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Role of lysosomal and cytosolic pH in the regulation of macrophage lysosomal enzyme secretion

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Rapid and parallel secretion of lysosomal β-*N*-acetylglucosaminidase and preloaded fluorescein-labelled dextran was initiated in macrophages by agents affecting intracellular pH (methylamine, chlorpromazine, and the ionophores monensin and nigericin). In order to evaluate the relative role of changes in lysosomal and cytosolic pH, these parameters were monitored by using pH-sensitive fluorescent probes [fluorescein-labelled dextran or 2′,7′-bis(carboxyethyl)-5(6)-carboxyfluorescein]. All agents except chlorpromazine caused large increases in lysosomal pH under conditions where they induced secretion. By varying extracellular pH and ion composition, the changes in lysosomal and cytosolic pH could be dissociated. Secretion was then found to be significantly modulated by changes in cytosolic pH, being enhanced by alkalinization and severely inhibited by cytosolic acidification. However, changes in cytosolic pH in the absence of stimulus were unable to initiate secretion. Dissociation of the effects on lysosomal and cytosolic pH was also achieved by combining stimuli with either nigericin or acetate. Further support for a role of intracellular pH in the control of lysosomal enzyme secretion was provided by experiments where bicarbonate was included in the medium. The present study demonstrates that an increase in lysosomal pH is sufficient to initiate lysosomal enzyme secretion in macrophages and provides evidence for a significant regulatory role of cytosolic pH.

INTRODUCTION

Macrophages play an important role in the various stages of an inflammatory response by generating and releasing mediators such as eicosanoids and cytokines and by secreting lysosomal hydrolases [1–4]. In an acidic environment, such as certain inflammatory foci, the latter may cause tissue damage and/or contribute to tissue remodelling. Activated macrophages have also been shown to seal off an acidic extracellular compartment through adherence [5], much like osteoclasts which, after adhesion to bone, create an acidic resorptive lacuna into which lysosomal enzymes appear to be secreted [5–7]. However, the means by which the secretion of lysosomal enzymes is regulated remains largely unknown.

Previous studies have demonstrated that massive secretion of preformed lysosomal enzymes from macrophages can be elicited by some amines [8–11] and monensin [12]. These agents are known to increase the pH of acidic organelles [13–15], but could also affect cytosolic pH. In the present study, changes in lysosomal and cytosolic pH have been dissociated in order to elucidate their relative role in the control of lysosomal enzyme secretion.

MATERIALS AND METHODS

Materials

4-Methylumbelliferyl *N*-acetyl-β-D-glucosaminide, fluorescein isothiocyanate–dextran (FD; average $M_r = 42000$; 9 mmol of fluorescein isothiocyanate/mol of glucose residue), monensin, nigericin, chlorpromazine and NADH were purchased from Sigma. Methylammonium chloride was obtained from Merck. Valinomycin, 2′,7′-bis(carboxyethyl)-5(6)-carboxyfluorescein (BCECF) and its tetra-acetoxymethyl ester (BCECF-AM) were from Molecular Probes, Eugene, OR, U.S.A. All materials for cell culture were purchased from Flow Laboratories.

Experimental media

Na^+-based solution with nominal bicarbonate (Na-medium) contained: NaCl, 127 mM; KH_2PO_4, 1.2 mM; KCl, 5.4 mM; $MgSO_4$, 0.8 mM; $CaCl_2$, 1.8 mM; glucose, 5.6 mM; Hepes, 10 mM. Adjustment of pH to the indicated value was performed at 37 °C.

In Na^+-free solution (Ch-medium) NaCl was replaced by choline chloride.

Solution based on presumed intracellular ionic composition (K-medium) contained: KCl, 127 mM; NaH_2PO_4, 1.2 mM; NaCl, 5.4 mM; and was otherwise identical with Na-medium.

Bicarbonate-containing solution (Na/HCO_3-medium) was identical with Na-medium except for replacement of 18.5 mM-NaCl by $NaHCO_3$ and omission of Hepes. pH was adjusted to the indicated value by varying the CO_2 partial pressure at 37 °C and was verified by pH-electrode and by comparing the fluorescence ratio of BCECF in Na/HCO_3-medium with that obtained in medium devoid of bicarbonate.

Macrophage culture and stimulation

Resident cells were harvested from female outbred NMRI mice (ALAB, Stockholm, Sweden) by peritoneal lavage with 4 ml of Medium 199 containing 1% heat-inactivated foetal-bovine serum. After plating on to 35 mm-diam. tissue-culture dishes (supplemented with coverglasses in experiments where intracellular pH was measured) and 2 h incubation in an humidified atmosphere of 5% CO_2 in air at 37 °C, non-adherent cells were removed, and to each well was added 2 ml of Medium 199 containing 10% serum.

After 14–22 h culture, this medium was exchanged for Na/HCO_3-medium 30 min before addition of stimuli. In experiments performed in nominal bicarbonate, medium was changed after incubation without serum for 20 min to Na-medium under atmospheric CO_2 partial pressure. The final experimental medium was applied 7 min before application of stimulus-containing medium.

Abbreviations used: NAG, *N*-acetyl-β-D-glucosaminidase (EC 3.2.1.30); FD, fluorescein isothiocyanate–dextran; BCECF-AM, 2′,7′-bis(carboxyethyl)-5(6)-carboxyfluorescein tetra-acetoxymethyl ester; pH_e, extracellular pH.

Stimuli were dissolved in dimethyl sulphoxide (chlorpromazine), ethanol (monensin, nigericin and valinomycin) or water, and were added to the experimental media in a volume never exceeding 0.5%. When millimolar concentration of a stimulus was applied, osmotic compensation by decreasing the major salt in the medium did not significantly affect the studied responses.

Enzyme assays

At the end of the experiments, the culture media were collected and the cells lysed with 1 ml of 0.1% (v/v) Triton X-100 and scraped off with a silicone-rubber policeman. The samples were centrifuged at 5000 rev./min for 5 min, and the supernatants collected and put on ice.

