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CHARACTERIZATION OF THE PLASMA AND MITOCHONDRIAL MEMBRANE POTENTIALS OF ALVEOLAR TYPE II CELLS BY THE USE OF IONIC PROBES

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The lipophilic cation triphenylmethylphosphonium ($TPMP^+$) and the potassium analog Rb^+, were used to monitor the membrane potential ($\Delta\Psi$) of freshly isolated rabbit type II alveolar epithelial cells. Type II cells were found to accumulate $TPMP^+$ rapidly at 37°C in Hanks' balanced-salt solution with 5 μM tetraphenyl boron, but this accumulation was partially due to non-membrane potential dependent binding of $TPMP^+$ to the cell. Lysophosphatidylcholine (lysoPC) was found to abolish $\Delta\Psi$ and permitted correction for bound $TPMP^+$ or Rb^+. $TPMP^+$ remaining in the cell following correction for binding represents the sum of mitochondrial and plasma membrane potential dependent accumulation. The accumulation of Rb^+ by the type II cell was found to be independent of the mitochondrial membrane potential and indicated a trans-plasma membrane Rb^+ distribution potential of -62.9 ± 4 mV. A similar value was obtained by estimating the plasma membrane potential dependent accumulation of $TPMP^+$ in type II cells whose mitochondria were depolarized with carbonylcyanide *m*-chlorophenylhydrazone (CCCP). The release of $TPMP^+$ due to CCCP treatment also permitted an estimation for the trans-mitochondrial membrane potential of -141.8 ± 10 mV. These techniques of membrane potential measurements were found to be sensitive to changes in $\Delta\Psi$ induced by a number of inhibitors and ionophores. The ability to measure the membrane potential of the type II pneumocyte, and the changes caused by various agents, should be useful in characterizing the functional responses of this pulmonary surfactant producing cell.

Introduction

The alveolar type II cell is responsible for the synthesis and secretion of surfactant in the lung [1,2]. This function is of critical importance to air breathing animals due to the role of surfactant in lowering alveolar surface tension and permitting alveolar expansion and stability [3–5]. In the past decade, techniques have been developed which allow the isolation of a relatively pure population of type II cells from among the approximately 40 different cell types in the lung [6–9]. This ability has led to new investigations into biochemical processes involved in regulation of type II cell functions, particularly those related to pulmonary surfactant metabolism [10]. However, little information is presently available concerning the electrophysiology of this epithelial cell.

Recent evidence has indicated that the trans-membrane electrical potential ($\Delta\Psi$) is intimately involved in the response of various cell types to

Abbreviations: CCCP, carbonyl cyanide *m*-chlorophenylhydrazone; TPB^-, tetraphenylboron anion; $TPMP^+$, triphenylmethylphosphonium ion; $\Delta\Psi_m$, trans-mitochondrial membrane potential; $\Delta\Psi_p$, trans-plasma membrane potential.

stimulatory agents such as insulin [11,12] and adrenergic agents [13,14]. These studies have demonstrated a specific interaction between cellular activation and changes in the distribution of ions responsible for the generation of $\Delta\Psi$ [15]. In this investigation we attempted to evaluate quantitatively the steady-state membrane potential of the isolated type II cell in order to better understand how stimulatory agents and lung injury might directly affect type II cell function.

The measurement of a membrane potential in isolated cells has been approached by a variety of methods. Direct measurements by microelectrode impalement of the plasma membrane are highly quantitative [16] yet extremely difficult in small cells (smaller than 12 μm) such as type II cells. Indirect methods such as fluorescent cyanine dyes have been found useful for qualitative evaluation of rapid fluctuations in $\Delta\Psi$ relative to a resting value [17–19], but the accuracy of dye methods is questionable in terms of quantitative evaluation of the steady-state membrane potential.

In the present study we used a method for measurement of $\Delta\Psi$ based on the intracellular accumulation of radiolabeled permeable ions. Specifically, measurements are made of the accumulation of the monovalent cations triphenylmethylphosphonium ([^{3}H]TPMP$^+$) and $^{86}Rb^+$ in isolated type II cells. TPMP$^+$ is a permeant lipophilic cation which has been used for the measurement of $\Delta\Psi$ in a number of cellular systems [20–23]. The potassium analog, $^{86}Rb^+$ is used in conjunction with TPMP$^+$ to distinguish between plasma membrane and mitochondrial membrane potential dependent accumulation of each ion [24]. These methods were applied to measure the membrane potentials across both the plasma and mitochondrial membrane of freshly isolated type II cells, and were used further to investigate the response of these membrane potentials to known depolarizing agents.

Methods and Materials

Isolation and purification of type II cells

Type II cells were isolated from the lungs of male New Zealand white rabbits (2–4 kg) and purified from the crude mixture of cells by centrifugation on a discontinuous Ficoll gradient as previously described [8]. For the present study, cell counts and viability were determined by hemocytometer counts and Trypan blue exclusion, respectively. Type II cells isolated by this procedure have previously been shown to exhibit metabolic activity consistent with the minimization of cell injury during the isolation process [8,25]. Purity of the isolated cells was determined by a modified Papanicolaou staining procedure on air dried smears. Type II cells were also routinely characterized by both light and electron microscopic observation.

