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Dissociation Between Phosphodiesterase Inhibition and Antiproliferative Effects of Phosphodiesterase Inhibitors on the Dami Cell Line

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ABSTRACT. Phosphodiesterase (PDE) inhibitors were shown to inhibit proliferation of various cell types. The present investigation was designed to study the activity of selective PDE inhibitors (8-MeoMIX, milrinone, trequinsin, rolipram, RO-201724, zaprinast, and MY-5445) on the proliferation of the Dami cell line in relation to their effects on cAMP levels and PDE isoenzymes isolated from Dami cells. All compounds, except 8-MeoMIX, elicited antiproliferative effects. Trequinsin, RO-201724, and MY-5445 (100 μM) were found to inhibit cell growth up to 60%, 83%, and 85%, respectively; milrinone, rolipram and zaprinast elicited only weak effects (19–21% at 100 μM). Their growth-inhibitory effects could not be related to their effects on cAMP levels. In addition, although PDE type III and IV inhibitors potentiated cAMP formation due to adenylycyclase activation, no potentiation could be observed when considering their antiproliferative effect. Separation and characterization of PDE of Dami cells revealed the existence of types III, IV, and V isoenzymes. The PDE inhibition found for the PDE inhibitors could not explain their antiproliferative effects. The lack of correlation with cAMP concentrations or PDE inhibition and the high concentrations needed to elicit antiproliferative effects suggest the implication of other parameters, such as cytotoxicity or lipophilicity, or other targets in addition to PDE for the PDE inhibitors tested. Lipophilicity did not seem to be of importance in antiproliferative effects. In contrast, cytotoxic effects, in particular those of trequinsin and MY-5445, could partially explain their negative action on cell growth. BIOCHEM PHARMACOL 53;8:1141–1147, 1997. © 1997 Elsevier Science Inc.

KEY WORDS. Dami cell line; PDE inhibitors; PDE isoenzymes; proliferation; cAMP; megakaryoblast

cAMP¶ is known to be involved in the proliferation of different cell types. It can be a positive intracellular signal for cell growth, as shown for many differentiated cells [1, 2] as well as a negative messenger as described for various cancer cells [2–5]. Rises in intracellular cAMP levels can be induced by inhibitors of the cAMP-degrading enzymes, the PDE. Previously, studies have shown that nonselective PDE inhibitors of the methylxanthine type [3, 5–10], pyrimidopyrimidines [11–13] and imidazo[1,2-a]pyrazine derivatives [14] could display antiproliferative effects on normal and cancer cell lines. At present, little is known about the activity of selective PDE inhibitors [15–17] on cell proliferation. Studies have been carried out with different selective-type I, III, IV, V, or mixed-type III/IV PDE inhibitors on various cell lines [18–23]. All PDE inhibitors of type III [19–23] as well as those of type IV [18, 19, 21–23] and of mixed type III/IV [19, 20] were shown to inhibit cell growth of the different cell types tested. These antiproliferative activities have been correlated to intracellular cAMP levels and to the PDE inhibitory potency of these compounds [19–21, 23].

In the present study, we have investigated the effects of a series of selective PDE inhibitors (Table 1) on the proliferation of the human megakaryoblastic leukemic Dami cell line [24], which was found to be negatively regulated by cAMP [5], in relation to their ability to increase intracellular cAMP levels and their PDE inhibitory potency toward isoenzymes isolated from Dami cells.

MATERIALS AND METHODS

Materials

Dami cells were obtained from the American type culture collection (CRL 9792) (ATCC, Rockville, MD). Horse serum was purchased from Techgen International (Les Ulis, France), and cell culture medium and PBS Dulbecco's were from Gibco BRL (Eragny, France).

Trequinsin, milrinone, RO-201724, rolipram, zaprinast

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¶ *Abbreviations*: PDE, phosphodiesterase; cAMP, cyclic 3′, 5′-adenosine monophosphate; cGMP, guanosine 3′,5′-cyclic monophosphate; PKA, cAMP-dependent protein kinase; LDH, lactate dehydrogenase, PGE1, prostaglandin E1.