NAG was determined at 37 °C in a SLM spectrofluorimeter (model 8000 C), with wavelengths for excitation and emission set at 341 and 447 nm respectively [16]. A sample (200 μl) of either medium or cell lysate was added to 1.8 ml of 0.25 mM-4-methylumbelliferyl *N*-acetyl-β-D-glucosaminide in 0.25 M-sodium citrate buffer (final pH 4.8). These conditions were found to represent optimal pH and saturating substrate concentration for the enzyme. The increase in fluorescence was linear with time and taken as a measure of NAG activity. The total enzyme activity per culture remained constant during the course of the experiment and averaged 0.5 nmol of reaction product/min. In Figures and Tables secretion is expressed as a percentage of this total enzyme activity.

Lactate dehydrogenase (EC 1.1.1.27) activity in media relative to cell lysates was taken as an indication of cellular integrity [17] and was negligible under all experimental conditions described. This enzyme was routinely assayed by a modification of a method described previously [18].

The release of enzymes from stimulated cells was always corrected for the release observed in controls (NAG, $< 2\%$; lactate dehydrogenase, $< 5\%$). All enzyme determinations were performed on the day of the experiment.

Measurement of lysosomal pH with FD

Changes in lysosomal pH were assessed from changes in fluorescence ratio essentially as described previously [13]. After cells had been allowed to adhere to 12 mm x 12 mm coverglasses, they were cultured in Medium 199 containing 10% serum and 0.5 mg of FD/ml for approx. 16 h. The FD used was devoid of free fluorescein isothiocyanate, as assessed by gel-permeation h.p.l.c. After FD loading, the coverglasses were further incubated for 30 min in FD-free medium to ensure that lysosomes were the major localization of endocytosed dextran. When inspected by fluorescence microscopy, the cells displayed a distinct granular perinuclear fluorescence consistent with lysosomal localization of FD.

Experiments were performed in a SLM (model 8000 C) spectrofluorimeter at 37 °C with 2 ml of experimental medium in the cuvette. The coverglass was mounted at an approx. 30° angle relative to the light beam in a special holder in a standard fluorimeter cuvette equipped with magnetic stirring. Before each experiment, a coverglass with cells not loaded with FD, but otherwise similarly treated, was used to determine a background fluorescence to be subtracted. During the experiment the medium was continuously exchanged by perfusion at a flow rate of approx. 1 ml/min. This was essential for removal of liberated probe. Addition of stimuli was performed by perfusion at a 10-fold increased flow rate during 1 min. This increase in flow rate changed neither temperature nor pH appreciably.

The fluorescence ratio, with wavelengths for excitation set at 497/456 nm and for emission at 518 nm, was recorded and translated into pH values on the basis of ratios obtained in FD-containing K-media of various pH. Calibration by addition of nigericin (10 μM) to FD-loaded cells in K-media of various pH was also attempted, but minor deviations were found from the ratio obtained in K-media containing FD.

Measurement of cytosolic pH with BCECF

Cells adherent to coverglasses were loaded with BCECF by incubation with 0.5 μM-BCECF-AM for 10 min. When cells were then inspected by fluorescence microscopy, they displayed a homogeneous non-granular fluorescence, consistent with a cytosolic localization of the indicator. After extensive washing, the cover slips were mounted in the fluorimeter as described above for FD-loaded cells, but with wavelengths for excitation set at 506/456 nm and for emission at 527 nm. Subtraction of background fluorescence and perfusion with experimental media were performed as described above for FD-loaded cells. The changes in fluorescence ratio were taken to reflect changes in cytosolic pH [19], with calibration of the ratio performed as described for FD-loaded cells. Ratios obtained by calibration with nigericin *in situ* did not deviate from ratios obtained in K-media containing BCECF.

RESULTS

Methylamine, monensin and nigericin as secretagogues

In agreement with earlier findings [8,9], methylamine caused secretion of NAG from macrophages in a dose-dependent manner (Fig. 1*a*). Secretion of NAG was also induced by NH_4Cl (results not shown). These agents are known to raise the pH of acidic organelles in macrophages ([13,14]; see also below), but may in addition raise cytosolic pH and interfere with enzymic processes in other cell compartments [10]. If the amine-induced secretion of NAG were causally related to an increase in either lysosomal or cytosolic pH, one would expect that such increases brought about by other mechanisms would also lead to secretion of lysosomal enzyme. Univalent-cation ionophores were employed for this purpose. Furthermore, the ionophores enabled us to induce differentiated changes in cytosolic and lysosomal pH.

The Na^+/H^+-ionophore monensin (in Na-medium), at 1–10 μM concentration, induced considerable secretion of NAG (Fig. 1*b*). Also, the K^+/H^+-ionophore nigericin induced secretion in a medium high in KCl (Fig. 1*c*), but not in ordinary Na-medium (see below). On the other hand, the K^+-ionophore valinomycin (5 μM) did not cause secretion of NAG in either KCl-based medium or Na-medium. Thus changes in membrane potential, expected to occur in K-medium or on addition of valinomycin, were not sufficient to induce NAG secretion. Valinomycin was also unable to change lysosomal or cytosolic pH (results not shown).

