Synergistic Inhibition of Vascular Smooth Muscle Migration by Phosphodiesterase 3 and Phosphodiesterase 4 Inhibitors

Daniel Palmer, Keith Tsoi, Donald H. Maurice

Abstract—Cyclic nucleotide phosphodiesterases (PDEs) hydrolyze cAMP or cGMP and terminate their signaling. Two important families of PDEs that regulate cAMP signaling in cardiovascular tissues are the cGMP-inhibited PDEs (PDE3) and the cAMP-specific PDEs (PDE4). In this study, we have used a combination of an in vitro motility assay and a sensitive method for the measurement of cAMP in order to determine the relative roles of PDE3 and of PDE4 in the regulation of cAMP-mediated inhibition of VSMC migration. Our data demonstrate that forskolin, an activator of adenylyl cyclases, causes concentration-dependent inhibition of platelet-derived growth factor–induced VSMC migration. Incubation of cultured VSMCs with a PDE4-selective inhibitor, Ro 20-1724, markedly potentiated both the antimigratory effect and the increase in cAMP caused by forskolin. Cilostamide, a PDE3-selective compound, did not affect either the antimigratory activity of forskolin or its ability to increase cAMP. Cilostamide and Ro 20-1724 interacted synergistically to potentiate the inhibition of VSMC migration by forskolin and caused a supra-additive increase in cAMP. These data are consistent with an important role for both PDE3 and PDE4 in the regulation of cAMP-mediated inhibition of VSMC migration. (Circ Res. 1998;82:852-861.)

Key Words: cAMP • cyclic nucleotide phosphodiesterase • vascular smooth muscle • migration • platelet-derived growth factor

Cell migration is vital in the processes of embryogenesis, wound healing, and bone remodeling and also in pathologies underlying diseases such as cancer.1,2 As it pertains to vascular biology, VSMC migration, from the medial to the intimal layer, has been strongly implicated in the development of atherosclerotic plaques and in the neointimal thickening found in restenosing arteries after balloon angioplasty.3,4 Migration of VSMCs can be induced by a number of blood-borne or vascular cell–secreted factors, including PDGF,6 angiostatin II,7 transforming growth factor-β,8 insulin-like growth factor-1,9 fibroblast growth factor-2 (basic fibroblast growth factor),10 vitronectin,11 fibronectin,12 and oxidized LDL.14

Recently, a growing body of evidence has emerged implicating cAMP in the inhibition of VSMC migration.5,12,13,15-17 Specifically, studies using lipophilic structural analogues of cAMP5,12,15,17 and activators of adenylyl cyclases5,12,15-17 have demonstrated that an increase in cAMP positively correlates with the inhibition of VSMC migration. Furthermore, the downregulation of the major effector of cAMP, cAMP-dependent protein kinase (PKA), abrogates inhibition of VSMC by forskolin.16

CAMP signaling in mammalian cells is terminated by cyclic nucleotide PDEs, a multifamily class of enzymes that catalyze the hydrolysis of cyclic nucleotides to 5'-nucleotide monophosphates, which do not activate cAMP effector proteins.15,19 Seven distinct PDE families (PDE1 to PDE7) have been designated, with each discriminated on the basis of several criteria, including kinetic and regulatory properties as well as molecular sequence.7 To date, ∼30 PDE isoforms have been identified.9 Of the PDE families identified in VSMCs, members of the PDE3 (cGMP-inhibited) and PDE4 (cAMP-specific) families have been shown to contribute to the regulation of cAMP signaling and its impact on VSMC function.20-25 More specifically, inhibitors of PDE3 or PDE4 activities increase VSMC cAMP, and PDE3 inhibitors have marked effects on VSMC contraction-relaxation coupling. Although some reports have identified calmodulin-stimulated PDE activity (PDE1) in homogenates of blood vessels7,20,21,23 and PDE1 has been shown to hydrolyze cAMP when this cyclic nucleotide is present at high concentration,19 vascular effects of selective PDE1 inhibitors, such as vinpocetine,26 do not correlate positively with inhibition of PDE1 activity and may relate to other effects attributable to this compound.27,28 Low level PDE2 activity has been isolated only once from porcine VSMCs, and no functional significance has been attributed to this activity in VSMCs.20

Several studies have correlated an inhibition of PDE3 activity in VSMCs with relaxation of aortic strips.29-31 In addition, Maurice et al32 have demonstrated that the selective
PDE3 inhibitor cilostamide synergizes with the β-adrenergic receptor agonist isoproterenol to increase relaxation of rat aorta. In contrast, selective PDE4 inhibitors are ineffective at eliciting relaxation of vascular smooth muscle in the absence of a functional endothelium despite the fact that they represent a significant portion of cAMP-PDE activity in the aorta.25,32 Combinations of isoproterenol and selective PDE4 inhibitors, however, relax vascular smooth muscle in a synergistic fashion.24,25 PDE3 and PDE4 inhibitors in combination also synergize to relax VSMCs.29,32

Whereas PDE3 inhibitors have potent vasorelaxant properties, they are relatively ineffective at attenuating VSMC proliferation.33,34 However, selective inhibition of PDE3 isozymes has been reported to potentiate the antiproliferative effects of forskolin, a direct activator of adenylyl cyclases.34 Like PDE3 inhibition, PDE4 inhibition could only significantly limit VSMC proliferation in the presence of activators of adenylyl cyclases.34,35 It is noteworthy that PDE4 inhibition potentiated the effects of forskolin to a greater extent than did PDE3 inhibition, consistent with the relative contribution of PDE3 and PDE4 activities in cultured VSMCs.36 In addition, PDE3 and PDE4 inhibitors have been shown to interact synergistically, as they do in the process of relaxation, to attenuate VSMC proliferation in VSMCs37 and in A10 cells, an immortalized VSMC-like cell line.37

Although a significant number of studies have focused on elucidating the role of PDE3 and PDE4 isozymes in the regulation of VSMC contraction and proliferation, a paucity of information exists on the contribution of these enzymes in the process of cAMP-mediated inhibition of VSMC migration. Furthermore, the differential capacity for specific PDE families to impact on VSMC function, as indicated by the disparate effects of PDE3 and PDE4 inhibitors on contraction and proliferation, has similarly not been suitably addressed as it applies to VSMC migration. Consequently, in the present study, PDGF-induced rat aortic VSMC migration was quantified in the presence or absence of combinations of cilostamide (a selective PDE3 inhibitor),38 Ro 20-1724 (a selective PDE4 inhibitor),39 IBMX (a nonselective PDE inhibitor),40 and forskolin to explore the respective roles of PDE3 and PDE4 in cAMP-mediated inhibition of VSMC migration. Similar combinations of these agents were used to relate the levels of cAMP in the VSMCs to observed modulations of migratory ability.