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TABLE 1. Selective PDE inhibitors used

PDE inhibitor*	Type
8-MeoMIX	I
Milrinone, trequinsin	III
Rolipram, RO-201724	IV
MY-5445, zaprinast	V

* Classification as proposed by Beavo (17).

and MY-5445 were purchased from Biomol (Tebu, le Perray en Yvelines, France) and 8-MeoMIX from LC-laboratories (Meudon, France-Biochem, France). The cAMP ^{125}I-radioimmunoassay kit was from Immunotech (Marseilles, France) and the CytoTox 96 kit from Promega (Charbonnières, France). Radiochemicals were obtained from Amersham (Les Ulis, France). Other reagents were purchased from Sigma (St. Quentin Fallavier, France).

Cells and Cell Culture Conditions

Cells were grown in suspension in RPMI 1640 medium supplemented with 10% heat-inactivated horse serum, 2 mM glutamine, 1 mM sodium pyruvate, and 0.4% nonessential amino acids. They were incubated in disposable sterile Erlenmeyer flasks (Corning, Polylabo, Strasbourg, France) at 37°C with a humidified atmosphere of 5% CO_2. Cultures were fed every 2–3 days by partial replacement of spent medium.

Proliferation Studies

Cellular proliferation was studied by placing cells in the exponential phase of growth in microwells (Nunc, Polylabo, Strasbourg, France) at a starting concentration of 2×10^5 cells/mL. Culture conditions were as described above. Cells were incubated with the products to be tested for 48 hr and cell proliferation was determined by cell counting performed using a Coulter Counter ZM (Coultronics S. A., Margency, France) equipped with a 140-μm orifice tube, and calibrated with 14 μm latex particles.

Cell Viability

Cell viability was determined by the use of the CytoTox 96 nonradioactive assay, which allows the quantitative spectrophotometric measurement of LDH activity [25]. Cells were seeded and incubated with the different compounds for 48 hr as described in Vittet *et al.* [5].

Cyclic AMP Assays

Samples for determination of intracellular cAMP concentrations by a radioimmunoassay kit were prepared as follows. Cells were washed with PBS (158 mM NaCl; 2.7 mM KCl; 1.5 mM KH_2PO_4; 8 mM Na_2HPO_4; 0.5 mM $MgCl_2$; 0.9 mM $CaCl_2$; 5.55 mM glucose, pH 7.4), resuspended to a final concentration of 2.0×10^6 cells/mL and preincubated for 5 min at 30°C before addition of compounds to be tested. Incubations were performed at 30°C for 10 min and terminated by adding 60% cold trichloroacetic acid to a final concentration of 10%. After mixing, each sample was spun at 14,000 *g* for 2 min and the supernatants were neutralized with KOH (3 mM) and buffered with HEPES (150 mM). The suspension was kept in an ice bath for 10 min and the precipitate was removed by centrifugation at 2500 *g* for 5 min. The neutralized extracts were stored at −20°C. cAMP concentration was assayed according to the manufacturers' recommendations.

Preparation of Cell Extracts and High-Speed Supernatant

Cells were separated from culture medium by centrifugation at 150 *g* for 5 min. After 2 washes with PBS Dulbecco's buffer without calcium, magnesium, or sodium bicarbonate, the cell pellet (10^8 cells) was resuspended in 2 mL extraction buffer (10 mM Tris-HCl, 2 mM MgCl2, 10 μM Antipain, 10 μM Leupeptin, 10 μM Pepstatin A, 5 mM β-mercaptoethanol, 200 μM phenylmethylsulfonyl fluoride, pH 7.5), homogenized, and centrifuged at 1000 *g* for 15 min. The supernatants were centrifuged at 100,000 *g* for 60 min to obtain the high-speed supernatant.

