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SPECTROSCOPIC STUDIES OF RAT MAST CELLS, MOUSE MASTOCYTOMA CELLS, AND COMPOUND 48/80—III

EVIDENCE FOR A PROTEIN BINDING SITE FOR COMPOUND 48/80

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Abstract—The interaction of compound 48/80 with mast cells, mastocytoma cells, isolated membranes and liposomes has been studied using electron spin resonance (e.s.r.) and light-scattering techniques. Unlike spin-labeled stearic acid, spin-labeled 48/80 (SL-48/80) bound to mastocytoma cells in a manner that was unaffected by the non-ionic detergent, Triton X-100. In contrast, sodium dodecylsulfate (SDS), a protein denaturant, altered the SL-48/80 binding site(s), causing an apparent increase in the motion of the spin label. Treatment of SL-48/80-labeled mast cells with trypsin also increased the apparent motion of the bound label. Binding of 48/80 to mast cells, mastocytoma cells, erythrocyte ghosts, and isolated mastocytoma cell membranes increased their respective light-scattering properties. In contrast, the Rayleigh scatter of protein-free liposomes was unaffected by 48/80 treatment. Cationic, anionic, and non-ionic detergents also increased the light-scattering properties of mastocytoma cells, in contrast to the effect of several other cationic drugs, including histamine liberators. Increased light-scattering occurred after 48/80 treatment of inhibited mast cells (treated at 5°), indicating that the effect was not a consequence of degranulation. Untreated cells heated above 40° showed a similar increase in light scattering that became irreversible after reaching 45°. This indicates that cells treated with 48/80 resemble those in which the proteins have been denatured by heat. The light-scattering and the e.s.r. data, therefore, are consistent with the hypothesis that 48/80 binds to proteins in biological membranes.

High molecular weight polymers, which constitute the active fractions of compound 48/80, bind to several natural and artificial membrane systems [1,2]. Our previous studies, using both unlabeled and spin-labeled 48/80, have shown that, although 48/80 does not cause a permanent change in the fluidity of membrane lipids [3], it does bind to both mast cells and mastocytoma cells with high affinity [4]. Since the binding of 48/80 to mast cells initiates membrane fusion that is outwardly similar to the allergic reaction and may also be a model for the secretory response [5], the molecular mechanism of action of this agent is of considerable interest.

In this study, the interactions of compound 48/80 with mast cells, mastocytoma cells, isolated membranes, and liposomes have been compared. Using e.s.r.‡ we have shown that the spectrum of SL-48/80 bound to cell membranes is significantly different from that of spin-labeled fatty acids which intercalate among the membrane lipids. We have also studied the light-scattering properties of intact cells, isolated membranes, and liposomes in the presence and absence of 48/80. The data are consistent with the idea that 48/80 binds to the membrane proteins of mastocytoma cells and mast cells.

MATERIALS AND METHODS

Cell and membrane preparation. Rat peritoneal and pleural cells, purified mast cells, and mastocytoma cells were obtained as described previously [3]. Mastocytoma cell membranes were prepared from mastocytoma cells that had been washed three times. The cells were lysed by freeze-thawing and sonication. They were then centrifuged at 400 *g* for 10 min at 1–2° and the pellet was discarded. The supernatant fraction was centrifuged at 5000 *g* for 20 min at 1–2° and the pellet was discarded. The membranes in the supernatant fraction were pelleted by centrifugation at 50,000 *g* for 50 min. The supernatant fraction was discarded and the pellet was resuspended in 10 mM phosphate buffer (pH 7.4). Examination of this preparation using light microscopy indicated that it contained a crude fraction of membranes. The membrane lipids were extracted by shaking an aqueous membrane suspension with 20 vol. of chloroform–methanol (2:1) for 15 min in a separatory funnel. Distilled water was then added and the mixture was shaken for 15 min more. After phase separation, the organic phase was collected and the solvent was evaporated. The lipids were suspended in phosphate buffer (10 mM, pH 7.4) and sonicated with a Kontes Ultrasonic Cell Disrupter (set on 5) for 1 min.

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‡ Abbreviations: e.s.r., electron spin resonance; 5-DSA, 5-doxyl stearic acid; SDS, sodium dodecylsulfate; SL-48/80, spin-labeled 48/80; and NEM, *N*-ethylmaleimide.

