0026-895X/93/051027-09$03.00/0

MOLECULAR PHARMACOLOGY, 44:1027-1035

Modulation of Rat Thymocyte Proliferative Response Through the Inhibition of Different Cyclic Nucleotide Phosphodiesterase Isoforms by Means of Selective Inhibitors and cGMP-Elevating Agents

P. MARCOZ, A. F. PRIGENT, M. LAGARDE, and G. NÉMOZ
Unité INSERM 352, Chimie Biologique INSA-Lyon, Villeurbanne, France
Received April 26, 1993; Accepted August 16, 1993

SUMMARY

We have investigated the role played by cyclic nucleotide phosphodiesterases (EC 3.1.4.17) in the control of T-lymphocyte response to mitogenic agents by their ability to influence the cellular level of cAMP. The importance of this messenger as a negative regulator in this cell type is well established. Multiple isoenzymes of phosphodiesterase were fractionated from the cytosol of rat thymic lymphocytes by high performance liquid chromatography on an anion exchange column. In addition to the type II, III, IV isoforms that we have already described [Valette *et al.*, *Biochem. Biophys. Res. Commun.* **169**:864–872 (1990)], a phosphodiesterase fraction sharing several of the characteristics of type V, cGMP-binding phosphodiesterase, was detected. Non-isoform-selective inhibitors of phosphodiesterase such as dipyridamole, papaverine, and methyl-isobutylxanthine were able to totally prevent the proliferative response of thymocytes to stimulation by the mitogenic lectin concanavalin A. In contrast, the selective inhibitor of type IV phosphodiesterases rolipram induced a rather moderate inhibition of proliferation, not exceeding 60%; and the selective inhibitors of type III and type V phosphodiesterases, milrinone and M&B 22,948, respectively, displayed only marginal inhibitory effects. The association of the type III and IV phosphodiesterase inhibitors produced synergistic inhibition of proliferation, which could then be almost totally suppressed. These inhibitory effects on cell multiplication were reflected at the level of the cell cAMP content; only rolipram was able to induce a significant (approximately 50%) increase in cAMP, and this increase was potentiated by the presence of milrinone, reaching almost 100%. The type V phosphodiesterase selective inhibitor M&B 22,948 displayed similar properties to those of milrinone, which suggests that it indirectly inhibited the type III, cGMP-inhibitable isoenzyme, by inducing a cGMP rise. This hypothesis was supported by evidence of a significant raising effect of M&B 22,948 on cGMP level, and by the ability of a cGMP-elevating agent, sodium nitroprusside, to mimic the synergistic effects of milrinone associated with rolipram. Furthermore, 8-bromo-cGMP, a potent activator of cGMP-dependent protein kinase, which showed only weak inhibitory effects on thymic type III phosphodiesterase, failed to alter the effects of rolipram on the cell proliferation. These results allow us to delineate a role for types III, IV, and V phosphodiesterase in the control of cAMP level during the proliferative response of thymic lymphocytes. They also suggest that endogenously formed cGMP might participate in the regulation of cAMP level in the cells by means of the inhibition of the type III phosphodiesterase. cGMP thus appears to be a potential physiological negative effector of the lymphocyte mitogenic response.

There is now general agreement that cAMP is a negative regulator of T-lymphocyte response to stimulation by specific antigens or mitogens (1). Indeed, elevation of cAMP levels is able to hamper the T-lymphocyte mitogenic process at several points, from the very precocious phase of the response (2, 3) as well as in the later, interleukin-2-promoted steps (4, 5). The rise in cAMP in T-cells, and the ensuing inhibition of proliferative response, can be induced by agonists of receptors activating adenylate cyclase, such as β-adrenergic agents, prostaglandins E_1 and E_2, adenosine, or by activators of adenylate cyclase which bypass the receptors such as cholera toxin and forskolin (4–7), or finally by inhibitors of the cAMP-degradating enzymes, the phosphodiesterases (8).

The negative effects of cAMP in T-lymphocytes have been shown to be mediated by the cAMP-dependent protein kinase isotype I (9). One of the earliest inhibitory effects of cAMP is its ability to counteract the phosphoinositide-specific phospholipase C activation induced by the stimulation of the T-cell antigen receptor complex. The inhibition mechanism has been unraveled recently: the activated protein kinase A proves able to phosphorylate phospholipase C-γ1 at a precise serine residue, thus preventing the *src*-like tyrosine kinase-catalyzed phos-

ABBREVIATIONS: 8-Br-cGMP, 8-bromo-cGMP; MIX, 1-methyl-3-isobutylxanthine; HPLC, high pressure liquid chromatography; TdR, thymidine; EDTA, ethylenediaminetetraacetic acid.

phorylation of the enzyme at multiple tyrosine residues, and thereby impairing its activation (10).

cGMP is a second messenger whose role in the regulation of physiological processes appears increasingly important. However, its implication in the control of the lymphoproliferative response is much less clear. The fact that a transient increase in cGMP level induced by mitogens has often been observed in T-cells, in addition to observations that some agents supporting mitogen-induced proliferation also increase cGMP (reviewed in ref. 11), has led to the proposal that cGMP is an initial proliferation signal. However, other authors did not observe any effect of mitogens on cGMP level, and failed to promote mitogen-induced lymphoproliferation in the presence of cGMP-elevating agents such as sodium nitroprusside (12, 13), which renders the above hypothesis seriously questionable.

Both cAMP and cGMP physiological effects are terminated by the action of phosphodiesterases. This enzyme system has recently been shown to be as complex in T-lymphocytes as it is in most other cell types, in that it is constituted by several isoforms of different specificities toward one or the other cyclic nucleotide (14–18). We have continued the characterization of thymic T-lymphocyte phosphodiesterase equipment by bringing some new data concerning separated isoenzyme forms.

In some tissues, the selective implication of one of the phosphodiesterase isoforms in a precise physiological process has been determined. For example, the antilipolytic effect of insulin in adipocytes is related to the selective stimulation of a type III[1] phosphodiesterase mediated by a phosphorylation process (20). In frog cardiomyocyte, cGMP has been shown to antagonize the cAMP-induced increase in slow inward calcium current by means of the activation of a type II, cGMP-activatable phosphodiesterase (21). Similarly, in the adrenal cortex, atrial natriuretic peptide decreases the cAMP-dependent steroidogenesis by inducing a rise in intracellular cGMP level, which stimulates the cAMP hydrolysis by type II phosphodiesterase (22). In platelets, the antiaggregatory action of nitrovasodilators has been shown to be attributable to their ability to raise cGMP level, and thereby to inhibit cAMP hydrolysis by the type III, cGMP-inhibited phosphodiesterase, which results in an accumulation of the antiaggregatory messenger cAMP (23).

In lymphocytes, the role played by each of the different phosphodiesterase isoforms in the proliferative response is still undefined. We have shown previously that mitogenic stimulation of thymocytes induces an early rise (at 10–30 min) of cAMP-phosphodiesterase activity, primarily attributable to stimulation of type IV isoforms (14), which suggests a role for these enzymes in the first steps of the response. Hurwitz *et al.* (18) have shown that the *de novo* synthesis of a type I phosphodiesterase takes place after 14 hr of stimulation of bovine peripheral lymphocytes, raising the possibility that this isoform is involved in the later phases of the response.

To delineate more precisely the implication of the different isoforms of phosphodiesterase in the response, we studied the effect on lectin-induced thymocyte proliferation of various selective phosphodiesterase inhibitors. As a type III isoenzyme, a characteristic of which is inhibition by cGMP, proved implicated, we extended our investigations to agents acting on the cGMP system.

