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REGULAR ARTICLE

Katherine B. Pryzwansky · Victoria J. Madden

Type 4A cAMP-specific phosphodiesterase is stored in granules of human neutrophils and eosinophils

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Abstract Persistent elevations of cAMP levels are generally accompanied by an inhibition of granulocyte functions. Phosphodiesterases play a critical role in regulating intracellular levels of cAMP. The expression of three isoforms of type 4 cAMP-specific phosphodiesterase (PDE4) in neutrophils suggests diversity of isoform localization and targeting in regulating cell function. The sites of cAMP regulation in granulocytes by the PDE4A isoform were investigated by immunoelectron microscopy. PDE4A was localized uniformly in all granule classes of eosinophils, but was restricted in neutrophils to a subset of myeloperoxidase (MPO)-containing granules that were round or elongated with a central crystalloid core. Granulocytes were stimulated with fMLP to investigate the sites of PDE4A targeting during cell activation. In neutrophils, fMLP induced a rapid (1 min) translocation of granules containing PDE4A to the plasmalemma, where some PDE4A and MPO were exocytosed. In these cells, PDE4A labeling within granules was focal and no longer homogeneous. While immunogold labeling of PDE4A was reduced after fMLP stimulation, staining of MPO-containing granules remained high. Extracellular release of PDE4A was also observed in eosinophils stimulated with fMLP. Morphometry revealed that Au labeling was significantly reduced within 1 min, and that there was a shift in PDE4A localization within eosinophil granules from the crystalline core to the matrix. Fluctuations of cAMP levels and ectoprotein kinase activity with PKA properties occur in blood under normal and pathological conditions. The exclusive localization of PDE4A within granules of neutrophils and eosinophils suggests that PDE4A may function to downregulate cAMP signaling at the cell membrane and/or in the extracellular space at the time of granule release.

Keywords Immunogold · cAMP-dependent protein kinase · fMLP · Myeloperoxidase · Lactoferrin · Human

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K. B. Pryzwansky (✉)
Department of Pathology and Laboratory Medicine,
Brinkhous-Bullitt Building,
University of North Carolina,
Chapel Hill, NC 27599-7525, USA
e-mail: pryz@earthlink.net
Tel.: +1-919-9295122
Fax: +1-919-9660704

Introduction

It is well established that cAMP is a key second messenger in regulating granulocyte functions. The levels of cAMP are under exquisite control in granulocytes, as persistent elevations of intracellular cAMP levels are generally accompanied by an inhibition of cell function (Coffey 1992). The only means for inactivating cAMP in cells is by hydrolysis of cAMP to 5'-AMP by cAMP phosphodiesterases (PDE). The predominant PDE in granulocytes belongs to the PDE4 family or cAMP-specific PDE (Smolen and Geosits 1984; Grady and Thomas 1986; Wright et al. 1990; Souness et al. 1991; Verghese et al. 1995a; Wang et al. 1999). PDE4, coupled with the well-defined role of cAMP as a generalized suppressor of immune and inflammatory cell activity, has led to the recognition that PDE play a critical role in regulating the function of these cells. Thus, specific PDE4 inhibitors are currently being investigated as anti-inflammatory and anti-asthmatic drugs because of their inhibitory effects on neutrophil and eosinophil functions. For example, the PDE4-specific inhibitors rolipram and RO 20-1724 inhibit neutrophil adhesion, granule secretion, and superoxide generation when activated with a variety of agonists including fMLP, LTB4, C5a, platelet-activating factor, A23187, TNFα, and GM-CSF, but not PMA (Nielson et al. 1990; Wright et al. 1990; Derian et al. 1995; Ottonello et al. 1995a; Bengis-Garber and Gruener 1996). Furthermore, a greater inhibition of these functions is observed when neutrophils are treated with both a PDE4 inhibitor and a cAMP-elevating agent such as PGE2 or forskolin (Nourshargh and Hoult 1986; Coffey 1992; Ottonello et al. 1995a, 1995b). Similarly, PDE4-

specific inhibitors downregulate eosinophil functions (Plaut et al. 1980; Dent et al. 1991; Souness et al. 1991; Newsholme and Schwartz 1993; Underwood et al. 1993; Santamaria et al. 1997).

There are at least four genes that encode for the PDE4 family in humans and are designated PDE4A, 4B, 4C, and 4D. Neutrophils and mononuclear phagocytes express PDE4A, 4B, and 4D (Verghese et al. 1995b; Manning et al. 1996; Pryzwansky et al. 1998; Wang et al. 1999). The PDE4 isoforms expressed in eosinophils have not been characterized. There is convincing evidence that the functioning of cAMP within cells may be compartmentalized and regulated through the interaction of cAMP-dependent protein kinase (PKA) with a family of specific PKA-binding proteins (Michel and Scott 2002). Furthermore, the catalytic activity of PDE4 isoforms can be regulated by intracellular targeting by isoform-specific N-terminal regions (Houslay et al. 1997; Beard et al. 2002). The expression of at least three isoforms for PDE4 in neutrophils, and the evidence that PDE4 activity is found in both the particulate (30%) and soluble (70%) fractions of neutrophil sonicates, suggests that the sites of cAMP degradation also may be compartmentalized (Grady and Thomas 1986; Souness et al. 1991; Peachell et al. 1992). For example, in neutrophils and mononuclear phagocytes, cAMP, PKA, and PDE4 are compartmentalized at the nascent phagosome during phagocytosis (Pryzwansky et al. 1981, 1998). We and others proposed that the targeting of cAMP receptor proteins at the nascent phagosome may function to regulate actin polymerization/depolymerization during phagocytosis (Pryzwansky et al. 1998; Ydrenius et al. 2000).

The loci and mechanisms involved in the inhibitory effects of cAMP elevations in granulocytes are of interest, but remain poorly understood. In order to understand the cellular mechanisms that regulate cAMP signaling, it is important to identify the intracellular targeting sites of cAMP receptor proteins. Immunocytochemistry provides a means to localize regulatory proteins without the inherent artifacts that accompany biochemical or immunological identification of targeting sites after cell lysis. Therefore, we investigated the localization of PDE4A in granulocytes at the ultrastructural level. We show that PDE4A is localized exclusively within cytoplasmic granules of neutrophils and eosinophils, and that fMLP induced the translocation of PDE4A-containing granules to the plasmalemma where some PDE4A was exocytosed. We suggest that the exclusive localization of PDE4A within granules of neutrophils and eosinophils may function to downregulate cAMP signaling at the cell membrane and/or in the extracellular space at the time of granule fusion with the plasmalemma.

