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Rat Mast Cell High Affinity Cyclic Nucleotide Phosphodiesterases: Separation and Inhibitory Effects of Two Antiallergic Agents

HÅKAN BERGSTRAND,[1] BRITTA LUNDQUIST, AND ANNIKA SCHURMANN

Research and Development Laboratories, AB Draco,[2] Fack, S-221 01 Lund, Sweden

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SUMMARY

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Rat mast cells, harvested from the thoracic and peritoneal cavities and purified to at least 90% by density gradient centrifugation in 38% bovine serum albumin (BSA) were examined for cyclic nucleotide phosphodiesterase activities. The soluble fraction (200,000 × *g*) of disrupted mast cells was found to contain approximately 80% of the total activity at a substrate concentration of 0.13 μM or 6.0 μM cyclic AMP or 0.12 μM cyclic GMP. It could be separated by DEAE-Sephadex chromatography into two major fractions (I_{MC} and III_{MC}) and one minor fraction (II_{MC}). Fraction I_{MC} contained phosphodiesterase activities with high apparent affinity for both cyclic GMP and cyclic AMP (app. K_m values in the order of 0.1 to 0.2 μM), cyclic GMP being the preferred substrate. Fraction III_{MC} hydrolyzed primarily cyclic AMP; the app. K_m value was 0.4 μM. The particulate fraction (200,000 × *g*) of rat mast cells, which was found to contain approximately 20% of the total activity at the indicated substrate concentrations, hydrolyzed cyclic AMP and cyclic GMP with similar apparent K_m values (~0.5 μM) and maximum velocities. The phosphodiesterase activity pattern of supernatants from nonpurified thoracic and peritoneal cells was different from that of mast cells, indicating that contaminating cells are not the source of the two major phosphodiesterase fractions in mast cell supernatants. The antiallergic agents disodiumcromoglycate (DSCG) and 2-o-propoxyphenyl-8-azapurin-6-one (M&B 22,948) inhibited the activity of the separated mast cell cyclic AMP phosphodiesterase activities. The lowest I_{50} values observed were 250 μM and 10 μM, respectively, i.e., concentrations that exceed those sufficient for inhibition of antigen-induced histamine release from mast cells.

INTRODUCTION

Compounds selectively inhibiting cyclic nucleotide phosphodiesterase(s) in a specified tissue or cell might possibly be utilized as therapeutic agents in certain kinds of disease states affecting that tissue/cell (1–5). Some clinically effective drugs with unknown biochemical mode of action might be suspected of exerting their effects through the inhibition of a cyclic AMP phosphodiesterase activity. Disodiumcromoglycate (DSCG) is an anti-asthmatic drug which inhibits antigen-induced release of histamine from mast cells. Substantial indirect evidence indicates that a number of agents presumably raising the level of cylic AMP inhibit immunologically-induced release of histamine from mast cells (for critical review, see ref. 6). Studies *in vitro* on the effect of DSCG on (i) cyclic AMP phosphodiesterase from various sources (7),

[1] To whom all correspondence should be addressed
[2] Subsidiary to AB Astra, Södertälje, Sweden

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and on (ii) antigen and phospholipase-A-induced rat mast cell degranulation led Taylor *et al.* (8, 9) to suggest the existence of a mast cell specific cyclic AMP phosphodiesterase which could be the target of the drug. Patients treated with the drug were then shown to have decreased cyclic AMP phosphodiesterase activity (10). Doubts have been expressed concerning the validity of this proposed mechanism of action of DSCG (11–15). For instance, DSCG was found to be a more potent inhibitor of cyclic GMP phosphodiesterase activities in human lung than of cyclic AMP phosphodiesterases (14, 15). However, phosphodiesterases are known to differ markedly in properties between various cells/tissues (1, 4, 5, 16–20). Therefore, previous work might not have examined the effect of DSCG with the relevant enzyme(s). The present report describes initial results on the partial preparation and characterization of "low K_m"-cyclic nucleotide phosphodiesterase activities from purified rat mast cell populations and examines the inhibitory effect of some antiallergic agents and compound 48/80 on these enzyme activities.

