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REV 2871* (CHBZ): A POTENT ANTIALLERGIC AGENT WITH A NOVEL MECHANISM OF ACTION

II. STUDIES ON THE MECHANISM OF ACTION

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Abstract—REV 2871 (CHBZ) was taken up by rat mast cells and human leukocytes in a specific and saturable manner. The compound can be hydrolyzed by a granule-associated enzyme in the mast cell to an ionic metabolite (REV 3579) whose *in vitro* profile is identical to that of disodium cromoglycate (DSCG). REV 3579, although achieving millimolar concentrations inside cells incubated with CHBZ, was not itself taken up by rat mast cells or human leukocytes. The unusual *in vitro* activity of CHBZ is postulated to arise from the fact that it is a prodrug for delivering a DSCG-like drug to the interior of a secretory cell. The internalized drug apparently exerts a more general and longer-lived inhibition of the secretory process than it can by acting on exterior membrane receptors. CHBZ thus represents a novel drug for studying anaphylactic responses *in vitro*.

Much has been learned recently concerning early biochemical events involved in the immunologically mediated secretion of histamine from rat and human mast cells and human basophils. Bridging of the IgE receptor elicits an immediate increase in phospholipid methylation and a transient rise in cellular levels of cAMP [1], which may be parallel responses to the cross-linkage of the IgE receptor [2]. These events are followed by the influx of calcium into the cell, thereby activating phospholipases which hydrolytically release intracellular arachidonic acid [3]. The antiallergic drug disodium cromoglycate (DSGG§) [4] inhibits the uptake of calcium by mast cells [5], and has been reported to bind to a specific binding site on the outer membrane of rat basophilic leukemia cells (RBL-2H3) and mast cells [6, 7]. These authors have also shown that DSCG binds calcium in methanol. More recently, Mazurek *et al.* [8] have proposed that the DSCG-binding site constitutes the IgE-receptor-coupled calcium channel in RBL-2H3 cells. DSCG has been shown recently in mast cells to induce the phosphorylation of a 78,000 dalton protein, which is thought to be part of the endogenous secretion-control system [9, 10].

In the preceding paper [11], the novel activity profile of REV 2871 (2-ethoxyethyl 5-chlorobenzoxazole-2-carboxylate; eclazolast; CHBZ) as an inhibitor of immunologically and non-immunologically mediated secretion of histamine from rat mast cells, human basophils and guinea pig lung was compared and contrasted with those of DSCG and proxicromil.‖ In this paper we describe investigations into the mechanism of action of CHBZ *in vitro*, particularly with respect to its locus of activity and the active form of the drug.

MATERIALS AND METHODS

Chemicals. CHBZ and REV 3579, in unlabeled and ^{14}C-labeled forms, were synthesized in the Medicinal Chemistry Department at the Revlon Health Care Group¶; combusion analyses were consistent with the assigned structures (see Fig. 1). The [^{14}C]substitution was at the number 2 position of the oxazole ring of CHBZ and REV 3579. Radiolabeled CHBZ and REV 3579 had specific activities of 5.18 and 6.27 μCi/mg respectively; [^{14}C]Rev 3579 was sometimes generated by a 20-sec treatment of [^{14}C]CHBZ with 1 mM NaOH followed by neutralization. Metrizamide was purchased from the Accurate Chemical Corp. (Hicksville, NY). Solvents and other chemicals were of the highest grade commercially available.

Rat peritoneal cells and purification of mast cells. Rat peritoneal cells were obtained by peritoneal lavage with cell isolation buffer (150 mM NaCl, 2.7 mM KCl, 1 mM $CaCl_2$, 0.1% calf skin gelatin and 1 mM NaH_2PO_4, pH 7.0) of rats anesthetized with CO_2 followed by decapitation. Mast cells were purified by isopycnic sedimentation through Metrizamide according to Coutts *et al.* [13].

* Also designated in the literature as RHC 2871.

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§ Abbreviations: CHBZ, 2-ethoxyethyl 5-chlorobenzoxazole-2-carboxylate; DMSO, dimethyl sulfoxide; DSCG, disodium cromoglycate; EDTA, ethylenediamine tetraacetic acid; and HEPES, *N*-2-hydroxyethyl-piperazine-*N'*-2-ethanesulfonic acid.

‖ A comparison of the *in vitro* profile of CHBZ with that of DSCG will involve the use of terms "DSCG-like" and "non-DSCG-like" or "CHBZ-like". Although these terms are explained in this and the preceding paper [11], a more complete discussion of the use of *in vitro* models of anaphylaxis to profile inhibitors of mediator release is given by Weinryb *et al.* [12].

¶ Revlon Health Care Group R & D was merged into Rorer Central Research in 1986.

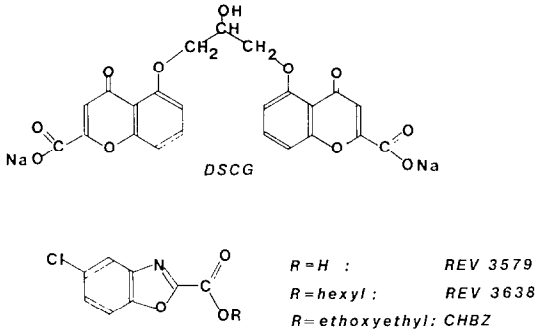

Fig. 1. Structures of DSCG-like compounds (DSCG and REV 3579) and non DSCG-like compounds (REV 3638 and CHBZ) discussed in this paper.

Human leukocytes. Leukocytes were separated from venous blood of volunteers according to Khandwala *et al.* [14].

