JOURNAL OF CELLULAR PHYSIOLOGY 135:169–178 (1988)

Osmotic Forces Are Not Critical for Ca^{2+}-Induced Secretion From Permeabilized Human Neutrophils

SALLY JO STOEHR AND JAMES E. SMOLEN*

Departments of Pediatric Hematology-Oncology (S.J.S., J.E.S.), and Pathology (J.E.S.), The University of Michigan Medical Center, Ann Arbor, Michigan 48109

In order to examine the role of osmotic forces in degranulation, the effects of solutes and osmolality on granule secretion were explored using both FMLP-stimulated, intact neutrophils and Ca^{2+}-stimulated, permeabilized cells. We employed a HEPES-based buffer system which was supplemented with: a) permeant (KCl or NaCl) or impermeant (Na-isethionate or choline-Cl) ions, or b) permeant (urea) or impermeant (sucrose) uncharged solutes. Intact and permeabilized cells had significantly different solute requirements for degranulation. FMLP-stimulated release from intact cells was supported by NaCl or Na-isethionate > KCl > choline-Cl or sucrose > urea. In contrast, the rank order of Ca^{2+}-stimulated release from permeabilized cells was choline-Cl > Na-isethionate, KCl, or NaCl > sucrose > urea. Hypo-osmotic conditions caused increased levels of background granule release from both intact and permeabilized neutrophils. However, hypo-osmolality inhibited both FMLP-stimulated degranulation from intact cells and Ca^{2+}-induced release from permeabilized neutrophils. While hyperosmotic conditions inhibited stimulated release from intact cells, this inhibition was much less pronounced in permeabilized cells when the granules were directly exposed to these solutions. In fact, hyperosmotic sucrose greatly enhanced Ca^{2+}-induced secretion. Although isolated specific and azurophil granules showed some lytic tendencies in hypo-osmotic buffers, the overall stability of the isolated granules did not indicate that swelling alone could effect degranulation. These results suggest that degranulation in permeabilized cells is neither due to nor driven by simple osmotic forces (under resting or stimulated conditions) and emphasize differences obtained by bathing both the granules and plasma membrane (as opposed to membranes alone) in various solutes.

Activation of intact neutrophils by stimuli such as the chemotactic peptide N-formyl-l-methionyl-l-leucyl-phenylalanine (FMLP) results in changes in membrane potential, fluxes of ions across the membrane, mobilization of intracellular Ca^{2+}, fusion of granule membranes with the plasma membrane, and discharge of granule contents into the extracellular space or the phagosome. While these events have been described in detail with respect to timing and contributing biochemical factors, the actual events of fusion and discharge of granule constituents are relatively unknown.

Degranulation from intact neutrophils is highly dependent on the ionic content of the extracellular milieu; replacement of Na^+ with $choline^+$ or K^+ greatly reduces lysosomal enzyme release from neutrophils exposed to concanavalin A or immune complexes (Korchak and Weissmann, 1980). Preincubation of neutrophils with anion channel blockers prior to stimulation markedly inhibits or eliminates exocytosis (Korchak et al., 1982).

Active or passive fluxes of ions across plasma and granule membranes can be accompanied by the passive flux of water. In view of this, changes in osmolality have been proposed to play a role in promoting vesicle-plasmalemma membrane fusion and exocytosis. This hypothesis is supported by observations that fusion of artificially constructed black lipid bilayers requires osmotic gradients across the bilayers (Fisher and Parker, 1984), and that phospholipid vesicles fuse with bilayer membranes upon osmotic swelling (Cohen et al., 1980). Furthermore, degranulation by sea urchin eggs (Zimmerberg et al., 1985), neutrophils (Yassin et al., 1985), and chromaffin cells (Hampton and Holz, 1983) can be inhibited by hypertonicity. In accord with this postulated osmotic fragility, isolated chromaffin granules placed in hypo-osmotic solutions (200 mOs) release more than 70% of their contents (Hampton and Holz, 1983), whereas granules within intact cells exposed to similar hypo-osmotic conditions are much more stable, releasing only about 25% of their contents into the intracellular milieu.

Permeabilization of the plasma membranes of cells using a variety of means has allowed direct manipulation of the intracellular space (Knight and Baker,

Received July 6, 1987; accepted December 9, 1987.

*To whom reprint requests/correspondence should be addressed.

1982; Dunn and Holz, 1983; Wilson and Kirshner, 1983; Smolen et al., 1986; Howell and Gomperts, 1987). While intact cells possess two barriers to solutes and water which might have an effect on secretory events, only the granule membranes could remain osmotically active in the permeabilized cell. Thus, the permeabilized cell system permits the direct testing of the effects of different solutes and changes in osmolality on the pertinent organelles, the granules.

Variable results have been obtained on the effects of hyperosmolality on cultured chromaffin cells permeabilized with digitonin. Holz and Senter (1987) observed little inhibition of Ca^{2+}-induced secretion from permeabilized chromaffin cells preincubated in hyperosmotic sucrose buffers up to 1000 mOs, while K-glutamate or Na-isethionate were more potent inhibitors of the Ca^{2+}-induced response. Ladona et al. (1987) observed 50% inhibition of Ca^{2+}-induced secretion at 600 mOsM sucrose from cultured cells permeabilized with either digitonin or a-toxin. These results more closely parallel data obtained from chromaffin cells permeabilized with intense electric fields, where hypertonic sucrose buffers in the 700 mOsM range caused 50% inhibition of Ca^{2+}-induced secretion (Knight and Baker, 1982).

Using a number of solutes which were permeable or impermeable to the membranes of neutrophils, we wished to assess possible differences in solute requirements for degranulation from both intact and permeabilized neutrophils. We also wished to conduct comprehensive studies over a wide range of solute concentrations and compositions to confirm the effects of hyperosmolality on intact cells and to see if permeabilized cells, which have only the granule membranes intact, would be similarly affected. Comparisons of degranulation in both cell systems could provide valuable information on the requirements for solute flux and osmotic stress for granule discharge.

We report here that intact and permeabilized cells had significantly different solute requirements for secretion. It was expected that if osmotic stress were involved in exocytosis, hypo-osmolality would increase stimulated degranulation from both the intact and permeabilized cells. In contrast, we found that hypo-osmotic conditions inhibited stimulated degranulation in both systems. Furthermore, the osmotic stress hypothesis would predict that hyperosmotic conditions should inhibit degranulation. While this prediction held for intact cells, hyperosmotic solutions did not greatly reduce Ca^{2+}-induced secretion from permeabilized neutrophils. Finally, while isolated specific and azurophil granules were somewhat unstable when incubated in hypo-osmolar buffers, granule lysis was not extensive, indicating that swelling, by itself, was insufficient to explain the degranulation step. Thus, our data do not support the hypothesis that osmotic stress may contribute to the processes of fusion and degranulation.

MATERIALS AND METHODS

Reagents

Digitonin (Lot 62F-0135), choline-Cl, isethionic acid (sodium salt), cytochalasin b, and FMLP were purchased from Sigma Chemical Co., St. Louis, MO. Ultrapure sucrose was obtained from International Technologies, New Haven, CT. Urea was obtained from Fisher Scientific, Fair Lawn, NJ, and Percoll was purchased from Pharmacia, Piscataway, NJ. All other chemicals were reagent grade.

Cells

For intact and permeabilized cell studies, neutrophils were isolated from heparinized blood of healthy, adult donors by standard techniques of Hypaque-Ficoll gradients (Boyum, 1968) followed by dextran sedimentation and hypotonic lysis of remaining red cells (Zurier et al., 1973). Concentrated stocks of KCl, NaCl, Na-isethionate, choline-Cl, urea, and sucrose were prepared in the presence of 30 mM K^+-HEPES and 1 mM EGTA (pH 7.0). These stocks were diluted to the desired molarities with a 30 mM K^+-HEPES, 1 mM EGTA solution (pH 7.0).

