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STIMULUS-RESPONSE COUPLING IN THE HUMAN NEUTROPHIL

TRANSMEMBRANE POTENTIAL AND THE ROLE OF EXTRACELLULAR Na^+

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Summary

Receptor-ligand interactions at the surface of the human neutrophil induce lysosomal enzyme release and the generation of O_2^-, responses which are anteceded by changes in the membrane potential ($\Delta\psi$) as measured by [^{3}H]-triphenylmethylphosphonium ion distribution. Surface stimuli (immune complexes, concanavalin A) initiated a rapid (less than 10 s) hyperpolarization response by both normal and cytochalasin B-treated cells. Replacement of extracellular Na^+ with either K^+ or choline depressed O_2^- generation and lysosomal enzyme release in neutrophils exposed to concanavalin A or immune complexes. Replacement of Na^+ with K^+ led to a substantial fall in resting membrane potential, whereas replacement of Na^+ with choline did not. Thus, depression of O_2^- generation and lysosomal enzyme release in Na^+-free medium were specifically due to a lack of extracellular Na^+ and not to depolarization of the membrane. Although it has been shown that extracellular Na^+, and possibly an influx of Na^+, is required for optimal neutrophil function, neither depolarization nor Na^+ influx per se was sufficient to activate fully these cells, since the Na^+ ionophore, monensin, was not an effective stimulus for β-glucuronidase release or O_2^- generation. The hyperpolarization response of neutrophils exposed to immune complexes and to concanavalin A was greatly diminished in both high [K^+] and [choline] buffers. Thus, extracellular Na^+ was required for an optimal membrane potential response to receptor-ligand interaction. Since O_2^- generation and lysosomal enzyme release in response to the Ca^{2+} ionophore, A23187, were also reduced in the absence of extracellular Na^+, it was concluded that extracellular Na^+ was also required after induction of Ca^{2+} fluxes. Ouabain (1 mM) had no effect on O_2^- generation, lysosomal

enzyme release or the hyperpolarization response to immune complexes, indicating that the hyperpolarization observed on stimulation cannot be due to the action of the electrogenic pump, ($Na^+ + K^+$)-ATPase. The experiments indicate that extracellular Na^+ is required (1) in the $\Delta\psi$ response triggered by receptor-ligand interaction, and (2) at a step(s) subsequent to Ca^{2+} fluxes and common to O_2^- generation and lysosomal enzyme release.

Introduction

The neutrophil responds to a single stimulus at its surface by undergoing chemotaxis, phagocytosis, superoxide anion (O_2^-) generation and degranulation. It is not clear how a single stimulus triggers multiple responses, and what the steps are in the signalling process. Studies of similar signalling processes which mediate stimulus-response coupling in a broad range of cell types (both excitable and non-excitable) have generally shown that a change in membrane potential is one of the earliest events in cell activation [1—6], followed by an influx or intracellular release of Ca^{2+} [7].

We have previously measured the transmembrane potential in the human neutrophil by means of the lipophilic cation, triphenylmethylphosphonium ion ($TPMP^+$) [8]. The resting potential of these cells is —25 mV and is dependent on the potassium concentration across the membrane since a high extracellular potassium concentration causes depolarization. Neutrophils stimulated with either concanavalin A or immune complexes undergo a prompt hyperpolarization of the membrane which is not dependent on extracellular Ca^{2+} or the presence of cytochalasin B [8]. The hyperpolarization response precedes by many seconds the onset of O_2^- generation [8—10] and lysosomal enzyme release [11]. Both O_2^- generation [8,10] and lysosomal enzyme release [12,13] are dependent on extracellular Ca^{2+} for optimal expression, and can be stimulated directly by an influx of Ca^{2+} as in neutrophils exposed to the calcium ionophore, A23187 [13].

In this communication we assess the role of the transmembrane potential in stimulus-response coupling in the neutrophil. Moreover, extracellular Na^+ has been shown to play an essential role in chemotaxis and lysosomal enzyme release in rabbit neutrophils stimulated with a chemotactic peptide, and stimulation of these cells is accompanied by increased Na^+ fluxes [14,15]. Consequently, we have also studied the role of Na^+ in the control of membrane potential changes and the subsequent release of lysosomal enzymes and O_2^- generation in human neutrophils.

Materials and Methods

Preparation of neutrophil suspensions. Neutrophil suspensions containing 98 ± 1% neutrophils were prepared from heparinized venous blood (10 units of heparin per ml of blood) obtained from healthy adult donors. Standard techniques (Hypaque/Ficoll gradients) were used [16] followed by dextran sedimentation and hypotonic lysis. The cells were suspended in Krebs-Ringer-Hepes buffer, pH 7.45, having the ionic composition Na^+ (150 mM), K^+

(5 mM), Ca^{2+} (1.3 mM), Mg^{2+} (1.2 mM), Cl^- (155 mM), Hepes (10 mM). This buffer was used throughout unless otherwise indicated. In buffers where Na^+ was omitted, an equal volume of equimolar choline chloride or KCl was substituted.

