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HYDROLYSIS OF ARGININE AND TYROSINE ESTERS IN MAST CELLS EXPOSED TO EPINEPHRINE

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Abstract—Acting in a dose-dependent fashion *in vitro*, *l*-epinephrine caused rat peritoneal fluid cells to hydrolyze substituted arginine (BAEE, TAME) and tyrosine (BTEE) esters. Cell activation was complete within 5 min; repeated washing of activated cells did not result in loss of their BAEE-hydrolyzing capacity. BTEE-hydrolyzing capacity fell to pre-activation levels within 10 min, even in the absence of washing. This loss may be the result of an interaction of BTEE-esterase with cell-borne inhibitory factors. 8-Br-cGMP or noradrenaline acted like epinephrine. diBu-cAMP was inactive but inhibited cell activation by epinephrine or cGMP. Neither epinephrine, 8-Br-cGMP nor diBu-cAMP released histamine. Exposure of peritoneal cells to a hypotonic salt medium caused histamine release but not activation of esterase. However, hypotonically pretreated cells responded to epinephrine in the same way as normal cells. Diisopropyl-fluorophosphate prevented esterase activity on TAME and BTEE. Following differential centrifugation of peritoneal fluid cells, epinephrine-sensitive esterase was found only in mast cells; eosinophils, lymphocytes and monocytes were inactive. Compound 48/80, although less active than epinephrine, was also able to activate mast cells. The concentrations required were ten times higher than those needed to cause substantial release of histamine. The appearance of esterase activity in epinephrine-treated rat mast cells seems to be the outcome of cGMP-dependent changes, associated with reversible morphological alterations, but not with the release of histamine.

Much of the considerable research devoted to mast cells is due to the recognition that they are sites of origin of several mediators of allergic and inflammatory responses of tissues [1]. Most of these agents are of low or intermediate molecular weight, and although they may trigger enzymatic processes, they have not been shown to have enzymatic activity of their own. Nevertheless, mast cells obtained from the peritoneal cavity of the rat have been shown to contain a chymotrypsin-like enzyme [2], a 'chymase' [3], which has been purified, characterized, and found to closely resemble pancreatic chymotrypsin [4, 5]. No role has been found for this enzyme in histamine release in anaphylaxis [6] or by chemical mediators [7].

Although a trypsin-like enzyme, capable of hydrolyzing substituted basic amino acid esters, has been found in rat circulatory basophils [8] and in skin mast cells of man and dog [9], a similar enzyme could not be demonstrated in rat mast cells. We have, in part, confirmed these findings, by showing that free mast cells, extracted from the peritoneal cavity of the rat, have only minimal hydrolytic activity on substituted arginine esters such as BAEE or TAME.* This activity, however, became clearly demonstrable following exposure of the cells to epinephrine. Very significant increases in the ability of the cell to hydrolyze substituted tyrosine esters, i.e. typical chymotrypsin substrates, were also induced by the catecholamine. It was felt that characterization of a process leading to the activation, by an ubiquitous endogenous mediator, of latent tryptic and chymotryptic mast cell enzymes might open new views on the pathological and physiological roles of these cells. Presented here are results of studies on time and concentration dependence, cell specificity, relationship to the histamine-releasing process, and reproducibility by cyclic nucleotides, of the activation of mast cell esterases by epinephrine. These studies complement previous results [10] showing that mast cell-containing rat peritoneal fluid cells, after stimulation by epinephrine and, to a lesser extent, by compound 48/80, become capable of causing the activation of a rat plasma arginine ester esterase with kallikrein-like properties, as evidenced by its ability to cause the consumption of plasma kininogen.

MATERIALS AND METHODS

Male, 180–250 g Wistar rats were used in this study. Peritoneal fluid cells were collected by flushing the abdominal cavity with 10 ml of Krebs–Ringer phosphate buffer, pH 7.3, centrifuging (5 min, 900 *g*), and resuspending the cells in adequate volumes of buffer. Mast cells were separated from other peritoneal cells using a 38% BSA solution as a density barrier against the penetration of such cells during controlled centrifugation [11]. Repeated washing of mast cells contained in the BSA layer led to a partial loss of their ability to respond to epinephrine. A single wash using the recommended MCS buffer [11] was, therefore, employed. Mast cells were quantitatively recovered; in fifteen experiments, they com-

* The following abbreviations are used in the text: BAEE, *N*-benzoyl-arginine-ethyl-ester; BTEE, *N*-benzoyl-tyrosine-ethyl-ester; TAME, *N*-*p*-toluenesulfonyl-arginine-methyl-ester; BSA, bovine serum albumin; DFP, diisopropyl-fluorophosphate; diBu-cAMP, N^6-2′-*O*-dibutyril-cyclic 3′,5′-adenosine monophosphate; 8-Br-cGMP, 8 bromo-cyclic 3′,5′-guanosine monophosphate; and TCA, trichloroacetic acid.