Effects on lysosomal and cytosolic pH

As shown in Fig. 2(*a*), increasing concentrations of methylamine induced a progressive increase in the fluorescence ratio of cells preloaded with FD. This reflects an increase in lysosomal pH, since FD has been shown by subcellular fractionation to co-localize with lysosomal enzyme [20], and since FD and NAG were released roughly in parallel from loaded cells in response to all stimuli employed in the present study (Table 1). Like methylamine, monensin also caused a concentration-dependent increase in lysosomal pH, and the effect of 10 μM-monensin was even larger than that of 20 mM-methylamine (Fig. 2*b*). At lower concentrations, the addition of both methylamine and monensin resulted in larger increases in lysosomal pH than with either agent separately, but not so at higher concentrations (result not shown). Also, secretion of NAG in response to combinations of methylamine and monensin became less than additive as the concentration of either or both was increased (result not shown),

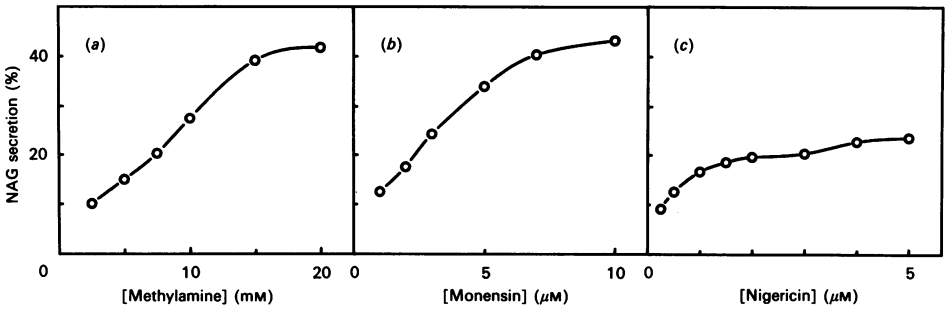

Fig. 1. Methylamine, monensin and nigericin as secretagogues

NAG secretion in response to (*a*) methylamine in Na-medium, (*b*) monensin in Na-medium and (*c*) nigericin in K-medium is shown. Results are representative of 8, 13 and 3 separate experiments respectively. The culture media were supplemented with stimuli of the indicated concentration (final pH 7.2). Incubation time was 30 min.

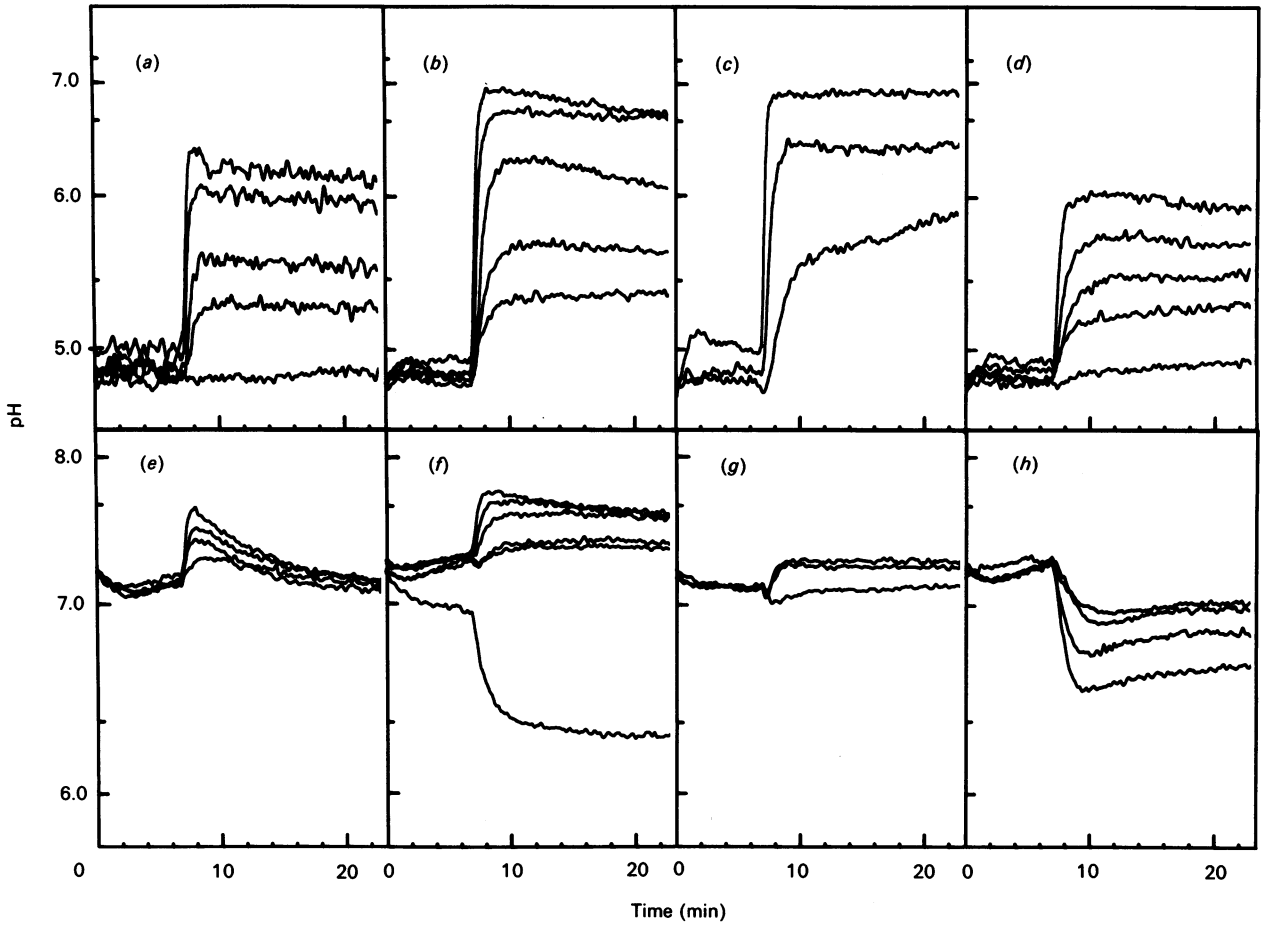

Fig. 2. Methylamine-, monensin- and nigericin-induced changes in lysosomal and cytosolic pH

Lysosomal (*a–d*) and cytosolic (*e–h*) pH changes induced at pH_e 7.2 by addition of methylamine in Na-medium (*a*, *e*), monensin in Na-medium (or Ch-medium) (*b*, *f*), nigericin in K-medium (*c*, *g*) or nigericin in Na-medium (*d*, *h*). Concentrations of stimuli applied at 7 min were (from lowest trace to uppermost): (*a*) no addition, 2.5, 5, 10 and 20 mM-methylamine in Na-medium; (*b*) 0.5, 1, 2, 5 and 10 μM-monensin in Na-medium; (*c*) 0.1, 0.5 and 5 μM-nigericin in K-medium; (*d*) 0.05, 0.125, 0.25, 0.5 and 1 μM-nigericin in Na-medium; (*e*) 2.5, 5, 10 and 20 mM-methylamine in Na-medium; (*f*) 10 μM-monensin in Ch-medium, 0.5, 1, 2, 5 and 10 μM-monensin in Na-medium; (*g*) 1, 5 and 10 μM-nigericin in K-medium; (*h*) 0.125, 0.25, 0.5 and 1 μM-nigericin in Na-medium.

which is consistent with a similar mechanism of action of these agents.

