CONTRIBUTION TO THE MEMBRANE POTENTIAL OF THE ELECTROGENIC Na^+,K^+-PUMP IN GUINEA PIG POLYMORPHONUCLEAR LEUKOCYTES

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1. Introduction

Changes in the membrane potential have been shown to play an important role in the initiation and regulation of a variety of polymorphonuclear leukocyte (PMN) functions [1–5]. Nevertheless, little is established about the origin of the membrane potential or the factors which determine the membrane potential. Since microelectrodes are not applicable to PMN due to their small size, efforts to measure the membrane potential must rely on indirect techniques. The lipophilic cations, such as TPP^+(tetraphenyl phosphonium) and $TPMP^+$(triphenylmethyl phosphonium), have now been utilized successfully in a number of biological systems [2,6–11]. This approach has been first introduced by Skulachev and co-workers [6] and is based on a passive equilibration of these ions with an electrical potential across the membrane. We have devised a selective electrode for TPP^+, which enables us to monitor membrane potentials continuously in mitochondria [12], and *Escherichia coli* cells [13]. Here, we report the application of the electrode method and show that the Na^+,K^+-pump (i.e., Na^+,K^+-ATPase) contributes to the membrane potential in guinea pig PMN.

2. Methods

2.1. *Preparation of PMN*

PMNs were obtained from guinea pig peritoneal cavity with a slight modification of the method in [14]. The peritoneal exudate was collected 18 h after intraperitoneal injection of 30 ml 2.0% sodium caseinate suspended in sterile isotonic saline. The exudate was washed once with isotonic ammonium chloride solution for hemolyzing red blood cells. PMNs were then washed once with a modified Hanks' buffer solution and resuspended in this solution. The composition of the modified Hanks' buffer used is 124 mM NaCl, 4.9 mM KCl, 0.64 mM Na_2HPO_4, 0.66 mM KH_2PO_4, 0.76 mM $CaCl_2$, 0.76 mM $MgCl_2$, 15.2 mM $NaHCO_3$, 20 mM Hepes (*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid) adjusted to pH 7.4 with Tris (Tris(hydroxymethyl)aminomethane) base, and 5.5 mM glucose. This buffer was used throughout unless otherwise indicated. The K^+-free medium was prepared by substituting NaCl for KCl and NaH_2PO_4 for KH_2PO_4.

2.2. *Uptake of TPP^+*

TPP^+ accumulation was monitored continuously with a TPP^+-selective electrode. The construction and properties of a TPP^+-selective electrode were described in [12]. Reactions were initiated by adding 200–300 μl ($5–8 \times 10^7$ cells) of washed cells to 4.0 ml of a modified Hanks' buffer solution containing 0.1 mM TPP^+. The temperature of the system was kept constant at the desired temperature, and the cells were maintained in suspension by agitating with magnetic stirring bar.

2.3. *Calculation of the membrane potential*

The membrane potential ($\Delta\psi$) can be calculated from the shift of the electrode potential provided that TPP^+ is passively distributed in accordance with the Nernst equation. When the electrode potential shifts by ΔE from the base line, $\Delta\psi$ is given by the following equation:

$$\Delta\psi = 2.3(RT/F)\log(v/V) - 2.3(RT/F)\log[10^{F\Delta E/2.3RT} - 1] \quad (1)$$

where v and V stand for the volume of PMNs and of the medium, respectively, and R, T and F have their usual thermodynamic significance. The base line is the electrode potential before addition of cells.

2.4. *Determination of cellular ATP level*

ATP was assayed by the luciferin–luciferase method using an Aminco -Chemglow apparatus. Cellular ATP was extracted by boiling for 5 min. The precipitate was removed by centrifugation (13 000 × *g*), and an aliquot of the supernatant was used for the determination of ATP. Protein was measured by Lowry method [15] with bovine serum albumin as a standard.