$TPMP^+$ distribution measurements. Since neutrophils are too small for the direct measurement of membrane potential by microelectrodes, an indirect method was used, employing the lipid-soluble cation, triphenylmethylphosphonium ion ($TPMP^+$). Uptake of $TPMP^+$ was measured by a minor modification of the method of Schuldiner and Kaback [17]. Neutrophils were incubated in buffer at 37°C in the presence of 100 μM [^{3}H]$TPMP^+$ (120 Ci/mol, New England Nuclear) in a final volume of 100 μl. Incubation and assays were carried out as previously described [8]. The immune complex, bovine serum albumin/anti-bovine serum albumin, was prepared according to the method of Ward and Zvaifler [18] with an anti-bovine albumin IgG (Cappel) to albumin (Sigma) ratio of 5 : 1. Concanavalin A was purchased from Sigma, and monensin was a gift from R. Hamill (Eli Lilly).

Measurement of superoxide generation. Duplicate reaction mixtures were incubated with 5.0 μg/ml cytochalasin B (Aldrich) in 0.1% dimethylsulfoxide and 75 μM horse heart ferricytochrome *c* (Type III, Sigma) for 10 min at 37°C. Reference cuvettes contained in addition 10 μg superoxide dismutase (Miles Laboratories). Concanavalin A (30 μg) or immune complex (300 μg protein) was added at zero time, and the reduction of cytochrome *c* monitored continuously at 550 nm in a Beckman model 25 spectrophotometer using the superoxide dismutase-containing cuvette as a reference. O_2^- generation expressed as nmol cytochrome *c* reduced/mg protein per 5 min was calculated using an absorbance coefficient of 21.1 mM at 550 nm (reduced-oxidized). The lag period was determined by extrapolating the linear portion of the curve back to zero O_2^- generation.

Measurement of lysosomal enzyme release. The extracellular release of the neutrophil granule-associated enzymes, lysozyme and β-glucuronidase, was measured as previously described [19,20] in duplicate reaction mixtures identical to those employed for the determination of O_2^- generation, but in the absence of cytochrome *c*. After incubation, the reaction mixtures were centrifuged in the cold (755 × *g* for 10 min) and cell-free supernatants removed for enzyme assays.

β-Glucoronidase was determined after 18 h incubation with phenolphthalein glucuronidate (Sigma) as substrate [19]. Lysozyme was determined as the rate of lysis of *Micrococcus lysodeikticus* (Worthington Biochemical Corp., Freehold, NJ) measured by the decrease in absorbance at 450 nm [21]. Total enzyme activities were determined simultaneously in duplicate reaction mixtures containing the detergent, Triton X-100 (0.2%; Rohm and Haas Co., Philadelphia, PA).

Cell viability was determined by monitoring lactate dehydrogenase release according to the method of Wacker et al. [22], and also by eosin Y exclusion. Experimental values for lactate dehydrogenase release did not exceed 3%.

Concanavalin A binding to neutrophils. For these experiments 0.15 μCi [^{3}H]concanavalin A (0.25 μg; New England Nuclear) plus 30 μg unlabelled concanavalin A were added to $5 \cdot 10^6$ neutrophils. After 5 min of incubation,

the neutrophils were centrifuged (155 × *g* for 10 min), washed three times with ice-cold buffer, and allowed to dry at room temperature. The dried pellets were digested with 0.5 ml of 0.2 N NaOH for 2 h at 46° C, followed by the addition of 0.2 ml of 3.0% acetic acid and 0.5 ml of distilled water. Aliquots (1.0 ml) were added to 15 ml of Bray's solution (New England Nuclear) and counted in a Beckman LS-7000 liquid scintillation counter. Results have been expressed as counts per min per mg protein.

Measurements of Na^+ influx. Influx of $^{22}Na^+$ was measured by a centrifugation technique. An aliquot of a cell suspension (50 μl) was layered on top of a 150 μl layer of Versilube F 50 (Harwick Chemical) and a 50 μl cushion of 12% sucrose in a 400 μl capacity microcentrifuge tube. Cells were separated from medium by centrifugation for 20 s in a Beckman microcentrifuge. The tip, containing cells in the sucrose cushion, was excised and its radioactivity was measured in 10 ml of Filtron X (National Diagnostics). Correction for entrainment was made by measurements with [^{14}C]inulin. Incubations were carried out at 37° C.

Protein was determined by using the method of Lowry et al. [23] using lysozyme as the standard. Student's *t*-test was used for statistical analysis; mean values ± S.E. are given where appropriate .

Results

Effect of extracellular Na^+ on O_2^- generation. Previous work has demonstrated that the generation of O_2^- in human neutrophils stimulated with concanavalin A or immune complex is anteceded by a change in membrane potential ($\Delta\psi$) [8], suggesting that the membrane potential, or the distribution of ions that it reflects, could play an important role in neutrophil function. When the neutrophils were depolarized by substitution of all Na^+ in the buffer with K^+ (K^+ buffer) a profound depression of O_2^- generation was found (Fig. 1). Substitution of Na^+ with choline ions (choline buffer), which does not abolish the K^+ gradient and therefore should not cause a pronounced depolarization, depressed O_2^- generation even more, both in concanavalin A- and in immune complex-stimulated cells (Fig. 1). Therefore, the depression of O_2^- generation was specifically due to a lack of Na^+ and not to depolarization of the cells. Addition of Na^+ to neutrophils in a choline buffer restored their ability to generate O_2^-. A dose-response curve for the effect of $[Na^+]_{out}$ on O_2^- generation was obtained by varying the proportions of Na^+ to choline in the buffer (Fig. 2). Increasing concentrations of Na^+, to approx. 75 mM Na^+, which is half the concentration of Na^+ in the standard Krebs-Ringer buffer used for these experiments, caused a progressive increase in O_2^- generation.