[1] Phosphodiesterase nomenclature is from Ref. 19.

Experimental Procedures

Materials. Glutamine, penicillin, streptomycin, lymphocyte separation medium J-Prep, RPMI 1640, and fetal calf serum were from J-Bio (Les Ulis, France). cAMP, cGMP, gelatin from porcine skin, concanavalin A, 8-phenyltheophylline, 8-Br-cGMP, sodium nitroprusside, MIX, papaverine, milrinone, adenosine deaminase (type VI), and snake venom (*Ophiophagus hannah*) were from Sigma-Chimie (St-Quentin-Fallavier, France). Rolipram [4-(3-cyclopentyloxy, 4-methoxyphenyl)-2-pyrrolidone] was a gift from Schering (Berlin, Germany). M&B 22,948 (2–0-propoxyphenyl-8-azapurin-6-one) was a gift from May & Baker (Dagenham, U.K.). Dipyridamole was purchased from Boehringer Mannheim (Meylan, France). cAMP ^{125}I-radioimmunoassay kit was from E.R.I.A. Diagnostics Pasteur (Marnes-la-Coquette, France). [U-^{14}C]Guanosine was from Dositek (Orsay, France). [8-^{3}H]cAMP, [8-^{3}H]cGMP, [U-^{14}C]adenosine, [*methyl*-^{3}H]TdR, cGMP ^{125}I-radioimmunoassay kit were from Amersham (Les Ulis, France).

Preparation of rat thymocytes. Male Sprague-Dawley rats (250–400 g) were sacrificed by decapitation. Thymus glands were removed and placed into 0.15 M NaCl medium, cleaned of adherent connective tissues, and gently dilacerated in a loose-fitting glass/glass homogenizer. Tissue remnants were removed by filtration of the cell suspension through a nylon gauze. Thymic lymphocytes were separated by gradient centrifugation on J-Prep medium. The cells were washed with 0.15 M NaCl, and cell density was adjusted with RPMI 1640 medium. All steps were carried out at room temperature. Under such conditions, cell viability was consistently greater than 90% as revealed by the trypan blue exclusion test. The homogeneity of cell preparations was routinely assessed by phase contrast microscopy examination, and by the May-Grünwald-Giemsa staining method. Contamination was consistently found to be less than 1% for platelets, and less than 0.25% for macrophages.

Characterization of the cell population was also performed by specific labeling of T-lymphocytes with a fluorescent monoclonal antibody directed against the Thy-1 surface antigen (OX 7, Sera-Lab, Crawley Down, England). Analysis by fluorescence-activated cell sorter (Epics Profile, Coulter) detected 98% positive cells.

Preparation of thymocyte cytosolic fraction and fractionation of phosphodiesterase isoforms by ion exchange high performance liquid chromatography. Procedure was performed as described by Marcoz *et al.* (15). Thymocytes were lyzed in hypotonic buffer (10 mM Tris, 1 mM $MgCl_2$, pH 7.2) in the presence of protease inhibitors (2 μg/ml pepstatin A, 40 units/ml aprotinin, 1 mM phenymethylsulfonyl fluoride) and homogenized in a tight-fitting Dounce homogenizer. The homogenate was mixed with 4 volumes of 0.25 M sucrose and centrifuged at 130,000 × *g* for 45 min. The resulting supernatant (40 ml, 20–30 mg of proteins) was loaded on a Mono-Q HPLC anion exchange column (Pharmacia, St-Quentin-en-Yvelines, France) equilibrated in 50 mM Tris/HCl, 1 mM EDTA, 7 mM 2-mercaptoethanol, 0.1 mM phenylmethylsulfonyl fluoride, pH 7.5. Phosphodiesterase isoforms were successively eluted by using a step-by-step NaCl gradient in the same buffer (see Fig. 1).

Phosphodiesterase assay, determination of IC_{50} values of phosphodiesterase inhibitors, and cGMP binding measurement. Enzyme activities were determined as reported in ref. 14, in the presence of 0.5 mg/ml gelatin as a stabilizer. Inhibitors were dissolved in dimethyl sulfoxide as 100 mM stock solutions, and subsequently diluted in 40 mM Tris/HCl, pH 8, buffer to provide the desired drug concentration. Final dimethyl sulfoxide concentration (0.1%) was shown to negligibly affect enzyme activities. IC_{50} values were determined by plotting the percentage of residual enzyme activity versus the logarithmic concentration of the inhibitor. Binding of [^{3}H]cGMP on HPLC eluate protein fractions was evaluated as described in ref. 24.

Determination of the cyclic nucleotide content of thymocytes. All cell suspensions were allowed to rest for at least 1 hr at 37° before the beginning of the experiments. 0.8 ml of thymocyte suspension (cell density: 8 × 10^6 cells/ml) were incubated for 30 min at 37° in RPMI 1640 supplemented or not with the tested compound in 10 ml borosil-

icate glass tubes (Corning). In every case, dimethyl sulfoxide was at 0.1% final concentration. Cells were eventually stimulated by the addition of 5 μg/ml concanavalin A, and allowed to incubate for an additional period of time, as indicated. Incubation was terminated by boiling the samples for 2 min. Proteins were removed by centrifugation (4500 × *g* for 15 min), supernatants were diluted as required in the immunoassay buffers, and cAMP and cGMP were assayed according to the manufacturers' recommendations, using the acetylation procedures.

[^{3}H]TdR incorporation into thymocytes. Measurements of cell proliferation were made by determining the incorporation of [^{3}H]TdR into cellular DNA. Thymocytes were cultivated in 96-well flat-bottomed microtiter plates (Costar). Each well contained, in a total volume of 0.25 ml, 2.5 × 10^5 cells in RPMI 1640 supplemented with 2 g/liter $NaHCO_3$, 2 mM glutamine, 100 units/ml penicillin, 1 g/liter streptomycin, and 10% fetal calf serum. Phosphodiesterase inhibitors were diluted in RPMI 1640 medium to give the desired concentrations, and a final dimethyl sulfoxide concentration of 0.1%, which did not affect the proliferative response. The cells were stimulated by the addition of 1 μg/ml concanavalin A, and were placed in humidified atmosphere of 95% air/5% CO_2 at 37°. 0.1 μCi of [^{3}H]TdR was added for the last 18 hr of a 72-hr culture. The cells were harvested using an automatic cell harvester (Automach 2000, Dynatech) on glass fiber filters, and the radioactivity was measured by liquid scintillation counting of the dried filters. Measurements were repeated 8 times for the control values, 4 times for all the other assays. TdR incorporation in stimulated cells amounted to approximately 2 × 10^4 cpm per well. Incorporation in unstimulated cells was found negligible (<200 cpm). The proliferation data are given in the figures as means ± standard error, referring to the variability observed in the typical experiment shown. All the proliferation inhibition experiments were repeated three times with similar results. 95% confidence intervals for IC_{50} values were calculated by linear regression as described in ref. 25. Interaction between two drugs in the inhibition of proliferation was evidenced by the method of Pöch and Holzmann (26), which consists in the comparison of the dose-response curve observed for the two drugs simultaneously present with a theoretical curve which fits a simply additive model. This latter curve is deduced from the curve observed with one of the drugs alone, by assuming that a fixed dose of the second drug purely acts as an equipotent dose of the first drug. For clarity, only the theoretical curve corresponding to a single dose of the second drug is shown on the figures.

None of the tested compounds was able to modify the viability of the cultured cells.