EXPERIMENTAL PROCEDURE

Chemicals. Cyclic [^{3}H]AMP (40 Ci/mmole) and cyclic [^{3}H]GMP (8 Ci/mmole) were purchased from New England Nuclear Corporation. Unlabelled cyclic nucleotides (cyclic AMP, cyclic GMP), 5′-nucleotidase (lot 66C-9570), bovine serum albumin (BSA[3]; fraction V powder), and compound 48/80 were the products of Sigma Chemical Co.; Fisons Ltd. supplied disodiumcromoglycate; and May & Baker Ltd. 2-o-propoxyphenyl-8-azapurin-6-one (M&B 22,948). Heparin was from Vitrum, Stockholm, Sweden. Water, double distilled from glass, was used throughout.

Buffers. Buffer A (for phosphodiesterase separation and assay) consisted of 40 mM Tris-HCl pH 8 (4°) made 5 mM in $MgCl_2$, 3.75 mM in 2-mercaptoethanol, and 10 μM in $CaCl_2$. Buffer MCM is the Mast Cell Medium described by Sullivan *et al.* (21) supplemented with 0.9 mM $MgCl_2$.

[3] The abbreviations used are: BSA, bovine serum albumin; DSCG, disodiumcromoglycate; MCM, mast cell medium.

Purification of mast cells. Sprague-Dawley rats weighing about 250 g were obtained locally. The method of Sullivan *et al.* (21), slightly modified, was used for harvesting cells from the thoracic and peritoneal cavities. A total of 20 ml cold (4°C) MCM (without Ca^{2+}) was used to collect the cells from each animal; toluidine blue staining and differential counting indicated that mast cells constituted 5 to 10% of the total cell number. Cells from 10 animals were combined (samples visibly contaminated with erythrocytes were discarded) and collected by centrifugation at ambient temperature in disposable polystyrol tubes (50 × *g* for 10 min). Nine-tenths of the total cell population obtained were purified by centrifugation at ambient temperature in a BSA gradient according to Sullivan *et al.* (21). The final mast cell preparation regularly contained less than 10% contaminating cells. The yield of mast cells from each animal amounted to approximately 10^6. These figures closely agree with those of Sullivan *et al.* (21). The remaining tenth of the crude cell population—referred to as a sham purified sample of the total cell population—was similarly treated except that the BSA-solution at gradient centrifugation was replaced by MCM. After one wash in MCM, the cells were disrupted by resuspension in 25 μl buffer A per 10^6 cells, incubated at 4°C for 1 hour, and frozen. Four to five batches of disrupted cells (normally a total of 50 to 75 × 10^6 mast cells at a purity of more than 90%, or a corresponding amount of sham purified cells, obtained from a total of 40 to 50 animals) which had been stored at −20°C for less than one (mast cells) or two weeks (sham purified cells) were thawed and combined with the aid of an additional 25 μl buffer A per 10^6 cells. Centrifugation was performed at 200,000 × *g* for 60 min at 4°C. The pellet was resuspended in 1 ml of buffer A with the aid of a Potter-Elvehjem homogenizer with a tight fitting teflon pestle. At this stage, microscopic examination of the suspension failed to reveal any intact cells. After centrifugation (200,000 × *g* for 60 min), the resulting supernatant was combined with the previous one; the pellet was resuspended in 40 μl of the buffer A made

0.5% in BSA per 10^6 cells. Aliquots of the supernatant were removed for examination of (i) total soluble phosphodiesterase activity at different substrate concentrations and (ii) protein concentration according to the procedure of Lowry *et al.* (22); BSA served as standard protein. The main part of the supernatant was subjected to ion exchange chromatographic separation.