Materials and Methods

Materials

Recombinant human PDGF-BB, DMEM, HBSS, trypsin-EDTA solution, penicillin-streptomycin antibiotic mixture, bovine calf serum, and FBS were purchased from Gibco BRL. Forskolin, 8-bromo-cAMP, and 1,9-dideoxyforskolin were obtained from Research Biochemicals International. Ro 20-1724, bovine brain calcium-modulin, and vinpocetine were acquired from Calbiochem-Novabachem Corp. HEPE, IBMX, EDTA, EGTA, dithiothreitol, phenylmethylsulfonyl fluoride, benzamidine HCl monohydrate (benzamidine), Tris-HCl, sodium chloride, and Triton X-100 were from ICN Biomedicals, Inc. The P-nitrophenyl phosphate tablets were acquired from Sigma-Aldrich, Ltd. Cilostamide was generously provided by Dr. H. Hidaka (Nagoya University School of Medicine, Nagoya, Japan). EHNA was from Biomol, Transwell cell culture chamber inserts (polycarbonate, tissue culture–treated, 6.5-mm diameter, and 8.0-μm pore size) were from the Corning Costar Corp. Trypan blue and Giemsa stain were purchased from BDH Chemicals. Affi-gel 601, column supports, gelatin, Dowex 50 (200 to 400 mesh), and aluminum oxide (alumina) were obtained from Bio-Rad Laboratories. Leupeptin was obtained from Boehringer-Mannheim. The BCA protein assay and bovine serum albumin were purchased from Pierce. [3H]Hypoxanthine (24.1 Ci/mmol), [3H]cAMP (27 Ci/mmol), and [3H]-ammonium (590.4 mCi/mmol) were from NEN Life Science Products. [3H]cAMP (283 mCi/mmol) was obtained from Amer sham Life Science. All other items and chemicals (reagent grade) were obtained from Fisher Scientific.

Cell Culture

Primary cultures of rat aortic VSMCs (after isolation from rat aorta as previously described)42 were a generous gift from Dr S.C. Pang (Department of Anatomy and Cell Biology, Queen’s University, Kingston, Canada). The identity of the cells was confirmed by immunohistochemical detection of smooth muscle-specific actin. VSMCs were routinely cultured in DMEM supplemented with 10% fetal bovine serum, 100 U/mL penicillin, and 100 μg/mL streptomycin in a 37°C, 95% air/5% CO2, humidified atmosphere. To dissociate the cells for subculturing, VSMCs were washed once in calcium- and magnesium-free HBSS, treated with 0.05% trypsin and 0.53 mmol/L EDTA for 2 to 5 minutes, and resuspended in growth medium. VSMCs were seeded in 75-cm2 flasks with 105 cells in 15 mL of medium per flask. In all experiments, VSMCs of passages 7 through 16 were used.

cAMP PDE Activity Assay

The cyclic nucleotide PDE activity in homogenates of cultured VSMCs was assayed using a modification of the method of Davis and Daly.42 Briefly, cultures of VSMCs were homogenized in ice-cold lysis buffer (50 mmol/L Tris-HCl [pH 7.4], 5 mmol/L magnesium chloride, 150 mmol/L sodium chloride, 1 mmol/L EDTA, 5 mmol/L benzamidine, 1 mmol/L dithiothreitol, 1 μmol/L leupeptin, 100 μmol/L phenylmethylsulfonyl fluoride, and 1% [vol/vol] Triton X-100). The homogenate was centrifuged at 10 000g for 3 minutes, and the supernatant was used for activity determinations. A sample of the homogenate (containing ~5 μg of protein) was added to reaction buffer (50 mmol/L Tris-HCl [pH 7.4], 5 mmol/L magnesium chloride, 100 μmol/L EDTA, and 0.1 mmol [~10 000 000 dpm] [3H]cAMP) in the presence of calcium (50 μmol/L) along with calmodulin (10 μmol/L), vinpocetine (2 to 200 μmol/L), EHNA (10 μmol/L), cilostamide (1 μmol/L), Ro 20-1724 (10 μmol/L), IBMX (500 μmol/L), or vehicle (DMSO or water) such that the reactions were carried out in a total volume of 100 μL. The samples were incubated at 37°C for 30 minutes, and the reactions were halted by the addition of 50 μL of ice-cold 0.5 mol/L EDTA (pH 8.0). To correct for recovery, 50 μL [3H]-cAMP (~1600 dpm) and 0.2 mL HEPES-NaCl buffer (0.10 mol/L sodium chloride and 0.10 mol/L HEPES [pH 8.5]) were added to the samples before purification of the nucleoside 5'-monophosphate reaction product. Products were purified via chromatography using a polyacrylamide-borateon gel column (Affi-gel 601 Bio-Rad, 1-mL bed volume). After the columns were washed with 8 mL of HEPES-NaCl buffer, the samples were applied. The columns were washed four times with 2 mL of HEPES-NaCl buffer and equilibrated with 1 mL of 0.05 mol/L sodium acetate (pH 4.8). The radiolabeled nucleoside 5'-monophosphate was eluted with 4 mL of 0.05 mol/L sodium acetate (pH 4.8) and quantified using liquid scintillation counting. The eluted [3H]cAMP was corrected for recov-
ery of [\(^{14}\)C]cAMP and normalized for the total protein used in the assay, and the total cAMP-hydrolyzing activity in the sample was expressed as picomoles per minute per milligram protein. The BCA protein assay (Pierce) was used (according to the manufacturer’s protocol with bovine serum albumin as the standard) to determine the total protein concentration of each sample. The activities are representative of at least three determinations for each agent(s).

