Factors determining the plasma-membrane potential of lymphocytes

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1. Lymphocytes from pig mesenteric lymph node have low permeability to K^+ (Rb^+), Na^+ and Cl^- . None of these ions is in Nernst equilibrium with the plasma-membrane potential ($\Delta\psi_p$). 2. $\Delta\psi_p$ can be calculated from the transmembrane distribution of the permeant cation methyltriphenylphosphonium ($TPMP^+$) in the presence of the uncoupler carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone (FCCP) to abolish uptake into intracellular mitochondria. In normal culture medium $\Delta\psi_p$ is 56 mV. 3. A similar potential is found in T-enriched pig cells and in mouse thymocytes. 4. The contribution of electrogenic ($Na^+ + K^+$)ATPase to $\Delta\psi_p$ is about 7 mV. 5. The remainder of the lymphocyte $\Delta\psi_p$ is a polyionic potential set up by K^+ and Cl^- with a permeability coefficient for Cl^- of similar magnitude to that for K^+ .

Qualitative studies of the lymphocyte $\Delta\psi_p$ have shown changes on mitogenic stimulation. Such changes may be important in the mechanism of cell division. Shapiro *et al.* (1979) observed a depolarization of human peripheral lymphocytes a few minutes after treatment with mitogen. In contrast, Tsien *et al.* (1982) showed a very early hyperpolarization of mouse thymocytes. Kiefer *et al.* (1980) report a depolarization of mouse spleen cells followed by a slower hyperpolarization. Despite this work a reliable quantitative study of the lymphocyte $\Delta\psi_p$ throughout the course of mitogenic stimulation has not yet been performed. Such a study would clearly contribute towards a greater understanding of the events leading to cell division.

Attempts to measure the $\Delta\psi_p$ of lymphocytes have been made by a variety of techniques. Microelectrode measurements yielded mean values for human peripheral small lymphocytes of 12 mV (Taki, 1970). Rink *et al.* (1980) employed fluorescent probes to give an estimate of 60 mV for resting

mouse spleen lymphocytes. Iversen (1976) proposed a value of 35 mV for resting rat thymocytes, based on measurements of Cl^- distribution across the plasma membrane. Studies on the distribution of the radiolabelled lipophilic ions $TPMP^+$, TPP^+ and SCN^- have yielded values for the lymphocyte resting potential ranging from 35 to 65 mV (Deutsch *et al.*, 1979b; Kiefer *et al.*, 1980). Clearly the methods used in recent years to quantify the resting lymphocyte $\Delta\psi_p$ have yielded disparate results.

Characterization of the lymphocyte $\Delta\psi_p$, in terms of the factors determining it, has only partly been achieved. Studies on $^{42}K^+$ and $^{86}Rb^+$ influx have indicated a low plasma-membrane K^+ permeability (Lichtman *et al.*, 1972; Owens & Kaplan, 1980). Rink *et al.* (1980) observed little effect of valinomycin, a K^+ ionophore, on the fluorescent dye signal of mouse spleen lymphocytes. This led them to describe the lymphocyte $\Delta\psi_p$ as a K^+ -dominated potential. However, this is consistent with low plasma-membrane K^+ permeability only if the permeabilities of other ions such as Na^+ and Cl^- are negligible in comparison.

Measurement of the $\Delta\psi_p$ of resting lymphocytes and elucidation of the factors that establish it would provide a sound basis for further investigation of effects of mitogens. In the present paper we introduce a reliable method of determining the resting $\Delta\psi_p$ of pig mesenteric lymphocytes and report a value of 56 mV in normal culture medium. In addition we present a characterization of this potential in terms of both the components resulting from ion concentration gradients existing across a selectively permeable plasma membrane in accordance with the constant field equation (Goldman,

Abbreviations used: $\Delta\psi_p$, difference in electrical potential across the plasma membrane (positive outside), given a positive sign throughout the present paper; $\Delta\psi_m$, difference in electrical potential across the mitochondrial membrane; $\Delta\psi_{ATPase}$, difference in electrical potential due to activity of electrogenic ($Na^+ + K^+$)ATPase; $\Delta\psi_{ion}$, difference in electrical potential due to passive ion fluxes; E_K , E_{Na} , E_{Cl} , calculated diffusion potential for K^+ , Na^+ or Cl^- ; $TPMP^+$, methyltriphenylphosphonium; TPP^+ , tetraphenylphosphonium; FCCP, carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone; SITS, 4-acetamido-4'-isothiocyanatostilbene-2,2'-bis-sulphonic acid; Hepes, 4-(2-hydroxyethyl)-1-piperazine-ethanesulphonic acid.

1943; Hodgkin & Katz, 1949) and an electrogenic component due to $(\text{Na}^+ + \text{K}^+)\text{ATPase}$ activity.

