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Plasma membrane potential of neutrophils generated by the Na^+ pump

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The plasma membrane potential of human neutrophils was monitored using the anionic dye oxonol-V. The cells maintain a potential of -75 ± 17 mV when suspended in physiological saline solutions. The cells are scarcely depolarized by extracellular K^+ and the depolarization induced by the chemotactic peptide fMet-Leu-Phe is of similar magnitude for cells suspended in 5 or 155 mM K^+. Neutrophils are, however, depolarized by suspension in K^+-free media or after treatment with ouabain. Neutrophils catalyse Na^+-H^+ exchange and possess other electroneutral ion transport systems. We propose that the neutrophil membrane potential is generated by an electrogenic Na^+ pump, that osmotic stability is achieved by electroneutral ion transport systems and that electrical stability is maintained by anion leakage. Similar mechanisms may also operate in other biological membranes.

Introduction

The plasma membrane potential of most animal cells is often assumed to be set by K^+ diffusion with a minor contribution from the Na^+ pump [1–4]. The observation that a membrane potential in Lettré cells, at steady state for Na^+ and K^+, of -55 mV is insensitive to alteration of extracellular K^+, Na^+ or Cl^- [5], but is collapsed by ouabain [6], has led to the suggestion [5,6] that the Na^+ pump is the principal contributor to membrane potential provided various balancing mechanisms operate to maintain electrical and osmotic stability. The coupling of Na^+ pump activity with Na^+-H^+ exchange provides the cells with what is, in effect, a H^+ pump. The implication that H^+ extrusion contributes to plasma membrane potential (as it does to the membrane potential of mitochondria [7], lysozomes [8] and chromaffin granules [9,10]) has also been made in respect of the closely-related Ehrlich ascites cells (Ref. 11; see also Ref. 6). The ouabain-sensitivity of membrane potential in human neutrophils [12–14] suggests that these cells may generate membrane potential by a similar mechanism; we show here that this is indeed the case, and make the general proposition that any cell with a low and similar permeability for K^+ and Na^+ can generate its membrane potential through the continuous action of the Na^+ pump, provided it possesses (i) neutral Na^+ and K^+ return mechanisms, (ii) Na^+-H^+ exchange and (iii) an adequate permeability to anions.

Methods and Materials

Human neutrophils were prepared from 'buffy coat' suspensions (kindly donated by S.W. Thames Regional Blood Transfusion Service) using the method described by Segal et al. [15]. The cells were stored as a 40% (v/v) suspension in medium H199 (Flow Laboratories) at room temperature

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Abbreviations: FCCP, carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulphonic acid.

prior to use. On occasions neutrophils were prepared by a procedure that obviated the osmotic lysis step: the buffy coat was diluted 1:1 with phosphate-buffered saline and layered onto lymphopaque (Pharmacia) which was itself layered onto lymphopaque to which ficoll had been added to give a final concentration of 10.7 g/100 ml. After centrifuging the tubes at $1000 \times g$ for 20 min at room temperature lymphocytes were collected from the upper interface and neutrophils, plus a few contaminating erythrocytes, were collected from the lower interface; the red cells pelleted at the bottom of the tubes. The neutrophil layer was washed with phosphate-buffered saline and the cells resuspended in medium H199 as above.

Membrane potential was monitored using the oxonol dye method described by Bashford et al. [6,16–18]. Briefly, cells were diluted 100-fold (to give a 0.4% v/v suspension) into a medium containing 150 mM NaCl, 5 mM KCl, 5 mM glucose, 1 mM $MgCl_2$ (or 1 mM $MgSO_4$), 5 mM Hepes pH adjusted to 7.4 with NaOH (Hepes-buffered saline) and 2.5 μM oxonol-V. Membrane potential is registered by the absorbance difference $A_{630\text{-}590}$, an increase indicating depolarisation and a decrease indicating a hyperpolarisation [6,16–18]. The dye signal was calibrated using valinomycin and K^+ or FCCP and H^+ as described previously (Ref. 6, 16–18; and see Fig. 1a).