prised 73 ± 2 per cent of the cells of the albumin fraction and less than 0.1 per cent of the cells which collected at the albumin–buffer interface. The cells of this fraction were also recovered in quantitative yield. Upon differential count (Giemsa stain), they were found to contain lymphocytes, eosinophils and monocytes in the same relative distribution in which such cells existed in the original peritoneal cavity washings; this indicated that the fractionation procedure employed segregated only mast cells. To generate esterolytic activity, 5–10 × 10^4 purified or non-fractionated mast cells were incubated for 5 min at 37° in 100 μl of Krebs–Ringer phosphate buffer, pH 7.3, under air, in a Dubnoff metabolic incubator at a shaking cycle of 125/min. To determine esterolytic activity, 100 μl of a suspension of control or activated cells were added to 900 μl of 10 mM BAEE or TAME substrates in 0.15 M Tris buffer, pH 7.8, and were incubated for 30 min at 37° in the Dubnoff shaker. None of the activators used, i.e. epinephrine, 48/80 or 8-Br-cGMP, affected the stability of the ester substrates, when tested in the absence of cells. Reactions were terminated by adding 10% TCA to a final concentration of 4%. The extent of hydrolysis was estimated by determining the percentage of substrate remaining after incubation. The colorimetric procedure recommended by Brown [12], based on the hydroxamate reaction, was followed. Preliminary trials using trypsin and chymotrypsin showed that, for up to 50 per cent substrate consumption, the extent of hydrolysis was a linear function of both enzyme concentration and time. Experiments employing cells were followed for a period yielding, at most, hydrolysis of 30 per cent of the substrate present. When BTEE was employed, incubation and assay mixtures contained 50% (w/w) methanol; considerable release of histamine, but no generation of chymotrypsin-like activity took place under the influence of this solvent. Histamine release was estimated from the values of histamine remaining in cells after incubation. The amine was quantitatively liberated from the centrifuged cells by heating for 10 min at 90° in 0.1 M HCl, and was assayed on the atropinized guinea pig ileum. Mast cells were counted in a Neubauer chamber, after staining with 1:5000 toluidine blue in saline.

Drugs. *l*-Epinephrine, compound 48/80, diBu-cAMP, 8-Br-cGMP, BAEE, TAME, BTEE, BSA (fraction V), trypsin (Type III), and α-chymotrypsin (Type II) were obtained from the Sigma Chemical Co., St. Louis, MO, and DFP from the Aldrich Chemical Co., Milwaukee, WI. Stock solutions of epinephrine (1 mg/ml) were prepared by dissolving the amine in 1 M HCl to pH 5–6. Appropriate dilutions were prepared just prior to use. BSA was stored in a vacuum dessicator in the presence of dry $CaCl_2$. Its protein concentration was periodically controlled by comparison with a standard of crystalline bovine serum albumin (Sigma), using the biuret method. DFP was dissolved in methanol and subsequently diluted to the desired concentration with buffer.

RESULTS

Figure 1 shows that, within 2 min following the incubation of peritoneal fluid cells with *l*-epinephrine, a marked ability to hydrolyze both the substituted arginine ester, TAME, and the substituted tyrosine ester, BTEE, developed in the cell suspension. Both activities were maximal after 5 or 10 min of incubation, but thereafter followed a different course. While TAME-esterase remained unchanged for a period of 40 min, BTEE-esterase activity was lost within 10–20 min.

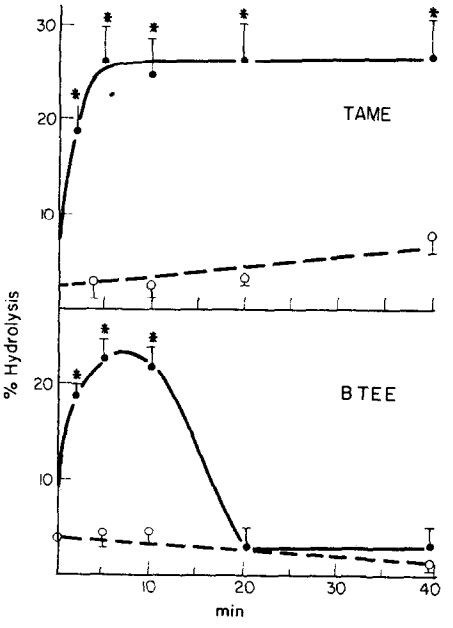

Fig. 1. Time course of the development of TAME and BTEE hydrolyzing activities in rat peritoneal fluid cells incubated in 2.7×10^{-6} M *l*-epinephrine (●), or in buffer (○). Results are averages of four experiments. An asterisk (*) indicates a statistically significant effect of epinephrine. Following activation for the periods shown, cells were allowed to act on the substrates for 30 min.

Figure 2 shows that the effect of epinephrine-activated cells on either BAEE or BTEE followed a non-linear course over the time interval examined; cells not exposed to epinephrine were ineffective with either substrate. The exponential character of the lines describing hydrolysis by activated cells contrasted with the linear pattern of hydrolysis observed (not shown) when trypsin and chymotrypsin were tested on their respective substrates. The 30-min period of activity of epinephrine-activated cells, chosen for the remaining experiments, represents the maximal time interval over which such activity could be demonstrated.

Figure 3 compares the efficiency of epinephrine with that of the histamine releaser compound 48/80, in generating BAEE and BTEE hydrolysis in rat peritoneal fluid cell suspensions following a 5-min incubation period. The molarity units used for compound 48/80 correspond to the content of the monomeric building unit, *p*-methoxyphenethyl methyl amine, of this compound. Epinephrine was considerably more effective as an esterase activator than compound 48/80 over the range of concentrations examined.

To establish the nature of the cells contained in

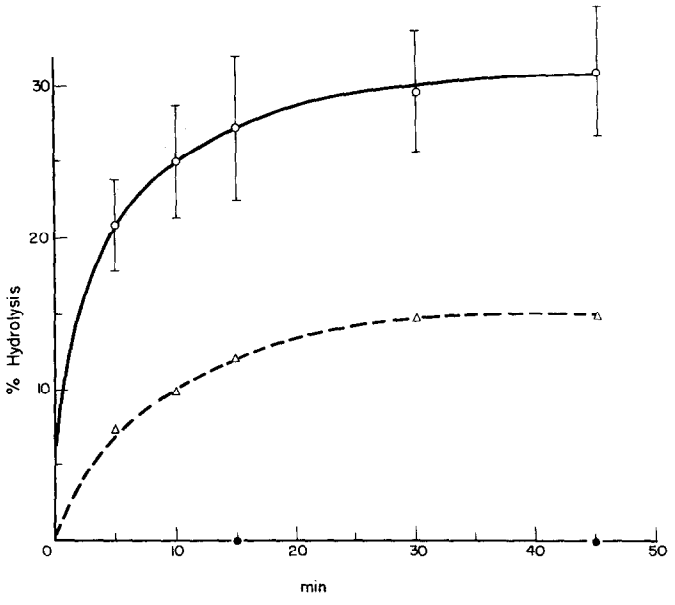

Fig. 2. Time course of the action on BAEE and BTEE of epinephrine pre-activated (5 min, 2.7×10^{-6} M catecholamine) rat peritoneal fluid cells. Key: (○——○) activity on BAEE (four experiments); and (△---△) activity on BTEE (single experiment). Activities of cells preincubated 5 min in buffer (●) were uniformly zero.