Separation of PDE Isoenzymes

PDE isoenzymes were separated from the high-speed supernatant by anion exchange chromatography. The supernatant (1600 μg protein) was applied to a DEAE-Sepharose CL-6B column (Sigma, St. Quentin Fallavier, France) preequilibrated with the extraction buffer (described above) containing 0.1 M Na acetate. PDE isoenzymes were successively eluted by using a step-by-step Na acetate gradient in the same buffer (see Fig. 4) and collected as 1 mL fractions. For storage at −20°C, ethylene glycol was added to a final concentration of 30% (v/v).

Protein concentration was determined as described by Lowry *et al.* [26].

PDE Assay

The PDE activity of cell extracts was determined by the method of Thompson *et al.* [27], modified by Cook *et al.* [28]. Assays were performed at 37°C in a total volume of 100 μL. Each tube contained 25 μL of each fraction, 50 μL of assay buffer (final concentration of 40 mM Tris-HCl, 2.5 mM $MgCl_2$, 3.75 mM β-mercaptoethanol, 0.2 μCi [^{3}H]-cAMP or cGMP, 1 μM cAMP, or cGMP, pH 8.0) and 25 μL PDE inhibitor or its respective solvent. Following 30 min incubation, the reaction was stopped by transferring to a bath of boiling water for 3 min. After cooling on ice, 20 μL of 1 mg/mL *Ophiophagus hannah* venom was added and the reaction mixture was incubated at 37°C for 10 min. Unreacted [^{3}H]-cAMP or [^{3}H]-cGMP was removed by the

addition of 400 μL of a 35% suspension of Dowex 1 × 8–400 μL resin and incubation on ice for 30 min. After centrifugation (2500 g, 5 min), 200 μL of the supernatant was removed for liquid scintillation counting. Less than 10% of the tritiated cyclic nucleotide was hydrolyzed in any assay.

The concentration of the PDE inhibitors that causes 50% PDE inhibition (IC_{50}) was determined from concentration-response curves. At least two concentration-response curves were generated for each agent.

Statistical Evaluation

Results are means ± SE Statistical differences were appreciated by Student's *t*-test or Withney-*U* test (cAMP assay). $P < 0.05$ was considered as significant. IC_{50} values were calculated using computer software [29] and lipophilicity values (logP) were evaluated as described by Viswanadhan et al. [30].

RESULTS

Effects of the PDE Inhibitors on Dami Cell Proliferation

None of the compounds affected cell growth at concentrations below 1 μM (1–100 nM). RO-201724, trequinsin, and MY-5445 induced a potent concentration-dependent cell growth inhibition at the highest concentration assayed (100 μM) up to 60%, 83%, and 85%, respectively (Fig. 1, Table 2). For milrinone, rolipram and zaprinast, only weak significant growth inhibitions of 19.2% ± 1.2, 21.5% ± 2.8 and 21.2% ± 1.9, respectively, were found at the highest concentration (100 μM) tested. 8-MeoMIX was devoid of any effect. Due to the poor compound solubility, 100 μM could not be exceeded.

