The cAMP-specific phosphodiesterase PDE4A5 is cleaved downstream of its SH3-interaction domain by caspase-3: consequences for altered intracellular distribution.

by

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Abstract.

The unique N-terminal region of the cAMP specific phosphodiesterase PDE4A5, which confers an ability to bind to certain protein SH3 domains, is cleaved during apoptosis in both Rat-1 fibroblasts and PC12 cells. Cleavage was abolished by both the caspase-inhibitor z-VAD-CHO and the caspase-3 selective inhibitor, z-DEVD-CHO, but not the caspase-1 selective inhibitor, z-YVAD-CHO. Caspase-3 treatment of PDE4A5, expressed either transiently in COS cells or generated in vitro by coupled transcription translation, generated a similar cleavage product of 100kDa compared to the native 110kDa PDE4A5. This product could be detected immunochemically with an antibody raised to a C-terminal PDE4A5 peptide but not an antibody raised to the N-terminus of PDE4A5, indicating that caspase-3 caused N-terminal cleavage of PDE4A5. Deletion of the putative caspase-3 cleavage site, DAVD\textsuperscript{69-72}, in PDE4A5, or generation of either the asp72ala or the asp69ala mutants, ablated the ability of caspase-3 to cause cleavage. The PDE4A8 isoform, which lacks the DAVD motif, was not cleaved either during apoptosis or by caspase-3 treatment. A variety of other recombinant caspases caused little or no cleavage of PDE4A5. The N-terminal truncate PDE4A5-ΔP3 was engineered to mimic the caspase cleaved product of PDE4A5. This showed altered catalytic activity and, unlike PDE4A5, was unable to interact with the SH3 domain of the tyrosyl kinase, LYN. Confocal immunofluorescence analysis showed that whilst both PDE4A5 and PDE4A5-ΔP3 were localized at cell cortical regions (ruffles), the distinct perinuclear association noted for both PDE4A5 and LYN was not seen for PDE4A5-ΔP3. Staurosporine-induced apoptosis caused a marked redistribution of PDE4A5 but not PDE4A8 in stably transfected Rat-1 cells. The PDE4 selective inhibitor, rolipram together with the adenylyl cyclase activator, forskolin caused a synergistic increase in the apoptosis of Rat-1 cells. Overexpression of PDE4A5 in Rat-1 cells protected against staurosporine-induced apoptosis in contrast to overexpression of PDE4A8 which potentiated apoptosis. We suggest that PDE4A5 may be the sole PDE4 family member to provide a substrate for caspase-3 cleavage and that this serves to remove the SH3 binding domain that is unique to this isoform within the PDE4A family and to alter its intracellular targeting.

Abbreviations.

rolipram, 4-\{3-(cyclopentoxyl)-4-methoxyphenyl\}-2-pyrrolidone; PDE, cyclic 3’, 5’ AMP phosphodiesterase; PKA, protein kinase A;
Apoptosis, a morphologically distinguished form of programmed cell death, is an essential process for the development and maintenance of homeostasis in multi-cellular organisms (1). Cells undergoing apoptosis exhibit a characteristic series of dramatic cellular changes, including DNA cleavage, nuclear breakdown, cell membrane blebbing and fragmentation of cytoplasmic organelles. Ultimately, these events lead to the fragmentation of the cell, generating apoptotic bodies that are swiftly engulfed by neighbouring cells within tissues, thereby preventing an inflammatory response. A wide variety of stimuli can initiate apoptosis. These include cellular stresses such as UV irradiation, heat shock and DNA damage as well as extracellular signals such as peptide growth factors, interleukins and cytokines. These diverse stimuli converge on a family of cysteine proteases, called caspases (2-6), which have homology to the CED-3 death gene product of the nematode, *C. elegans* and the interleukin-1B-converting enzyme (ICE). Mammalian homologues, despite their shared requirement for cleavage after aspartate residues, are highly specific in their substrate preferences with the sequence of three residues immediately amino-terminal to the aspartate cleavage site conferring specificity. Caspases are synthesized in the cell as inactive forms and are activated via autoproteolysis at internal caspase cleavage sites in a cascading process similar to that of the immune complement system. They can be divided into initiator caspases and downstream (effector) caspases such as the ubiquitously expressed caspase-3 (CPP32), which are implicated in the specific cleavage of cellular proteins essential for the structured dismantling of the cell during apoptosis, (3-5). Cellular substrates cleaved by effector caspases during the apoptotic pathway can be divided into two main groups. The first group includes those proteins which are considered to be regulators of apoptosis and that are either activated or inactivated by caspase cleavage. The second group includes members of housekeeping or structural protein families whose cleavage is required for the disassembly of the cell (3-5).

The ubiquitous second messenger, cAMP appears to modulate apoptotic signals in a wide variety of cell types. Agents elevating cAMP levels have been shown to induce, augment or inhibit apoptosis in a cell-type specific fashion (7-12). However, as yet, the molecular mechanisms underlying these cAMP-mediated events remain to be elucidated.

The control of intracellular cAMP levels is achieved by a complex signalling system involving multi-enzyme families of adenylyl cyclases, protein kinase A (PKA) and cAMP phosphodiesterases (PDE) (13). A seemingly important facet of this signalling system is the phenomenon of cAMP compartmentalisation observed in a number of different cell types (13-17). This can arise through the distinct intracellular localisation of separate adenylyl cyclase isoforms, the specific intracellular targeting of cAMP specific PDE isoforms via alternatively spliced N-terminals regions and the binding of PKA isoforms to specific anchoring proteins. The sole route for cAMP degradation in cells is through the action of cAMP phosphodiesterases (16,18-20). These enzymes are encoded by a large multigene family which differ in regulatory properties and intracellular
localisation. As such, they are poised to exert a profound regulatory effect on cAMP cell signalling. Recently, rolipram, a selective inhibitor of the PDE4 cAMP specific phosphodiesterase enzyme family has been shown to induce apoptosis in chronic lymphocytic leukemia (CLL) cells (7). This has led to the suggestion of the use of PDE4-selective inhibitors as a therapeutic approach to the treatment of lymphoid malignancies (7) in addition to their proposed use in inflammatory diseases such as asthma, rheumatoid arthritis and AIDS (21-23). Over 16 different PDE4, cAMP-specific enzymes have been identified (16). These are encoded by 4 separate genes (A, B, C and D), each of which generates a number of isoforms through alternative mRNA splicing. PDE4 isoenzymes are uniquely characterised by UCR1 and UCR2 regions located between the isoform-specific N-terminal region and the catalytic unit. The ‘long’ PDE4 isoforms, such as PDE4A5 and PDE4A8, possess both UCR1 and UCR2 whilst short isoforms, such as PDE4A1, lack UCR1 (16). Each splice variant is then characterised by a unique N-terminal region which appears to confer intracellular targeting (24) and interaction with anchor/scaffold proteins (25,26). Thus, for example, the N-terminus of PDE4D5 isoform confers an ability to interact with the scaffold protein RACK1 (25) and the N-terminus of PDE4A5 isoform confers an ability to interact with the SH3 domains of specific proteins, such as that of the SRC family tyrosyl kinase LYN (27). Here we show that the N-terminal region of PDE4A5 uniquely possesses a cleavage site for caspase-3. Cleavage at this site is shown to occur in cells undergoing apoptosis as well as in vitro using recombinant caspase-3. An N-terminal truncated form of PDE4A5, which lacks the region cleaved by caspase-3, had altered activity and was unable to interact with the SH3 domain of the tyrosyl kinase LYN. We show that caspase-3 cleavage of PDE4A5 alters the intracellular distribution of PDE4A5 and suggest that this may have consequences for compartmentalised cAMP signalling.
Materials and Methods

Protease inhibitor tablets were obtained from Boehringer Mannheim (Germany). Peptide caspase inhibitors were obtained from Calbiochem, Anti-mouse IgG horseradish peroxidase and [\(^3\)H]-cyclic AMP and ECL reagent were from Amersham. Dithiothreitol, Triton X-100 and \(N--{1-(2,3-\text{Dioleoyloxy})propyl}-N,N,N-,\text{trimethylammonium methylsulfate} \) (DOTAP) was obtained from Boehringer Mannheim. Bradford reagent was from Bio-Rad (Herts, UK). Rolipram was a kind gift from Schering. All other materials were from Sigma (Poole, UK).