Uptake of CHBZ by mast cells and human leukocytes. The uptake of ^{14}C-labeled CHBZ by rat peritoneal cells, purified rat mast cells and human leukocytes was determined as follows. Rat peritoneal cells, mast cells purified over Metrizamide or human leukocytes ($3.5 \cdot 10^6$ mast cells or leukocytes/ml; 60 μl) were suspended in assay buffer (10 mM piperazine-*N*,*N*'-bis 2-ethane sulfonic acid, 2.7 mM KCl, 150 mM NaCl, 1 mM $CaCl_2$ and 0.1% gelatin, pH 7.0) and were incubated with test compound (in DMSO; final concentration of DMSO was 1% in the assay tube) or buffer for 5 min at 37°. [^{14}C]CHBZ or [^{14}C]REV 3579 (7.5 mM in DMSO, diluted with assay buffer just before addition to achieve a final concentration of 1–100 μM) was added and the incubation was continued for an additional 5 min at 37°. The final volume was 0.5 ml. The assay tubes were then placed on ice, and the cells were immediately washed twice with 10 ml of ice-cold cell isolation buffer. The washed cells were resuspended in 0.5 ml of 0.04 M NaOH and placed in boiling water for 5 min. The content of each tube was mixed with 10 ml of scintillation fluid, and the radioactivity was determined by scintillation spectrometry.

Preparation of crude rat mast cell esterase. Mixed peritoneal cells, harvested from ten rats, were purified over Metrizamide. Purified cells were counted, resuspended in 1.0 ml of 10 mM *N*-morpholinoethane sulfonic acid (pH = 6.0), and sonicated. The sonicate was centrifuged at 8000 *g* for 5 min, the supernatant fraction was collected and saved, and the pellet was resuspended in 1.0 ml of *N*-morpholinoethane sulfonic acid. The esterase content was assayed spectrophotometrically at 25° in a pH 7.6 buffer containing 0.1 M NaH_2PO_4, 1 mM dithiothreitol, 1 mM EDTA, and 150 μM CHBZ. Approximately 4×10^5 cell equivalents per 1.0 ml volume were used in the kinetic study, and the hydrolysis was observed at 314 or 275 nm.

Calculation of half-lives. The hydrolysis of CHBZ to REV 3579 was analyzed as a pseudo-first-order reaction [15], according to equation (1), where $CHBZ_0$ is the concentration of CHBZ at 0 time, k_{app} is the apparent rate constant, A^0_{314} and A^t_{314} are the initial absorbance and absorbance at time t (314 nm), respectively, and 0.0038 is a constant to convert optical changes to nmoles/ml of hydrolyzed ester.

$$k_{app}\, t = \ln (CHBZ_0/[CHBZ_0 + (A^t_{314} - A^0_{314})/0.0038]) \quad (1)$$

Data from these reactions were analyzed by least squares.

Isolation of esterase from mast cell granules. The procedure followed for the isolation of secretory granules from mast cells and the dissociation of chymase from the heparin core was essentially as described by Yurt and Austen [16]. Briefly, rat peritoneal mast cells were purified over Metrizamide as described above (*ca.* 85% purity). The washed cells (14×10^6) were resuspended in distilled water, adjusted to pH 7.1 with 20 μM NaOH, at a cell density of 10^6/ml. After agitation for 5 min at room temperature (RT), the suspension was centrifuged at 400 *g* for 10 min to remove large cellular debris, and then sedimented at 3000 *g* for 20 min at 4°. The pellet was washed in 2.0 ml of 10 mM NaH_2PO_4 (pH 7.1) for 15 min at RT, and repelleted at 3000 *g* for 20 min (low salt wash). The pellet was then resuspended in 1.0 ml of a pH 7.1 solution of 1.0 M NaCl and 50 mM HEPES, and stired for 25 min at RT. The sample was frozen and thawed six times, sonicated briefly, and then sedimented at 13,000 *g* for 10 min; the supernatant fraction (high salt wash, containing the solubilized granule) was saved for assay.

Fractionation of the cellular constituents of rat mast cells. Metrizamide-purified rat mast cells (85%; *ca.* 12×10^6 mast cells) were incubated in 2.0 ml of cell isolation buffer containing 30 μM [^{14}C]CHBZ for 10 min at 37°. The labeled cells were washed twice with 10.0 ml of cell isolation buffer at RT and were resuspended in 1.0 ml of the same buffer. Aliquots of 20 μl were taken for the determination of cell-associated radioactivity. The remaining cells were sonicated and divided into 5×100 μl aliquots, diluted with 900 μl of cell isolation buffer, and centrifuged at 100 to 100,000 *g* for 40 min.

All supernatant fractions were divided into two equal portions of 400 μl each. Pellets were resuspended in 1.0 ml of cell isolation buffer, and two 400-μl aliquots were taken for counting. All aliquots were treated with 20 μl of 1 M NaOH, and boiled for 5 min, followed by the addition of 10 ml of scintillation fluid. The radioactivity content was determined by scintillation spectrometry.

RESULTS

Uptake of [^{14}C]CHBZ and [^{14}C]REV 3579 by rat peritoneal cells, rat mast cells and human leukocytes. [^{14}C]CHBZ and [^{14}C]REV 3579 (each 100 μM) were incubated with equal numbers (3×10^5/tube) of both unpurified (*ca.* 10%) and purified (82%) mast cells. Substantially more ester (CHBZ) than acid (REV 3579) was able to cross cell membranes ($P < 0.005$); over 50-fold more radioactivity remained associated with washed mast cells when incubated with CHBZ (7.9 ± 1.1 nmoles/10^6 cells) than with REV 3579 (0.15 ± 0.07 nmoles/10^6 cells). This ratio was over 100 for mixed peritoneal cells.