Effect of substituting extracellular Na^+ with different monovalent cations. The ability of a series of monovalent cations to replace Na^+ in the medium during O_2^- generation by neutrophils was examined (Table I). A level of 30 mM Na^+/120 mM choline was chosen since this concentration supported approx. 50% of optimal O_2^- generation, and equimolar amounts of the various monovalent ions were substituted for Na^+. Using immune complex bovine serum albumin/anti-bovine serum albumin as a stimulus, monovalent cations stimulated O_2^- generation to varying degrees, the order of activity being $Na^+ \simeq NH_4^+$

EFFECT OF MONOVALENT CATIONS ON O_2^- GENERATION BY HUMAN PMN

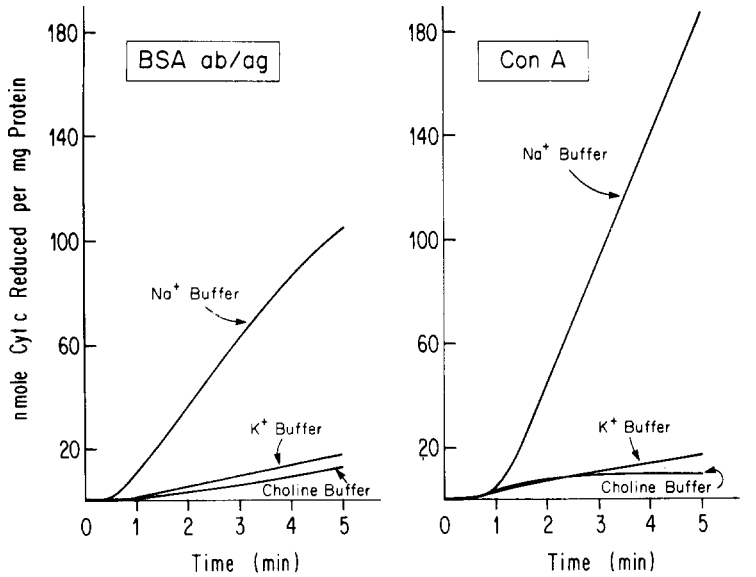


Fig. 1. Effect of monovalent cations on O_2^- generation by human neutrophils, stimulated with immune complex bovine serum albumin/anti-bovine serum albumin (BSA ab/ag, 300 μg protein/ml) or concanavalin A (Con A, 30 μg/ml). Continuous recording at 550 nm and 37°C. Na^+ buffer, standard Krebs-Ringer-Hepes buffer (150 mM Na^+); K^+ buffer, all Na^+ substituted with K^+; choline buffer, all Na^+ substituted with choline$^+$.

EFFECT OF $[Na^+]$ ON O_2^- GENERATION BY HUMAN PMN STIMULATED BY BSA ab/ag

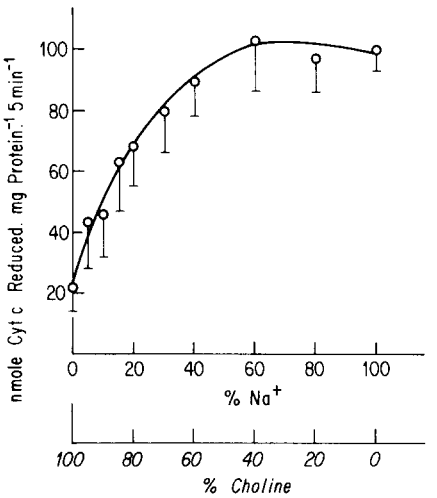


Fig. 2. Effect of extracellular Na^+ concentration on O_2^- generation by human neutrophils stimulated with immune complex bovine serum albumin/anti-bovine serum albumin (BSA ab/ag). Extracellular Na^+ concentration was changed by varying the proportion of Na^+ buffer (150 mM Na^+) to choline buffer (150 mM choline).

TABLE I

EFFECTIVENESS OF DIFFERENT MONOVALENT CATIONS AT REPLACING Na^+ (30 mM) IN THE GENERATION OF O_2^- BY HUMAN NEUTROPHILS STIMULATED BY BOVINE SERUM ALBUMIN/ANTI-BOVINE SERUM ALBUMIN

Values of O_2^- generation are expressed as nmol cytochrome *c* reduced/mg protein per 5 min (mean ± S.E., n = 3). Lag period values are expressed in min (mean ± S.E., *n* = 3).

Monovalent cation	O_2^- generation	Lag period
choline	18.6 ± 2.8	0.80 ± 0.12
K^+	26.4 ± 4.0	0.92 ± 0.24
Li^+	29.6 ± 1.6	0.72 ± 0.10
Cs^+	33.3 ± 5.5	0.84 ± 0.20
Rb^+	37.1 ± 7.2	0.71 ± 0.17
NH_4^+	48.9 ± 11.5	0.52 ± 0.01
Na^+	52.8 ± 7.7	0.42 ± 0.01

$>Rb^+ > C_s^+ > Li^+ > K^+ >$choline. The lag period for the onset of O_2^- generation was also affected by the species of monovalent ions in the buffer. The lag period was longest in buffer containing choline or K^+. The shortest lag periods were found for the ions stimulating the highest activity of O_2^- generation, namely Na^+ and NH_4^+. Thus, both the time of onset, i.e., the coupling process between stimulus and response, and the rate of O_2^- generation itself were affected by the species of monovalent cations present in the medium.