DEAE-Sephadex chromatography was performed at 4°C with columns prepared in Pasteur pipettes (gel bed dimensions 6 × 0.5 cm) (Fig. 2 legend gives details). Active fractions were pooled, concentrated approximately 5 times by ultrafiltration (Amicon UM 10 under N_2 pressure), and divided into small aliquots which were lyophilized and stored at −20°C until examined. Reconstitution was performed with cold distilled water.

Assay of phosphodiesterase activity. The Thompson and Appleman (23) procedure was used with some modifications (specified in detail elsewhere) (24, 25). In order to minimize enzyme consumption, the volume of each component in the reaction mixture—and the components added thereafter—was scaled down five times in the present work so that (i) the total reaction mixture consisted of a volume of 100 μl and (ii) 100 μl aliquots of the supernatant after Bio-Rad addition was examined for radioactivity.

RESULTS

Phosphodiesterase Activity of Disrupted Cell Supernatants and Particulate Fractions

Table 1 lists the total phosphodiesterase activity recorded for the supernatant and the particulate fractions of the purified mast cells and also for the sham purified cells. The activities are expressed as pmoles of cyclic nucleotide hydrolyzed per 10^6 cells and min at 25°. The mast cell supernatants contain 4.3 ± 0.6 mg protein per ml (mean ± SEM of five experiments). We estimate that, at most, 0.5–1 mg/ml protein can be accounted for by contaminating BSA and gelatin originating from the density gradient and the MCM. Thus, based on the figures given in Table 1, a specific activity for the soluble mast cell cyclic AMP phosphodiesterase at 0.13 μM in the order of 50 pmoles/min and mg protein can be calculated.

Table 1 also shows that approximately 20% of the total activity is recovered with the particulate fraction. It is noticeable that the sham purified cells show a cyclic AMP phosphodiesterase activity comparable to or higher than that of purified mast cells, whereas the cyclic GMP phosphodiesterase activity seems to be higher for the mast cells than for the total cell population.

DEAE-Sephadex chromatography. The main part of the soluble phosphodiesterase activity from the mast cell and the sham purified cell populations was subjected to chromatography on DEAE-Sephadex. The obtained activity patterns showed at least two major and one minor component for the mast cells and one major and two minor for the total cell population (Fig. 1). Concerning mast cell phosphodiesterases, fraction I_{MC}, at low substrate concentrations, hydrolyzes primarily cyclic GMP, whereas fractions II_{MC} and III_{MC} hydrolyze primar-

TABLE 1

Cyclic nucleotide phosphodiesterase activity in the soluble (200,000 × g) and particulate fractions of a purified mast cell population and an analogously sham purified total cell population from the peritoneal and thoracic cavities

The activity is expressed as pmoles cyclic nucleotide hydrolyzed per 10^6 cells and min at 25°C. Figures given are means ± SEM of five experiments. Figures in parentheses are percentages of total activity recovered.

Substrate	Cell fraction	Cell type	
		Mast cells	Total cells
0.12 μM cGMP	Soluble	5.40 ± 0.53 (82)	2.90 ± 0.17 (92)
	Particulate	1.17 ± 0.17 (18)	0.26 ± 0.19 (8)
0.13 μM cAMP	Soluble	9.68 ± 1.03 (85)	8.68 ± 1.07 (90)
	Particulate	1.70 ± 0.19 (15)	0.93 ± 0.25 (10)
6.01 μM cAMP	Soluble	44.67 ± 7.9 (78)	70.72 ± 14.3 (89)
	Particulate	12.53 ± 2.51 (22)	8.84 ± 2.12 (11)

