Use of a Lipophilic Cation to Monitor Electrical Membrane Potential in the Intact Rat Lens

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Purpose. Tetraphenylphosphonium (TPP^+) is a permeant lipophilic cation that accumulates in cultured cells and tissues as a function of the electrical membrane potential across the plasma membrane. This study was undertaken to determine whether TPP^+ can be used for assessing membrane potential in intact lenses in organ culture.

Methods. Rat lenses were cultured in media containing 10 μM TPP^+ and a tracer level of ^{3}H-TPP^+ for various times. ^{3}H-TPP^+ levels in whole lenses or dissected portions of lenses were determined by liquid scintillation counting. Ionophores, transport inhibitors, and neurotransmitters were also added to investigate their effects on TPP^+ uptake.

Results. Incubation of lenses in low-K^+ balanced salt solution and TC-199 medium, containing physiological concentrations of Na^+ and K^+, led to a biphasic accumulation of TPP^+ in the lens that approached equilibrium by 12 to 16 hours of culture. The TPP^+ equilibrated within 1 hour in the epithelium but penetrated more slowly into the fiber mass. The steady state level of TPP^+ accumulation in the lens was depressed by 90% when the lenses were cultured in a medium containing high K^+. The calculated membrane potential for the normal rat lens in TC-199 was -75 ± 3 mV. Monensin (1 μM) and nigericin (1 μM), Na^+H^+ and K^+H^+ exchangers respectively, as well as the protonophore carbonylcyanide-*m*-chlorophenylhydrazone (CCCP, 10 μM) and the calcium ionophore A23187 (10 μM), abolished TPP^+ accumulation and caused cloudiness of the lenses. The neurotransmitter acetylcholine at 50 μM decreased TPP^+ accumulation in the lens, but this effect could be prevented by simultaneous application of 1 mM atropine.

Conclusions. TPP^+ accumulation can be used as an indicator of changes in membrane potential in intact lenses, but because of the long time required to reach steady state, its utility is limited. The slow accumulation of TPP^+ and its slow efflux from the lens under conditions known to depolarize membranes are consistent with a diffusion barrier in the deep cortex and nucleus of the lens. (*Invest Ophthalmol Vis Sci.* 2000;41:482–487)

The lens is a syncytial structure consisting primarily of tightly packed, elongated fiber cells oriented from pole to pole and interconnected by gap junctions. The inner mature fiber cells are almost completely devoid of organelles but contain abundant amounts of the lens crystallins. This very high protein concentration is indispensable to the refractive function of the lens. A single layer of epithelial cells actively transports sodium and potassium caps the anterior hemisphere.[1] These cells control their volume by maintaining a negative resting membrane potential that opposes the diffusion of permeant anions into the cytoplasm. This gradient requires metabolic energy. Adenosine-5′-triphosphate is hydrolyzed by NaK-ATPase, which keeps the concentration of intracellular Na^+ low and K^+ high. The cellular membrane conductance is made selective for K^+ by expression of K^+ channels, and thus a negative resting voltage results. A majority of the fiber cells have no functional Na^+K^+ pumps, and no evidence of K^+-selective channels has been observed.[1] Therefore, the resting electrical membrane potential ($\Delta\psi$) is thought to be maintained primarily by the epithelial layer.

Microelectrodes provide a direct means of measuring transmembrane electrical potentials and intracellular ion activities. However, other methods are also widely used for determining the electrical membrane potential in mitochondria and cells.[2–4] Organic lipophilic ions with a diffuse charge distribution such as tetraphenylphosphonium ion (TPP^+) have found widespread use since they were first introduced by Skulachev.[5] Being permeable but charged compounds, they distribute passively across the plasma membrane according to the Nernst equation. Thus, the measurement of their distribution in steady state conditions yields the electrical membrane potential across the plasma membrane.[2] These ions have not been used to measure the $\Delta\psi$ in the lens. Because the electrical membrane potential and the ion concentration gradients of the lens are determined by a single layer of cells, it seemed an appropriate system for the determination of $\Delta\psi$ using TPP^+. The TPP^+ accumulation and distribution in the lens were studied in detail, and the effects of several ionophores and other agents on TPP^+ uptake by the lens were also investigated.