Part of this work has been published previously in abstract form (Pryzwansky et al. 2001).

Materials and methods

Granulocyte isolation

Granulocytes were isolated from human peripheral blood collected in 0.38% sodium citrate by density gradient centrifugation in Polymorphprep (Nycomed, Oslo, Norway). The cells were resuspended at 2.5×10^6 cells/ml in Gey's balanced salts containing 1.5 mM $CaCl_2$, 1 mM $MgCl_2$, and 0.3 mM $MgSO_4$ (GBSS), and supplemented with 10% human type AB serum. The cells were layered onto glass coverslips for 15 min at 37°C. The non-adherent cells were removed and the monolayer was washed 2 times with GBSS to remove the serum. Cells were >98% viable by trypan blue exclusion and consisted of approximately 96% neutrophils, with the remaining cells consisting of eosinophils.

Immunofluorescence microscopy

Cell monolayers were stimulated with 0.1 μM fMLP (Peninsula Lab., Belmont, CA) from 1 to 5 min at 37°C. The cells were fixed as previously described and stained with mouse anti-PDE4A for 30 min, washed in PBS, and stained with FITC goat anti-mouse IgG (Chemicon, Temecula, CA) (Pryzwansky and Merricks 1998). Cells were mounted in polyvinyl alcohol and viewed on a Leitz fluorescence microscope. Photomicrographs were captured on a Sony Optronics DEI-470 video monitor and printed on a Sony video color printer.

The monoclonal antibody against PDE4A (clone 66C12H, IgG1) was a gift from Dr. Sharon Wolda (ICOS Inc., Bothell, WA). Antibody specificity was demonstrated by adsorption of the antibody with purified recombinant PDE4A, PDE4B, and PDE4D. Staining of neutrophils with anti-PDE4A was blocked only by adsorption with recombinant PDE4A. In addition, antibody staining was observed only in yeast spheroplasts induced to express its respective isoform, and not with an unrelated isoform. Staining of yeast induced to express a specific isoform was blocked when the antibody was adsorbed only by its specific isoform (Pryzwansky et al. 1998).

Immunoelectron microscopy

Monolayers of unstimulated granulocytes, or cells stimulated with 0.1 μM fMLP, were microwave processed for electron microscopy as previously described (Madden 1998). The monolayers were placed in a fixative containing 2% paraformaldehyde and 0.5% glutaraldehyde in 0.1 M sodium cacodylate buffer (pH 7.4), cooled to 10°C, and microwaved in a Pelco Model 3440 MAX laboratory microwave oven with a power output of 800 W (Ted Pella, Inc., Redding, CA) for approximately 40 s until a temperature of 40°C was attained. Following rinses in cacodylate buffer, the cells were microwaved in 1% osmium tetroxide in 0.1 M cacodylate buffer (pH 7.4) for 2 min at 40°C. The monolayers were dehydrated with acetone using short 45 s irradiation cycles for each dehydration concentration with a temperature limitation of 45°C, and then infiltrated in two steps with LR White resin-hard grade (Ted Pella, Inc) for 10 min at 50°C. Coverslips were inverted onto gelatin capsules overfilled with LR White resin and polymerized overnight at room temperature using a long-wave (365 nm) UV lamp. Ultrathin sections (80 nm) mounted on nickel grids were treated with saturated sodium metaperiodate for 10 min, blocked with 5% normal goat serum for 15 min, and incubated for 1 h at room temperature with one of three primary antibodies: 0.1 μg/ml mouse anti-PDE4A (clone 66C12H, gift from ICOS), 0.7 μg/ml rabbit anti-human granulocyte MPO [#A0398, lot #096(101) DAKO, Carpinteria, CA], or 3.6 μg/ml rabbit anti-human lactoferrin (DAKO). Negative controls were run concurrently, consisting of normal mouse IgG subtype 3 (isotype of PDE4A monoclonal antibody) or normal rabbit immunoglobulins (DAKO) at the same dilution. Cells were incubated for 1 h at room temperature with either 1:50 10 nm Au-goat anti-mouse IgG + IgM ($F(ab')_2$) or 1:100 5 nm Au-goat

anti-rabbit IgG ($F(ab')_2$) (BBI Intl., Ted Pella). Sections were poststained with uranyl acetate and lead citrate and observed on a LEO EM-910 transmission electron microscope at 80 kV (LEO Electron Microscopy, Thornwood, NY).

Morphometry

Photomicrographs of cells from three experiments were taken on a LEO EM910 transmission electron microscope equipped with a Gatan Bioscan model 792 digital camera system and Digital Micrograph 3.4.3 acquisition software (Gatan, Inc., Pleasanton, CA, USA). Images were photographed at 25,000 magnification and saved as 1024×1024 gray-scale TIFF format images. The micrographs were imported into Image J (version 1.28, National Institutes of Health, Bethesda, MD, http://rsb.info.nih.gov/ij) to quantify the Au particles. Intact, membrane-bound eosinophilic granules with visible crystalline inclusions were chosen for counting in 15 unstimulated and 15 fMLP-activated eosinophils. One hundred granules per condition were counted with the data collected as number of Au particles in the granule matrix, number of Au particles on the crystalloid core, and total number of Au particles per granule. These values were imported into Excel spreadsheets (Microsoft Excel X for Mac), where statistical calculations were performed using Student's *t*-test.

