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SELECTIVE INHIBITION BY NPT 15392 OF LYMPHOCYTE CYCLIC GMP PHOSPHODIESTERASE

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Abstract—Cyclic nucleotide phosphodiesterases were measured in mouse spleen and thymus lymphocyte membranes and soluble fractions and in extracts of canine tracheal smooth muscle. The immunostimulant erythro-9(2-hydroxy,3-nonyl) hypoxanthine (NPT 15392) was found to be a potent and relatively selective inhibitor of mouse lymphocyte cyclic GMP phosphodiesterase, with IC_{50} values 15–180 times greater for cyclic AMP than cyclic GMP phosphodiesterases. The greatest inhibition by NPT 15392 was found using 10 μM substrate, and inhibition was greater in membrane than soluble forms of phosphodiesterase. Spleen soluble enzymes were separated by DEAE Bio-Gel A column into six peaks. A major form of cyclic GMP phosphodiesterase was inhibited effectively by NPT 15392 in a competitive manner (K_i = 50 μM). Cyclic AMP phosphodiesterase activity in the same fraction, but representing only a fifth of the total activity, was also inhibited (K_i = 70 μM). Other soluble enzymes were not affected significantly. Membrane bound enzymes were solubilized and separated into three peaks. One with high affinity for cyclic GMP was strongly inhibited (K_i = 10 μM) by NPT 15392. Inosine and isoprinosine were one-tenth to one-hundredth as effective as NPT 15392 as cyclic nucleotide phosphodiesterase inhibitors. Incubation of mouse splenic lymphocytes with NPT 15392 for 48 hr resulted in enzymes with altered responsiveness to the drug in broken cell assays: inhibition of cyclic GMP hydrolysis was enhanced while that of cyclic AMP hydrolysis was decreased. Among three separated and characterized forms of tracheal smooth muscle phosphodiesterase, NPT 15392 inhibited the low K_m cyclic GMP phosphodiesterase 6–10 times more effectively than the other enzymes. These data suggest that the immunopharmacologic activities of NPT 15392 may include specific cyclic GMP phosphodiesterase inhibition as one of several possible mechanisms.

Erythro-9(2-hydroxy,3-nonyl) hypoxanthine (NPT 15392) is a potent immunostimulant in animals and man [1–4]. It induces prothymocyte differentiation and augments helper, suppressor, and proliferative functions of mouse T lymphocytes *in vitro*. While mechanisms of immunopotentiation are incompletely understood, many immunopotentiators increase the ratio of cyclic GMP to cyclic AMP [4, 5]. We found no effects of NPT 15392 to modify lymphocyte guanylate or adenylate cyclase activity. However, this compound was found to be a potent and relatively selective inhibitor of lymphocyte cyclic GMP phosphodiesterase.

Several authors [6–8] have described multiple forms of cyclic AMP and cyclic GMP phosphodiesterases in the soluble fraction of mouse splenic and thymus lymphocytes, and our data support these findings. Our experiments with both membrane and soluble mouse spleen and thymus enzymes and phosphodiesterases isolated from canine tracheal smooth muscle and the effects of NPT 15392, inosine, and isoprinosine on these activities are described in this paper.

MATERIALS AND METHODS

Male mice (BALB C/BYJ, C57BL/6J, and BDFI) were obtained from Jackson Laboratories. At age 8–12 weeks, they were killed by cervical dislocation. Spleen and thymus tissues were minced in cold RPMI 1640 medium, passed through fine steel mesh followed by aspiration through a 26G syringe needle. After allowing clumps of cells to settle, the cell suspensions were centrifuged at 1000 rpm for 10 min, and the cells were resuspended in homogenizing buffer: 50 mM 4-(2-hydroxyethyl)-1-piperazine-ethanesulfonic acid (Hepes), pH 7.6, 1 mM $MgCl_2$ and 1 mM dithiothreitol. Cells were broken by 100 strokes with a hand-operated tight-fitting Dounce homogenizer and centrifuged at 48,000 *g* for 1 hr. Membranes were resuspended in homogenizing buffer.

