THE INTERNAL pH OF MAST CELL GRANULES

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1. Introduction

Mast cells, which are found in all anatomical structures of the body except the blood and lymph, possess one of the largest contents of the biogenic amines histamine and serotonin (5-hydroxytryptamine) found within mammalian cells [1,2]. These amines, along with large amounts of the anionic mucopolysaccharide heparin and proteolytic enzymes, are stored in high concentration within the osmophilic storage granules of the mast cell [3,4]. Varying in size from 300–800 nm, the spherical mast cell granules can be easily viewed by light microscopy [5]. According to [4] histamine is synthesized from histidine in the cytosol of the mast cell via the enzyme histidine decarboxylase, however fluorescence microscopy has established that histamine is localized in high concentration only in the granules [5]. Although this evidence suggests active histamine accumulation into mast cell granules against an apparent concentration gradient, the precise mechanism by which histamine and serotonin are transported and stored is unknown.

Considerable similarity exists between the mast cell granule, the chromaffin granule, and the platelet dense granule with respect to the existence of a high content of basic biogenic amines, the presence of large M_r acidic molecules implicated in the storage complex, the presence of associated peptide hormones, and the release by a stimulus coupling mechanism [1,6,7]. Chromaffin granules and platelet granules, which store large quantities of catecholamines and serotonin, respectively, are remarkable in that they both maintain a distinctly acidic intragranular pH of 5.5 even after isolation and resuspension at neutral pH [6–8]. There is good evidence that this intravesicular acidic pH is generated by a H^+-translocating ATPase within the granule membrane; the resulting ΔpH is maintained by the low H^+ permeability of both granule membranes, and by their high internal buffering capacity [7,9,10]. Because the existence of a ΔpH across the membrane of these granules has been shown to provide a chemiosmotic gradient which drives uptake of biogenic amines [9], the structural and biochemical similarities between chromaffin granules, platelet granules and mast cell granules prompted this investigation as to whether mast cell granules might also maintain a distinctly acidic interior. To take advantage of the unusually large size of the granules, and because they are not as easily isolated with full biologic activity as the granules of the chromaffin cell or of platelets, the internal pH of mast cell granules was determined within intact mast cells using two different established techniques: the fluorescent distribution of the pH-sensitive dyes 9-amino acridine (9-AA) and atebrin, and the radioisotopic distribution of the ΔpH indicator [^{14}C]-methylamine.

2. Materials and methods

2.1. *Isolation of mast cells*

Intact mast cells were isolated from male Sprague-Dawley rats (250–300 g) using a modification of the method in [11]. Briefly, the rats were decapitated and bled and 6 ml buffered NaCl (0.9% NaCl, 2 mM $MgCl_2$, 2 mM EGTA and 6 mM phosphate buffer, at pH 7.0) were injected into the abdominal cavity. After 1 min gentle massage, the fluid was withdrawn with a plastic pipettor and was layered onto step gradients of 30% Ficoll, 0.9% NaCl and 6 mM phosphate buffer, at pH 7.0. Centrifugation at 3000 × *g* for 25 min yielded a loose pellet which was carefully resuspended, washed twice in a solution of 0.9% NaCl, 1 mM glucose, and 6 mM phosphate buffer, at pH 7.0;

and stored at 4°C for ⩽30 min before use in experiments.

2.2. *Phase contrast microscopy*

Mast cells suspended in buffered NaCl were examined under a Nikon 100 X glycerol objective on an Aus Jena'Fluoval' microscope (Optical Apparatus Co., Ardmore, PA) equipped with phase contrast and epi-fluorescence optics and a mercury arc lamp. Excitation wavelengths of 420 nm for 9-AA and 450 nm for atebrin were utilized. Emission was observed through a G247 guard filter. Micrographs were taken with a Nikon Microflex (M-35FA) system and Kodak Tri-X film (exposure time 16 s for fluorescence photomicrographs). A Farrand photomultiplier (Farrand Optical Co., Valhalla, NY) equipped with a grating MSA monochromator was set at 550 nm and connected to a strip chart recorder for some experiments.