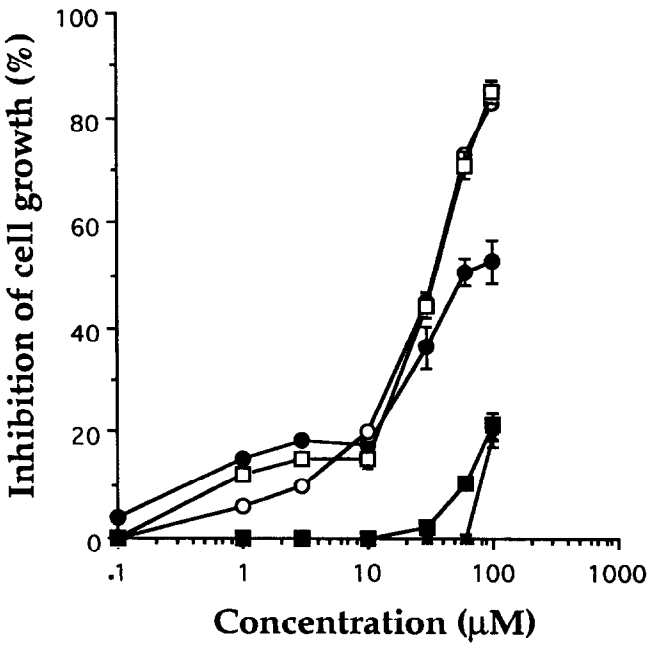

FIG. 1. Concentration-dependent activity of RO-201724 (●) trequinsin (○), MY5445 (□), rolipram (■) and Milrinone (×) on Dami cell growth. Growth inhibition is expressed in percent related to control. Each point represents the mean ± SE of 3 experiments performed in duplicate. Each value of duplicate is based on 4 cell counts.

Determination of Cytotoxicity

We observed a significant increase in LDH release from cells induced by trequinsin and MY-5445 at 10 μM and above (Fig. 2A, B). A correlation (R = 0.93) between LDH release and inhibition of cell proliferation was found for these compounds. 100 μM rolipram also presented significant cytotoxic activity (14.8 ± 1.3 vs 8.5 ± 0.8), whereas neither 100 μM RO-201724, milrinone, now zaprinast significantly increased LDH release (results not shown).

Effects of the PDE Inhibitors on Intracellular cAMP Levels in the Presence and Absence of Prostaglandin E1 (PGE1)

To look for a potential relationship between cell growth inhibition and intracellular cAMP concentrations, we investigated the effect of the compounds on cAMP concentration in the presence and absence of the adenylyl cyclase stimulator PGE1. PGE1 was tested at 0.1 μM, a concentration that induced only a low cAMP increase in Dami cells (37.7 ± 11.4 pmol/million cells). In the absence of PGE1, only milrinone and trequinsin significantly increased cAMP concentration (Fig. 3A). All compounds tested, except 8-MeoMIX, MY-5445, and zaprinast, potentiated cAMP formation in the presence of PGE1 (Fig. 3B).

Effects of Milrinone and RO-201724 on Dami Cell Growth in the Presence and Absence of PGE1

To investigate whether or not the antiproliferative effects of milrinone and RO-201724 were potentiated in the presence of adenylyl cyclase stimulation, we compared their activity on cell growth after 48 hr incubation at 100 μM and 10 μM (only RO-201724) in the presence and absence of PGE1 (Table 3). PGE1 (0.1 μM) elicited weak proper antiproliferative effects (11.6% ± 0.5) and did not potentiate the antiproliferative effect of 100 μM Milrinone or RO-201724 (10 and 100 μM). Only additive effects on cell growth inhibition were observed for these compounds. Furthermore, we did not observe any potentiation of the effect of RO-201724 at 0.1 and 1 μM in the presence of PGE1 (data not shown).

Distribution of Cyclic Nucleotide PDE Activity in Dami Cells

Dami cells contained both cAMP PDE and cGMP PDE activities. Over 85% of total cAMP and 85% of total cGMP PDE activity was found in the cytosolic fraction.

TABLE 2. Effects of PDE inhibitors on PDE isoenzymes from Dami cytosol and on cell growth inhibition (G.I.)

Compounds†	log P	-log (IC_{50})*			
		G.I.	Type III (peak 3)	Type IV (peak 2)	Type V (peak 1a/b)
Trequinsin	1.97	4.6 ± 0.1	8.2 ± 0.2	5.8 ± 0.3	N.D.‡
Milrinone	−0.57	<4.0	5.2 ± 0.1	4.7 ± 0.1	N.D.‡
Rolipram	1.66	<4.0	<4.0	5.2 ± 0.1	N.D.‡
RO-201724	1.81	4.3 ± 0.0	<4.0	4.1 ± 0.2	N.D.‡
Zaprinast	1.36	<4.0	N.D.‡	N.D.‡	5.4 ± 0.1
MY-5445	6.58	4.5 ± 0.0	N.D.‡	N.D.‡	<5.0

* Values are the means ± SE of N = 7–10 experiments performed in triplicate, † Compounds were assayed in the presence of 1 μM cAMP or cGMP as substrate ‡ ND, not determined.