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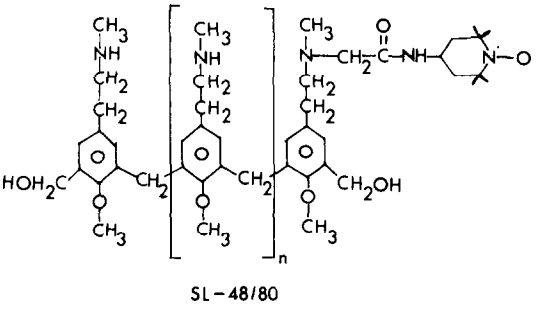
Human erythrocyte ghosts were prepared by the method of Dodge *et al.* [6] and the sulfhydryl groups were labeled with spin-labeled maleimide using the procedure of Sandburg *et al.* [7]. Protein (2.5 to 3.0 mg/ml) was measured according to the method of Lowry *et al.* [8]. This stock solution was diluted 1:10 for light-scattering measurements. Liposomes were prepared from a Human Erythrocyte Type Phospholipids Kit 6202 [2.4 μM lecithin (bovine), 2.0 μM sphingomyelin (bovine), 1.0 μM phosphatidyl serine (bovine), 0.08 μM cardiolipin (bovine), and 0.08 μM phosphatidyl inositol (plant); P-L Biochemicals, Inc., Milwaukee, WI]. The chloroform was evaporated from 1 ml of lipid solution that was then resuspended in phosphate buffer and sonicated in the same manner as described above.

Chemicals. SL-48/80 and unlabeled 48/80 were synthesized as described previously [4]. Spin-labeled stearic acid (5-DSA) and 4-maleimido-2,2,6,6-tetramethylpiperidinooxyl were purchased from Syva Associates, Palo Alto, CA.



Spectral measurements. Electron spin resonance spectra were recorded as described previously [3] using a Varian E-4 spectrometer or a Varian E-109 spectrometer equipped with a TM_{110} cavity.

The 90° light scatter from cell and membrane preparations was measured using a Perkin–Elmer SPF-44A spectrophotofluorimeter equipped with a corrected spectra accessory. The samples were