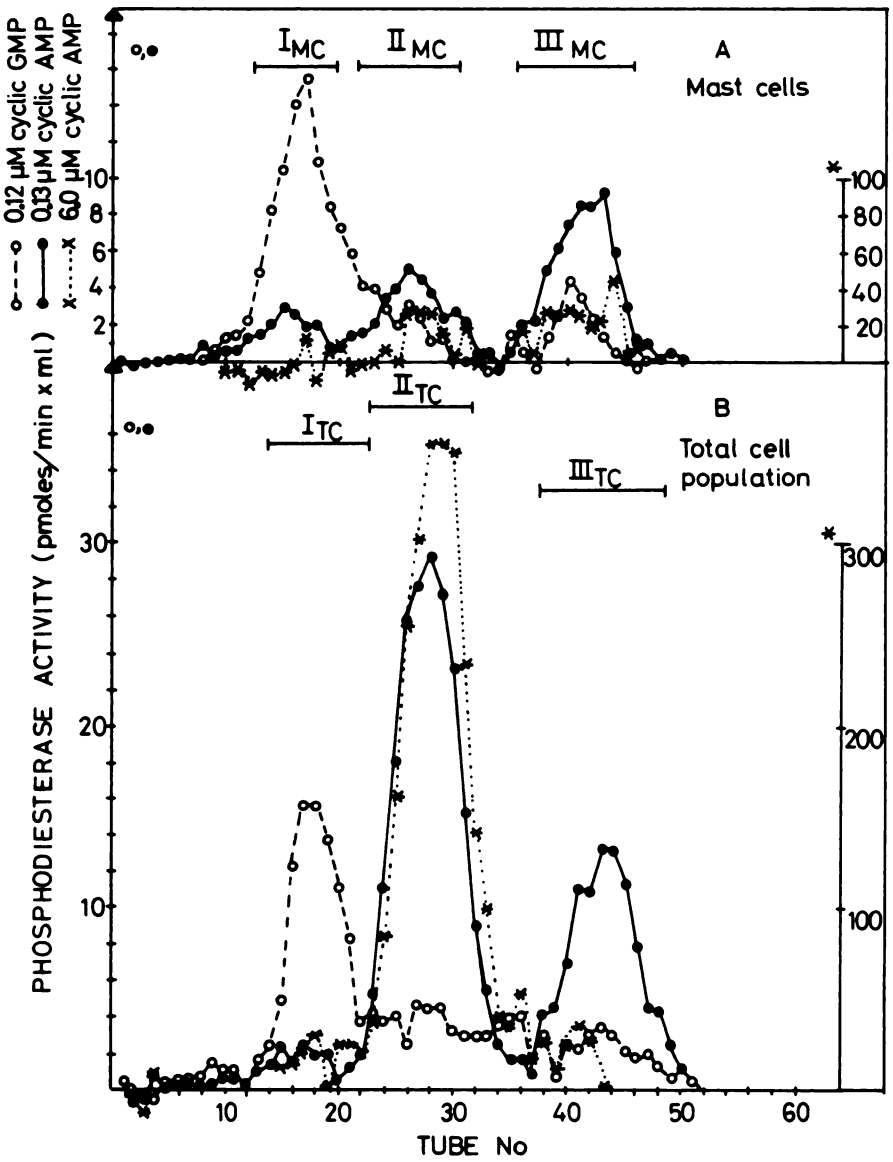

FIG. 1. *Chromatography on DEAE-Sephadex at 4°C of the phosphodiesterase activity of a soluble extract (200,000 × g) of 6 × 10⁷ purified mast cells (A) or of 7 × 10⁷ cells from the corresponding sham purified total cell population (B).*

Gel bed dimensions 6 × 0.5 cm. Elution was performed with a linearly increasing concentration of NaCl ranging from 0.05 to 0.5 M in buffer A (total volume of gradient 200 ml). Flow rate: Approximately 6 ml per hour; fractions were collected each half hour in tubes containing 0.2 ml of buffer A made 0.05 mM in NaCl and 25 mg/ml in BSA. Ninety milliliter aliquots from each reaction tube were equilibrated for a few minutes at 25°C and were then examined for phosphodiesterase activity at the indicated substrate concentrations. The reaction was initiated by the addition of 10 μl of substrate. Blanks were run with boiled aliquots (100°C for 2 minutes followed by centrifugation) from each second or third tube (blanks for intermediate tubes were obtained by intrapolation).