Osmolalities of each buffer were obtained by direct measurement on an advanced digimatic osmometer (Advanced Instruments, Inc., Model 3D-II, Needham Heights, MA) which measures osmolality on the basis of freezing point depression. Neutrophils bathed in an iso-osmotic phosphate buffer containing 138 mM NaCl, 2.7 mM KCl, 8.1 mM Na_2HPO_4, 1.5 mM KH_2PO_4, 0.6 mM $CaCl_2$, and 1.0 mM $MgCl_2$, pH 7.4 (PiCM), served as controls for intact cells suspended in other buffers. In general, 5×10^6 cells/ml were preincubated for 5 minutes with the desired solutes in K^+-HEPES buffer with 5 ug/ml cytochalasin b, 1.6 mM $CaCl_2$, and 1 mM sodium azide. Cytochalasin b was included to produce maximal secretion from this system. Azide was added to prevent destruction of vitamin B-12 binding protein by secreted myeloperoxidase. FMLP (10^{-7} M) was added, and incubation was continued for an additional 5 min. The cell-free media were assayed for B-glucuronidase (Brittinger et al., 1968), lysozyme (Worthington Enzyme Manual, 1972), vitamin B-12 binding protein (Smith and Peters, 1982), and lactate dehydrogenase (Wacker et al., 1956).

For studies of permeabilized cells, neutrophils (25×10^6/ml) were suspended in an iso-osmolar buffer containing 100 mM KCl, 20 mM NaCl, 30 mM HEPES, and 1 mM EGTA, pH 7.0 (Buffer K) and pre-warmed to 37°C for 10 min. Digitonin (10 μg/ml) was then added, and the cells were incubated an additional 25 min (Smolen et al., 1986). Aliquots of the permeabilized cells were transferred to tubes containing the prewarmed buffers of choice, with or without 60 μM free Ca^{2+}, the stimulus for degranulation. The cell suspensions were incubated for 7 min at 37°C, centrifuged, and the supernatants harvested for analyses. Ca^{2+}-induced enzyme release from cells in the HEPES-based buffer, brought to iso-osmolarity with KCl, served as the 100% control.

Cell fractionation

Neutrophils isolated for cell fractionation were prepared from normal human blood, with acid citrate dextrose as the anti-coagulant, by techniques described by Curnutte and Babior (1974). The neutrophils were disrupted and the specific and azurophil granules were purified using discontinuous Percoll gradients (Borregaard et al., 1983). Granules were resuspended in Buffer K at $100–125 \times 10^6$ cell equivalents/ml. Aliquots of the granule suspensions ($20–25 \times 10^6$ cell

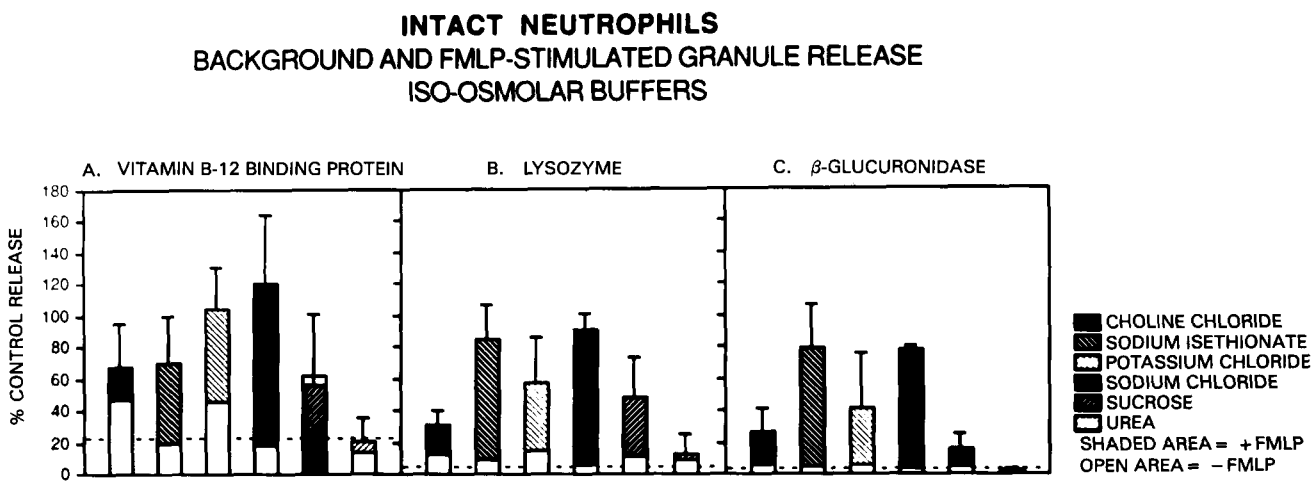

Fig. 1. Effects of different solutes on background and FMLP-stimulated release of granule markers from intact neutrophils. Intact neutrophils (5×10^6/ml) were preincubated for 5 min at 37°C in iso-osmolar buffers containing 120 mM of the solute of choice, 30 mM K^+-HEPES, 1 mM EGTA, (pH 7.0 with KCl) supplemented with 1.6 mM $CaCl_2$, 1 mM azide, and 5 μg/ml cytochalasin b. Actual mOs for each buffer were choline-Cl (250), Na-isethionate (260), KCl (260), NaCl (255), sucrose (295), and urea (275). Stimulation of the cells with FMLP (10^{-7} M) was for an additional 5 min. Supernatants of the pelleted samples were assayed for vitamin B-12 binding protein (Panel **A**), lysozyme (Panel **B**), and B-glucuronidase (Panel **C**). Neutrophils suspended in PiCM, supplemented with azide and cytochalasin b, and stimulated with FMLP served as the 100% control (FMLP stimulation of neutrophils in PiCM resulted in release of 23.7 ± 6.3% of total cellular vitamin B-12 binding protein, 59.5 ± 10.7% lysozyme, and 36.4 ± 11.0% B-glucuronidase, n = 10). Background release in PiCM (------) is expressed here as a percentage of stimulated release (background release averaged 5.1 ± 3.3% of total cellular vitamin B-12 binding protein, 3.8 ± 1.6% lysozyme, and 1.8 ± 0.8% B-glucuronidase, n = 10). Background release of granule constituents incubated in experimental buffers alone is shown by the open bars, and FMLP-stimulated release by the solid bars (all panels). The results are given as means of at least three experiments, and SD's are illustrated for the FMLP-stimulated samples.

eq/0.2 ml) were then transferred to the buffers of choice, and incubated 7 min at 37°C. Granules were separated from the supernatants by centrifugation at 1500g for 20 min at 4°C. Aliquots of the supernatants were assayed for vitamin B-12 binding protein, myeloperoxidase (Bretz and Baggiolini, 1974), and lysozyme content. Tritonized samples of the isolated granules served as 100% controls.

RESULTS

Solute composition

We first wished to compare the effects on degranulation of several solutes, both permeant and impermeant, charged and uncharged, at iso-osmolar concentrations. We selected NaCl (permeant ions) to mimic the extracellular environment and KCl (permeant ions) to mimic the intracellular milieu. Na-isethionate was used to replace Cl^- with an impermeant anion, and choline-Cl to substitute for Na^+ or K^+ as an impermeant cation. Sucrose was used as an impermeant, uncharged solute, and urea as a permeant, uncharged solute.