Effect of extracellular Na^+ on lysosomal enzyme release and O_2^- generation stimulated by immune complex bovine serum albumin/anti-bovine serum albumin. Since O_2^- generation stimulated by both concanavalin A and immune complex bovine serum albumin/anti-bovine serum albumin was highly dependent on extracellular Na^+, it was important to determine if other functions, such as lysosomal enzyme release which are simultaneously stimulated by concanavalin A and immune complex, were similarly affected by the concentration of extracellular Na^+. The effect upon these neutrophil responses of substituting all Na^+ in the medium with choline ions is shown in Table II. In neutrophils stimulated with immune complex, release of both β-glucuronidase and lysozyme was markedly dependent on the presence of Na^+ in the extracellular medium, although not to the same extent as O_2^- generation. A similar dependence on extracellular Na^+ has also been shown for lysosomal enzyme release by rabbit and human neutrophils stimulated with the chemotactic peptide, formylmethionylleucylphenylalanine [15,24].

Na^+ influx as a stimulus: effect of monensin on lysosomal enzyme release and O_2^- generation. Since extracellular Na^+ was required in neutrophil activation, it appeared possible that an influx of Na^+ could trigger secretion. Monensin is a linear polyether ionophore which is selective for Na^+. Monensin was shown to cause an influx of $^{22}Na^+$ into cytochalasin B-treated neutrophils (Table III). Addition of monensin in the concentration range $1 \cdot 10^{-7}$—$1 \cdot 10^{-5}$ M to neutrophils did not elicit O_2^- generation (Table III) or β-glucuronidase release. However, at concentrations of $1 \cdot 10^{-6}$ and $1 \cdot 10^{-5}$ M monensin elicited a release of small amounts of lysozyme.

Role of extracellular Na^+ in the $\Delta\psi$ response. The transmembrane potential of human neutrophils in Na^+, high $[K^+]$ and choline buffers was determined

TABLE II

ROLE OF EXTRACELLULAR Na^+ IN LYSOSOMAL ENZYME RELEASE AND O_2^- GENERATION

BSA, bovine serum albumin. Values of release are expressed as % (mean ± S.E.; number (n) of determinations in parentheses). O_2^- generation is expressed as nmol cytochrome c reduced/mg protein per 5 min (mean ± S.E. (n)). P determined by Student's t-test.

	β-Glucuronidase release	Lysozyme release	O_2^- generation
BSA/anti-BSA (300 μg) Na^+ buffer	9.5 ± 1.0 (10)	17.2 ± 1.1 (11)	99.9 ± 7.2 (10)
BSA/anti-BSA (300 μg) Choline buffer	4.1 ± 1.2 (6) $P < 0.005$	6.3 ± 2.3 (6) $P < 0.001$	21.4 ± 7.7 (8) $P < 0.001$
A23187 ($5 \cdot 10^{-6}$ M) Na^+ buffer	16.3 ± 2.5 (6)	34.1 ± 2.6 (6)	34.7 ± 5.7 (7)
A23187 ($5 \cdot 10^{-6}$ M) choline buffer	3.5 ± 1.5 (5) $P < 0.005$	12.6 ± 4.7 (5) $P < 0.005$	12.7 ± 3.3 (6) $P < 0.001$

by measuring $TPMP^+$ uptake (Fig. 3A). Taking the neutrophils in high $[K^+]$ buffer as $\Delta\psi = 0$, and using a previously determined value for the intracellular volume of 3.97 μl/mg protein [8], it was possible to calculate the resting $\Delta\psi$ in the different buffers. The $\Delta\psi_{Na^+}$ value was calculated to be −25.1 mV, while $\Delta\psi_{choline}$ was −20.5 mV. Thus, while substitution of Na^+ with K^+ profoundly depolarized the neutrophils, substitution of Na^+ with choline had only a small depolarizing effect.

Addition of the insoluble immune complex, bovine serum albumin/anti-bovine serum albumin, to neutrophils in the Na^+ buffer led to a rapid hyperpolarization, peaking at 1 min, as demonstrated before [8]. In the choline and K^+ buffers, the hyperpolarization response to immune complex led to a long, slow hyperpolarization. Similar results were obtained for neutrophils stimulated with concanavalin A (Fig. 3B). Thus, extracellular Na^+ is not required for the maintenance of the resting membrane potential, but it is essential for an optimal hyperpolarization response in stimulated cells.

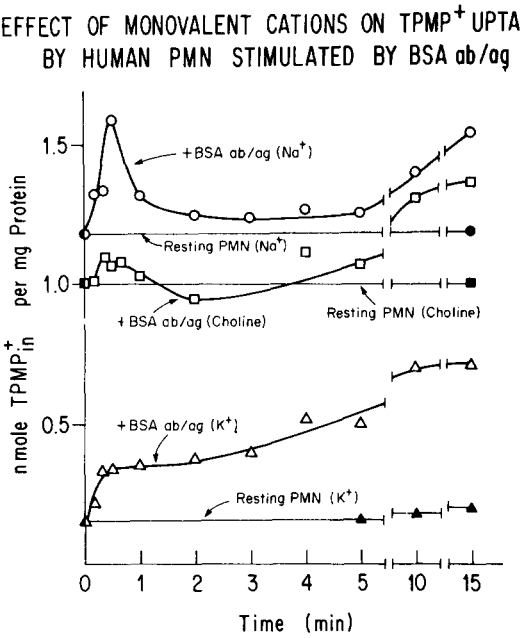
Effect of ionic milieu on concanavalin A binding to neutrophils. Since extracellular Na^+ has been shown to affect receptor-ligand binding in other cell types [3,25], it seemed possible that the depressed hyperpolarization response could be due to a decrease in receptor-ligand binding in the low