Cell Na^+ and K^+ were determined after pelleting 200 μl samples of the 0.4% cell suspension through oil (2 parts di-*n*-butylphthalate and 1 part dinonylphthalate) in a Beckman microfuge B. After the removal of the supernatant medium and the oil the centrifuge tubes were blotted dry and the cell pellets dispersed by sonication in 0.1 M lithium nitrate/0.1 M H_2SO_4 (BDH, Poole) and the ions were assayed by atomic absorption spectroscopy. The concentration of cellular ions was obtained directly from the pellet ion/pellet H_2O ratio, the latter being determined as the difference between wet and dry pellet weight [17].

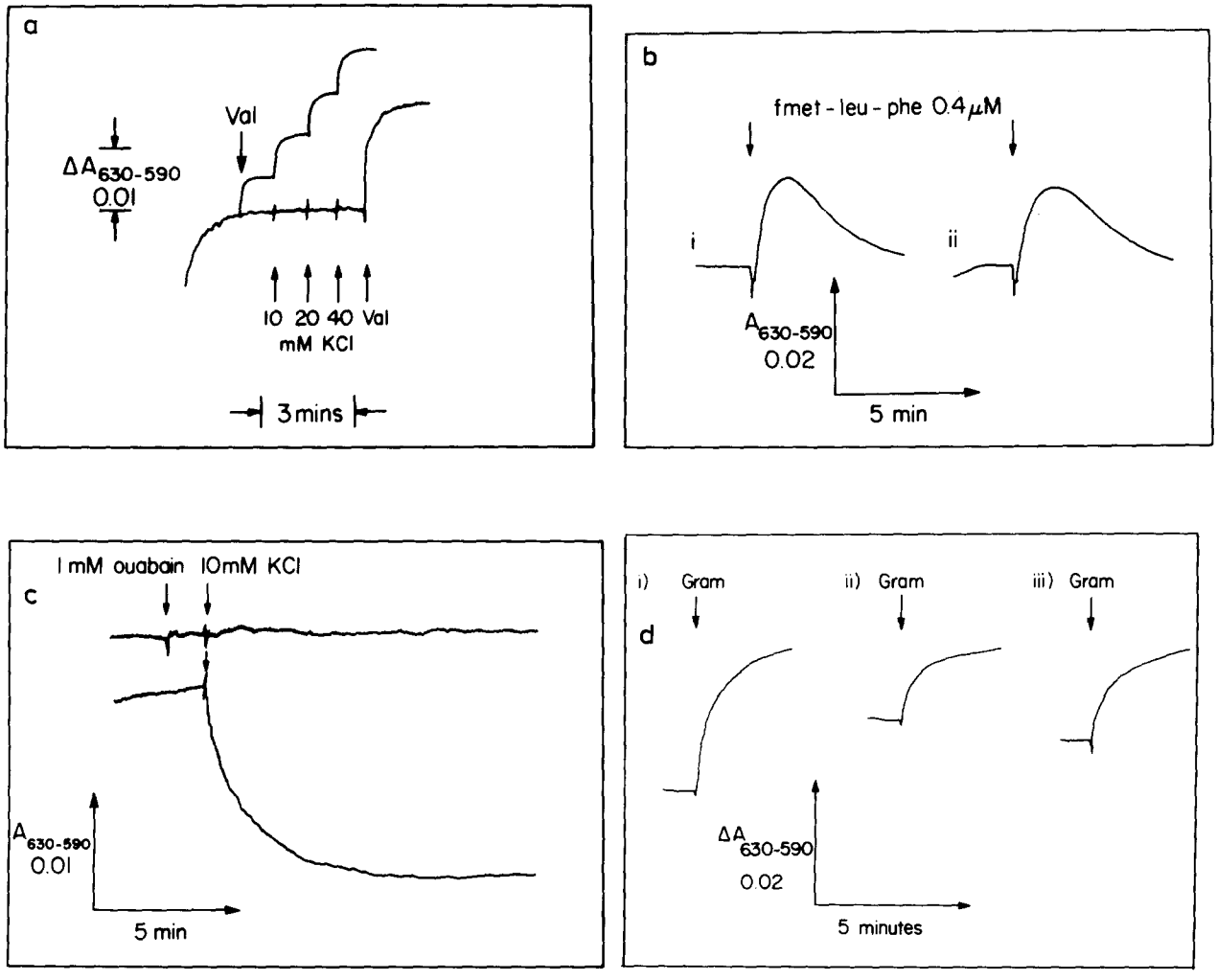
Extracellular pH was recorded with a Corning semi-micro combination electrode (Type 19) and a Corning model 125 pH meter. Intracellular pH was monitored using the permeant indicator neutral red and highly-buffered media such that only pH changes in the intramembranous compartment(s) are indicated [19,20]; intracellular pH is indicated by the absorbance difference $A_{445\text{-}540}$, a decrease indicating acidification. All absorption measurements were made with a Johnson Research Foundation compensated fluorimeter/spectrophotometer [17].