Results are presented in all Figures (except Fig. 9) as proportions of granule constituent release compared to defined, stimulated control samples, not total granular content. The number of variables examined in typical experiments required combining results from several individual experiments. Substantial day-to-day variability existed in the percentage of total granule release when intact neutrophils were stimulated with FMLP. Likewise, the permeabilization technique itself varied daily in its effectiveness and subsequent Ca^{2+}-induced release correlated with the degree of permeabilization (Smolen et al., 1986). Thus, normalization of the data as percentages of stimulated controls was necessary and allowed us to compile results from several experiments.

Figure 1 shows background and FMLP-stimulated release of vitamin B-12 binding protein (Panel A), lysozyme (B), and B-glucuronidase (C) from intact neutrophils incubated in the various iso-osmolar buffers. All data are given as percentages of FMLP-stimulated release from control cells incubated in PiCM. Cytochalasin b was employed so that azurophil granule secretion could be obtained.

In most cases, background release of all three granule markers (open bars) was comparable to that observed in control cells (dashed line). However, iso-osmolar choline-Cl, KCl, and sucrose buffers supported higher levels of background vitamin B-12 binding protein release (from specific granules) than the control (Panel A).

NaCl, the buffer which most closely mimics the extracellular milieu of the cell, supported nearly optimal FMLP-stimulated release of all granule markers (Panels A–C). When the chloride ion was replaced with isethionate, an optimal response was still observed. KCl supported only half-maximal stimulated degranulation from both specific (vitamin B-12 binding protein and lysozyme) and azurophil (lysozyme and B-glucuronidase) granules. Choline-Cl was a poor substitute for Na^+; sucrose was comparable to choline-Cl. Urea failed to support any FMLP-stimulated degranulation (Panels A–C). Thus, optimal degranulation from intact neutrophils required an ionic-based buffer and, specifically, extracellular Na^+. The permeable cation K^+ was only somewhat effective as a substitute for Na^+. When cations were absent (sucrose and urea) or cation

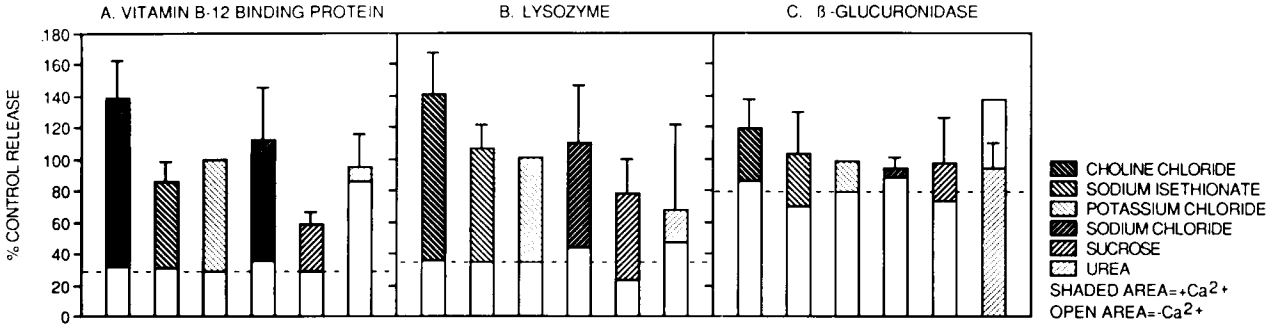

Fig. 2. Effects of different solutes on background and Ca^{2+}-induced release of granule markers from permeabilized neutrophils. Neutrophils (25×10^6/ml in Buffer K) were permeabilized with 10 μg/ml digitonin for 25 min at 37°C. Aliquots of the cell suspensions (5×10^6 in 0.2 ml) were added to 0.8 ml of pre-warmed iso-osmolar buffers containing 120 mM of the solute of choice, 30 mM K^+-HEPES, 1 mM EGTA, (pH 7.0 with KCl) with or without 60 μM free Ca^{2+}. For actual mOs of each buffer, see the legend for Figure 1. Incubation of the cell suspensions was continued for an additional 7 min. Supernatants of the pelleted samples were assayed for vitamin B-12 binding protein (Panel **A**), lysozyme (Panel **B**), and B-glucuronidase (Panel **C**). Neutrophils suspended in K^+-HEPES based buffer, brought to iso-osmolarity with KCl and supplemented with Ca^{2+}, served as the 100% control of (Ca^{2+}-induced release from permeabilized neutrophils in this buffer averaged 24.0 ± 8.0% of total cellular vitamin B-12 binding protein, 18.5 ± 9.3% lysozyme, and 21.8 ± 11.1% B-glucuronidase, n = 19). Background release in iso-osmolar KCL-HEPES buffer (-------) is expressed here as a percentage of Ca^{2+}-induced release (background release averaged 6.9 ± 2.8% of total cellular vitamin B-12 binding protein, 6.3 ± 4.0% lysozyme, and 17.2 ± 9.8% B-glucuronidase, n = 19). Background release of granule constituents is shown by the open bars, and Ca^{2+}-stimulated release by the solid bars (all panels). The results are given as means of at least three experiments, and SDs are illustrated for the Ca^{2+}-stimulated samples.

influx was prevented (choline-Cl), degranulation was severely inhibited. The reduction of anionic fluxes alone (Na-isethionate) did not inhibit the ability of the neutrophils to secrete.

Figure 2 shows background and Ca^{2+}-induced granule release from permeabilized neutrophils in various iso-osmolar buffers. All data are given as percentages of Ca^{2+}-stimulated release from permeabilized neutrophils exposed to the HEPES-based buffer, brought to iso-osmolarity with KCl, and supplemented with 1.6 mM $CaCl_2$ (to assure that excess Ca^{2+} remained after partial chelation with EGTA) to stimulate degranulation.

Urea was the only solute which caused a significant increase in background granule marker release. Ca^{2+}-induced vitamin B-12 binding protein and lysozyme release was substantial in all ionic buffers and in sucrose (Panels A and B, first 5 bars). In general, stimulated release was supported according to the following rank order: choline-Cl > Na-isethionate, KCl, or NaCl > sucrose. This hierarchy of support for granule discharge in these buffers was, however, different and much less pronounced than that observed for intact neutrophils stimulated with FMLP. Urea failed to support significant Ca^{2+}-induced degranulation over background (Panels A–C). Ca^{2+}-induced B-glucuronidase release from azurophil granules was modest under the conditions employed; this was expected since azurophil granule release from permeabilized neutrophils generally requires higher Ca^{2+} concentrations than employed here (Smolen et al., 1986). However, similar hierarchical patterns were observed. Therefore, ionic buffers of any kind supported degranulation from permeabilized neutrophils, though permeability of the ions was unimportant.

Osmolality

We extended our investigations of stimulated degranulation from the two cell systems to investigate whether changes in osmolality (along with the already observed differences in solute requirements) might affect degranulation differently. We adjusted our buffer systems to observe degranulation from intact and permeabilized neutrophils at concentrations ranging from 90 to 680 mOs. We wished to verify the inhibitory effects of hyperosmolality on intact cells, and to systematically investigate osmotic effects on permeabilized cells.

Figures 3, 4, and 5 show background and net FMLP-stimulated release of granule markers from intact neutrophils bathed in buffers of various osmolal strengths. Once again, all data are expressed as a percentage of the FMLP-stimulated control (iso-osmolar PiCM). Background release (panels A and B, all Figs.) represents granule constituent release as a percentage of this control for each condition. Net release (panels C and D, all Figs.) is the difference between the FMLP-stimulated sample (as a proportion of the control) and the illustrated backgrounds. Hypo-osmotic conditions caused extensive background release of vitamin B-12 binding protein, and to a lesser extent, lysozyme, from intact cells (Figs. 3 and 4, Panels A and B), an observation consistent with the theory of osmotically-induced granule swelling.