Results

Characterization of the phosphodiesterase isoforms of thymocyte cytosol. As we already reported (15), fractionation of rat thymic cytosol on a strong anion exchange HPLC column results in the efficient separation of five phosphodiesterase forms (Fig. 1). Characterization of the isoforms from peaks numbered 1 to 4 in the figure has already been described in a previous report (14). The enzyme of peak 1 hydrolyzes cAMP and cGMP, and cAMP hydrolysis is strongly stimulated in the presence of cGMP. This enzyme thus belongs to the cGMP-stimulatable type (type II according to ref. 19). The enzyme forms eluted in peaks 2 and 3 hydrolyze cAMP very specifically and are highly sensitive to the inhibitor rolipram. Both thus belong to the rolipram-sensitive type (type IV). The form of peak 4 proves relatively specific for cAMP hydrolysis. Its activity is inhibited by low concentrations of cGMP, and by the cardiotonic compound milrinone. It thus belongs to the cGMP-inhibited type (type III). An additional peak numbered 1a, eluted at a low salt concentration, contains both cGMP- and cAMP-hydrolyzing activities.

Characterization of the 1a enzyme form was attempted. The corresponding eluate fractions were able to significantly bind cGMP. This binding was stimulated (+31%), in the presence of 2 mM MIX. By comparison, the other cGMP-hydrolyzing form (peak 1, cGMP-stimulated form), was totally unable to bind cGMP under the same conditions (Table 1). Reference phosphodiesterase inhibitory compounds M&B 22,948 and dipyridamole strongly inhibited the cGMP-hydrolyzing activity of peak 1a, but were inactive (M&B 22,948) or 4 times less potent (dipyridamole) on peak 1 activity (Table 1). MIX-stimulatable binding of cGMP, and potent inhibition by dipyridamole and M&B 22,948 are the reported characteristics of a peculiar phosphodiesterase isoform observed in platelets and lung tissue, which selectively hydrolyzes cGMP and is referred to as type V phosphodiesterase (27). The cGMP-hydrolyzing activity of peak 1a may thus be brought together with this type V isoform. However, as the phosphodiesterase 1a from thymocytes hydrolyzes efficiently cAMP (Fig. 1) it might be hypothesized that its cAMP-hydrolyzing activity is due to a co-eluting phosphodiesterase of different nature. Attempts to separate cGMP- and cAMP-hydrolyzing activities by using a gently sloping linear salt gradient at the beginning of the elution procedure (up to the 0.26 M NaCl step in the procedure of Fig. 1) were unsuccessful (not shown). The cAMP-phosphodiesterase activity of peak 1a was seen to be insensitive to inhibition by M&B 22,948 (Table 2) and rolipram (IC_{50}>310 μM). It was also insensitive to calcium chelation or calmodulin addition, and in contrast to peak 1 enzyme, it was not affected by micromolar concentrations of cGMP (not shown). Contamination by a degradative product of the highly proteolysis-sensitive calmodulin-activatable isoform (type I) is very unlikely, as resting normal T-cells seem to be devoid of such an enzyme form (8, 14–18).[2] A peculiar phosphodiesterase type described in pig liver cytosol (28) is eluted at a low salt concentration from anion exchange columns and, although displaying a preferential activity on pyrimidine cyclic nucleotides, it also efficiently hydrolyzes cAMP and cGMP. This so-called cCMP-phosphodiesterase is atypically independent from Mg^{2+} ions for its activity, and insensitive to the aspecific phosphodiesterase inhibitor MIX. In sharp contrast, the thymocyte form 1a displayed no detectable activity in the absence of free Mg^{2+} and was markedly inhibited by MIX (IC_{50} = 12 μM). A similarity with the liver cCMP-phosphodiesterase form may thus be clearly ruled out.

Effect of phosphodiesterase inhibitors on concanavalin A-induced thymocyte proliferation. Non-isoform-selective phosphodiesterase inhibitors such as dipyridamole (which, in addition to its potent effect on cGMP hydrolysis by type V enzyme, non-negligibly affects cAMP hydrolysis by the other forms), papaverine and MIX, were able to strongly affect proliferation as they were capable of totally preventing TdR incorporation into concanavalin A-stimulated cells, at the highest concentrations employed (Fig. 2). However, their potencies greatly varied, as IC_{50} values were of 0.19 μM, 5.4 μM, and 68 μM, respectively, which parallels, for papaverine and MIX, the order of their potencies as inhibitors of the different cAMP-phosphodiesterases (Table 2). Concerning dipyridamole, its potent inhibitory effect on TdR incorporation can hardly be explained solely by the potency of its effects on the various

[2] Authors' observations.

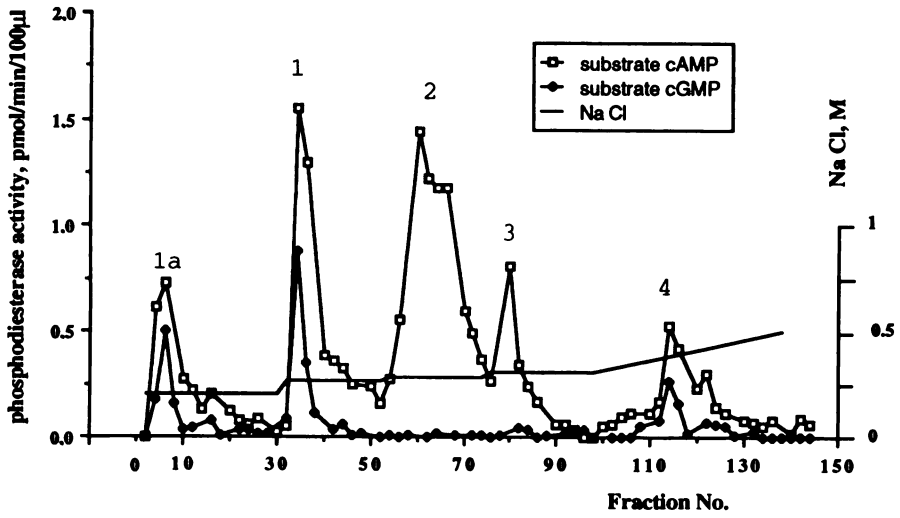

Fig. 1. Anion exchange HPLC fractionation profile of cyclic nucleotide phosphodiesterase from thymocyte cytosol. Phosphodiesterase activity was assayed with 0.25 µM cAMP (□) or cGMP (◆) as substrate. The shape of the NaCl gradient is shown. Fraction volume was 0.9 ml. The activity yields were usually 70–90% for cAMP-phosphodiesterase, and 70–80% for cGMP-phosphodiesterase.

TABLE 1

Comparison of the characteristics of the two phosphodiesterase forms hydrolyzing cGMP in thymocyte cytosol

	HPLC peak	
	1a	1
cAMP-PDE activity[a]	0.431	0.726
cGMP-PDE activity[a]	0.170	0.242
cGMP binding[b] – MIX	0.180	0.008
cGMP binding[b] + MIX	0.234	0.007
IC_{50},[c] cGMP as substrate		
M&B 22,948	5.0	>100
Dipyridamole	0.94	4.0

[a] In picomoles hydrolyzed per min per 100 µl of enzyme from the pooled fractions 3 to 13 (peak 1a) and 32 to 44 (peak 1) in the experiment in Fig. 1. PDE = phosphodiesterase.
[b] In picomoles bound per 100 µl of enzyme, without and with 2 mM MIX added.
[c] In micromolar; substrate concentration was 0.25 µM.