Experimental

Materials

$^{86}\text{RbCl}$, Na^{36}Cl , $[^3\text{H}]\text{inulin}$ and $^3\text{H}_2\text{O}$ were from The Radiochemical Centre, Amersham, Bucks. U.K. $[^3\text{H}]\text{TPMP}^+$ bromide was from New England Nuclear Chemicals, Dreieich, Germany. RPMI 1640 culture medium was from Flow Laboratories, Irvine, Scotland, U.K. Bongkreic acid was a gift from Dr. P. J. F. Henderson of this Department. Valinomycin, ouabain and FCCP were from Sigma (London) Chemical Co., Kingston upon Thames, Surrey, U.K. Oligomycin and antimycin A were from Boehringer Corp. (London), Lewes, East Sussex, U.K. Fluorescein isothiocyanate-conjugated rabbit anti-(pig immunoglobulin G) was from Miles Laboratories, Slough, U.K. Bis-oxonol [bis-(1,3-diethylthio-barbiturate)-trimethine oxonol] was a gift from Dr. T. J. Rink, Department of Physiology, University of Cambridge. All other reagents were of analytical grade.

Preparation of pig mesenteric lymphocytes

Lymph nodes were dissected from the mesentery of a freshly slaughtered pig. Lymphocytes were teased out through a nylon sieve into standard medium (RPMI 1640 without glutamine, containing 10 mM- or, in some experiments, 50 mM-Hepes). Erythrocytes and dead cells were removed by centrifugation through 6% Ficoll (Pharmacia) containing 16.7% Isopaque (Nygaard) at 1200 *g* for 15 min. The interfacial layer was removed and washed by further centrifugation at 1200 *g* for 5 min. The cells were finally resuspended and kept at room temperature at a concentration of $(2-6) \times 10^7$ cells $\cdot \text{ml}^{-1}$. Viability (>85%) was assessed by exclusion of sodium eosin.

T-enriched pig mesenteric lymphocytes were prepared on continuous density gradients of 45% Percoll (Pharmacia) made iso-osmotic with standard RPMI medium. Gradients were spun at 18000 rev./min for 10 min at 20°C in an MSE 18 centrifuge. T-enriched cells were collected from the lower end of each gradient. After washing and resuspension into fresh medium the cells were typed for B-cell immunoglobulin staining by a fluorescein isothiocyanate-conjugated anti-immunoglobulin immunofluorescence assay. Less than 12% of T-enriched cells stained as B-cells. Non-enriched cells were 45% B.

Preparation of mouse thymocytes

Balb/c mice, aged between 6 and 8 weeks, were killed by cervical dislocation and the thymus was removed, washed in ethanol and placed in standard

RPMI medium. Cells were teased out, washed by centrifugation at 1200 *g* for 5 min and resuspended in fresh medium to a cell density of $(2-6) \times 10^7$ cells $\cdot \text{ml}^{-1}$.

Determination of $^{86}\text{Rb}^+$, $^{36}\text{Cl}^-$ and $[^3\text{H}]\text{TPMP}^+$ accumulation by pig lymphocytes

Cells were routinely pre-incubated for 30 min at 37°C in standard medium (RPMI 1640, 10 or 50 mM-Hepes, pH 7.4) at a concentration of $(2-6) \times 10^7$ cells $\cdot \text{ml}^{-1}$. This medium contains 8 mM- K^+ , 111 mM- Cl^- and 113 mM- Na^+ . An alternative medium was used for experiments involving iso-osmotic substitutions, as described in the Figure legends. At the start of each experiment cells were mixed with an equal volume of medium with appropriate additions. Final concentrations of these were: 3 μM -tetraphenylboron, 0.1–1.0 μCi of $[^3\text{H}]\text{TPMP}^+/\text{ml}$ (20–50 nM) and either 0.02 μCi of $^{86}\text{RbCl}/\text{ml}$ or 0.09 μCi of $\text{Na}^{36}\text{Cl}/\text{ml}$. Other additions are described in the Figure legends. Samples (1 ml) were taken in duplicate at relevant times for centrifugation in an Eppendorf bench centrifuge for 2 min. Samples (250 μl) of supernatant were removed to 15 ml of scintillant containing 4 g of 2,5-diphenyloxazole, 10 mg of 1,4-bis-(5-phenyloxazol-2-yl)benzene, 0.33 litre of Triton X-100 and 0.67 litre of toluene per litre. Water (1 ml) was added to each vial to give a stable mixture. The remaining supernatant was decanted and the residual liquid removed with a tissue. Each pellet was resuspended in 40 μl of 20% (v/v) Lubrol and the base of the centrifuge tube containing the entire pellet was cut off into scintillant. Vials were counted for radioactivity in a dual-channel liquid-scintillation spectrometer. ^3H counts were corrected for crossover from ^{86}Rb or ^{36}Cl as appropriate.