Migration Assay

VSMC migration assays were performed using a modified Boyden’s chamber. Briefly, a confluent monolayer of VSMCs was washed with a 500 \(\mu\)L final volume of PBS and then treated with 0.05\% trypsin and 0.53 \(\mu\)mol/L EDTA for 2 to 5 minutes to detach the cells from the substratum. VSMCs were sequentially washed with growth medium and with DMEM supplemented with 0.5\% FBS. Isolated cells were resuspended in DMEM/0.5\% FBS to a concentration of 6.7 \times 10^5 cells/mL, as determined using a hemocytometer. Viability of the cells used in the assay was determined by trypsin blue exclusion, and viability was always >90\%. VSMCs were allowed to equilibrate in DMEM/0.5\% FBS for 1 hour before use. Transwell inserts (6.5-mm diameter, 8\-\mu\texttext{m} pores) were immersed in a DMEM/0.5\% FBS (500 \(\mu\)L aliquot of gelatin to dry. Approximately 2 \times 10^5 VSMCs, in a 300 \(\mu\)L aliquot of the DMEM/0.5\% FBS suspension, were added to the upper chamber of the Transwell inserts, and DMEM/0.5\% FBS (500 \(\mu\)L) was added to the lower chamber (beneath the insert). After a 1-hour incubation at 37\°C, under tissue-culturing conditions, individual inserts were transferred to separate wells in a 24-well cluster plate in which 500 \(\mu\)L of DMEM/0.5\% FBS containing the chemoattractant factor PDGF-BB (10 \(\mu\)g/mL) or vehicle (0.1 \(\mu\)g/mL acetic acid) was present. When tested, foroksin (1 to 100 \(\mu\)mol/L), 1,9-dideoxyforoksin (10 \(\mu\)mol/L), cilostamide (1 \(\mu\)mol/L), Ro 20-1724 (10 \(\mu\)mol/L), IBMX (500 \(\mu\)mol/L), 8-bromo-cAMP (1 \(\mu\)mol/L), or combinations of these agents were added to the lower chamber with PDGF. The vehicle (DMSO), at 0.2\% of the total volume, was added to the lower chamber in all experiments. Transwell apparatuses were incubated for 6 hours in a 37\°C, 95\% air/5\% CO\(_2\) humidified atmosphere. After which time, cells remaining on the upper face of the membrane were removed by scraping with cotton swabs. VSMCs that had migrated to the lower face of the membrane were fixed for 12 to 16 hours in 10\% (wt/vol) paraformaldehyde in PBS at 4\°C and stained with Giemsa stain for 1 hour. Membranes were washed in PBS and removed from their support, and the number of migrating cells was measured by light microscopy. Stained cells possessing a distinct nucleus and multiple projections that had clearly exited the pores of the filter were counted in eight random fields of view (magnification \(\times\)200), such that the VSMC migrational activity was determined as cells per field of view. To ensure that effects on VSMC migration were not due to toxicity, VSMC suspensions were seeded in wells of a 24-well cluster plate, which was precoated with a 0.25\% gelatin (wt/vol) and incubated for 1 hour at 37\°C and stained with Giemsa stain for 1 hour. Membranes were washed in PBS and removed from their support, and the number of migrating cells was measured by light microscopy. Stained cells possessing a distinct nucleus and multiple projections that had clearly exited the pores of the filter were counted in eight random fields of view (magnification \(\times\)200), such that the VSMC migrational activity was determined as cells per field of view. This result was consistent with the absence of calcium (50 \(\mu\)mol/L)/calmodulin (10 U)–stimulated activity in these homogenates (Table). Similarly, EHNA (10 \(\mu\)mol/L), a selective inhibitor of PDE2,\(^{47}\) was ineffective in inhibiting VSMC cAMP-PDE activity, validating our previous work.\(^{36}\) Also consistent with our previous work,\(^{23}\) cilostamide (1 \(\mu\)mol/L) and Ro 20-1724 (10 \(\mu\)mol/L), selective PDE3 and PDE4 inhibitors, respectively, inhibited VSMC cAMP-PDE activity by \(\sim\)16\% and 40\% individually and were strictly additive when combined (Table). The addition of vinpocetine (2 to 200 \(\mu\)mol/L) to this combination of cilostamide and Ro 20-1724 did not further inhibit the cAMP-PDE activity, further attesting to the absence of PDE1 in these cells (Table). The broad-spectrum

Measurement of cAMP in Cultured VSMCs

A confluent 75-cm\(^2\) flask of VSMCs was incubated with fresh growth medium supplemented with 20 \(\mu\)Ci/L [\(^{3}\)H]hypoxanthine for 16 hours. As previously described,\(^{26}\) the incubation of cultured VSMCs with [\(^{3}\)H]hypoxanthine allows for the homogeneous labeling of both the ATP and GTP metabolic pools. Labeled VSMCs were washed with calcium- and magnesium-free HBSS and subsequently treated with 0.05\% trypsin and 0.53 \(\mu\)mol/L EDTA for 2 to 5 minutes to detach the VSMCs from the flask. Dissociated cells were washed, resuspended in DMEM/0.5\% FBS, and incubated in a 37\°C, 95\% air/5\% CO\(_2\) humidified atmosphere for an hour. Labeled VSMCs (500 \(\mu\)L, \(\times\)10^5 dpm) were seeded in 24-well cluster plates that had been precoated with DMEM supplemented with 0.25\% gelatin (wt/vol) and incubated for 1 hour at 37\°C and 95\% air/5\% CO\(_2\). After the last equilibration period, cells were treated with forskolin (1 to 100 \(\mu\)mol/L), cilostamide (1 \(\mu\)mol/L), Ro 20-1724 (10 \(\mu\)mol/L), or IBMX (500 \(\mu\)mol/L), alone or in combination, for 30-minute, 1-hour, and 6-hour intervals. The drug vehicle (DMSO) was added to each well and represented no more than 0.2\% of the total volume. Incubations were terminated by the addition of 0.5 \(\mu\)L of ice-cold 10\% trichloroacetic acid, and \(\sim\)1000 dpm of [\(^{3}\)H]cAMP was added to each sample as an internal standard. cAMP was isolated and purified via sequential column chromatography using neutral alumina and Dowex 50 resin columns. [\(^{3}\)H]cAMP and [\(^{3}\)H]cAMP amounts were determined using liquid scintillation as described previously.\(^{46}\) After correction for recovery, the [\(^{3}\)H]-cAMP present in the individual wells was expressed as a percentage of the total \(^{3}\)H in each well. Individual treatments were assayed in triplicate at least three independent experiments unless otherwise indicated.