Results

PDE4A is localized within granules of neutrophils and eosinophils

The localization of PDE4A was investigated in adherent granulocytes by immunogold electron microscopy. Prominent immunogold staining of PDE4A was observed exclusively in cytoplasmic granules of neutrophils and eosinophils (Fig. 1). In neutrophils, the localization of PDE4A was restricted to a subpopulation of granules (Fig. 1a). Unlike neutrophils, PDE4A in eosinophils was localized uniformly in all granule categories with no preference of localization for the crystalline core or granule matrix (Fig. 1b). These findings were consistent in cell preparations from three different individuals. The staining of cells for PDE4A was specific, as there was low background reactivity of gold in all treatments of controls. The secondary antibody gold control showed very low levels of labeling in the nucleus, cytoplasm, granule membrane, or granule matrix. In addition, minimal labeling was observed when the antibody was adsorbed with recombinant PDE4A (Fig. 1c) or with normal mouse IgG subtype 3 (isotype of PDE4A monoclonal antibody, not shown), indicating little non-selective binding of primary or secondary antibodies. Thus, PDE4A is stored within all cytoplasmic granules of eosinophils, but is confined to a granule subset in neutrophils.

To identify the granule class containing PDE4A in neutrophils, cells were stained simultaneously for PDE4A and lactoferrin (LF) or PDE4A and myeloperoxidase (MPO), markers of specific and azurophil granules, respectively (Borregaard and Cowland 1997). In cells stained for PDE4A and LF, PDE4A was localized exclusively in round and crystalloid granules lacking LF (Fig. 2b). Dual staining of cells for PDE4A and MPO demonstrated that while there were many granules that were labeled for MPO, only a small population of granules contained both MPO and PDE4A (Fig. 2a). This subclass of azurophil granules was round or elongated with a central crystalloid core. The localization of PDE4A in the absence of MPO was rare. Therefore, in neutrophils, PDE4A resides within a subpopulation of azurophil granules.

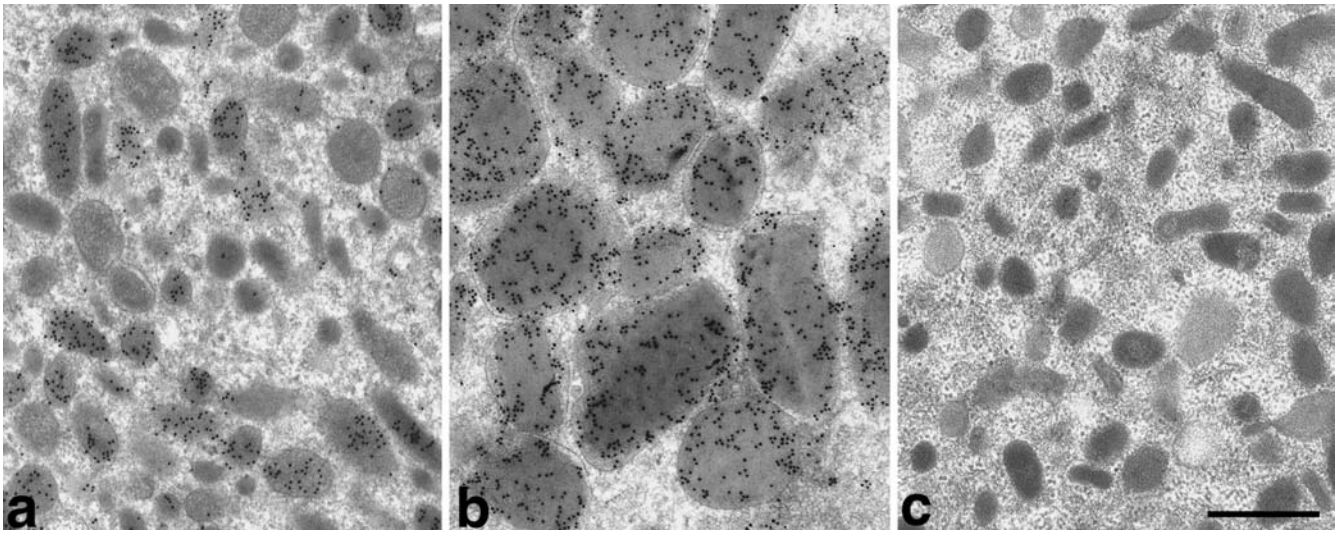

Fig. 1a–c Immunogold localization of PDE4A in adherent unstimulated granulocytes. PDE4A was localized within a subset of granules in neutrophils **(a)** and within the crystalloid and small granules of eosinophils **(b)**. No staining was observed with antibody adsorbed with recombinant PDE4A in neutrophils **(c)** or in eosinophils (not shown). *Scale bar* 0.25 μm