TABLE 2

IC_{50} values, in micromolar, of different inhibitors, on the various phosphodiesterase isoforms fractionated from thymocyte cytosol, assayed with 0.25 µM cAMP as substrate

Compounds tested	HPLC peaks				
	1a	1	2	3	4
MIX	12	11	12	4.7	3.5
Papaverine	11	1.1	2.0	1.5	0.79
Dipyridamole	19	6.3	8.9	14	35.5
M&B 22,948	>100	>100	ND[a]	ND	ND
8-Br-cGMP	NE[b]	+55%[c]	≫100	NE	>100

[a] ND, not determined.
[b] NE, no effect, up to 10^{-4} M.
[c] 55% of activation at 10^{-4} M.

cAMP-hydrolyzing forms (the IC_{50} are roughly similar or higher than those observed with MIX; Table 2). One could have suspected that its well documented ability to inhibit the transport of adenosine would have induced an accumulation in the extracellular medium of the nucleoside, which could have acted on adenylate cyclase activator A2 receptors, thereby promoting a cAMP rise in synergy with phosphodiesterase inhibition. This hypothesis has to be ruled out, as addition in the culture medium of the aspecific adenosine receptor antagonist 8-phenyltheophylline or the adenosine-metabolizing enzyme adenosine deaminase did not modify the IC_{50} value of dipyridamole on TdR incorporation (Fig. 2).

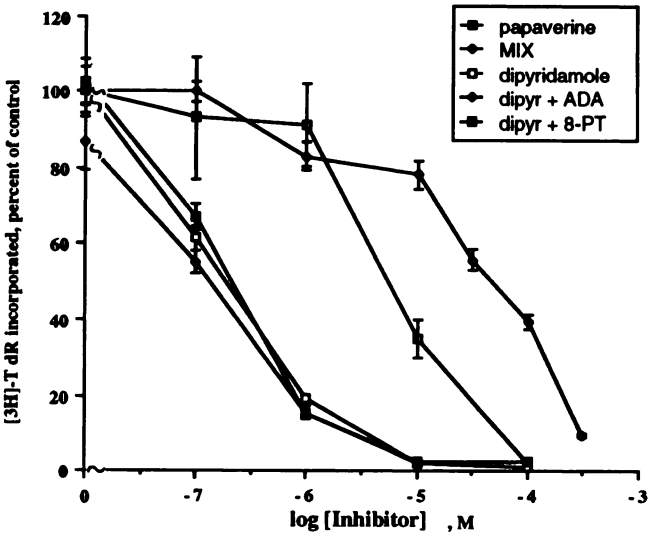

Fig. 2. Inhibition of thymocyte proliferation by non-isoform-specific phosphodiesterase inhibitors. Dipyridamole (*dipyr*) was assayed alone, or in the presence of 10 units/ml adenosine deaminase (*ADA*), or 2 µM 8-phenyltheophylline (*8-PT*). IC_{50} values and their confidence interval limits were in µM: MIX, 68 (59–79); papaverine, 5.4 (3.1–6.9); dipyridamole, 0.19 (0.15–0.23); dipyridamole + ADA, 0.20 (0.17–0.24); dipyridamole + 8-PT, 0.20 (0.17–0.25).

The antiproliferative effects of the selective phosphodiesterase inhibitors rolipram, milrinone, M&B 22,948, which affect type IV, type III, and type V phosphodiesterases, respectively, by contrast were much more restricted (Figs. 3 and 5). Rolipram was the most efficient of them, with an IC_{50} value of 37.0 ± 6.0 µM (standard error, $n = 9$ separate experiments). Its dose-effect curve tended to plateau near 50% inhibition, and at the highest dose (10^{-4} M), its effect did not exceed 60% inhibition. Milrinone and M&B 22,948 proved markedly less efficient than rolipram. Both showed no significant effect up to a 10^{-6} M concentration. At 10^{-5} M, inhibition of proliferation was minimal: 11.3 ± 3.6% (standard error, $n = 7$ separate experiments) for milrinone, and 12.0 ± 4.9% (standard error, $n = 10$ separate experiments) for M&B 22,948. At the highest dose tested (10^{-4} M), inhibition attained 38.0 ± 3.6% (standard error, $n = 6$ separate experiments) with milrinone, and 29.7 ± 7.4% (standard error, $n = 9$ separate experiments) with M&B 22,948.

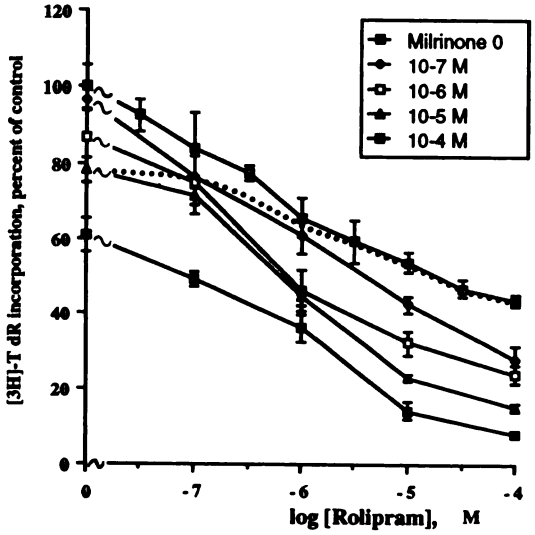

Fig. 3. Inhibition of thymocyte proliferation by the association of rolipram and milrinone. The theoretical additive dose-response curve for 10^{-5} M milrinone (. . . .) was calculated as described in Ref. 26. IC_{50} values and their confidence interval limits were, in μM: 0 milrinone, 21.9 (10.4–46.0); 10^{-7} M milrinone, 5.2 (2.9–9.2); 10^{-6} M milrinone, 2.1 (1.1–4.0); 10^{-5} M milrinone, 2.0 (1.5–2.6); 10^{-4} M milrinone, 1.4 (0.86–2.2).

On the whole, it seems likely that the selective inhibition of one particular isotype of phosphodiesterase does not provide a sufficient rise of cAMP level to totally prevent the progress of mitogenesis.

Effect of the association of type III and IV phosphodiesterase inhibitors on thymocyte proliferation and cAMP level. The association of the type III and IV phosphodiesterase inhibitors milrinone and rolipram induced clearly overadditive antiproliferative effects, as evidenced by the comparison of the experimental curve with the theoretical additive concentration-response curve constructed for a 10^{-5} M milrinone concentration in Fig. 3. The synergism between the two drugs is exemplified by the significant displacement of the IC_{50} value of rolipram from 22 μM to 2.1 μM, in the presence of 10^{-6} M milrinone, a concentration at which the latter drug alone is devoid of significant effect. A 10^{-5} M concentration of both drugs strongly inhibited proliferation (by 80%), and the inhibition was almost 100% at 10^{-4} M.

cAMP levels attained in the presence of phosphodiesterase inhibitors were determined in thymocytes stimulated or not by the mitogen concanavalin A (Fig. 4). In stimulated as well as unstimulated cells, 10^{-5} M rolipram induced a significant but moderate (+46–50%) rise in cAMP, whereas 10^{-5} M milrinone failed to modify cAMP level. Together, the two inhibitors induced a markedly enhanced cAMP rise, reaching +90–130% more than control. These data thus closely parallel the antiproliferative effects reported in the preceeding paragraph, and establish the existence of a synergistic effect of the two drugs on cAMP accumulation.