Statistical Analysis

Data are presented as mean±SEM of at least three independent experiments unless otherwise indicated. Statistical differences between results were determined using unpaired ANOVA, with Dunnett or Tukey-Kramer multiple comparison post hoc tests or unpaired Student \(t\) tests as indicated. A value of \(P<0.05\) was considered statistically significant.

Results

Effects of Selective PDE Inhibitors on VSMC cAMP-Hydrolyzing PDE Activity

To determine which isozymes represented major components of the total cAMP-hydrolyzing PDE (cAMP-PDE) activity and to permit for a rational interpretation as to which enzymes would likely be of significant importance in the regulation of cAMP-mediated inhibition of VSMC migration, cAMP-PDE activity from VSMC homogenates was assayed in the presence and absence of various PDE inhibitors (Table). Vinpocetine, a putative selective inhibitor of PDE1 isozymes, had no significant effect on total PDE activity over the range of concentrations used (2 to 200 \(\mu\)mol/L, Table). This result was consistent with the absence of calcium (50 \(\mu\)mol/L)/calmodulin (10 U)–stimulated activity in these homogenates (Table). Similarly, EHNA (10 \(\mu\)mol/L), a selective inhibitor of PDE2,\(^{47}\) was ineffective in inhibiting VSMC cAMP-PDE activity, validating our previous work.\(^{36}\) Also consistent with our previous work,\(^{23}\) cilostamide (1 \(\mu\)mol/L) and Ro 20-1724 (10 \(\mu\)mol/L), selective PDE3 and PDE4 inhibitors, respectively, inhibited VSMC cAMP-PDE activity by \(\sim\)16\% and 40\% individually and were strictly additive when combined (Table). The addition of vinpocetine (2 to 200 \(\mu\)mol/L) to this combination of cilostamide and Ro 20-1724 did not further inhibit the cAMP-PDE activity, further attesting to the absence of PDE1 in these cells (Table).
### Modulation of cAMP-Hydrolyzing PDE Activity in VSMC Homogenates

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Experiment 1</th>
<th>Experiment 2</th>
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<tbody>
<tr>
<td>Vehicle</td>
<td>74±7</td>
<td>79±5</td>
</tr>
<tr>
<td>Vinpocetine 2 μmol/L</td>
<td>67±3</td>
<td>...</td>
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<tr>
<td>20 μmol/L</td>
<td>60±4</td>
<td>...</td>
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<tr>
<td>200 μmol/L</td>
<td>62±6</td>
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<tr>
<td>Calcium (50 μmol/L) and calmodulin (10 μU)</td>
<td>...</td>
<td>90±5</td>
</tr>
<tr>
<td>EHNA (10 μmol/L)</td>
<td>75±3</td>
<td>...</td>
</tr>
<tr>
<td>Cilostamide (1 μmol/L)</td>
<td>56±7*</td>
<td>...</td>
</tr>
<tr>
<td>Ro 20-1724 (10 μmol/L)</td>
<td>44±2*</td>
<td>...</td>
</tr>
<tr>
<td>Cilostamide (1 μmol/L) and Ro 20-1724 (10 μmol/L)</td>
<td>29±5*</td>
<td>...</td>
</tr>
<tr>
<td>Cilostamide (1 μmol/L), Ro 20-1724 (10 μmol/L) and vinpocetine (2 μmol/L)</td>
<td>32±5*</td>
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</tr>
<tr>
<td>Cilostamide (1 μmol/L), Ro 20-1724 (10 μmol/L) and vinpocetine (20 μmol/L)</td>
<td>32±2*</td>
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</tr>
<tr>
<td>Cilostamide (1 μmol/L), Ro 20-1724 (10 μmol/L) and vinpocetine (200 μmol/L)</td>
<td>31±4*</td>
<td>...</td>
</tr>
<tr>
<td>IBMX (500 μmol/L)</td>
<td>6±3*</td>
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</tbody>
</table>

Depicted are representative experiments for the determinations of PDE activity, as described in "Materials and Methods." Values are mean±SEM. *P<.05 compared with vehicle-treated VSMC cAMP-PDE activity as determined by unpaired one-way ANOVA and Dunnett multiple comparisons test.

PDE inhibitor IBMX (500 μmol/L) inhibited virtually all of the cAMP-PDE activity in these homogenates (Table). Thus, only PDE3 and PDE4 isozymes contribute significantly to the hydrolysis of cAMP in our VSMCs.

### PDE3 and PDE4 Inhibitors Potentiate Forskolin-Mediated Inhibition of PDGF-Induced VSMC Migration

Consistent with earlier reports,15-17 PDGF-BB caused a concentration-dependent increase in the migration of cultured rat aortic VSMCs when assayed using a modification of Boyden's chamber method.7 For our experiments, 10 ng/mL PDGF-BB was chosen, since this concentration gave an ≈50% maximal stimulation. In our studies, this concentration of PDGF-BB stimulated VSMC migration by ≈4-fold from a basal migration of 32.9±13.4 to 121.9±14.1 cells per field of view. Forskolin, an activator of adenylyl cyclases, inhibited PDGF-induced migration of cultured rat aortic VSMCs (Figure 1) and caused marked changes in the morphology of these cells (see below). VSMC migration to 10 ng/mL PDGF was not inhibited by incubation with the lowest concentration of forskolin used in our studies (1 μmol/L). However, higher concentrations of forskolin (10 or 100 μmol/L) did inhibit PDGF-induced migration by ≈21% and 58%, respectively (Figure 1). In both experiments in which it was measured, 8-bromo-cAMP (1 mmol/L) also inhibited PDGF-induced migration, whereas 1, 9-dideoxyforskolin (10 μmol/L), a structural analogue of forskolin that does not activate adenylyl cyclases, had no effect on PDGF-induced migration (not shown). These data are consistent with prior reports of forskolin-mediated inhibition of PDGF-induced VSMC migration and support the hypothesis that cAMP mediates these effects.