rat peritoneal fluid responsible for esterase generation, the cells were submitted to differential centrifugation [11]. The results presented in Table 1 show that only mast cells could generate arginine or tyrosine ester hydrolase activity following exposure to either epinephrine or compound 48/80. Other peritoneal fluid cells, employed at the same concentration at which they had been present in incubates of non-fractionated cells, were not activated by either 48/80 or epinephrine; differential counting had shown that their qualitative distribution was not different from that prevailing prior to fractionation. A surprising, unexplained finding shown in Table 1 was the higher esterase activity with BAEE caused by compound 48/80 in purified mast cells, compared to corresponding activity in non-fractionated cells. It is not clear to what this may be due. Although, following fractionation, mast cells were washed to remove excess fractionation medium, traces of this fluid must have been added together with the cells to the substrates. The fractionation medium which was employed [11] contained bovine serum albumin and heparin, which are a potential source of esterases and a rat plasma esterase activator respectively [13]. Nevertheless, the enhanced activity of purified mast cells cannot be caused by esterolytic effects of contaminating fractionation medium *per se*; additional experiments showed it to be devoid of esterolytic activity with either BAEE or BTEE. Also, had this medium been, by itself, the cause of the observed effect, one would expect it also to affect the control or epinephrine-activated cells. The results shown in Table 1 indicate that this was not the case, and one is led to conclude that the purification procedure, which was employed, specifically sensitized mast cells to the esterase-generating activity of compound 48/80.

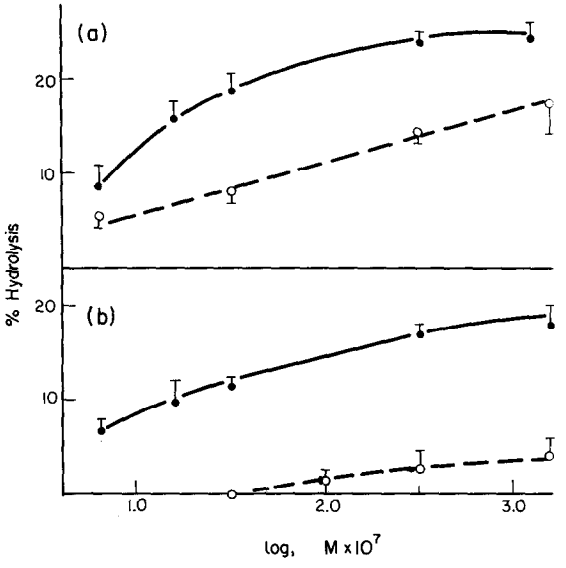

Fig. 3. Esterolytic activity on BAEE (panel a) and on BTEE (panel b) acquired by peritoneal fluid cells after 5 min exposure to different concentrations of epinephrine (●), or compound 48/80 (○). Values shown are averages of four experiments; they have been corrected for activities shown by controls incubated in buffer, which were as follows: 0.25 + 0.25 per cent (N = 8) for BAEE, and 1.81 per cent (N = 8) for BTEE. Concentrations of activators are expressed in terms of the log of the molarity $\times 10^7$ at which these substances were present during incubation.

The effects on BAEE and BTEE of epinephrine-treated peritoneal fluid cell suspensions bore a relationship to their mast cell content. Figure 4 illustrates these findings and suggests that factors retarding the process appear at higher mast cell concentrations.

Table 1. Mast cell origin of the esterolytic activities generated by *l*-epinephrine or compound 48/80 in rat peritoneal fluid cells*

Source	Cells in incubates Content per ml × 10^{-3} Mast cells	Non-mast cells	Treatment	Activity (% hydrolysis) BAEE	BTEE
Non-fractionated peritoneal fluid	41.2 ± 2.4	1287 ± 60	Control	0.0	2.7 ± 0.2
			Epinephrine	23.5 ± 2.7†	14.6 ± 2.1†
			48/80	10.4 ± 1.5†	6.9 ± 1.9†
Albumin layer	41.2 ± 2.2	15 ± 2	Control	7.4 ± 1.1	3.4 ± 0.9
			Epinephrine	27.2 ± 3.6†	22.5 ± 1.0†
			48/80	44.5 ± 3.6†	25.0 ± 1.0†
Interfacial layer	0.0	1275 ± 55	Control	1.3 ± 0.5	3.6 ± 0.8
			Epinephrine	3.8 ± 0.3	3.8 ± 0.7
			48/80	7.4 ± 1.0†	3.4 ± 0.9

* After centrifugation, the lower (albumin) layer was diluted 10-fold with MCM medium [11], and its cells were sedimented by a 5-min centrifugation at 60 *g*, washed once with fresh medium, and suspended in the desired amount of Krebs–Ringer phosphate buffer. Non-mast cells, collected at the interface between the albumin and buffer layers, were removed by careful aspiration, sedimented by centrifugation, washed and resuspended in buffer. Esterolytic activity was generated by incubating the different cell fractions for 5 min in the presence of epinephrine (2.7×10^{-6} M) or compound 48/80 (5.0 μg/ml). Results refer to averages of four experiments.