Characterization of the PDE Isoforms of Dami Cytosol

Four peaks of cyclic nucleotide PDE activity were separated by Na acetate gradient elution of the cytosolic fraction (Fig. 4). Peaks 1a and 1b revealed specific hydrolytic activity for cGMP. This activity was not stimulated by Ca^{2+}-calmoduline (results not shown) and was inhibited by zaprinast and MY-5445 (Table 2). According to Beavo and Reifsnyder [17, Table 1], these characteristics apply to PDE type V. The isoenzymes eluted in peaks 2 and 3 preferentially hydrolyzed cAMP. The isoenzymes eluted in peak 2 were insensitive to cGMP (10 μM), not stimulated by Ca^{2+}-calmoduline (results not shown), and were primarily inhibited by rolipram and RO-201724 (Table 1). These are specific characteristics for type IV isoenzymes. The specific PDE type III inhibitors milrinone and trequinsin showed preferences for the isoenzymes of peak 3 (Table 2), which were also inhibited by cGMP (38.1% ± 0.6 at 10 μM; 52.4% ± 3.3 at 1 μM). The trequinsin IC_{50} for the cAMP hydrolytic activity of peak 3 was 1000 times lower than that of peak 2. These characteristics apply to PDE type III.

DISCUSSION

The PDE type I and V inhibitors tested in our study presented only marginal (zaprinast) or undetectable (8-MeoMIX) effects on Dami cell proliferation at the highest concentration tested, which was consistent with studies from other groups [18, 23]. Cell growth inhibition elicited by MY-5445 appears essentially related to its cytotoxic effect.

PDE inhibitors type III and IV have shown antiproliferative effects on Dami cell proliferation. Among them, trequinsin and RO-201724 inhibited cell growth in a concentration-dependent manner. Milrinone and rolipram presented only a weak antiproliferative effect at the highest concentrations tested. These results are in agreement with other studies that have investigated the activity of different selective type III, IV, or mixed type III/IV PDE inhibitors on proliferation of lymphocytes [18, 19], smooth muscle cells [20–22], B16 murine melanoma and human MCF-7 mammary carcinoma cell lines [23]. In these studies, all PDE inhibitors of type III, SK&F 94836 [19, 21], milrinone [18], trequinsin [21], CI-930, and cilostamide [22], as well as