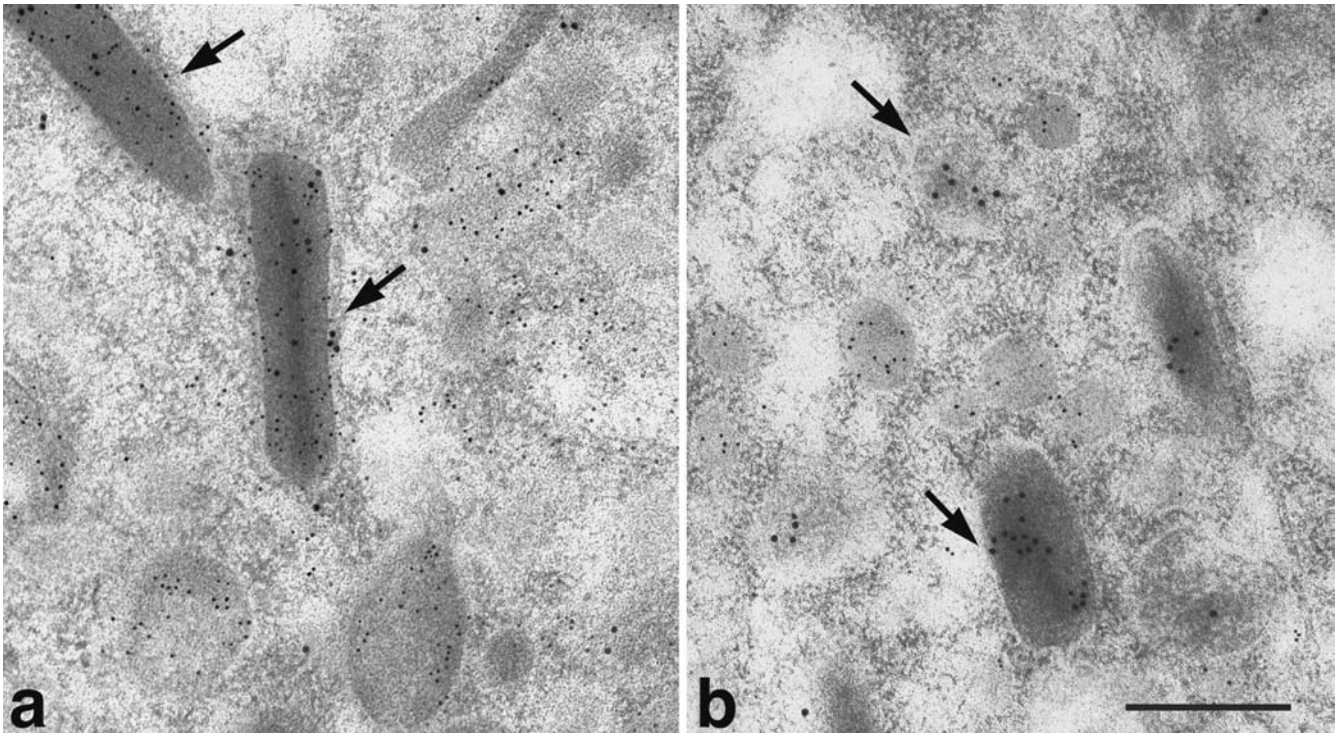

Fig. 2a, b Immunogold localization of PDE4A (10 nm Au) with MPO (5 nm Au) or LF (5 nm Au) in adherent unstimulated neutrophils. PDE4A was co-localized within a subset of MPO-containing granules (**a,** *arrows*). In cells dual stained for PDE4A and LF, PDE4A was localized in electron-dense and non-electron-dense granules devoid of LF (**b,** *arrows*). *Scale bar* 0.25 μm

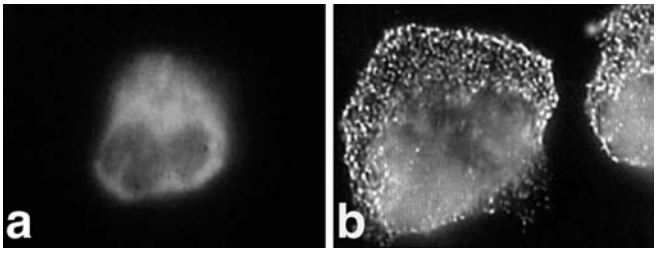

Fig. 3a, b Immunofluorescence localization of PDE4A in unstimulated neutrophils and cells stimulated with fMLP for 1 min. PDE4A was localized in the cytoplasm of unstimulated neutrophils **(a)**. A granule-staining pattern was not apparent because of the density of the cell body at this magnification. However, fMLP induced cell spreading and revealed granular staining of PDE4A in the cytosol **(b)**. Granular staining was pronounced near the cell membrane, suggesting that PDE4A-containing granules move to the plasmalemma during cell activation. ×2500

Sites of targeting of PDE4A in fMLP-stimulated neutrophils

The compartmentalization of PDE4A to cytoplasmic granules suggested that PDE4A might regulate cAMP levels at the time of granule secretion. Therefore, it was of interest to determine the site(s) of targeting of PDE4A in activated granulocytes. Adherent cells were stimulated for 1–5 min with concentrations of the chemotactic peptide fMLP (0.1 μM) that induce granule secretion in the absence of cytochalasins (Pryzwansky and Merricks 1998). Immunofluorescence microscopy was performed first to investigate the predominant sites of PDE4A localization after cell activation. Cells were then processed for immunoelectron microscopy to resolve the sites of PDE4A targeting at the ultrastructural level.

Unstimulated neutrophils were small and round, and immunofluorescence microscopy indicated that the cytoplasm was the primary site of PDE4A localization (Fig. 3a). Stimulation of neutrophils with fMLP for 1 min induced neutrophils to spread. In these well-spread cells, a particulate (granular) staining pattern of PDE4A was revealed in the cytosol (Fig. 3b), supporting our observations that PDE4A is localized in granules (Fig. 1a). In addition, there was pronounced granular staining of PDE4A near the cell margin, suggesting that PDE4A-containing granules moved to the plasmalemma during activation with fMLP.

Immunogold electron microscopy was used to resolve the subcellular localization of PDE4A in neutrophils during activation with fMLP. When neutrophil monolayers were stimulated with fMLP for 1 or 2.5 min, PDE4A-containing granules redistributed to sites proximal to the plasma membrane (Fig. 4). These data are in agreement with the immunofluorescence microscopy observations (Fig. 3b). Examination of granules after neutrophil activation revealed that PDE4A was no longer uniformly distributed (Fig. 5). Within 1 min, clusters of immunogold labeled PDE4A were observed within one area of the granule matrix, whereas the distribution of MPO within

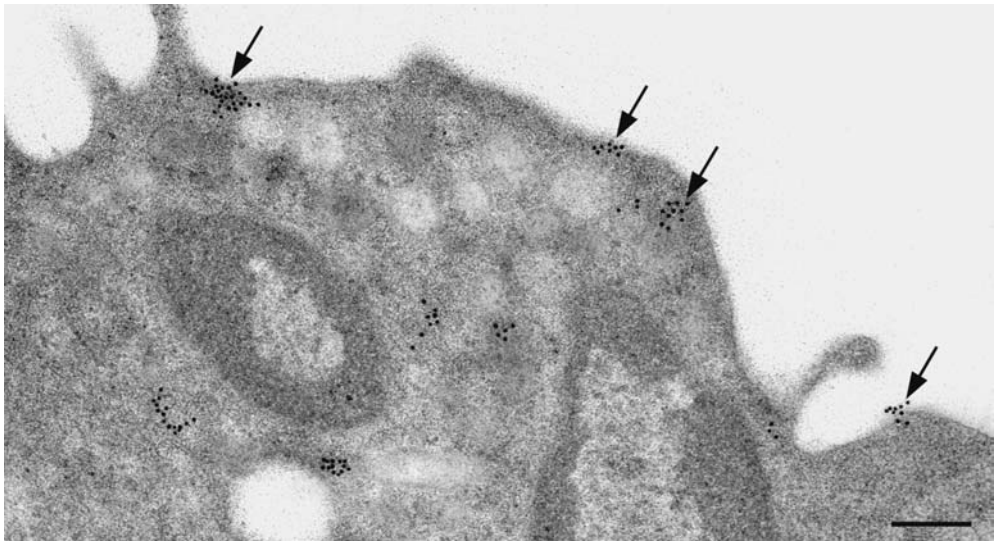

Fig. 4 Immunogold localization of PDE4A (10 nm Au) in an adherent neutrophil stimulated with fMLP for 2.5 min. Clusters of PDE4A-labeled Au particles were observed near the plasma membrane (*arrows*). *Scale bar* 0.25 μm