Nigericin was found to raise lysosomal pH in both K^+- and Na^+-based medium (Figs. 2*c* and 2*d*). The increases were larger in K^+-medium and occurred at lower concentrations than those required for monensin.

Thus the dose–response relationships for NAG secretion and for the increase in lysosomal pH were quite similar with methylamine, monensin and nigericin (medium high in K^+), but not with nigericin in Na-medium.

Methylamine caused a dose-related, although transient, increase in cytosolic pH (Fig. 2*e*). The increase in cytosolic pH in

Table 1. Parallel release of NAG and FD in response to various stimuli

Experiments were conducted on FD-loaded cells for 30 min at pH_e 7.20, unless otherwise indicated. The amount of NAG secreted did not differ from that by parallel cultures not loaded. The data presented are representative of five separate experiments.

	Release (% of total)							
	Na-medium		Ch-medium		K-medium		Na/HCO_3-medium	
	NAG	FD	NAG	FD	NAG	FD	NAG	FD
Control	0.0	0.7	0.0	1.9	0.6	6.0		
Monensin (5 μM)	33.2	31.9	1.2	6.3			10.1	13.6
Monensin* (5 μM)							19.1	19.8
Nigericin (0.5 μM)	5.1	10.5						
Nigericin (5 μM)					28.2	20.5		
Methylamine* (10 mM)	47.6	56.9					46.4	52.3
Chlorpromazine (20 μM)	5.2	8.9						

* Experiment conducted at pH_e 7.60.

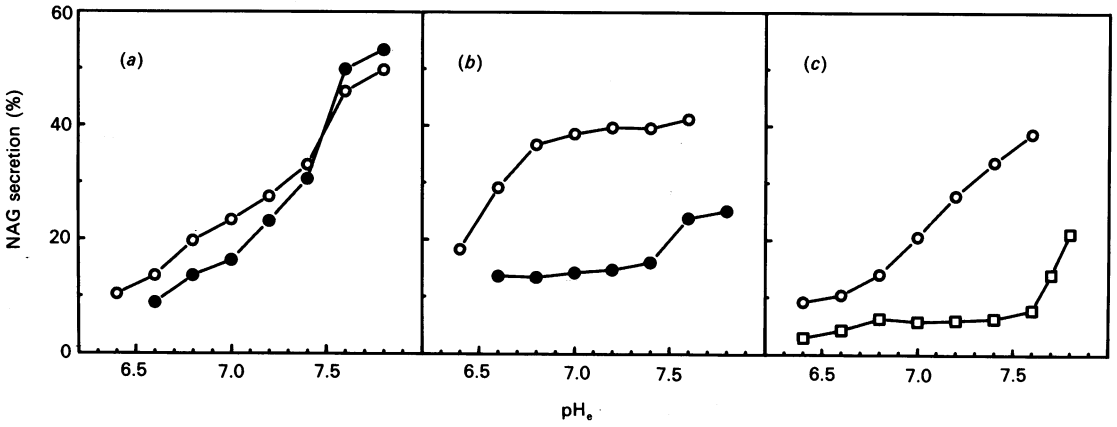

Fig. 3. pH_e-dependence of NAG secretion induced by methylamine, monensin and nigericin: effect of bicarbonate

NAG secretion in response to (*a*) 10 mM-methylamine in Na-medium (○) or Na/HCO_3-medium (●), (*b*) 10 μM-monensin in Na-medium (○) or Na/HCO_3-medium (●), and (*c*) 5 μM-nigericin in K-medium (○) or 0.5 μM-nigericin in Na-Medium (□) are shown. Results are representative of 17, 6, 17, 7, 9 and 3 similar experiments respectively. Incubation time was 30 min at the indicated pH_e, for Na/HCO_3-medium under a gas phase with controlled pCO_2, individually set for each value of pH as described in the Materials and methods section.

response to monensin was also dose-related, but was sustained during the whole period of recording (Fig. 2*f*). In Na^+-free medium, monensin induced instead a pronounced decrease in cytosolic pH (Fig. 2*f*), and under these conditions the secretion of NAG was severely inhibited (Table 1). In a medium high in KCl, nigericin (1–10 μM) caused a limited but sustained increase in cytosolic pH (Fig. 2*g*). In contrast, cytosolic pH was depressed in Na-medium already at 0.1 μM-nigericin (Fig. 2*h*).

These results suggest that an increase in lysosomal pH may be causally related to the secretion of lysosomal enzyme, if acidification of the cytosol exerts an inhibitory effect.

Dependence on extracellular pH of NAG secretion, and the effect of bicarbonate

In attempts to define further the relative role of lysosomal and cytosolic pH in the secretory response to amines and ionophores, the influence of extracellular pH (pH_e) was assessed. Fig. 3 demonstrates the dependence on pH_e of NAG secretion induced by methylamine (Fig. 3*a*), monensin (Fig. 3*b*) or nigericin (Fig. 3*c*). Methylamine-induced secretion increased steeply in the range pH 7.2–7.6, whereas the response to monensin (in the absence of bicarbonate) developed in the pH range 6.4–7.0 and remained unchanged above pH 7.0. In K-medium nigericin-induced secretion of NAG increased in the pH range 6.8–7.4 (Fig. 3*c*). Thus the pH-dependence for nigericin in KCl-based medium was displaced by approx. 0.4 pH unit towards the alkaline side compared with that for monensin in Na-medium. In the latter medium nigericin did not cause NAG secretion, except at more alkaline pH (Fig. 3*c*).