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MATERIALS AND METHODS

Lens Culture

All experimental procedures were performed in accordance with the tenets of the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research. Mixed populations (male and female) of Sprague–Dawley rats (75–100 g) were obtained from Taconic Farms (Germantown, NY). Rats were killed by CO_2 asphyxiation, and lenses were carefully dissected by a posterior approach. Each rat lens was incubated in 2.0 ml modified TC-199 medium, as previously reported,[6] and the integrity of all lenses placed in culture was confirmed by quantification of protein leakage into the medium.[7] The undamaged lenses were transferred into fresh media containing 10 μM TPP^+ and a tracer level of ^{3}H-TPP^+ (80,000 cpm/ml, 34.0 Ci/mmol) and incubated at 37°C in a 5% CO_2 atmosphere for the designated time. Ionophores and reagents selected were dissolved in water or ethanol and added to each well to the final concentrations indicated to investigate their effect on TPP^+ uptake.

Chemicals and Solutions

TPP^+ was from Aldrich (Milwaukee, WI), tetra[^{3}H] phenylphosphonium bromide (34.0 Ci/mmol) was from Amersham Life Sciences (Arlington Heights, IL), and L-glutamic acid was from Pierce (Rockford, IL). Carbonylcyanide-*m*-chlorophenylhydrazone (CCCP), atropine, acetylcholine chloride, γ-amino-*n*-butyric acid (GABA), A23187, monensin, nigericin, ouabain, veratridine, *N*-methyl-D-glucamine (NMDG), and other reagents were from Sigma (St. Louis, MO).

The standard medium for lens culture was TC-199 modified as previously described.[6] Basic balanced salt solution (low-K^+ medium) contained 115 mM NaCl, 4 mM KCl, 30 mM $NaHCO_3$, 0.3mM Na_2HPO_4, 0.32 mM KH_2PO_4, 0.58 mM $MgSO_4$, 5.5 mM glucose, and 1.5 mM $CaCl_2$. The medium was adjusted to 298 ± 2 mOsm with sodium chloride. For high-K^+ medium, 115 mM KCl, 4 mM NaCl, and 30 mM $KHCO_3$ were used in place of 115 mM NaCl, 4 mM KCl, and 30 mM $NaHCO_3$, respectively, and the medium was adjusted to 298 ± 2 mOsm with potassium chloride. The remaining components were the same as in the low-K^+ medium. For Na^+-free media, choline chloride or NMDG was used in place of Na^+, and 25 mM Tris-HEPES (pH 7.4) was used as a buffer. These media were adjusted to 298 ± 2 mOsm with choline chloride and NMDG, respectively, and to pH 7.4 with Tris.

CCCP, veratridine, A23187, ouabain, nigericin, and monensin were dissolved in ethanol. The final concentration of ethanol in the tissue culture system was 1% by volume, and an equal amount of ethanol was added to the control samples. GABA and glutamate were dissolved in TC-199 medium immediately before use.

TPP^+ Uptake

Lenses were harvested at times indicated, rinsed briefly in phosphate-buffered saline (PBS), and blotted on filter paper before weighing. The water content of the normal rat lens of the age used was taken as 62% of wet weight.[8] Each lens was homogenized in 1 ml 0.1 N NaOH, an aliquot (0.1 ml) of the supernatant was added to 5 ml scintillation fluid, and radioactivity was measured by liquid scintillation spectrometry (counting efficiency, 46.4% for ^{3}H). To assess the distribution of TPP^+ in different regions of the lens, lenses were dissected into three portions: capsule epithelium, cortex, and nucleus; and each portion was weighed. The lens nucleus, which was separated from cortex on the basis of physiological hardness, averaged approximately 40% of total lens weight. Each portion was homogenized in 0.1 N NaOH, and an aliquot of each supernatant was transferred to a counting vial for measurement of radioactivity by liquid scintillation spectrometry.

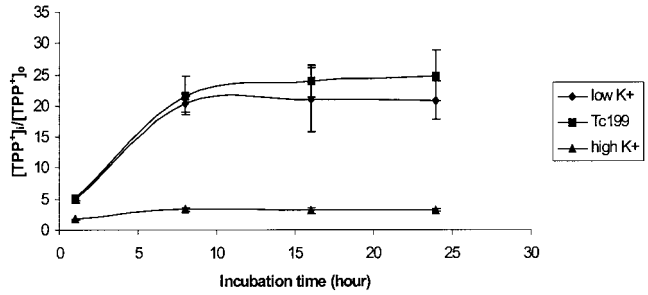

FIGURE 1. Accumulation of TPP^+ in rat lenses incubated in low-K^+ or high-K^+ balanced salt solution and modified TC-199 medium. TPP^+ (10 μM) was added to the medium, and the lenses were harvested as described in the Materials and Methods section. TPP^+_{in} was calculated assuming water content of 62% of wet weight in the lens. Data are presented as means ± SE for a representative experiment in which each group contained at least four lenses. The entire experiment was repeated at least twice.

Statistics

The data reported are from representative experiments in which each point was derived from a minimum of four lenses. Each entire experiment was repeated at least twice. Statistical analyses of the data were performed using Student's *t*-test.