To ascertain the role(s) of cAMP-PDEs in regulating this forskolin-mediated effect, inhibition of migration was also measured in the presence of inhibitors of the major cAMP-PDE activities expressed in these cells. Whether used alone or in combination, neither the selective PDE3 inhibitor cilostamide (1 μmol/L), the selective PDE4 inhibitor Ro 20-1724 (10 μmol/L), nor the broad-spectrum PDE inhibitor IBMX (500 μmol/L) inhibited PDGF-induced migration (not shown). In contrast to their effects alone, addition of some of these agents with forskolin markedly augmented the ability of forskolin to inhibit PDGF-induced migration (Figure 1). In our experiments, a combination of 1 μmol/L forskolin and 10 μmol/L Ro 20-1724, two agents that when used alone had no effect on PDGF-induced migration, caused a 30% decrease in migration in response to PDGF-BB. Although the addition of cilostamide did not increase the antimigratory effect of forskolin at this concentration, it potentiated the effects of a combination of forskolin and Ro 20-1724 by a further 38%, leading to a 68% total inhibition. Notably, this synergistic potentiation of the effects of forskolin by cilostamide and Ro 20-1724 was not significantly different from that caused by a combination of IBMX and 1 μmol/L forskolin. In fact, IBMX and 1 μmol/L forskolin inhibited migration in our studies by ≈76%.

Incubation of VSMCs with a combination of 10 μmol/L forskolin and cilostamide did not result in a greater inhibition
of migration than was achieved with this dose of forskolin alone (Figure 1). Potentiation of the inhibitory effect on VSMC migration by 10 μmol/L forskolin was, however, observed when Ro 20-1724 was added. Thus, whereas 10 μmol/L forskolin inhibited PDGF-induced migration by 21%, the combination of Ro 20-1724 and this concentration of forskolin resulted in a 63% inhibition of the effect of PDGF-BB. This represented a 3-fold potentiation of the effect of forskolin alone. Together, cilostamide and Ro 20-1724 synergistically enhanced the effects of 10 μmol/L forskolin such that this combination further reduced VSMC migration to 80%, a 1.3-fold potentiation. It is notable that the extent to which cilostamide was able to increase the inhibitory effects of Ro 20-1724 and forskolin was diminished at the higher dose of forskolin (38% increase with 1 μmol/L and 17% with 10 μmol/L forskolin). As with the lower dose of forskolin, IBMX substantially augmented the inhibition mediated by 10 μmol/L forskolin (from 21% to 76%), an enhancement equivalent to that mediated by the addition of both cilostamide and Ro 20-1724 to this concentration of forskolin. To ensure that nonspecific toxicological effects were not responsible for differences in VSMC migration observed after the addition of cAMP-elevating agents, VSMC survival was assessed using two separate tests. Under no circumstances were differences observed in VSMC survival after incubation of VSMC with the tested compounds, either alone or in combination, with or without cAMP-elevating agents used to inhibit PDGF-induced migration were measured at any time point, whereas IBMX (500 μmol/L) incubation of VSMC caused a doubling of cAMP (Figure 3). Forskolin (1 μmol/L) caused time-dependent increases in cAMP, which reached a plateau between 30 minutes and 1 hour (Figures 2 and 3). Ro 20-1724, the PDE4 inhibitor, augmented the forskolin-induced increase in cAMP by 5.1-, 7.8-, and 5.6-fold at the 30-, 60-, and 360-minute incubations, respectively. Although cilostamide did not potentiate the increases in cAMP caused by any concentration of forskolin, it caused a further increase in cAMP when combined with forskolin and Ro 20-1724 (Figure 3). Thus, over the 30-minute, 1-hour, and 6-hour time intervals, the combination of PDE3 and PDE4 inhibitors potentiated the forskolin-induced increase in cAMP by 7.0-, 7.9-, and 6.6-fold, respectively. Similarly, the inclusion of IBMX resulted in a marked potentiation of the forskolin-mediated increase in cAMP such that the effects of forskolin were 6.8-, 10-, and 9.9-fold larger than those caused by forskolin alone at the three time points. Incubation with 10 μmol/L or 100 μmol/L forskolin increased VSMC cAMP levels by 50- and 300-fold, respectively (not shown). In addition, IBMX augmented this increase in cAMP levels mediated by 10 μmol/L forskolin by 2.2-fold after 30 minutes (not shown).

Potentiation of Forskolin-Induced Increases in VSMC cAMP by PDE Inhibitors

cAMP levels in VSMCs treated with the combinations of the agents used to inhibit PDGF-induced migration were measured at 30 minutes, 1 hour, and 6 hours (Figures 2 and 3). Under our conditions, neither cilostamide (1 μmol/L) nor Ro 20-1724 (10 μmol/L) alone caused a significant increase in cAMP levels in VSMCs at any time point, whereas IBMX (500 μmol/L) incubation of VSMC caused a doubling of cAMP (Figure 3). Forskolin (1 μmol/L) caused time-dependent increases in cAMP, which reached a plateau between 30 minutes and 1 hour (Figures 2 and 3). Ro 20-1724, the PDE4 inhibitor, augmented the forskolin-induced increase in cAMP by 5.1-, 7.8-, and 5.6-fold at the 30-, 60-, and 360-minute incubations, respectively. Although cilostamide did not potentiate the increases in cAMP caused by any concentration of forskolin, it caused a further increase in cAMP when combined with forskolin and Ro 20-1724 (Figure 3). Thus, over the 30-minute, 1-hour, and 6-hour time intervals, the combination of PDE3 and PDE4 inhibitors potentiated the forskolin-induced increase in cAMP by 7.0-, 7.9-, and 6.6-fold, respectively. Similarly, the inclusion of IBMX resulted in a marked potentiation of the forskolin-mediated increase in cAMP such that the effects of forskolin were 6.8-, 10-, and 9.9-fold larger than those caused by forskolin alone at the three time points. Incubation with 10 μmol/L or 100 μmol/L forskolin increased VSMC cAMP levels by 50- and 300-fold, respectively (not shown). In addition, IBMX augmented this increase in cAMP levels mediated by 10 μmol/L forskolin by 2.2-fold after 30 minutes (not shown).