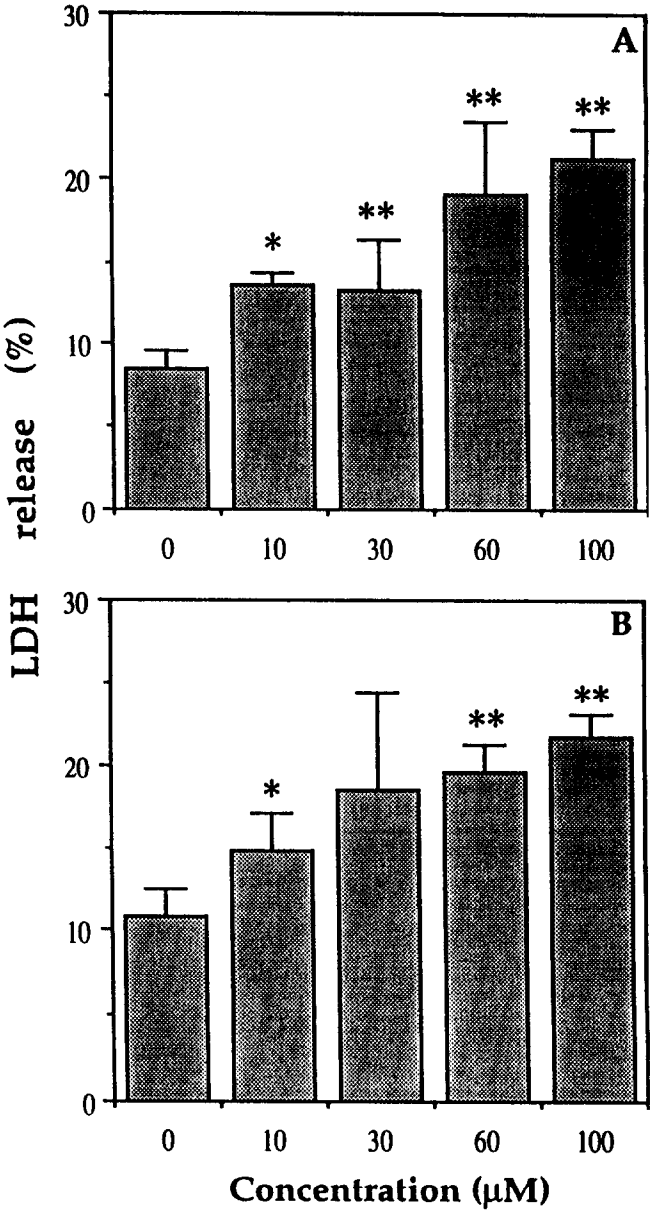

FIG. 2. Concentration-dependent activity of (A) Trequinsin, (B) MY5445, and their respective controls on LDH release from Dami cells after 48-hr incubation. Histograms are the means and vertical bars indicate the SE of at least 4 experiments performed in triplicate. Significantly different from controls: **$P < 0.01$, *$P < 0.05$.

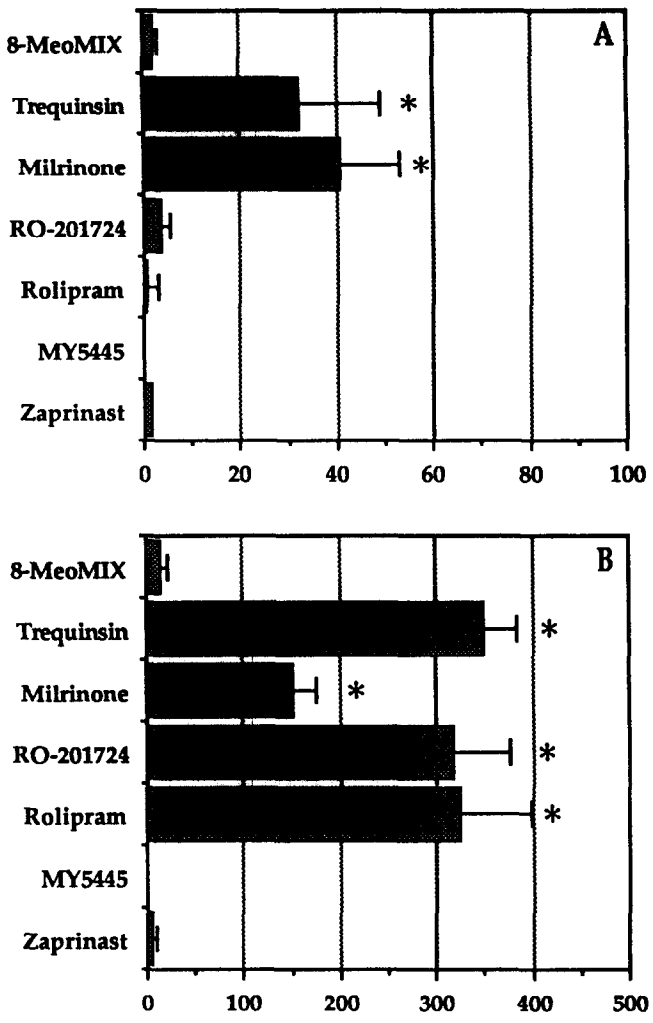

FIG. 3. Effects of 100 μM of the PDE inhibitors on intracellular cAMP levels (A) in absence, and (B) in presence of 0.1 μM PGE1. Histograms are the means and horizontal bars indicate the SE of 3 to 5 experiments performed in duplicate. *P < 0.01.

those of type IV, DC-TA-46 [23], rolipram, and RO-201724 [18, 19, 21, 22] and those of mixed type III/IV, zardaverine and cilostazol [19, 20], were shown to inhibit cell growth.