RESULTS

TPP^+ Accumulation

Intact rat lenses incubated in low-K^+ medium or modified TC-199 media, containing physiological concentrations of Na^+ and K^+ (i.e., 145 mM Na^+ and 5 mM K^+), accumulated TPP^+ from the culture medium against a concentration gradient. The accumulation increased during the first few hours, achieving a steady state level by 12 to 16 hours, which was maintained for at least an additional 10 hours. No statistical difference was noted between low-K^+ and modified TC-199 media (Fig. 1). In contrast, when the lenses were cultured in a medium containing high K^+, the steady state level of TPP^+ accumulation was depressed by 90%. Because TPP^+ was readily lost from the lenses under various conditions (see later description), it became apparent that the steady state levels were achieved by free TPP^+ rather than by conversion of the TPP^+ into a stable adduct with lens protein. Because direct electrical measurements have shown that the $\Delta\psi$ of the lens is mainly due to the potassium diffusion gradient,[1] TPP^+ accumulation in high-K^+ medium is obviously unrelated to the $\Delta\psi$ across the lens. Thus, by subtracting the values obtained for TPP^+ accumulation at high external K^+ concentrations, that component of the total accumulation due to the $\Delta\psi$ in the lens can be approximated (i.e., $[TTP^+]^{low\ K+} - [TPP^+]^{high\ K+} = [TPP^+]^{corrected}$). When the concentration ratio calculated in this manner was inserted

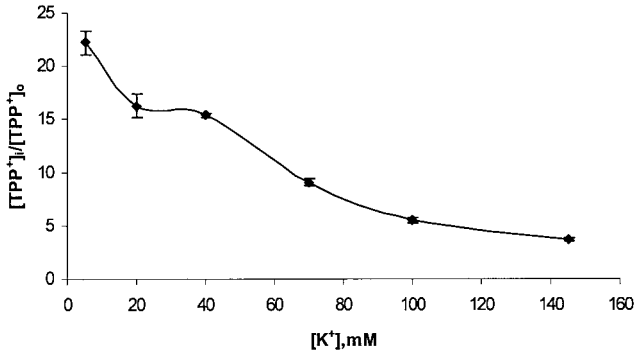

FIGURE 2. Effect of K^+ concentration in the medium on TPP^+ accumulation in rat lenses. Rat lenses were incubated in the presence of 10 μM TPP^+ for 18 hours, and TPP^+ uptake was measured as described in the Materials and Methods section. Data are presented as means ± SE ($n \geq 4$).

into the Nernst equation ($\Delta\psi = -61 \log [TPP^+]_{corrected}/[TPP^+]_{out}$), a $\Delta\psi$ value of -75 ± 3 mV was obtained. This value is comparable to those obtained using other methods.[9]

Because $\Delta\psi$ in the lens is largely dependent on K^+ diffusion potential, the effect of external K^+ on the accumulation of TPP^+ was investigated in detail. Because K^+ concentration was increased from 5 mM (low K^+) to 145mM (high K^+) the steady state level of the TPP^+ accumulation ratio (i.e., $[TPP^+]_{in}/[TPP^+]_{out}$) in the lens decreased (Fig. 2). The reduction in TPP^+ accumulation ratio with increased external K^+ was biphasic, reaching a first plateau at 20 mM and second at approximately 120 mM. Perhaps at least two compartments exist for TPP^+ accumulation in the lens, one highly sensitive to external K^+, and another less sensitive. The presence of two compartments for TPP^+ uptake in the lens was confirmed by determining TPP^+ uptake in different regions of the lens (see later description).

In TC-199 medium (5 mM K^+), the TPP^+ was taken up by the epithelium relatively fast, so that a steady state level was reached within an hour of incubation (Fig. 3A). In contrast, the accumulation of TPP^+ by the fiber mass was a slower process, and steady state levels were not observed even at 8 hours. Analyses of epithelium, cortex, and nucleus indicated that at the time the steady state was reached in the epithelium, the fiber cells in the cortex were accumulating TPP^+ rapidly (Fig. 3B). The nuclear fiber cells were last and slowest to accumulate TPP^+. This gradual accumulation of TPP^+ contributed to the slope of the curve in Figure 3A particularly from hour 4 to hour 8. In this time range, although cortical cells may still be accumulating TPP^+, the nuclear cells were taking up the TPP^+ more rapidly than the cortical ones. By the 28-hour time point, 95% of the total TPP^+ in the lens was present in the cortex and nucleus.