Stable expression of PDE4A5 and PDE4A8 in rat-1 fibroblasts

Rat-1 fibroblasts were obtained from M. Frame (Beatson Institute, Glasgow) and were cultured at 37°C in an atmosphere of 5% \(\text{CO}_2\)/95% air in a complete growth medium containing DMEM (1% v/v), penicillin (10\(^4\) units/ml), streptomycin (10\(^4\) µg/ml), 2mM glutamine and supplemented with 10% (v/v) fetal calf serum. Cells were transfected with previously described constructs encoding PDE4A5 (pSV.SPORT-PDE4A5) (28) and PDE4A8 (pCDNA3-PDE4A) (29). This was done as described before by us (30). Briefly cells were plated at 60% confluence on 10cm plates 24h prior to transfection. A ‘transfection mix’ of 70µl of the transfection reagent DOTAP and 10µg of DNA in total volume of 500µl of HBS (20mM Hepes, 150mM NaCl, pH7.4) was prepared and incubated at room temperature for 10min. After addition of the transfection mix to the cells, the cells were incubated for 66h with one change of medium and then transfected cells were selected by inclusion of 1mg/ml geneticin G418 sulphate in the growth medium. Transfected colonies were individually picked into multi-well cell culture dishes, expanded and maintained.

PC12 cell culture and differentiation

PC12 cells were obtained from ECACC. They were maintained routinely in Dulbecco’s modified Eagles medium containing penicillin (10\(^4\) units/ml), streptomycin (10\(^4\) µg/ml), 2mM glutamine and supplemented with 10% horse serum and 5% (v/v) fetal calf serum. Cells were differentiated by seeding onto collagen coated 10cm plates and grown for 24h in serum containing medium. On reaching 70% confluency cells were incubated for 7-10 days in 2% horse serum containing growth medium supplemented with 100ng/ml NGF. To induce apoptosis by NGF withdrawal, cells were washed three times before incubating in serum-free medium.

Preparation of triton soluble cell extracts

Cells were harvested by first washing in ice cold PBS and then scraping them into lysis buffer (25mM Hepes/2.5mM EDTA/ 50mM NaCl/ 50mM NaF/ 30mM sodium pyrophosphate/ 10% glycerol/ 1% Triton X-100, pH7.5) with added protease inhibitors. They were then incubated end-on-end at 4°C for 30 min. Unbroken cells and debris were then discarded following centrifugation at 1000g for 10mins.
**Immunoprecipitation**

This was done using a modified method of that described previously by us (31). Briefly triton soluble extracts of cells were prepared and 300µg of protein in 750µl of lysis buffer was pre-cleared on 30µl of washed protein A beads, for 30min at 4°C. 15µl of antibody was added to the pre-cleared cell lysate and the sample was incubated, end-over-end, for 2h at 4°C. 50µl of washed protein A beads were added and the sample mixed for 1h at 4°C. Following this, the sample was centrifuged in a microfuge at 12,000 x g at 4°C for 5min, the pellet was washed twice in lysis buffer and twice in phosphate buffered saline solution containing protease inhibitors before the pellet was boiled in Laemmli buffer (32).

**Transfection and sub-cellular fractionation of Cos-7 cells**

Cos-7 cells were maintained and transfected essentially as described previously by us (33). Cos-7 cells were seeded at approximately 33% confluency onto 10cm diameter plates. Immediately before transfection the culture medium was replaced with 5ml of Dulbecco’s modified Eagles medium supplemented with 10% (v/v) Newborn Calf serum together with 0.1 mM chloroquine. 10µg DNA was diluted to 250µl with TE buffer (10mM Tris, 0.1 mM EDTA, pH 7.6) and 200µl 10mg/ml DEAE dextran was then added. The mixture was incubated at room temperature for 15 min before addition to the culture medium. Cells were incubated at 37°C/5%CO₂ for 3-4h before the medium was aspirated and the cells shocked for 2min with 10% dimethyl sulphoxide in a phosphate buffered saline solution. The culture was then rinsed twice in phosphate-buffered saline solution before Dulbecco’s modified Eagles medium containing 10% fetal calf serum was added, and the cells were incubated at 37°C in a 5%CO₂ atmosphere for 72h. Disruption of cells and the isolation of particulate and high-speed supernatant fractions were done as described in detail previously by us (33). Fractionation was carried out in KHEM buffer (50mM KCl / 50mM Hepes, pH 7.2/ 10mM EGTA, 1.92mM MgCl₂) containing protease inhibitors.

**Treatment of rat-1 cells with staurosporine and peptide caspase inhibitors**

Cells to be treated with staurosporine were grown to confluency and then serum starved overnight before treatment. Staurosporine (1µM) was added to cells in complete growth medium and the cells incubated for the times indicated at 37°C/ 5% CO₂. Where peptide caspase inhibitors were used these were added at the concentrations indicated 3h prior to the addition of staurosporine.

**SDS/PAGE and Western Blotting**

8% acrylamide gels were used and the samples boiled for 5min after being resuspended in Laemmli buffer. Gels were run at 8mA/gel overnight or 50mA/gel for 4-5h with cooling. For detection of transfected PDE by Western blotting, 2-50µg protein samples were separated by SDS-PAGE and then transferred to nitrocellulose before being immunoblotted using specific antisera. Labeled bands were identified by using anti-rabbit peroxidase linked IgG and the Amersham ECL Western blotting was used as a visualization protocol. The peptide antisera used in this study have
been described in detail previously by us (28-31,34). The C-terminal antiserum was generated to
detect specifically the C-terminal region found in common to all of the rodent PDE4A isoforms. The
N-terminal antiserum is able to detect specifically the N-terminal region of the isoform PDE4A5 but
not that of other PDE4A isoforms.

**PDE assay**

Cyclic nucleotide phosphodiesterase activity was assayed by a modification of the two-step
procedure of Thompson and Appleman (35) as previously described by us (36). All assays were
conducted at 30˚C and in all experiments a freshly prepared slurry of Dowex:H₂O:ethanol (1:1:1) was
used for determination of activities. Initial rates were taken from linear time courses of activity. All
kinetics measurements were performed as previously described by us (33). To define Kₘ and Vₘₐₓ
values, data from PDE assays were done over a range of cAMP substrate concentrations and then
analyzed as before (33) by computer fitting to the hyperbolic form of the Michaelis-Menten equation
using an iterative least squares procedure (Ultrafit; with Marquardt algorithm, robust fit, experimental
errors supplied; Biosoft, Cambridge, UK). As described before in some detail (26,28,37), relative
Vₘₐₓ values were obtained by using equal amounts of PDE4A immunoreactive protein in assays as
determined using the PDE4A-specific C-terminal antiserum. Total PDE4 activity in cells was
determined at a substrate concentration of 1µM cAMP and defined as that amount of PDE activity
which could be inhibited by the addition of 10µM-rolipram. This is a concentration at which
rolipram serves as a PDE4 selective inhibitor and can completely inhibit PDE4 activity (16).