Measurement of type II cell volume

Total type II cell volume was measured using electronic cell sizing based on Coulter counter determinations of freshly isolated cells equilibrated for 15 min at 37°C in Hanks' balanced-salt solution. The fraction of the total cell volume representative of the exchangeable water compartment was evaluated based on established techniques for the determination of intracellular and extracellular space [26]. In brief, type II cells were equilibrated in Hanks' salt solution for 15 min at 37°C, and were then suspended at a concentration of $1.5 \cdot 10^6$ cells/ml in Hanks' salt solution containing either [^{3}H]H_2O (1 μCi/ml) or [^{14}C]sucrose (0.5 μCi/ml). After thorough mixing and incubation at 37°C with [^{3}H]H_2O for 15 min or [^{14}C]sucrose for 3 min, 0.5 ml of the cell suspension was layered upon 0.3 ml of an oil cushion (6 parts dibutylphthalate to 1 part mineral oil, v/v, $d = 1.02$) in a 1.5 ml plastic microfuge tube. The tubes were then spun for 90 s in a Beckman Model B microfuge. An aliquot of the supernatant was saved to determine radioactivity and the rest discarded to isolate the cell pellet. The radioactivity associated with the cell pellet was analyzed by cutting off the bottom of the tube below the oil meniscus. The tube tip and pellet were then added to 1 ml of 0.1 M NaOH in a glass scintillation vial. Following overnight incubation at room temperature to allow complete cell lysis, the total radioactivity was measured by liquid scintillation spectrometry in a Beckman LS700-liquid scintillator system. The total volume of the pellet was determined from the [^{3}H]H_2O content, and the extracellular space from the [^{14}C]sucrose content.

The intracellular space was taken as the difference between total and extracellular volumes.

Uptake of [^{3}H]TPMP$^+$ and ^{86}Rb$^+$

Under conditions identical to those in which cell volume determinations were made, uptake of $^{86}Rb^+$ was initiated by the addition of a known volume of 5 mM RbCl in water with an $^{86}Rb^+$ specific activity of (0.6 μCi/ml). [^{3}H]TPMP$^+$ uptake was initiated by first equilibrating cells with 1–5 μM sodium tetraphenylboron (TPB^-) in Hanks' salt solution at 37°C for 15 min. This pre-treatment with TPB^- was used to enhance the rate of cellular uptake of $TPMP^+$ to its steady-state value, as described in detail later. A known volume of a working solution of 1 mM triphenylmethylphosphonium bromide ($TPMP^+$) in H_2O with [^{3}H]TPMP$^+$ activity of (20.0 μCi/ml) was then added to the cell suspension. All uptake experiments for either Rb^+ or $TPMP^+$ were conducted in a 37°C water bath with periodic mixing. The amount of [^{3}H]TPMP$^+$ or $^{86}Rb^+$ in the type II cell pellet was determined by separation through an oil cushion as described above for the measurement of cell volume.

To further characterize the membrane potential of type II cells, a number of experiments were carried out involving exposures to various agents during the course of [^{3}H]TPMP$^+$ or $^{86}Rb^+$ uptake. The specific agents used, each known to affect cell membranes or membrane potential in other cell systems included: 5 μM CCCP, 1 mM ouabain, 2 μM valinomycin, 100 μM 2,4-dinitrophenol, 5 μg/ml oligomycin, 2 mM KCN, and 100 μM A23187. All stock solutions of these agents were prepared in absolute ethanol (except KCN which was in H_2O) and mixed with the cell suspensions to give a final ethanol concentration of not more than 1%. Control measurements of $TPMP^+$ or Rb^+ uptake were done using approx. 1% ethanol.

Cell permeabilization

For accurate measurement of membrane potential, it was necessary to separate the membrane potential dependent accumulation of $TPMP^+$ or Rb^+ from the uptake which was due to binding of these radiolabeled ions to cellular constituents. Binding was determined by permeabilizing the cell to abolish the electropotential gradient across the cell membrane. Nystatin has been used in this capacity by others [27] but we found relatively little effect of nystatin on the membrane potential of isolated type II cells. Miller et al. [28] and Castellot [29] have described a method for reversibly permeabilizing cells by the use of lysophosphatidylcholine. This method was newly adapted here in order to obtain a background value for $TPMP^+$ or Rb^+ accumulation by type II cells. This accumulation represented the binding of $TPMP^+$ or Rb^+ in the absence of a trans-membrane electrical potential. Following incubation with [^{3}H]TPMP$^+$ or $^{86}Rb^+$ in Hanks' salt solution at 37°C, aliquots of the type II suspension were quickly removed and exposed to 10–12 μg/ml lysophosphatidylcholine at 37°C for 3–5 min. The cell suspension was then transferred to microfuge tubes, spun through the oil cushion, and radioactivity in the cell pellet determined as described earlier. Triplicate samples of lysophosphatidylcholine treated type II cells were taken at each point at which total [^{3}H]TPMP$^+$ or $^{86}Rb^+$ accumulation was determined. The total DNA in cell pellets was analyzed by the method of Labarca and Paigen [30] and total protein was determined by the method of Lowry et al. [31].

Calculation of membrane potentials

The transmembrane electrical potential ($\Delta\Psi$) can, in principle, be calculated based on the distribution of any freely permeable cationic probe across the cell membrane. The steady-state value for $\Delta\Psi$ is given by the Nernst relation in the form:

$$\Delta\Psi = \frac{RT}{F} \ln \frac{[C^+]}{[C^+]_{in}^{out}}$$

where $[C^+]_{out}$ is the concentration of cation in the solution outside of the membrane and $[C^+]_{in}$ is the concentration of the cation in the compartment contained by the membrane. The quotient of these terms (the distribution ratio) represents the ability of a negative $\Delta\Psi$ to accumulate the positively charged probe. Since a membrane potential exists across both the plasma membrane and the mitochondrial membrane in the type II cell, it was necessary to distinguish the value for $[C^+]_{in}$ within the mitochondrial compartment from that within

the cytoplasmic compartment. In this report we present an approach for determining the distribution ratios for [^{3}H]TPMP$^+$ and ^{86}Rb$^+$ across both the mitochondrial and plasma membranes. An analysis of the distribution ratios established by both of these ionic probes was then applied towards the estimation of the mitochondrial and plasma membrane potential of the type II cell.