Parallel determination of intra- and extra-cellular pellet spaces were performed with each experiment. After pre-incubation, as described above, cells were incubated with 0.02 μCi of $^{86}\text{RbCl}/\text{ml}$ and either 0.8 μCi of $[^3\text{H}]\text{inulin}/\text{ml}$ (56 μM) or 1.5 μCi of $^3\text{H}_2\text{O}/\text{ml}$. Samples (1 ml) were taken at 30 min and treated as above. Determination of the inulin-permeable pellet volume allowed a correction for extracellular space to be made to TPMP^+ , Rb^+ and Cl^- apparent spaces. Accumulation ratios were then calculated by dividing these corrected spaces by the intracellular volume, measured as the inulin-impermeable $^3\text{H}_2\text{O}$ -permeable space. With this technique the extracellular space comprised about 50% of the total pellet volume. The intracellular volume for pig cells was found to be 178 ± 17 (mean $\pm$ S.D.) fl/cell.

Measurement of extra- and intra-cellular K^+

Cells were pre-incubated for 30 min at 37°C in standard RPMI medium at a concentration of

$(1-3) \times 10^7$ cells $\cdot$ ml $^{-1}$. Samples (1 ml) were taken at defined times after the start of the experiment and centrifuged for 2 min in an Eppendorf bench centrifuge. After sampling and removal of contaminating supernatant as before, each pellet was resuspended in 100 μ l of water. Trichloroacetic acid (900 μ l) was then added and the precipitated protein was sedimented by centrifugation for 4 min. Supernatant samples from both centrifugation steps were assayed for K $^{+}$ content in a Perkin-Elmer atomic-absorption spectrophotometer 380. Intra- and extra-cellular volumes were determined in parallel experiments as described above.

ATP assays

Intracellular ATP levels were measured by a luciferin-luciferase assay in a DuPont 760 luminescence biometer.

Immunofluorescence assays

Cells (1.5×10^5 in 50 μ l of medium) were incubated for 10 min at room temperature in the presence of 2 μ l of fluorescein isothiocyanate-conjugated rabbit anti-(pig immunoglobulin G). Medium (1 ml) was then added and the cells were sedimented in an Eppendorf bench centrifuge for 10 s. The pellet was resuspended in 50 μ l of medium and the percentage of fluorescence labelled B-cells was assessed by viewing the cells under a Leitz Wetzlar fluorescence microscope.

Fluorescence experiments using bis-oxonol

These experiments were done in a Perkin-Elmer MPF 44A spectrophotometer by the method of Rink *et al.* (1980). Bis-oxonol in dimethyl sulphoxide was added to 3 ml of standard RPMI medium in a quartz cell maintained at 37°C to a final concentration of 150 nM. The fluorescence signal of the solution was recorded with excitation and emission at 540 nm and 580 nm and slit widths of 10 nm. Cells (6×10^5) were added after a steady signal had been achieved. Other additions are described in the legend to Fig. 7.

Results

The suitability of $^{86}\text{Rb}^{+}$ (an analogue of K $^{+}$) and $^{36}\text{Cl}^{-}$ as indicators of $\Delta\psi_p$ was investigated. For one or other of these tracers to be useful cells must have a high Rb $^{+}$ or Cl $^{-}$ permeability so that the distribution of these ions across the plasma membrane approximates to that predicted by the Nernst equation as follows:

$$\Delta\psi_p = 2.3 \frac{RT}{F} \log \frac{[\text{Rb}^{+}]_i}{[\text{Rb}^{+}]_e}$$

or

$$\Delta\psi_p = 2.3 \frac{RT}{F} \log \frac{[\text{Cl}^{-}]_e}{[\text{Cl}^{-}]_i}$$

where subscripts i and e refer to intra- and extra-cellular ion concentrations.

$^{86}\text{Rb}^{+}$ distribution

Fig. 1(a) shows the rate of $^{86}\text{Rb}^{+}$ uptake into pig lymphocytes to be substantially increased by the presence of valinomycin. Steady-state $^{86}\text{Rb}^{+}$ distribution with valinomycin was achieved within 30 min, whereas control Rb $^{+}$ distribution was far from equilibrium with the K $^{+}$ gradient even at 55 min. Such observations show the plasma membrane to be relatively impermeable to Rb $^{+}$ and hence to K $^{+}$. $^{86}\text{Rb}^{+}$ is therefore unsuitable as a probe for the measurement of $\Delta\psi_p$ in lymphocytes. The increased Rb $^{+}$ accumulation in the presence of valinomycin is not due to Rb $^{+}$ uptake by mitochondria which will be depolarized in cytosolic [K $^{+}$] (136 mM) when valinomycin is added.