† Statistically significant ($P < 0.05$) effect of epinephrine or of compound 48/80.

This effect appeared especially noticeable when the hydrolysis of BTEE was followed. In view of these results, and since epinephrine or 48/80-generated esterase activities had been found to be associated only with mast cells, further investigations were performed with non-fractionated suspensions of rat peritoneal fluid cells.

Figure 5 shows that, although some of the esterolytic action on BAEE generated by epinephrine in mast cells was lost into the medium during incubation, the major part of it remained bound to the cells in a manner firm enough to withstand removal by repeated washing and centrifugation. The centrifugation conditions employed in the present study (5 min, 900 *g*) were similar to those followed by Lagunoff [14], and it is quite possible that, as proposed by this author, mast cell esterases reside in granules contained either within or adhering to such cells. BTEE esterolytic activity in cells decreased after each washing; since it was not recovered in the supernatant fractions, it appeared to have been progressively inactivated during incubation. A similar instability of this enzyme occurred in the absence of washing (Fig. 1).

Figure 6 illustrates that, at concentrations capable of generating cell esterases, epinephrine failed to cause histamine release from mast cells. Compound 48/80, on the other hand, produced a high release

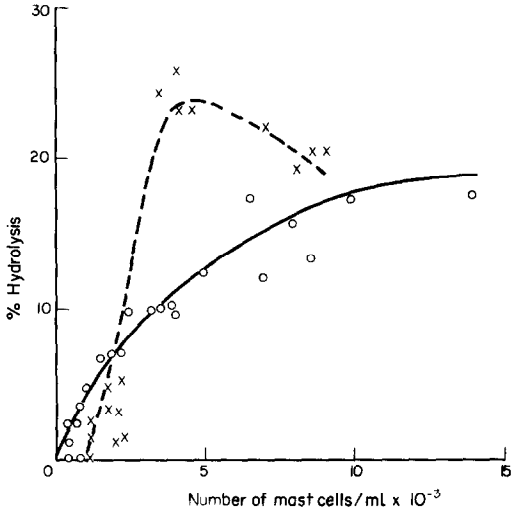

Fig. 4. Relationship between mast cell content and activity on BAEE (○) or BTEE (×) of peritoneal fluid cell suspensions which had been incubated for 5 min with 2.7×10^{-6} M epinephrine. Mast cells comprised between 2.3 and 8.3 per cent (mean 4.5 ± 0.6; N = 12) of peritoneal fluid cells present.

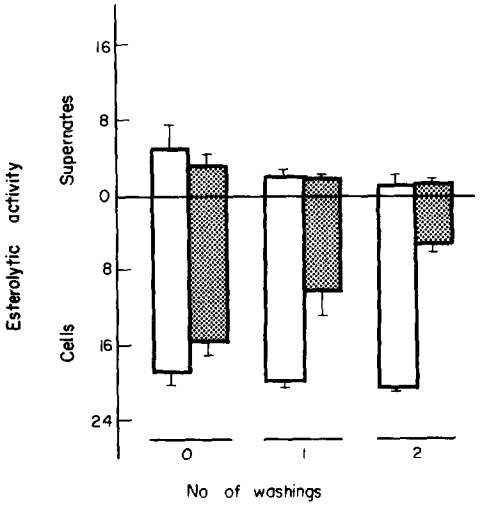

Fig. 5. Effect of washing by repeated centrifugation (5 min, 900 *g*) and resuspension in fresh medium (1 ml) on the esterolytic activity on BAEE □ or BTEE ▨ of rat peritoneal fluid mast cells which had been incubated for 5 min with 2.7×10^{-6} M epinephrine.

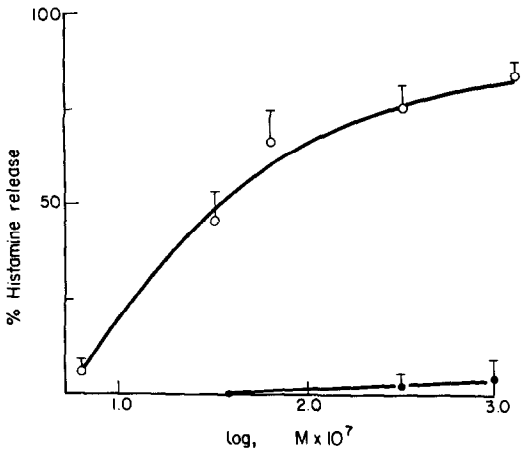

Fig. 6. Histamine release from rat peritoneal fluid mast cells after a 10-min exposure to different concentrations of epinephrine (●), or compound 48/80 (○). Values presented are averages of four experiments. Controls incubated in the absence of activators failed to release measurable amounts of histamine. Concentrations are expressed in terms of the log of the molarity $\times 10^7$ at which the active substances were present during incubation. In the case of compound 48/80, they are based on the content of *p*-methoxyphenethyl methyl amine of the compound.

of the amine at concentrations generating slight BAEE-esterolytic or BTEE-esterolytic activities.

The presence of epinephrine failed to inhibit the histamine-releasing action of compound 48/80. Additional experiments showed that, while 1 μg/ml of 48/80 released 57.7 ± 1.5 per cent of the amine from mast cells, the presence of epinephrine (2.7×10^{-6} M) caused this release to amount to 57.6 ± 5.6 per cent (N = 7), a statistically non-significant difference. A 10-fold increase in the concentration of the catecholamine did not change this result.