However, net FMLP-stimulated granule release was inhibited under both hypo-osmotic and hyperosmotic

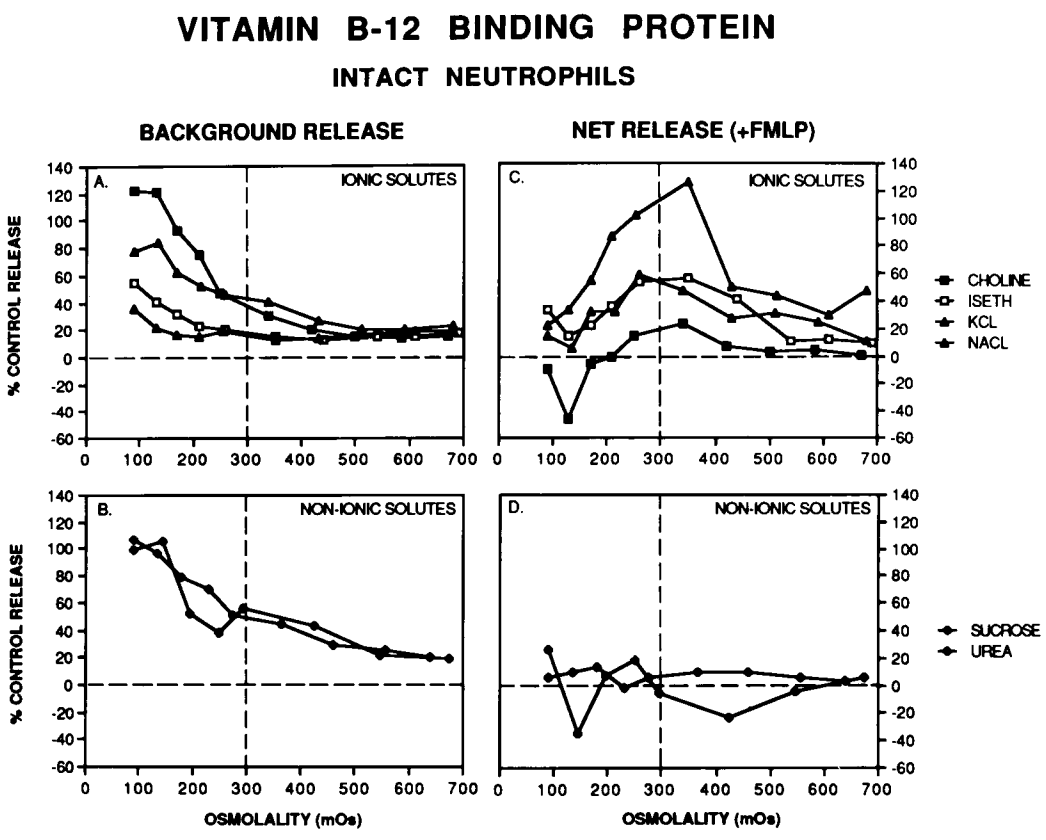

Fig. 3. Effects of osmolality of various solutes on background and FMLP-stimulated vitamin B-12 binding protein release from intact neutrophils. Intact neutrophils (5×10^6/ml) were preincubated for 5 min at 37°C in buffers of various solutes and osmolalities, containing 30 mM K^+-HEPES, 1 mM EGTA, (pH 7.0 with KOH), 1.6 mM $CaCl_2$, 1 mM azide, and 5 μg/ml cytochalasin b. Stimulation of the cells with FMLP (10^{-7} M) was for an additional 5 min. Supernatants of the pelleted samples were assayed for vitamin B-12 binding protein, a specific granule marker. Neutrophils suspended in PiCM, supplemented with azide and cytochalasin b, and stimulated with FMLP served as the 100% control. Background vitamin B-12 binding protein release from ionic buffers (as a percentage of this control) is shown in Panel **A**, and background release from non-ionic buffers in Panel **B**. Net FMLP-stimulated release (% control release-background release) is shown for ionic buffers in Panel **C** and for non-ionic buffers in Panel **D**. The results are means of at least three experiments.

conditions when the intact cells were bathed in ionic buffers (Panel C of Figs. 3–5). Cells exposed to sucrose reacted similarly, with the exception of vitamin B-12 binding protein (Panel D of Figs. 3–5). Failure of sucrose to support stimulated release of this constituent is not easily explained. While it was recognized that background vitamin B-12 release under highly hypo-osmotic conditions was significant (up to 33% of the total vitamin B-12 binding protein present), most of this marker remained in the cells and FMLP-stimulation could have subsequently triggered additional release. Urea was the only solute which blocked all FMLP-stimulated degranulation; this was not likely to be due to the effects of urea on the FMLP receptor (Yassin et al., 1985).

Figures 6, 7, and 8 show background and net Ca^{2+}-induced release of granule markers from permeabilized neutrophils (expressed as percentages of the defined control samples) in solutions of various osmolal strengths. Like intact cells, hypo-osmotic, ionic buffers caused background release of granule markers (Panel A, Figs. 6–8).

Net Ca^{2+}-induced release from permeabilized cells, like net FMLP-stimulated release from intact cells, was inhibited under hypo-osmotic conditions, an observation contrary to the simple osmotic hypothesis. Most significantly, only weak inhibition of Ca^{2+}-induced lysozyme degranulation was observed under hyperosmotic conditions (Fig. 7, Panels C and D). Surprisingly, hyperosmotic sucrose buffers greatly enhanced Ca^{2+}-induced granule discharge (Panel D, Figs. 6–8), an effect which has no parallel with intact cells. Urea appeared to be lytic when it was directly bathing the granules, reflected in the high background release of granule constituents; Ca^{2+}-induced granule release in urea was constant or even lower than the backgrounds (Panel D, Figs. 6–8). These situations are likely to be due to the ability of Ca^{2+} to stabilize membranes against lysis induced by hypotonicity or urea.

We also examined the effects of osmolarity on specific and azurophil granules isolated from neutrophils by means of cell fractionation and purification on discontinuous Percoll gradients. Figure 9 shows the release of constituents from granules incubated in hypo-osmolar, iso-osmolar, and hyperosmolar KCl, sucrose, or urea. Granules bathed in Na-isethionate, choline-Cl, or NaCl reacted similarly to those in KCl, and these data are not shown. Ca^{2+}_{fr} (60 μM) was