Table 1. Uptake of 100 μM [^{14}C]CHBZ and [^{14}C]REV 3579 by mixed rat peritoneal cells and purified rat mast cells, before and after purification

Cells	CHBZ uptake* (nmoles/10^6 cells)	REV 3579 uptake* (nmoles/10^6 cells)	CHBZ/REV 3579 ratio
Mixed peritoneal cells†	7.9 ± 0.2	0.15 ± 0.01‡	53
Mast cells preincubated with labeled compounds, followed by washing and purification over Metrizamide (94% purity)	5.5 ± 1.1	0.040 ± 0.028	137
Mast cells purified over metrizamide (85% purity) followed by incubation with labeled compounds and then washed	4.01 ± 0.03§	0.039 ± 0.007	103

* Values are means ± SD.
† Preparation contained *ca.* 10% mast cells.
‡ Significantly different from CHBZ uptake ($P < 0.005$).
§ No difference from cells purified after incubation with labeled material ($P < 0.05$).

The uptake of [^{14}C]CHBZ and [^{14}C]REV 3579 by rat peritoneal cells, followed by purification of the mast cells by isopycnic sedimentation through metrizamide, was compared with the uptake by mast cells which were *first* purified and then incubated with labeled compounds. All cells were taken from the same pool of mixed peritoneal cells. These data are summarized in Table 1. Once more, CHBZ was 50- to 100-fold more avidly taken up by cells than was its acid analogue, REV 3579. Statistically equivalent amounts of radiolabeled material were associated with washed mast cells when they were incubated with [^{14}C]CHBZ before *or* after purification over Metrizamide ($P < 0.05$).

Titration of rat mast cells and human leukocytes with CHBZ. Other experiments (data not shown) had also demonstrated that human leukocytes took up the ester [^{14}C]CHBZ but not the acid (REV 3579), similar to the mast cell system. Leukocytes contain approximately 1% basophils (which, like the mast cell system, are capable of IgE-mediated release of histamine), which are purified only with low yield and great difficulty, so no experiments were performed to measured the uptake of CHBZ by basophils.

Up to this point all uptake experiments had been carried out with a single concentration of ^{14}C-labeled ligand. Therefore, both purified rat mast cells and human leukocytes were incubated for 5-min periods with increasing concentrations of [^{14}C]CHBZ. Titration curves were constructed for nmoles of CHBZ incorporated per million cells versus concentration of CHBZ, and are displayed in Fig. 2. Uptake achieved a plateau at concentrations >200 μM for both preparations of cells.

Kinetics of uptake. The rates at which [^{14}C]CHBZ is absorbed and concentrated in purified rat mast cells and human leukocytes were measured in parallel experiments. A maximum uptake of the compound was achieved between 5 and 15 min of incubation before washing both preparations of cells (Fig. 3). The rate of loss of labeled material with longer periods of incubation was quite gradual, but was relatively more rapid with human leukocytes than with purified mast cells.

Competition of uptake of [^{14}C]CHBZ by unlabeled chlorbenzolate. Mast cells were incubated with increasing concentrations (100–300 μM) of CHBZ for 5 min, followed by the addition of [^{14}C]CHBZ (26 μM) for an additional 5 min. As a control, another group of cells was also preincubated with 300 μM REV 3579 before the addition of [^{14}C]CHBZ. The cell-associated radioactivity was then determined. The percent inhibition of nmoles of [^{14}C]CHBZ taken up per million mast cells is shown in Fig. 4. Whereas unlabeled REV 2871 inhibited uptake of [^{14}C]REV 2871, REV 3579 provided no competition, i.e. did not inhibit uptake of the ester.

Averaged data for uptake and inhibition of uptake of CHBZ. A large number of experiments have been performed in measuring uptake of [^{14}C]CHBZ both by rat mast cells and, to a lesser extent, by human leukocytes. The actual concentration of labeled CHBZ used in each experiment varied slightly (15–30 μM), and the nmoles taken up per 10^6 cells varied more significantly. Consequently, the data were normalized by dividing the nmoles/10^6 cells by the micromolar concentration of [^{14}C]CHBZ in the experiment. In addition, the mean percent inhibition given by 300 μM unlabeled CHBZ, when measured, was also determined. On the average, the preincubation of 10^6 mast cells or human leukocytes with 25 μM [^{14}C]CHBZ led to the uptake of 4.9 ± 0.3 (N = 44) or 6 ± 1 (N = 10) (mean ± SE) nmoles of the ester respectively. Preincubation of mast cells or human leukocytes with 300 μM CHBZ inhibited uptake of labeled CHBZ by 90 ± 0.3 and 68 ± 3% (N = 9) respectively.

One might well argue that the inhibition of uptake by unlabeled CHBZ was merely a non-specific, isotope-dilution phenomenon. This possibility was tested by incubating purified mast cells with increasing concentrations of CHBZ for 5 min, followed by washing the cells twice with 2 ml buffer, and then adding [^{14}C]CHBZ. The resultant inhibition curve (Fig. 5) shows that CHBZ was a less effective inhibitor of uptake of ^{14}C-labeled CHBZ by mast cells in this protocol. Nevertheless, concentration-dependent inhibition was observed, with an apparent plateau at 48 ± 1% inhibition occurring at 300 μM CHBZ.