**Confocal analyses and digital deconvolution.**

Cos-7 cells were plated out onto coverslips (18x18mm) at about 40% confluency 48h after
transfection. After another 24h the cells were fixed for 30 min in 4% paraformaldehyde in Tris-
buffered saline (TBS). In the case of staurosporine treated Rat-1 fibroblasts, cells were plated onto
coverslips at 30% confluency. After 24h these cells were serum starved overnight before treating
with 1µM-staurosporine for the time indicated, all cells were in culture for the same length of time
and were fixed simultaneously in 4% paraformaldehyde. Fixed cells were permeabilised with 3
changes of 0.2% Triton in TBS for 15 min and, following four 5 min blocking incubations with 20%
bovine serum albumin, were labelled for 2h with polyclonal antibodies raised against specific peptide sequences of the C-terminal region of PDE4A5 (28,34). Labelling was
detected using a FITC- or TRITC-conjugated goat anti-rabbit IgG for 1h. Co-staining of cells was
achieved using a monoclonal mouse anti-LYN antibody (Transduction laboratories, Lexington, KY))
at a dilution of 1:100. Localisation of proteins was visualized using the complementary FITC- or
TRITC-conjugated goat anti-mouse IgG to the polyclonal staining. All incubations were carried out
at room temperature. Cells were visualized using a laser-scanning confocal microscope using an
Axiovert 100 microscope with a X63/1.4NA plan apochromat lens, as described previously by us.
Deconvolution of images and three-dimensional reconstruction was performed using an Improvision Open-Lab package (kindly on loan from Improvision Labs).

**Deletion mutagenesis and point mutations**

Site directed mutagenesis was performed using a Quickchange DNA mutagenesis kit (Stratagene) according to the manufacturers instructions. All mutagenesis and deletion constructs were confirmed by DNA sequencing.

**Apoptosis Assays**

Apoptotic cells were quantified using annexin V flow cytometry or qualified using DNA cleavage analysis. Annexin V flow cytometry exploits the fact that there occurs a loss of membrane lipid asymmetry in early apoptosis. This results in the exposure of phosphatidylserine on the outer leaflet of the plasma membrane, which can then be detected. All cells were serum-starved for 24h before adding ligands to evaluate action upon apoptosis. These ligands were either treated with staurosporine (1µM), forskolin (50µM), IBMX (100µM) or rolipram (10µM) over the indicated time-period. Briefly, cells were removed from culture plates by trypsinising and then, following washing once in ice-cold phosphate buffered saline, they were resuspended in binding buffer (10mM Hepes/NaOH, pH7.4, 140mM NaCl, 2.5mM CaCl₂). They were then stained with 5µl FITC-conjugated annexin V (Pharminogen) as well as being counterstained with 0.5µg propidium iodide (Calbiochem), in order to allow necrotic cells to be excluded. The cells were subsequently analyzed using a Becton Dickinson FACSCAN. DNA ladder isolation was performed using the Oncogene Suicide Track Kit (Oncogene Research Products, Cambridge) as described in the manufacturer’s instructions.

**Caspase-3 assay**

Caspase-3 activity was assayed using the Calbiochem Caspase-3 Cellular Activity Assay Kit as described in the manufacturer’s instructions.

**Cell free translation**

*In vitro* transcription/translation was performed using the single tube protein system 3 (Novagen) as described in the manufacturers instructions. Radiolabelled species were generated using ³⁵S-methionine as described before by us (38).

**In vitro caspase cleavage assays**

All recombinant caspases were generated in an active form as described before by one of us in some detail (2). 10-50µg of cytosolic Cos-7 cell lysate expressing the indicated PDE4A isoforms or 2µl of the TNT reaction mixture containing *in vitro* translated PDE4A5, were incubated for 2h at 37°C with various recombinant caspases. As described previously (39), in order to normalise for the amount of mature caspase in each instance, equal concentrations (147ng) of the p20 active subunit were added in a final volume of 25µl complete KHEM. Resultant protein was retained for PDE assays or boiled in Laemmli solution for Western Blotting.
Pull-down assays with GST-fusion Proteins

This was performed essentially as described previously by us in some detail (26,27). Briefly, equal volumes of slurry containing 400µg of fusion protein immobilized on glutathione-agarose were pelleted and the supernatants were discarded. The pellets were resuspended in 100µg of crude cytosol of Cos-7 cells, diluted to a final volume of 200µl in KHEM buffer containing 1mM dithiothreitol and a protease inhibitor mix (complete KHEM). The immobilized fusion protein and cytosol were incubated together for 10min “end-over-end” at 4˚C. The beads were then collected by centrifugation for 5s at high speed in a bench-top centrifuge, and the supernatant was retained as the unbound fraction. The beads were held on ice and washed three times with 400µl of complete KHEM over a 15min period. These washes were pooled with the unbound fraction and aliquots were taken for PDE assay and Western blotting. Bound PDE was eluted from the beads by incubating three times in 100µl of elution buffer (10mM glutathione, 50mM Tris-HCL, pH 8.0) for 10min at 4˚C. The eluted fractions were pooled and aliquots were taken for PDE assay and Western blotting.

Presentation of results

Except where indicated all results are presented in triplicate or are representative of results found in at least three separate experiments.
Results

The rat PDE4A gene encodes at least three different isoforms, which differ from each other by their unique N-terminal regions (16,29), namely PDE4A1, PDE4A5 and PDE4A8 (Fig. 1). Of these, PDE4A5 is the most commonly expressed product known to date, with expression of PDE4A1 being restricted essentially to the brain and PDE4A8 to the testis (16). The engineered ‘core’ PDE4A enzyme, lacking the alternatively spliced N-terminal and UCR regions, can be expressed as a fully active species that is found entirely in the cytosol fraction of cells. In contrast to this, PDE4A1 is entirely membrane-associated (24,34) whilst PDE4A5 (28) and PDE4A8 (29) are found both in the cytosol and particulate fractions of cells. It is believed that the N-terminal regions of these various splice variants are responsible for association with cellular particulate fractions (16).

Caspase-3 is known to require a motif of the form $D^4X^3X^2D^1$ to act; where asp at position 1 defines the site for caspase cleavage and asp at position 4 determines the specificity for cleavage by caspase-3 (40). Caspase-3 also has a preference for a hydrophobic residue at position 2 in this sequence motif but tolerates a wide range of amino acids at position 3.

Analysis of the primary sequence of all PDE4 isoenzymes known to date indicates that PDE4A5, and its human homologue PDE4A4 (16), are the sole PDE4 isoforms which exhibit potential sites for cleavage by the effector caspase, caspase-3. In PDE4A5 there are two putative sites of caspase-3 cleavage within adjoining sequence motifs, DA$V^72$ and DTGD$^75$. These are both located within the alternatively spliced N-terminal region of PDE4A5 (Fig. 1).

PDE4A5 undergoes cleavage upon induction of apoptosis in Rat-1 cells.

We wished to determine if PDE4A5 could be cleaved by caspase-3 in cells undergoing apoptosis. Rat-1 fibroblasts express PDE4A5 as their sole PDE4A isoform. However, PDE isoforms have extremely high specific activities and are expressed at very low protein levels in cells (16,20,41). This makes them notoriously difficult to analyze. In order to obviate this problem we established a Rat-1 cell line, called Rat-1-6R8, which was stably transfected so as to overexpress PDE4A5. The total PDE4 activity in native Rat-1 cells, assayed at 1µM cAMP substrate concentration, was 10 ± 2pmol/min/mg whilst in Rat-1-6R8 cells the PDE4 activity was elevated some 70-fold to 694 ± 70pmol/min/ug. This indicated that in the Rat-1-6R8 transfected cell line the major PDE4 activity was due to the transfected PDE4A5 enzyme. Consistent with this, PDE4A5 was now readily detectable as a 110kDa species upon Western blotting of the transfected Rat-1-6R8 cell line (see below). This is the size at which PDE4A5 is found when expressed endogenously and also when transiently expressed in COS cells (28). We were unable to detect any increase in other PDE4 species in these transfected cells, consistent with the increase in PDE4 activity in the Rat-1-6R8 cell line being due predominantly to the transfected PDE4A5 (data not shown).