The magnitude of the K $^{+}$ concentration gradient was determined by two different methods. Measurement of both the steady-state $^{86}\text{Rb}^{+}$ accumulation ratio in the presence of valinomycin and the K $^{+}$ distribution by atomic absorption gave a value of 17 (Fig. 1b). In a K $^{+}$ -permeable cell this is equivalent to

Fig. 1. Rb $^{+}$ uptake and K $^{+}$ concentration gradient in pig lymphocytes

The accumulation ratio for ^{86}Rb and the K $^{+}$ distribution (by atomic-absorption spectroscopy) were measured as described in the Experimental section. (a) $\circ$, ^{86}Rb uptake control; $\square$, with 0.5 mM-ouabain; $\triangle$, with 50 μM -valinomycin; (b) $\circ$, ^{86}Rb uptake control; $\triangle$, with 5 μM -valinomycin; $\blacktriangle$, K $^{+}$ distribution measured by atomic-absorption spectroscopy.

a K^+ diffusion potential (E_K) of 76 mV. This may or may not approximate to $\Delta\psi_p$ in the lymphocyte, depending on the relative permeabilities of other ions. Valinomycin at $50\mu M$ was required in these experiments to make the plasma membrane totally permeable to K^+ (Fig. 2). The value of 17 for the Rb^+ accumulation ratio in the presence of valinomycin gives a value of 136 mM for intracellular free $[K^+]$. This is confirmed by the direct measurements of K^+ distribution (Fig. 1b).

Fig. 1(a) shows that ouabain inhibits approx. 50% of the control Rb^+ influx into pig lymphocytes. This decrease in rate can be attributed to inhibition of a K^+/Rb^+ exchange associated with the $(Na^+ + K^+)ATPase$ (Glynn *et al.*, 1971). A similar effect on K^+ influx rate was seen with oligomycin present. Ouabain and oligomycin sensitivity of the rat thymocyte $(Na^+ + K^+)ATPase$ has previously been observed by Lichtman *et al.* (1972).

The control $^{86}Rb^+$ accumulation time course presented in Fig. 1(a) is biphasic. Our $^{86}Rb^+$ uptake studies on pig lymphocyte subpopulations prepared on continuous Percoll gradients have shown that the initial flux can be attributed to $^{86}Rb^+$ uptake into a minor population of small cells. Any possible effects of the large lymphocyte nucleus on total cell cation accumulation have been eliminated. Tracer studies on nuclei *in situ* demonstrated that there exists no significant potential or K^+ gradient across the nuclear membrane (S. M. Felber & M. D. Brand, unpublished work).

$^{36}Cl^-$ distribution

Cl^- distribution across the plasma membrane may

Fig. 2. Effect of valinomycin concentration on Rb^+ accumulation ratio

$^{86}Rb^+$ uptake was assayed at 45 min as described in the Experimental section.

reflect $\Delta\psi_p$ providing no Cl^- pumps exist. Fig. 3 shows that $^{36}Cl^-$ equilibrated across the lymphocyte plasma membrane in about 30 min. The $^{36}Cl^-$ accumulation ratio was 0.27, giving a value for intracellular free $[Cl^-]$ of 30 mM. This corresponds to an E_{Cl} of 35 mV, in close agreement with the value obtained by Iversen (1976) measured by colorimetric titration. To discover whether the Cl^- distribution actually reflected $\Delta\psi_p$, valinomycin was added to clamp $\Delta\psi_p$ at E_K (76 mV). Under these conditions the $^{36}Cl^-$ accumulation ratio rose to 0.37 (Fig. 3), not 0.06 as predicted by the Nernst equation, assuming $^{36}Cl^-$ to have equilibrated with $\Delta\psi_p$. This result is incompatible with Cl^- having solely a passive distribution across the lymphocyte plasma membrane and provides evidence for an inwardly directed Cl^- pump.

SITS is an inhibitor of the well-characterized Cl^-/HCO_3^- exchange in erythrocytes (Cabantchik *et al.*, 1978; Knauf, 1979) and in mouse ascites cells (Aull *et al.*, 1977). It was found to have no effect on control Cl^- steady-state distribution at concentrations ranging from 10 to $60\mu M$ (results not shown). Cl^- distribution in the lymphocyte is therefore kept from reaching equilibrium with $\Delta\psi_p$ by the presence of a SITS-insensitive pump and cannot be used to estimate $\Delta\psi_p$.

Measurement of $\Delta\psi_p$ using TPMP $^+$

Since neither Rb^+ nor Cl^- distribution may be used to estimate $\Delta\psi_p$, we have employed TPMP $^+$ for this purpose. TPMP $^+$ is a lipophilic cation that will passively distribute across both the plasma and mitochondrial membranes in accordance with $\Delta\psi$ (Deutsch *et al.*, 1979a; Hoek *et al.*, 1980; Scott & Nicholls, 1980; Davis *et al.*, 1981). TPMP $^+$ uptake is accelerated in the presence of tetraphenylboron

Fig. 3. Cl^- uptake into pig lymphocytes

The $^{36}Cl^-$ accumulation ratio was measured as described in the Experimental section. Valinomycin ($50\mu M$) was added immediately after the cells where indicated.

anion which catalyses entry of the cation by forming a neutral ion pair, the permeability of which is not hindered by the electric dipole potential across lipid bilayers (Stark, 1980). It should be possible to utilize TPMP^+ solely as a probe of $\Delta\psi_p$, providing $\Delta\psi_m$ is abolished completely.