Phosphodiesterase assays were conducted at 37° for 10 min in 0.1 ml of the above buffer with 5–25 μg of membrane or soluble protein, 10 mM $MgCl_2$ and 0.1 to 100 μM [^{3}H]cyclic GMP or [^{3}H]cyclic AMP (New England Nuclear), using the two-step procedure of Thompson *et al.* [9]. Substrates were purified by AG1 (Bio Rad) formate columns [10]. Results were expressed as pmoles of cyclic nucleotide hydrolyzed per min per mg protein. Rates were apparently linear with respect to time and protein content, provided the amount of substrate converted was in the range of 1–20%.

NPT 15392 was provided by Dr. Alfredo Giner-Sorolla, University of South Florida College of Medicine. It was dissolved in a small volume of 1 N NaOH and diluted with buffer before use. Other inhibitors were dissolved in buffer. Isoprinosine (a complex of *p*-acetamidobenzoic acid, *N*,*N*-dimethylamino-2-

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Table 1. Specific activities of lymphocyte phosphodiesterases*

Mouse strain	Cyclic GMP (pmoles/min/mg protein)				Cyclic AMP (pmoles/min/mg protein)			
	Spleen		Thymus		Spleen		Thymus	
	Sol	Memb	Sol	Memb	Sol	Memb	Sol	Memb
BALB C/BYJ	10,200	2,940	1,860	610	3,210	530	1,610	200
C57BL/6J	1,530	920	350	260	910	370	320	150
BDF1	2,010	420	ND	ND	1,410	590	ND	ND

* Data were obtained after a 10-min incubation with 10 μM substrate. Mean values of seven experiments (C57BL/6J) or two experiments (BALB C/BYJ, DBF1 strains of mice) are shown. Variations between experiments ranged from 20 to 70%. Abbreviations: Sol, soluble; Memb, membrane-derived enzyme; and ND, not done.

propanol and inosine, 3:3:1 molar ratio) was provided by Newport Pharmaceuticals. Inhibitors were added to enzyme preparations 5–10 min before substrate.

RESULTS

Experiments were performed with lymphocytes from spleens and thymus of three strains of mice which had different levels of phosphodiesterase activity (Table 1). Specific activities were greater in spleen than thymus in agreement with Winchurch *et al.* [6]. Membrane enzyme activities were appreciable in all of these cells, ranging from 0.12 to 0.74 of the cytosol activities. Specific activities for hydrolysis of cyclic GMP were greater than for cyclic AMP in all cases except spleen membrane of BDF1 mice. No stimulation of any of these activities was observed with calcium and calmodulin (provided by Dr. Charles Brostrom), and no inhibition was obtained with trifluoperazine, an inhibitor of calmodulin effects.

Mouse lymphoid tissues contain two or more phosphodiesterases for each nucleotide, with different affinities for substrate [6–8]. Our experiments were performed with crude, unseparated mixtures of these enzymes which showed anomalous kinetics. We found the most potent inhibition of mouse lymphocyte cyclic GMP phosphodiesterases by NPT 15392 to occur at the 10 μM substrate level (Figs. 1–4). This suggests that the drug acts selectively on an enzyme of intermediate affinity. The relatively weak effect of NPT 15392 on cyclic AMP phosphodiesterase in membrane and soluble preparations from mouse spleen, compared with the effect on cyclic GMP phosphodiesterase, is illustrated in Fig. 5. Data for both spleen and thymus are summarized in Table 2. The concentration of NPT 15392 causing 50% inhibition (IC_{50}) of cyclic GMP (10 μM) breakdown ranged from 6 to 19 μM, compared to 110–2100 μM for cyclic AMP hydrolysis. NPT 15392 was a more effective inhibitor of membrane than soluble phosphodiesterases for both cyclic AMP and cyclic GMP. By way of comparison with a commonly used phosphodiesterase inhibitor, 3-isobutyl-l-methylxanthine (IBMX) inhibited all phosphodiesterase enzymes with IC_{50} values of 2–5 μM and showed no apparent selectivity for cyclic GMP or cyclic AMP hydrolysis (data not shown).