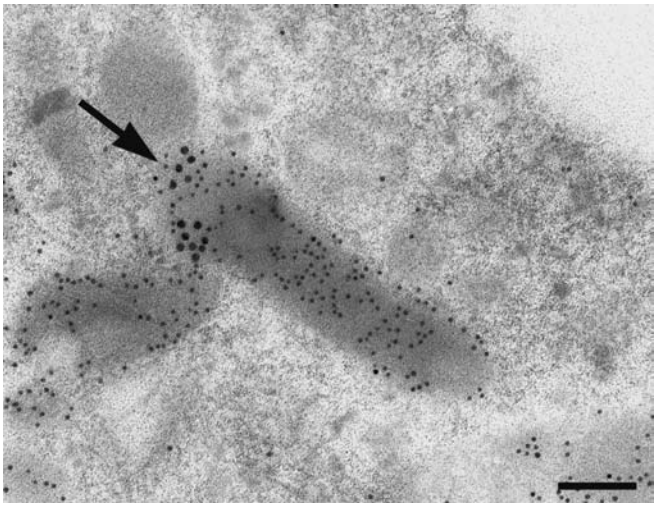

Fig. 5 Immunogold localization of PDE4A (10 nm) and MPO (5 nm) in a neutrophil stimulated with fMLP for 1 min. While MPO was uniformly distributed within the granules, clusters of PDE4A were observed at one region of the granule matrix (*arrow*), suggesting that PDE4A and MPO are organized within different compartments. *Scale bar* 0.1 μm

granules remained uniform. In addition, immunogold labeling for PDE4A and MPO was observed on residual granule material in the extracellular space (Fig. 6). While immunogold labeling of PDE4A was reduced in neutrophils after fMLP stimulation, staining of MPO-containing granules remained high (Fig. 6). The prominent staining of MPO within granules is consistent with our previous studies, indicating that only 15% of the total MPO is released from adherent neutrophils stimulated with fMLP, and that granule release is essentially complete within 2.5 min (Wyatt et al. 1993; Pryzwansky and Merricks 1998). Thus, only a small population of MPO-containing granules is exocytosed, and this azurophil granule subset contains PDE4A.

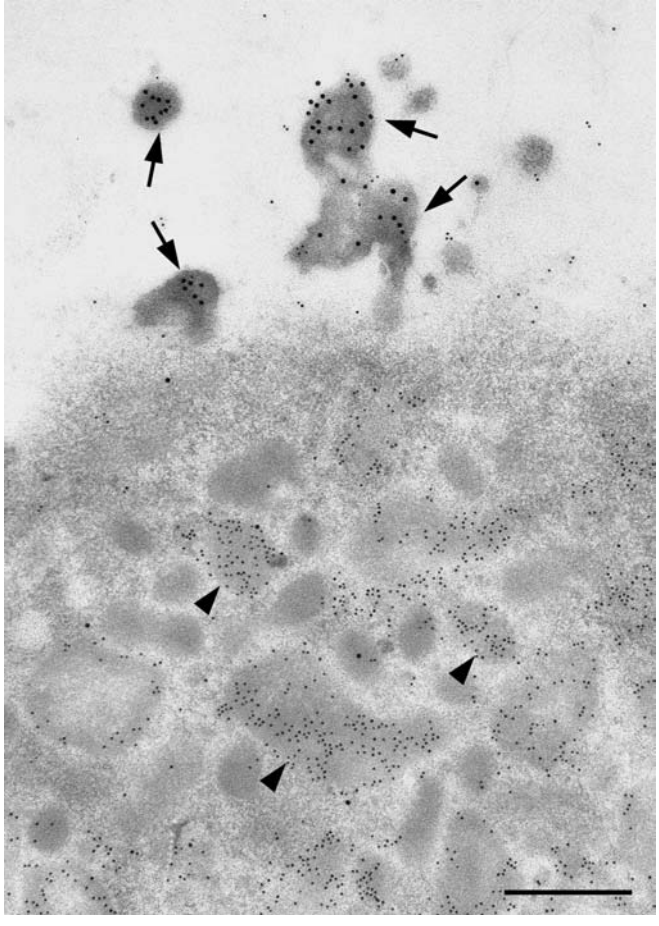

Fig. 6 Immunogold localization of PDE4A (10 nm Au) and MPO (5 nm Au) in an adherent neutrophil stimulated with fMLP for 2.5 min. FMLP stimulated the exocytosis of PDE4A and MPO from intracellular stores. Residual granule material labeled with PDE4A and MPO (*arrows*) was observed in the extracellular space. However, the contents of many MPO-containing granules were not exocytosed (*arrowheads*), suggesting that only a small population of MPO-containing granules was released. *Scale bar* 0.25 μm