In order to assess the effect of apoptosis on PDE4A5, Rat-1-6R8 cells were challenged with the potent inducer of apoptosis, staurosporine (42,43). The time-dependence of apoptosis was
assessed by (i) measurement of caspase-3 activity, (ii) annexin-V flow cytometry to detect phosphatidyl serine in the outer leaflet of the plasma membrane and (iii) DNA laddering. It was evident from such studies (Fig. 2) that apoptosis had begun in these cells some 2h after exposure to staurosporine. Staurosporine-treatment of Rat-1-6R8 cells triggered the time-dependent generation of a PDE4A immunoreactive species some 100kDa in size (Fig. 3; panel A). This was accompanied by an apparent reduction in the amount of the immunoreactive 110kDa PDE4A5 isoform (Fig. 3; panel A), suggesting that it was cleavage of PDE4A5 that generated the 100kDa product. The production of this cleaved species was apparent around 1- 2h after challenge of Rat-1-6R8 cells with staurosporine and its levels continued to rise for at least 24h (Fig. 2; panel B).

The 100kDa species was immunodetected using an antiserum raised to the C-terminal region of PDE4A5 (Fig 3; panel A). Unlike PDE4A5, however, this 100kDa species could not be detected using an antiserum specific for the unique N-terminal region of PDE4A5 (Fig. 3; panel C). This suggests that staurosporine-induced apoptosis in Rat-1-6R8 cells caused cleavage within the N-terminal region of PDE4A5, implying an isoform-specific action. To investigate such a possibility we generated a further rat-1 cell-line (Rat-1-39B3) that was stably transfected to express a different long PDE4A isoform, namely PDE4A8. The total PDE4 activity of the Rat-1-39B3 transfected cell line was 200 ± 22pmol/min/ug protein. This indicated that the major PDE activity in Rat-1-39B3 cells was due to PDE4A8. Consistent with this, western blotting indicated the expression of a novel 98 kDa PDE4A species in these cells (Fig. 3; panel D) which co-migrated with PDE4A8 expressed transiently in COS7 cells (29). The sequence of the PDE4A8 isoform is identical to PDE4A5 except for a domain swap at the extreme N-terminus. Thus the first 102 amino acids of PDE4A5 sequence are replaced by a novel sequence of 21 residues in PDE4A8 (Fig. 1) which does not exhibit either a putative caspase-3 cleavage site or an SH3-binding site. Whilst staurosporine-treatment caused apoptosis in Rat-1-39B3 cells (data not shown), in marked contrast to Rat-1-6R8 cells staurosporine treatment did not generate any novel PDE4A immunoreactive species (Fig. 3; panel D).

A caspase-3 inhibitor prevents cleavage of PDE4A5 in Rat-1-6R8 cells.

The, presumed, cleaved PDE4A5 species observed in staurosporine-treated Rat-1-6R8 cells was around 10kDa smaller in size than full length PDE4A5. This is of the order expected for caspase-3 cleavage of PDE4A5 occurring at either D72 or D75 (Fig. 1). Consistent with this, we observed (Fig. 3A) that pre-treatment of Rat-1-6R8 cells with the broad-range, cell-permeable, cysteine protease inhibitor, z-VAD-fmk (4), abolished the appearance of the 100kDa species. In addition to this, the cell-permeable z-DEVD-CHO peptide, which selectively inhibits members of the caspase-3 family, was able to prevent (Fig. 3; panel E, track 3) the staurosporine-induced production of the 100kDa species. In contrast to this, treatment with the caspase-1 selective inhibitor, z-YVAD-CHO (4) failed to block generation of the 100kDa species (Fig. 3; panel E, track 4). These data indicate that the 100kDa species generated in Rat-1-6R8 cells upon staurosporine-induced apoptosis
is a species of PDE4A5 which has been N-terminally cleaved by caspase-3. Consistent with this, caspase-3 activity increased in Rat-1-6R8 cells over a similar timecourse (Fig. 2) to that of generation of the cleaved PDE4A5 species (Fig. 3).

Caspase-induced cleavage of PDE4A5 was also seen in Rat-1-6R8 cells challenged with etoposide to elicit apoptosis and in COS-7 cells transiently transfected with PDE4A5 where apoptosis was elicited by either staurosporine or etoposide (data not shown).

_PDE4A5 is cleaved in differentiated PC12 cells undergoing NGF withdrawal._

In order to examine whether natively expressed PDE4A5 was similarly cleaved upon apoptosis we used differentiated rat pheochromocytoma (PC12) cells that undergo apoptosis following NGF withdrawal (44,45). These cells were shown here to natively express PDE4A5 which can be detected by both C-terminal PDE4A specific antiserum (Fig. 3; panel F, track 1, upper arrow) and the N-terminal PDE4A5-specific antiserum (data not shown). Withdrawal of NGF from the culture medium generated a faster migrating PDE4A immunoreactive species some 10kDa smaller in size than PDE4A5 (Fig. 3; panel F, track 2, lower arrow). This faster migrating species was not detected using the PDE4A5-specific antiserum (data not shown), consistent with N-terminal cleavage of PDE4A5.

_Caspase-3 cleaves PDE4A5 within its unique N-terminal region._

To determine whether caspase-3 could elicit the cleavage of PDE4A5 _in vitro_, lysates of COS cells, which transiently expressed this isoform, were challenged with recombinant active caspase-3. This produced a single 100kDa species which could be detected using the PDE4A specific C-terminal antiserum but not the PDE4A5-specific N-terminal antiserum (Fig.4A). Consistent with this being due to the N-terminal, isoform-specific, cleavage of PDE4A5, treatment with recombinant caspase-3 caused no cleavage of other splice variants of the PDE4A gene, namely PDE4A1A and PDE4A8 (Fig. 4A).

PDE4A5 contains two putative sites for caspase-3 cleavage within its unique N-terminal region, namely D<sup>69</sup>AVD<sup>72</sup> and D<sup>72</sup>TGD<sup>75</sup>. Within the DXXD cleavage motif specific for caspase cleavage of substrate proteins it is the aspartate immediately amino-terminal to the cleavage point that is essential for cleavage by caspases. To define which of these motifs directed caspase-3 cleavage we generated three mutant forms of PDE4A5, expressed them in COS7 cells and treated lysates with recombinant, active caspase-3. Unlike wild-type PDE4A5, both the asp69ala (4A5-AAVD) and asp72ala (4A5-DAVA) mutant forms of PDE4A5 were resistant to cleavage by caspase-3, whilst the asp75ala (4A5-DTGA) mutant was still cleaved (Fig. 4B, C). This indicates that the target motif is DAVD<sup>72</sup> and is consistent with caspase-3 favouring a hydrophobic residue on the C-terminal side of the first aspartate residue, given here by alanine in DAVD (3,4,40). To provide further support for this, we effected the deletion of the entire DAVD<sup>72</sup> motif. As might be predicted, this was completely insensitive to cleavage by recombinant caspase-3 (Fig. 4B).
Intriguingly a band co-migrating with the 100kDa caspase-cleaved product of PDE4A5 was also present at low levels in the untreated control samples of PDE4A5 (Fig. 4A, B) and was increasingly evident after expression over longer periods subsequent to transfection (Fig. 4D). This species was not, however, evident in either the deletion mutant, PDE4A5-ΔDAVD (Fig. 4B), or the caspase-3 resistant point mutants (Fig. 4B, C). We suggest that some endogenous cleavage of PDE4A5 by caspase-3 may occur in COS cells during the transfection period and that this is not seen in these caspase-3 resistant mutant forms.