To evaluate the nature of inhibition by NPT 15392, spleen phosphodiesterases were separated on a DEAE Bio-Gel A column. We observed six peaks

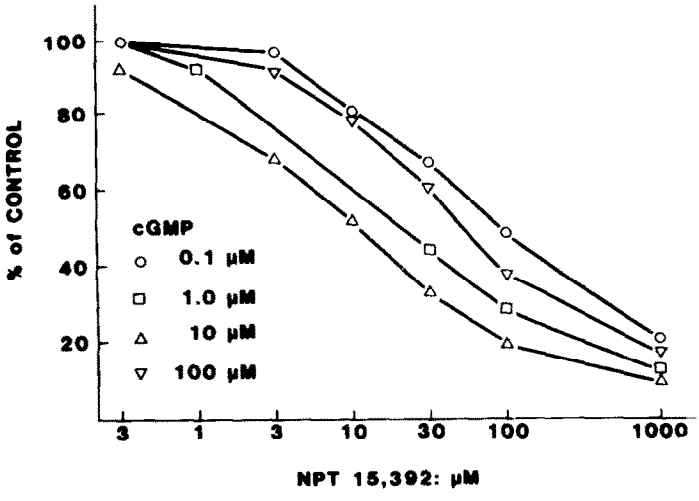

Fig. 1. Inhibition of mouse spleen membrane cyclic GMP phosphodiesterase by NPT 15392. Each point represents the mean of six experiments with C57BL/6J mice. Differences between inhibition at 10 μM cyclic GMP and other concentrations of substrate are significant from 3 to 100 μM NPT 15392. Error bars were eliminated in this and the following figures in the interest of clarity.

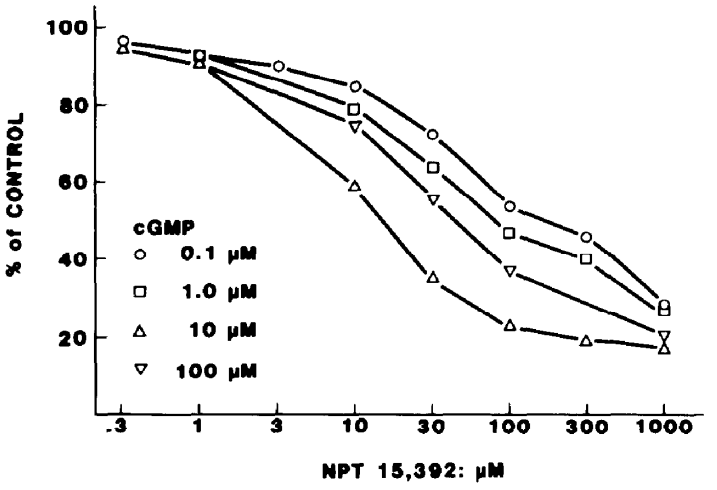

Fig. 2. Inhibition of mouse spleen soluble cyclic GMP phosphodiesterase by NPT 15392. Each point represents the mean of six experiments with C57BL/6J mice. Differences between inhibition at 10 μM cyclic GMP and other concentrations of substrate are significant from 10 to 300 μM NPT 15392.