Since Cl^-/HCO_3^- exchange is considered to be the predominant protective mechanism against cytosolic alkalinization [21], the effect on NAG secretion of inclusion of bicarbonate in the medium was assessed. The presence of bicarbonate hardly affected the pH_e-dependence of methylamine-induced secretion (Fig. 3*a*), but the secretory response to monensin was depressed considerably, with an apparent alkaline shift in pH_e-dependence of approx. 1 pH unit (Fig. 3*b*).

Effect of extracellular pH and bicarbonate on the changes in lysosomal and cytosolic pH

The increase in lysosomal pH in response to either monensin (Fig. 4*b*) or nigericin (Figs. 4*c* and 4*d*) was large already at

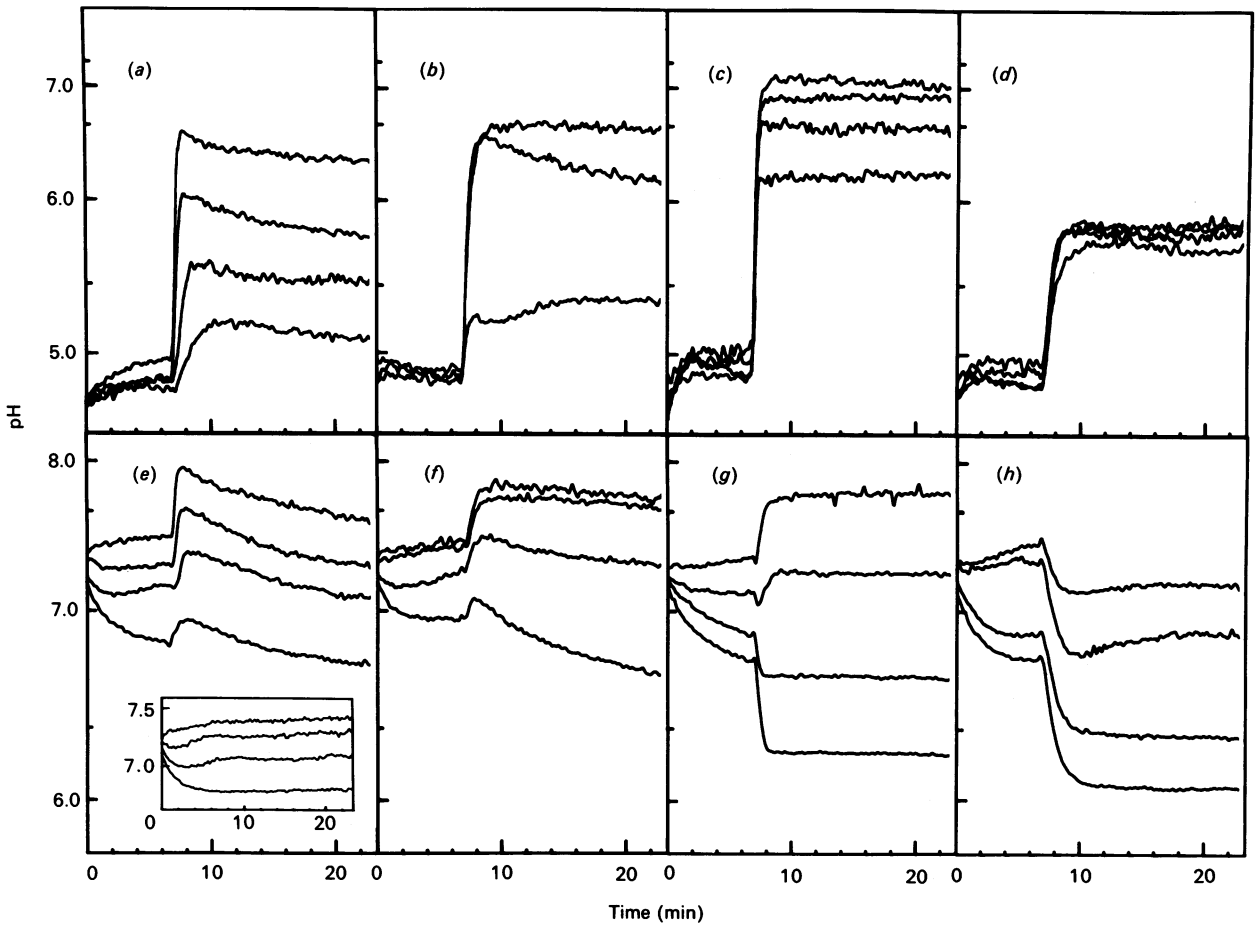

Fig. 4. pH_e-dependence of methylamine-, monensin- and nigericin-induced changes in lysosomal and cytosolic pH

pH_e-dependence of lysosomal (*a–d*) and cytosolic (*e–h*) pH changes induced by addition of methylamine (10 mM) in Na-medium (*a*, *e*), monensin (5 μM) in Na-medium (or 10 μM in Ch-medium) (*b*, *f*), nigericin (5 μM) in K-medium (*c*, *g*) or nigericin (0.5 μM) in Na-medium (*d*, *h*). pH values of the experimental media were (from lowest trace to uppermost): (*b*) 7.2 (Ch-medium), 6.4 and 7.8 (Na-medium); (*a*, *c–h*) 6.4, 6.8, 7.2 and 7.6. In (*e*) the inset shows the effect of pH_e on cytosolic pH with no stimulus added.

pH_e 6.4, corresponding very poorly to the pH_e-dependence for NAG secretion (see above) and clearly indicating that an increase in lysosomal pH may not always lead to secretion. It should be noted that the pH-sensitivity of FD decreases at higher pH. In contrast, methylamine induced a gradual increase in lysosomal pH with increasing pH_e (Fig. 4*a*), but also here the increase in lysosomal pH showed a somewhat different dependence on pH_e from that of the secretion of NAG.