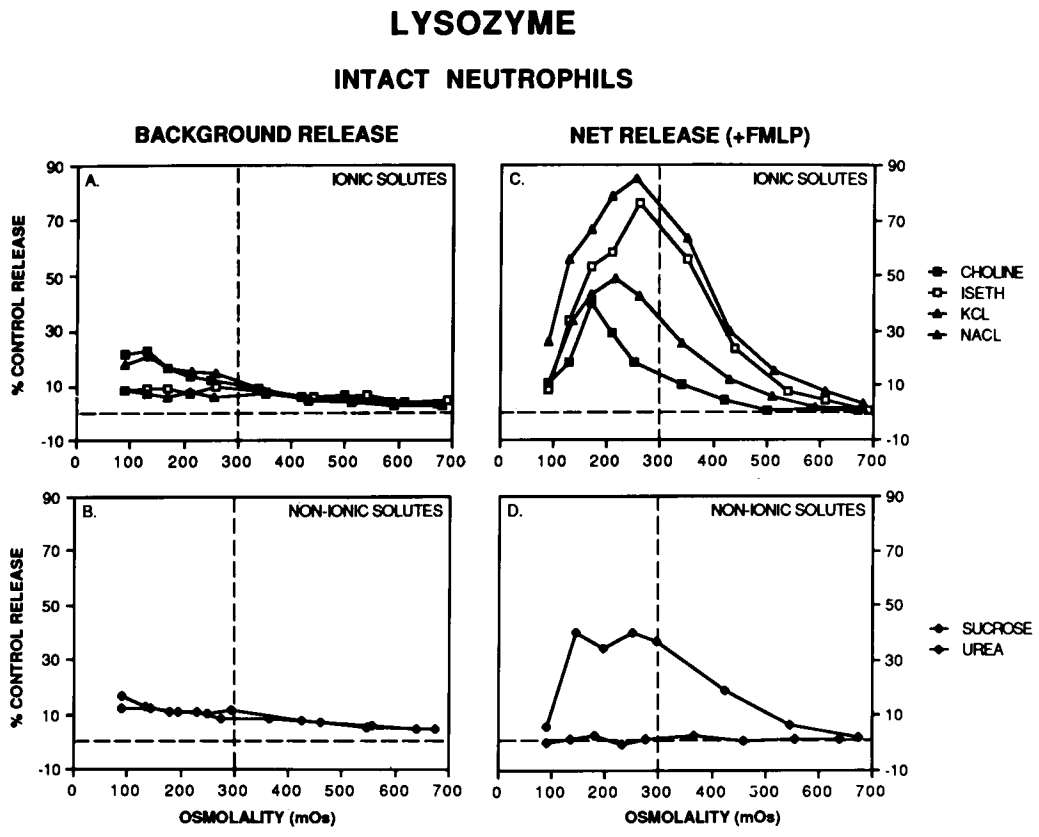

Fig. 4. Effects of osmolality of various solutes on background and FMLP-stimulated lysozyme release from intact neutrophils. Intact neutrophils (5×10^6/ml) were treated as outlined in the legend to Figure 3. Supernatants of the pelleted samples were assayed for lysozyme, a constituent of both specific and azurophil granules.

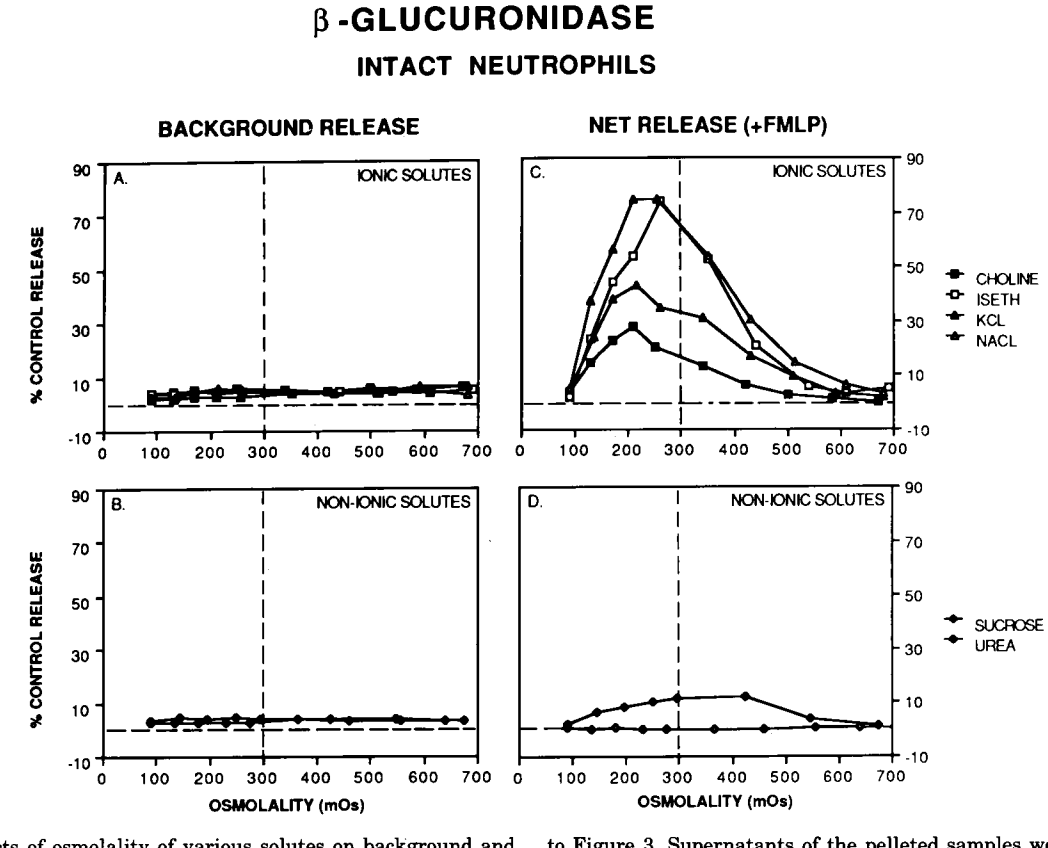

Fig. 5. Effects of osmolality of various solutes on background and FMLP-stimulated B-glucuronidase release from intact neutrophils. Intact neutrophils (5×10^6/ml) were treated as outlined in the legend to Figure 3. Supernatants of the pelleted samples were assayed for B-glucuronidase, an enzyme found exclusively in azurophil granules.