Materials

[^{3}H]Triphenylmethylphosphonium bromide, [^{86}Rb]rubidium chloride, tritiated water, and [^{14}C]sucrose were purchased from New England Nuclear, Boston, MA. Rubidium chloride, 99.9% pure salt, was obtained from ICN Pharmaceuticals, Inc., Plainview, NY. CCCP was purchased from US Biochemical Corp., Cleveland, Ohio. All other inhibitors and ionophores as well as sodium tetraphenylboron, triphenylmethylphosphonium bromide, and Trypsin and DNAase for type II cell isolations were obtained from Sigma Chemical Co., St. Louis, MO. The elastase used for the isolation of type II cells was purchased from Worthington Diagnostic Systems, Freehold, NY. Media used in the preparation of type II cells were purchased from GIBCO, Grand Island, NY.

Results and Discussion

Accumulation of TPMP$^+$ by type II cells

The use of a lipophilic cationic probe such as TPMP$^+$ to calculate the membrane potential of a cell requires the probe to be freely permeable to the cell membrane. The results shown in Fig. 1 illustrate the rate of uptake of TPMP$^+$ by a freshly isolated type II cell suspension. Although the cells are permeable to TPMP$^+$, the rate of intracellular accumulation is very slow. Following 120 min of incubation no apparent steady-state accumulation value was reached. This slow accumulation rate of TPMP$^+$ has been reported previously for other cell types, and has been corrected for by the addition of a lipophilic anion tetraphenylboron (TPB$^-$) [20–22]. Fig. 1 shows that the addition of various concentrations of TPB$^-$ to the incubation medium influenced both the rate and total amount of TPMP$^+$ accumulated by the type II cell. In the

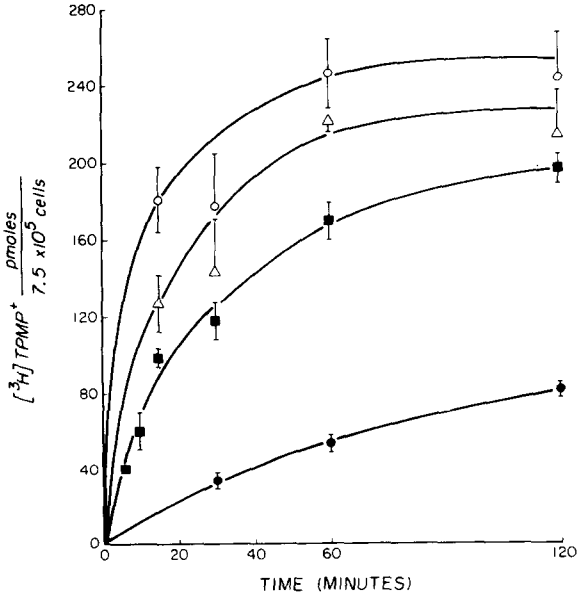

Fig. 1. Effect of TPB$^-$ on total uptake of TPMP$^+$ by type II cells. Type II cells were incuabted at 37°C with 7 μM [^{3}H]TPMP$^+$ and with TPB$^-$ concentrations of 0 (●), 1 μM (■), 2 μM (△), and 5 μM (○). Each point represents the mean ± S.E. of total TPMP$^+$ uptake in the cell pellets from triplicate samples taken from three to five separate cell preparations.

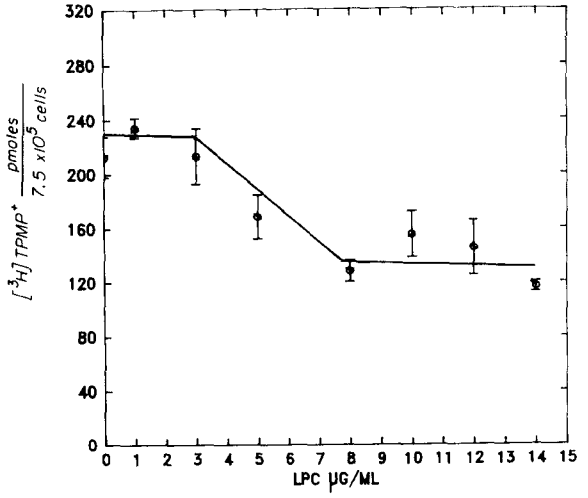

Fig. 2. TPMP$^+$ binding by lysophosphatidylcholine (LPC)-treated type II cells. Type II cells were incubated for 30 min at 37°C with 7 μM TPMP$^+$ and 5 μM TPB$^-$ prior to a 5 min treatment with lysophosphatidylcholine. Total accumulation in the pellet was determined immediately following lysophosphatidylcholine treatment. Each point represents the mean ± S.E. of triplicate samples taken from three to five separate cell preparations.

presence of 5 μM TPB^-, type II cells were found to accumulate $TPMP^+$ rapidly within the first 15 min of incubation. However, the apparent steady-state accumulation of $TPMP^+$ expressed by the plateau phase of the curve was not constant, and varied with the concentration of TPB^- in the incubation medium. These results indicated that TPB^- either increased total $TPMP^+$ uptake through a hyperpolarizing effect on $\Delta\Psi_p$, or increased the total $TPMP^+$ accumulated by non-membrane potential dependent binding of $TPMP^+$ to the cell. To distinguish between these two effects, it is necessary to obtain a control value for $TPMP^+$ accumulation at a $\Delta\Psi_p$ of 0 mV [27].