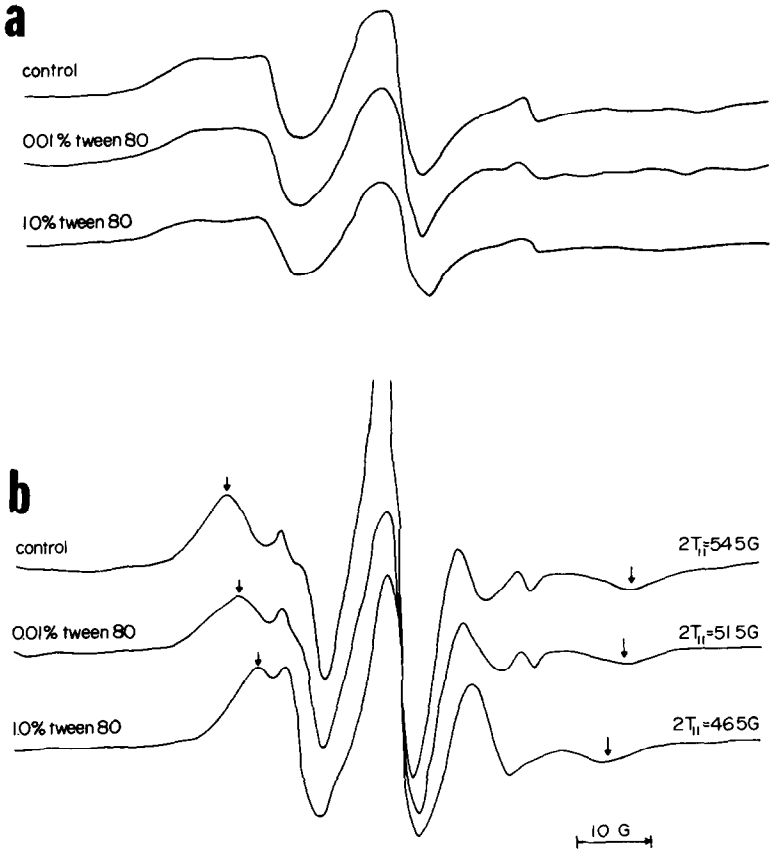


Fig. 1. Effect of Tween-80 on the e.s.r. signal from 5-DSA and SL-48/80 bound to mastocytoma cells. Panel a: SL-48/80 (500 μg/ml) was added to the washed (3) mastocytoma cells (10^7) which were then incubated for 5 min. The cells were centrifuged and resuspended in fresh Locke's solution. The control scan was recorded and Tween-80 was added to reach the concentrations indicated (intermediate concentrations were also recorded and the spectra were similar to the above). Panel b: 5-DSA (1×10^{-5} M) was added to washed (3) mastocytoma cells (10^7) and the e.s.r. spectra were scanned. Tween-80 was then added to reach the concentrations indicated (intermediate concentrations were also recorded, which showed a progressive increase in $2T_{\parallel}$ with increasing concentration of Tween-80).

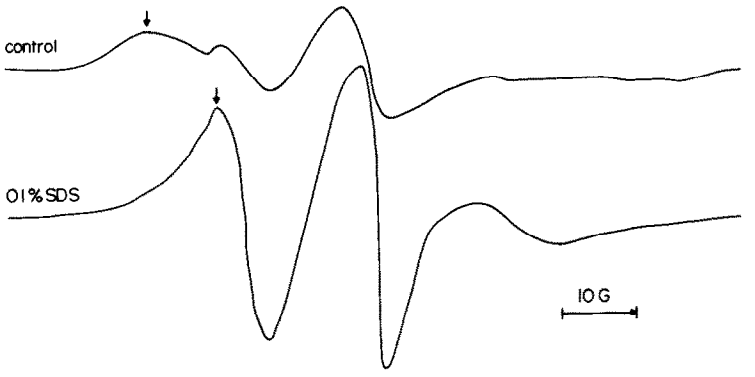


Fig. 2. Effect of SDS on SL-48/80 bound to mastocytoma cells. Mastocytoma cells (10^7) were labeled with SL-48/80 (125 μg/ml) for 5 min, centrifuged, and resuspended in fresh Locke's solution. The control signal was scanned, SDS was added and the signal was re-scanned immediately.

scanned from 200 nm to about 450 nm with both monochromators set at the same wavelength so that only Rayleigh scatter was measured.

RESULTS

*Electron spin resonance studies.*The e.s.r. spectrum of SL-48/80 bound to mastocytoma cells (Fig. 1a) was similar to that obtained in our previous study [4]. The apparent motion of the spin label was unaffected by treatment of the cells with the non-ionic detergent, Tween-80 (Fig. 1a). In contrast, the motion of the intercalated fatty acid spin label, 5-DSA, was increased significantly by the detergent (Fig. 1b) as evidenced by a decrease in the separation ($2T_{11}$) between the outer hyperfine extrema. It should be noted that 5-DSA was not displaced from the membranes by Tween-80 since the supernatant fraction was devoid of any e.s.r. signal. SDS, an anionic detergent, caused a large increase in the motion of SL-48/80 bound to the cells (Fig. 2). The supernatant fraction was also free of SL-48/80, indicating that the observed changes were not a result of displacement or of detergent solubilization but rather were a result of an alteration in the characteristics of the bound SL-48/80.

The spectrum of SL-48/80 dissolved in Locke's solution is shown in Fig. 3A. Attempts to extract this spin label into heptane were unsuccessful (Fig. 3B). When the SL-48/80 was bound to mastocytoma