Macrophage cytosolic pH was to some extent sensitive to changes in pH_e in the absence of ionophore or amine, but steady-state levels were reached within 5–6 min (Fig. 4*e*, inset). Methylamine and ionophores were therefore introduced 7 min after the change in pH_e. Methylamine- and monensin-induced increases in cytosolic pH were affected in a similar manner by changes in pH_e (Figs. 4*e* and 4*f*), although the increases occurring in response to monensin were better maintained with time than those induced by the amine, in particular at and above pH_e 7.2. In contrast, nigericin led to dramatic changes in cytosolic pH that were quite different in K-medium (Fig. 4*g*) and Na-medium (Fig. 4*h*) and that were very sensitive to pH_e. In K-medium cytosolic pH was increased by nigericin at and above pH_e 7.2, whereas it was decreased at and below pH_e 6.8. In Na-medium nigericin decreased cytosolic pH irrespective of pH_e. Below pH_e of approx. 7.0 (K-medium) and in the whole range of pH_e (Na-medium) respectively, the decrease in cytosolic pH may provide an explanation for the relative inability of nigericin to induce NAG secretion and further evidence that a decrease in cytosolic pH is inhibitory to lysosomal enzyme secretion.

The very different effects of bicarbonate on methylamine- and monensin-induced secretion (Figs. 3*a* and 3*b*) were accompanied by corresponding differences with regard to the changes in lysosomal and cytosolic pH. Thus bicarbonate did not affect methylamine-induced changes in either lysosomal pH (Fig. 5*a*; cf. Fig. 2*a*) or cytosolic pH (Fig. 5*c*; cf. Fig. 2*e*), whereas the increases in lysosomal and cytosolic pH induced by monensin were depressed (Figs. 5*b* and 5*d*; cf. Figs. 2*b* and 2*f*).

Inhibition of NAG secretion by nigericin and acetate

Further evidence that the secretion of lysosomal NAG is sensitive to changes in cytosolic pH is provided by the data in Table 2. When macrophages were exposed to a combination of nigericin and methylamine in Na-medium, the increase in lysosomal pH was larger than with either agent added separately, and cytosolic pH was decreased to the same extent as with nigericin alone (results not shown). Under these conditions the secretion of NAG was significantly less than with methylamine alone. Similarly, acetate, which was without effect on lysosomal pH, but depressed cytosolic pH, caused significant inhibition of NAG secretion induced by either monensin or methylamine.

Chlorpromazine-induced NAG secretion and changes in lysosomal and cytosolic pH

Chlorpromazine inhibits the secretion of lysosomal enzymes and mediators from granulocytes, platelets and mast cells [22–25], but was instead found to be a potent inducer of lysosomal enzyme secretion in macrophages (Fig. 6*a*), in particular at pH_e in the range 7.2–7.8 (Fig. 6*b*). However, chlorpromazine gave rise to only minor increases in lysosomal pH (Fig. 7*a*) and limited increases in cytosolic pH as well (Fig. 7*c*). Although chlorpromazine induced somewhat larger rises in lysosomal and cytosolic pH at pH_e in the alkaline range (Figs. 7*b* and 7*d*), the increases were still small compared with those seen with other stimuli. When cells were exposed to chlorpromazine in the presence of either monensin or nigericin in Na-medium, the secretory response was enhanced significantly (Fig. 6*a*). Under these conditions, increases in lysosomal pH were significantly larger than when ionophores were applied alone (results not shown). In contrast, changes in cytosolic pH were similar. Thus the rise in lysosomal pH most likely contributes to the enhancement of NAG secretion. Chlorpromazine-induced secretion was also sensitive to changes in cytosolic pH, however. As shown in Table 2, acetate, which depressed cytosolic pH in combination with chlorpromazine but left the small increase in lysosomal pH unaffected, led to inhibition of secretion.

Finally, under no conditions used in the present study was the secretion of NAG accompanied by any significant release of lactate dehydrogenase from the cells.

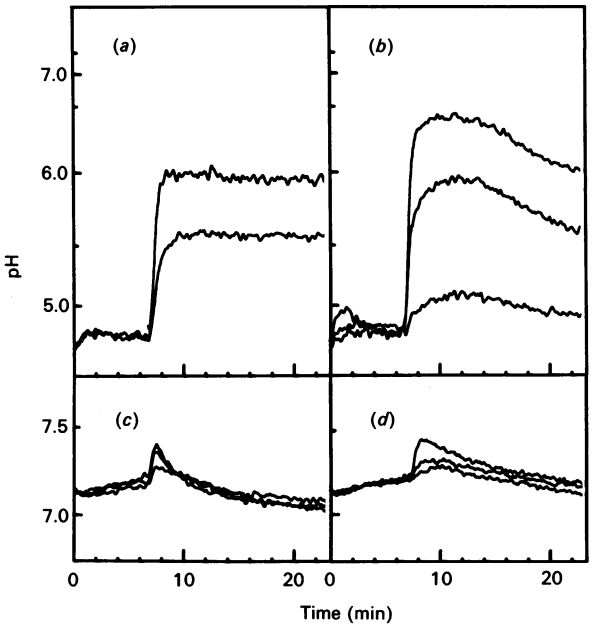

Fig. 5. Methylamine- and monensin-induced changes in lysosomal and cytosolic pH in the presence of bicarbonate

Lysosomal (*a*, *b*) and cytosolic (*c*, *d*) pH changes induced in Na/HCO_3-medium, pH_e 7.2 (gas phase with appropriate pCO_2), by addition of (from lowest trace to uppermost): (*a*) 5 and 10 mM-methylamine; (*b*) 0.5, 2 and 5 μM-monensin; (*c*) 5, 10 and 20 mM-methylamine; (*d*) 2, 5 and 10 μM-monensin.