Table 2 shows that osmotic injury of mast cells, caused by exposure to hypotonic medium (0.05 M Tris buffer), results in appreciable loss of histamine, but the cells did not develop the capacity to hydrolyze BAEE. Similarly (Fig. 7), treatment of mast cells with amounts of compound 48/80, which caused intense release of histamine and modest activation of TAME esterase, did not prevent the cells from showing additional esterolytic activity following exposure to epinephrine.

Sensitivity to DFP is a characteristic property of active serine-proteases such as trypsin and chymotrypsin. As shown in Table 3, the esterolytic activities generated by epinephrine in rat mast cells were inhibited extensively by 5×10^{-3} M DFP.

Figure 8 shows that the bromine analogue of cGMP produced considerable activation of BAEE

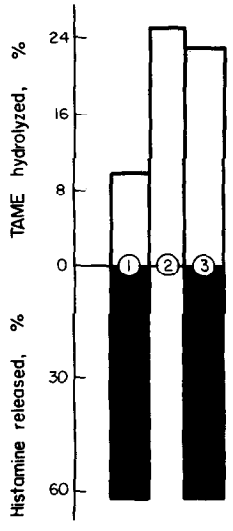

Fig. 7. Generation of TAME-esterase and release of histamine in rat peritoneal fluid mast cells treated in succession with 5.0 μg/ml of compound 48/80 and 2.7×10^{-6} M epinephrine. Bar 1: cells exposed only to 48/80; bar 2: cells exposed only to epinephrine; and bar 3: cells exposed first to 48/80 and then to epinephrine. Values are averages of two experiments. Each drug was allowed to act on cells for 10 min.

Table 2. Effect of the permanence of peritoneal fluid mast cells in a hypotonic medium on their histamine content and their esterolytic activity prior to and after subsequent incubation with epinephrine*

Cells Pretreatment	Treatment	Histamine loss (%)	Esterolytic activity (% BAEE consumed)
Hypotonic medium,† 10 min, 37°		46.0 ± 8.0	1.6 ± 0.7
Hypotonic medium,† 10 min, 37°	Epinephrine		20.0 ± 1.8‡
Isotonic medium,§ 10 min, 37°		6.0 ± 2.9	2.5 ± 0.8
Isotonic medium,§ 10 min, 37°	Epinephrine		17.3 ± 2.0‡

* Following pretreatment (10 min at 37°), cells were centrifuged, resuspended in Krebs–Ringer phosphate buffer, and incubated for 5 min at 37° in the presence or the absence of epinephrine (2.7×10^{-6} M). Results are averages of six experiments.

† 0.05 M Tris, pH 7.8.

‡ Statistically significant ($P < 0.01$) effect of epinephrine.

§ 0.15 M Tris, pH 7.8.

Table 3. Sensitivity to DFP of TAME and BTEE esterase activities generated by epinephrine in peritoneal fluid cells of the rat*

Incubation	Activity (% consumed) TAME	BTEE
Epinephrine (2.7×10^{-6} M)	20.2 ± 3.4	23.5 ± 4.6
DFP (5×10^{-3} M) + epinephrine	4.9 ± 1.4†	3.7 ± 1.0†

* Peritoneal fluid cells, suspended in Krebs–Ringer phosphate buffer, were incubated for 15 min at 37°. After triple centrifugation and washing, esterolytic activity was tested on each substrate.

† Statistically significant ($P \leq 0.01$) effect of DFP. Results are averages of six experiments.

and BTEE peritoneal fluid cell esterases. In contrast, the dibutyryl analogue of cAMP, while incapable of generating such activities, inhibited their generation by either 8-Br-cGMP or epinephrine. As shown in Table 4, neither 8-Br-cGMP nor diBu-cAMP released histamine from rat peritoneal fluid cells. Additional experiments (not presented), employing the fractionation procedure described in Table 1, showed that esterase generation by 8-Br-cGMP was only found in the mast cell fraction of rat peritoneal fluid cells. Thus, the effect of 8-Br-cGMP on mast cell esterases resembles that of epinephrine, regarding both its independence from the histamine-releasing process and its sensitivity to inhibition by cAMP.

Table 5 shows that, although capable of activating rat peritoneal fluid mast cells, neither *l*-norepinephrine nor *l*-isoprenaline was as effective in this sense as *l*-epinephrine; *dl*-isoprenaline was less effective, even when employed at twice the concentration of the endogenous amines.

No evidence identifying the action of *l*-epinephrine as directed toward either alpha- or beta-adrenergic receptors on the mast cell was obtained. The results, shown in the second part of Table 5, indicate that

Table 4. Histamine content of rat peritoneal fluid cells following incubation with 8-Br-cGMP, diBu-cAMP or compound 48/80*

Incubation	Histamine content† (μg/ml)
8-Br-cGMP (10^{-4} M)	4.0 ± 0.4 (6)
diBu-cAMP (10^{-4} M)	4.0 ± 0.4 (3)
Compound 48/80 (1 μg/ml)	1.8 ± 0.3‡ (3)
Buffer	4.0 ± 0.4 (6)

* Incubations were performed in Krebs–Ringer phosphate buffer, for 10 min at 37°. Figures in parentheses represent the number of experiments performed.

† Expressed in terms of histamine diphosphate; cell suspensions contained 2×10^{-5} ± 15% mast cells/ml.

‡ Statistically significant ($P < 0.05$) effect of compound 48/80.