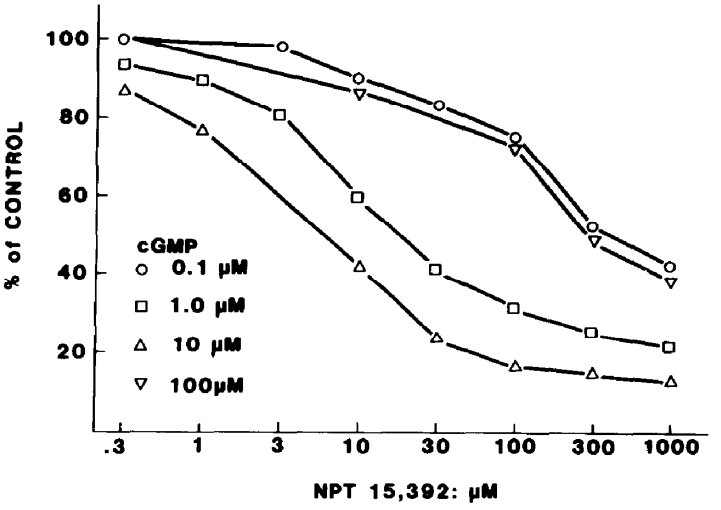

Fig. 3. Inhibition of mouse thymocyte membrane cyclic GMP phosphodiesterase by NPT 15392. Each point represents the mean of three experiments with C57BL/6J mice. Differences between inhibition at 10 μM cyclic GMP and other concentrations of substrate are significant from 1 to 1000 μM 15392.

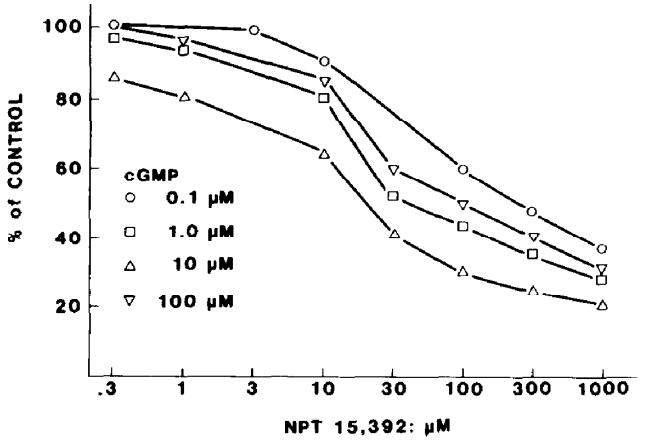

Fig. 4. Inhibition of mouse thymocyte soluble cyclic GMP phosphodiesterase by NPT 15392. Each point represents the mean of three experiments with C57BL/6J mice. Differences between inhibition at 10 μM cyclic GMP and other concentrations of substrate are significant from 1 to 100 μM NPT 15392.

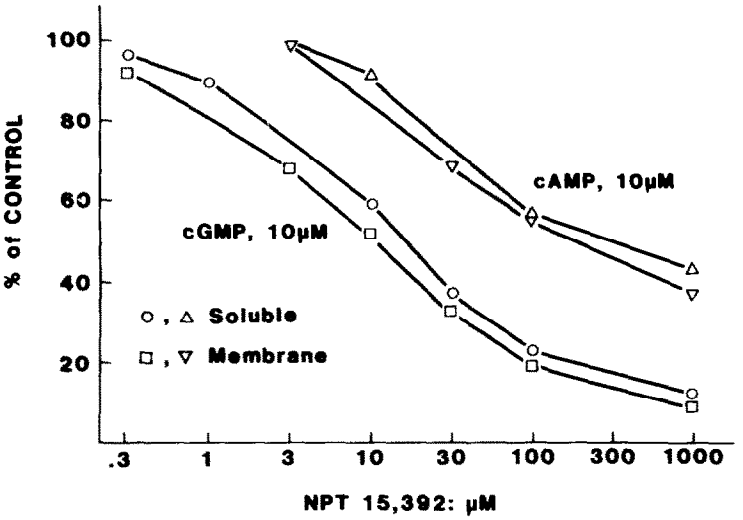

Fig. 5. Comparison of C57BL/6J mouse spleen cyclic AMP and cyclic GMP phosphodiesterase inhibition by NPT 15392. Each point is the mean of six experiments.