Effects of the association of type IV and V phosphodiesterase inhibitors on thymocyte proliferation and cAMP level. Associating type IV (rolipram) and type V (M&B 22,948) phosphodiesterase inhibitors induced synergistic effects on thymocyte proliferation in a manner very similar to that observed with rolipram and milrinone (Fig. 5). Thus, at 10^{-5} M M&B 22,948, a concentration inducing only minor effects on

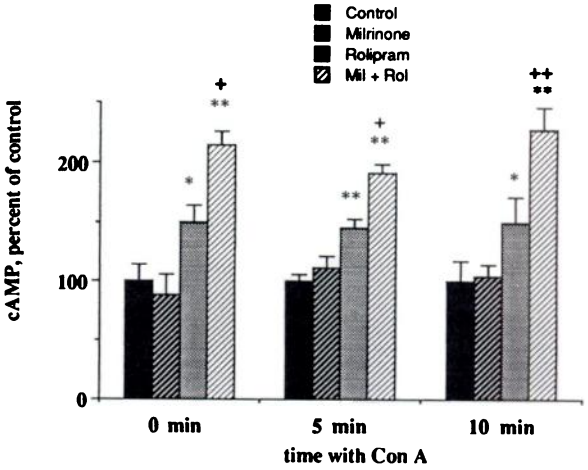

Fig. 4. cAMP content of thymocytes in the presence of 10^{-5} M milrinone, 10^{-5} M rolipram, or 10^{-5} M of both inhibitors (*Mil + Rol*), measured in unstimulated cells, or 5 and 10 min after stimulation by concanavalin A (*Con A*). Controls (without inhibitor added) were assayed in triplicate; the other determinations were done in duplicate. The mean control values at 0, 5, and 10 min stimulation were: 0.73, 0.74, 0.67 pmol of cAMP/10^6 cells, respectively. For each time, data were submitted to ANOVA and Scheffé F-test. *, significantly different from control, $p < 5\%$; **, significantly different from control, $p < 1\%$; +, significantly different from rolipram alone, $p < 5\%$; ++, significantly different from rolipram alone, $p < 1\%$. Two independent experiments were performed and gave similar results.

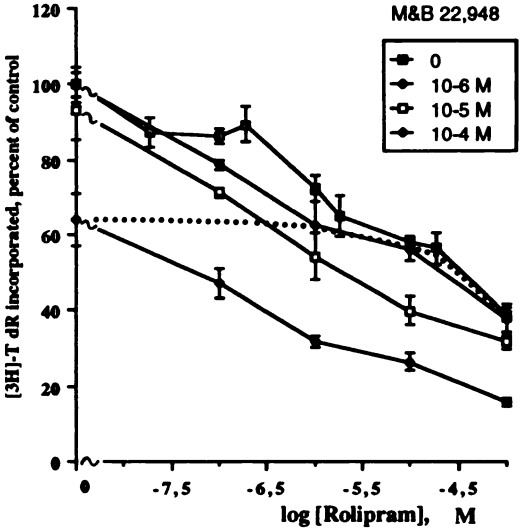

Fig. 5. Inhibition of thymocyte proliferation by the association of rolipram and M&B 22,948. The theoretical additive dose-response curve for 10^{-4} M M&B22,948 (. . . .) was calculated as described in Ref. 26. IC_{50} values and their confidence interval limits were, in μM: 0 M&B 22,948, 28.9 (14.5–57.3); 10^{-6} M M&B 22,948, 15.4 (5.4–43.6); 10^{-5} M M&B 22,948, 3.5 (1.5–8.4); 10^{-4} M M&B 22,948, 2.1 (1.2–3.8).

proliferation, the IC_{50} of rolipram was significantly shifted from 29 to 3.5 μM.

Concerning cAMP levels, synergism was also evident between the two drugs, as 3×10^{-5} M M&B 22,948, which had no effect by itself, significantly augmented the effect of 10^{-5} M rolipram, the association inducing a 94% increase in cAMP level (Fig. 6).

These synergistic effects between rolipram and M&B 22,948 were unexpected, as M&B 22,948 was shown to affect only cGMP hydrolysis by peak 1a enzyme (Tables 1 and 2), and as such, was not supposed to induce a rise in cAMP. An interpre-

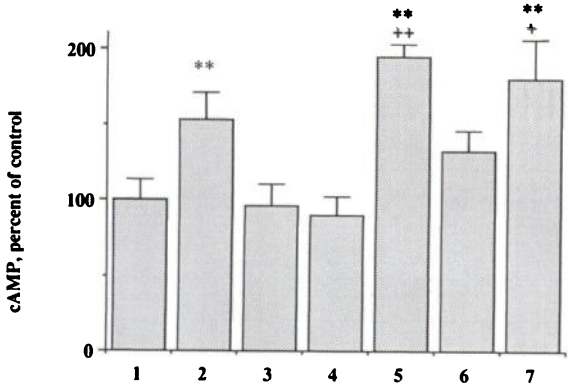

Fig. 6. cAMP content of thymocytes in the presence of various agents, 10 min after stimulation by concanavalin A. All the tested compounds were present at a 10^{-5} M concentration, except M&B 22,948 (3×10^{-5} M). Control (without added agent): $n = 7$, other conditions: $n = 4$. The mean control value was 0.67 pmol of cAMP/10^6 cells. Data were analyzed by ANOVA and Scheffé F-test: **, significantly different from control, $p < 1\%$; or *t*-test: +, significantly different from rolipram alone, $p = 2\%$; ++, significantly different from rolipram alone, $p < 1\%$. *1*, control cells; *2*, +Rolipram; *3*, +M&B 22,948; *4*, +sodium nitroprusside; *5*, +Rolipram + M&B 22,948; *6*, +Milrinone + M&B 22,948; *7*, +Rolipram + sodium nitroprusside.

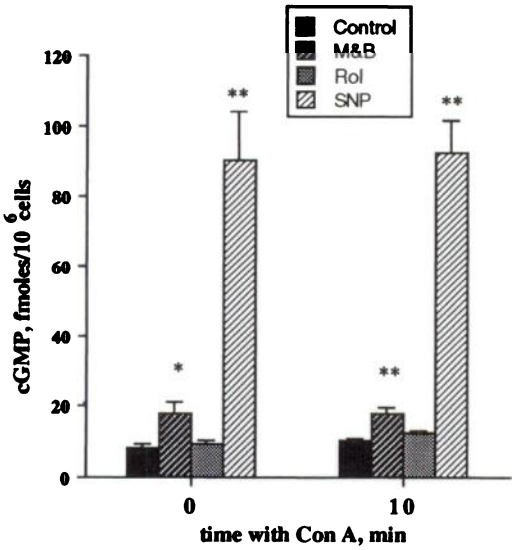

Fig. 7. cGMP content of thymocytes in the presence of 3×10^{-5} M M&B 22,948 (*M&B*), 10^{-5} M rolipram (*Rol*), or 10^{-5} M sodium nitroprusside (*SNP*), without stimulation, or 10 min after concanavalin A (*Con A*) stimulation. Measurements were made in triplicate. Data were analyzed by the Kruskal-Wallis H-test. *, significantly different from control (without addition), $p < 1\%$; **, $p < 1‰$.

tation of these results is that M&B 22,948 induces a rise in intracellular cGMP level, which would in turn alter the cAMP-hydrolyzing activity of the type III phosphodiesterase present in the cells, this isoform presenting the property of being potently inhibited by cGMP (14). One would thus, in the presence of type V and type IV inhibitors, reproduce the situation generated by the simultaneous presence of type III and type IV inhibitors. The ability of M&B 22,948 to significantly raise cGMP level in unstimulated or stimulated thymocytes was verified (Fig. 7). Under the same conditions, rolipram had no significant effect.

Lack of synergism between type III and type V inhibitors. If the above hypothesis holds true, one should expect a lack of synergism between milrinone and M&B 22,948, both compounds acting on type III phosphodiesterase, the former directly, the latter indirectly via the generation of cGMP accumulation. The effects of the association of milrinone and M&B 22,948 on the proliferative response of thymocytes were thus studied (Fig. 8). These experiments clearly denoted an absence of synergism between the two compounds, up to a milrinone concentration of 10^{-5} M, as experimental data fitted well with the theoretical curves of simply additive effects. At 10^{-4} M milrinone, a concentration at which the inhibitor is no longer specific for type III phosphodiesterase, but also affects the type IV forms (not shown), a mild overadditive antiproliferative effect can be noted for M&B 22,948 concentrations higher than 10^{-5} M.