Fig. 4 presents a time course of TPMP^+ accumulation into pig lymphocytes. Steady-state distribution of the cation was achieved within 30 min at an accumulation ratio normally exceeding 80. The mitochondrial component of the total TPMP^+ accumulation was removed by adding the uncoupler FCCP at concentrations greater than $2\text{ }\mu\text{M}$ to give a residual accumulation of 10-fold across the plasma membrane (Fig. 5a). Substitution of the TPMP^+ accumulation ratio into the Nernst equation then yields $\Delta\psi_p$.

$\Delta\psi_p$ can be measured by this technique within 2 min of incubation as shown in Fig. 4. TPMP^+ concentrations below 200 nM were found to have no deleterious effect on TPMP^+ uptake into the cytosol, measured at 45 min (Fig. 5b). Tetraphenylboron anion concentrations of between 3 and $6\text{ }\mu\text{M}$ were optimal for equilibration of TPMP^+ across the plasma membrane within 10 min (Fig. 5c).

Table 1 shows that other uncouplers reduced total TPMP^+ accumulation to give a residual component of similar magnitude to that measured in the presence of FCCP. Thus the effect of FCCP was correctly attributed to its uncoupling action. The combination of the mitochondrial respiratory chain inhibitor antimycin and bongkreik acid, an inhibitor of the adenine nucleotide translocase (Henderson & Lardy, 1970) did not reduce TPMP^+ accumulation to the same extent as FCCP. A similar effect was seen with 1 mM-KCN (not shown). This

Fig. 4. TPMP^+ uptake into pig lymphocytes. The $[\text{H}]\text{TPMP}^+$ accumulation ratio was measured as described in the Experimental section. FCCP ($10\text{ }\mu\text{M}$) was present where indicated.

Fig. 5. Effects of $[\text{FCCP}]$, $[\text{TPMP}^+]$ and $[\text{tetraphenylboron anion}]$ on TPMP^+ accumulation ratio. The $[\text{H}]\text{TPMP}^+$ accumulation ratio was measured as described in the Experimental section. (a) Uptake measured at 40 min with $[\text{FCCP}]$ varied; (b) uptake measured at 40 min with $5\text{ }\mu\text{M-FCCP}$ present and $[\text{TPMP}^+]$ varied; (c) uptake measured at 10 min, with $5\text{ }\mu\text{M-FCCP}$ present and $[\text{tetraphenylboron anion}]$ varied.

difference may be due to residual antimycin- and cyanide-insensitive respiration under these conditions. Alternatively it may be due to TPMP^+ uptake into the nucleus. However, this is unlikely since it is abolished by uncoupler.

Table 1 also shows that both ouabain and oligomycin slightly decreased TPMP^+ accumulation in the presence of FCCP within 4 min. No further decrease was observed at 16 min. This effect of ouabain and oligomycin can be interpreted as inhibition of the $(\text{Na}^+ + \text{K}^+)\text{ATPase}$. This has been shown to be electrogenic in the erythrocyte (Sen &

Table 1. *Effect of various reagents on TPMP⁺ accumulation ratio*

[³H]TPMP⁺ accumulation ratio was measured as described in the Experimental section at 30 min (at 4 min and 16 min for oligomycin and ouabain experiments). Reagents were: 5 μ M-FCCP; 4 μ g of oligomycin/10⁷ cells; 0.2 mM-ouabain; 0.4 μ M-antimycin; 4 μ M-bongkreikic acid (BKA); 10 μ M-carbonyl-cyanide *m*-chlorophenylhydrazine (CCCP); 10 μ M-tetrachlorotrifluorobenzimidazole (TTFB); and 5.0 μ l of ethanol/ml. This was the highest ethanol concentration added in any of the experiments in the present paper, and had little effect on TPMP⁺ uptake.

Addition	[³ H]TPMP ⁺ accumulation ratio
Control	83.2 $\pm$ 8.3 (<i>n</i> = 17)
+FCCP	10.1 $\pm$ 0.4 (<i>n</i> = 14)
+FCCP + oligomycin	8.38 $\pm$ 0.12 (<i>n</i> = 4)*
+FCCP + ouabain	8.04 $\pm$ 0.36 (<i>n</i> = 4)*
+antimycin + BKA	50.3
+CCCP	11.7
+TTFB	10.9
+Ethanol	75.0

* *P* (compared with '+FCCP') < 0.05.