Table 2. Inhibition of lymphocyte phosphodiesterase by NPT 15392*

Mouse strain	IC_{50} (μM) Cyclic GMP				IC_{50} (μM) Cyclic AMP				Ratio of IC_{50} values Cyclic AMP:Cyclic GMP			
	Spleen		Thymus		Spleen		Thymus		Spleen		Thymus	
	Sol	Memb	Sol	Memb	Sol	Memb	Sol	Memb	Sol	Memb	Sol	Memb
BALB C/BYJ	11	6	17	9	2000	240	2100	820	182	40	124	91
C57BL/6J	14	11	18	6	550	164	1600	500	39	15	89	83
BDF1	19	8	ND	ND	115	110	ND	ND	6	14	ND	ND

* Data are IC_{50} values obtained at a 10 μM substrate concentration giving maximal inhibition of phosphodiesterases in soluble and membrane fractions of spleen and thymus. Mean values from four experiments (C57BL/6J) or two experiments each (BALB C/BYJ, BDF1) are shown. Variations between experiments ranged from 10 to 30%. Abbreviations: Sol, soluble; Memb, membrane-derived enzyme; and ND, not done.

Table 3. Column separation of spleen phosphodiesterases*

	Cyclic GMP			Cyclic AMP		
	SA	K_m (μM)	K_i (μM)	SA	K_m (μM)	K_i (μM)
Soluble peak						
I	600	2.1	100	0		
II	2050	15	50	190	1.2	70
III	0			173	2.7	NI
IV	0			98	ND	ND
V	700	280	NI	0		
VI	51	ND	ND	990	3.6	NI
Membrane peak						
I	886	3.7	10			
II				644	2.5	110
III	360	2.3	50			

* Splenic lymphocytes of twelve BALB/C males, 14-weeks-old, were homogenized in 40 mM Hepes·Na, pH 7.5, 4 mM beta-mercaptoethanol, 5 mM $MgCl_2$, 10 μM $CaCl_2$ and 1 mM phenylmethylsulfonyl fluoride. The supernatant fraction (48,000 g) was separated on a 0.7 × 23 cm DEAE Bio-Gel A (Bio Rad) column according to Polson *et al.* [11] using a linear gradient of NaCl in the above buffer. Membrane phosphodiesterases were solubilized with 1% Triton X-100 (30 min at 0°) in the same buffer and separated as above. Abbreviations: SA, specific activity (pmoles/min/mg protein) at 10 μM substrate; K_m, apparent Michaelis constant (μM) determined by reciprocal plot of $1/v$ vs $1/s$ with 0.1 to 100 μM cyclic nucleotides; K_i, inhibitor constant determined by Dixon plot [12] for NPT 15392, 1–1000 μM; NI, negligible inhibition by NPT 15392; and ND, not done.

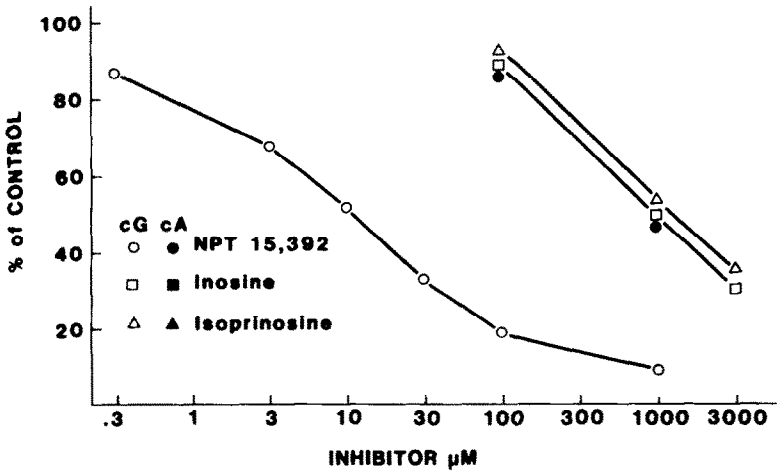

Fig. 6. Comparison of mouse C57BL/6J spleen membrane phosphodiesterase inhibition by NPT 15392, inosine, and isoprinosine. Data are means of two experiments with 10 μM substrates.