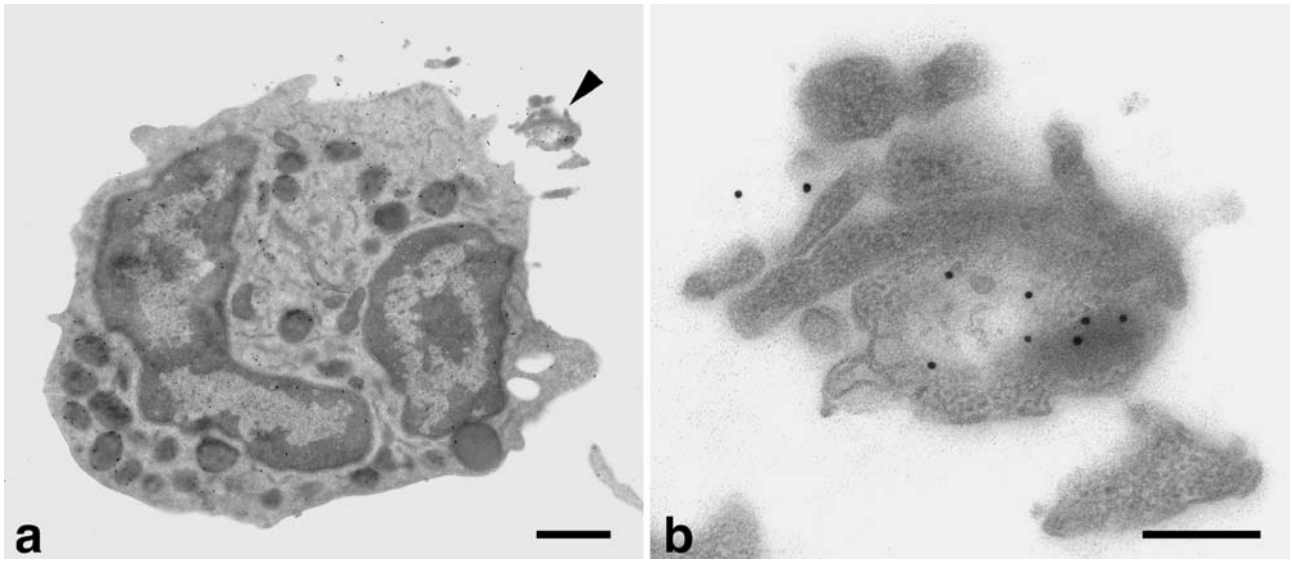

Fig. 7a, b Immunogold localization of PDE4A (10 nm Au) in an eosinophil stimulated with fMLP for 1 min. FMLP stimulated the exocytosis of PDE4A (*arrowhead*) from internal stores **(a)**. In these activated cells, labeling of PDE4A was observed on residual granule material in the extracellular space (**b,** high magnification of area near *arrowhead*). *Scale bars* 1 μm **(a),** 0.25 μm **(b)**

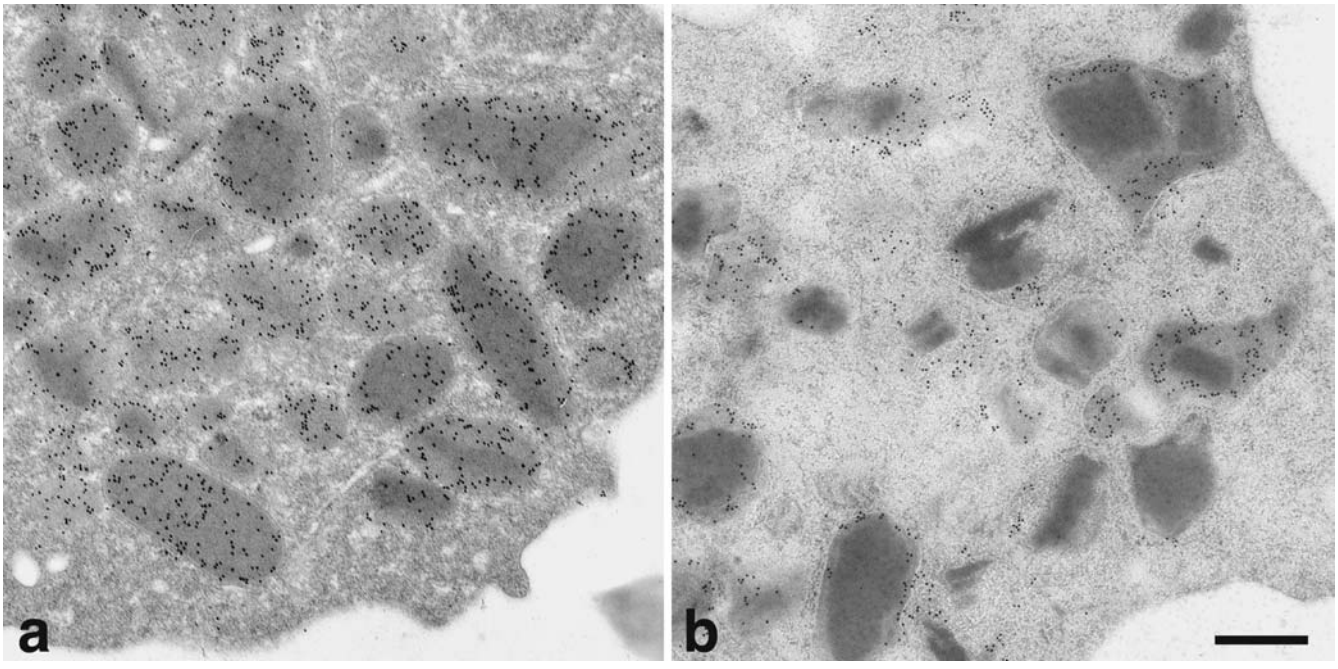

Fig. 8a, b Immunogold localization of PDE4A (10 nm Au) in unstimulated and fMLP-stimulated eosinophils. In unstimulated eosinophils **(a)** Au labeling was evenly distributed in the granule crystal and matrix. However, after stimulation with fMLP for 1 min **(b)**, Au labeling was observed primarily in the granule matrix, and there was an overall decrease of Au labeling in granules. *Scale bar* 0.25 μm

Sites of targeting of PDE4A in FMLP-stimulated eosinophils

Similar to neutrophils, PDE4A was rapidly exocytosed by eosinophils after stimulation with fMLP (Fig. 7). Within 1 min, granular material labeled with PDE4A was observed in the extracellular space (Fig. 7b). Au labeling for PDE4A was noticeably reduced in activated eosinophils when compared with unstimulated cells stained under the same conditions (Fig. 8). In addition, the distribution of Au label in granules was no longer homogeneous, but was more prominent in the granule matrix devoid of the crystalline core. Morphometry demonstrated that there was a significant decrease in

Table 1 Distribution of PDE4A Au label in eosinophil granules of control cells and cells stimulated with fMLP for 1 min

	Matrix	Crystal	Total[a]
Control	42.7%	57.3%	2241
fMLP	89.2%	10.8%	1785

[a] Label in matrix and crystal; $P_{t\text{-test}}<0.03$

immunogold labeling of PDE4A in granules after 1 min stimulation with fMLP, and that there was a shift in localization of PDE4A within granules (Table 1). In unstimulated eosinophils, labeling of PDE4A was distributed in the granule matrix (43%) and crystalline core (57%). In eosinophils stimulated with fMLP, PDE4A was primarily localized in the granule matrix (89%) and not the crystalline core (11%). Thus, fMLP induced the release of PDE4A from granules of eosinophils into the extracellular space, and cell activation was accompanied by an overall decrease in PDE4A labeling in granules, particularly from the crystalloid area of the granule.