TABLE III

MONENSIN AS A STIMULUS IN NEUTROPHILS

Values of release expressed as % (mean ± S.E., n = 4). Values of O_2^- generation expressed as nmol cytochrome c reduced/5 min per mg protein (mean ± S.E., n = 3). Na^+ influx expressed as nmol Na^+/5 min per mg protein (mean ± S.E., n = 3)

Monensin (μM)	β-Glucuronidase release	Lysozyme release	O_2^- generation	Na^+ influx
0	0	0	0	1.9 ± 0.5
0.1	0.3 ± 0.2	1.6 ± 0.8	0 ± 0	3.1 ± 0.4
1.0	0.7 ± 0.4	5.2 ± 1.5	0 ± 0	3.5 ± 0.4
10	2.2 ± 0.4	5.7 ± 1.2	0 ± 0	4.3 ± 0.3



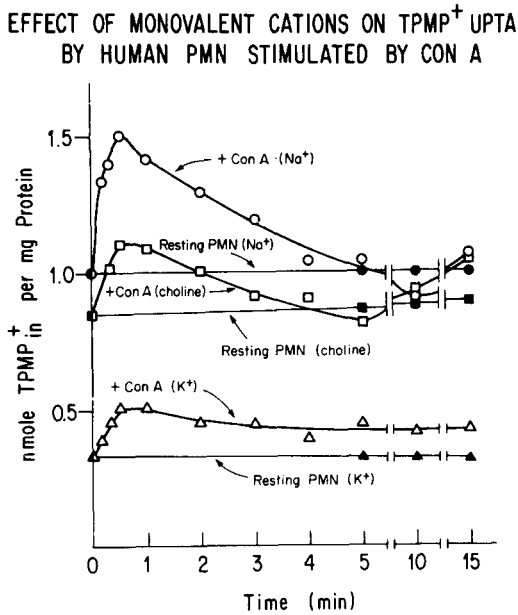


Fig. 3. Effect of monovalent cations on $TPMP^+$ uptake in human neutrophils (PMN). Cells were pre-equilibrated with [^{3}H]$TPMP^+$ before addition of stimulus (zero time). (A) Stimulus, immune complex bovine serum albumin/anti-bovine serum albumin (BSA ab/ag, 60 μg protein). ●, resting cells in Na^+ buffer; ○, stimulated cells in Na^+ buffer; ■, resting cells in choline buffer; □, stimulated cells in choline buffer; ▲, resting cells in K^+ buffer; △, stimulated cells in K^+ buffer. (B) Stimulus, concanavalin A (con A, 30 μg). ●, resting cells in Na^+ buffer; ○, stimulated cells in Na^+ buffer; ■, resting cells in choline buffer; □, stimulated cells in choline buffer; ▲, resting cells in K^+ buffer; △, stimulated cells in K^+ buffer.

TABLE IV

EFFECT OF VARIOUS MONOVALENT CATIONS ON CONCANAVALIN A BINDING TO HUMAN NEUTROPHILS

Results are expressed as cpm/mg protein (mean ± S.E., $n = 3$)

Buffer	Cytochalasin B	Concanavalin A binding	
		0°C	37°C
Na^+ buffer	—	16 769 ± 2122	31 533 ± 408
	+	20 098 ± 1057	49 022 ± 584
High [K^+] buffer	—	19 622 ± 1145	28 063 ± 401
	+	22 238 ± 740	36 634 ± 363
Choline buffer	—	24 929 ± 1855	46 032 ± 1605
	+	24 531 ± 1105	59 808 ± 964

[Na^+] buffers. Using ^{3}H-labeled concanavalin A, it was possible to test the effect of the various buffers, i.e., Na^+, K^+ and choline buffers, on the binding of concanavalin A to the neutrophils (Table IV). The substitution of Na^+ by K^+ had no significant effect on concanavalin A binding at 0°C, while at 37°C binding was reduced in the high [K^+] buffer. In contrast, in the choline buffer, concanavalin A binding was increased slightly at 0°C and to a marked extent at 37°C. In all buffers more concanavalin A was bound to cytochalasin B-treated cells at 37°C, probably due to greater availability of binding sites.

Effect of extracellular Na^+ on lysosomal enzyme release and O_2^- generation stimulated by A23187. Release of β-glucuronidase and lysozyme, and the generation of O_2^- can also be stimulated by the calcium ionophore, A23187, in the presence of Ca^{2+}, which bypasses receptor-ligand interaction by directly provoking fluxes of Ca^{2+}. In neutrophils stimulated with $5 \cdot 10^{-6}$ M A23187, the presence of extracellular Na^+ was also required for optimal release of the lysosomal enzymes, and for the generation of O_2^- (Table II). Thus, extracellular Na^+ is required for both degranulation and O_2^- generation at a step after the induction of Ca^{2+} fluxes.