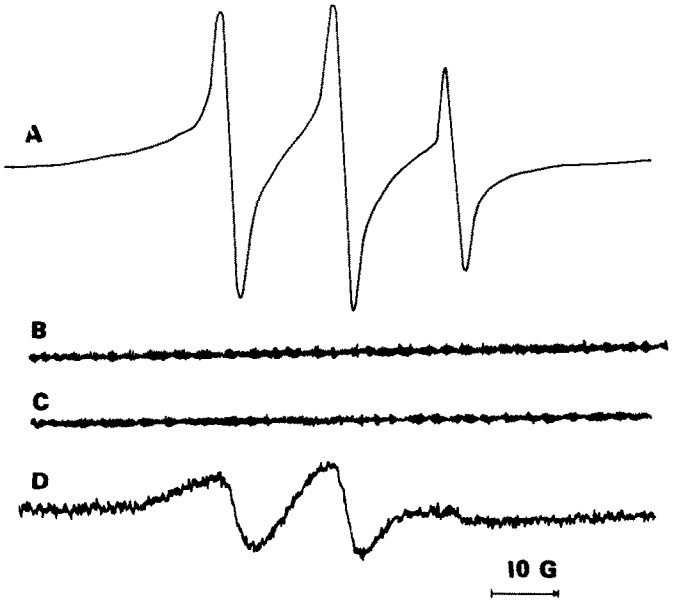


Fig. 3. Heptane extraction of SL-48/80. (A) represents the e.s.r. spectrum of SL-48/80 (100 μg/ml) in phosphate buffer (10 mM, pH 7.4). Heptane (spectral grade) was added (1:1), and the mixture was vortexed for 5 min. After phase separation, the organic phase was scanned (B). SL-48/80 was bound to mastocytoma cells as described in Fig. 2. Heptane (1:1) was added, and the cells were vortexed for 5 min. After phase separation, the organic phase was scanned (C). SL-48/80 was bound to "erythrocyte-type" liposomes as described in Fig. 2. Heptane (1:1) was then added, and the liposomes were vortexed for 5 min. After phase separation, the organic phase was scanned (D).

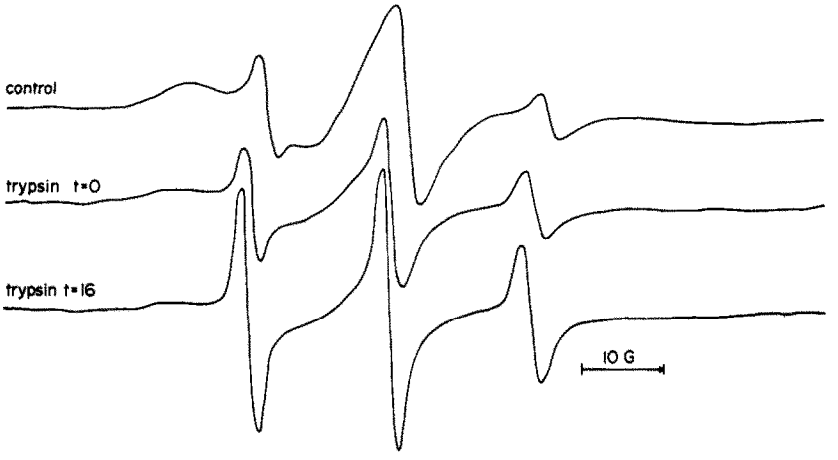


Fig. 4. Effect of trypsin on NEM-SL erythrocyte ghosts. Fresh ghosts were labeled with NEM-SL as described in Materials and Methods. The washed ghosts were resuspended in Locke's solution and the control e.s.r. signal was scanned. Trypsin (15 μg/ml) was added, and the e.s.r. signal was scanned at various intervals.

cells, it was also not possible to extract it in its complexed form into the same organic solvent (Fig. 3C). In contrast, when SL-48/80 was bound to liposomes, it was extracted into heptane, presumably bound to one of the lipids (Fig. 3D). The e.s.r. spectrum of this extracted SL-48/80 was identical to that observed in the intact liposomes.

Spin-labeled *N*-ethylmaleimide was covalently bound to the sulfhydryl groups of erythrocyte ghost membrane proteins [7], and the free label was removed by several washings. Figure 4 illustrates a spectrum typical of this label, showing two distinct populations of bound labels with different mobilities. Almost immediately after the addition of trypsin, the signal from the highly immobilized labels began to disappear, and after 16 min it was almost entirely absent. A similar experiment was done using purified mast cells labeled with SL-48/80. Trypsin treatment of these cells caused the e.s.r. spectrum to assume a configuration typical of the free drug (Fig. 5). This indicated that after proteolysis, the mast cells were unable to retain the bound SL-48/80.