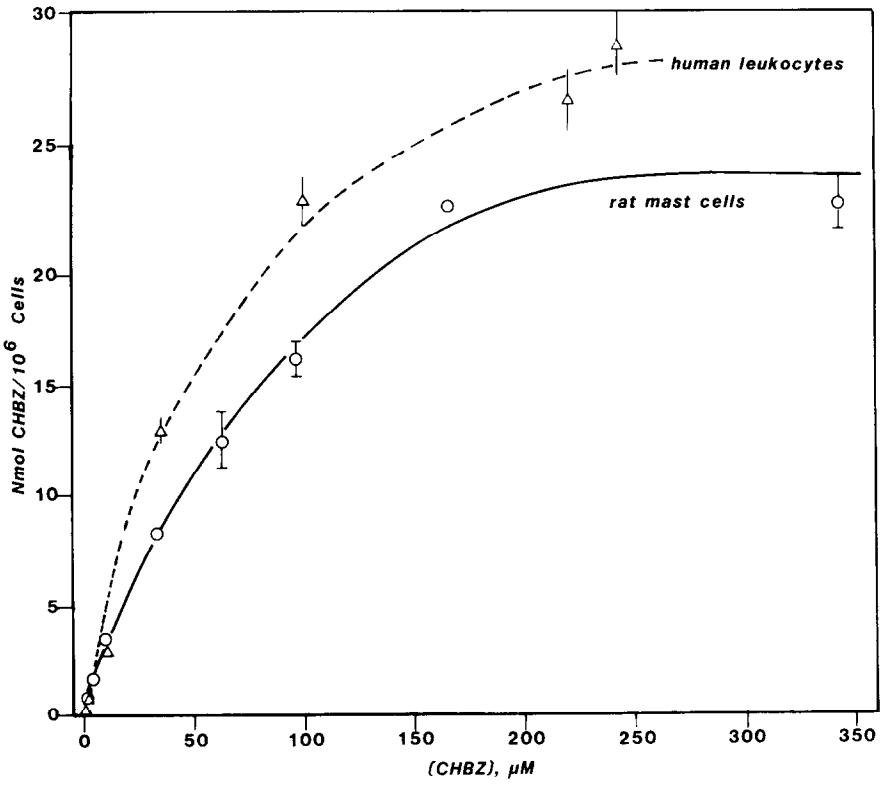

Fig. 2. Uptake of [^{14}C]CHBZ by purified rat peritoneal mast cells (○) and human leukocytes (△). The mean purity ± SD of mast cells was 89 ± 4%.

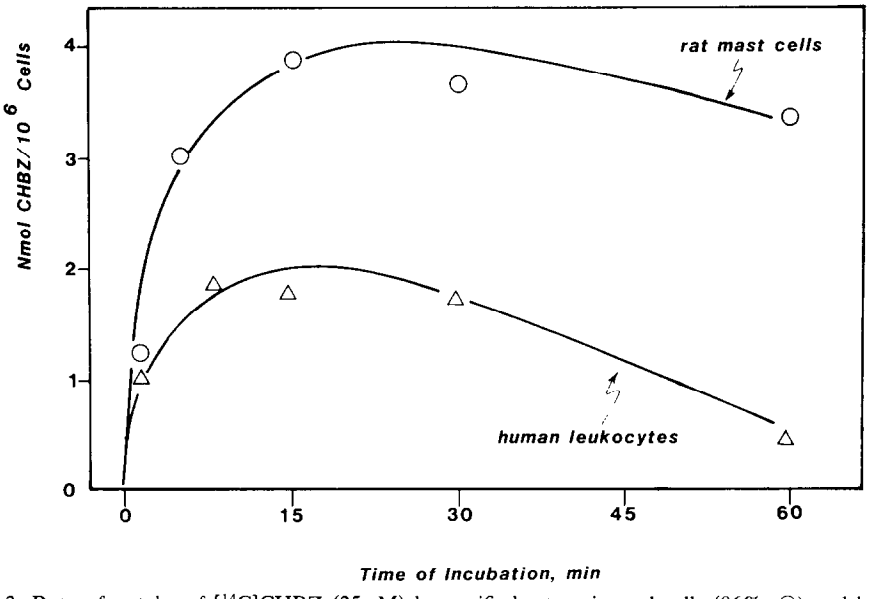

Fig. 3. Rate of uptake of [^{14}C]CHBZ (25 μM) by purified rat peritoneal cells (96%; ○) and human leukocytes (△).

Competition by an ester analogue. As additional evidence to support the proposal that the competitive inhibition of uptake of [^{14}C]CHBZ by unlabeled CHBZ is specific, a hexyl ester analogue of CHBZ, REV 3638, was employed as the inhibitor. The structure of REV 3638 is given in Fig. 1 and the results of this assay are shown in Fig. 6. REV 3638, which is a different compound than CHBZ and would not inhibit non-specific uptake by isotopic dilution, clearly did inhibit uptake, reaching a maximum of 66% inhibition at 100 μM. Higher inhibition was not observed, likely due to the low solubility of the compound. By comparison, 100 μM CHBZ inhibited uptake only slightly more, 71%.

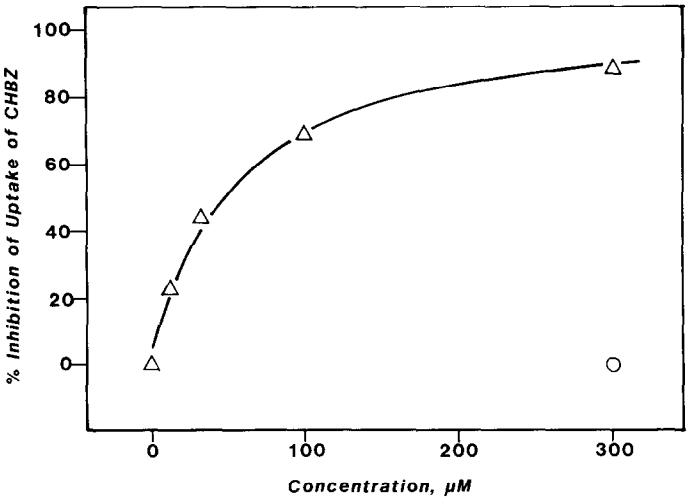

Fig. 4. Inhibition of uptake of [^{14}C]CHBZ (26 μM) by purified rat peritoneal mast cells (73%) by unlabeled CHBZ (△) and REV 3579 (○). Untreated cells took up 3.69 ± 0.03 nmoles CHBZ per million cells.