Post, 1964) and in nerve and muscle (Thomas, 1972). Inhibition of the enzyme will immediately abolish only electrogenic effects; collapse of ion gradients and diffusion potentials will occur more slowly. The lymphocyte $\Delta\psi_p$ therefore has a small component of around 7 mV due to electrogenicity of the (Na⁺ + K⁺)ATPase ($\Delta\psi_{ATPase}$).

Binding of TPMP⁺

To quantify $\Delta\psi_p$ by following lipophilic ion distribution, any binding of TPMP⁺ to cytoplasmic components must be assessed. To do this we determined the TPMP⁺ accumulation ratio at several known values of $\Delta\psi_p$, which was clamped by adding valinomycin and varying the external K⁺ concentration. Fig. 6 presents the relationship between experimental and theoretical TPMP⁺ accumulation ratios. The graph has a slope of 1.0 and an intercept equivalent to a 1.8-fold accumulation ratio. Thus there is a small constant potential-independent binding of TPMP⁺ to lymphocytes, but no significant extra potential-dependent binding.

$\Delta\psi_p$ in the absence of valinomycin was then calculated from the experiment in Table 1, using the Nernst equation with a correction for the constant binding component:

$$\Delta\psi_p = 2.3 \frac{RT}{F} \log \left(\frac{[\text{TPMP}^+]_i^{\text{FCCP}}}{[\text{TPMP}^+]_e} - 1.8 \right)$$

where 2.3 *RT*/*F* has the value of 61.5 at 37°C.

Fig. 6. *Relationship between measured and theoretical TPMP⁺ accumulation ratio*

Medium (1.25 mM-CaCl₂, 0.8 mM-MgSO₄, 10 mM-Hepes, 1 mM-Na₂HPO₄, 5.5 mM-glucose, 3 μ M-tetra-phenylboron anion, 50 nM-[³H]TPMP⁺ (0.2 μ Ci/ml), 0.02 μ Ci of ⁸⁶RbCl/ml, and various amounts of NaCl and KCl to an Na⁺ + K⁺ concentration of 132 mM) was brought to pH 7.4 with NaOH and incubated at 37°C. Cells in RPMI medium were diluted 1:10 into this medium to give a final cell concentration of 3 $\times$ 10⁷ cells $\cdot$ ml⁻¹, and 50 μ M-valinomycin was added. The accumulation ratios were measured at 30 min as described in the Experimental section. The theoretical TPMP⁺ accumulation ratio was calculated for each extracellular K⁺ concentration from the Nernst equation assuming $\Delta\psi_p = E_K$. The open circle shows the result for data from similar experiments carried out in RPMI medium. The line shown was calculated by linear regression.

The value obtained for the $\Delta\psi_p$ of pig mesenteric lymphocytes was 56.5 $\pm$ 1.3 mV (mean $\pm$ S.E.M.; *n* = 14).

Effects of FCCP on $\Delta\psi_p$

It was important to show that the addition of FCCP to lymphocytes did not itself perturb $\Delta\psi_p$.

Measurement of lymphocyte ATP levels after FCCP treatment showed that the control level of 500 amol $\cdot$ cell⁻¹ dropped to and was maintained at 45% after 5 min. This decrease is not large enough to alter $\Delta\psi_p$ by affecting the plasma-membrane (Na⁺ + K⁺)ATPase since this has a *K_m* for ATP of around 100–200 μ M (Hexum *et al.*, 1970).

A more direct qualitative study of the effects of FCCP on $\Delta\psi_p$ is presented in Fig. 7. Here we have employed the fluorescent probe bis-oxonol to mon-

Fig. 7. Effect of FCCP on $\Delta\psi_p$ measured with bis-oxonol

Fluorescence was measured as described in the Experimental section. Additions, where shown, were: 150 nM-bis-oxonol; 2×10^5 pig lymphocytes/ml; 130 mM-KCl; 130 mM-NaCl; 10 μ M-FCCP. $\Delta\psi_p$, calculated as shown in the text, is 57 mV in the control, 63 mV after NaCl addition and 28 mV after KCl addition.

itor any changes in $\Delta\psi_p$. Fig. 7(a) shows a large depolarization of the cells on addition of KCl, as reported by Rink *et al.* (1980) and a small hyperpolarization on addition of NaCl. This provides a crude calibration of the dye signal and also suggests that both K^+ and Cl^- contribute to $\Delta\psi_p$ (see below). Fig. 7(b) shows that FCCP had no discernible effect on $\Delta\psi_p$ after the first few minutes. Our measurement of the lymphocyte $\Delta\psi_p$ with TPMP⁺ in the presence of FCCP is therefore valid.

Other cell preparations

Fig. 8 presents time courses of TPMP⁺ accumulation, in the presence of FCCP and oligomycin, into various lymphocyte preparations. The potential in the presence of oligomycin ($\Delta\psi_{ion}$) did not appear to differ markedly between these lymphocyte subpopulations and species.