In order to examine the characteristics of the caspase-3 cleaved product of PDE4A5 without stimulating apoptosis, we engineered a truncated version of PDE4A5, called PDE4A5-ΔP3, whose initiation codon was positioned at D72. This also provides a model for examining the properties of the cleaved PDE4A5 species in the absence of any contaminating full-length protein. PDE4A5-ΔP3 was expressed in COS-7 cells and lysates subjected to western blotting with the C-terminal PDE4A-specific antiserum. This identified that PDE4A5-ΔP3 co-migrated with the more rapidly migrating 100kDa PDE4A species generated in staurosporine-treated Rat-1-6R8 cells (Fig. 4E). However, like the cleaved PDE4A5 product, PDE4A5-ΔP3 was not immunodetected using the N-terminal PDE4A-specific antiserum (data not shown).

Probing PDE4A5 for cleavage by a panel of recombinant caspases.

In order to determine if there was selectivity regarding the ability of caspases to cause cleavage of PDE4A5 in vitro, we screened the ability of a series of active caspases to act on this enzyme.

As caspases act on different substrates then there is always going to be an inherent problem in trying to decide what amount of enzyme to take. As a pragmatic approach to this we normalized for the amount of mature caspases by using equal concentrations of the active p20 subunit whilst choosing a concentration that has been demonstrated to allow the evaluated caspases to act on specific substrates (39). To determine the susceptibility of PDE4A5 to cleavage by these various caspases we then took two independent approaches. These involved screening both in vitro translated 35S-labelled PDE4A5 and also PDE4A5 expressed in the lysates of transiently transfected COS cells against a panel of recombinant caspases (Fig. 5). Using both sources of PDE4A5 we noted that it was only caspase-3 which could cleave PDE4A5 to completion over the time of this experiment, although some degree of cleavage was also seen with caspase-7, particularly using the TNT generated PDE4A5 (Fig. 5). This is perhaps not too surprising given that these caspases belong to the same family of CPP32 caspases and both have specificity for a DXXD directed cleavage site (4). Nevertheless, it is clear from the analysis of action on COS cell-generated PDE4A5 that caspase-3 is likely to be more effective than caspase-7 in eliciting breakdown of PDE4A5 in intact cells (Fig. 5; panel B). Whilst PDE4A5 contains a number of potential caspase-1 cleavage sites, present mainly in the core catalytic region and towards the C-terminal region, it was clear (Fig. 5) that no alteration in
the levels of PDE4A5 occurred when treated with caspase-1. This suggests that such sites are not available for cleavage by this enzyme. It is also possible that some degree of cleavage of PDE4A5 was evident with caspase-2 and caspase-6 (Fig. 5). However, this was very small compared to that seen using caspase-3. No cleavage products other than the indicated 100kDa species could be seen with the various other caspases used in the panel, suggesting that the DAVD site undergoes very specific cleavage during the apoptotic pathway and that this is likely to be directed predominantly, if not exclusively, through the action of caspase-3.

**Properties of PDE4A5-ΔP3, an N-terminal truncate which mimics caspase-3 cleaved PDE4A5.**

Lysis of transfected COS cells transiently expressing PDE4A5 shows that this enzyme is distributed some 64 ± 1% in the cytosol (high speed supernatant) fraction and some 36 ± 4% in the particulate fraction. Similar analysis of PDE4A5-ΔP3, the N-terminal truncate engineered to reflect the caspase-3 cleaved form of PDE4A5, showed it to be distributed as 78 ± 4% in the cytosol and 22 ± 3% in the particulate fraction.

The \( K_m \) values for cAMP hydrolysis were 2.4 ± 0.7uM and 8.0 ± 0.9uM for PDE4A5 and PDE4A5-ΔP3, respectively, as found in the cytosol fraction and were 2.4 ± 0.7uM and 3.0 ± 0.4 uM for PDE4A5 and PDE4A5-ΔP3, respectively, as found in the high speed P2 particulate fraction.

It has been demonstrated previously that the \( V_{\text{max}} \) value of PDE4A5 is considerably smaller than that of both the short PDE4A1 isoform and met26-RD1, a truncate lacking both UCR and N-terminal regions (28). Using a quantitative ELISA method to immunodetect the C-terminal region of these PDE4A enzymes (see Methods), we noted here that the soluble, N-terminally truncated PDE4A5-ΔP3 species exhibited a relative \( V_{\text{max}} \) value which was 1.7 ± 0.2 – fold that of soluble PDE4A5. However, in marked contrast to this, in the P2 particulate fraction we observed that the relative \( V_{\text{max}} \) value of PDE4A5-ΔP3 was 0.4 ± 0.2 – fold of that of particulate PDE4A5 (data are means ± SD, n=3 separate experiments). These data suggest that PDE4A5-ΔP3 shows functional differences in its catalytic unit compared to PDE4A5 and that these are seemingly influenced by its subcellular localisation.

**PDE4A5 interaction with the SH3 domain of LYN.**

We have shown previously (27) that PDE4A5 can interact with the SH3 domains of certain proteins and shows particular specificity for those of SRC family tyrosyl kinases, in particular LYN and FYN. Such an interaction was not seen with other rat PDE4A isoforms. This led us to conclude (27) that SH3 domains may interact with the PXXPXXR motifs found at the extreme N-terminus of PDE4A5 (Fig. 1). Consistent with this, a fusion protein reflecting the N-terminal region of PDE4A5 was able to interact with the SH3 domains of certain proteins (27). It is thus of some potential significance that caspase-3 cleavage of PDE4A5 would be presumed to generate (Fig. 1) a catalytically active N-terminally truncated species that would be unable to interact with SH3-domain
containing proteins due to the loss of the PXXPXXR motifs found at the extreme N-terminus of PDE4A5. To be able to investigate this directly we analyzed the N-terminal truncate, PDE4A5-ΔP3 which reflects the caspase-3 cleaved form of PDE4A5. Here we show (Fig. 6) that, unlike PDE4A5, PDE4A5-ΔP3 was unable to bind LYN-SH3. This mirrored the PDE4A8 isoform, which lacks potential SH3 binding domains in its N-terminal region, and which was also unable to bind to the SH3 domain of LYN (Fig. 6). As the LYN SH3 domain binds to the N-terminal region of PDE4A5 we wished to determine whether such an interaction could affect the ability of caspase-3 to cleave PDE4A5. To evaluate this we expressed PDE4A5 in COS cells and then complexed the soluble form of this enzyme with LYN-SH3 prior to challenge with caspase-3. As can be seen in Fig. 6, caspase-3 was still able to cleave PDE4A5 when complexed to the SH3 domain of the SRC-family tyrosyl kinase, LYN.

*The intracellular localisation of PDE4A5-ΔP3 in COS cells as determined by laser scanning confocal microscopy.*

We have shown previously that PDE4A isoforms, which differ solely by virtue of their unique N-terminal regions, exhibit very different intracellular distributions (16,24,28-30,34,38,46). We therefore examined whether PDE4A5-ΔP3, which reflects the caspase-3 cleavage product of PDE4A5, showed an altered intracellular distribution in COS cells compared to PDE4A5 itself. To do this, COS-7 cells, expressing either full-length PDE4A5 or PDE4A5-ΔP3, were analyzed using an immunofluorescence procedure with detection by laser scanning confocal microscopy (Fig. 7). It is apparent from both total projection analysis and from analysis of single sections through the cell that PDE4A5 immunofluorescence is highly organized within an ordered network throughout the cell. The main foci of interaction appear to be a pronounced staining seen around the nuclear membrane, namely the perinuclear region, and also at cortical regions of the plasma membrane (Fig. 7; middle panels). This pattern of staining is similar to that seen for the human homologue of PDE4A5, namely PDE4A4 (33), which interacts with LYN in COS7 cells (26). Indeed, as found with PDE4A5, analysis of LYN immunofluorescence identified a pronounced perinuclear staining as well in these cells (Fig. 7; upper panels). In contrast to this, PDE4A5-ΔP3 showed a very different form of subcellular distribution (Fig 7; lower panels). In particular there was loss of definition of localisation around both the nuclear membrane and the perinuclear region of the cell. Whilst retaining somewhat distinct staining around the plasma membrane this was no longer limited to the cortical regions but was more evenly distributed. In addition the ordered networking seen throughout PDE4A5 transfected cells was less apparent in PDE4A5-ΔP3 transfectants as was the similarity to the distribution of LYN-SH3.
Redistribution of PDE4A5 in staurosporine-treated Rat-1-6R8 cells.