2.3. *Measurement of ΔpH*

The equilibrium distribution of [^{14}C]methylamine (8.7 μM) was utilized to measure the internal pH of mast cell granules. This method is highly reproducible in a variety of subcellular organelles, with minimal binding observed, and has been described in depth [10]. Extravesicular water space in the pellet was determined using [^{14}C]polydextran (0.19 mg/ml) and $^{3}H_2O$(4.2 mg/ml). When the measured pH was more basic than the pH of the incubation medium 5,5-di[^{14}C]methyl-2,4-oxazolidedione (DMO) was utilized.

2.4. *Materials*

All reagents were purchased from Sigma Chemical Co., St Louis, MO. [^{14}C]methylamine (48.1 mCi/nmol, [^{14}C]polydextran (1.11 mCi/g), [^{14}C]DMO and $^{3}H_2O$ (1 mCi/g) were purchased from New England Nuclear Co., Boston, MA.

3. Results and discussion

The monoamine dye 9-AA is a fluorescent compound that as a weak base accumulates inside an acidic intragranular space by diffusing across the membrane in the uncharged form, to be reprontonated in the acidic interior. It had been suggested to behave as an ideal pH indicator with a high degree of specificity and has been used with good success to measure the ΔpH in a variety of organelles [12–15].

In the presence of 8 μM 9-AA, phase contrast micrographs of mast cells reveal a well-delineated cell membrane surrounding a dense population of discrete intracellular granules (fig.1); parallel fluorescence micrographs demonstrate that at pH 7.5 the granules accumulated large amounts of 9-AA with respect to the nucleus, cytosol, and extracellular medium. The granules thus appear to be distinctly acidic inside. This conclusion is confirmed by the step-wise decrease in granule fluorescence observed when the same cell was perfused with incubation media of successively lower pH (6.5 and 5.5). That no granule fluorescence remained at pH 5.5 is good evidence that the internal pH of the mast cell granules is between 6.5 and 5.5.

As discussed in [16], non-specific binding of a fluorescent dye to cell structures can be evaluated by comparing the fluorescence in the presence and absence of nigericin in high concentrations of NH_4^+, agents which cause perturbation of the ΔpH. Like 9-AA, NH_4^+ distributes across biological membranes in the uncharged form in response to a ΔpH (acidic interior), and when present in high concentrations

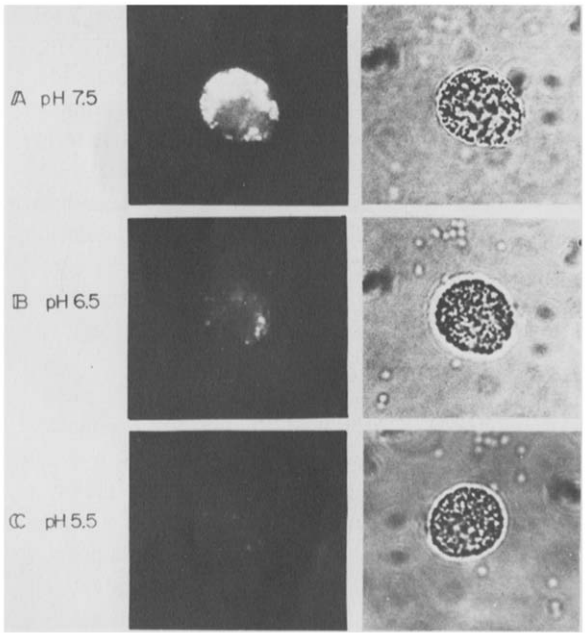

Fig.1. Fluorescence and phase contrast photomicrographs at varying extracellular pH levels. Freshly isolated mast cells were suspended in a solution of 0.9% NaCl, 2 mM KH_2PO_4, 8 μM 9-AA-HCl and 1 mM glucose at pH 7.5. When indicated, the cells were then perfused for 5 min in the above solution at pH 6.5 and pH 5.5. The same cell is pictured for all three external pH values (X 100). Temperature was 26°C.