The effect of the association of 10^{-5} M milrinone and 3.10^{-5} M M&B 22,948 on the cAMP level of thymocytes was also evaluated (Fig. 6). This was observed to be limited, as a non-significant 26–33% rise was detected, which reflects well the weak inhibitory effect of the associated compounds at the same concentrations on proliferation (−30%).

Synergism between the type IV phosphodiesterase inhibitor rolipram and a cGMP-elevating agent, sodium nitroprusside. To challenge the hypothesis that M&B 22,948 acts via a cGMP rise, we studied the interaction between rolipram and sodium nitroprusside, a compound known to induce substantial rises of cGMP in a variety of cell types, including lymphocytes, independently of phosphodiesterase inhibition (Fig. 9). Nitroprusside was weakly antiproliferative by itself (−30% at 10^{-4} M). However, it strongly potentiated the antiproliferative effect of rolipram. The IC_{50} value of rolipram was significantly shifted from 45 μM (rolipram alone) to 0.7 μM (in the presence of 10^{-5} M nitroprusside). In the presence of 10^{-4} M nitroprusside, 10^{-5} M rolipram almost completely inhibited proliferation (−94%).

In close parallel with the effects observed on proliferation, 10^{-5} M nitroprusside alone was unable to modify cAMP level in thymocytes, whereas it significantly increased the effect of 10^{-5} M rolipram, the combination of the two drugs inducing a 80% rise in cAMP level (Fig. 6).

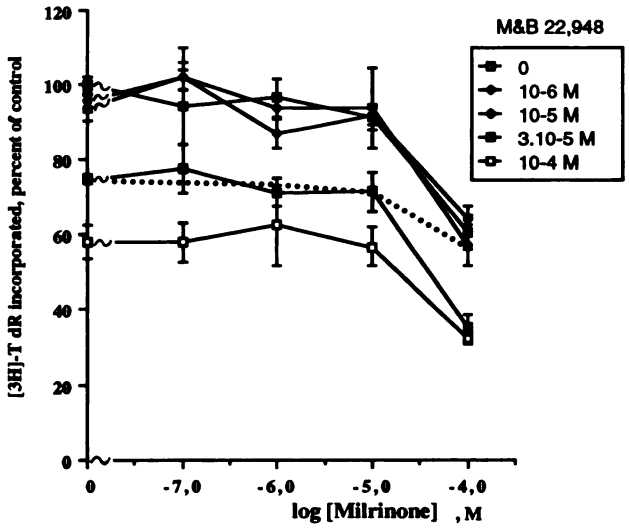

Fig. 8. Inhibition of thymocyte proliferation by the association of milrinone and M&B 22,948. The theoretical additive dose-response curve for 3×10^{-5} M M&B 22,948 (. . . .) was calculated as described in ref. 26.

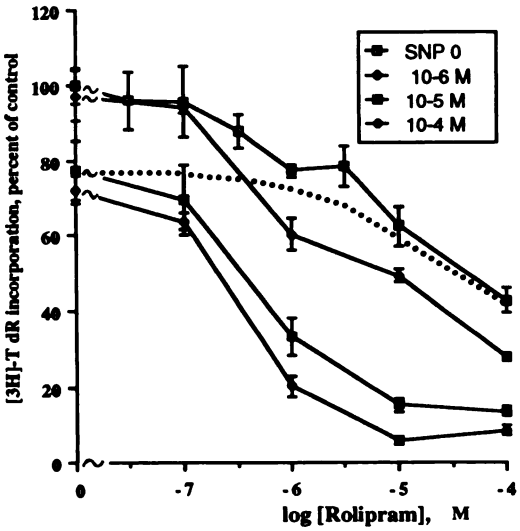

Fig. 9. Inhibition of thymocyte proliferation by the association of rolipram and sodium nitroprusside (*SNP*). The theoretical additive dose-response curve for 10^{-5} M SNP (. . . .) was calculated as described in ref. 26. IC_{50} values and their confidence interval limits were, in μM: 0 SNP, 44.6 (22.3–89.6); 10^{-6} M SNP, 6.5 (4.0–10.8); 10^{-5} M SNP, 0.70 (0.27–1.8); 10^{-4} M SNP, 0.43 (0.35–0.47).

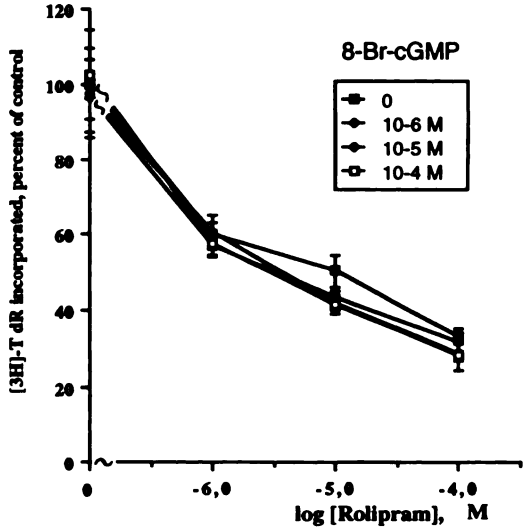

Fig. 10. Lack of effect of 8-Br-cGMP on the inhibition of thymocyte proliferation induced by rolipram. IC_{50} values and their confidence interval limits were, in μM: 0 8-Br-cGMP, 7.1 (3.5–14.4); 10^{-6} M 8-Br-cGMP, 4.8 (2.7–8.3); 10^{-5} M 8-Br-cGMP, 4.5 (2.8–7.3); 10^{-4} M 8-Br-cGMP, 3.2 (1.6–6.4).

Nitroprusside effect on cGMP level was measured in stimulated and unstimulated thymocytes. It induced in both cases a large increase in cGMP (Fig. 7).

Effect of the protein kinase G activator 8-Br-cGMP on the proliferation of lymphocytes. To confirm that endogenously generated cGMP does not exert its antiproliferative action by stimulating the cGMP-dependent protein kinases, but rather by inhibiting type III phosphodiesterase, we studied the effects on proliferation of 8-Br-cGMP, a potent activator of protein kinases G which has no inhibitory effect on thymocyte phosphodiesterase III up to a 10 μM concentration (Table 2). 8-Br-cGMP neither exhibited antiproliferative effects by itself, nor did it potentiate the effects of rolipram, as no significant IC_{50} shift could be detected in its presence (Fig. 10).

Discussion

As it has been widely reported previously for differently stimulated T-cells of various origins (8), non-isoform-selective phosphodiesterase inhibitors such as dipyridamole, papaverine, MIX are able to thoroughly inhibit the proliferative response of rat thymic lymphocytes stimulated by concanavalin A. This property is most likely related to the intracellular accumulation of cAMP induced by suppressing the degradative activity of the various phosphodiesterase isoforms present in the cells. This is true at least for papaverine and MIX, as their effects on proliferation parallel their potencies as inhibitors of the various isoforms. Concerning dipyridamole, its moderate effects on cAMP hydrolysis can hardly account alone for its potent inhibitory action on [^{3}H]TdR incorporation. However, it has been reported that it can synergize with the cyclase stimulator dimethyl prostaglandin E_2 (29), which establishes that phosphodiesterase inhibition participates in its antiproliferative effect. An effect of dipyridamole via the accumulation of adenosine in the extracellular medium may be excluded on the basis of our results. Additional effects might be involved, as it has already been shown to inhibit the entry of [^{3}H]TdR into cells (30).