3. Results

Fig.1 shows the time course of TPP^+ uptake at different temperatures. The upward shift means a decrease in TPP^+ concentration in the medium, i.e., uptake by PMNs. TPP^+ uptake reached a maximum in ~30 min at 37°C, and then spontaneous efflux of accumulated TPP^+ occurred. The slower rate of TPP^+ uptake than that by mitochondria and by *E. coli* cells [12,13] is due to the lower permeability of PMN cell membrane to TPP^+ in comparison with the mitochondrial and *E. coli* cell membranes. The reason of the efflux is not clear at present. Decreasing the ambient temperature reduced both the rate and extent of TPP^+ uptake. Significant TPP^+ uptake was observed only at >15°C, showing that the membrane potential is temperature-dependent, presumably due to the connection with metabolic energy. No TPP^+ uptake was observed in the cell which had been disrupted by freezing and thawing or by sonication (not shown). This suggests that TPP^+-uptake requires membrane integrity which is consistent with the idea that TPP^+ is accumulated electrophoretically by the membrane potential (inside negative).

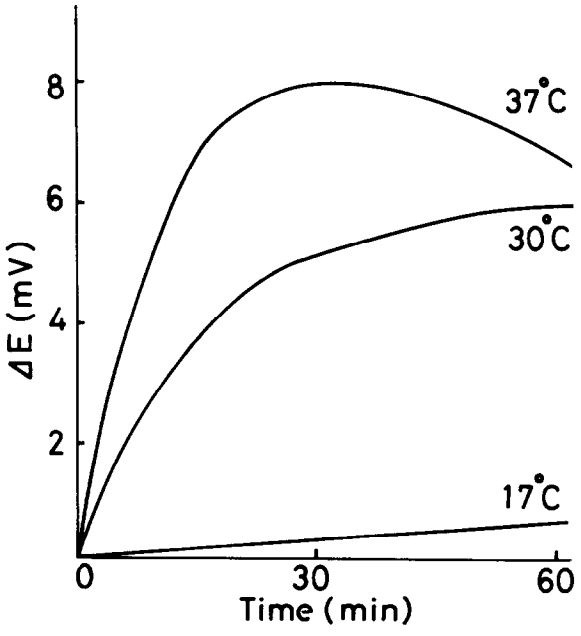

Fig.1. Time course of the uptake of TPP^+ by PMNs at different temperatures. Cells (2 × 10^7/ml) were incubated at the indicated temperature in a modified Hanks' buffer. TPP^+ uptake was monitored with a TPP^+-electrode as in section 2. The value of (ΔE) given on the ordinate represents the electrode potential difference before and after cells were introduced into the medium.

Fig.2 shows the effect of ouabain on TPP^+ accumulation. Addition of ouabain (10^{-4} M) to PMNs suspended in the normal medium resulted in an efflux of TPP^+, suggesting the contribution of Na^+,K^+-pump to the membrane potential. Since Na^+,K^+-pump requires the presence of extracellular K^+ to operate, we have tried to examine the TPP^+ accumulation in K^+-free medium. As shown in fig.2, the amount of TPP^+ accumulated by PMNs was smaller than that suspended in the normal buffer. In addition, the level obtained in K^+-free buffer was the same as that observed in the normal medium containing enough amount

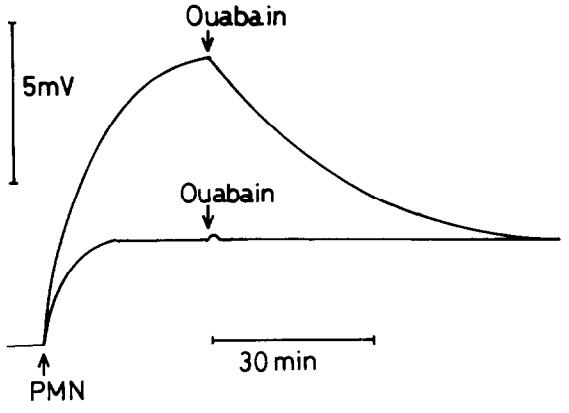

Fig.2. Effects of ouabain on TPP^+ accumulation. Cells (1.8 × 10^7/ml), washed in K^+-free buffer, were suspended in normal buffer (upper), or K^+-free buffer (lower) at 37°C. Ouabain (10^{-4} M final conc.) was added where indicated. The vertical bar represents a change of 5 mV in the TPP^+-electrode.

of ouabain. The effect of ouabain was not observed when PMNs were suspended in K^+-free medium. These findings are consistent with the idea that Na^+, K^+-pump contributes to the membrane potential of PMN.