Light-scattering studies. Examination of normal mast cells and mastocytoma cells using dark field microscopy showed that the cells became more opaque after addition of 48/80 under the cover slip. This observation suggested that changes in the light-scattering properties of mast cells may be related to the initiation of membrane fusion and histamine secretion. The effects of 48/80 on light scattering by mastocytoma cells, purified mast cells, mastocytoma cell membranes, erythrocyte ghosts, liposomes prepared from mastocytoma cells, and liposomes ("erythrocyte-type", see Materials and Methods) are shown in Fig. 6. The light-scattering properties of the intact cells and of the protein-containing cell membranes were increased significantly by treatment with 48/80. This effect was not due to cell aggregation as determined by light microscopy. In contrast, the light-scattering properties of both mastocytoma cell membrane liposomes and "erythrocyte-type" liposomes were unaffected by 48/80 treatment. It should be noted that the variations in the light-scattering properties among the different preparations at wavelengths less than 300 nm were probably due to the absorption of light by the varying amounts of nucleic acid (260 nm) or protein (280 nm) present in each preparation, and the absorption of light at 280 nm by compound 48/80. The increase in light scattering at 305 nm was dose dependent over the 48/80 concentration range which was pharmacologically active (Fig. 7), indicating that this phenomenon may bear a close relationship to the mechanism of 48/80-induced histamine secretion.

Other surface active agents with different properties were also tested for their effects on light scattering. In these studies mastocytoma cells were used to avoid potential artifacts in the light-scattering measurements caused by the liberation of free granules from normal mast cells. Table 1 shows that Zephiran (benzalkonium chloride), a cationic detergent, SDS, an anionic detergent, and Triton X-100,

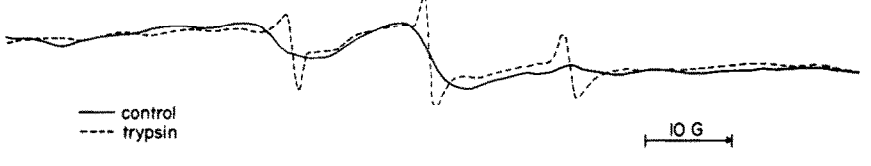


Fig. 5. Effect of trypsin on the e.s.r. signal from SL-48/80 bound to purified mast cells. Purified mast cells (10^6) were labeled with SL-48/80 (10 μg/ml) at 4° for 5 min. The cells were resuspended in fresh Locke's solution (0.5 ml) at 23°, and the e.s.r. spectra were scanned (solid line). Trypsin (10 μg) was added and the spectra were scanned immediately (broken line).

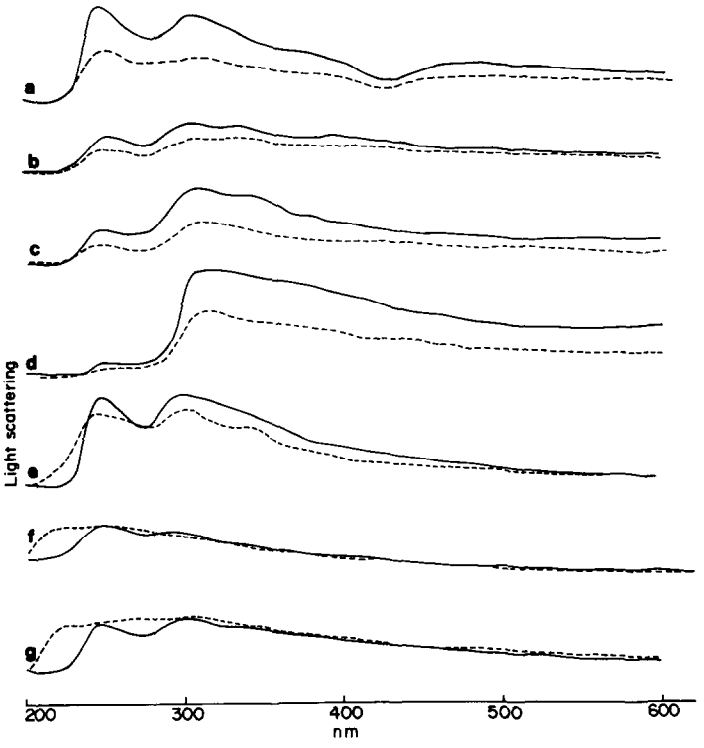


Fig. 6. Effect of 48/80 on the light scattered by intact biological systems and liposomes. The experimental conditions are described in Materials and Methods. Key: (a) erythrocyte ghosts; (b) purified mast cells; (c) mixed peritoneal cells; (d) mastocytoma cells; (e) mastocytoma cell membranes; (f) liposomes; and (g) liposomes derived from mastocytoma cell membranes. The dotted lines represent control spectra; the solid lines are the spectra scanned immediately after the addition of 48/80 (10 μg/ml).