Characterization of $\Delta\psi_p$

In the present work, studies on the distribution of radiolabelled ions across the plasma membrane have revealed that the lymphocyte $\Delta\psi_p$ is neither set by E_K nor reflected by E_{Cl} and that the contribution to $\Delta\psi_p$ by electrogenic ($Na^+ + K^+$)ATPase activity is significant. $\Delta\psi_p$ therefore has two components, one determined by the electrogenic nature of the ATPase ($\Delta\psi_{ATPase}$), the other potential ($\Delta\psi_{ion}$) described by the constant field equation (Goldman, 1943; Hodgkin & Katz, 1949):

$$\Delta\psi_p = \Delta\psi_{ATPase} + 2.3 \frac{RT}{F} \times \log \left(\frac{P_K[K^+]_i + P_{Cl}[Cl^-]_e + P_{Na}[Na^+]_i}{P_K[K^+]_e + P_{Cl}[Cl^-]_i + P_{Na}[Na^+]_e} \right)$$

where P_K , P_{Cl} and P_{Na} are constant field permeability coefficients and $2.3 RT/F$ has the value 61.5 at 37°C.

Fig. 8. TPMP⁺ accumulation by different lymphocyte preparations

[³H]TPMP⁺ accumulation was measured as described in the Experimental section, in the presence of 10 μ M-FCCP and 4 μ g of oligomycin/ 10^7 cells. ●, Pig mesenteric lymphocytes; ▲, T-enriched pig mesenteric lymphocytes; ■, mouse thymocytes. All TPMP⁺ accumulation ratios are corrected for binding.

Fig. 9. Effects of extracellular $[K^+]$ and $[Cl^-]$ on $\Delta\psi_{ion}$. Medium $[0.4\text{ mM-Ca}(\text{NO}_3)_2, 0.4\text{ mM-MgSO}_4, 11\text{ mM-glucose}, 1.0\text{ mM-Na}_2\text{HPO}_4, 10\text{ mM-Hepes}, 3\text{ }\mu\text{M-tetraphenylboron anion}, 50\text{ nM-}[^3\text{H}]TPMP^+ (0.1\text{ }\mu\text{Ci/ml}), 0.02\text{ }\mu\text{Ci of }^{86}\text{RbCl/ml}, 5\text{ }\mu\text{M-FCCP and }200\text{ }\mu\text{M-ouabain with Na}^+, K^+, Cl^- \text{ and gluconate added in various proportions as indicated below}]$ was brought to pH 7.4 with NaOH and incubated at 37°C . Cells in RPMI medium were diluted 1:5 into this medium to give a final concentration of 2.5×10^7 cells/ml $^{-1}$. The $[^3\text{H}]TPMP^+$ accumulation ratio was measured at 5 min as described in the Experimental section. (a) $[Cl^-] = 115\text{ mM}$, $[K^+]$ and $[Na^+]$ varied, $[K^+] + [Na^+] = 124\text{ mM}$; (b) $[K^+] = 8\text{ mM}$, $[Na^+] = 116\text{ mM}$, $[Cl^-]$ and $[gluconate^-]$ varied, $[Cl^-] + [gluconate^-] = 115\text{ mM}$. The broken lines show the theoretical curves obtained assuming the Goldman-Hodgkin-Katz equation applies, using different P_{Cl}/P_K ratios as indicated by each line. $\circ$, TPMP $^+$ accumulation ratio determined in RPMI medium ($8\text{ mM-K}^+, 113\text{ mM-Na}^+$ and 111 mM-Cl^-). All TPMP $^+$ accumulation ratios are corrected for binding as described in the text.

The relative contributions of K^+ , Cl^- and Na^+ to the lymphocyte $\Delta\psi_p$ were assessed by measuring $\Delta\psi_p$ in synthetic media of various ion concentrations by iso-osmotic substitution, in the presence of ouabain to abolish $\Delta\psi_{ATPase}$.

Alteration of the extracellular Na^+ concentration by iso-osmotic substitution with choline had no effect on $\Delta\psi_{ion}$ (results not shown), an expected result, since cells are generally very Na^+ -impermeable and the value of $\Delta\psi_p$ ($+56\text{ mV}$) is far from E_{Na} (-34 mV).

Fig. 9 shows that $\Delta\psi_{ion}$ was very dependent on extracellular $[K^+]$ and partially dependent on extracellular $[Cl^-]$. It also shows theoretical lines calculated from the constant field equation with P_{Na} set at zero, using different P_{Cl}/P_K ratios. The best fit between experimental and theoretical data occurred at a P_{Cl}/P_K ratio of around 1.0. Thus it is Cl^- fluxes that contribute to $\Delta\psi_p$ in the lymphocyte and account for the deviations from a simple K^+ -dominated potential.