Although most PDE inhibitors tested here elicited cell-growth inhibition, very high concentrations up to 100 μM were needed to induce that effect. Moreover, such high concentrations of selective PDE inhibitors have already been described to be necessary to induce cell-growth inhibition [18, 21, 22].

In view of the high concentrations needed, it appeared difficult to explain this antiproliferative activity by their PDE-inhibitory potency. To clarify this point, we investigated if the antiproliferative activities of the PDE inhibitors were related to their effects on intracellular cAMP. Our results indicated no correlation between these two parameters. Indeed, rolipram and RO-201724 inhibited cell growth at the highest concentration tested without affecting intracellular cAMP levels. In contrast, trequinsin and milrinone induced intracellular cAMP levels in the same manner, but exhibiting quite different effects on cell proliferation (*i.e.* marginal for milrinone and potent for trequinsin. In addition, although the effects of the PDE type III and IV inhibitors on intracellular cAMP levels were potentiated in the presence of PGE1, their antiproliferative activity was only added to those of PGE1, which confirms a lack of correlation between cell-growth inhibition and intracellular cAMP levels.

TABLE 3. Effects of selective PDE inhibitors on Dami cell growth

Compounds	G.I. (%)* In absence of PGE1	G.I. (%)* In presence of PGE1 (0.1 μM)
PGE1	–	11.6 ± 0.5
Milrinone†	18.7 ± 0.7	34.3 ± 3.3
RO-201724†	39.5 ± 7.9	57.2 ± 2.4
RO-201724‡	24.9 ± 2.0	42.8 ± 2.8

* G.I., growth inhibition, values are means ± SE, N = 4–8.
† 100 μM.
‡ 10 μM.

The need to verify a potential relationship between antiproliferative activity and PDE inhibitory potency led us to isolate the PDE isoenzymes from Dami cells.

Separation of these isoenzymes revealed the presence of a peak of cGMP-specific hydrolyzing isoenzymes, charac-

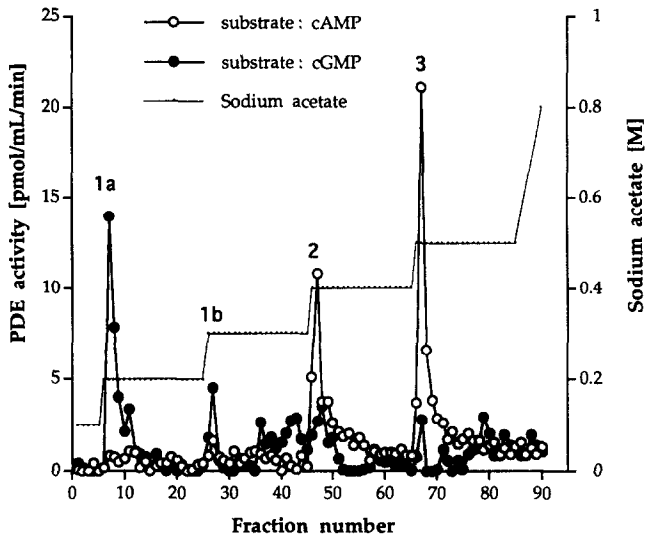

FIG. 4. DEAE-Sepharose-CL-6B anion exchange chromatography of PDE activity of Dami cytosol: cAMP-PDE activity was assayed with 1 μM cAMP (○) and cGMP-PDE activity with 1 μM cGMP (●) as substrate. The shape of the Na acetate gradient is shown. Fractions of 1 mL were collected and 30% ethylene glycol (v/v) was added. The activity yield of the 90 fractions collected represents 80% of total cAMP-PDE and 90% of total cGMP-PDE.

terized by the inhibitory effects of zaprinast and MY-5445 as PDE type V, as well as the presence of 2 peaks of cAMP-specific hydrolyzing isoenzymes. In view of the inhibitory effects of the type III and IV inhibitors, we characterized these isoenzymes as type III and IV. These results were confirmed by the observation that cGMP selectively inhibited PDE isoenzymes characterized as type III, whereas it presented no effect on those of type IV.