By contrast, the isoform-selective phosphodiesterase inhibitors exhibit relatively moderate inhibitory effects on the proliferative response of thymocytes. The type IV phosphodiesterase inhibitor rolipram was seen to be the most active, its maximal inhibitory effect approximating 60%, whereas the type III phosphodiesterase inhibitor milrinone had little or no effect up to a 10^{-5} M concentration. This result demonstrates a predominant role for the type IV isoenzymes over the type III isoenzyme in the control of cAMP level in thymocytes. This is evidenced by the ability of rolipram, but not milrinone, to induce a significant rise in intracellular cAMP in the absence of adenylate cyclase stimulation. Associating two selective inhibitors, rolipram and milrinone, each of them having limited effects, allowed much more marked inhibition of proliferation and cAMP rise, the two compounds obviously acting in synergy.

This synergy may likely be explained by the necessity of almost complete suppression of the degradation of cAMP to induce an efficiently antiproliferative cAMP accumulation without stimulating cAMP synthesis. The basal activity of adenylate cyclase may indeed be low enough to require the simultaneous inhibition of type III and IV phosphodiesterases to lower the cAMP hydrolysis rate markedly below the synthesis rate. A potentiation of the antiproliferative effects of type III and IV phosphodiesterase inhibitors CI-930 and Ro 20–1724 has been suggested by a report of Robicseck *et al.* (17) studying human peripheral T-cell proliferation.

It appears that the drug effects we observed on cAMP levels and lymphoproliferative response are closely linked, which fits well the widely accepted concept of cAMP being a negative effector of lymphocyte mitogenic response. However, it is noticeable that the variations in cAMP level capable of inducing antiproliferative effects are of very limited extent; thus, a 50% rise in cell cAMP has significant negative consequences on proliferation (approximately −50%), and a rise of about 100% abrogates the major part of the response. This supports the idea that protein kinases A which mediate the action of cAMP in T-cells (9) are activated at relatively low cAMP concentrations, and that physiologically active cAMP levels are thus

comprised in a narrow range, due to the amplifying effect of the kinase A-catalyzed phosphorylation step (31). Confirmation of this point will require the measurement of cAMP-dependent protein kinase activity ratios.

Another conclusion that ensues from the ability of type III and IV phosphodiesterase inhibitors to have synergistic positive effects on cAMP level is that the two enzymes share the same cAMP pool in the intact cell, and that no compartmentalization of cAMP is detected, in contrast to conclusions reported for heart tissue (32).

We report in this paper the presence in thymic lymphocytes of a phosphodiesterase isoform which shares the characteristics of a type V enzyme, especially a strong sensitivity of cGMP hydrolysis to inhibition by M&B 22,948. Somewhat surprising was our observation that M&B 22,948 could exhibit synergism with rolipram in inhibiting lymphoproliferation as well as in raising cAMP levels. Indeed, this compound is not expected to directly provoke a rise in cAMP, but rather of cGMP, which we verified experimentally. The hypothesis that the M&B 22,948-induced cGMP rise can in turn inhibit type III phosphodiesterase and thus mimic the conditions where a synergism with rolipram can take place provides a likely interpretation of these results. Several experimental data support this hypothesis:

1. A compound such as sodium nitroprusside which induces large rises of cGMP in thymocytes, through a mechanism different from phosphodiesterase inhibition, i.e., guanylate cyclase stimulation by generation of intracellular nitric oxide, is able to reproduce the effects of M&B 22,948 on proliferative response as well as on cAMP levels.
2. The lack of synergism between M&B 22,948 and milrinone suggests that both compounds finally act (through different mechanisms) on the same target, type III phosphodiesterase, and thus can not have, in association, greater effects than each compound alone.
3. A cGMP analog, 8-Br-cGMP, which has no phosphodiesterase-inhibiting properties at the concentrations used, did not reproduce the synergistic actions of cGMP-elevating agents, such as M&B 22,948 or nitroprusside, associated to rolipram. This strongly suggests that endogenously produced cGMP is not acting on proliferation through kinase G activation, but rather by means of type III phosphodiesterase inhibition.

The work reported here allows us to delineate the role played by three types of phosphodiesterases in the control of cAMP level in thymic lymphocytes; type III, IV, and V enzymes all participate in certain circumstances in the determination of cAMP level, and consequently, influence the efficiency of the proliferative response. The existence of a type V phosphodiesterase in lymphocytes and its involvement in the control of cell response has not been reported before.

One aspect which is of major interest to us is the recognition of a role for cGMP in the control of lymphoproliferation. There has long been controversy concerning the implication of cGMP in the proliferative response of lymphocytes. Our observations allow us to propose that endogenously formed cGMP, although devoid of effect on proliferation by itself, would be able, in the presence of a cAMP-elevating agent, to potentiate the accumulation of cAMP by means of inhibition of the type III phosphodiesterase present in lymphocytes. cGMP would thus potentiate the negative influence of cAMP on the response, and as such, appears to be an antiproliferative signal.

This finding is potentially important in the physiological context of the inhibitory effect exerted by macrophages on lymphocytes in various species. Some authors (33, 34) have shown that this suppressive effect of macrophages stimulated by cytokines is related to their ability to generate nitric oxide by the oxidative pathway of arginine. As nitric oxide is able to diffuse across cellular membranes, it is likely that it elevates cGMP levels in lymphocytes. It may thereby potentiate the negative influence of cAMP-elevating stimuli, such as prostaglandins. Other mechanisms have been evoked for the cytostatic effect of nitric oxide on various cell types (e.g., tumoral cells): iron loss, inhibition of mitochondrial respiration, inhibition of the citric acid enzyme aconitase, and inhibition of DNA synthesis. The present results suggest that the alteration of cAMP level in lymphocytes, via the modulation of type III phosphodiesterase activity by cGMP, might also constitute a physiological device contributing to the control of lymphocyte proliferation by macrophages.

There thus appears to be considerable pharmacological potential for modulating lymphocyte response through the selective inhibition of one or several phosphodiesterase isoforms, and through the alteration of nitric oxide generation by use of inhibitors of arginine oxidative pathway or compounds able to liberate nitric oxide.