Effect of Na^+ flux modulators on O_2^- generation and lysosomal enzyme release. Neutrophils were treated with a variety of cation transport inhibitors that have been shown to alter Na^+ fluxes in neutrophils and other cells. The effect was studied of these inhibitors on lysosomal enzyme release and superoxide anion generation induced by the immune complex, bovine serum albumin/anti-bovine serum albumin (Table V). Ouabain, which classically inhibits ($Na^+ + K^+$)-ATPase and thereby the active extrusion of Na^+ from the cell, has been shown to inhibit active K^+ uptake in the neutrophil [26]. Ouabain neither affected lysosomal enzyme release nor generation of superoxide anion. Diphenylhydantoin, which acts either via stimulation of ($Na^+ + K^+$)-ATPase or by inhibiting passive Na^+ fluxes [27], had a small inhibitory effect on lysosomal enzyme release but none on O_2^- generation.

The potassium-sparing diuretic, amiloride, blocks passive Na^+ influxes in the distal nephron as well as in other epithelial tissues [28]. Amiloride ($1 \cdot 10^{-4}$ M) had a small but significant inhibitory effect on lysosomal enzyme release from immune complex-stimulated neutrophils. In contrast, amiloride

TABLE V

EFFECT OF TRANSPORT INHIBITORS ON LYSOSOMAL ENZYME RELEASE AND O_2^- GENERATION

Cells were preincubated for 10 min at 37°C with cytochalasin B and the appropriate Na^+-flux modulator. Results on enzyme release expressed as % of control (±S.E., *n* in parentheses), with values for control being β-glucuronidase 9.0 ± 1.3% release, lysozyme 17.8 ± 1.2% release, and O_2^- generation 99.9 ± 7.2 nmol cytochrome *c* reduced/mg protein per 5 min. *P* determined by Student's *t*-test

Additions	β-Glucuronidase	Lysozyme	O_2^- generation
Control	100	100	100
Ouabain ($1 \cdot 10^{-3}$ M)	101.5 ± 4.3 (3)	95.2 ± 5.9 (3)	96.7 ± 2.5 (3)
Diphenylhydantoin ($1 \cdot 10^{-3}$ M)	71.5 ± 4.2 (4)	82.5 ± 5.6 (4)	95.9 ± 3.2 (3)
	$P < 0.005$	$P < 0.05$	
Furosemide ($1 \cdot 10^{-3}$ M)	77.6 ± 5.7 (4)	75.6 ± 2.3 (4)	101.0 ± 10.1 (4)
	$P < 0.025$	$P < 0.0025$	
Amiloride ($1 \cdot 10^{-4}$ M)	75.7 ± 7.2 (4)	66.7 ± 10.8 (4)	99.6 ± 9.0 (4)
	$P < 0.025$	$P < 0.05$	

had no effect on O_2^- generation by neutrophils. The diuretic, furosemide, caused a small inhibition of lysosomal enzyme release, but did not significantly affect superoxide anion generation (Table V).

Effect of Na^+ flux modulators on resting $\Delta\psi$ and the $\Delta\psi$ response to a stimulus. The effect of the different Na^+ flux modulators on the resting $\Delta\psi$ (measured by $TPMP^+$ uptake) is shown in Table VI. After 10 min preincubation, ouabain had no effect on the resting membrane potential, however, incubation at 37°C for 60 min in the presence of 1 mM ouabain did lead to a significant depolarization, 0.70 ± 0.06 nmol $TPMP^+_{in}$/mg protein as compared to 1.09 ± 0.15 nmol $TPMP^+_{in}$/mg protein for the control cells. When the neutrophils were stimulated by the addition of the immune complex, bovine serum albumin/anti-bovine serum albumin, ouabain had no effect on the hyperpolarization response. Diphenylhydantoin significantly raised the resting membrane potential of the neutrophils, but did not affect the cells' ability to hyperpolarize on the addition of the immune complex.

TABLE VI

EFFECT OF Na^+ FLUX MODULATORS ON $TPMP^+$ UPTAKE OF RESTING AND STIMULATED NEUTROPHILS

BSA, bovine serum albumin. $TPMP^+$, triphenylmethylphosphorium ion. Transport inhibitors added 10 min before addition of bovine serum albumin/anti-bovine serum albumin. Values of uptake are expressed as nmol $TPMP^+_{in}$/mg protein (mean ± S.E.). *P* determined from Student's *t*-test

Additions	Uptake of $TPMP^+$	
	−BSA/anti-BSA	+BSA/anti-BSA for 1 min
Control	1.09 ± 0.05	1.47 ± 0.06 ($n = 10$)
Ouabain ($1 \cdot 10^{-3}$ M)	1.16 ± 0.13	1.42 ± 0.13 ($n = 4$)
Diphenylhydantoin ($5 \cdot 10^{-4}$ M)	1.34 ± 0.14	1.67 ± 0.20 ($n = 3$)
	$P < 0.05$	
Amiloride ($1 \cdot 10^{-4}$ M)	1.26 ± 0.11	1.40 ± 0.16 ($n = 4$)
Furosemide ($1 \cdot 10^{-3}$ M)	1.09 ± 0.10	1.40 ± 0.16 ($n = 5$)

Amiloride caused a slight hyperpolarization of the resting neutrophil, as would be expected for an agent that reduces passive Na^+ influx. However, amiloride did not prevent the increase in $TPMP^+$ uptake subsequent to stimulation with immune complex. Furosemide had no effect on the $TPMP^+$ uptake of resting or immune complex-stimulated cells.