Scale on left-hand ordinate refers to phosphodiesterase activity (in pmoles/min and ml) at 0.13 μM cyclic AMP or 0.12 μM cyclic GMP (represented by the symbols ● and ○, respectively. Scale on right-hand ordinate refer to phosphodiesterase activity (in pmoles/min and ml) at 6.0 μM cyclic AMP (represented by the symbol *).

ily cyclic AMP. With the sham purified cells, at low substrate concentrations, fraction I_{TC} seems to be specific for cyclic GMP, and fractions II_{TC} and III_{TC} for cyclic AMP; fraction II_{TC} is much more active than is fraction III_{TC} with 6 μM cyclic AMP as a substrate.

Activity patterns closely similar to those recorded in Fig. 1 were obtained with two other preparations of each cell population.

Estimates of the kinetic parameters of separated mast cell phosphodiesterase. The activity of the separated mast cell phosphodiesterases was examined at low substrate concentrations according to previously described principles (24, 25). Figure 2A shows the reciprocal plots of the data recorded with fraction I_{MC}; Fig. 2B shows those recorded at cyclic AMP hydrolysis by fractions II_{MC} and III_{MC}. With fraction II_{MC}, there was no indication of nonlinear kinetics; the activity of fraction III_{MC} was not examined at substrate concentrations exceeding 1 μM. With fraction III_{MC}, the app K_m for hydrolysis of cyclic GMP is 0.26 μM; the corresponding V_{max} value is 2.2 pmoles/min 10^6 cells. The phosphodiesterase activity of the washed and resuspended particulate fraction of the disrupted mast cells hydrolyzed cyclic AMP and cyclic