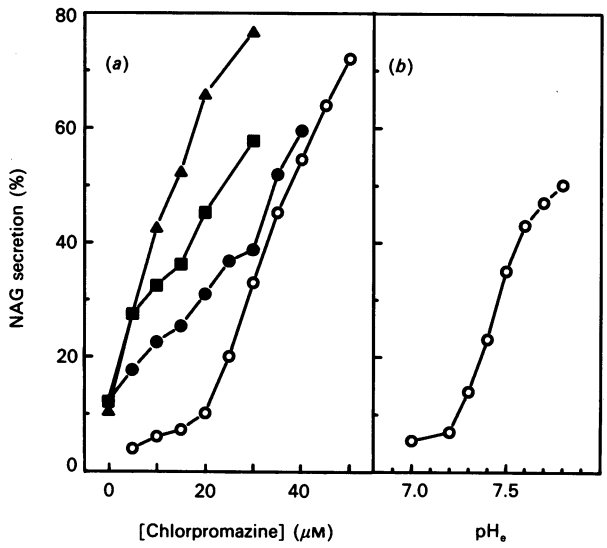

Fig. 6. Chlorpromazine as secretagogue, alone and in combination with methylamine, monensin or nigericin: pH_e-dependence of NAG secretion induced by chlorpromazine

NAG secretion in response to (*a*) indicated concentrations of chlorpromazine alone (○) or in combination with 5 mM methylamine (●), 1 μM-monensin (■) or 0.5 μM-nigericin (▲). In (*b*) pH_e-dependence of NAG secretion induced by 20 μM-chlorpromazine is shown. Results are representative of 14, 4, 4, 5 and 7 similar experiments respectively. Incubation time was 30 min in Na-medium either at pH_e 7.2 (*a*) or as indicated (*b*).

Table 2. Effect of combinations of stimuli on NAG secretion

Macrophage cultures were incubated in the presence of either agent A or agent B or a combination of A + B for 30 min in Na-medium at pH_e 7.20, unless otherwise indicated. The effect of combining the two agents is expressed as the fraction of additive effect on NAG secretion. The data presented are representative of at least three separate experiments.

Agent A		Agent B		Agent A+B	
	NAG secretion (% of total)		NAG secretion (% of total)	NAG secretion (% of total)	Fraction of additive effect
Nigericin (0.5 μM)	10.5	Methylamine (5 mM)	19.3	13.1	0.44
Nigericin (0.5 μM)	10.5	Methylamine (10 mM)	35.4	23.7	0.52
Acetate (30 mM)	2.9	Monensin (5 μM)	36.9	15.6	0.39
Acetate (30 mM)	2.9	Methylamine (10 mM)	35.4	8.1	0.21
Acetate (30 mM)*	2.9	Methylamine (10 mM)*	54.7	22.9	0.40
Acetate (30 mM)*	2.9	Chlorpromazine (20 μM)*	49.2	19.0	0.36

* Experiment conducted at pH_e 7.60.

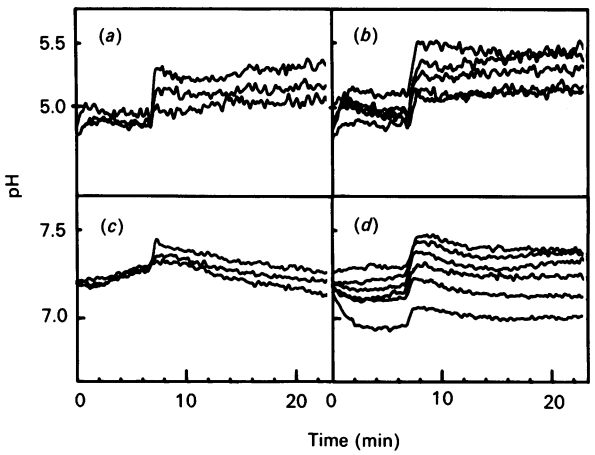

Fig. 7. Chlorpromazine-induced changes in lysosomal and cytosolic pH: dependence on extracellular pH

Lysosomal (*a*, *b*) and cytosolic (*c*, *d*) pH changes induced in Na-medium by addition of (from lowest trace to uppermost): (*a*, *c*) 10, 20 and 40 μM-chlorpromazine, pH_e 7.2; (*b*, *d*) 20 μM-chlorpromazine at pH_e 6.8, 7.0, 7.2, 7.4, 7.6 and 7.8.

DISCUSSION

Agents raising the pH of acidic organelles of cells generally exert an inhibitory effect on constitutive secretion. They also inhibit the intracellular sorting and receptor-mediated uptake of lysosomal enzymes, owing to interference with pH-dependent ligand–receptor dissociation [10,11,26,27]. In macrophages certain amines [8–11] and monensin [12] have, in addition, been shown to induce rapid and extensive secretion of preformed lysosomal enzymes. It has also been shown that this secretion is insensitive to protein-synthesis inhibitors [28] and that the secreted enzyme is of the processed mature type [11]. The rate and extent of NAG secretion from macrophages and the concomitant release of preloaded FD, shown here, provide further evidence that preformed enzyme is released by exocytosis of lysosomal contents. Such secretion, although much less extensive, can be induced also in fibroblasts [27].