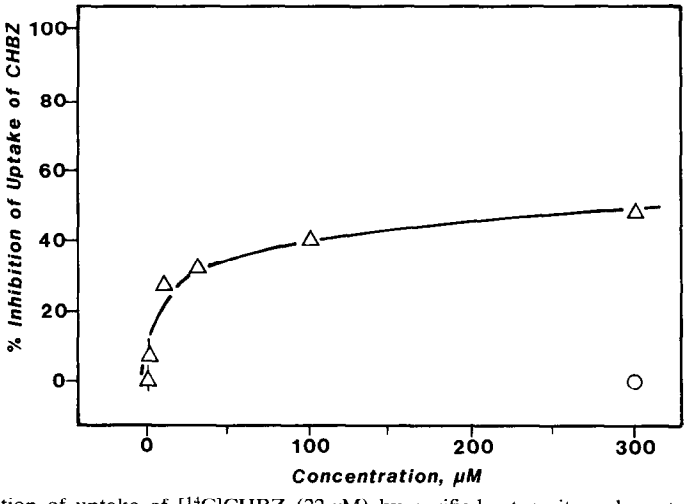

Fig. 5. Inhibition of uptake of [^{14}C]CHBZ (22 μM) by purified rat peritoneal mast cells (93%) by unlabeled CHBZ (△) and REV 3579 (○) after washing the cells following 5-min preincubation with inhibitor. Untreated cells took up 5.15 ± 0.05 nmoles CHBZ per million cells.

Inhibition of uptake by acylating or alkylating agents. A concern arose that the inhibition of uptake of CHBZ might be due to non-specific acylation or alkylation of cellular components, in spite of the fact that the metabolite of CHBZ, REV 3579, remains soluble in the cell (see below). This hypothesis was tested by examining the effect of acetylsalicylic acid (1–300 μM) and iodoacetamide (30 μM) on the uptake assay. No significant inhibition by either compound (<22% inhibition at any concentration) was observed.

Localization of [^{14}C]REV 3579 within the mast cells. Purified rat peritoneal mast cells were incubated with 30 μM [^{14}C]CHBZ, and the cells were washed and sonicated. The sonicate was sedimented at several different speeds (100, 1,000, 3,000, 15,000 and 100,000 *g*) to separate the cellular constituents into different fractions. The amount of radioactivity associated with each pelleted fraction and its supernatant fraction was determined. The results show that >98% of the radioactivity taken up by the cells remained soluble inside the cell, i.e. was not covalently bound to any sedimentable subcellular structure (>12.6 nmoles/10^6 cell equilvalents in the supernatant fraction and ≤0.26 nmoles/10^6 cell equivalents in any pellet).

Hydrolysis of CHBZ in mast cells. The purified mast cell was examined for the presence of an enzymatic activity that could account for the rapid conversion of CHBZ to REV 3579. This was accomplished by measuring the change in absorbance at 314 nm of solutions of CHBZ; a decrease in absorbance at wavelengths between 250 and 320 nm was associated with hydrolysis. It was initially found that crude sonicates of purified rat mast cells caused a decrease in absorbance at 314 nm. This was not a general property of proteases, since of six commercially available enzymes examined (trypsin, papain, chymotrypsin, ficin, pronase and elastase) at a final concentration of ≥0.1 mg/ml, only pronase

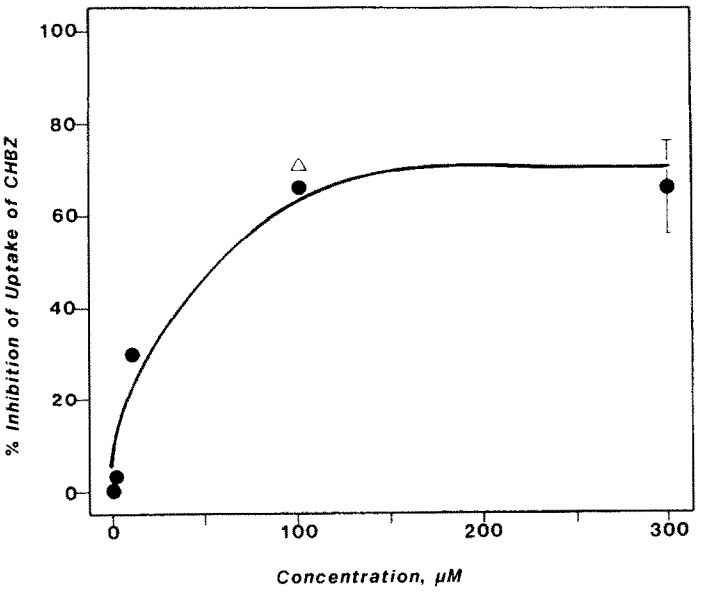

Fig. 6. Inhibition of uptake of [^{14}C]CHBZ (26 μM) by purified rat peritoneal mast cells (88%) by REV 3638 (●), the hexylester analogue of CHBZ, and unlabeled CHBZ (△); control cells took up 5.22 ± 0.06 nmoles of CHBZ per million cells.

and elastase showed esterolytic activity toward CBHZ (data not shown). Additional experiments at pH 7.6 with sonicates of mixed peritoneal and purified mast cells from the rat showed that: (a) the esterase activity in the purified mast cells (81% mast cells) sedimented at 8000 *g* and was not soluble under these conditions (8000 *g* supernatant from 4×10^5 equivalents of purified cells gave an initial rate of hydrolysis equal to the spontaneous rate, <2.6 nmoles/min; the resuspended 8000 *g* pellet from the same number of cell equivalents gave an initial rate of hydrolysis of 39.2 nmoles/min) and (b) esterase activity was also present in the 8000 *g* pellet of sonicated mixed peritoneal cells (roughly 8% mast cells), but in approximately 40-fold lower amounts (5-fold more cell equivalents gave one-eighth of the initial hydrolysis rate).