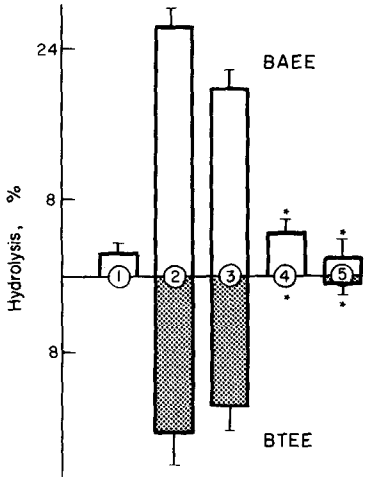

Fig. 8. Effect of 8-Br-cGMP, epinephrine and diBu-cAMP on the generation of BAEE and BTEE esterase activities in rat peritoneal fluid cells. Bar 1: cells exposed to diBu-cAMP, 10^{-4} M; bar 2: cells exposed to 8-Br-cGMP, 10^{-4} M; bar 3: cells exposed to epinephrine, 2.7×10^{-6} M; and bars 4 and 5: cells exposed to 8-Br-cGMP or epinephrine, respectively, in the presence of diBu-cAMP. Values are averages of four experiments. Each drug or pair of drugs was allowed to act on cells for 5 min at 37°. An asterisk (*) indicates a statistically significant effect of diBu-cAMP.

Table 5. Effect of sympathomimetic agonists and antagonists on the generation of BAEE esterase activity in rat peritoneal fluid cells*

Drug	Concn (M)	Activity (% BAEE consumed)
Control		1.9 ± 0.5 (8)
Agonists		
l-Epinephrine	2.7×10^{-6}	22.0 ± 1.8† (4)
l-Norepinephrine	2.7×10^{-6}	10.1 ± 0.8† (4)
l-Isoprenaline	2.7×10^{-6}	15.9 ± 1.0† (4)
dl-Isoprenaline	5.4×10^{-6}	8.5 ± 1.1† (4)
Antagonists		
l-Epinephrine + Phenoxybenzamine	2.7×10^{-6} + 1.8×10^{-5}	3.5 ± 0.5‡ (3)
l-Epinephrine + Propranolol	2.7×10^{-6} + 1.8×10^{-5}	6.2 ± 0.6‡ (3)

* Cells were incubated for 5 min with adrenergic agonists; antagonists were added 10 min prior to epinephrine. Figures in parentheses refer to the number of experiments performed.

† Statistically significant ($P < 0.05$) effect of agonists.

‡ Statistically significant ($P < 0.05$) effect of antagonists of epinephrine.

either phenoxybenzamine or propranolol at equimolar concentration prevented the mast cell BAEE-esterase activating action of the catecholamine.

DISCUSSION

The present results refer to hitherto undescribed responses of rat peritoneal fluid cells to epinephrine and compound 48/80, consisting of esterolytic activity toward aromatic and basic amino-acid esters. Since the peritoneal cavity of the rat contains at least four cell types (lymphocytes, monocytes, eosinophils and mast cells), an early objective of this study was the identification of the cell or cells providing the activated enzymes. For this purpose, mixed peritoneal cells were submitted to a fractionation procedure which segregates mast cells from other cells by evoking their selective sedimentation into a dense bovine serum albumin solution. It could be clearly shown that mast cells were responsible for the increased ability to hydrolyze the BAEE or BTEE, which was developed by peritoneal fluid cells in response to epinephrine or 48/80. Following fractionation in concentrated bovine serum albumin medium, mast cells proved to be considerably more responsive in terms of esterase activation, especially to compound 48/80, than they had been prior to fractionation. Since this effect was not due to the direct action of components of the medium, alternative ways of explaining it have to be sought. Mast cells harvested from the peritoneal cavity of rats represent a mixture of pre-existing free cells plus cells dislodged from loose binding to the surface of the peritoneal membranes [15]. It is conceivable that such cells present vacant protein and/or heparin binding sites which, unless filled, prevent cells from full interaction with compound 48/80. Exposure to the fractionation medium may replenish such sites, thus enhancing the sensitivity of the cells to the esterase-activating action of compound 48/80. This interpretation receives a certain amount of support from the observations of Uvnäs and Thon [16], which show that, unlike non-fractionated cells, mast cells separated in a high density medium (Ficoll) can only react to compound 48/80 with release of histamine when either cat serum or human serum albumin is incorporated into the cell-suspending medium. As in our experiments, the reason for the activating effects of such apparently quite unrelated proteins is unknown. Inhibitory influences from non-mast cell sources, present to a greater extent in non-fractionated peritoneal fluid cell suspensions, may also be contributing to the observed effects.

The mast cells are the most important site of storage and release of histamine in the body. The generation of esterase activity by epinephrine occurred without a concomitant release of histamine. Cells which had lost a substantial part of their bound histamine after exposure to a hypotonic medium were not esterolytically activated; they preserved their esterases in a latent state. They could be activated by secondary exposure to epinephrine. The esterases which are the subject of this study may reside in hydrophobically shielded structures, possibly within lipoid membranes, unaffected by a hypotonic environment. This suggestion receives support from results showing that, after activation by epinephrine, the esterases are not easily detached from the cell even by repeated washing. Unlike histamine, they are apparently fixed constituents of the mast cell.