(1–50 mM) a large influx of ammonia results in a dose-dependent alkalinization of the internal space, and a corresponding decrease in the ΔpH as the internal buffering capacity is exceeded. When isolated mast cells were incubated with 9-AA and various concentrations of NH_4^+ at pH 7.5, a dose-dependent decrease in the fluorescence was observed with nearly complete elimination of the granule fluorescence observed with the addition of 30 mM NH_4^+ (not shown). The same experiment was repeated using atebrin, a diamine dye analogous to 9-AA [17,18], to corroborate this effect (fig.2). At the same concentration of dye (8 μM), however, the granules were brilliantly fluorescent with the measured fluorescence clearly saturated under identical conditions. For this reason, 0.8 μM atebrin was utilized in subsequent experiments, a 10-fold dilution. This observation can be explained by the fact that since atebrin is a diamine, the distribution of the dye across the membrane of the granule is equal to the square of the ratio of the $[H^+]$ [13]. As with 9-AA, atebrin fluorescence was localized predominately to the granules and excluded from the nucleus, cytosol, and extracellular medium (fig.2). The addition of ammonia resulted in a dose-dependent decrease in fluorescence similar to that observed when the distribution of 9-AA was measured. In addition to illustrating the low degree of specific atebrin binding by the granules, the evidence again suggests that the internal pH of the granules is distinctly acidic. Moreover, since the ΔpH was completely collapsed only by large ammonia concentrations, the internal buffering capacity of the granules appears to be quite high. As is also noted with chromaffin and platelet granules (R. J., S. C., A. S., unpublished), upon the addition of large amounts of ammonia, some swelling of the mast cell granules occurred; but this was not thought to be of sufficient magnitude to account for the fluorescence degree. Moreover, in the presence of nigericin, a carboxylic acid ionophone which catalyzes an electroneutral exchange of K^+ and H^+, atebrin fluorescence within the granules was almost completely eliminated with minimal swelling produced (not shown).

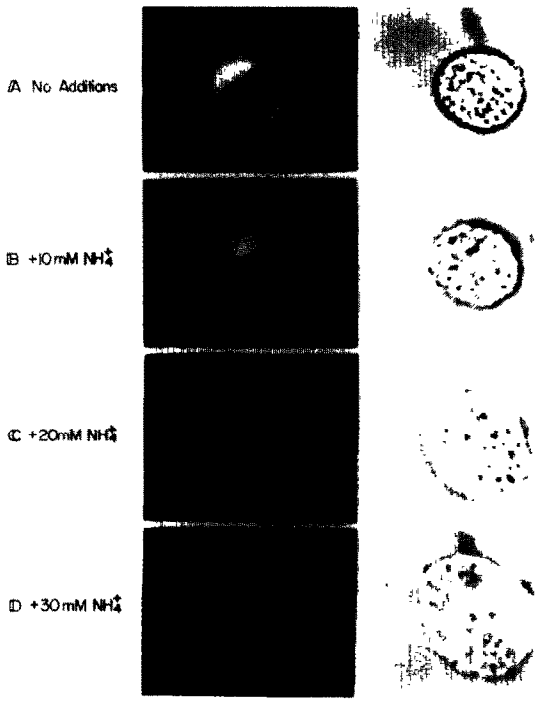

Fig.2. Fluorescence and phase contrast photomicrographs at varying extracellular $[NH_4^+]$cs. Mast cells were incubated as in fig.1, except that 0.8 μM atebrin–di-HCl was substituted for 8 μM 9-AA. As the extracellular pH was held constant at 7.5, the cells were successively perfused for 5 min with incubation solution containing increasing concentrations of $(NH_4)_2SO_4$ as indicated. The same cell is pictured throughout the perfusion process (× 100). Temperature was 26°C.

The effect of increasing concentrations of NH_4^+ on granule fluorescence at pH 7.5 was further quantitated with a photomultiplier equipped with a grating monochromator attached to the microscope ocular (not shown), and fluorescence at 550 nm was found to be proportional to the external ammonia concentration. The observed effects of ammonia and nigericin thus support the conclusion that mast cell granules maintain an acidic interior.

