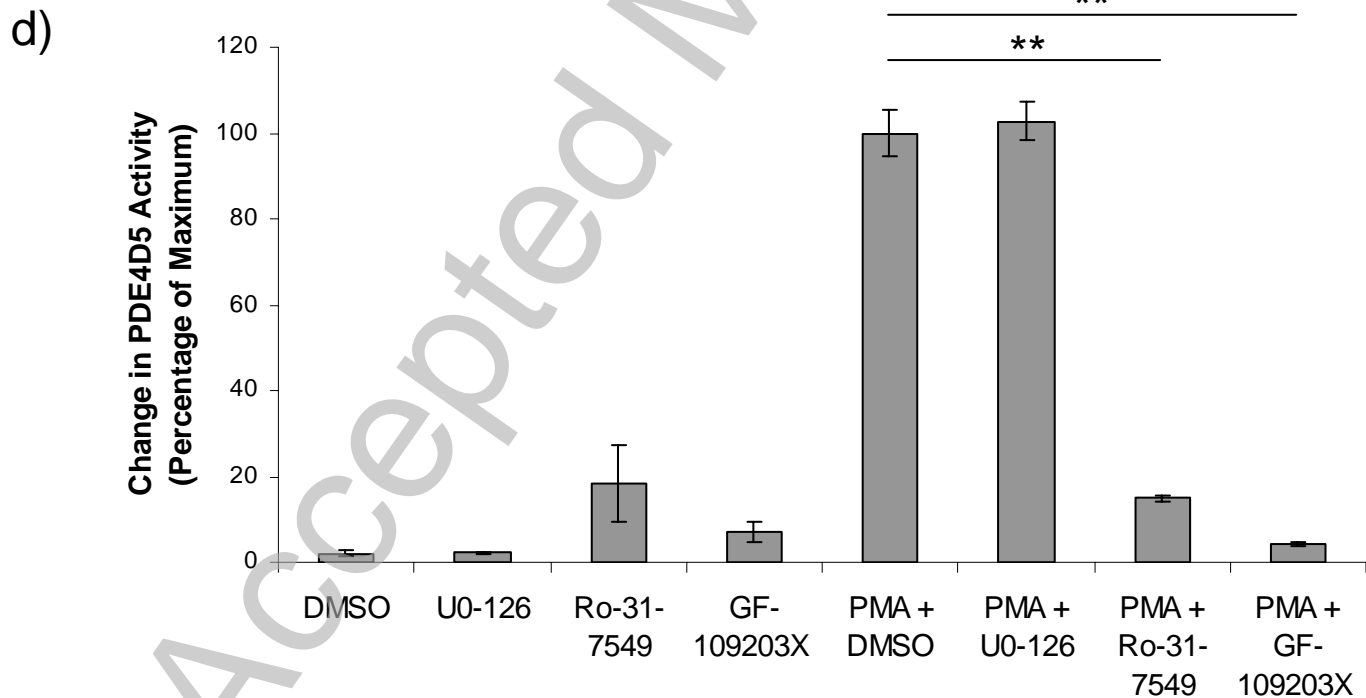
THIS IS NOT THE VERSION OF RECORD - see doi:10.1042/BJ20101010