Discussion

It has been suggested that changes in membrane potential and associated ion fluxes are critical for the initiation of stimulus-secretion coupling in neutrophils [8,29,30]. The immune complex, bovine serum albumin/anti-bovine serum albumin, and concanavalin A caused a large and rapid hyperpolarization response when they interacted with the neutrophil membrane, a response which had previously been shown to occur in the presence or absence of cytochalasin B [8]. If there is an initial depolarization of the membrane, as suggested by Naccache et al. [29], it must occur too rapidly to be detected by the $TPMP^+$-distribution method.

The ionic milieu is of crucial importance in neutrophil function. When neutrophils were depolarized by suspension in a high $[K^+]$ buffer (i.e., all Na^+ replaced by K^+), generation of O_2^- was profoundly depressed. If, however, all extracellular Na^+ was replaced with choline ions, the resting membrane potential was not appreciably affected, but generation of O_2^- and lysosomal enzyme release were similarly depressed. Thus, the absence of extracellular Na^+ per se rather than a depolarization of the membrane potential was responsible for the depression of O_2^- generation and lysosomal enzyme release. A similar requirement for extracellular Na^+ for lysosomal enzyme release [15] has been demonstrated in rabbit neutrophils and for chemotaxis in rabbit [14] and human neutrophils [31], but not for phagocytosis [32].

The reduced O_2^- generation seen in the absence of extracellular Na^+ was shown to be reversible. At 30 mM Na^+, half-maximal O_2^- generation was restored. A series of monovalent cations at a concentration of 30 mM had the ability to restore O_2^- generation in the order of $Na^+ \simeq NH_4^+ > Rb^+ > Cs^+ > Li^+ > K^+ >$choline. It is of interest to note that the most effective ions in restoring O_2^- generation, in particular NH_4^+, also shortened the lag period. Not only was the rate of O_2^- generation itself accelerated in the presence of Na^+ (and NH_4^+) but the rate of activation of the O_2^- -generating system was also accelerated. The activity of NH_4^+ in replacing Na^+ in the medium suggests that Na^+ could be important in regulating intracellular pH.

In considering the nature of the requirement for extracellular Na^+ in neutrophil activation, it seemed possible that an Na^+ influx could trigger secretion. Na^+ influxes, as well as effluxes, have been shown to occur in activated rabbit neutrophils [29,33] as well as in exocrine and endocrine pancreatic cells [34,35]. In the neutrophil, the Na^+ ionophore, monensin, did not provoke β-glucuronidase release or O_2^- generation although a small amount of lysozyme was secreted. An influx of Na^+ per se is evidently not sufficient to fully activate the neutrophil, although an influx of Na^+ may be an essential step in the activation process.

Extracellular Na^+ was shown to be required for the initial events in neutro-

phil activation, i.e., prior to the Ca^{2+} influx. In the depolarizing buffer (high $[K^+]$), the resting membrane potential was profoundly depressed. On stimulation with concanavalin A or immune complex, the neutrophils in high $[K^+]$ buffer showed only a small, slow, protracted hyperpolarization in contrast to the large, rapid hyperpolarization observed in an Na^+ buffer. In choline buffer there was only a small drop in resting membrane potential, but on stimulation with concanavalin A or immune complex the hyperpolarization response was substantially diminished. Binding studies with tritiated concanavalin A demonstrated a slightly enhanced binding in the choline buffer compared to binding in the Na^+ buffer, and a slightly reduced binding in the high $[K^+]$ buffer. While the decreased O_2^- generation and $\Delta\psi$ response in the K^+ buffer could be partially due to a reduction in receptor-ligand binding, this cannot be the case in the choline buffer. Thus, for cells stimulated with concanavalin A, extracellular Na^+ was required subsequent to the receptor-ligand binding step.

The differences in binding of concanavalin A to the neutrophil membranes in the presence and absence of extracellular Na^+ suggested that changes in the ionic milieu could cause conformational changes in membrane proteins. The ionic milieu has been shown to affect receptor-ligand binding in other cell types [3,25], altering both receptor number and affinity. Indeed, Romeo et al. [36] have shown (by means of the fluorescent dye, 8-anilino-1-naphthalene-sulfonate) that rapid changes in the conformation of membrane protein follows exposure of guinea-pig neutrophils to polystyrene spherules. If these conformational changes are an essential part of the activation process in the neutrophils, then the absence of extracellular Na^+ might change the conformation of membrane glycoproteins so as to favor ligand-concanavalin A binding but to inhibit the transitions necessary for the transmission of the signal and activation of the cells.

When the initial step of ligand-receptor binding leading to hyperpolarization was bypassed by use of the Ca^{2+} ionophore, A23187, a marked depression of O_2^- generation and lysosomal enzyme release was still found in the absence of extracellular Na^+. This indicates that extracellular Na^+ is required for both O_2^- generation and enzyme release, at a step subsequent to induced Ca^{2+} fluxes.

In an attempt to define the possible role of Na^+ fluxes in neutrophil activation, the effect of a series of Na^+-flux modulators on neutrophil function was investigated. The most specific inhibitor of Na^+ efflux was the cardiac glycoside, ouabain, which inhibits the electrogenic pump, $(Na^+ + K^+)$-ATPase. Ouabain has been shown to inhibit 60% of the K^+ influx in the human neutrophil [26]. Ouabain (after 10 min preincubation) did not inhibit either O_2^- generation or lysosomal enzyme release. The resting membrane potential of the neutrophil was depolarized only after prolonged incubation in 1 mM ouabain. This provides indirect evidence for the existence of an $(Na^+ + K^+)$-ATPase which is important in the maintenance of the (Na^+, K^+) gradient across the membrane. This enzyme has been demonstrated by a direct assay in the rabbit peritoneal neutrophil [37] but was not demonstrable by direct assay in the human neutrophil [38,39]. Since ouabain also has no significant effect on the hyperpolarization response to a stimulus, this hyperpolarization cannot be due to the action of the electrogenic pump, $(Na^+ + K^+)$-ATPase.