It is noteworthy that only rolipram—trequinsin elicited a good PDE III–PDE IV discriminating potency, RO-201724—milrinone being less selective. However, milrinone presented the same inhibitory potency on the PDE III isoenzymes when used in the presence of 10 μM rolipram (results not shown). All these observations show a satisfactory separation of type III and IV isoenzymes. However, the IC_{50} values observed here were 6 to 36 times higher than those described for other systems [23, 31–36]. Both high IC_{50} values and discriminating potencies of RO-201724 and milrinone revealed quite unusual reactivities for the PDE isoenzymes isolated from Dami cells that may be related to the existence of different genetic subtypes, as has already been described for type III and IV isoenzymes [37]. When comparing IC_{50} values for PDE and growth inhibitions, there appears at least a 2-log difference and no correlation between the two parameters. Indeed, RO-201724 elicited equipotent antiproliferative effects in the 1–10 μM range, and its PDE inhibitory potency varied from 0 to 38.5%. Furthermore, rolipram and milrinone did not affect cell growth at lower concentrations (from 1 to 10 μM), but presented PDE inhibitory effects from 30 to 58.6% and from 17.8 to 62.5% in the same concentration range, respectively. In addition, trequinsin inhibited PDE type III at nM concentration (IC_{50} = 6 nM) without affecting cell proliferation at these concentrations. Studies from other authors have also shown discrepancies between the PDE and growth IC_{50} values [18, 21, 22] of PDE inhibitors, although their growth-inhibitory effect has been related to their PDE-inhibitory potency.

In summary, it appears that the effects of the selective PDE inhibitors tested on Dami PDE isoenzyme activity and on intracellular cAMP levels could not totally explain proliferation responses. These results are in contrast to other studies realized with selective PDE inhibitors. The antiproliferative effect of the type IV inhibitor DC-TA-46 has been correlated to intracellular cAMP levels and to its PDE-inhibitory potency [23]. Furthermore, for the type III inhibitor SK&F 94836 and type IV inhibitor rolipram, alone or in combination, as well as for cilostazol and zardaverine, increased cAMP levels were reported to mediate the inhibitory effect on cell growth [19–21]. This discrepancy between our results and those found previously suggests the implication of other parameters, in addition to PDE inhibition, for the PDE inhibitors tested, such as cytotoxicity and differences in lipophilicity, and targets other than PDEs. The potent antiproliferative effects of trequinsin and MY-5445 seem to be partially related to their cytotoxic effects. Although high concentrations of PDE inhibitors have been used in some studies to induce potent effects on cell growth inhibition [18, 22], no such cytotoxic effects have been reported. Evaluation of the lipophilicity values of the PDE inhibitors has indicated an absence of linkage between this parameter and cell-growth inhibitory effects.

PDE inhibitors have also been reported to have other targets in addition to PDEs, in particular, cAMP-dependent protein kinase (PKA) [38] or Gi protein [39]. Furthermore, there is evidence for a "cross-talk" between PDEs and other signalling systems that could regulate PDE activity and/or expression [40]. It has been suggested that such interactions or other targets modulate the PDE inhibitory activity of RO-201724, MY-5445 and zaprinast [41–43].

This study shows that some of the selective PDE inhibitors tested exhibited inhibitory effects on the growth of the Dami cancer cell line. However, in view of the high concentrations needed to elicit cell-growth inhibition and the absence of any correlation with intracellular cAMP levels or selective PDE-inhibitory potency, it appears that side effects of the PDE inhibitors also participate in Dami cell-growth inhibition.

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