of activity, summarized in Table 3. The major cyclic GMP phosphodiesterase peak II had an intermediate K_m, accounted for about one-half the total soluble activity, and was inhibited by NPT 15392 in a competitive manner with a K_i value of 50 μM as determined by a Dixon plot. A cyclic AMP phosphodiesterase occurred in the same fraction and was similarly inhibited (K_i = 70 μM). This enzyme constituted about one-fifth of the total soluble activity. The higher affinity cyclic GMP phosphodiesterase in peak 1 was inhibited less effectively by NPT 15392, and the other soluble enzymes were not affected significantly by the drug. These data may account for the greater effectiveness of NPT 15392 on phosphodiesterase measured with 10 μM cyclic GMP observed in unseparated mixtures.

The membrane bound phosphodiesterases were readily solubilized by treatment with the nonionic detergent Triton X-100. Two cyclic GMP phosphodiesterases were separated and found to have similar low K_m values. The more active of these enzymes was inhibited by NPT 15392 in a competitive manner and to a greater extent than any of the soluble enzymes, reflecting the greater sensitivity of the crude membrane enzymes as shown in Table 2. The reason for the greater effectiveness of NPT 15392 at 10 μM cyclic GMP in crude membranes is not apparent in the data derived from separated enzymes. Exact calculations of the contributions of each form to the total enzyme in unfractionated mixtures are not possible, due to artifacts including differential labilities of the different forms of phosphodiesterases.

It was of interest to compare NPT 15392 with isoprinosine and inosine, which also contain the hypoxanthine nucleus. We found that the effects of isoprinosine and inosine were nearly identical and were about 1/100 and 1/10 of the effects of NPT 15392 on both phosphodiesterases of C57BL/6J mouse spleen membranes (Fig. 6) and soluble preparations (Fig. 7) respectively. IC_{50} Values for a similar comparison with cells from BALB C/BYJ mouse are tabulated in Table 4.

Despite the great selectivity of NPT 15392 at 10 μM to inhibit cyclic GMP hydrolysis, the immunological effects of this drug are noted at much lower concentrations [2–4] and may not be readily explained by the above data. More appropriate experiments

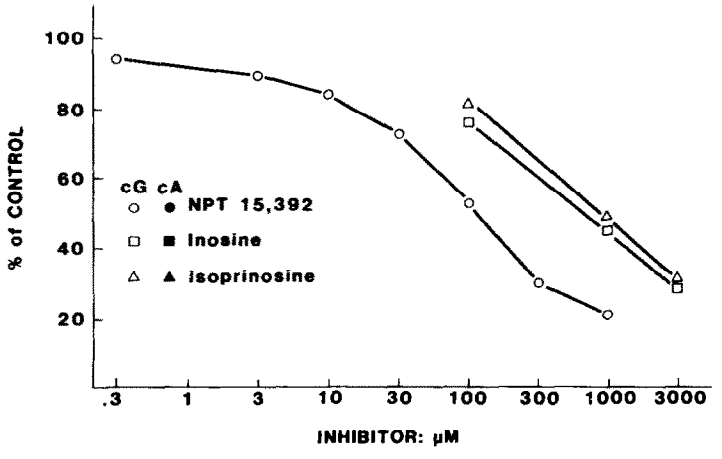

Fig. 7. Comparison of C57BL/6J mouse spleen soluble phosphodiesterase inhibition by NPT 15392, inosine, and isoprinosine. Data are means of two experiments with 10 μM substrates.