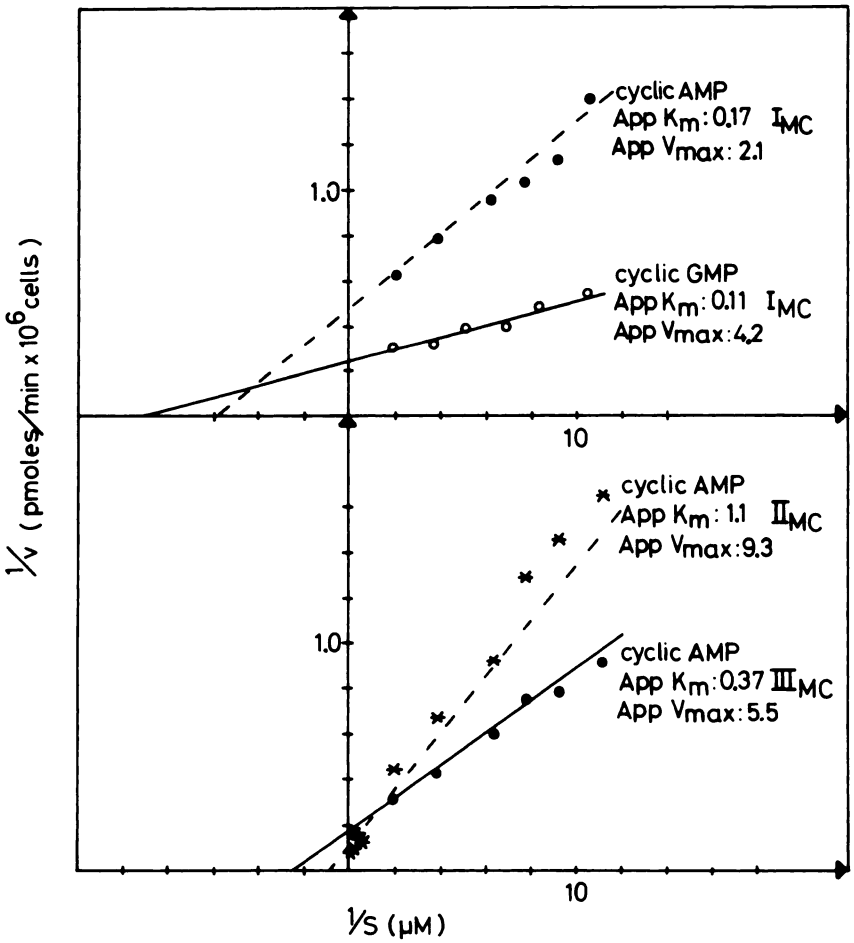

FIG. 2. *Estimates of the kinetic parameters of mast cell phosphodiesterases*

Assay of phosphodiesterase activity of each specified DEAE-Sephadex fraction (see Fig. 1A) was performed at 25°C in buffer A made 0.5% in BSA using a fixed reaction time of 30 min. The graph shows the double reciprocal plot of velocity data. Apparent K_m and V_{max} values were obtained according to previously detailed principles (24, 25). V_{max} values are expressed as pmoles/min × 10^6 cells initially employed for enzyme preparation.

GMP at a comparable degree; app K_m values recorded were 0.5 μM (cyclic AMP) and 0.4 μM (cyclic GMP), and the corresponding V_{max} values were 1.6 (cyclic AMP) and 1.4 (cyclic GMP) pmoles/min 10^6 cells.

With phosphodiesterases prepared from the supernatant of the total cell populations, the following K_m values were recorded. Fraction I_{TC}: 0.32 μM (cGMP), fraction II_{TC}: 4.34 (cAMP). The activity of fraction III_{TC} was not examined.

Inhibition of activity of partially purified phosphodiesterases. The inhibitory capacity of some selected compounds on the activity of the purified mast cell phosphodiesterases were examined according to principles previously employed with enzymes prepared from human lung (14). Table 2 summarizes the obtained results.

DISCUSSION

We previously showed that partially purified human lung tissue cyclic nucleotide phosphodiesterases are inhibited by some antiallergic agents in a way which does not readily conform to the proposal that such compounds act through inhibition of a cyclic AMP phosphodiesterase activity (14). These results were recorded with enzyme activities not necessarily reflecting the relevant mast cell phosphodiesterases. Therefore we set out to separate rat mast cell phosphodiesterase activities in quantities sufficient to permit examination of these enzymes with respect to inhibitory effects of some antiallergic agents.

Working with human peripheral lymphocytes and monocytes, Thompson *et al.* (19) recently demonstrated the importance of using extensively purified cell populations at the characterization of the phosphodiesterase activity of the pertinent cell type. In the present work we examined mast cells purified to at least 90%. Attempts to obtain a higher degree of purity by repeated density gradient centrifugation were unsuccessful. Therefore we chose to compare the results obtained with purified mast cells with those obtained with the total cell population to see whether phosphodiesterases of the mast cell population could be accounted for by contaminating cells.

With the mast cell supernatant, it is possible to demonstrate both cyclic AMP and cyclic GMP phosphodiesterase activities. The recorded specific activity of the former (at 0.13 μM or 6 μM cyclic AMP) is similar to that which can be calculated from data of Fredholm *et al.* (26) but approximately 10 and 100 times higher than the corresponding figures that we have calculated from data of Sullivan and Parker (27) and Johnson *et al.*, (28), respectively. The reasons for these discrepancies are not clear, although differences in technique could contribute.