In the present study we have tried to elucidate the relative role of changes in lysosomal and cytosolic pH in the initiation and regulation of macrophage lysosomal-enzyme secretion. By employing, in addition to methylamine, the proton-translocating ionophores monensin and nigericin, conditions were found where changes in lysosomal and cytosolic pH could be dissociated. Changes in cytosolic pH itself could be dissociated from the initiation of secretion by simply elevating pH_e in the absence of stimuli. On the other hand, massive release of enzyme was always accompanied by elevated lysosomal pH, and the similar dose–response relationship for secretion and elevation of lysosomal pH (except for nigericin in Na-medium) indicates a causal relationship. The mechanism(s) responsible for such a relationship is, however, not known. In addition to the change in lysosomal proton concentration, exposure to methylamine and, possibly, also to the ionophores could lead to osmotic effects on the lysosomes. Thus rapid permeation of methylamine through the lysosomal membrane would be followed by a slower accumulation, driven by lysosomal H^+-pumping and protonation of the amine. Accumulation of amine has been shown to occur in macrophages as well as in other cell types [29,30]. In accordance with the notion that an osmotic load could enhance secretion, the release of platelet lysosomal enzymes in response to thrombin was progressively enhanced by preincubation with amine [31]. A certain potentiation of methylamine- or chlorpromazine-induced secretion was observed in macrophages when an osmotic load on lysosomes was established by preincubation with sucrose (80 mM) overnight (results not shown). Monensin on its own causes swelling of organelles, notably the *trans*-Golgi cisternae [32], but monensin-induced enzyme secretion was not affected by sucrose pretreatment. Furthermore, vacuolization of lysosomes is not in itself sufficient to induce secretion of lysosomal enzyme, as shown by a lack of secretion in response to sucrose [9].

Secretion of lysosomal enzyme induced by agents that elevate lysosomal pH was significantly affected by changes in cytosolic pH. This was evidenced by comparing the pH_e-dependence of secretion with that of cytosolic pH changes induced by monensin and nigericin, and by the effects of nigericin or acetate when applied in combination with other stimuli. These results indicate that secretion is both inhibited by a decrease in cytosolic pH below a presumed physiological resting level and promoted by a rise above that level. It should be emphasized that large changes in cytosolic pH, as in some of our experimental conditions, were not necessary for a significant effect on NAG secretion. For example, the difference in pH_e-dependence for nigericin-induced (K-medium) and monensin-induced (Na-medium) secretion indicates that a change in cytosolic pH of even 0.1 unit in the range pH 6.8–7.2 leads to a measurable change in secretion.

The possibility that monensin-induced changes in cytosolic Na^+/K^+ ratio, rather than pH, could be responsible for the effects on NAG secretion appears unlikely. First, nigericin in Na-medium should affect the Na^+/K^+ ratio similarly, as shown in platelets [33], but secretion was affected differently and more in line with the effect on cytosolic pH. Secondly, nigericin (K-medium) and methylamine (Na-medium) induced secretion, without possibly affecting cation concentrations to a comparable extent.

The effect of including bicarbonate in the experimental medium provides further evidence for a connection between secretion and intracellular pH changes. In bicarbonate-containing medium, the monensin-induced increase in cytosolic pH was severely depressed, as was the secretory response, probably owing to counteraction of the alkalinization by Cl^-/HCO_3^- exchange. The surprising finding that the increase in lysosomal pH in response to monensin was also depressed indicates that bicarbonate affects lysosomal acidification, either directly by a mechanism as yet unidentified, or by enhancing lysosomal H^+-ATPase, as has been reported for H^+-ATPase reconstituted into liposomes [34]. The lack of effect on methylamine-induced secretion and pH changes could be explained by increased uptake of amine, compensating for increased buffer capacity and bicarbonate-dependent pH-regulation. Also, the methylamine-induced increase in cytosolic pH is already transient in the absence of bicarbonate. Corresponding effects of bicarbonate were seen on the cytosolic acidification induced by acetate and nigericin in Na-medium. The decrease in cytosolic pH when acetate was applied was transient and independent of bicarbonate, whereas the nigericin-induced decrease in cytosolic pH became transient in the presence of bicarbonate (not shown).

One possible target for the sensitivity of secretion to cytosolic pH changes would be the microtubular system. Lysosomes accumulate in the perinuclear region adjacent to the microtubule-organizing centre, and are often observed in contact with and possibly moving along microtubules [35]. Furthermore, significant redistribution of lysosomes within macrophages in response to treatments that would cause changes in cytosolic pH has recently been reported [36]. Acidifying protocols were found to counteract perinuclear accumulation and to result in reversible dispersion and apparent decrease in size of lysosomes, whereas alkalinizing protocols, shown here to promote exocytosis, had an opposite effect [36]. This may indicate that exocytosis of lyso-

somal contents occurs primarily close to the perinuclear region. So far, there is no evidence for directed secretion in macrophages towards either the adherent aspect of the cell or that facing the culture medium.

Chlorpromazine, an amphiphilic tertiary amine that inhibits many secretory responses [22–25], was in macrophages instead found to induce secretion of lysosomal enzyme with a marked dependence on pH_e. However, only minor changes occurred in either cytosolic or lysosomal pH in response to chlorpromazine, indicating that the secretory response involves some additional, possibly pH-sensitive, mechanism. Nevertheless, also here a cytosolic pH-dependence and a promoting effect of a rise in lysosomal pH were observed. Chlorpromazine has been shown to exert numerous effects in cell-free and cellular systems. It was early recognized to be a Ca^{2+}-dependent calmodulin antagonist [37], but has later been shown to inhibit a number of membrane-associated processes, such as the activation of protein kinase C [38], the mobilization of arachidonic acid [39] and the generation of superoxide [40]. Probably, these effects are secondary to its partitioning into cellular membranes [41], where it intercalates and may affect lipid packing, surface charge and ionic interactions in the membrane. How this relates to the ability of chlorpromazine to induce secretion of lysosomal enzyme in macrophages, as shown here, is not clear.

Whether the initiating effect on lysosomal-enzyme secretion of a rise in lysosomal pH and/or the cytosolic pH-dependence hold also for stimuli relevant to the situation *in vivo* remains to be investigated. Since macrophages would be expected to encounter somewhat acidic environments, the inhibition of enzyme secretion by acidification of the macrophage cytosol might act to limit this process. Alternatively, the cellular response to relevant agents involves regulatory changes that either cause a shift in the pH-dependence of the secretory process or make the macrophage able to maintain cytosolic pH also in an acidic environment.

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