Table 4. Inhibition of phosphodiesterases by hypoxanthine derivatives*

	IC_{50} (μM) Cyclic GMP	Cyclic AMP
Membrane		
NPT 15392	6	280
Isoprinosine	1200	2600
Inosine	940	3000
Soluble		
NPT 15392	11	2300
Isoprinosine	940	>6000
Inosine	680	6000

* Results are means of duplicate determinations from a single experiment with BALB C/BYJ mouse spleen, using 10 μM substrates.

would involve prolonged incubation of cells with lower concentrations of NPT 15392. The results of two such experiments are summarized in Table 5. C57BL/6J spleen cells, freed of monocytes by glass adherence, were incubated at 10^7 cells/ml in RPM1 1640 medium + 5% fetal calf serum at 37° for 48 hr, in the presence or absence of 1 μg/ml (3 μM) NPT 15392. NPT 15392 preincubation had minimal effects on the enzyme activities assayed in the absence of the drug. This is in contrast to the increases caused by prolonged incubation with mitogens observed by Epstein *et al.* [13] and also noted by us. Interestingly, enzymes from cells that had been preincubated in the presence of NPT 15392 now showed greatly altered responses to the drug added during the assay: a 2-fold increase in inhibition of cyclic GMP phosphodiesterase by NPT 15392 and a reduced sensitivity of cyclic AMP phosphodiesterase to the compound were observed following the 48-hr preincubation with NPT 15392.

To answer questions regarding tissue specificity of action of NPT 15392 as a phosphodiesterase inhibitor, experiments were performed with several forms of phosphodiesterase extracted and separated from dog tracheal smooth muscle [11]. One form (Peak I) has high affinity for cyclic GMP, another (Peak V) for cyclic AMP, and another (Peak II) has low affinities for both. We tested the inhibitory capacity of NPT 15392 on these enzymes and found, in agreement with the lymphocyte studies, much greater effects on cyclic GMP- than on cyclic AMP-phosphodiesterase (Table 6). Peak I contained a high affinity cyclic GMP phosphodiesterase which was inhibited by NPT 15392 nearly ten times as effectively as the high affinity cyclic AMP hydrolyzing enzyme in Peak V. The low affinity Peak II responded poorly to NPT 15392 inhibition using either cyclic GMP or cyclic AMP as substrate. This enzyme also showed unusually weak inhibition by 3-isobutyl-1-methylxanthine.

Table 6. Canine tracheal smooth muscle phosphodiesterase*

	K_m (μM)	NPT 15392 K_i (μM)	IBMX K_i (μM)
Peak I, cGMP	0.3	55	2.3
Peak II, cGMP	3.3	365	102
Peak II, cAMP	4.1	295	57
Peak V, cAMP	0.6	515	4.7

* Results are means of three experiments. K_m values were determined with 0.1 to 100 μM substrate and K_i with 0.1 to 100 μM substrate and 1–100 μM inhibitor.

DISCUSSION

The availability of phosphodiesterase inhibitors selective for cyclic GMP would be of great benefit in research on cyclic GMP mechanisms in biology, and particularly in lymphocyte activation and function [4, 5]. Hypoxanthine is a very weak inhibitor of phosphodiesterases, with IC_{50} values of 4.5 and 3.2 mM for brain cyclic GMP and cyclic AMP phosphodiesterases respectively [14]. Inosine is about ten times more potent, but still shows no selectivity. Several 1,3-disubstituted and 1,3,8-trisubstituted xanthine derivatives have been shown to inhibit a calmodulin-sensitive cyclic GMP phosphodiesterase more effectively than the cyclic AMP specific form of the enzyme in pig coronary artery [15]. The present data with the immunostimulant NPT 15392 constitute the first report of a 9-substituted hypoxanthine with significant selectivity for inhibiting cyclic GMP phosphodiesterase. The effects of inosine and the immunostimulant iso-

Table 5. Effects of splenic cell culture with NPT 15392 on phosphodiesterases*

Enzyme	Substrate	Ratio NPT 15392:Control Specific activity	IC_{50}, NPT 15392
Soluble	cGMP	1.23 ± 0.04	0.57 ± 0.12
Membrane	cGMP	0.97 ± 0.12	0.65 ± 0.18
Soluble	cAMP	1.48 ± 0.20	1.47 ± 0.37
Membrane	cAMP	0.91 ± 0.08	1.49 ± 0.39