Fig. 2 illustrates how permeabilization by lysophosphatidylcholine affected the final steady-state value for $TPMP^+$ accumulation by a type II cell suspension. In the low concentration range of lysophosphatidylcholine (from 0.1 to 3 μg/ml) no appreciable loss of $TPMP^+$ was seen from the cell pellet. At higher concentrations of lysophosphatidylcholine, there was a significant loss of $TPMP^+$ associated with the type II cell pellet until a new constant value for $TPMP^+$ accumulation was reached at 8–15 μg/ml lysophosphatidylcholine. This decrease in $TPMP^+$ accumulation corresponded to a large increase in the uptake of Trypan blue from less than 5% to greater than 95%. Furthermore, the additional treatment of lysophosphatidylcholine treated cells with mitochondrial uncouplers such as CCCP or DNP, or treatment of type II cells with valinomycin in a high K^+ containing medium, did not result in any further loss of $TPMP^+$ from the type II cell pellet. The absence of any further effect from these depolarizing agents indicates a maximal depolarization by treatment with lysophosphatidylcholine alone. These results are therefore consistent with a depolarization of the type II cell membrane potential toward 0 mV in the presence of 8–15 μg/ml lysophosphatidylcholine. The DNA and protein content of the cell pellet remained constant throughout a lysophosphatidylcholine concentration range of 0.1–15 μg/ml. Microscopic and electronic cell sizing observations of these cells indicated that the cells were intact up to a concentration of 15 μg/ml lysophosphatidylcholine, but began to burst at concentrations greater than this. Therefore, at a lysophosphatidylcholine concentration of 8–15 μg/ml, the $[^3H]TPMP^+$ in the cell pellet represented uptake (binding) by intact, depolarized cells, and was suitable for comparison to untreated cells.

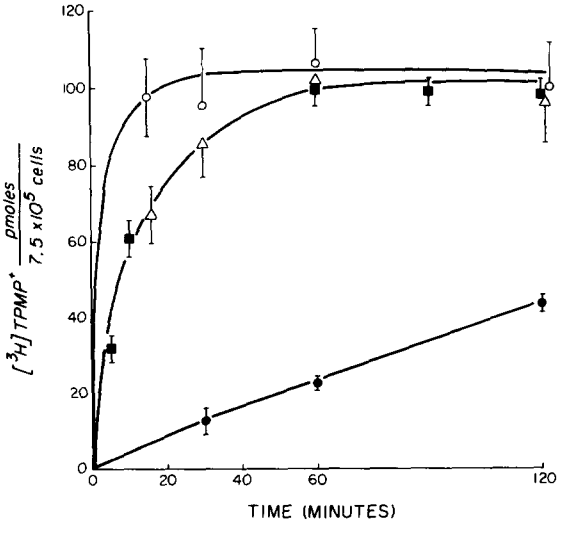

Fig. 3. Effect of TPB^- on $TPMP^+$ uptake by type II cells after correction for $TPMP^+$ binding. Type II cells were incubated at 37°C with 7 μM $[^3H]TPMP^+$ and with TPB^- concentrations of 0 (●), 1 μM (■), 2 μM (△), and 5 μM (○). Following incubation with $TPMP^+ \pm TPB^-$, for the indicated time periods, an aliquot of the cell suspension was removed and treated with 12 μg/ml lysophosphatidylcholine for 5 min. Each point represents the difference in the means ± S.E. between control and lysophosphatidylcholine-treated cell pellets from triplicate samples of three to six separate cell preparations.

As a result of the data in Fig. 2, the amount of $[^3H]TPMP^+$ accumulated by lysophosphatidylcholine-treated cells (12 μg/ml) was used to define the amount of non-membrane potential dependent binding of $TPMP^+$ to type II cells. This non-membrane potential dependent binding of $TPMP^+$ was found not to be a constant value but to vary according to the time of incubation with $TPMP^+$ and the concentration of TPB^- in the incubation medium. When $TPMP^+$ binding was subtracted from the corresponding values for total accumulation in Fig. 1, the results in Fig. 3 were generated. These results show that initially TPB^- affected the membrane potential dependent accumulation of $TPMP^+$ by increasing the initial rate of the $TPMP^+$ uptake phase of the accumulation curve. In contrast to the rate data in Fig. 1, the plateau phase shown in Fig. 3 remains con-

stant for at least 60 min and thus gives a meaningful steady-state value for $TPMP^+$ accumulation by type II cells in Fig. 3. The steady-state accumulation value of 95 pmol $TPMP^+$ per $7.5 \cdot 10^5$ cells was found to be independent of TPB^- concentration from 1 to 5 μM in the incubation medium. These results demonstrated that TPB^- assisted a rapid distribution of $TPMP^+$ across the type II cell membrane, but did not affect the final amount of accumulation which was due specifically to the membrane potential. Consequently, TPB^- did not affect the final calculation of $\Delta\Psi$ based on this final amount of $TPMP^+$ accumulation, and its use with $TPMP^+$ was a permissible combination for the measurement of type II cell membrane potential.