Additional experiments demonstrated that: (a) no catalysis was observable with the mast cell enzyme at pH 6.0 (b) the rate of hydrolysis was not measurably affected by the inclusion of the product, REV 3579, and (c) the absorbance after completion of the hydrolysis was equivalent to the absorbance of a mixture of the same concentration of REV 3579 and mast cell enzyme. To investigate the cellular localization of the esterolytic activity seen in these experiments, secretory granules from lysed, purified rat peritoneal mast cells were washed with low and high salt solutions, and the fractions were assayed for esterolytic activity (Table 2). Under the gentle conditions of opening the cells by osmotic shock in distilled water and washing the granules, all esterolytic activity (above the buffer-catalyzed hydrolysis rate of $0.0075A_{314}$/min, relative rate of 1.0) was associated with the secretory granules and eluted with high salt in a manner similar to the mast cell chymase.

Additional preparations of granule-associated CHBZ esterase were examined in more detail by kinetic analysis. Fig. 7A shows the hydrolysis of 150 μM CHBZ by granule-associated esterase from purified (85%) mast cells (3×10^5 cell-equivalents/assay) at 25°. CHBZ was rapidly (half-life of 4.4 min) and completely hydrolyzed to absorbance values equivalent to that of 150 μM REV 3579 plus the same amount of enzyme. The addition of a second bolus of CHBZ was accompanied by an immediate rise in absorbance at 314 nm, followed by a similar decrease in absorbance due to hydrolysis (half-life of 3.9 min), demonstrating catalytic turnover by the enzyme preparation. The pseudo-first-order plots for these curves are shown in Fig. 7B. The average half-life ± SD calculated from five experiments performed at 25° with granule-associated enzyme from rat mast cells was 4.76 ± 0.97 min. The spontaneous rate of hydrolysis under the same conditions (pH 7.6, 25°) yielded a half-life of 30.6 ± 12.5 min, giving a ratio of half-lives of roughly 6-fold as a measure of enzyme-catalyzed hydrolysis. The rate of hydrolysis was faster when the granule-associated esterase was assayed with CHBZ at 37° (half-life of 2.47 min). The buffer-catalyzed rate also increased, but the

Table 2. Hydrolysis of CHBZ (150 μM) by components of purified rat mast cells (85% purity)

Source of enzyme	Relative rate of hydrolysis*
Blank (no enzyme)	1.0
Cell debris (400 *g* pellet after osmotic shock)	1.0
Supernatant from low salt (10 mM sodium phosphate) wash of granules	1.0
Supernatant from high salt (1.0 M NaCl) wash of granules	7.2

* Rate of spontaneous hydrolysis in buffer = 1.

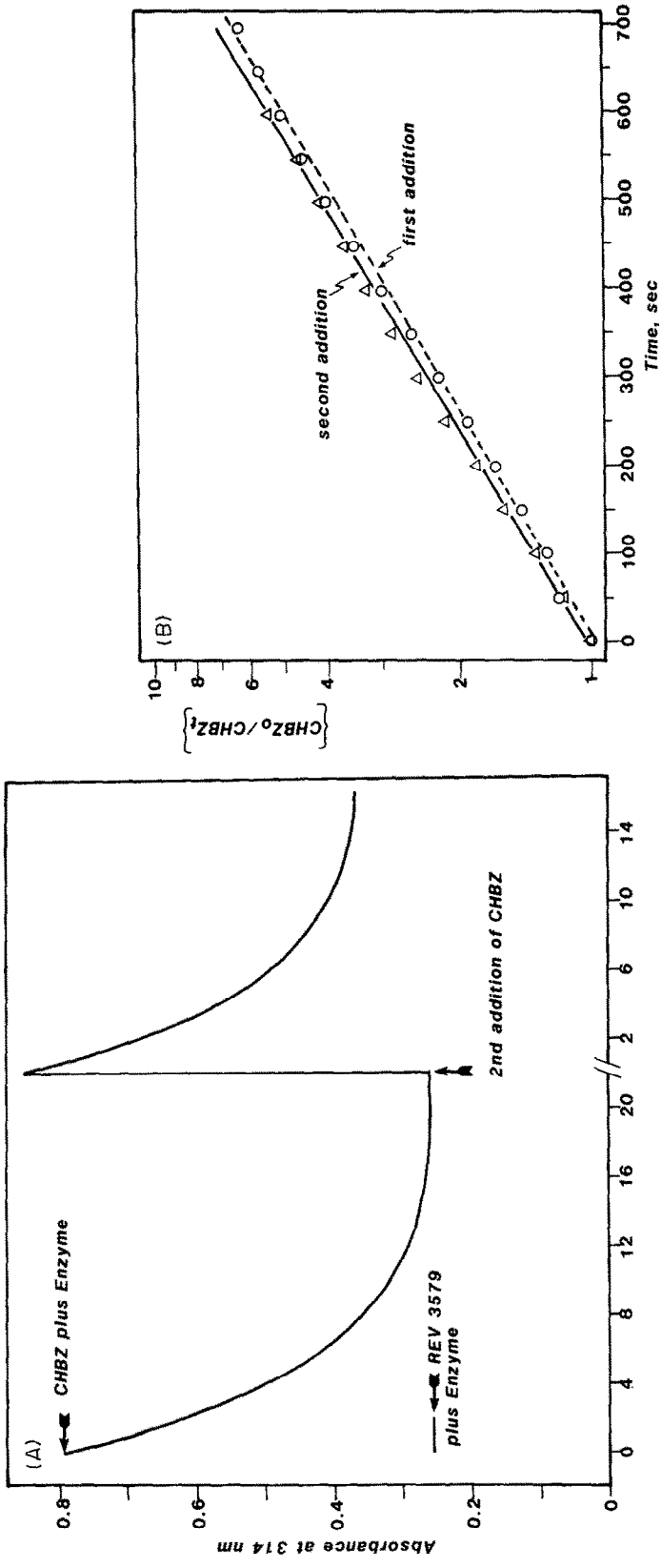

Fig. 7. Hydrolysis of CHBZ (150 μM) by esterase activity obtained from granules of purified rat peritoneal mast cells (3×10^5 cell equivalents per ml) at 25°. (A) Ultraviolet tracings as a function of time after addition of enzyme; addition of 150 μM CHBZ was followed by a second addition after reaction was complete. (B) Pseudo-first-order plots of data in part A.