TABLE 2

Inhibitory effects of various compounds on the activity of different cyclic nucleotide phosphodiesterases from rat mast cells

	I_{50}[a]				
	Fraction I_{MC}		Fraction II_{MC}	Fraction III_{MC}	Fraction P_{MC}[c]
	0.12 μM cGMP	0.13 μM cAMP	6 μM cAMP	0.13 μM cAMP	0.13 μM cAMP
Compound 48/80[b]	1000	1000	50	50	>1000
Disodiumcromoglycate	250	1000	3000	1000	630
M&B 22,948	1.0	10	>10	>10	Not determined

[a] The I_{50} value is the concentration (in μM) of each compound that produces 50% inhibition of the activity at the indicated substrate concentration. Each figure represents the results obtained with the enzyme preparations of Fig. 1A. Activity determinations were performed in duplicate at five different concentrations of inhibitor and I_{50} values were determined graphically according to principles described previously (ref. 14). Figures closely corresponding to those recorded in Table 2 were obtained with another set of enzyme preparations.

[b] Figures given for compound 48/80 represent concentration in μg/ml.

[c] Fraction P_{MC} is the particulate fraction of disrupted mast cells.

With supernatants from both purified mast cells and the sham purified total cell population, DEAE-Sephadex chromatography separates the cyclic nucleotide phosphodiesterase activity into at least three different fractions. However, the phosphodiesterase pattern obtained with the mast cell supernatant differs from that of the sham purified cells. The last-mentioned enzyme source contains, as the major phosphodiesterase activity, cyclic AMP hydrolyzing enzyme activity with an apparent K_m value of approximately 5 μM (fraction II_{TC}). A phosphodiesterase activity with these characteristics is virtually absent from the mast cell supernatant although such an enzyme form might constitute part of the activity of fraction II_{MC}. This finding indicates that unless there is an unidentified cell type which co-purifies with the mast cells, the pattern of phosphodiesterase activity attributed to mast cells does not originate from a contaminating cell type.

The following properties were found with the mast cell phosphodiesterase activities. The cyclic GMP hydrolyzing activity of fraction I_{MC} expresses an apparent substrate affinity which is very high. Inhibition of the cyclic GMP hydrolyzing activity of fraction I_{MC} by DSCG and M&B 22,948 resembles inhibition of the lung cyclic GMP specific enzyme with these compounds (see ref. 14). Fraction I_{MC} also hydrolyzes cyclic AMP but both the apparent affinity and maximum velocity and the inhibitory potency of M&B 22,948 with this substrate are lower than with cyclic GMP. We suspect that the activity of fraction I_{MC} should be attributed to at least two enzyme forms; one high affinity cyclic GMP specific phosphodiesterase activity resembling the lung fraction I (24, 25) and one nonspecific high affinity enzyme similar to the lung fraction Ia/Ic (25). The activity of fraction III_{MC} shows some resemblance to the high affinity cyclic AMP hydrolyzing lung enzyme III_b (24) but the mast cell enzyme exhibits a higher relative cyclic GMP hydrolyzing activity and is less sensitive than the lung enzyme to inhibition with compound 48/80. The activity of fraction II_{MC} shows an apparent K_m value for cyclic AMP of approximately 1 μM. It is not clear whether this activity represents one or more mast cell phosphodiesterases or originates from contaminating cells. M&B 22,948 and DSCG inhibit antigen-induced histamine release from rat mast cells with I_{50} values amounting to approximately 0.1–1 and 5–10 μM, respectively (12, 29, 30, and our unpublished results). We conclude that the inhibitory potency of these compounds for rat mast cell cyclic AMP hydrolyzing enzymes, as recorded in the present work, does not support the proposal that these compounds act through inhibition of cyclic AMP phosphodiesterase activity. The physiological relevance, if any, of the cyclic GMP phosphodiesterase inhibitory effect of M&B 22,948 and DSCG is still to be established.