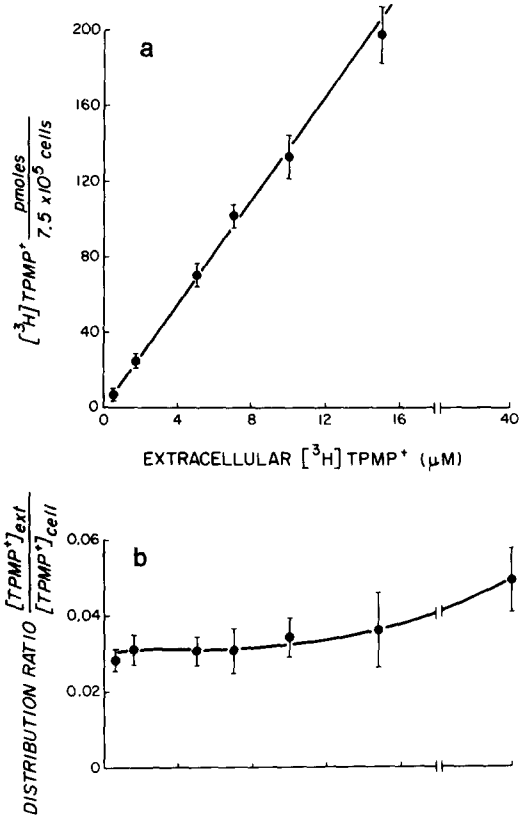

Fig. 4. Effect of extracellular $TPMP^+$ on type II cells. The accumulation of $TPMP^+$ was measured following a 20 min incubation at 37°C with various concentrations of $TPMP^+$ and 5 μM TPB^-. (a) [^{3}H]$TPMP^+$ accumulation after correction for non-membrane potential dependent $TPMP^+$ binding is expressed as a function of the extracellular concentration of [^{3}H]$TPMP^+$ in the incubation media. (b) The concentration of $TPMP^+$ in the extracellular solution ($[TPMP^+]_{ext}$) was divided by the concentration calculated for $TPMP^+$ in the type II cell ($[TPMP^+]_{cell}$) and expressed as a distribution ratio. $[TPMP^+]_{cell}$ was calculated based on the accumulation of $TPMP^+$ seen in (a) and a type II cell intracellular volume of 0.442 μl per $7.5 \cdot 10^5$ cells. The distribution ratio is expressed as a function of the extracellular concentration of [^{3}H]$TPMP^+$ with each point representing the mean ± S.E. from triplicate samples of three separate cell preparations.

Fig. 4a shows that the steady-state value for $TPMP^+$ accumulation by a type II cell suspension varied linearly with the concentration of $TPMP^+$ in the incubation medium in the range of 1 to 16 μM. This linear relationship is equivalent to a constant distribution ratio for the concentration of $TPMP^+$ in the extracellular and intracellular compartments (Fig. 4b). This behavior is consistent with the hypothesis that $TPMP^+$ uptake (corrected for binding) is a passive process and, within the concentration range used here, not saturable (i.e., the membrane potential is unaffected by $TPMP^+$). At external $TPMP^+$ concentrations higher than 16 μM there was an increase in the distribution ratio, presumably due to a direct effect of high $TPMP^+$ concentrations on $\Delta\Psi$. As a result of the data in Figs. 3 and 4, the conditions for $TPMP^+$ accumulation in subsequent experiments were standardized to a 30 min incubation with 5 μM TPB^- and 7 μM external $TPMP^+$.

Accumulation of Rb^+ by type II cells

To calculate a plasma membrane potential based on the steady-state distribution ratio of a cationic probe such as $TPMP^+$, it is necessary to distinguish how much of the total steady-state accumulation is due to the trans-plasma membrane potential ($\Delta\Psi_p$) as opposed to the trans-mitochondrial membrane potential ($\Delta\Psi_m$). High values have been reported for transmitochondrial membrane potentials in other cell types (130–165 mV) [22,24,32] and we expected a significant fraction of the total cell $TPMP^+$ to be contained in the type II cell mitochondria since $TPMP^+$ has been shown to be freely permeable to the inner mitochondrial membrane [11,20]. To evaluate the mitochondrial accumulation of $TPMP^+$, a second radiolabeled probe ($^{86}Rb^+$), was used. Unlike the lipophilic ion $TPMP^+$, the Rb^+ is selectively permeable and will not passively enter the inner mitochondrial membrane [24]. Therefore, its ac-

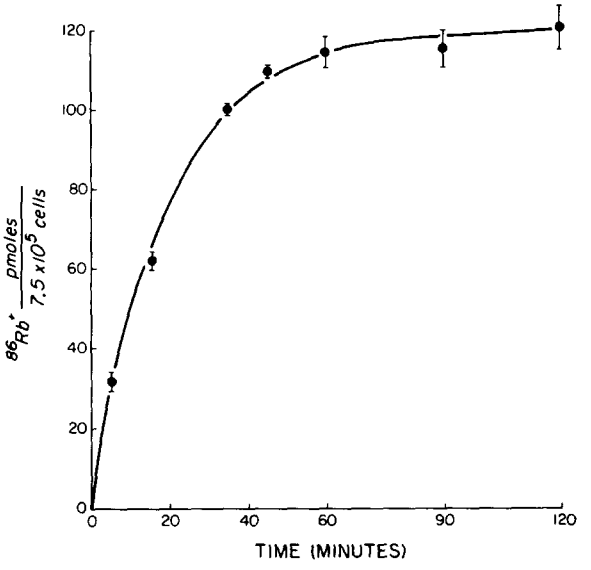

Fig. 5. $^{86}Rb^{+}$ accumulation by type II cells. The accumulation of 25 μM $^{86}Rb^{+}$ by type II cells at 37°C was measured and expressed as a function of time of incubation with Rb^{+}. Each point represents the mean ± S.E. for the difference between total Rb^{+} accumulation and Rb^{+} accumulated following 12 μg/ml lysophosphatidylcholine treatment of triplicate samples from three or four separate determinations.