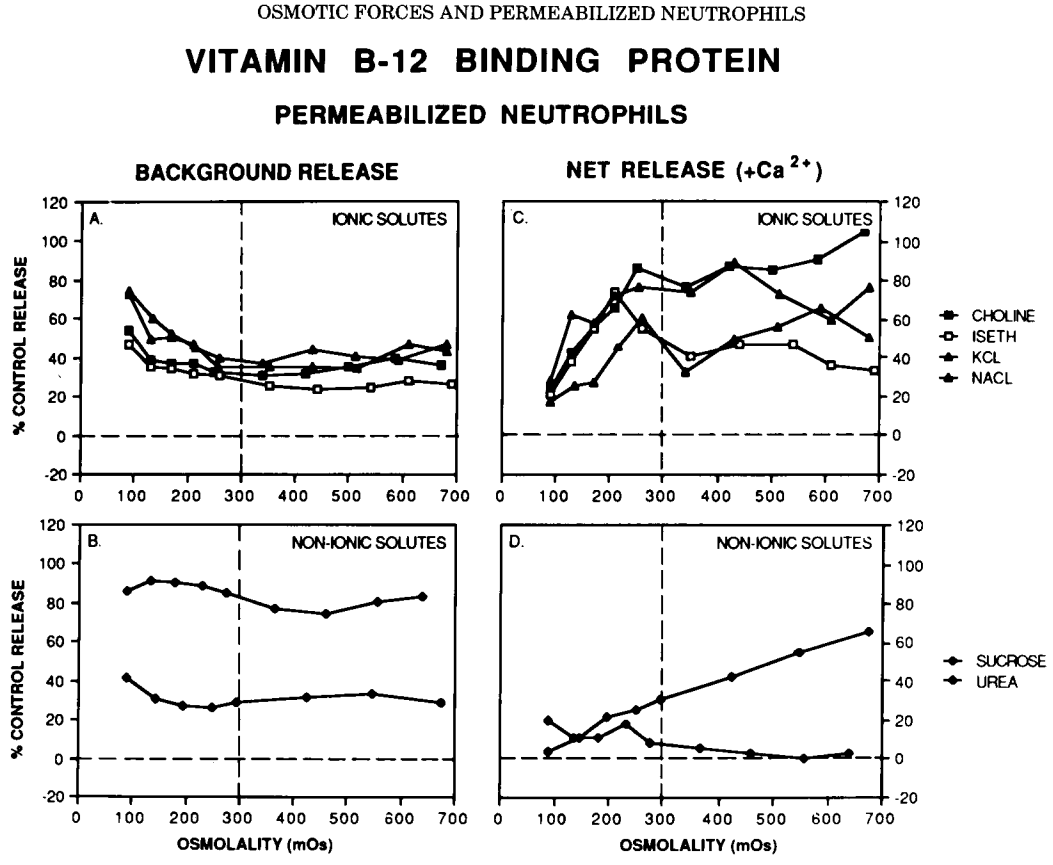

Fig. 6. Effects of osmolality of various solutes on background and Ca^{2+}-induced vitamin B-12 binding protein release from permeabilized neutrophils. Neutrophils (25×10^6/ml in Buffer K) were permeabilized with 10 μg/ml digitonin for 25 min at 37°C. Aliquots of the cell suspensions (5×10^6 in 0.2 ml) were added to 0.8 ml of pre-warmed buffers of various solutes and osmolal strengths, containing 30 mM K^+-HEPES, 1 mM EGTA, (pH 7.0 with KOH), with or without 60 μM free Ca^{2+}. Incubation of the cell suspensions was continued for an additional 7 min. Supernatants of the pelleted samples were assayed for vitamin B-12 binding protein. Neutrophils suspended in the HEPES-based buffer, brought to isotonicity with KCl and supplemented with 60 μM free Ca^{2+} served as the 100% control. Background vitamin B-12 binding protein release from ionic buffers (as a percentage of this control) is shown in Panel **A**, and background release from non-ionic buffers in Panel **B**. Net Ca^{2+}-stimulated release (% control release-background release) is shown for ionic buffers in Panel **C** and for non-ionic buffers in Panel **D**. The results are means of at least three experiments.

added to one-half the samples to serve as a control for Ca^{2+}-induced release routinely observed from permeabilized cells. In general, granule marker release was greater under hypo-osmotic conditions than from granules within permeabilized neutrophils exposed to similar conditions (permeabilized neutrophils released up to 35% of their total B-glucuronidase under highly hypo-osmotic conditions, and lesser amounts of lysozyme or vitamin B-12 binding protein, shown in Figures 6–8 as percent control). Lysozyme release from both specific and azurophil granules incubated in hypo-osmolar KCl or sucrose was greater than release under iso-osmolar conditions (Panels A and C). However, this trend was less pronounced or absent for vitamin B-12 binding protein (specific granules, Panel B) or myeloperoxidase (azurophil granules, Panel D). Hyperosmotic conditions did not, for the most part, reduce granule discharge; rather, release of specific granule markers increased (Panels A and B). The increases in granule marker release (except myeloperoxidase) with urea concentration indicated that this solute was lysing the granules. The addition of 60 μM free Ca^{2+} significantly increased or decreased discharge of granule constituents in some instances ($p < .05$). However, the addition of Ca^{2+} to granules bathed in hyperosmotic sucrose did not significantly increase granule discharge. Thus, interactions between hyperosmotic sucrose, Ca^{2+}, and the isolated granules were inadequate to explain the phenomenon observed in permeabilized cells; namely, that hyperosmotic sucrose and Ca^{2+} greatly enhanced granule secretion.

DISCUSSION

A comparison of the data on stimulated degranulation from intact and permeabilized neutrophils in the various iso-osmolar buffers indicated that intact and permeabilized cells had significantly different solute requirements for secretion. Na^+ appeared to be an essential constituent for FMLP-stimulated granule release from intact cells, in agreement with previous reports to this effect (Showell et al., 1977; Korchak et al., 1982), while all the ionic buffers supported Ca^{2+}-induced degranulation from permeabilized neutrophils rather equally. Replacement of Na^+ with either the permeant cation K^+ or the impermeant cation $choline^+$ resulted in a decreased response in the intact cell system, but the elimination of Cl^- was not inhibitory. The observed optimal secretory response of intact neutrophils incubated in Na-isethionate buffer brings into question the specificity of previously investigated

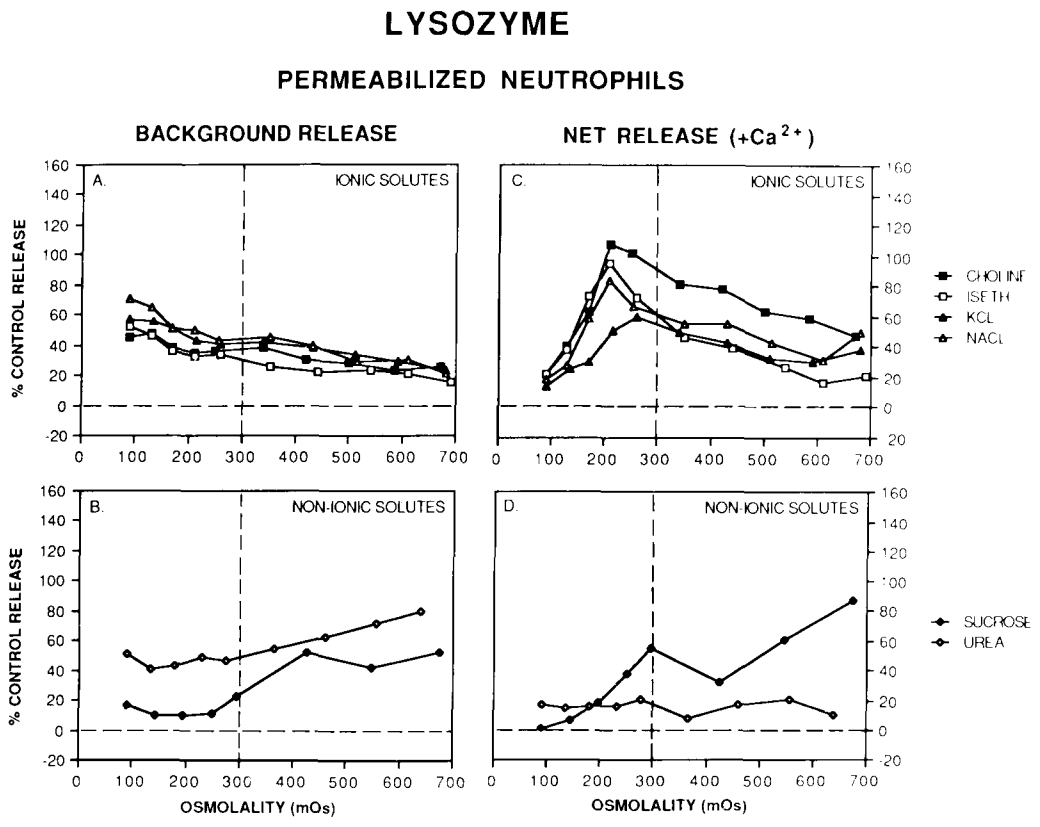

Fig. 7. Effects of osmolality of various solutes on background and Ca^{2+}-induced lysozyme release from permeabilized neutrophils. Neutrophils were treated as outlined in the legend to Figure 6. Supernatants of the pelleted samples were assayed for lysozyme.

anion channel blockers, SITS and DIDS (Korchak et al., 1982). The uncharged, impermeant solute sucrose supported only minimal degranulation from intact or permeabilized neutrophils, and urea was consistently inhibitory in both cell systems. These latter results suggest that substantial concentrations of ionic solutes are required for secretion.

If an osmotic component of granule discharge were important in secretion, then we would have expected that hypo-osmolality would enhance degranulation from permeabilized cells and, perhaps, intact cells as well; instead, we observed the opposite response. While hypo-osmolality caused increased levels of background granule release from both intact and permeabilized neutrophils, hypo-osmolality actually inhibited stimulated degranulation.