Oxonol-V was the kind gift of Dr. B. Chance, Johnson Research Foundation, University of Pennsylvania; amiloride was donated by Professor J.G. Widdicombe, Department of Physiology, St George's Hospital Medical School.

Results and Discussion

Human neutrophils have a membrane potential, assessed with oxonol-V and calibrated either with valinomycin and K^+ (see Fig. 1a) or FCCP and protons [6,16–18], of -75 ± 17 mV (S.D.; $n = 16$). Fig. 1a shows that membrane potential is insensitive to the addition of K^+, unless the K^+ ionophore valinomycin [21,22] is added first. Indeed cells are sufficiently polarized in 155 mM KCl, for the chemotactic peptide fMet-Leu-Phe [12] to depolarize them to the same extent as it does when they are in 5 mM KCl (Fig. 1b). In contrast, cells depolarize completely in K^+-free medium; they become polarized by the addition of K^+, in a ouabain-sensitive manner (Fig. 1c). Furthermore addition of ouabain depolarizes cells suspended in their normal medium, which contains 5 mM KCl, without any detectable change in their cellular ion content (Fig. 1e). Greatly increasing the plasma membrane permeability to Na^+ and K^+ by the addition of gramicidin [21,23] also depolarizes neutrophils (Fig. 1d). These results are compatible with the notion that human neutrophils have so few K^+ channels that their plasma membrane potential is set almost entirely by an electrogenic Na^+ pump. Preliminary experiments with rat neutrophils using the patch-clamp technique indicate that such cells indeed have very few K^+ (or other ion) channels (Fernandez, J. and Neher, E., Göttingen, personal communication).

The neutrophil preparations used for the experiments reported in Fig. 1 had intracellular K^+ concentrations in the range 50–70 mM and a $Na^+ + K^+$ content of 155 ± 21 mM (S.E.; $n = 6$). Similar values have been reported for preparations which include an osmotic lysis step (induced either by NH_4Cl or by reduced osmolarity) to remove