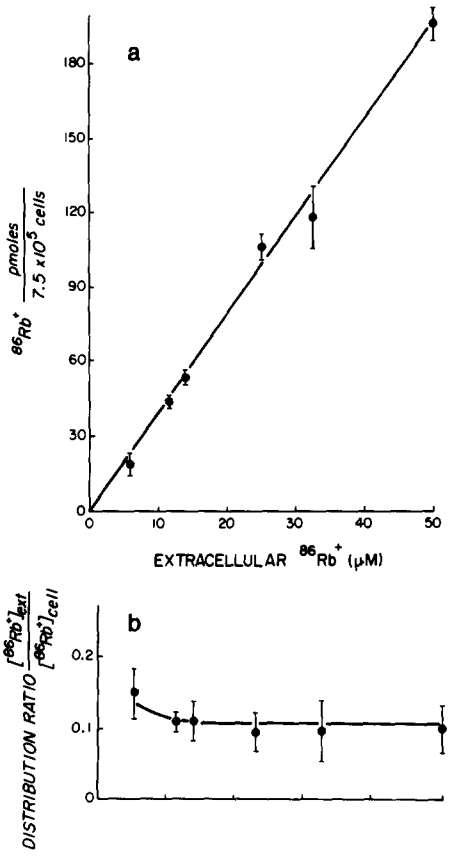

Fig. 6. Effect of extracellular $^{86}Rb^{+}$ on type II cells. The accumulation of $^{86}Rb^{+}$ by type II cells was measured following a 60 min incubation with extracellular $^{86}Rb^{+}$ at 37°C. (a) Accumulation of $^{86}Rb^{+}$ after correction for binding is expressed as a function of extracellular $^{86}Rb^{+}$. (b) The distribution ratio for $^{86}Rb^{+}$ was calculated based on the accumulation of $^{86}Rb^{+}$ seen in (a) and a type II cell intracellular volume of 0.442 μl per $7.5 \cdot 10^{5}$ cells. Each point represents the mean ± S.E. for triplicate samples of two or three separate cell preparations.

cumulation should be independent of the mitochondrial membrane potential. As an analogue of K^{+}, Rb^{+} has been used in other cell systems [24,20] to give an estimate of the K^{+} diffusion potential across the plasma membrane.

The accumulation of $^{86}Rb^{+}$ by freshly isolated type II cells is depicted in Fig. 5. A steady-state accumulation of $^{86}Rb^{+}$ occurred after 60 min of incubation with a $^{86}Rb^{+}$ extracellular concentration of 25 μM. Fig. 6a demonstrates a linear relationship between the extracellular concentration of $^{86}Rb^{+}$ and intracellular $^{86}Rb^{+}$ as measured at steady state in the type II cell. As described previously with [^{3}H]TPMP^{+} accumulation, the distribution ratio of extracellular $^{86}Rb^{+}$ to intracellular $^{86}Rb^{+}$ (Fig. 6b) is a constant over the range of 10 to 50 μM extracellular $^{86}Rb^{+}$. Thus, Rb^{+}, as well as $TPMP^{+}$, can be used at low concentrations which will not directly affect the measured value for the membrane potential of the type II cell.

Determination of the plasma membrane potential

The steady state distribution ratios depicted in Figs. 4 and 6 are based on identical incubation conditions in which the concentration of intracellular probe was calculated from the volume of intracellular space and the membrane potential specific accumulation at steady state. Based on [^{3}H]H_2O and [^{14}C]sucrose measurements (see Methods and Materials), the mean value found for type II cell intracellular space was 0.422 μl per $7.5 \cdot 10^{5}$ cells. The mean total volume of the type II cell based on electronic cell sizing was 0.542 μl per $7.5 \cdot 10^{5}$ cells. These volume measurements were found not to be affected significantly by the concentrations of $TPMP^{+}$, Rb^{+}, or TPB^{-} used in the present study.

To test the hypothesis that Rb^+ accumulation reflects the trans-plasma membrane potential while $TPMP^+$ accumulation reflects both the plasma and mitochondrial membrane potentials, type II cells were treated with the mitochondrial uncoupler CCCP for 5 min following the steady-state accumulation of $TPMP^+$ or Rb^+. This CCCP treatment dissipates the mitochondrial electropotential gradient [32,33]. Therefore, following treatment with CCCP, the mitochondrial contribution to the total accumulation of either $^{86}Rb^+$ or $TPMP^+$ will be eliminated.

Experiments conducted on type II cells which had accumulated $^{86}Rb^+$ to steady state showed no significant change in the total accumulation of $^{86}Rb^+$ after mitochondrial depolarization with CCCP. At an extracellular concentration of 25 μM $^{86}Rb^+$, the total $^{86}Rb^+$ accumulated by type II cells after correction for non-membrane potential binding was 111 ± 9 pmol per $7.5 \cdot 10^5$ cells. The lack of any change in the accumulation of $^{86}Rb^+$ following CCCP treatment confirms the inability of $^{86}Rb^+$ to be accumulated by the trans-mitochondrial potential gradient. If the total $^{86}Rb^+$ accumulation is then assumed to be due to the trans-plasma membrane potential alone, the Nernst equation (see Methods and Materials) predicts a Rb^+ distribution potential of -62.9 ± 4 mV.

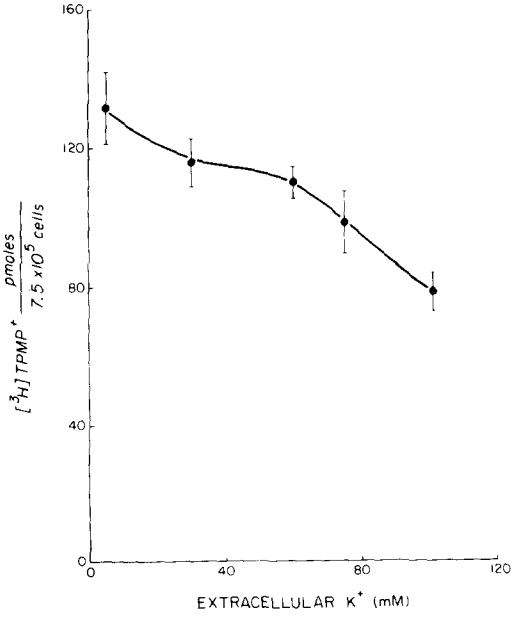

Fig. 7. Effect of extracellular $[K^+]$ on the steady state accumulation of $[^3H]TPMP^+$. Type II cells equilibrated at 37°C in a modified Hanks' salt solution containing 40 mM NaCl, and choline$^+$ plus K^+ equal to 140 mM. Cells were incubated with 5 μM TPB^- and 7 μM $TPMP^+$ for 20 min. Each point represents the mean ± S.E. of the total $[^3H]TPMP^+$ contained in the cell pellet of triplicate samples from two to four separate cell preparations.