Discussion

In granulocytes bioactive molecules are compartmentalized within subsets of cytoplasmic granules that are designed for delivery of bactericidal, hydrolytic and proteolytic molecules directly into the phagosome or extracellular space. We demonstrate that one isoform of the PDE4 family (PDE4A) is localized within cytoplasmic granules of neutrophils and eosinophils. PDE4A was stored in all cytoplasmic granules of eosinophils. However, in neutrophils, PDE4A was confined to a subset of azurophil granules. The finding of a regulatory molecule in a subgranule population of azurophil granules is unique, as modulators of inflammation have been restricted to secondary and tertiary granules (Borregaard and Cowland 1997). The presence of a distinct cytosolic compartment for a signal transduction regulatory molecule in both neutrophils and eosinophils suggests a common mechanism for cAMP regulation by PDE4A.

There is biochemical and ultrastructural evidence for granule heterogeneity in neutrophils. The granules of neutrophils can be resolved into 13 fractions by Percoll density centrifugation (Rice et al. 1986; Borregaard and Cowland 1997). Morphologically, azurophil granules are heterogeneous, and show diversity in size, shape, electron density, and content (Rice et al. 1986; Borregaard and Cowland 1997). Stereo high-voltage electron microscopy of whole-mount preparations of neutrophils reveals that azurophil granules with the same size and shape may be interconnected to each other by fine strands or filaments (Pryzwansky and Breton-Gorius 1985), and that the heterogeneity in granule shape may be a consequence of granule movement (Pryzwansky 1987). In this report, we observed that a subset of azurophil granules was labeled for PDE4A. Labeling of PDE4A and MPO was homogeneous and predominantly distributed in crystalloid granules, with some labeling in small, dense granules. There is evidence for compartmental localization of biochemical content within granules. For example, the large azurophil granules have a peripheral distribution of MPO, bactericidal permeability increasing protein (BPI), cathepsin G, and elastase, suggesting differences in composition between the peripheral and central parts (Rice et al. 1987; Egesten et al. 1994). Furthermore, in the small and nucleated (crystalloid) azurophil granules, BPI is associated at the rim of the granules, the crystals label with proteinase 3, and a homogeneous distribution is observed for MPO, elastase, and cathepsin G (Egesten et al. 1994). PDE4A is most likely stored in this latter granule subset. It is estimated that one-third of the azurophil granule population contains elastase (Damiano et al. 1988).

We found that PDE4A was localized in all granule classes of eosinophils. Eosinophils synthesize and secrete a variety of inflammatory and regulatory cytokines. TNFα, IL-5, and GM-CSF are stored in eosinophil granules (Beil et al. 1993; Dubucquoi et al. 1994; Levi-Schaffer et al. 1995). Similar to PDE4A, GM-CSF is stored in crystalloid granules, granule cores, and small granules (Levi-Schaffer et al. 1995). The finding that PDE4A is also localized in eosinophil granules implies a role for this enzyme in regulating cAMP signaling at the time of granule release.

We demonstrated by immunofluorescence microscopy that PDE4A, PDE4B, and PDE4D were localized in the cytoplasm of peripheral blood neutrophils (Pryzwansky et al. 1998). As with PDE4A, ultrastructural analysis will be required to resolve the specific site(s) of localization for PDE4B and PDE4D. Others have reported that PDE4B accounted for essentially all of the total PDE4 mRNA in human peripheral blood neutrophils (Wang et al. 1999). The PDE4B gene was constitutively expressed in neutrophils, and unlike monocytes was unaffected by treatment of cells with LPS or Il-10 (Wang et al. 1999). Consistent with our observations that PDE4D is expressed in neutrophils (Pryzwansky et al. 1998), some mRNA activity was detected for PDE4D (Wang et al. 1999). However, low mRNA activity was detected for PDE4A or PDE4C. Evidence is convincing that granule proteins are synthesized and packaged in immature granulocytes during myelopoiesis in the bone marrow (Borregaard and Cowland 1997). Since PDE4A is stored in granules of mature neutrophils, it will be important to investigate mRNA activity for PDE4A in immature bone marrow myeloid cells.

In neutrophils activated with fMLP, Au labeling of PDE4A was observed in clusters near the cell margin, and PDE4A and MPO were observed on residual granular material in the extracellular space. In addition, a focal concentration of PDE4A, but not MPO, was evident in granules of activated neutrophils. PDE4A was also exocytosed by fMLP-stimulated eosinophils. Clustering of PDE4A Au labeling in granules was not observed in activated eosinophils. However, morphometry demonstrated that labeling of PDE4A was reduced, and there was a shift in PDE4A localization within granules from

the crystalline core to the matrix. The significance of the shift in PDE4A label within eosinophil granules is not understood. In neutrophils, we observed a reorganization of complement receptor 3 (CR3) and LF in specific granules of PMA-stimulated neutrophils (Pryzwansky et al. 1991). Stereo high-voltage immunogold electron microscopy of unsectioned neutrophils demonstrated that the redistribution of CR3 and LF within granules took place at sites of specific granule fusion with the plasma membrane where CR3 was inserted into the plasma membrane and residual CR3 was shed into the extracellular medium on granular material containing LF. There is evidence that LF and CR3 are localized within different compartments of specific granules (Bainton et al. 1987). The reorganization of PDE4A within granules during fMLP stimulation suggests that, similar to LF and CR3, PDE4A and MPO reside within different granule compartments of neutrophils.