We undertook a temporal analysis of PDE4A5 distribution within staurosporine-treated 6R8 and 39B3 cells. In 6R8 cells, PDE4A5 immunofluorescence was localized predominantly to the perinuclear region (Fig. 8a), whereas in 39B3 cells, PDE4A8 immunofluorescence was diffuse throughout the cell (Fig. 8b). However, some 0.5h after treatment of 6R8 cells with staurosporine, PDE4A5 began to lose definition of the distinct subcellular staining with little immunofluorescence at the cell nuclear membrane. As staurosporine-treated 6R8 cells changed their morphology the PDE4A5 staining became more and more diffuse. These stages of redistribution away from the ordered network of staining through the cell and at the nuclear membrane appeared to mirror the differential staining seen in COS 7 cells transfected with PDE4A5 as compared to the N-terminally truncated species, PDE4A5ΔP3 (Fig. 7). In contrast to this, the distribution of PDE4A8 was unchanged in staurosporine-treated 39B3 cells (Fig. 8b). This shows that the apoptosis-induced redistribution of PDE4A5 was not just a general action on PDE4A isoforms but is specific to PDE4A5.

Apoptosis in Rat-1 fibroblasts.

We show here that incubation (24h) of Rat-1 fibroblasts with either the adenylyl cyclase activator forskolin (50uM) or the non-specific PDE inhibitor IBMX (100uM) or the PDE4-selective inhibitor, rolipram (10uM) caused little increase in apoptosis (Fig. 9a). However, the combination of forskolin with either of these PDE inhibitors gave rise to a marked increase in apoptosis (Fig. 9a). It is clear from these results that activation of adenylyl cyclase alone is insufficient to promote apoptosis, presumably because of active cAMP degradation by PDE activity in these cells. That PDE inhibitors alone did not promote apoptosis indicates that basal adenylyl cyclase activity is low. Consistent with this, the addition of the non-selective PDE inhibitor, IBMX now allowed forskolin stimulation of adenylyl cyclase to promote apoptosis (Fig. 9a). Interestingly, rolipram was equally as effective as IBMX, suggesting that PDE4 activity has an important role in the regulation of apoptosis in Rat-1 fibroblasts.

We also determined (Fig. 9b) whether the timecourse of staurosporine-induced apoptosis was altered in the cell lines that stably over-expressed either PDE4A5 (6R8 cells) or PDE4A8 (39B3 cells). It would appear that overexpression of PDE4A5 can protect against staurosporine-induced apoptosis as only 50% of 6R8 cells underwent apoptosis after a 24h challenge with staurosporine compared to 83% of wild-type cells (Fig. 9b). In marked contrast to this, the onset of staurosporine-induced apoptosis appeared to be potentiated by the over-expression of PDE4A8, with 70% of 39B3 cells undergoing apoptosis some 4h after exposure to staurosporine as compared to only 40% of wild-type cells (Fig. 9b). These data add further support to the notion that PDE4 activity can play a major role in the regulation of apoptosis in Rat-1 cells.
Conclusion

Apoptosis is of fundamental importance in the control of a number of biological systems including the regulation of normal cell turnover, the immune response, embryonic development and chemical induced cell death. Caspases, a family of cysteine dependent aspartate specific proteases have a pivotal role within this cell death machinery, with the executioner enzymes, such as caspase 3 mediating the breakdown of a number of key substrates involved in mediating cellular responses characteristic of apoptosis (1,3,4,40). The full extent of these substrate proteins, however, is not known and identification of these could provide important clues as to the execution of the apoptotic process.

Cyclic AMP, a ubiquitously expressed second messenger, has been implicated in the control of apoptosis in a number of systems (7-12). The sole route to the degradation of this signalling molecule is attributed to the family of cAMP-specific phosphodiesterases (PDEs), which are thus poised to exert profound regulatory roles (16,18-20). Thus, inhibitors that are selective for different PDE isoenzymes regulate distinct cellular responses and serve as therapeutic agents (16,18-20). Indeed, PDE4 selective inhibitors have been shown to serve as potent and selective anti-inflammatory agents (16,21). Recently, however, the archetypal PDE4 selective inhibitor rolipram has been reported to induce apoptosis in chronic lymphocytic leukemia (CLL) cells and CD19+ B cells (7) as well as HL60 cells (47). Nevertheless, to date there have been no reports as to whether any members of the multigene PDE4 family are affected by the process of apoptosis itself. Of the 16 known isoenzymes that form the PDE4 enzyme family, only PDE4A5 has a cleavage site for the downstream effector of apoptosis, namely caspase-3. Here we show that this PDE4A isoform can indeed be cleaved by caspase-3 and that such cleavage occurs at a single site, asp72. This action of caspase-3 can also be observed both in vitro using recombinant caspase-3 and also in intact cells. Indeed, the N-terminal cleavage of natively expressed PDE4A5 was also evident in differentiated PC-12 cells when apoptosis was initiated by NGF withdrawal. In addition, we also demonstrated such an occurrence in a Rat-1 cell line (6R8) stably transfected with PDE4A5 where apoptosis was induced using staurosporine. This apoptosis-induced cleavage of PDE4A5 was blocked using both a non-selective caspase inhibitor as well as a selective caspase-3 inhibitor, confirming the role of PDE4A5 as a substrate for caspase-3 in intact cells as well as in vitro. Indeed the cleaved PDE4A5 product was detected within 1-2h of staurosporine treatment of Rat-1-6R8 cells, a point when 80% of cells, as compared to controls, were still viable. Thus cleavage of PDE4A5 seems to occur as an early event in the apoptotic process of cell death. Indeed, the appearance of the 100kDa PDE4A5 cleavage product appeared to follow activation of caspase-3 and to precede the onset of apoptosis itself.

We have previously shown (27) that PDE4A5 can interact with the SH3 domains of SRC family protein kinases, in particular that of LYN. This was suggested to occur through interaction with PxxPxxR motifs located within the unique N-terminal region of PDE4A5. We show here that...
the association of PDE4A5 with LYN-SH3 did not prevent caspase-3 causing the cleavage of PDE4A5. However, the caspase-3 mediated generation of a truncated form of PDE4A5 failed to bind to LYN-SH3 as evaluated using the truncate PDE4A5-ΔP3 which was used to model the caspase-3 generated species. This is consistent with the removal from PDE4A5 of the extreme N-terminal part of the alternatively spliced region, which contains the putative SH3-interaction sites (27).

The unique N-terminal region of PDE4A enzymes is believed to determine their intracellular distribution (16). Interestingly, we see here that whilst caspase-3 cleavage of PDE4A5 caused the loss of around 70% of the unique N-terminal region, it did not ablate the ability of PDE4A5 to bind to cell particulate fractions as evaluated using PDE4A5-ΔP3. However, PDE4A5-ΔP3 exhibited a profound change in its intracellular distribution, as assessed by immunofluorescence analysis, compared to PDE4A5 (Fig. 7). Thus PDE4A5 immunofluorescence appeared to be highly organized within an ordered network throughout the cell with pronounced staining in both the perinuclear region and also at cortical regions of the plasma membrane (Fig. 7). This perinuclear staining closely mimicked that seen for LYN expressed natively in these COS cells (Fig. 7). Intriguingly, PDE4A5-ΔP3, which lacks the ability to interact with the SH3 domain of LYN showed a loss of this highly ordered perinuclear staining (Fig. 7). This would be consistent with an association of PDE4A5 with LYN causing targeting to the perinuclear region and the inability of PDE4A5-ΔP3 to interact with LYN-SH3 underpinning the failure of this truncate to show such a distinctive localisation within the perinuclear region.