a non-ionic detergent, all increased the light-scattering properties of mastocytoma cells. The cationic detergent was more highly effective in this respect than either the anionic or the non-ionic detergent. In addition to the detergents, various cationic chemicals were tested for both their own effects on light scattering and for the abilities to block the effects of 48/80. The scattering properties of mastocytoma cells treated with tyramine, decamethonium, decamethylene diamine and the 48/80 monomer were unchanged, and none of these drugs inhibited the response to 48/80. The histamine liberators, morphine, curare, and ionophore A23187 did not increase the light scattering nor did they inhibit the subsequent effects of 48/80 on the light-scattering properties of mastocytoma cells (data not shown).

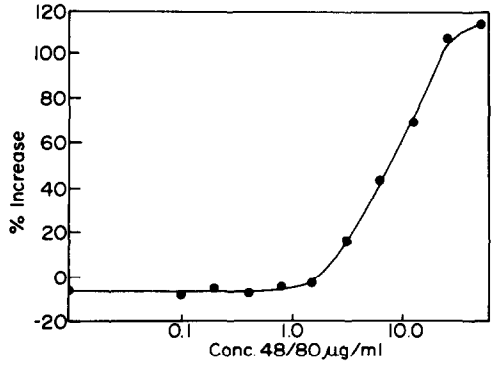


Fig. 7. Concentration–response curve of the effect of 48/80 on light scattering by mastocytoma cells (2×10^5/ml). The experimental conditions were described in Materials and Methods.

Table 1. Effect of surface-active agents on Rayleigh scatter by mastocytoma cells*

Detergent	% Increase
Triton X-100	
0.005%	49
0.05%	80
0.5%	72
Zephiran Cl	
0.001%	8
0.002%	29
0.01%	527
SDS	
0.0001%	20
0.001%	22
0.01%	85

* Rayleigh scattering was measured by determining the height of the spectrum of scattered light at 305 nm (where light was not absorbed by proteins or nucleic acids). In each sample, 2×10^5 cells/ml were treated with the detergents to reach the final concentrations indicated. Each measurement was made at 22° immediately after the addition of the detergent, and the per cent increase was based on the height of the control spectrum for each sample.

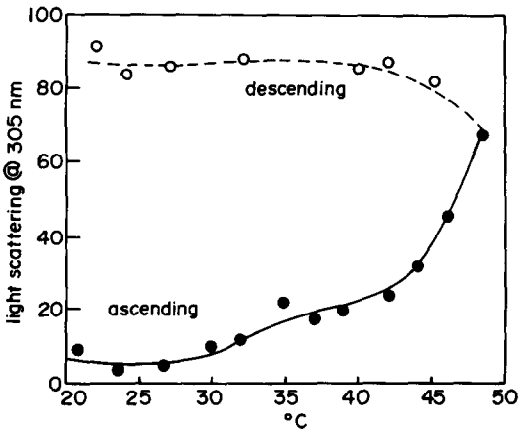


Fig. 8. Effect of temperature on light scattering by mastocytoma cells. The Rayleigh scatter at 305 nm was measured on the same sample at the temperature indicated. The sample was then cooled gradually and the light scattering was measured.

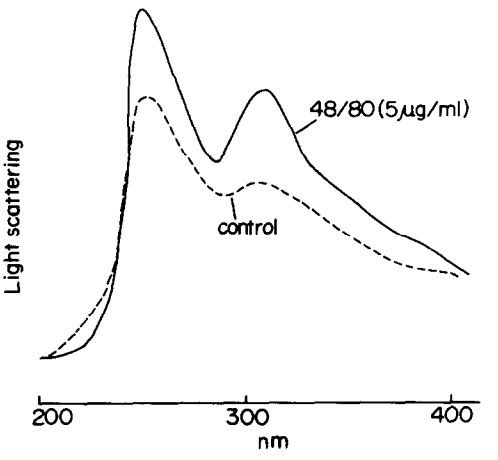


Fig. 9. Effect of temperature on light scattering by purified mast cells at 4°. Purified mast cells (10^6/ml) were cooled to 4° and the light scattering was measured. Compound 48/80 was added immediately after the control measurements were taken and the light scattering was again measured. Dry N_2 was blown on the cuvette to prevent condensation, and the temperature was monitored throughout the experiment.