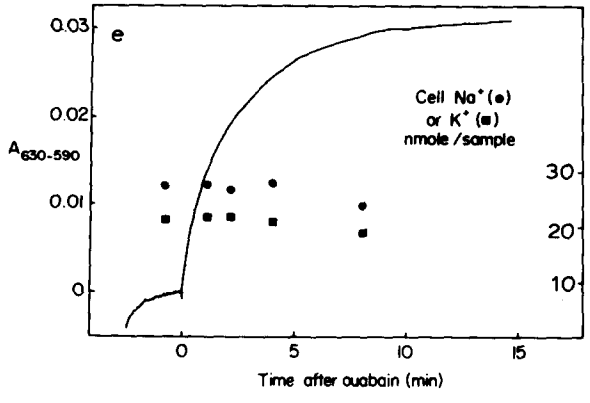


Fig. 1. Monitoring of plasma membrane potential in human neutrophils using oxonol-V. Neutrophils, 0.4% v/v, suspended in 5 mM glucose, 5 mM Hepes, 1 mM $MgCl_2$, 2.5 μM oxonol-V, pH adjusted to 7.4 with NaOH at 37°C and (a) 150 mM NaCl; 5 mM KCl; (b) (i) 150 mM NaCl, 5 mM KCl, (ii) 155 mM KCl; (c) 150 mM NaCl; (d) 5 mM KCl and (i) 150 mM NaCl, (ii) 75 mM Na_2SO_4, 75 mM mannitol, (iii) 150 mM sodium lactate; (e) 150 mM NaCl, 5 mM KCl. Additions were made to give the final concentrations indicated; in (a) valinomycin was 1 μg/ml, in (d) gramicidin was 2 μg/ml, ouabain was added to the upper trace only in (d) and at 1 mM in (e). Membrane potential (V) can be calculated from the equation: $V = (-RT/F)\ln([K^+]_{np}/[K^+]_i)$ where R is the gas constant, T the absolute temperature, F the Faraday constant, $[K^+]_i$ the intracellular K^+ concentration and $[K^+]_{np}$ the 'null-point', extracellular K^+ concentration at which addition of valinomycin causes no change in the absorbance of oxonol-treated cells [6,16–18]. For the experiment illustrated in (a) the 'null-point' (upper trace) obtained by extrapolation was 3 mM , $[K^+]_i$ was 51 mM and V was −76 mV.

contaminating red cells (see, for example, Refs. 24 and 25). Neutrophils prepared in homologous plasma, however, have much higher K^+ content, 120 mM [25]. We have repeated our experiments with neutrophils not subjected to osmotic shock (see Methods and Materials) and such cells show properties similar to those indicated in Fig. 1. The 'non-shocked' preparations retain a $Na^+ + K^+$ content of about 155 mM and a cell K^+ of 101 ± 5 mM (S.D.; $n = 9$). The absence of K^+-sensitivity of the membrane potential of our neutrophil preparations contrasts with other reports which show that neutrophil membrane potential can be K^+-sensitive [14,26,27]. However, in those experiments, using radiolabelled triphenylmethylphosphonium ($TPMP^+$), the calibration procedure assumed that the potential was zero in high K^+-media in order to correct for the mitochondrial contribution to $TPMP^+$ accumulation; in the absence of this 'correction' the K^+-dependence of $TPMP^+$ is markedly reduced [26] and, even in its presence, the K^+-dependence falls far short of the 61.5 mV per decade expected from the Nernst relationship [14]. In agreement with these reports we have noticed, in some experiments, a partial, variable K^+-sensitivity of our neutrophil preparations. Thus we find that 80 mM K^+ in the medium depolarizes neutrophils by $37 \pm 25\%$ (S.D.; $n = 14$) of the extent expected for a perfectly K^+-sensitive membrane and as found in the presence of valinomycin. The reasons for the variability of the response are not fully understood but contributing factors are the heterogeneity of neutrophil preparations [27] and the differential induction of K^+-channel activity by the incubation procedures employed. In the latter regard, the omission of Ca^{2+} from our final incubation (but Ca^{2+} is present at 1.35 mM in the stock cell suspension) may be a factor if neutrophils possess significant numbers of Ca^{2+}-activated K^+-channels. The apparent dearth of channels in rat neutrophils (see above) speaks against this possibility and we re-emphasize that whether or not neutrophils, like mast cells [28], can be persuaded to express channel activity our data show that under many circumstances they can exhibit a substantial plasma membrane potential that is sustained by the electrogenic cation pump (Ref. 13, Figs. 1c, e) and which does not depend on cation diffusion.