Morphological Characterization of Migrated VSMCs

Photomicrographs depicting changes in the appearances of VSMCs incubated with the various pharmacological agents studied are shown (Figure 4). Panels a and b show VSMCs that had migrated in the absence and in the presence of 10 μmol/L forskolin alone. Potentiation of the inhibitory effect on VSMC migration by 10 μmol/L forskolin was, however, observed when Ro 20-1724 was added. Thus, whereas 10 μmol/L forskolin inhibited PDGF-induced migration by 21%, the combination of Ro 20-1724 and this concentration of forskolin resulted in a 63% inhibition of the effect of PDGF-BB. This represented a 3-fold potentiation of the effect of forskolin alone. Together, cilostamide and Ro 20-1724 synergistically enhanced the effects of 10 μmol/L forskolin such that this combination further reduced VSMC migration to 80%, a 1.3-fold potentiation. It is notable that the extent to which cilostamide was able to increase the inhibitory effects of Ro 20-1724 and forskolin was diminished at the higher dose of forskolin (38% increase with 1 μmol/L and 17% with 10 μmol/L forskolin). As with the lower dose of forskolin, IBMX substantially augmented the inhibition mediated by 10 μmol/L forskolin (from 21% to 76%), an enhancement equivalent to that mediated by the addition of both cilostamide and Ro 20-1724 to this concentration of forskolin. To ensure that nonspecific toxicological effects were not responsible for differences in VSMC migration observed after the addition of cAMP-elevating agents, VSMC survival was assessed using two separate tests. Under no circumstances were differences observed in VSMC survival after incubations with the tested compounds, either alone or in combination, with or without cAMP-elevating agents used to inhibit PDGF-induced migration were measured at any time point, whereas IBMX (500 μmol/L) incubation of VSMC caused a doubling of cAMP (Figure 3). Forskolin (1 μmol/L) caused time-dependent increases in cAMP, which reached a plateau between 30 minutes and 1 hour (Figures 2 and 3). Ro 20-1724, the PDE4 inhibitor, augmented the forskolin-induced increase in cAMP by 5.1-, 7.8-, and 5.6-fold at the 30-, 60-, and 360-minute incubations, respectively. Although cilostamide did not potentiate the increases in cAMP caused by any concentration of forskolin, it caused a further increase in cAMP when combined with forskolin and Ro 20-1724 (Figure 3). Thus, over the 30-minute, 1-hour, and 6-hour time intervals, the combination of PDE3 and PDE4 inhibitors potentiated the forskolin-induced increase in cAMP by 7.0-, 7.9-, and 6.6-fold, respectively. Similarly, the inclusion of IBMX resulted in a marked potentiation of the forskolin-mediated increase in cAMP such that the effects of forskolin were 6.8-, 10-, and 9.9-fold larger than those caused by forskolin alone at the three time points. Incubation with 10 μmol/L or 100 μmol/L forskolin increased VSMC cAMP levels by 50- and 300-fold, respectively (not shown). In addition, IBMX augmented this increase in cAMP levels mediated by 10 μmol/L forskolin by 2.2-fold after 30 minutes (not shown).

**Figure 2.** Effects of the PDE inhibitors cilostamide, Ro 20-1724, and IBMX on time-dependent forskolin-induced increase in VSMC cAMP. Intracellular cAMP was measured after incubation of [3H]hypoxanthine-prelabeled VSMCs with forskolin (1 μmol/L) in the presence or absence of cilostamide (1 μmol/L), Ro 20-1724 (10 μmol/L), and IBMX (500 μmol/L). [3H]cAMP values are expressed as a percentage of the total [3H]. Values are mean±SEM from triplicate determinations in a representative experiment. Similar results were obtained in at least five determinations. All values were significantly different (P<.05) from forskolin alone, with the exception of the combination of forskolin and cilostamide. Values determined for incubations of forskolin plus Ro 20-1724 were only significantly different (P<.05) from forskolin plus Ro 20-1724 plus cilostamide at the 30-minute time point. Unpaired Student t test was used for analyses.

**Figure 3.** Effects of PDE inhibitors, in the presence of 1 μmol/L forskolin, on VSMC cAMP levels. [3H]cAMP was determined after incubation of [3H]hypoxanthine-labeled VSMCs with forskolin (1 μmol/L) in the presence of at least eight determinations in three independent experiments. [*P<.05 compared with basal (DMSO). #P<.05 compared with 1 μmol/L forskolin alone. **P<.05 compared with the combination of forskolin and Ro 20-1724. Unpaired Student t test was used for analyses.**
ng/mL PDGF-BB, respectively. In both instances, cells are well spread out over the membrane surface and display distinct lamellipodia and pseudopodia. Addition of 1 μmol/L forskolin (panel c) caused little change in cell morphology or in cell number. Although incubation of VSMCs with the combination of forskolin (1 μmol/L) and cilostamide (panel d) did not result in fewer cells migrating in response to PDGF-BB, some cells appeared to develop a more spindly appearance characterized by multiple, thin, branch NG processes and a compact cell body. Addition of Ro 20-1724 with forskolin (1 μmol/L) resulted in a notable decrease in the number of cells that had migrated to the lower face of the membrane and also resulted in a large number exhibiting the spindly appearance (panel e). In combination, cilostamide and Ro 20-1724, in the presence of forskolin (1 μmol/L), substantially decreased the number of cells that had migrated, and of those that were present, all displayed the spindly morphology (panel f). Furthermore, a substantial number of VSMCs under these conditions were seen to remain within the pores of the filter. The spindly appearance was also observed with the concurrent incubation of VSMCs with forskolin (1 μmol/L) and IBMX (panel g) or with higher concentrations (10 μmol/L) of forskolin (panel h). Since incubation of VSMCs with 1,9'-dideoxyforskolin, the inactive forskolin analogue, did not result in the appearance of spindly cells or in inhibition of migration (not shown) and since 1 mmol/L 8-bromo-cAMP mimicked both of these effects of forskolin (not shown), it is reasonable to propose that both phenomenon were cAMP-mediated. Of further note, this morphology was also seen to be adopted by VSMCs on the upper face of the Boyden’s chamber membrane under the conditions of the assay as well as by VSMCs under standard culturing conditions when treated with forskolin and 8-bromo-cAMP, suggesting that the morphology is not necessarily limited to migrating cells.