In contrast to the accumulation of $^{86}Rb^+$, the accumulation of $TPMP^+$ by type II cells was extremely sensitive to treatment with CCCP. Under control conditions of 7 μM extracellular $[^3H]$ $TPMP^+$, type II cells accumulated $TPMP^+$ to a steady-state value of 100 ± 7 pmol per $7.5 \cdot 10^5$ cells (after correction for non-membrane potential dependent binding). Immediately following treatment with CCCP, $TPMP^+$ accumulation decreased to 35 ± 9 pmol per $7.5 \cdot 10^5$ cells. Thus, it would appear that $TPMP^+$ was substantially accumulated by the mitochondria of the type II cell, and that CCCP resulted in release of this fraction of $TPMP^+$ accumulated by the trans-mitochondrial membrane potential. The $TPMP^+$ remaining after CCCP treatment can be substituted into the Nernst equation to give a value of -63 ± 9 mV. This value agrees closely with the value obtained based on the Rb^+ distribution potential, and thus indicates the $TPMP^+$ remaining after CCCP treatment can be attributed purely to the plasma membrane potential.

Estimation of the mitochondrial membrane potential

The mitochondrial membrane potential is defined by the Nernst equation with the distribution ratio based on the cytosolic concentration of the probe for $[C^+]_{out}$ and the intramitochondrial concentration for $[C^+]_{in}$. The major problem with the use of the equation in this form concerns the necessity for an accurate measure of the volume of the mitochondrial compartment. The results of the previous section demonstrated a 65% loss of $TPMP^+$ by type II cells following CCCP treatment. Since CCCP did not result in any loss of $^{86}Rb^+$, it is reasonable to assume that $CCCP^+$ did not affect the plasma membrane potential. Therefore 65% (65 pmol), of the $TPMP^+$ accumulation can be attributed to the mitochondrial compartment. However, to determine a distribution ratio of $TPMP^+$ across the mitochondrial membrane, it is necessary to know the concentration of $TPMP^+$

in mitochondria, and this requires knowledge of mitochondrial volume.

Morphologic evidence indicates that the intramitochondrial volume is a small fraction of cytoplasmic volume in type II cells [8]. Consequently, the cytoplasmic volume can be approximated as the total measured intracellular volume. If the mitochondrial volume (V_m) is assumed as some percent (e.g., 1%) of the total cytoplasmic volume, the concentration of $TPMP^+$ in the mitochondria ($[C^+]_{in}$) can be estimated. The mitochondrial membrane potential, $\Delta\Psi_m$, can then be calculated from the Nernst equation based on a value for $[C^+]_{out}$ which is equal to the cytoplasmic concentration of $TPMP^+$. By this method $\Delta\Psi_m$ is estimated to be -141.8 ± 10 mV if V_m is 1% of intracellular volume. This value for the mitochondrial membrane potential in type II cells is similar to those calculated for the mitochondrial membrane potential in a number of different cell systems [23,24,32]. Unfortunately, this method of determining the mitochondrial membrane potential is strongly dependent on the estimate of mitochondrial volume. A 2-fold error in the value for V_m results in an 18 mV change in the magnitude of $\Delta\Psi_m$ based on the $TPMP^+$ accumulations found here. However, even though the absolute magnitude of $\Delta\Psi_m$ is approximate, accurate evaluation of the relative effect of various agents on the mitochondrial membrane potential is possible since these do not require knowledge of V_m but only of relative changes in the accumulation of $TPMP^+$. Thus, although $TPMP^+$ presents difficulties in calculating an absolute value for either the plasma or mitochondrial membrane potential due to its accumulation in both compartments, it is very useful as a relative indicator of mitochondrial or plasma membrane responses. The results of a number of experiments involving plasma membrane and/or mitochondrial membrane potential changes in type II cells are now described.

Effect of extracellular K^+ on $TPMP^+$ accumulation

Type II cells were equilibrated for 15 min at 37°C in modified solutions of Hanks' medium which contained 40 mM Na^+ and various concentrations of K^+. The osmolarity of the solution was maintained at a constant value by substitution of K^+ for $choline^+$. Increasing the extracellular concentration of K^+ by this method has been reported by several investigators [15,22,26] to depolarize cells in which the plasma membrane potential is strongly dependent upon a K^+ diffusion potential. Fig. 7 illustrates the effect of K^+ on the total steady-state accumulation of $TPMP^+$ in type II cells and the results demonstrate the sensitivity of $TPMP^+$ to monitor changes in the plasma membrane potential. The remaining $TPMP^+$ shown in the cells at high extracellular K^+ is presumably due in part to non-membrane potential dependent binding and in part to the mitochondrial accumulation of $TPMP^+$ since $\Delta\Psi_m$ is not strongly dependent on the K^+ concentration gradient in the absence of a K^+ ionophore [24]. This result tends to further support the validity of using $TPMP^+$ accumulation to detect changes in the plasma membrane potential of the type II cell. It is not appropriate however, to make a direct comparison of type II cells in this system containing a low concentration of Na^+ (40 mM) to cells incubated in normal Hanks' salt solution since the contribution of Na^+ to the membrane potential of the type II cell is at present unknown.

Effects of ionophores and inhibitors on $TPMP^+$ accumulation

The effects of several inhibitors and ionophores on the steady-state accumulation of $TPMP^+$ by type II cells are summarized in Table I. Before exposure to the experimental agent, type II cells were first allowed to reach the normal steady-state value for $TPMP^+$ accumulation (incubation time 30 min). Following 5 and 30 min of exposure to each agent, aliquots of the cell suspension were removed and analyzed for $TPMP^+$ accumulation.