Human neutrophils contain electroneutral Na^+ and K^+ exchange mechanisms, as evidenced by the fact that the internal cation content responds rapidly to alteration of external cation concentration even though the membrane potential may not alter [6] (Fig. 2; see also Fig. 1). The nature of the electroneutral Na^+ and K^+ movements remains to be established but may include the volume-regulatory movements of Na^+, K^+ and Cl^- described by Ellory and others [29]. That neutrophils contain an Na^+-H^+ exchange mechanism is illustrated in Fig. 3. Panel A shows that the addition of extracellular Na^+ to cells suspended in low-Na^+ medium increases the rate of H^+ extrusion; the increase is completely blocked by amiloride [30,31]. Panel B of Fig. 3 shows that when neutrophil cytoplasm is acidified, by the addition of magnesium or sodium acetate to the medium (acidification of the cell milieu occurs because the undissociated acetic acid present enters cells rapidly [22] and subsequently dissociates), the restoration of the original cell pH requires extracellular Na^+ and is inhibited by amiloride. Other workers [31] have recently reported an amiloride-sensitive, Na^+-H^+ counter transport system in human neutrophils similar to the one described here.

The generation of membrane potential by an outward cation current requires the existence of a balancing return current; without it, cells would hyperpolarize to the point of dielectric breakdown of the membrane [32]. Such a current can be provided either by a backward leakage of cations, as in the case of H^+ movements in mitochondria [33] or photosynthetic bacteria [34], or by an outward leakage of anions, as in the case of lactate and bicarbonate leakage from Lettré cells [6]. In the case of human neutrophils, at least part of the balancing current appears to be an outward anion leakage: this is indicated by the observation that cells are less polarized when they are suspended in media which contain lactate or sulphate than when they are suspended in conventional chloride-containing media (Fig. 1d). These results suggest that neutrophils may have a significant permeability to chloride. However, chloride entry does not determine the potential because the removal of K^+ from, or the addition of ouabain to, the medium depolarizes the cells (Fig. 1) without a change in the chloride content of the cells, which is ap-

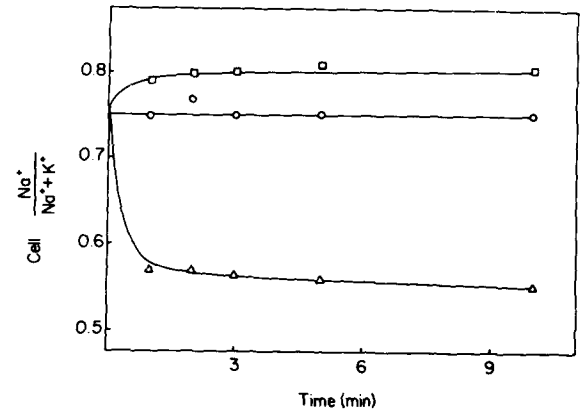
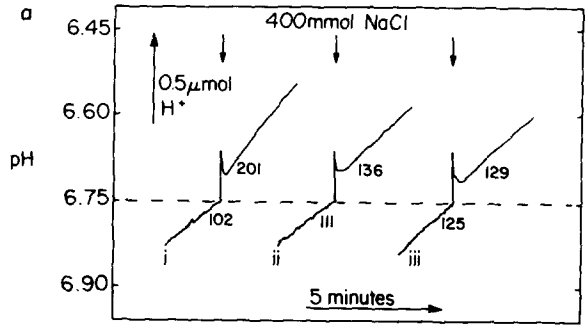


Fig. 2. Electroneutral cation movements in human neutrophils. 40% neutrophils in Hepes-buffered saline were diluted 1 : 100 in a similar medium (○), a medium with KCl replaced by NaCl (□) or a medium with NaCl replaced by KCl (△) all at 37°C. Cell cations were determined at the times indicated after diluting the cells.