The relation between epinephrine-activated mast cell chymotrypsin and mast cell 'chymase' [3] has not been determined. The latter is predominantly contained in a granule-rich fraction of mast cell sonicates or lysates [3, 5] which also contain most of the bound histamine of the cell. Nevertheless, no evidence of a concomitant release of histamine and chymase from 48/80-treated or otherwise stimulated intact mast cells seems to have been reported. Our results clearly show that mast cell esterase activation and histamine release are independent events; since electron-microscopic studies [17, 18] have suggested the existence of at least two types of granules in mast cells, the possibility that histamine and esterase reside in different granules deserves investigation. The release of histamine by compound 48/80 has been considered not to involve proteolytic mast cell enzymes, after it was shown [7] that DFP, a potent protease inhibitor, failed to block the process. Evidence of release of chymase from rat mast cells exposed to polymixin B, a histamine-releasing agent, has been reported [14]. The fraction of cell-bound enzyme released, however, was small in comparison to that of the histamine liberated. The release of these two mast cell components thus appeared not to be necessarily causally related. It has not been possible to determine whether the esterase activity arising in mast cells exposed to epinephrine, compound 48/80 or 8-Br-cGMP results from the generation of enzymes from inactive precursors or from an uncovering of active enzymes which, in the non-stimulated cells, are masked or otherwise prevented from gaining access to the substrate. The latter alternative may be the more likely; recent results (unpublished) have shown that morphological changes evoked by epinephrine in rat mesentery mast cells [19, 20] are reversible once the cell is removed from the influence of the catecholamine. It is possible that reversibility may explain the progressive loss of esterase activity observed in certain instances in epinephrine-treated cells. However, inhibition by products of hydrolysis or by other factors present in the incubates may also be occurring. It is of interest to note in this regard that mast cells seem to be the storage site of the Kunitz anti-protease also described under the name of Trasylol [21]. Further work will be required to clarify the reasons for the peculiar kinetic behavior of esterolytic factors arising from activated mast cells. Unless such data become available, results as clear-cut as those originating from work with soluble, pure mast cell enzymes dispersed in a homogeneous medium [4, 5] will not be obtained.

Although our results indicate the activation of a trypsin-like enzyme together with a chymotrypsin-like entity in rat mast cells, the existence of tryptic activity in such cells is not generally accepted [2]. This is to be expected; unstimulated peritoneal fluid cells do indeed have low to undetectable levels of arginine ester esterase. Darzynkiewicz and Barnard [22] have shown that tritiated DFP is preferentially

bound to sites located near or within the granules of the mast cell. These sites were considered to be the active centers of a chymotryptic enzyme, since considerable inhibition of labeling occurred in experiments in which DFP was employed together with chymotrypsin substrates such as BTEE or ATEE (*N*-acetyl-tyrosine-ester). Interestingly, the binding of DFP was also markedly reduced when, instead of these substrates, basic amino acid esters or amides, such as BAEE, TAME or BAA (*N*-benzoyl arginine amide), were used. Although these compounds are rather specific trypsin substrates, the findings did not lead the authors [22] to conclude that a trypsin-like enzyme exists in mast cells. Rather, they stated that the inhibitory effect of the substituted basic amino acid esters was due to the non-specific binding of substituted arginine substrates to the active center of mast cell chymase via the aromatic, *N*-benzoyl part of their molecules. Since in these experiments BAEE and TAME were more effective than either ATEE or BTEE in displacing labeled DFP, the binding of the basic amino acid esters must have been more intense than that of the chymotrypsin substrates proper. While this possibility cannot be disregarded, it seems equally valid to consider that the findings of Darzynkiewicz and Barnard [22], like ours, reflect the existence of tryptic enzymes in rat mast cells.

The effects of epinephrine were not reproduced, but were rather inhibited by diBu-cAMP, a compound considered to substitute adequately for endogenous cAMP in cell systems [23]. Sullivan *et al.* [11] have shown that increased levels of cAMP are achieved only after exposure of peritoneal fluid mast cells to concentrations of epinephrine 10- to 100-fold higher than those shown to be sufficient for the activation of mast cell esterases. Thus, although the most frequently described effect of epinephrine on cell metabolism is stimulation of adenyl cyclase [24], leading to increased levels of cell cAMP, this does not appear to be responsible for mast cell esterase activation.

In contrast to the cAMP derivative, the 8-bromo analogue of cGMP, reported [23] to be an adequate substitute for endogenous cGMP, reproduced the effect of epinephrine on mast cell esterases; like the catecholamine, its action was inhibited by diBu-cAMP. Changes in mast cell levels of cGMP, or perhaps of cGMP/cAMP ratios [25], may initiate effects of epinephrine leading to esterase activation. Measurements of changes in mast cell cyclic nucleotide levels, accompanying the action of epinephrine, will be required to substantiate this hypothesis.

Although changes in cyclic nucleotide-dependent enzymes seem to be involved in the histamine-releasing process [26], no report of an initiation of this event by exogenous cyclic nucleotides or their analogues has been published. The present results confirm this by showing that neither diBu-cAMP nor 8-Br-cGMP treatment of mast cells led to the release of histamine. These results emphasize the biochemical differences existing between the esterase-activating and histamine-releasing processes of the mast cell.

Compound 48/80 was able to partially reproduce the esterase-generating effect of epinephrine or 8-Br-cGMP. Different authors [27, 28] have shown that, along with its histamine-releasing action, compound 48/80 induces a lowering of mast cell cAMP levels. It is possible that, by causing an imbalance of cGMP/cAMP ratios, compound 48/80 creates a condition to which the cell responds with activation of its tryptic and chymotryptic esterases. This effect is less complete than that of epinephrine, since cells pretreated with 48/80 were shown to generate additional amounts of esterases following exposure to the catecholamine.

l-Norepinephrine and *l*-isoprenaline were capable of activating mast cell BAEE esterase, although less efficiently than *l*-epinephrine; *dl*-isoprenaline was nearly inactive. The action of epinephrine was sensitive to inhibition by either phenoxybenzamine or propranolol. In previous studies [10] related to those presently reported, the action of epinephrine was examined in a system composed of rat plasma rendered reactive to the catecholamine by the presence of mast cell-containing peritoneal fluid cells. Epinephrine caused cell-mediated consumption of rat plasma kininogen and activation of plasma arginine ester hydrolase. These effects were partially reproduced by norepinephrine, but not by *dl*-isoprenaline; they were inhibited by propranolol and, somewhat more effectively, by phenoxybenzamine. The absence of a clear-cut defintion of the alpha- or beta-adrenergic nature of mast cell receptors for epinephrine was considered to reflect the none-too-selective pharmacological activity of propranolol, which includes the ability to affect the alpha-adrenergic receptors [29]. In view of the present results, it is probably best to admit that mast cell targets of the action of epinephrine behave like mixed alpha–beta receptors [30], presenting slight predominance of alpha-adrenergic properties.