Hyperosmolal buffers did inhibit FMLP-stimulated degranulation from intact neutrophils, as would be anticipated if swelling of the granules were important for effecting secretion. Similar observations have been made on intact chromaffin cells exposed to hyperosmotic sucrose, stachyose, or potassium glutamate (Hampton and Holz, 1983), platelets bathed in hyperosmotic NaCl or sucrose (Pollard et al., 1977), and sea urchin eggs in Na-sulfate, sucrose, or stachyose (Zimmerberg and Whitaker, 1985). However, inhibition was much less pronounced in permeabilized neutrophils when hyperosmolal solutions directly bathed the secretory organelles, an observation which correlates well with the chromaffin cell studies of Holz and Senter (1986). For charged solutes, it is conceivable that enhanced ionic strength could help maintain secretory responsiveness. Interestingly, hyperosmolal sucrose actually enhanced Ca^{2+}-induced degranulation from permeabilized neutrophils. Under iso-osmolal conditions, Holz and Senter (1986) observed a reduced requirement for Ca^{2+} if potassium glutamate was replaced with sucrose. Our own observed enhancement of Ca^{2+}-induced release at hyperosmotic concentrations of sucrose is currently being investigated.

Finally, our investigations of isolated granules in different osmolar buffers indicated that isolated granules were more sensitive to hypo-osmotic stress than granules within permeabilized cells. Isolated chromaffin granules exposed to hypo-osmotic conditions were comparatively less stable than granules within intact cells (Hampton and Holz, 1983). While swelling of the granules did, in fact, cause discharge of granule constituents, differences in lytic tendencies of different solutes of the same osmolar concentrations may be indicators of sensitivity to the solutes themselves rather than simple osmotic forces; indeed, specific granule discharge was actually increased under hyperosmotic conditions. Purification of the granules and elimination of the plasma membrane portion of the cells and their contents eliminated the increase in granule discharge which could be achieved in permeabilized cells by the addition of Ca^{2+}. Thus, Ca^{2+} continues to be a candidate for playing a role in the important event of fusion of the membranes which

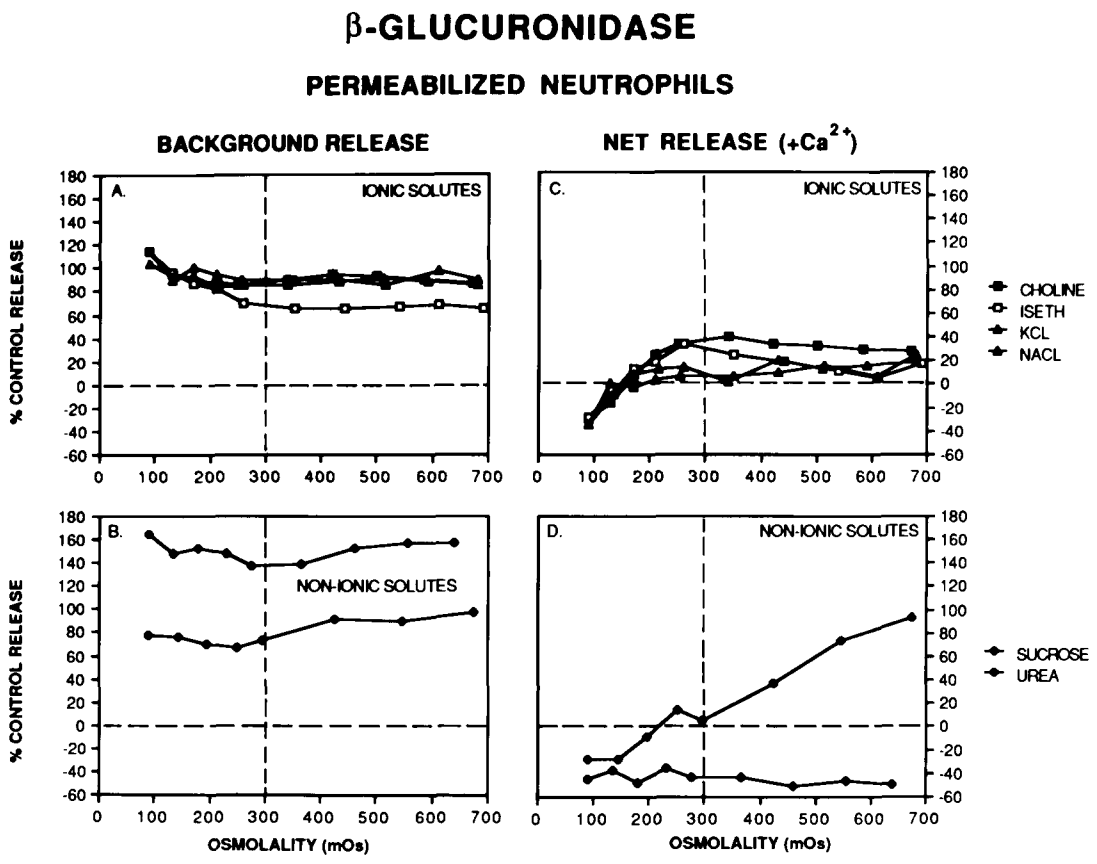

Fig. 8. Effects of osmolality of various solutes on background and Ca^{2+}-induced B-glucuronidase release from permeabilized neutrophils. Neutrophils were treated as outlined in the legend to Figure 6. Supernatants of the pelleted samples were assayed for B-glucuronidase.

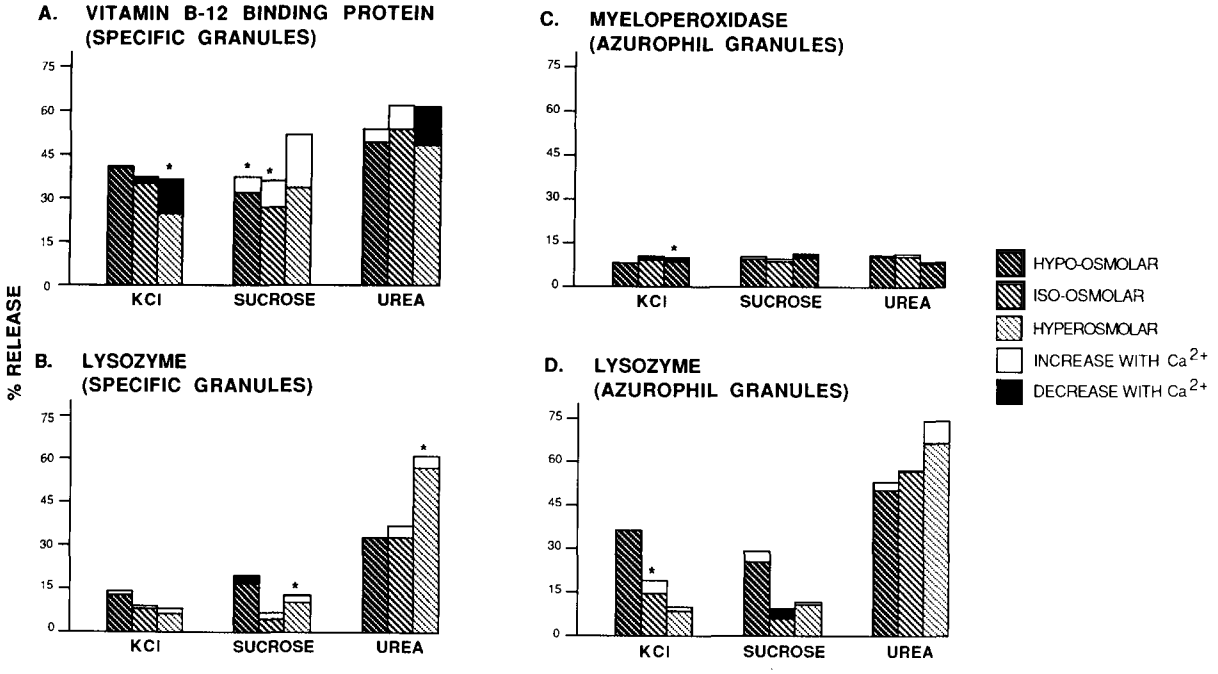

Fig. 9. Effects of hypo- and hyperosmolarity and different solutes on granule marker release from isolated specific and azurophil granules of human neutrophils. Granules were purified from neutrophils as described in "Materials and Methods." Granules (25×10^6 cell eq/0.2 ml in Buffer K) were added to 0.8 ml of pre-warmed buffers of various solutes and osmolar strengths, with or without 60 μM free Ca^{2+}. Incubation of the granules was continued for an additional 10 min. Supernatants of the pelleted samples were assayed for vitamin B-12 binding protein (Panel **A**), lysozyme (Panels **B** and **D**), and myeloperoxidase (Panel **C**). Total granule content (100%) was determined from Triton X-100-treated, sonicated samples. The addition of Ca^{2+} resulted in both decreases (shown as a black portion within the shaded bar) and increases (shown as an open bar above the shaded bar) in granule discharge. Significant change ($p < .05$) as indicated by *.

precedes degranulation. Swelling may promote later stages of the secretory response. Fusion of mast cell granules with the plasma membrane precedes swelling of the granules, and it has been proposed that in these cells, swelling may accompany movement of substances through an exocytotic pore and that osmotic stress could affect the size of this pore (Zimmerberg et al., 1987).