ratio of the buffer-catalyzed to enzyme-catalyzed half-life remained the same (6-fold).

DISCUSSION

Data presented in a companion paper by Khandwala *et al.* [11] have demonstrated the unique *in vitro* profile of CHBZ, a mediator-release inhibitor. CHBZ differs from the standard antiallergic drug DSCG in several important respects: it exerts long-lived inhibition of both immunologic and non-immunologic secretion of histamine from rat mast cells; it is not tachyphylactic nor cross-tachyphylactic with DSCG; and it inhibits both immunologic and non-immunologic release of histamine from human leukocytes.

Uptake of CHBZ. We have sought in the present work to investigate the molecular basis for CHBZ's antiallergic activity and its difference from DSCG. The first clue came from experiments in which CHBZ was found to be avidly concentrated by both rat peritoneal cells and purified rat mast cells. It has been reported that mast cells and basophils have a binding site for DSCG on their membranes, and that conjugates of DSCG that are physically incapable of entering the cells do nonetheless inhibit degranulation [6]. It has also been suggested that the DSCG-binding protein plays a role in the influx of calcium which accompanies IgE-mediated secretion [8, 17]. The action of DSCG in our hands is reversible, i.e. removed by washing the cells, so there is firm evidence that DSCG exerts its effect on secretion by a membrane-level interaction. We report here that, whereas the lipophilic ester, CHBZ, partitioned into mast cells, the uptake of the DSCG-like hydrolysis product, REV 3579, was 50- to 100-fold less (Table 1). Furthermore, the uptake of [^{14}C]CHBZ was long-lived, i.e. not removed by washing the cells. Indeed, when mixed peritoneal cells were incubated with CHBZ, washed and layered over Metrizamide, the purified mast cells obtained from the Metrizamide layer had as much or more radioactivity associated with them on a per cell basis as did purified mast cells that were incubated with CHBZ after purification. Thus, cell-associated radioactivity survived four washing steps and an isopycnic sedimentation. The high internal concentration of equivalents of CHBZ per cell thus suggests a major difference between CHBZ and DSCG. It was also found that mixed rat peritoneal cells and human leukocytes incorporated CHBZ to about the same extent as did purified mast cells. Because of our interest in IgE-mediated secretion and because of the ease with which rat peritoneal mast cells may be purified, we chose to perform most of our experiments with the mast cell system.

A noteworthy aspect of the uptake of CHBZ by both rat mast cells and human leukocytes is that the uptake reached saturation at approximately 200 μM drug (Fig. 2). These results implied that uptake was a finite process, more akin to a titration of binding sites than a simple partitioning of a drug based on hydrophobicity. When purified mast cells were incubated with [^{14}C]CHBZ for 5 min, followed by a short washing procedure and sonication of the cell pellet, the sole radioactive product found associated with the cells was the hydrolysis product REV 3579, a carboxylic acid [18]. Thus, although REV 3579, a compound with an *in vitro* profile identical to DSCG, is not taken up by cells, it is the sole product found inside mast cells incubated with CHBZ.

The uptake of CHBZ by rat mast cells and human leukocytes was dependent on time, requiring 5–10 min to reach a maximum. The kinetic curves in Fig. 3 display a remarkable resemblance to the dependence of percent inhibition of histamine release as a function of time of preincubation [11], which imply a parallelism of uptake and antisecretory phenomena. The fact that maximum uptake and inhibition of histamine release take several minutes to achieve also implies the existence of secondary events, e.g. enzymatic action, as part of the internalization and inhibitory actions. Diffusion-controlled processes, such as the binding of a small molecule to a receptor outside the cell, would be expected to occur much more rapidly at these concentrations.

If the cellular uptake of CHBZ is specific and saturable, one would expect that preincubation of cells with unlabeled drug would inhibit the incorporation of labeled metabolite inside the cell. This is indeed the case (Fig. 4), since the uptake can be inhibited completely by a 10-fold excess of unlabeled CHBZ. A significant finding is that neither REV 3579, the DSCG-like metabolite of CHBZ found inside the mast cell, nor any standard antiallergic agent tested, e.g. DSCG, PRD-92EA, FPL-55712, and FPL-57787 [18], was unable to inhibit the uptake of CHBZ by purified mast cells. The inhibition of incorporation of [^{14}C]CHBZ by the unlabeled molecule was not merely isotope dilution, for cells that were preincubated with the unlabeled drug followed by washing were still inhibited from taking up the radiolabeled analogue (Fig. 5). Greater inhibition in these wash-out experiments was seen when cells were preincubated for up to 60 min with unlabeled compound before washing the cells, again implying a time-dependent phenomenon.

Since the concept that the uptake of CHBZ is a specific and saturable phenomenon is important in the analysis of its mechanism of action, it was important to establish that the inhibition by unlabeled CHBZ represented a specific molecular interaction as well. Thus, it was found that different ester analogues of CHBZ also inhibited its uptake. For example, REV 3638, the hexyl ester of REV 3579, was equipotent to unlabeled CHBZ in inhibiting the uptake of the ^{14}C-labeled drug (Fig. 6). Indeed, it has become clear from experiments with several other compounds which have a similar non-DSCG-like biological profile to CHBZ but which are from different chemical classes, that inhibition of uptake of [^{14}C]CHBZ does not require compounds that are esters of 5-chlorobenzoxazole-5-carboxylic acid [18].