In this report, we show that fMLP induced the rapid (1 min) mobilization of a subset of azurophil granules to the plasmalemma, where some PDE4A and MPO was extruded into the extracellular space. Numerous MPO-containing granules remained intact in these activated cells (Fig. 6). We reported that only 15% of the total MPO content is exocytosed from fMLP-stimulated adherent neutrophils, and that cytochalasins are required to release 80% of the MPO content (Pryzwansky and Merricks 1998). Our finding in adherent fMLP-stimulated neutrophils that PDE4A-containing azurophil granules are mobilized to exocytose in the absence of cytochalasin B suggests that this subclass of granules functions early in activated neutrophils. Similarly, we showed by immunofluorescence microscopy that PDE4A was localized within 1 min at the nascent phagosome in neutrophils and macrophages during phagocytosis of zymosan (Pryzwansky et al. 1998). Thus, cAMP levels may be regulated early by PDE4A at the time of granule release near the site of granule fusion with the plasmalemma or phagosome. Since increases in cAMP levels downregulate granulocyte functions, including adhesion, chemotaxis, granule release and superoxide generation (Nourshargh and Hoult 1986; Dent et al. 1991; Harvath et al. 1991; Coffey 1992; Newsholme and Schwartz 1993; Derian et al. 1995), the translocation of PDE4A-containing granules to the plasmalemma or phagosome during phagocytosis may regulate granulocyte activation at the time of granule fusion. Cyclic AMP elevations may exert inhibitory effects directly on phospholipase D, arachidonic acid release, or Ca^{2+} influx, or at sites proximal to those involved in signal transduction (e.g., receptors, G proteins, PKA) (Verghese et al. 1985; Takenawa et al. 1986; Agwu et al. 1991).

Houslay and co-workers have investigated the biochemistry and functional role of the PDE4A enzyme. They reported splice variants within the N-terminal domain of the 4A family which conferred a variety of functional roles including membrane targeting, regulation of enzyme activity, and susceptibility to phosphorylation by PKA (Scotland and Houslay 1995; Shakur et al. 1995; Smith et al. 1996). The long form or core PDE4A enzyme was engineered and expressed as a freely soluble species. Spliced variants of the N-terminal domain of PDE4A conferred the subcellular localization and activity of the enzyme. For example, PDE4A1 conferred an exclusive membrane-associated location, and an 11-residue helical module in the unique N-terminal region of PDE4A1 allowed a calcium-triggered membrane association for its interaction with phosphatidic acid (Baillie et al. 2002). PDE4A5 was distributed in both the particulate and cytosol fractions, was bound to the SH3 domain of v-Src, and was localized to ruffles at the cell margin and to the perinuclear region of transiently expressed COS-7 cells (O'Connell et al. 1996; Beard et al. 2002). In this report, PDE4A was localized in cytoplasmic granules of neutrophils and eosinophils that were destined to target the plasmalemma during cell activation. These observations are in agreement with the biochemistry of the enzyme and its spliced variants.

Chemoattractants such as fMLP stimulate transient elevations of intracellular cAMP levels that peak 15–30 s after exposure to the chemotactic peptide, and return to baseline levels by 5 min (Simchowitz et al. 1980; Smolen and Weissmann 1981). The increase in cAMP precedes an increase in PDE4 activity, and there is evidence that cAMP is elevated by activating adenylyl cyclase rather than by inhibiting cAMP PDE (Grady and Thomas 1986; Iannone et al. 1989). Interestingly, neutrophils secrete cAMP under basal conditions and after exposure to chemoattractants and cAMP-elevating agents such as forskolin or PGE1 (Harvath et al. 1991). The significance for the egress of cAMP from neutrophils is unknown. We and others have proposed that local concentrations of cAMP in neutrophils may be elevated in compartments where the enzymes responsible for eliciting physiological changes are localized (Pryzwansky et al. 1981; Harvath et al. 1991; Pryzwansky et al. 1998). Furthermore, PKA-anchoring proteins allow for the localized activation of PKA within specialized compartments (Michel and Scott 2002). It is interesting to consider the observations that fluctuations of cAMP levels and ectoprotein kinase activity occur in the extracellular milieu of activated granulocytes and platelets as well as in blood under normal and pathological conditions (Dusenbery et al. 1988; Ehrlich et al. 1990; Skubitz et al. 1991; Skubitz and Goueli 1991; Teshima et al. 1997). During an inflammatory response, platelets exocytose PKA and ATP (Gordon 1986). Ecto-enzymes have been proposed as a mechanism for controlling local extracellular cell functions (Goding 2000). There is evidence for ectokinase activity of PKA in neutrophils and other cells (Dusenbery et al. 1988; Kubler et al. 1989). The extracellular cAMP, ATP and PKA may be involved in cell-to-cell interaction of cells during an inflammatory response (Emes and Crawford 1982; Dubyak et al. 1990; Ehrlich et al. 1990; Ward et al. 1990). For example, there is evidence for a cAMP-dependent platelet ectoprotein kinase with PKA properties that phosphorylates platelet glycoprotein IV (CD36), a thrombospondin- and collagen-binding site on platelets

(Hatmi et al. 1996). In addition, ectoprotein kinase activity with PKA properties has been detected on the cell surface of neutrophils, and vitronectin, a serum protein, was identified as a substrate for this kinase in the extracellular environment (Skubitz et al. 1991). These serum proteins are important for cell-cell interactions of platelets and neutrophils. The rate-limiting step for phosphorylation of ecto-PKA depends on ATP and cAMP. The focal release of PDE4A by neutrophils and eosinophils at sites of inflammation may serve as a mechanism to regulate cell-cell interaction by downregulating PKA phosphorylation of serum adhesion proteins by degrading cAMP.

In summary, evidence is convincing that the functioning of cAMP within cells may be compartmentalized and regulated through the interaction of PKA with a family of specific PKA-binding proteins (Michel and Scott 2002), and through the regulation of the catalytic activity of PDE4 isoforms by intracellular targeting by isoform specific N-terminal regions (Houslay et al. 1997; Beard et al. 2002). Future studies will be important to support the premise that there are functional differences in the localization and targeting of PDE4A, PDE4B, and PDE4D during granulocyte activation.

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