Discussion

The present study represents the first detailed investigation into the roles played by specific PDE families in the regulation of VSMC migration. Although previous reports have established that cAMP-elevating agents and structural analogues of cAMP are capable of inhibiting VSMC migration in response to various chemotactic agents, cAMP-PDE involvement in the regulation of this process has received considerably less attention. For our studies, cultured rat aortic VSMCs were used. We have previously characterized the cAMP-PDE activities present in these cells. In addition to this previous characterization, we have demonstrated here that the cells used in the present study expressed no detectable PDE1 activity as assessed by the inability of calcium-calmodulin to stimulate VSMC cAMP-PDE activity as well as the inability of a selective PDE1 inhibitor, vinpocetine, to affect the total cAMP-hydrolyzing activity when used alone or in combination with cilostamide and Ro 20-1724, selective PDE3 and PDE4 inhibitors, respectively. Although some reports have shown that vinpocetine can relax isolated blood vessels, the fact that this agent does not potentiate nitrovasodilator–or atrial natriuretic peptide–induced increases in vascular cGMP may perhaps indicate that PDE1 inhibition was not the molecular basis of its vasorelaxant properties. Consistent with our previous report, the lack of effect obtained using EHNA, a selective PDE2 inhibitor, attests to the lack of this activity in these cells. Also in agreement with our previous report, PDE3 and PDE4 activities accounted for >60% of the total cAMP-hydrolyzing activity in the VSMCs used in the present study. Furthermore, the near total inhibition of cAMP-PDE activity by IBMX was taken to mean that our cultured VSMCs did not express PDE7, a novel cAMP-specific PDE that is insensitive to IBMX. Because of these considerations, we chose to determine the relative roles of PDE3 and PDE4 in the regulation of VSMC migration by cAMP. A recent report has indicated that PDE4 isozymes exhibit differential sensitivity to classical PDE4 inhibitors, such as rolipram and Ro 20-1724. The rat aortic VSMCs used here predominantly express PDE4D (authors’ unpublished data, 1997), a PDE4 isozyme that is inhibited potently by Ro 20-1724 (IC50, ∼0.7 μmol/L), thus validating the selection and dose of our selective PDE4 inhibitor.

Cultured VSMCs are a preferred model for the present study of VSMC migration since they represent VSMCs in a synthetic phenotype similar to that found for migrating cells in vivo. VSMC migration, in the present study, was in response to the addition of the potent and pathophysiologically relevant chemotactic factor PDGF-BB. Selective inhibitors of PDE3 (cilostamide, 1 μmol/L) and PDE4 (Ro 20-1724, 10 μmol/L) isozymes, when used at concentrations that were selective and additive in terms of their effects on cAMP-PDE activity, had no effect on PDGF-induced VSMC migration when used alone or in combination. This result was consistent with a previous study by Tanaka et al., in which it was reported that a selective PDE3 inhibitor, E-1020, had no effect on serum-induced VSMC migration over concentrations ranging from 0.1 to 10 μmol/L. Furthermore, in the present study, a broad-spectrum PDE inhibitor, IBMX (500 μmol/L), was also ineffective at negatively modulating the migratory ability of VSMCs. The significant role for PDE3 and PDE4 activities was shown when inhibitors of these activities were combined with forskolin. More specifically, the present study has demonstrated that cAMP-mediated inhibition of VSMC migration is regulated by a complex interplay between these two PDEs. Thus, whereas cilostamide had a relatively modest impact on the concentration dependence of forskolin-induced inhibition of VSMC migration, Ro 20-1724 markedly potentiated this inhibitory effect of forskolin. Evidence for a significant interaction between PDE3 and PDE4 activities in modulating this cell function was demonstrated when addition of both of these selective PDE inhibitors caused a synergistic potentiation of forskolin-induced inhibition of VSMC migration. These data demonstrated that PDE3 and PDE4 inhibitors could interact synergistically to modulate cellular effects mediated by cAMP. Similar interactions have been reported in relation to the effects of these compounds on regulating relaxation-contraction coupling in VSMCs. In their study, synergism between PDE3 and PDE4 inhibitors was attributed to the supra-additive increases in cAMP in the tissues studied. In our experiments, a similar phenomenon was observed. Thus, Ro 20-1724 potentiated the forskolin-induced increase in cAMP.
Figure 4. Representative photomicrographs of migrated VSMCs. VSMC migration was in response to PDGF-BB (10 ng/mL) for 6 hours using a modification of the method of Boyden. Incubation conditions included 0.5% FBS (a), 10 ng/mL PDGF-BB (b), PDGF-BB and 1 μmol/L forskolin (c), PDGF-BB, 1 μmol/L forskolin, and 1 μmol/L cilostamide (d), PDGF-BB, 1 μmol/L forskolin, and 10 μmol/L Ro 20-1724 (e), PDGF-BB, 1 μmol/L forskolin, cilostamide, and Ro 20-1724 (f), PDGF-BB, 1 μmol/L forskolin, and 500 μmol/L IBMX (g), and PDGF-BB and 10 μmol/L forskolin (h). Original magnification ×200.
at all time points studied, and the addition of cilostamide further augmented these increases. Given that cilostamide was unable to potentiate forskolin-induced increases in cAMP, these data were consistent with an important role for PDE3 only when PDE4 was inhibited. These findings identify a significant role for PDE4 in modulating the antimigratory potential of cAMP, consistent with the VSMC cAMP-PDE activity attributable to PDE4 isozymes relative to other cAMP-hydrolyzing PDEs. This observation stands in marked contrast to the role played by PDE4 in the regulation of relaxation-contraction coupling of VSMCs.29

Our data demonstrate that although forskolin, when used alone or in the presence of selective PDE inhibitors, inhibited PDGF-stimulated VSMC migration and increased VSMC cAMP in a concentration-dependent manner, no relationship between the absolute level of cAMP generated by the various combinations of agents tested and their inhibitory potential existed. For example, although 100 μmol/L forskolin increased cAMP to a level ~10-fold that achieved by a combination of 1 μmol/L forskolin and 500 μmol/L IBMX, the resulting levels of inhibition of migration with these treatments were virtually identical. There exists at least two potential explanations for these findings. First, a coordinated regulation of PKA activity by adenylyl cyclase and cAMP-PDE activities could influence the steady-state concentration of cAMP required for full activation of PKA in cells in a manner independent of absolute cAMP levels.51 In support of this model, Deeg et al53 demonstrated that parotid gland amylase secretion was stimulated by a coordinated increase in both adenylyl cyclase and PDE activity such that the cells seemed to respond to an increase in cAMP metabolism even though the levels of cAMP did not change. In addition, subcellular colocalization of selected PDE and PKA isozymes may allow for a coordinated regulation of function.52 Second, the absence of a more significant inhibition of VSMC migration with combinations of agents giving rise to very large increases in cAMP may be due to a significant non-cAMP-inhibitable component of PDGF-induced migration. Also, our data demonstrate that there exists a threshold increase of cAMP that is required in treated VSMCs to mediate this inhibition of migration. For example, although 1 μmol/L forskolin significantly increased cAMP levels, this concentration did not result in any measurable change in migration. In seeming contradiction with this cAMP-mediated mechanism of inhibition, findings of Mooradian et al54 suggest that inhibition of adenylyl cyclase, via activation of a G, heterotrimeric GTP-binding protein, could promote inhibition of VSMC migration. However, given that Gα can modulate effectors other than adenylyl cyclases and that the βγ subunits are also able to effect changes in cell function, it is possible that this correlation of activity with function is not mechanistic.25