We would like to propose that there may well be at least two potential subcellular anchor sites for PDE4A5. One of these we suggest is an SH3 domain interaction site found at the extreme N-terminal region of PDE4A5 and which, at least in COS cells, confers an ability to target to the perinuclear region of these cells and appears to be of dominant importance. The other targeting site within PDE4A5 remains to be identified. We also noted (Fig. 8) that PDE4A5 was also predominantly localized to a distinct perinuclear region when stably expressed in the transfected RAT1-6R8 cell line and that the onset of apoptosis disrupted such targeting. It is tempting to speculate that a contributory factor in this relates to the caspase-3 cleavage of PDE4A5 serving to remove its SH3-domain interacting region. However, we cannot discount other contributory factors such as the re-distribution of the anchor species during the apoptotic process.

There is good evidence that in many, if not all, cell types cAMP signalling is compartmentalized (13-16). We would like then to suggest that caspase-3 action in the apoptotic process could lead to the disassociation of PDE4A5 from interaction with an SH3-domain containing anchor protein. Given the high specific activity of PDE enzymes this might be expected to lead to alterations in the local levels of cAMP and thus changes in regulatory activity. This may be of importance as cyclic AMP has been shown to regulate apoptosis in several cell types, inhibiting or stimulating the process depending on the cell type and stage of differentiation (7-12). Indeed, we
show here that rolipram inhibition of PDE4 activity in Rat-1 fibroblasts can stimulate apoptosis provided that adenylyl cyclase is activated, such as was achieved here using forskolin (Fig. 9). This suggests that the regulation of cAMP levels by PDE4 activity in Rat-1 cells may be of importance to the apoptotic process. Thus the combination of a decrease in activity of particulate-associated PDE4A5 activity, coupled with its redistribution, may contribute to the regulation of apoptosis in Rat-1 cells. Indeed, there is evidence that compartmentalized cAMP responses are involved in regulating apoptotic events in the case of gliotoxin induced apoptosis in thymocytes, a process which is mediated by phosphorylation of Histone H3 by specific pools of PKA (48). Also in human neutrophils it has been reported that the cAMP-induced delay of apoptosis is mediated specifically by just a fraction of the total PKA pool, namely involving type 1 PKA activation (49).

To begin to understand the roles of PDE4 enzymes in the apoptotic process we have here examined the effect of stable over-expression of two PDE4A isoenzymes, PDE4A5 and PDE4A8, on staurosporine-induced apoptosis in Rat-1 cells. These isoenzymes show very different patterns of tissue expression and intracellular distribution (16), implying functionally distinct roles. They differ only in their extreme N-terminal region which, in the case of PDE4A5, contains the SH3-binding site that we show here to be cleaved by caspase-3 action. Here we see a striking difference where the stable overexpression of these isoenzymes in Rat-1 cells leads to very different effects on staurosporine-induced apoptosis. Intriguingly, overexpression of PDE4A5 led to protection from staurosporine-induced apoptosis. This is consistent with our finding that the PDE4 selective inhibitor, rolipram can lead to apoptosis provided that adenylyl cyclase is stimulated. That this was not evident in the 39B3 cells that over-expressed PDE4A8 might suggest that the intracellular targeting of PDE4A5, and thus control of cAMP signalling in specific intracellular compartments, is important in defining the protective role of PDE4A5. Nevertheless, the mechanisms that provide controls upon apoptosis are many and complex and it will be a considerable challenge to identify the precise points at which compartmentalized cAMP signalling can afford regulation.

The present study provides novel insight into the properties of PDE4A5. Thus both natively expressed and recombinant PDE4A5 can be cleaved during apoptosis, a cellular process of major importance. Cleavage is due to caspase-3 action at a single site (asp72) found within the 256-residue N-terminal region that is unique to PDE4A5. Such cleavage leads to functional changes in PDE activity as well as the dynamic redistribution of PDE4A5 that we suggest here may be due to loss of the SH3 binding region found at the extreme N-terminus of PDE4A5. This indicates that at least two sites on PDE4A5 are responsible for membrane-association and intracellular targeting, one of which encompasses the SH3-binding extreme N-terminal region that is cleaved by caspase-3 action.

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Figure legends

Figure 1  Schematic representation of PDE4A isoforms.

The unique sequence of the extreme N-terminal region is shown for each of the three rat PDE4A isoforms. Of these, only the unique N-terminal region of PDE4A5 contains three PxxPxxR motifs, shown in bold type, which have been shown to provide interaction sites for SH3 domain binding (27). The potential motifs for caspase-3 cleavage found only in PDE4A5 are indicated as underlined text. The long isoforms, PDE4A5 and PDE4A8, exhibit both of the Upstream Conserved Regions known as UCR1 and UCR2. The short PDE4A1 isoform exhibits a truncated (C-terminal) part of the UCR2 region only. The rat PDE4A5 enzyme is characterized by the clone rpde6 (GenBank ™ accession number L27057); rat PDE4A8 by the clone rpde-39 (GenBank ™ accession number L36467); rat PDE4A1 by the clone RD1 (GenBank ™ accession number M26715 and J04554).

Figure 2. Time dependent activation of caspase-3 activity and apoptosis in response to staurosporine treatment of Rat-1-6R8 cells

Rat-1 6R8 cells were treated with 1µM staurosporine for the indicated times. Cells were then harvested for analysis of (A) caspase-3 activity (pmol/min/mg protein), (B) Annexin-V binding by FACS analysis (apoptotic cells as a %-age of control) and (C) DNA cleavage (laddering).

Figure 3. Apoptosis causes caspase-induced cleavage of an N-terminal region from PDE4A5.

Panel A. Rat-1-6R8 cells were treated with 1µM-staurosporine for the indicated times in order to induce apoptosis. An antiserum specific for the C-terminal region of PDE4A enzymes identified a single 110±3kDa immunoreactive species as indicated by the upper arrow in the zero time (0). Over the timecourse of the experiment a single additional, faster migrating immunoreactive species was observed (lower arrow). This migrated as a 100±2kDa species. Also shown is the effect of pre-incubating cells with the general caspase inhibitor ZVAD-fmk (50µM) for 3h prior to treatment with staurosporine for 24h.

Panel B. The production of the cleaved 100kDa species was detected as shown in panel A by immunoblotting. This was assessed by densitometric scanning (arbitrary units) as described before by us (33) and expressed here as a percentage conversion;

Panel C. This shows the identical blot to that described in panel A, except that it was stripped and probed with an antiserum that was specific for the N-terminal region of PDE4A5. In this instance a single, 110±2kDa immunoreactive species was evident (upper arrow), with no indication of any 100kDa immunoreactive species.

Panel D. This shows the single immunoreactive species of 98kDa evident in Rat-1-39B3 cells, transfected to express PDE4A8 detected using the C-terminal PDE4A specific antiserum (upper
arrow). No cleaved species were apparent upon treatment of these cells with 1µM-staurosporine for the indicated times. The lower arrow indicates the position a species some 10kDa less in size would be expected to migrate at. No species of any other size were evident on the gel (data not shown).

Panel E. Rat-1-6R8 cells were either untreated (lane 1) or treated with 1µM-staurosporine (lanes 2,3,4) prior to immunoblotting lysates with the C-terminal PDE4A-specific antiserum. In certain experiments cells were additionally pre-treated for 3 hours with the caspase-3 specific inhibitor DAVD-CHO (100µM) (lane 3) or with the caspase-1 specific inhibitor, z-YVAD-CHO (100µM) (lane 4).