According to eq. (1), the value of v, intracellular volume, is necessary to calculate the absolute value of the membrane potential. However, if we assume that the first term in eq. (1) remains constant, we can calculate the magnitude of the change in the membrane potential by addition of ouabain because the term cancells [13]. We have to pay attention to the uptake of the TPP^+ accumulated inside the cell by nuclei, although the membrane potential of nuclei is not known. This uptake can be treated as a change in the cellular volume, v in eq. (1)*. Then, the above procedure can eliminate the effect of distribution across nuclear membranes provided that the membrane potential of nuclei is unchanged by addition of ouabain, which may be the case. Fig.3 shows a dose–response curve so obtained, and a maximal change in the membrane potential is estimated to be ~30 mV. This value represents the contribution of ouabain-sensitive component (i.e., Na^+-K^+-pump) to the resting membrane potential.

Effects of a number of inhibitors on the uptake of TPP^+ and cellular ATP level are shown in table 1. Inhibition of glycolysis by NaF or 2-deoxyglucose (2-DG) led to a profound depletion of cellular ATP level and caused a significant decrease in TPP^+ accumulation. On the other hand, respiratory inhibitors (NaCN, NaN_3) had little effect on TPP^+ accumulation and on cellular ATP level. PMNs have few mitochondria and oxidative phosphorylation is thought to play a minor role in PMN energy metabolism [14]. Sulf-

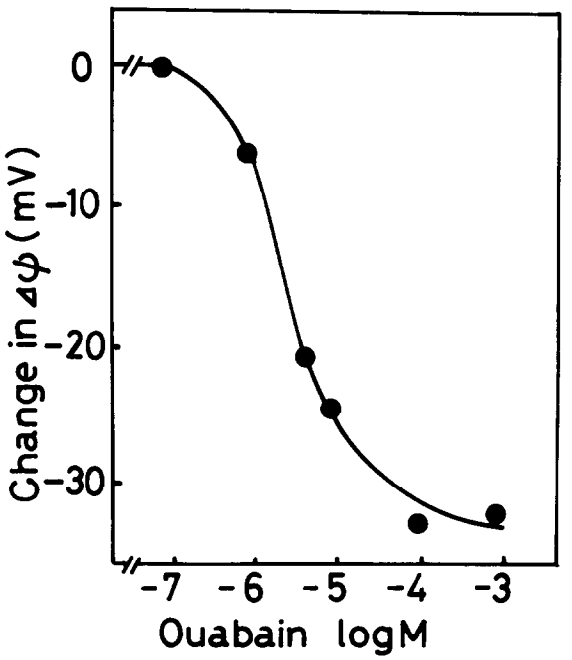

Fig.3. Dose–response curve for the effect of ouabain on the membrane potential ($\Delta\psi$). Ouabain at the desired concentration was added before the start of the experiment. After incubation for 30 min at 37°C, the value of the change in $\Delta\psi$ was calculated from ΔE in the absence and in the presence of ouabain. Each point represents the mean of duplicate or triplicate determinations.