REFERENCES

1. Appleman, M. M., Thompson, W. J., & Russell, T. R. (1973) *Adv. Cyclic Nucleotide Res.*, **3**, 65–98.
2. Amer, M. S., & Kreighbaum, W. E. (1975) *J. Pharm. Sci.*, **64**, 1–37.
3. Strada, S. J., & Pledger, W. J. (1975) in *Cyclic Nucleotides in Disease* (Weiss, B., Ed.), pp. 3–44, University Park Press, Baltimore.
4. Weiss, B. (1975) *Adv. Cyclic Nucleotide Res.*, **5**, 195–211.
5. Weiss, B., & Hait, W. (1977) *Ann. Rev. Pharmac. Tox.*, **17**, 441–478.
6. Parker, C. W., Sullivan, T. J., & Wedner, H. J. (1974) *Adv. Cyclic Nucleotide Res.*, **4**, 1–79.
7. Roy, A. C., & Warren, B. T. (1974) *Biochem. Pharmacol.*, **23**, 917–920.
8. Taylor, W. A., Francis, D. H., Sheldon, D., & Roitt, I. M. (1974) *Int. Arch. Allergy Appl. Immunol.*, **47**, 175–193.
9. Taylor, W. A., & Sheldon, D. (1974) *Int. Arch. Allergy Appl. Immunol.* **47**, 696–707.
10. Lavin, N., Rackelefsky, G. S., & Kaplan, S. A. (1976) *J. Allergy Clin. Immunol.*, **57**, 80–88.
11. Tateson, J. E., & Trist, D. G. (1976) *Life Sci.*, **18**, 153–162.
12. Vardey, C. J., & Skidmore, I. F. (1976) *Brit. Med. J.*, (ii), 369.
13. Barrett-Bee, K., and Henderson, W. (1976) *Biochem. Soc. Transactions*, **4**, 699–701.
14. Bergstrand, H., Kristoffersson, J., Lundquist, B., & Schurmann, A. (1977) *Mol. Pharmacol.*, **13**, 38–43.
15. Coulson, C. J., Ford, R. E., Marshall, S., Walker, J. L., Wooldridge, K. R. H., Bowden, K., & Coombs, T. J. (1977) *Nature* **265**, 545–547.
16. Uzunov, P., Shein, H. M., & Weiss, B. (1974) *Neuropharmacology* **13**, 377–391.
17. Kakiuchi, S., Yamazaki, R., Teshima, Y., Uenishi,

K., & Miyamoto, E. (1975) *Biochem. J.*, **146**, 109–120.
18. Hidaka, H., & Asano, T. (1976) *Biochim. Biophys. Acta*, **429**, 485–497.
19. Thompson, W. J., Ross, C. P., Pledger, W. J., Strada, S. J., Banner, R. L., & Hersh, E. M. (1976) *J. Biol. Chem.*, **251**, 4922–4929.
20. Ross, P. S., Manganiello, V. C., & Vaughan, M. (1977) *J. Biol. Chem.*, **252**, 1448–1452.
21. Sullivan, T. J., Parker, K. L., Stenson, W., & Parker, C. W. (1975) *J. Immunol.*, **114**, 1473–1479.
22. Lowry, O. H., Rosebrough, N. J., Farr, A. L., & Randall, R. J. (1951) *J. Biol. Chem.*, **193**, 265–275.
23. Thompson, W. J., & Appleman, M. M. (1971) *Biochemistry*, **10**, 311–316.
24. Bergstrand, H., & Lundquist, B. (1976) *Biochemistry*, **15**, 1727–1735.
25. Bergstrand, H., Lundquist, B., & Schurmann, A. (1978) *J. Biol. Chem.*, **253**, 1881–1891.
26. Fredholm, B. B., Guschin, I., Elwin, K., Schwab, G., & Uvnäs, B. (1976) *Biochem. Pharmacol.*, **25**, 1583–1588.
27. Sullivan, T. J., & Parker, C. W. (1973) *Biochem. Biophys. Res. Commun.*, **55**, 1334–1339.
28. Johnson, A. R., Moran, N. C., & Mayer, S. E. (1974) *J. Immunol.*, **112**, 511–519.
29. Garland, L. G., & Mongar, J. L. (1974) *Br. J. Pharmac.*, **50**, 137–143.
30. Herzig, D. J., & Kusner, E. J. (1975) *J. Pharmacol. Exp. Ther.*, **194**, 457–462.