References

1. Kammer, G. M. The adenylate cyclase-cAMP protein kinase A pathway and regulation of the immune response. *Immunol. Today* **9**:222–229 (1988).
2. Kaibuchi, K., Y. Takai, Y. Ogawa, S. Kimura, and Y. Nishizuka. Inhibitory action of adenosine 3′, 5′-monophosphate on phosphatidylinositol turnover: difference in tissue response. *Biochem. Biophys. Res. Commun.* **104**:105–112 (1982).
3. Patel, M. D., L. E. Samelson, and R. D. Klausner. Multiple kinases and signal transduction. Phosphorylation of the T-cell antigen receptor complex. *J. Biol. Chem.* **262**:5831–5838 (1987).
4. Farrar, W. L., S. W. Evans, U. R. Rapp, and J. L. Cleveland. Effects of antiproliferative cyclic AMP on interleukin 2-stimulated gene expression. *J. Immunol.* **139**:2075–2080 (1987).
5. Johnson, K. W., B. H. Davis, and K. A. Smith. cAMP antagonizes interleukin-2-promoted T-cell cycle progression at a discrete point in early G1. *Proc. Natl. Acad. Sci. U. S. A.* **85**:6072–6076 (1988).
6. Van Tits, L. J. H., M. C. Michel, H. J. Motulsky, A. S. Maisel, and O. E. Brodde. Cyclic AMP counteracts mitogen-induced inositol phosphate generation and increases in intracellular Ca^{2+} concentration in human lymphocytes. *Br. J. Pharmacol.* **103**:1288–1294 (1991).
7. Dornand, J., J. C. Bonnafous, and J. C. Mani. 5′-nucleotidase-adenylate cyclase relationships in mouse thymocytes. A re-evaluation of the effects of concanavalin A on cyclic AMP levels. *FEBS Lett.* **110**:30–34 (1980).
8. Epstein, P. M., and R. Hachisu. Cyclic nucleotide phosphodiesterase in normal and leukemic human lymphocytes and lymphoblasts. *Adv. Cyclic Nucleotide Protein Phosphorylation Res.* **16**:303–324 (1984).
9. Skalhegg, B. S., B. F. Landmark, S. O. Døskeland, V. Hansson, T. Lea, and T. Jahnsen. Cyclic AMP-dependent protein kinase type I mediates the inhibitory effects of 3′,5′-cyclic adenosine monophosphate on cell replication in human T lymphocytes. *J. Biol. Chem.* **267**:15707–15714 (1992).
10. Park, D. J., H. K. Min, and S. G. Rhee. Inhibition of CD3-linked phospholipase C by phorbol ester and by cAMP is associated with decreased phosphotyrosine and increased phosphoserine contents of PLC-γ 1. *J. Biol. Chem.* **267**:1496–1501 (1992).
11. Hadden, J. W., and R. G. Coffey. Early biochemical events in T-lymphocyte activation by mitogens. *Immunopharmacol. Rev.* **1**:273–376 (1990).
12. Atkinson, J. P., J. P. Kelly, A. Weiss, H. J. Wedner, and C. W. Parker. Enhanced intracellular cGMP concentrations and lectin-induced lymphocyte transformation. *J. Immunol.* **121**:2282–2291 (1978).
13. Kaever, V., and K. Resch. Role of cyclic nucleotides in lymphocyte activation. *Curr. Top. Membr. Transp.* **35**:375–398 (1990).
14. Valette, L., A. F. Prigent, G. Némoz, G. Anker, O. Macovschi, and M. Lagarde. Concanavalin A stimulates the rolipram-sensitive isoforms of cyclic nucleotide phosphodiesterase in rat thymic lymphocytes. *Biochem. Biophys. Res. Commun.* **169**:864–872 (1990).
15. Marcoz, P., G. Némoz, A. F. Prigent, and M. Lagarde. Phosphatidic acid stimulates the rolipram-sensitive cyclic nucleotide phosphodiesterase from rat thymocytes. *Biochim. Biophys. Acta.* **1176**:129–136 (1993).
16. Robicsek, S. A., J. J. Krzanowski, A. Szentivanyi, and J. B. Polson. High pressure liquid chromatography of cyclic nucleotide phosphodiesterase from

purified human T-lymphocytes. *Biochem. Biophys. Res. Commun.* **163**:554–560 (1989).
17. Robicsek, S. A., D. K. Blanchard, J. Y. Djeu, J. J. Krzanowski, A. Szentivanyi, and J. B. Polson. Multiple high affinity cAMP-phosphodiesterases in human lymphocytes. *Biochem. Pharmacol.* **42**:869–877 (1991).
18. Hurwitz, R. L., K. M. Hirsch, D. J. Clark, V. N. Holcombe, and M. Y. Hurwitz. Induction of a calcium/calmodulin-dependent phosphodiesterase during phytohemagglutinin-stimulated lymphocyte mitogenesis. *J. Biol. Chem.* **265**:8901–8907 (1990).
19. Beavo, J. A., and D. H. Reifsnejder. Primary sequence of cyclic nucleotide phosphodiesterase isozymes and the design of selective inhibitors. *Trends Pharmacol. Sci.* **11**:150–155 (1990).
20. Degerman, E., C. J. Smith, H. Tornqvist, V. Vasta, P. Belfrage, and V. Manganiello. Evidence that insulin and isoprenaline activate the cGMP-inhibited low Km cAMP phosphodiesterase in rat fat cells by phosphorylation. *Proc. Natl. Acad. Sci. USA* **87**:533–537 (1990).
21. Simmons, M. A., and H. C. Hartzell. Role of phosphodiesterase in regulation of calcium current in isolated cardiac myocytes. *Mol. Pharmacol.* **33**:664–671 (1988).
22. MacFarland, R. T., B. D. Zelus, and J. A. Beavo. High concentrations of a cGMP-stimulated phosphodiesterase mediate ANP-induced decreases in cAMP and steroidogenesis in adrenal glomerulosa cells. *J. Biol. Chem.* **266**:136–142 (1991).
23. Maurice, D. H., and R. J. Haslam. Molecular basis of the synergistic inhibition of platelet function by nitrovasodilators and activators of adenylate cyclase: inhibition of cyclic AMP breakdown by cyclic GMP. *Mol. Pharmacol.* **37**:671–681 (1990).
24. Hamet, P., and J. Tremblay. Platelet cGMP-binding phosphodiesterase. *Methods Enzymol.* **159**:710–722 (1988).
25. Brownlee, K. A. *Statistical Theory and Methodology in Sciences and Engineering.* John Wiley and Sons, New York, 1–590 (1967).
26. Pöch, G., and S. Holzmann. Quantitative estimation of overadditive and underadditive drug effects by means of theoretical, additive dose-response curves. *J. Pharmacol. Methods.* **4**:179–188 (1980).
27. Francis, S. H., M. K. Thomas, and J. D. Corbin. Cyclic GMP-binding cyclic GMP-specific phosphodiesterase from lung, in *Cyclic Nucleotide Phosphodiesterases: Structure, Regulation and Drug Action* (J. A. Beavo and M. D. Houslay, eds.) John Wiley, Chichester, United Kingdom, 117–135 (1990).
28. Helfman, D. M., N. Katoh, and J. F. Kuo. Purification and properties of cyclic CMP phosphodiesterase. *Adv. Cyclic Nucleotide Res.* **16**:403–416 (1984).
29. Ellis, N. K., G. P. Duffie, M. R. Young, and H. T. Wepsic. The effects of 16, 16-dimethyl PGE_2 and phosphodiesterase inhibitors on Con A blastogenic response and NK cytotoxic activity of mouse spleen cells. *J. Leukocyte Biol.* **47**:371–377 (1990).
30. Farmer, J. L., and M. D. Prager. Inhibition of lymphoproliferation by dipyridamole. *Biochem. Pharmacol.* **31**:1381–1386 (1982).
31. Nimmo, H. G. and P. Cohen. Hormonal control of protein posphorylation. *Adv. Cyclic Nucleotide Res.* **8**:145–266 (1977).
32. Weishaar, R. E., D. C. Kobilarz-Singer, R. P. Steffen, and H. R. Kaplan. Subclasses of cyclic AMP-specific phosphodiesterase in left ventricular muscle and their involvement in regulating myocardial contractility. *Circ. Res.* **61**:539–547 (1987).
33. Hoffman, R. A., J. M. Langrehr, T. R. Billiar, R. D. Curran, and R. L. Simmons. Alloantigen-induced activation of rat splenocytes is regulated by the oxidative metabolism of L-arginine. *J. Immunol.* **145**:2220–2226 (1990).
34. Mills, C. D. Molecular basis of "supressor" macrophages. Arginine metabolism via the nitric oxide synthetase pathway. *J. Immunol.* **146**:2719–2723 (1991).

Send reprint requests to: Dr. Georges Némoz, Chimie Biolgique (406), INSA, 69621 Villeurbanne Cedex, France.