Panel F. Differentiated PC12 cells were grown in either NGF-containing (lane 1) or NGF-free (lane 2) medium for 24h. PDE4A5 was immunoprecipitated from lysed cells using a C-terminal antiserum before Western blotting with the C-terminal PDE4 antiserum. The upper arrow indicates PDE4A5 and the lower arrow a species some 10kDa smaller in size.

**Figure 4. Cleavage of PDE4A5 by recombinant caspase-3.**

Panel (A). 10µg of lysate cell protein from Cos-7 cells transiently expressing PDE4A5, PDE4A8 or PDE4A1A was incubated for 2h at 37°C in the presence (+) or absence (-) of recombinant caspase-3 (147ng). Lysates from PDE4A5-transfected cells identified a major 110kDa species as detected by both the N- and C-terminal antisera as indicated. A minor 100kDa immunoreactive species was evident in lysates of PDE4A5-transfected cells when probed with the C-terminal PDE4A-specific antisera. Lysates from either PDE4A1 or PDE4A8 transfected cells yielded single immunoreactive species that migrated as 79kDa and 98kDa species, respectively.

Panel (B). 10µg of cell protein from cells expressing either wild-type PDE4A5, the internal deletion mutant PDE4A5-ΔDAVD72 or the point mutant PDE4A5-DAVA72 were separately incubated in either the presence (+) or absence (-) of recombinant caspase-3 prior to immunoblotting with PDE4A specific antiserum.

Panel (C) 10µg of cell protein from cells expressing either the point mutant PDE4A5-AAVD72 or the point mutant PDE4A5-DTGA75 were separately incubated in either the presence (+) or absence (-) of recombinant caspase-3 prior to immunoblotting with PDE4A specific antiserum.

Panel (D). Cos-7 cells were transiently transfected to express PDE4A5. At the indicated times after transfection, cells were harvested and 10µg of lysate cell protein taken for analysis by immunoblotting for PDE4A. The upper arrow indicates the major 110kDa that reflects PDE4A5 and the lower arrow the minor 100kDa immunoreactive species.

Panel (E). Here wild-type PDE4A5 (track 1) is compared to the N-terminal truncated species, PDE4A5-ΔP3 (track 2) when transiently expressed in Cos-7 cells. PDE4A immunoreactivity is analysed in untreated Rat-1-6R8 cells (track 3) and in Rat-1-6R8 cells treated with 1µM staurosporine (track 4).
Figure 5 Screening a library of recombinant caspases for their ability to cleave PDE4A5.

Panel A. This shows the action of the indicated forms of activated recombinant caspases on PDE4A5. PDE4A5 was generated as a $^{35}$S-methionine-labelled species by an in vitro transcription-translation procedure (38). The products of the experiments were incubated in the presence of a panel of active, recombinant caspases, subjected to SDS-PAGE and visualized by phosphorimaging.

Panel B. This shows the action of the indicated forms of activated recombinant caspases on PDE4A5 generated by transient transfection of COS cells. The products of these experiments were analyzed by immunoblotting.

Caspases used in this study are in each case identified here by their number. This is given under the indicated lane. The first track (-) indicates the control fraction of PDE4A5 which had not been treated with caspase-3. The upper arrow indicates the 110kDa PDE4A5, the lower arrow the position of the 100kDa cleaved product.

Figure 6. Caspase-3 action and the interaction of PDE4A5 with LYN-SH3.

Panel A shows immunoblotting of soluble PDE4A5 which either had been treated (+) or not (-) with caspase-3. Control (‘ctr’) experiments were done with no further additions. PDE4A5 was also complexed to the SH3 domain of LYN expressed as a GST fusion protein, as described before (27), prior to caspase challenge (‘Lyn-SH3’). In addition, GST was also mixed with PDE4A5 prior to caspase challenge (indicated as ‘GST’).

Panel B. This shows that PDE4A5ΔP3, unlike native PDE4A5, can no longer interact with LYN-SH3. A GST fusion protein of the SH3 domain of LYN was used to probe for binding to PDE4A5 expressed in COS7 cells as described before by us (27). This was set up such that around 40% of the PDE4A5 in the reaction mixture was ‘pulled down’ by the added GST-LYN-SH3 fusion protein. This procedure was repeated using identical amounts of either the N-terminal truncate, PDE4A5ΔP3 or the other long PDE4A isoform, PDE4A8. These showed little or no binding to GST-LYN-SH3 as the amount pulled down was comparable to that pulled down using GST, rather than GST-LYN-SH3, with all of these PDE4A constructs, namely 5-8% (range).

Figure 7. Confocal immunofluorescence analysis of PDE4A5 and PDE4A5ΔP3 expressed in COS cells.

The upper two panels (A) show total projections of two different cells immunostained for LYN. Note that the focus of LYN staining in these cells is at a discrete perinuclear area (red arrow).

The middle panels (B) show COS-7 cells which had been transiently transfected with full length PDE4A5. The first two colour pictures show total projections and the third black and white
picture a single section through the centre of a transfected cell. Note that the focus of PDE4A5 immunofluorescence in these cells is at both a discrete perinuclear area (red arrow) and at the cell cortical region (yellow arrow).

The lower panes (C) show COS-7 cells which had been transiently transfected with PDE4A5-ΔP3. The first two colour pictures show total projections and the third black and white picture a single section through the centre of a transfected cell. Note that the focus of PDE4A5-ΔP3 immunofluorescence in these cells is at the cell cortical region (yellow arrow) and in punctate structures throughout the cell cytosol.

These examples in each case are typical of cell staining patterns. All images were visualized as 0.2µm sections on a laser scanning confocal microscope.

Figure 8. PDE4A5 expressed in Rat-1-6R8 cells redistributes in a time dependent manner following staurosporine-induced apoptosis.

Cells were incubated with 1µM-staurosporine for the times indicated before being fixed and the PDE4A5 distribution in Rat-1-6R8 cells (Panel A) and PDE4A8 distribution in Rat-1-39B3 cells (Panel B) were visualized using a PDE4A-specific C-terminal antibody and anti-rabbit IgG conjugated to FITC. Immunostaining was visualized as 0.2µm sections on a laser scanning microscope with total projections being shown.

Figure 9. Apoptosis in Rat-1 cells.

(a) This analyses apoptosis in Rat-1 cells treated for 24h with agents that either activate adenyl cyclase, namely 50uM-forskolin (F) or inhibit cAMP phosphodiesterases, namely 100uM-IBMX (I) or 10uM-rolipram (R). In some instances forskolin was added together with either IBMX (F+I) or with rolipram (F+R). Control with no additions is shown (C).

(b) Wild type Rat-1 cells as well as PDE4A5- (6R8) and PDE4A8- (39B3) overexpressing Rat-1 cells were challenged with staurosporine (1µM) for 16h.

In both instances, apoptotic and necrotic cells were quantified using annexin V and propidium iodide flow cytometry, respectively, with data given as means ± SD of n=3 experiments. Shown are the percentage cells that have undergone apoptosis.
Unique N-terminal regions

PDE4A5 (rpde6)

MEPPAAPSERSLSLSLPGPREGQATLKKPPQHLWRQPRTPIRIQRGYPDSAERSETERS

PDE4A8 (rpde39)

PHRPIERA\textbf{DAVD}^{72}\textbf{TGD}\text{RPGLRTTRMSWPSSFHGTGGSSRR}^{102}

PDE4A1 (RD1)

MPSRKRLTLPRIFIVRKNGNS^{21}

MPLVDFCETCSKPWLVGWWDQ^{22}
Fig. 3

A

B

C

D

E

F

Production of cleaved product (% age conversion)

Time (h.)

staurosporine

Caspase inhibitor

- 1 2 3 4

D Y E V V A

1 + + +

2 - - -

F

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