* Results are means of two experiments with C57BL/6J mouse spleen cells. Cells were cultured at 10^7 cells/ml of RPMI 1640 medium + 5% fetal calf serum at 37° in a 5% CO_2 atmosphere for 48 hr in the absence or presence of 1 μg/ml NPT 15392 and washed; the enzymes were assayed in the absence or presence of NPT 15392 and 10 μM cyclic GMP or cyclic AMP. Results are expressed as ratios of specific activity and IC_{50} values for enzymes derived from cells pretreated with NPT 15392 to values of controls.

prinosine which contains inosine [1] were 10–100 times less potent. While this work was in progress, another purine-6-one was described as having a great selectivity for cyclic GMP phosphodiesterase [16]. This compound, M & B 22948, has a substituent on position 2 rather than 9.

Erythro-9-(2-hydroxy,3-nonyl) adenosine (EHNA), a compound structurally related to NPT 15392, inhibits immune functions, and most workers believe this is due to its capacity to inhibit adenosine deaminase [17]. However, Duncan *et al.* [18] have found recently that EHNA is also a potent inhibitor of cyclic AMP phosphodiesterase. They proposed that this effect to increase cyclic AMP levels might account for the immunoinhibitory effects of EHNA. An action of EHNA on cyclic GMP phosphodiesterase has yet to be determined.

Several forms of phosphodiesterase have been described in lymphocytes. Winchurch *et al.* [6], using a gel electrophoresis system, found two forms of cyclic AMP phosphodiesterase in mouse thymocytes, compared to four in B lymphocytes of mouse spleen. Hait and Weiss [7] further described the four forms of cyclic AMP phosphodiesterase in normal mouse splenic lymphocytes. Three of these also had cyclic GMP phosphodiesterase activity. We, therefore, expected that a greater degree of inhibition could be observed with a specific form of phosphodiesterase when separated from other enzymes. This approach was very successful in correlating the efficacy of cyclic AMP phosphodiesterase inhibitors with their ability to relax canine tracheal smooth muscle [11]. Using a column method to separate spleen phosphodiesterases, we found six peaks of soluble enzyme and three peaks of membrane bound enzyme. A major form of cyclic GMP phosphodiesterase, with an intermediate K_m, was inhibited more effectively than the other forms characterized by either higher or lower K_m values. Membrane phosphodiesterases were solubilized and separated, revealing one form of cyclic GMP phosphodiesterase that was inhibited by NPT 15392 to a greater extent than any of the soluble forms. These data are helpful in understanding the selective nature of inhibition by NPT 15392, although the dramatic effectiveness at 10 μM substrate compared to other concentrations of substrate is not explained completely. A direct comparison of the forms of phosphodiesterase separated by our DEAE Bio-Gel A method and by the electrophoresis method of Weiss and coworker [6, 7] is not feasible because of the different separation techniques. Our separation system does produce enzymes highly differentiated with respect to their responses to a moderately selective cyclic GMP phosphodiesterase inhibitor, and should be useful in further analyzing the relevance of immunopotentiator effects on cyclic nucleotide metabolism.

The immunopharmacologic relevance of the present findings remains to be shown. NPT 15392 induces T cell differentiation and augments a number of T lymphocyte functions at concentrations of 0.01 to 1 μg/ml, which are two to three orders of magnitude lower than the concentrations that inhibit cyclic GMP phosphodiesterase in mouse lymphocytes *in vitro* [2–4]. It is possible that in the 48-hr preincubation experiments NPT 15392 caused the selective increase in one form of the cyclic GMP hydrolyzing enzyme which is more susceptible to inhibition, and concomitantly the disappearance of a susceptible cyclic AMP·phosphodiesterase. We plan to examine the multiple forms of mouse lymphocyte cyclic phosphodiesterases in pretreated cells in order to address these possibilities.

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