In conclusion, while we verified and expanded upon earlier evidence which had been used to support the osmotic swelling theory of degranulation, the additional data provided here indicate that osmotic swelling is neither necessary nor supportive of degranulation in either intact or permeabilized neutrophils. We are further investigating ionic requirements for degranulation in the permeabilized cell system, again taking advantage of the fact that in this system, the pertinent organelles are directly exposed to the solutes in question.

ACKNOWLEDGMENT

This work was supported by NIH grant AM32471 (J.E.S.).

LITERATURE CITED

Borregaard, N., Heiple, J.M., Simons, E.R., and Clark, R.A. (1983) Subcellular localization of the b cytochrome component of the human neutrophil microbicidal oxidase: Translocation during activation. J. Cell Biol., *97*:52–61.

Boyum, A. (1968) Isolation of mononuclear cells and granulocytes from human blood. Isolation of mononuclear cells by centrifugation and of granulocytes by combining centrifugation and sedimentation at 1 G. Scand. J. Lab. Invest., *21*:77–89.

Bretz, U., and Baggiolini, M. (1974) Biochemical and morphological characterization of azurophil and specific granules of human neutrophilic polymorphonuclear leukocytes. J. Cell Biol., *63*:251–269.

Brittinger, G.R., Hirschhorn, R., Douglas, S.D., and Weissmann, G. (1968) Studies on lysosomes. XI. Characterization of hydrolase-rich fraction from human lymphocytes. J. Cell Biol., *37*:394–411.

Cohen, F.S., Zimmerberg, J., and Finkelstein, A. (1980) Fusion of phospholipid vesicles with planar phospholipid bilayer membranes. J. Gen. Physiol., *75*:251–270.

Curnutte, J.T., and Babior, B.M. (1974) Biological defense mechanisms. The effect of bacteria and serum on superoxide production by granulocytes. J. Clin. Invest., *53*:1662–1672.

Dunn, L.A., and Holz, R.W. (1983) Catecholamine secretion from digitonin-treated adrenal medullary chromaffin cells. J. Biol. Chem., *258*:4989–4993.

Fisher, L.R., and Parker, N.S. (1984) Osmotic control of bilayer fusion. Biophys. J., *46*:253–258.

Hampton, R.Y., and Holz, R.W. (1983) Effects of changes in osmolality on the stability and function of cultured chromaffin cells and the possible role of osmotic forces in exocytosis. J. Cell Biol., *96*:1082–1088.

Holz, R.W., and Senter, R.A. (1986) Effects of osmolality and ionic strength on secretion from adrenal chromaffin cells permeabilized with digitonin. J. Neurochem., *46*:1835–1842.

Howell, T.W., and Gomperts, B.D. (1987) Rat mast cells permeabilised with streptolysin O secrete histamine in response to Ca^{2+} at concentrations buffered in the micromolar range. Biochim. Biophys. Acta, *927*:177–183.

Knight, D.E., and Baker, P.F. (1982) Calcium-dependence of catecholamine release from bovine adrenal medullary cells after exposure to intense electric fields. J. Membr. Biol., *68*:107–140.

Korchak, H.M., and Weissmann, G. (1980) Stimulus-response coupling in the human neutrophil: Membrane potential changes and the role of extracellular Na^+. Biochim. Biophys. Acta, *601*:180–194.

Korchak, H.M., Eisenstat, B.A., Smolen, J.E., Rutherford, L.E., Dunham, P.B., and Weissmann, G. (1982) Stimulus-response coupling in the neutrophil: the role of anion fluxes in degranulation. J. Biol. Chem., *257*:6916–6922.

Ladona, M.G., Bader, M.F., and Aunis, D. (1987) Influence of hypertonic solutions on catecholamine release from intact and permeabilized cultured chromaffin cells. Biochim. Biophys. Acta, *927*:18–25.

Pollard, H.B., Tack-Goldman, K., Pazoles, C.J., Creutz, C.E., and Shulman, N.R. (1977) Evidence for control of serotonin secretion from human platelets by hydroxyl ion transport and osmotic lysis. Proc. Natl. Acad. Sci. U.S.A., *74*:5295–5299.

Showell, H.J., Naccache, P.H., Sha'afi, R.I., and Becker, E.L. (1977) The effects of extracellular K^+, Na^+, and Ca^{2+} on lysosomal enzyme secretion from polymorphonuclear leukocytes. J. Immunol., *119*:804–811.

Smith, G.P., and Peters, T.J. (1982) The release of granule components from human polymorphonuclear leukocytes in response to both phagocytic and chemical stimuli. Biochim. Biophys. Acta, *719*:304–308.

Smolen, J.E., Stoehr, S.J., and Boxer, L.A. (1986) Human neutrophils permeabilized with digitonin respond with lysosomal enzyme release when exposed to micromolar levels of free calcium. Biochim. Biophys. Acta, *886*:1–17.

Wacker, W.E.C., Ulmer, D.D., and Vallee, B.L. (1956) Metalloenzymes and myocardial infarction. II. Malic and lactic dehydrogenase activities and zinc ion concentration in serum. N. Eng. J. Med., *255*:449–456.

Wilson, S.P., and Kirshner, N. (1983) Calcium-evoked secretion from digitonin-permeabilized adrenal medullary chromaffin cells. J. Biol. Chem., *258*:4994–5000.

Worthington Enzyme Manual (1972) pp. 101–101.

Yassim, R., Shefcyk, J., White, J.R., Tao, W., Volpi, M., Molski, T.F.P., Naccache, P.H., Feinstein, M.B., and Sha'afi, R.I. (1985) Effects of chemotactic factors and other agents on the amounts of actin and a 65,000-mol-wt protein associated with the cytoskeleton of rabbit and human neutrophils. J. Cell Biol., *101*:182–188.

Zimmerberg, J., and Whitaker, M. (1985) Irreversible swelling of secretory granules during exocytosis caused by calcium. Nature, *315*:581–584.

Zimmerberg, J., Sardet, C., and Epel, D. (1985) Exocytosis of sea urchin egg cortical vesicles *in vitro* is retarded by hyperosmotic sucrose: kinetics of fusion monitored by quantitative light-scattering microscopy. J. Cell Biol., *101*:2398–2410.

Zimmerberg, J., Curran, M., Cohen, F.S., and Brodwick, M. (1987) Simultaneous electrical and optical measurements show that membrane fusion precedes secretory granule swelling during exocytosis of beige mouse mast cells. Proc. Natl. Acad. Sci. U.S.A., *84*:1585–1589.

Zurier, R.B., Hoffstein, S., and Weissmann, G. (1973) Cytochalasin B: effect on lysosomal enzyme release from human leukocytes. Proc. Natl. Acad. Sci. U.S.A., *70*:844–848.