What is the concentration of REV 3579 inside the cell? The titration curve for uptake of [^{14}C]CHBZ by purified rat mast cells (Fig. 2) allows a calculation of the intracellular concentration of REV 3579, the only radioactive product found associated with cells, after making an assumption about the average size and shape of a rat peritoneal mast cell. The typical peritoneal mast cell has a diameter of 15 microns

Table 3. *In vitro* profiles of DSCG-like and non-DSCG-like inhibitions of mediator release*

Class of compound	Inhibition of histamine release from: Rat mast cells: Immunologic 0 min	Immunologic 5 min	Non-immunologic 0 min	Non-immunologic 5 min	Human leukocytes	Guinea pig lung slices	Tachyphylaxis	Uptake by rat mast cells
DSCG-like agent	+	−	−	−	−	−	+	−
Non-DSCG-like agent (CHBZ)	+	+	+	+	+	−	−	+
Hybrid	+/−		+/−		+/−	+/−	+/−	+/−

* A plus sign (+) indicates inhibition, and a minus sign (−) indicates lack of inhibition.

and can be approximated by a sphere. One may then calculate the volume occupied by one million mast cells to be, on the average, $(15 \times 10^{-3}\,\text{mm})^3 \times 10^6/6 = 1.77\,\text{mm}^3$ or μl. When the added concentrations of REV 2871 are 1.6, 10 and 100 μM, the concentration of REV 3579 *inside* the cell is calculated to be 0.49, 2.0 and 9.2 mM respectively. The extent of the resulting concentration gradient is obtained by dividing the concentration of REV 3579 inside the cell by the concentration of [^{14}C]CHBZ outside the cells at the start of the experiment. This ratio varies from 300- to 38-fold, being greatest where the titration curve has the largest slope (at low concentrations of CHBZ). Since the concentration of CHBZ necessary to inhibit 50% of the IgE-mediated secretory response in *unpurified* rat peritoneal cells is about 2 μM [11], the I_{50} value for CHBZ equivalents (REV 3579) inside the cell is approximately 600 μM.

The avid and effectively irreversible uptake of CHBZ by mast cells and leukocytes thus results in a relatively high intracellular concentration of the DSCG-like metabolite REV 3579. The difference in the *in vitro* biological activity seen between DSCG and CHBZ apparently arises from the fact that DSCG operates from without, i.e. at the membrane level of the cell, while CHBZ is a prodrug for delivering a high concentration of a DSCG-like drug to the inside of the cell. Could the REV 3579 inside the mast cell be covalently attached to cellular components? This seemed unlikely since radioactive material extracted from CHBZ-treated mast cells chromatographed on thin layers as intact REV 3579. Additional experiments showed that only 1–2% of the radioactivity in CHBZ-treated cells was associated with sedimentable components. The ratio of supernatant to pellet radioactivity did not change significantly as a function of *g*-force used to pellet the components, which is consistent with the inference that virtually all incorporated REV 3579 is soluble.

Hydrolysis of CHBZ. How does CHBZ accomplish intracellular delivery of drug to this extent? The fact that no CHBZ could be detected in the washed mast cells prompted us to examine the cells for enzymes that might catalyze the hydrolysis of CHBZ to REV 3579. It was found that particulate material from sonicated mast cells was able to catalyze the conversion of CHBZ to REV 3579, and that mast cells contained about 40-fold more of this activity on a per cell basis than did other peritoneal cells. Furthermore, the resuspended pellet from purified mast cells (330,000 cell equivalents) catalyzed hydrolysis at a rate equivalent to 0.1 mg/ml of the non-specific protease pronase. Interestingly, no esterolytic activity was found in the supernatant fraction from sonicated cells, implying that the enzymatic activity was localized in a sedimentable organelle of the cell, e.g. the secretory granule. The concept was strengthened by reports of neutral proteolytic enzymes in granulocytes [19] and in mast cells [20, 21] which are associated with heparin and histamine markers (i.e. in the secretory granule). When granules from sonicated purified mast cells were isolated and the granule-associated enzymes were solubilized by use of 1 M sodium chloride (a procedure shown by Yurt and Austen [16], to release mast cell chymase from isolated granules), it was shown that the esterase activity against CHBZ was associated with the granule and could be eluted with high salt (Table 2; Fig. 7). The rat mast cell was thus shown to have a granule-associated enzyme that catalyzes the hydrolysis of CHBZ to REV 3579. One interpretation of these data is that the neutral ester can apparently cross the cellular membrane of the mast cell, while the acidic metabolite finds it much more difficult to diffuse back out, thus leading to a concentration gradient and a high concentration of REV 3579 inside the cell.

Comparison of CHBZ and DSCG. CHBZ, an ester derivative of a DSCG-like drug, is thus concentrated inside the mast cell in the form of its DSCG-like metabolite. The most logical explanation for the difference in the *in vitro* profiles between the two drugs is the difference in the loci of action, i.e. inside versus outside the cell, although the details as to how this happens are unclear. A general comparison of inhibitory properties of DSCG-like versus non-DSCG-like (i.e. CHBZ-like) compounds is summarized in Table 3. It is conceivable that "hybrid" molecules possessing a mixture of the properties from the two classes of agents might also exist. CHBZ thus represents a novel compound which can

be used in the analysis of IgE- and non-immunologically mediated secretion from mast cells and basophils, as well as a potentially useful therapeutic agent when administered directly to the target organ.

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