Figure 8 shows the effect of temperature on light scattering by mastocytoma cells. Untreated cells were gradually heated, and their light-scattering properties were measured at regular intervals. As the cells were heated, the intensity of the scattered light remained constant until about 40°. Between 40 and 50°, however, the scattered light increased considerably, and subsequent cooling of the cells did not restore the original scattering properties. Heat-treated cells (45°, 10 min) scattered up to 50 per cent more light when treated with 48/80 (Table 2).

To study purified mast cells, the potential artifact introduced by the liberation of granules was avoided by cooling the cells to 4°. This treatment inhibits the secretory process, although 48/80 can still activate the cell membrane. Cells treated in this manner will then degranulate normally after warming to about 15°. When purified mast cells were treated with 48/80 at 4°, the light-scattering properties were increased in a manner similar to that seen with the mastocytoma cells (Fig. 9).

DISCUSSION

In our previous study, we have shown that the spin-labeled analog of compound 48/80 is a useful tool for the study of its molecular mechanism of action. The e.s.r. signal from SL-48/80 bound to mastocytoma cells showed that its binding characteristics were complex and substantially different from those of 5-DSA. Although the interpretation of the e.s.r. spectrum of cell-bound SL-48/80, in terms of motion or order parameter, is difficult [4], the effects of various treatments on its binding to biological systems can be determined. Through the use of detergents, we have shown that disruption of the cell membranes increased the apparent motion of 5-DSA, which is thought to intercalate into the interface region among the fatty acid moieties of the phospholipids. The apparent increase in membrane

Table 2. Effect of heat treatment on the response of rat peritoneal cells to compound 48/80*

Treatment	Time (min)	Light scattering at 305 nm (arbitary units)	% Increase
Control cells	0	4.5	
	1	7.5	66
	10	10.5	130
	20	11.0	144
Heated cells	0	6.0	
	1	14.0	120
	10	20.0	230
	20	22.0	266

* Mixed peritoneal cells were washed and suspended in 3 ml of Locke's solution (2×10^5/ml). An equal quantity of cells from the same preparation was heated to 45° for 10 min. Light scattering in both samples was measured at 22° before and after the addition of compound 48/80 (10 μg/ml) at 1-, 10- and 20-min intervals.

fluidity was presumably due to detergent intercalation among the membrane lipids. The resulting disruption of the ordered state, therefore, allowed the spin-labeled stearic acid more freedom of motion. Since the binding characteristics of SL-48/80 were unaffected by non-ionic detergents, its interaction with cells must, therefore, differ fundamentally from the intercalative binding of 5-DSA. The ability of SDS to increase the motion of SL-48/80, however, indicates that a strong protein denaturing action was required to affect the binding. These data therefore suggest that SL-48/80 was bound tightly to a protein or proteins rather than being loosely intercalated among the membrane lipids.

Because SL-48/80 could not be extracted into heptane from its binding site on mastocytoma cell membranes, it seems unlikely that it is bound to membrane lipids. In contrast, although SL-48/80 itself is insoluble in heptane, it was readily extracted after binding to "erythrocyte-type" liposomes. Presumably, the SL-48/80 was bound to an anionic lipid molecule, possibly phosphatidyl serine [9], to form a neutral complex that was soluble in the organic solvent. Although SL-48/80 may form a complex with lipids in liposomes, the inability of the organic solvent to extract it from the cells indicates that it probably does not bind to anionic phospholipids in biological membranes.

The effect of trypsin on the signal from SL-48/80 bound to mast cells was compared to its effect on NEM spin-labeled erythrocyte ghosts. It has been well established that the primary binding sites for spin-labeled NEM derivatives are sulfhydryl groups on the membrane proteins [7]. As expected, therefore, trypsin treatment caused a dramatic increase in the motion of the maleimide spin label due to membrane protein hydrolysis. Since the same phenomenon was seen when SL-48/80-labeled mast cells were exposed to trypsin, this evidence further supports the hypothesis that the membrane binding site of 48/80 is a protein.