Figure 7

c) Antibody

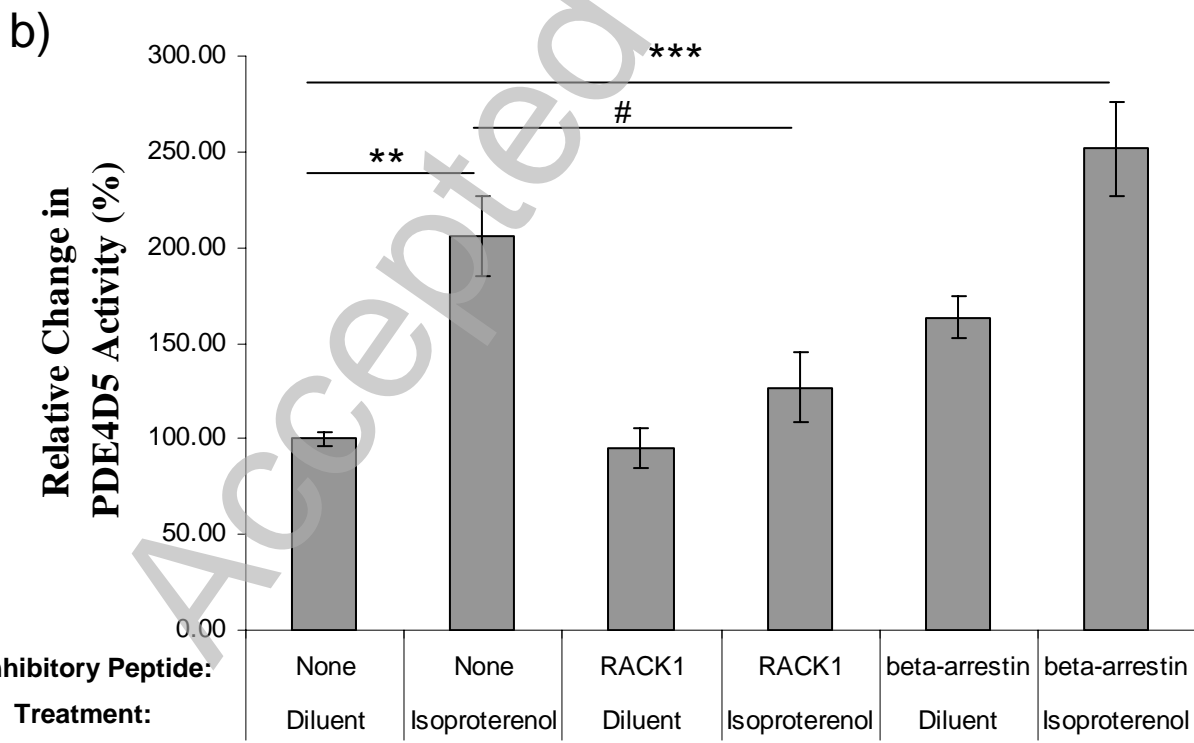
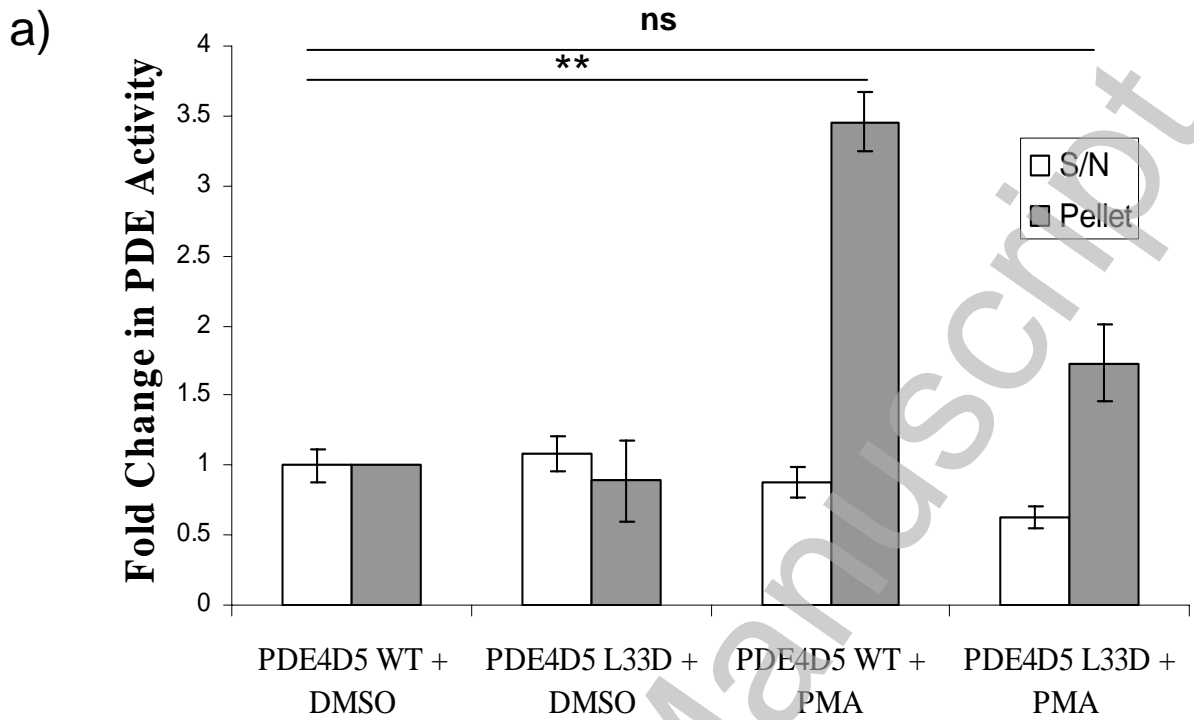


Inhibitor	-	U0	Ro	GFX	-	U0	Ro	GFX
PMA	-	-	-	-	+	+	+	+



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Figure 8



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