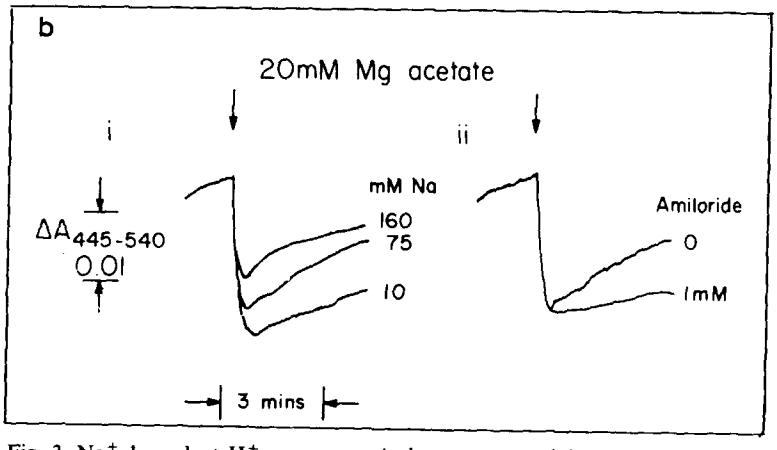


Fig. 3. Na^+-dependent H^+ movements in human neutrophils. (a) 0.4 ml of neutrophils (40% v/v in Hepes-buffered saline) were mixed with 10 ml of 290 mM sucrose, 10 mM mannitol, 1 mM $MgCl_2$, 0.5 mM Hepes, pH as indicated. NaCl was added as indicated to cells in the absence (i) and presence of 1 mM (ii) or 2 mM (iii) amiloride. The numbers beside each trace indicate the rate of H^+ appearance (*nmol/min*). (b) *0.8% neutrophils in 10 mM Hepes, 1 mM* $MgCl_2$, *10 μM neutral red*, pH adjusted to 7.4 with NaOH at 37°C and (i) mixtures of 200 mM mannitol, 50 mM sucrose and 155 mM NaCl to give the Na^+ concentration shown or (ii) 100 mM mannitol, 25 mM sucrose, 75 mM NaCl and amiloride as indicated. Magnesium acetate was added to give the final concentration shown.

proximately 80 mM (calculated from the equilibrium distribution of $^{36}Cl^-$). Furthermore at a potential of −75 mV, chloride would be expected to leave, not enter, cells and it is this outward flux that appears to provide the balancing current for the Na^+ pump. For cells to remain in an osmotic steady state under these conditions chloride must re-enter the cells electroneutrally; this may occur via the $Na^+ + K^+ + 2Cl^-$ pathway described by Geck et al. [35] for Ehrlich ascites cells or via the volume-regulating $Na^+ + Cl^-$ or $K^+ + Cl^-$ pathways found in many cell types [29]. Neutrophils, like Lettré cells [6], may also possess channels for organic anions, but the present experiments do not provide sufficient evidence for this assertion to be made.

Fig. 4 illustrates three different models for the maintenance of plasma membrane potential of cells. The models are not mutually exclusive and different cells may possess elements of one or more of them, in a ratio that can only be assessed by experiment. Fig. 4A shows the position of a membrane polarized by the sodium pump whose current is balanced by chloride efflux; osmotic steady state is preserved by electroneutral $Na^+ + K^+ + Cl^-$ pathways. Each turnover of the sodium pump exports one positive charge (as Na^+) from the cell; at the steady-state potential of −75 mV, this current is balanced by the leak of one anion

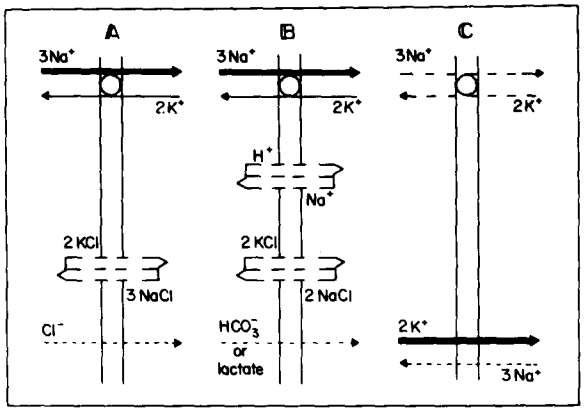


Fig. 4. Mechanisms by which cells may maintain plasma membrane potentials.