The ($Na^+ + K^+$)-ATPase appears not to play an active role in neutrophil activation; its function appears, rather, to be homeostatic, i.e, by maintenance of the Na^+ and K^+ gradient across the plasmalemma.

The effects of diphenylhydantoin, amiloride and furosemide in inhibiting lysosomal enzyme release, but not O_2^- generation, resemble those of anion channel blockers (e.g., 4-acetamido-4′-isothiocyanostilbene-2,2′-disulfonic acid (SITS), 4,4′-diisothiocyanostilbene-2,2′-disulfonic acid (DIDS) etc.) which have been shown to selectively inhibit lysosomal enzyme release in neutrophils [40]. Furosemide inhibits SO_4^{2-} fluxes in neutrophils (Korchak, H.M., Eisenstat, B.A. and Weissmann, G., unpublished observations) and amiloride inhibits Cl^- fluxes in frog skin [41]. An interrelationship between Na^+ fluxes and anion fluxes has been demonstrated, thus the anion flux inhibitor, 4-acetamido-4′-isothiocyanostilbene-2,2′-disulfonic acid (SITS), can inhibit Na^+ fluxes [42,43] and conversely the inhibition of Na^+ fluxes can inhibit the anion fluxes [41,44]. Interdependence between fluxes of Na^+ and anions may explain the inhibitory actions of diphenylhydantoin, amiloride and furosemide on lysosomal enzyme secretion from the neutrophil.

From the foregoing, we would propose a stimulus-response sequence for the neutrophil (Fig. 4), in which interaction of a stimulus with the membrane leads to a prompt hyperpolarization response. This change of $\Delta\psi$ precedes the onset of both O_2^- generation and lysosomal enzyme release by many seconds. Under conditions where ligand-triggered hyperpolarization is inhibited ($[Na^+]_{out} = 0$), both O_2^- generation and lysosomal enzyme release are also inhibited. The hyperpolarization response is not dependent on extracellular Ca^{2+}, however both lysosomal enzyme release and O_2^- generation are dependent on extracellular Ca^{2+} for an optimal response.

Extracellular Na^+ can modulate the neutrophil stimulus response sequence at a number of points. The presence of Na^+ is required for optimal expression of the hyperpolarization response. In addition it has been shown [24] that

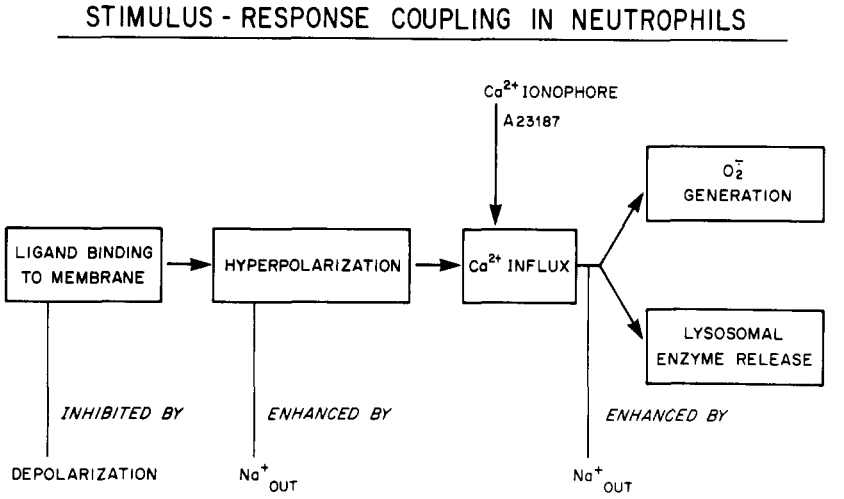


Fig. 4. Stimulus-response coupling in neutrophils—a working model.

Na^+ is required for the Ca^{2+} influx triggered by formylmethionylleucylphenylalanine in the human neutrophil. Extracellular Na^+ is also required for optimal lysosomal enzyme release and O_2^- generation at a step (or steps) subsequent to the Ca^{2+} influx. An influx of Na^+, as elicited by monensin, is not sufficient to activate the neutrophil fully. It is at present not known if the presence of extracellular Na^+ per se is sufficient, or if an Na^+ gradient across the membrane is required, for optimal neutrophil activity. Na^+ gradients can drive transport processes such as the active transport of amino acids and glucose, and in addition, there are a number of Na^+-Ca^{2+} exchange mechanisms which are important in the regulation of intracellular Ca^{2+}. A lack of extracellular Na^+ would then lead to a rise in intracellular Ca^{2+}. Alternatively, hyperpolarization response could be due to an increase in intracellular Ca^{2+}, i.e., a Gardos effect, and the presence of intracellular Na^+ might be essential for the Ca^{2+} mobilization process. The ability of NH_4^+ to substitute for Na^+ could be indicative of the importance of intracellular pH in maintaining neutrophil function, indicating a possible role for an Na^+-H^+ exchange mechanism in intracellular pH homeostasis. Alternatively, extracellular Na^+ might be essential to preserve the correct conformation of membrane proteins involved in the activation sequence of the neutrophil.

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