Table 1
Effect of various inhibitors on the uptake of TPP^+ and cellular ATP level

Inhibitor	(mM)	% of control	
		TPP^+ uptake	ATP content
None		100	100
2-DG	5	58	8
NaF	10	55	38
NaCN	1	89	89
NaN_3	1	88	97
NEM	0.5	25	2
PCMBS	0.1	35	90
Ouabain	0.1	36	95

Cells were incubated for 30 min at 37°C in a modified Hanks' buffer (glucose-free). The results are expressed as % of control values. Control cellular ATP was 3.0 nmol/mg protein

* When we consider the uptake of TPP^+ by nuclei, the amount of TPP^+ accumulated by PMNs, vC_{in}, is expressed by:

$$vC_{in} = (v - v_{nuc})C_{cyt} + v_{nuc}C_{nuc}$$

where v_{nuc}, C_{cyt} and C_{nuc} stand for the volume of nuclei, the TPP^+ concentration in cytoplasm and that in nuclei. The distribution of TPP^+ across the nuclear membrane is governed by the following Nernst equation:

$$C_{nuc} = C_{cyt}\exp(-F\Delta\psi_{nuc}/RT)$$

where $\Delta\psi_{nuc}$ is the potential difference across the nuclear membrane. Then, we obtain:

$$vC_{in} = [v - v_{nuc} + v_{nuc}\exp(-F\Delta\psi_{nuc}/RT)]C_{cyt}$$

showing that $[v - v_{nuc} + v_{nuc}\exp(-F\Delta\psi_{nuc}/RT)]$ should be used instead of v in eq. (1). However, we can calculate the change in the membrane potential by the procedure described in the text, because this term cancells provided that v_{nuc} and $\Delta\psi_{nuc}$ are constant

hydryl inhibitors, such as *N*-ethylmaleimide (NEM) and *p*-chloromercurybenzene sulfonic acid (PCMBS), which have been reported to inhibit Na^+,K^+-ATPase activity [16], markedly depressed TPP^+ accumulation. NEM markedly reduced cellular ATP level, whereas PCMBS has little effect. These results are consistent with the difference in the membrane permeability of the agents, i.e., the membrane is permeable to NEM but not to PCMBS.

4. Discussion

We show the the Na^+,K^+-pump contributes to the membrane potential of PMNs. The temperature dependence of TPP^+ uptake (fig.1) is consistent with this conclusion. However, the origin of the ouabain-insensitive component of the membrane potential is not clear at present.

Mammalian cell membranes have transport system that functions as a Na^+,K^+-pump which allows them to maintain a high level of intracellular K^+ and a low level of Na^+. In the red blood cell membrane the hydrolysis of each molecule of ATP by the Na^+,K^+-pump is accompanied by an outward movement of 3 Na^+ and an inward movement of 2 K^+ [17]. This inequality in an ion movement suggests that the Na^+, K^+-pump may be electrogenic; its action leads to a net movement of charge across the membrane. However, we cannot observe the ouabain-sensitive component in the membrane potential of red blood cells under physiological conditions, because high permeability of Cl^- determines the membrane potential. When the experiments were done under the condition of low anion permeability, the Na^+,K^+-pump contributed to the membrane potential of the red cell [18]: Membrane potential hyperpolarized when Na^+,K^+-pump was activated by addition of external K^+ and depolarized on subsequent addition of ouabain. As shown in fig.2, our result is the same as that in red cells carried out under these special conditions, suggesting that ion movement by the Na^+,K^+-pump is the major part of the ion-transport across the resting PMN cell membrane.

The pump was activated by the chemotactic peptide (f-Met–Leu–Phe) in [19]. Therefore, changes in the membrane potential associated with Na^+,K^+-pump activity should be of particular interest.

In [5] f-Met–Leu–Phe caused a change in 3,3′-dipentyloxacarbocyanine [diO-C_5-(3)] fluorescence (a membrane potential-sensitive probe). The response to f-Met–Leu–Phe was inhibited by ouabain (10^{-4} M) in a time-dependent fashion, but ouabain itself did not cause the fluorescence change, suggesting that the Na^+,K^+-pump makes no direct contribution to the resting membrane potential. This is contradictory to our results. Preliminary experiments using another fluorescent dye 3,3′-dipropylthiadicarbocyanine [diS-C_3-(5)] gave results essentially similar to those obtained by measurement of TPP^+ distribution (unpublished). The reason of this discrepancy remains unclear. The biological significance of an electrogenic Na^+,K^+-pump in guinea pig PMN awaits further investigation.

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