Rayleigh scatter was studied because it reflects fundamental properties of the cell membrane which can be examined in both intact cells and in isolated membranes. Light scattering by cell membranes depends on the molecular organization of the membrane which confers a certain degree of reflectivity on the system. Treatment of mast cells with pharmacologically active concentrations of 48/80 causes an increase in the opacity of the membranes, thereby increasing their light-scattering properties. This increased scattering was not a result of degranulation or particle release by the cells, since the increased scatter by 48/80 in mast cells could be demonstrated at 4°, when degranulation was inhibited. Furthermore, isolated mastocytoma cell membranes and erythrocyte ghosts also reacted in a similar manner. Although several cationic drugs, including the 48/80 monomer, were tested, the only other agents capable of increasing light scattering were detergents. Furthermore, other histamine liberators such as morphine or curare were unable to duplicate this effect of 48/80 or to inhibit it. This suggests that, in accord with our previous findings [4], morphine and curare do not compete effectively for 48/80 binding site(s).

Compound 48/80 acts on cell membranes in at least two distinct ways. At low concentrations (approximately 1.0 μg/ml) non-cytotoxic membrane activation occurs [10]. At very high concentrations (>100 μg/ml), however, the cell membranes are damaged due to the amphipathic nature of the molecule, which enables it to act as a detergent. The effect of 48/80 on light scattering occurs at concentrations within the minimal range of pharmacological activity, which is two orders of magnitude below the concentration that causes non-specific release. Therefore, although the increased light scattering caused by 48/80 mimics the effects seen with the detergents, there is no pharmacological evidence for a detergent-like action of the drug at the lower concentrations. Recently, Read and Kiefer [11] have shown that the cationic detergent benzalkonium chloride inhibits the action of 48/80 by competing for its binding sites on mast cells. This also occurred at concentrations below the lytic level, indicating that at low concentrations cationic detergents may bind to pharmacological receptor sites and may thus influence the subsequent action of drugs.

The non-cytotoxic secretory stimulus initiated by 48/80, however, may still depend on the amphipathic nature of the molecule. This type of molecular structure could enable 48/80 to associate simultaneously with both hydrophobic and hydrophilic sites on membrane proteins. Furthermore, the average size of the active 48/80 polymers is a hexamer [12, 13] which means that the drug could potentially cause non-covalent crosslinking of the membrane proteins. The increased light scatter may, therefore, result from a lateral phase separation of the membrane lipids induced by membrane protein denaturation and/or aggregation [14]. This could lead to a heterogeneous lipid distribution or domain formation, resulting in a change in the specific volume and refractive index of the lipid hydrocarbon region. Using scanning electron microscopy, Chi *et al.* [15] have found that mast cell intramembraneous particles tend to aggregate during histamine secretion, leaving areas of the membranes apparently smooth and free of particles. These authors have postulated that such smooth areas may be the primary point of attachment for the perigranular and plasma membranes prior to fusion. This initial particle aggregation may indeed be a primary event in the initiation of histamine secretion and the smooth areas may represent regions of altered lipid distribution.

Heat treatment is a method commonly used to inhibit mast cell degranulation. Its effectiveness is probably due to a random disorganization of membrane structure resulting from protein denaturation. This inhibition is permanent and the cells do not recover their sensitivity to histamine liberators after cooling. The effect of heat treatment on the light-scattering properties of mastocytoma cells was similar to that seen with 48/80. Both treatments resulted in an incremental increase in light scattering which was consistent with a progressive increase in protein denaturation. It is also interesting to note that heat treatment actually potentiated an additional 48/80-induced increase in light scattering, indicating that heat-treated cells may bind more 48/80. This would also be consistent with the theory

of a protein binding site, since proteins generally unfold during denaturation, exposing additional potential binding sites.

In conclusion, compound 48/80 binds to a variety of biological preparations ranging from intact cells to membrane lipid derived liposomes, and there is no evidence to suggest that there is anything unique about its binding to purified rat peritoneal mast cells. The data also indicate that 48/80 interacts in some way with the membrane proteins and that, even at low concentration, this interaction resembles thermal denaturation and detergent treatment. Although 48/80 can probably bind to a variety of protein sites, it is possible that compound 48/80 changes the properties of a specific molecule in a way that precipitates membrane fusion. Identification of the activating site and a more analytical treatment of 48/80 binding will depend on the purification of a single species of active polymer.

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