(presumably Cl^-) from the cell. To prevent cell shrinkage (because the effect of the pump has been to export $Na^+ + Cl^-$), and to maintain intracellular cation composition we propose that $3Na^+ + 3Cl^-$ enter and $2K^+ + 2Cl^-$ exit from the cells by electroneutral mechanisms either as salt movements as written or, in part, as cation exchanges. These latter processes are powered by the transmembrane gradients of Na^+ and K^+ which cells sustain in physiological media. This model is similar to the 'chloride pump' found in a number of epithelial tissues [36] except that in this case the cells do not have separate mucosal and serosal surfaces and Cl^- leaks back across the same membrane through which it enters the cell. This model explains, in part, the plasma membrane potential of neutrophils. In Fig. 4B the membrane is again polarized by the Na^+ pump but in this case, as found in Lettré cells, the balancing current is of organic anions; again osmotic steady state is preserved by electroneutral Na^+, K^+ and Cl^- movements driven by the cation gradients as discussed above; the tendency of the cells to acidify (because of the exit of organic ions but not of their associated H^+) is compensated by Na^+-H^+ exchange. The mechanism outlined in Fig. 4B couples the activity of the Na^+ pump to the requirement of cells to export metabolically-produced acid via the Na^+ gradient and Na^+-H^+ exchange; in effect the Na^+ pump is operating as a 'proton pump' in the same way as it is proposed to act as a 'chloride pump' [36] (see Fig. 4A). Neutrophils possess Na^+-H^+ exchange activity (Fig. 3 and Ref. 31) and may

TABLE I

PLASMA MEMBRANE POTENTIAL OF CELLS

Cell	Predominant mechanism setting potential *	Reference
Neutrophils	A (and B)	this work; 13
Lettré	B (and A)	6
Lymphocytes	C	6, 38
Excitable cells	C	text books

* Letters refer to the mechanisms illustrated in Fig. 4.

generate some of their plasma membrane potential by the mechanism shown in Fig. 4B. Fig. 4C illustrates the situation where plasma membrane potential is determined principally by K^+ diffusion balanced by Na^+ influx; in this case the Na^+ pump makes only a minor contribution to the potential, but provides the means whereby the cells maintain the cation gradients necessary to maintain their plasma membrane potential and osmotic stability [1-4] (but see also Ref. 37). This is the traditional view of the role of the Na^+ pump in cell membrane potential and occurs in many cell types including lymphocytes [6,38] and most excitable cells [1,2,4]. Cell types in which each of the mechanisms indicated in Fig. 4 contributes to plasma membrane potential are listed in Table I.

We propose that the models outlined in Fig. 4A and B are general ones that overcome previous reservations concerning the setting of membrane potential by an electrogenic Na^+ pump in cells at an ionic steady state [3,4]. The requirements, apart from the presence of an electrogenic Na^+ pump which appears to be ubiquitous in animal cells [39], are (i) a low permeability to K^+ and Na^+, (iii) the presence of Na^+-H^+ exchange and other electroneutral Na^+ and K^+ return mechanisms and (iii) an adequate permeability to anions such as lactate, bicarbonate or chloride. These processes are likely to be especially important in cells which do not possess specific channels for ion permeation such as freshly isolated macrophages [28]. The model in Fig. 4B depends in essence on a separation of acid extrusion into separate H^+ and organic anion pathways. That all viable cells excrete acid (greatly in excess of that required to maintain a negative inside membrane potential as depicted in Fig. 4B) as a result of metabolism, –whether aerobic or anaerobic, –is self-evident; it

is certainly compatible with the internal acidification of endosomes derived from the plasma membrane [39,40]. Since cells are thought to develop ion channels during ontogeny (Refs. 42, 43; see also Ref. 28), and perhaps to lose them during the progression towards malignancy, it is clearly of interest to examine the origin of membrane potential in cells at different stages of development.

Acknowledgements

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