In addition to characterizing the ability of forskolin and the PDE inhibitors to attenuate VSMC migration, we also examined the effect of these agents on VSMC morphology (Figure 4). As stated previously, PDGF-BB (10 ng/mL) caused a profound increase in the number of cells found on the lower face of the membrane (Figure 1). Consistent with a migratory phenotype, cells on the lower face of the membrane possessed prominent lamellipodia and pseudopodia (Figure 4b).12 Treatment with 1 μmol/L forskolin or with the PDE inhibitors alone did not reduce the number of cells present on the lower face of the membrane or migratory phenotype (Figure 4c).

When combined, this concentration of forskolin and either cilostamide or Ro 20-1724 gave rise to a spindly morphology in the cells that had migrated (Figure 4d and 4e). These cells were characterized as having a compact cell body with multiple, thin, branching processes. Also of note was the absence in these cells of the lamellipodia observed in cells that had migrated in the absence of these agents. A secondary observation was that under conditions in which the majority of the cells observed had a spindly appearance, a significant percentage of cells appeared to have remained within the membrane pores. Moreover, Bornfeldt et al56 have demonstrated a similar tendency for VSMCs to remain in the pores of a modified Boyden’s chamber apparatus when treated with sphingosine-1-phosphate, an agent that elevates cAMP through an as-yet-undetermined mechanism. The appearance of this morphology correlated with increases in intracellular cAMP. Consistent with this hypothesis is the observation that VSMCs treated with 10 μmol/L forskolin almost exclusively displayed this morphology (Figure 4h). Moreover, at a higher concentration (100 μmol/L), the same compound evoked not only a substantial decrease in the cell number but also a complete change in cell morphology to the spindly type for all VSMCs found on the lower face. As further support for a role for cAMP in mediating these changes in cell shape, 1,9-dideoxyforskolin (10 μmol/L) was unable to bring about a similar change in the appearance of these cells, whereas 1 mmol/L 8-bromo-cAMP did. Similarly, when potentiated by IBMX or the combination of selective PDE inhibitors (Figure 4f and 4g), VSMCs exclusively exhibited the spindly morphology. In relation to these findings, since recent reports have indicated that elevations in cAMP correlated with actin filament disassembly, this mechanism could, at least in part, explain the morphological changes in VSMCs observed under these conditions.15,56 The functional consequence of adopting this morphology is unclear, since treatments that induced this change in cell shape did not necessarily decrease the migratory ability of VSMCs (Figures 1, 4c, and 4d).

Given that agents that increase cAMP or cGMP can synergistically regulate VSMC function through effects on PDE3, the data presented in the present study have both physiological and pharmacological implications.25 Two endothelium-derived vasoactive agents that regulate VSMC function through cAMP or cGMP are prostacyclin and EDRA.25 Indeed, loss of endothelial cell function, which results in decreased release of these endothelium-derived vasoactive substances, has been shown to be an early event in the development of atherosclerosis, a process in which VSMC migration has a critical role.37 Of further note is the observation that balloon catheterization of rat aorta results in a selective upregulation of PDE4 activity, consistent with the data presented here, suggesting a prominent role for PDE4 isoenzymes in the regulation of cAMP-mediated inhibition of VSMC migration.58 The molecular basis for this cAMP-mediated inhibition of VSMC migration has not, however, been fully elucidated. Although some results16 would appear...
to support a central role for PKA as the primary effector for cAMP in this mechanism, others7 suggest the involvement of multiple systems. In this regard, the recent observation that cAMP can activate the cGMP-dependent protein kinase (PKG) represents one other possible avenue through which cAMP could act.59,60 Since PDGF-induced VSMC migration is ultimately dependent on increases in intracellular calcium,1,2,9 cAMP-mediated decreases in cytosolic calcium may contribute to the diminished migratory ability of VSMCs treated with cAMP-elevating agents.61 In addition, cAMP-mediated changes in cytoskeletal structures16,56 and alteration of gene expression via the cAMP-response element binding protein may also be important.62 In addition to these other possible mechanisms of inhibition, activation of PKA by cAMP-elevating agents has been shown to attenuate VSMC proliferation, presumably by negatively modulating the signaling by the MAP kinase pathway.61 A role for the MAP kinase pathway may also be involved in the process of cell motility as indicated by a recent study by Graf et al.64 Antisense oligodeoxynucleotides directed against mRNA for the MAP kinase pathway components ERK-1 and ERK-2 inhibited PDGF-BB-induced VSMC migration, suggesting an important role for the MAP kinase pathway in the signal transduction events regulating VSMC migration and the potential that cAMP could act to inhibit this process via this mechanism.

In conclusion, the present study demonstrates that selective PDE3 and PDE4 inhibitors can potentiate the cAMP-mediated antimigratory effects of forskolin in VSMCs. Since an inhibitor of PDE4 markedly potentiated the effects of forskolin in the present study, whereas a PDE3 inhibitor had more modest effects, and in light of the observation that PDE4 is upregulated in the aorta in response to balloon angioplasty, PDE4 inhibitors may represent a class of agents that are useful in limiting the VSMC migration occurring in response to endothelial damage but have limited impact on overall blood pressure. Further studies relating the generality of our observations to other activators of adenylyl cyclase, such as prostaglandins and β-adrenergic receptor agonists, should address this potential.

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Synergistic Inhibition of Vascular Smooth Muscle Cell Migration by Phosphodiesterase 3 and Phosphodiesterase 4 Inhibitors

Daniel Palmer, Keith Tsoi and Donald H. Maurice


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