As shown in Table I, the $(Na^+ + K^+)$-ATPase inhibitor ouabain resulted in a 20% decrease of $TPMP^+$ uptake in the type II cell by 5 min and almost a 40% decrease at 30 min. The early depolarization due to ouabain treatment indicates the possible presence of a electrogenic component to the plasma membrane potential in type II cells which is abolished by inhibition of the Na^+,K^+ pump activity.

Mitochondrial poisons such as CN^- and Oligomycin do not function through an immediate dissipation of transmembrane potentials in other cell systems [34]. Therefore, as expected, $TPMP^+$

accumulation in type II cells did not significantly decrease after 5 min of treatment with these agents. After 30 min of exposure, the results of Table I do show decreased $TPMP^+$ accumulation, which may reflect a depolarization of the mitochondria due to the inability of poisoned mitochondria to maintain a proton gradient, as well as possible effects on the plasma membrane potential due to a depletion of ATP in the cell.

The K^+ ionophore valinomycin [35], and the calcium ionophore A23187 [35,36], both resulted in a substantial release of $TPMP^+$ from type II cells. Valinomycin acts to increase the permeability of both the mitochondrial and plasma membrane to K^+ [35], and should thus give rise to two effects in our system. First, $\Delta\Psi_m$ should depolarize with the lower limit being the value seen with CCCP mitochondrial inhibition; second, $\Delta\Psi_p$ should approach a value set by the distribution ratio of K^+ across the plasma membrane. The results in Table I show that after 5 min of valinomycin exposure there was a release of $TPMP^+$ from the type II cell essentially identical to that seen after CCCP treatment. The $TPMP^+$ remaining in the type II cell at this point would apparently indicate that the K^+ distribution ratio is quite close to the plasma membrane potential seen after CCCP treatment. However, this does not necessarily mean that the plasma membrane potential after either CCCP or valinomycin treatment is equal to the normal K^+ distribution potential for this cell.

TABLE I

EFFECTS OF INHIBITORS AND IONOPHORES ON THE STEADY-STATE ACCUMULATION OF $TPMP^+$

Type II cells were incubated with 7 μM $TPMP^+$ and 5 μM TPB^- for 20 min and then treated with test compound or 1% ethanol as a vehicle control. Following 5 min or 30 min of treatment, [^{3}H]$TPMP^+$ accumulation in the type II cell was measured. The data are expressed as % control ± S.E. for the mean of triplicate determinations from at least three separate cell preparations.

Treatment	% cell-associated $TPMP^+$	
	5 min	30 min
Control	100	100
Oligomycin (5 μg/ml)	103 ± 6	73 ±13
KCN (2 mM)	107 ± 8	38 ±15
Ouabain (1 mM)	78 ±10	63 ± 7
Valinomycin (2 μm)	29 ± 4	1.0± 8
A23187 (100 μg/ml)	8.0± 9	–

The calcium ionophore A23187 was seen to have the most significant effect on the membrane potential of type II cells (Table I). The release of $TPMP^+$ suggests that this ionophore depolarized both the mitochondrial and plasma membranes by permitting movement of Ca^{2+} into the cell. In Ca^{2+} free medium this effect of A23187 was not found to be present (data not shown). Therefore, it appears that measurement of $TPMP^+$ accumulation could provide a sensitive indicator of calcium entry into the type II cell in future experiments.

In summary, the responses of isolated type II cells to the presence of valinomycin and high external K^+ are both consistent with the ability of $TPMP^+$ to detect changes in the plasma and mitochondrial membrane potentials of the type II cell. The accumulation of Rb^+, while not sensitive to mitochondrial membrane potential changes, offers the benefit of specifically detecting changes in the plasma membrane potential. Therefore, by independently investigating the membrane potential of type II cells with both ionic probes, one can detect an alteration in either membrane and distinguish a purely plasma membrane specific effect from the action of an agent which may influence both the plasma and mitochondrial membrane potential.

In the process of establishing these measurement methods for type II cells, several other points have been illustrated. First, the uptake of a cationic probe such as $TPMP^+$ by charged cells is not a process simply dependent on $\Delta\Psi_p$. The rate of uptake and approach to steady state must be considered, and if compounds are added to increase this rate (e.g., TPB^-) their effects on the cells of interest must be accounted for. For example, considerable care must be taken to correct for non-membrane potential dependent binding effects, or for uptake by other charged compartments in the cell. The mitochondrial component of $TPMP^+$ accumulation has been shown here to be a considerable factor in the type II cell system, with a mitochondrial potential which is larger in absolute magnitude than the plasma membrane potential. Possible effects on the measurement of $\Delta\Psi_p$ from other compartments such as Golgi, endoplasmic reticulum, or lamellar bodies are more

difficult to resolve. However, the apparent similarity between the Rb^+ measurements and the CCCP treated $TPMP^+$ measurements would tend to indicate that the contribution of these other compartments to the measurement of the membrane potential by $TPMP^+$ is minimal in this system.

A final point of interest concerns the effect of the calcium ionophore A23187 on type II cell membrane potential compared to the effect of this calcium ionophore on type II cell function. Mason et al. [7] have shown that surfactant release by type II cells occurs in response to A23187 treatment. Through the analysis of changes in $TPMP^+$ and Rb^+ accumulation, it may be possible to distinguish between the role of mitochondrial calcium release and the trans-plasma membrane calcium flux in surfactant secretion. Furthermore, other type II cell secretagogues such as phorbol myristate acetate, and β adrenergic agents, are under investigation in our laboratory as having a possible influence on the type II cell membrane potential. The ability to obtain an accurate measurement of both the plasma and mitochondrial membrane potential at steady state should prove to be of significant utility for the analysis of the type II cell's response to normal physiologic and toxic agents.

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