Newball *et al.* [31] reported recently that human peripheral leukocytes can be immunologically activated to release a BAEE-splitting enzyme which cleaves bradykininogen yielding kinin. In a preliminary report [32], we have shown that human whole blood acquires kininogen splitting activity following contact with epinephrine. Since blood basophils are in some respects the circulatory counterparts of tissue mast cells, it appears that the investigation of conditions leading to the activation of basophilic or mast cell esterases may reveal hitherto unknown functions of these cells, resulting not only from pathological stimuli, like the antigen–antibody reaction, but also from physiological influences, e.g. a catecholamine discharge.

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REFERENCES

1. K. F. Austen, S. I. Wasserman and E. J. Goetzl, in *Molecular and Biological Aspects of the Acute Allergic Reaction* (Eds. S. G. O. Johansson, K. Strandberg and B. Uvnäs), p. 293. Plenum Press, New York (1976).
2. E. P. Benditt and M. Arase, *J. exp. Med.* **110**, 451 (1959).

3. D. Lagunoff and E. P. Benditt, *Ann. N.Y. Acad. Sci.* **103**, 185 (1963).
4. J. Kawiak, W. H. Vensel, J. Komender and E. A. Barnard, *Biochim. biophys. Acta* **235**, 172 (1971).
5. R. Yurt and K. F. Austen, *J. exp. Med.* **146**, 1405 (1977).
6. B. A. V. Perera and J. L. Mongar, *Immunology* **6**, 478 (1963).
7. B. A. V. Perera and J. L. Mongar, *Immunology* **6**, 472 (1963).
8. D. Lagunoff, E. P. Benditt and R. Watts, *J. Histochem. Cytochem.* **10**, 672 (1962).
9. G. G. Glenner, V. K. Hopsu and L. A. Cohen, *J. Histochem. Cytochem.* **10**, 109 (1962).
10. A. M. Rothschild, A. Castania and R. S. B. Cordeiro, *Naunyn-Schmiedeberg's Arch. Pharmac.* **285**, 243 (1974).
11. T. J. Sullivan, K. L. Parker, W. Stenson and C. W. Parker, *J. Immun.* **114**, 1473 (1975).
12. M. E. Brown, *J. Lab. clin. Med.* **55**, 616 (1960).
13. A. M. Rothschild and L. A. Gascon, *Nature, Lond.* **212**, 1364 (1966).
14. D. Lagunoff, *Biochem. Pharmac.* **21**, 1889 (1972).
15. D. L. Wilhelm, L. C. J. Young and L. G. Watkins, *Agents Actions* **8**, 146 (1978).
16. B. Uvnäs and I. Thon, *Expl Cell Res.* **18**, 512 (1960).
17. D. Lagunoff, M. T. Phillips, O. A. Iseri and E. P. Benditt, *Lab. Invest.* **13**, 1331 (1964).
18. G. D. Bloom and O. Haegermark, *Acta physiol. scand.* **71**, 257 (1967).
19. M. P. Oliveira and A. M. Rothschild, *Nature, Lond.* **218**, 382 (1968).
20. A. M. Rothschild, E. L. Tamburús and A. Castania, *Com. VII Intern. Congr. Pharmac.*, Paris (1978).
21. H. Fritz, J. Kruck, I. Rüsse and H. G. Liebich, *Hoppe-Seyler's Z. physiol. Chem.* **360**, 437 (1979).
22. Z. Darzynkiewicz and E. A. Barnard, *Nature, Lond.* **213**, 1198 (1967).
23. L. N. Simon, D. A. Shuman and R. K. Robins, *Adv. Cyclic Nucleotide Res.* **3**, 225 (1973).
24. G. A. Robison, R. W. Butcher and E. W. Sutherland, in *Cyclic AMP* (Eds. G. A. Robison, R. W. Butcher and E. W. Sutherland), p. 146. Academic Press, New York (1971).
25. N. D. Goldberg, M. K. Haddox, S. E. Nicol, D. B. Glass, C. H. Sanford, F. A. Kuehl, Jr. and R. Estensen, *Adv. Cyclic Nucleotide Res.* **5**, 307 (1975).
26. B. B. Fredholm and B. Uvnäs, in *Molecular and Biological Aspects of the Acute Allergic Reaction* (Eds. S. G. O. Johansson, K. Strandberg and B. Uvnäs), p. 229. Plenum Press, New York (1976).
27. E. Gillespie, *Experientia* **29**, 447 (1973).
28. T. J. Sullivan, K. L. Parker, S. A. Eisen and C. W. Parker, *J. Immun.* **114**, 1480 (1975).
29. R. F. Furchgott, in *Handbook of Experimental Pharmacology* (Eds. H. Blaschko and E. Muscholl), Vol. 33, Chap. 9, p. 283. Springer, Berlin (1972).
30. G. A. Robison, R. W. Butcher and E. W. Sutherland, in *Cyclic AMP* (Eds. G. A. Robison, R. W. Butcher and E. W. Sutherland), p. 305. Academic Press, New York (1971).
31. H. H. Newball, R. W. Berninger, R. C. Talamo and L. M. Lichtenstein, *J. clin. Invest.* **64**, 457 (1979).
32. J. Assreuy Filho, C. A. Flores, F. Q. Cunha, M. A. Martins, H. N. Vasconcelos, R. S. B. Cordeiro and A. M. Rothschild, Comm. XXXI Ann. Meet. Braz. Assoc. Adv. Science, Fortaleza (1979). *Cienc. Cult.* **31**, 637 (1979).