After establishing in fluorescent photomicrographs that both pH indicator dyes did not accumulate within the nucleus or cytosol of mast cells but were taken up only by the granules, it was then possible to determine the internal granule pH by radioassay of the distribution of [^{14}C]methylamine, a small monamine whose accumulation into acidic granule interiors is again dictated by the magnitude of the ΔpH (reviewed [18]). As the external pH was varied from 5.5–8.5 (fig.3), the distribution of [^{14}C]methylamine was assayed using standard techniques to calculate the internal granule pH. Two assumptions were made: (i) the cell membrane was assumed to be permeable to protons; and (ii) the granules were estimated to occupy from 25–35% of the internal volume of the cell, based on electron micrographs in [11,19]. The calculated pH of the mast cell granules under these

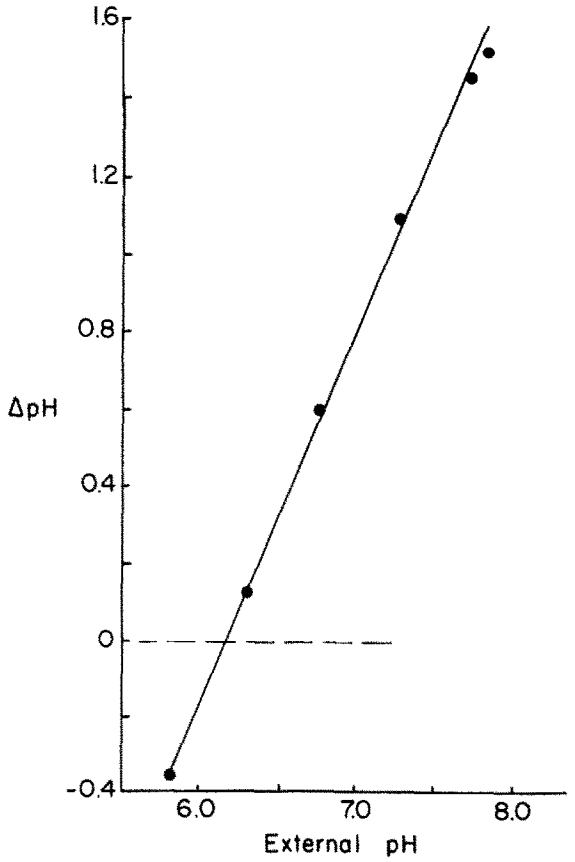

Fig.3. Distribution of [^{14}C]methylamine as a function of extracellular pH. Freshly isolated mast cells were suspended at 1.21 mg protein/ml in an incubation medium of 0.9% NaCl, 12.4 mM phosphate buffer, [^{14}C]methylamine and $^{3}H_2O$, at the indicated pH values. After 20 min, incubation samples (1.2 ml) were centrifuged and both supernatant and pellet were assayed for radioactivity using standard scintillation techniques as in section 2. Temperature was 24°C.

assumptions is 6.1. If the cell membrane is impermeable, however, or maintains only a moderate permeability to H^+, the calculated internal pH would be underestimated by at most 0.2 pH units. Contributions by other subcellular organelles which may be acidic (i.e., lysomes) or basic (i.e., mitochondria) inside are not thought to contribute significantly to the measurement due to the sparcity within the mast cell, because of the minute percentage of the total volume which they represent, and the small gradients involved.

These results have important physiological implications. That the cytosolic pH of most mammalian cells in pH 7.2–7.4 suggests that the intragranular pH (pH 6.1) of mast cell granules would be over 1.0 unit more acidic than that of the cytosol in vivo (ΔpH 1.0). Rapidly accumulating evidence shows that several kinds of subcellular organelles which store biogenic amines maintain an acidic internal pH [6,7, 20]. The protonmotive force, composed of a chemical component (ΔpH) and an electrical component ($\Delta\Psi$) is implicated as the driving force for amine accumulation into the subcellular storage organelles [9,10,21,22]. In the case of mast cells, the acidic interior of the storage granules may play a heretofore unsuspected central role in the mechanism of histamine and serotonin accumulation and storage. In addition, the low intragranular pH may help:

(i) To explain how such potent granular enzymes as chymase, kallikrein, arylsulphatase, and β-exoglycosidases are maintained in an inactive form prior to release [1];

(ii) To account for the complex formed between heparin and histamine which is stabilized at acidic pH values [23].

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