Discussion

In the present paper we show that the $\Delta\psi_p$ maintained by 'resting' pig lymphocytes in normal culture medium is 56 mV . A small contribution of 7 mV is made to this potential by the ouabain- and oligomycin-sensitive ($Na^+ + K^+$)ATPase ($\Delta\psi_{ATPase}$). This observation is consistent with the existence of an electrogenic Na^+ pump in the lymphocyte plasma

membrane as found in nerve and muscle (Thomas, 1972) and the erythrocyte (Sen & Post, 1964). The ionic component ($\Delta\psi_{ion}$) of the lymphocyte $\Delta\psi_p$ is described accurately by the constant field equation (Goldman, 1943; Hodgkin & Katz, 1949). It is a K^+ -dominated ionic potential with a significant Cl^- contribution. The lymphocyte therefore differs from excitable cells where $\Delta\psi_p$ is K^+ -dominated (Hodgkin, 1951; Adrian, 1956) and from cells such as erythrocytes, where $\Delta\psi_p$ is close to E_{Cl} (Lassen & Sten-Knudsen, 1968).

Any technique of $\Delta\psi_p$ determination that assumes the potential to approximate to either E_K or E_{Cl} is inappropriate in the light of this work. One such technique is that of measurement of passive Cl^- distribution by colorimetric titration, employed by Iversen (1976). Similarly measurement of ^{86}Rb distribution will not give a valid quantitative estimate of $\Delta\psi_p$.

Another important restriction in the use of radiolabelled ions is that accumulation by intracellular organelles must be considered and either abolished (as in the present work) or quantitatively assessed and corrected for. Deutsch *et al.* (1979b) measured total TPMP $^+$ accumulation into human peripheral lymphocytes from which they calculated a value of 52 mV for $\Delta\psi_p$. These workers chose to use a TPMP $^+$ concentration of $10\text{ }\mu\text{M}$. We find that this concentration is sufficient to cause substantial, but not complete, collapse of $\Delta\psi_m$ by excessive cation accumulation (S. M. Felber & M. D. Brand, unpublished work). Thus in the experiments of

Deutsch *et al.* (1979b) TPMP⁺ accumulation reflects $\Delta\psi_p$ plus an undetermined residual contribution from $\Delta\psi_m$. The validity of their measurement of $\Delta\psi_p$ is therefore highly questionable.

Kiefer *et al.* (1980) have estimated the $\Delta\psi_p$ of mouse spleen lymphocytes to be 65 mV using the lipophilic cation TPP⁺. They calculate $\Delta\psi_p$ after subtracting the TPP⁺ accumulation ratio measured in a high K⁺ medium from that measured in a low K⁺ medium. This correction contains two assumptions, both of which are inappropriate. First, they assume that $\Delta\psi_p$ is zero in high K⁺ medium. This is not true for a $\Delta\psi_p$ with a significant Cl⁻ component and will therefore lead to inaccuracy in the measured value of $\Delta\psi_p$. Second, and more important, they assume that the amount of TPP⁺ taken up into the mitochondria is constant even when $\Delta\psi_p$ is depolarized. This is clearly wrong, since depolarization of $\Delta\psi_p$ causes cytosolic [TPP⁺] and therefore mitochondrial [TPP⁺] to drop by an order of magnitude even if $\Delta\psi_m$ remains unchanged. Thus the correction applied by Kiefer *et al.* (1980) is not appropriate; not only will it give an erroneous value of $\Delta\psi_p$ but it is also not clear whether the changes in TPP⁺ accumulation that were seen during mitogenic stimulation were caused by changes in $\Delta\psi_m$, $\Delta\psi_p$ or both.

Techniques other than those employing radio-labelled ions have also been used to estimate the lymphocyte $\Delta\psi_p$. The low values of 12 mV obtained by microelectrode measurements (Taki, 1970) are likely to be gross underestimates, in the light of our work, probably due to damage of the cells during electrode insertion. Rink *et al.* (1980) have measured the $\Delta\psi_p$ of mouse spleen lymphocytes to be 60 mV, using the fluorescent dye 3,3'-dipropylthiadicarbocyanine, at the valinomycin null point, which occurred at an extracellular K⁺ concentration of 12–15 mM. Under their conditions we would predict the pig lymphocyte $\Delta\psi_p$ to be around 62 mV. This is good agreement, despite the species difference, although mouse thymocytes do have the same $\Delta\psi_p$ as pig lymphocytes (Fig. 8). However, another group of workers, using fluorescent dyes on rat thymocytes, reported the valinomycin null point to occur at 120 mM-K⁺, which is indicative of a much lower $\Delta\psi_p$ (Bramhall *et al.*, 1976).

Clearly most of the methods that have previously been applied to the quantitative determination of $\Delta\psi_p$ in lymphocytes are of doubtful validity. The TPMP⁺ distribution method we describe overcomes many of the difficulties faced by other workers and allows accurate assessment of $\Delta\psi_p$.

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