Effect of Ionophores and Transport Inhibitors on TPP^+ Uptake

Rat lenses were cultured in modified TC-199 medium in the presence of ionophores or other effectors to investigate the consequences of these chemicals on TPP^+ uptake by intact lenses. An intriguing result was the decrease in accumulation caused by monensin (1 μM) and nigericin (1 μM). Monensin, an ionophore that exchanges Na^+ and H^+ caused a drop of almost 60%. Monensin has been shown to increase intracellular Na^+,[10,11] stimulate the activity of NaK-ATPase,[12] cause hyperpolarization, and increase TPP^+ uptake in other cells.[2,13] Thus, the reduction in TPP^+ accumulation in the lens induced by this ionophore was somewhat surprising, although in other systems monensin has been reported to depolarize the membrane potential.[10,14,15] Nigericin, an ionophore exchanging K^+ and H^+, had an even greater effect at this concentration, inhibiting the TPP^+ accumulation by 87%. The protonophore CCCP and the calcium ionophore A23187, increase plasma membrane conductance to H^+ and Ca^{2+} ions, respectively. In agreement with these known properties, the addition of CCCP (10 μM) and A23187 (10 μM) caused a marked reduction in TPP^+ accumulation in the lens (Table 1) and cloudiness in the cultured lenses. These reductions represented the manifestation of a depolarization of the plasma membranes in the cell. The alkaloid veratridine is a compound that opens voltage-dependent Na^+ channels. Veratridine, which depolarizes all excitable cells,[10,14] did not affect TPP^+ accumulation in the lens (Table 1) suggesting the absence of these channels in this organ. The NaK-ATPase inhibitor ouabain (10 μM) also did not influence TPP^+ accumulation under the condition of this experiment.

Because the ion-specific ionophores monensin and nigericin produced such profound and unexpected effects on the cultured lens and because little information exists in the liter-

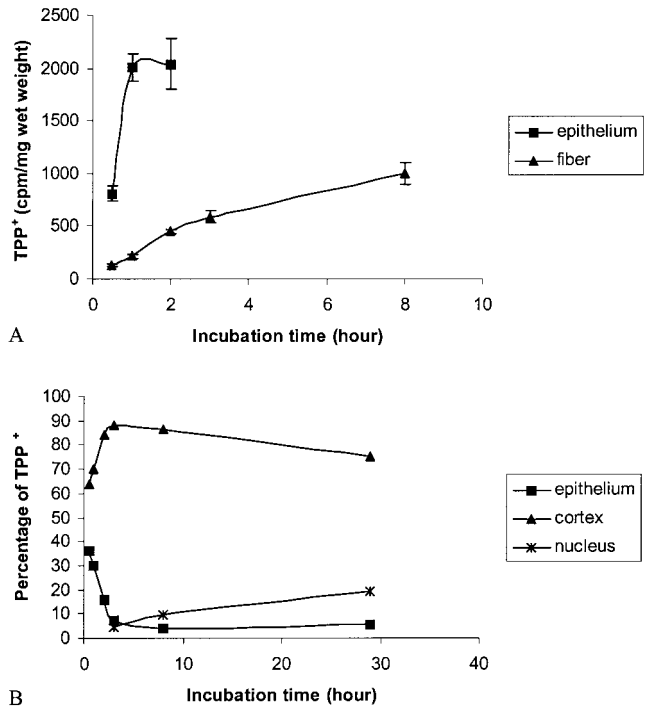

FIGURE 3. (**A**) TPP^+ accumulation in lens capsules and fibers. Rat lenses were incubated in TC-199 medium with 10 μM TPP^+ and were dissected at different time points. The TPP^+ uptake was measured as described in the Materials and Methods section. Data are presented as means ± SE ($n \geq 4$). (**B**) Percentage of TPP^+ uptake in different regions of the rat lens incubated in TC-199 medium with 10 μM TPP^+. Lenses were dissected, TPP^+ accumulation in each region was measured by liquid scintillation spectrometry, and total [^{3}H]TPP^+ counts in each region were then calculated. Data are presented as means. The SDs from at least four lenses were within 5% of the means.

TABLE 1. Effect of Ionophores and Transport Inhibitors on TPP^+ Uptake by Intact Rat Lens

Reagents	TPP^+_{in} (μM)	TPP^+ Accumulation (% of control)
Control	204 ± 12	100 ± 6
10 μM ouabain	186 ± 15	91 ± 8
1 μM veratridine	171 ± 15	84 ± 7
10 μM A23187	29 ± 5*	14 ± 3*
10 μM CCCP	88 ± 10*	43 ± 5*
1 μM monensin	84 ± 12*	41 ± 6*
1 μM nigericin	27 ± 3*	13 ± 1*

These data were from a representative experiment in which the lenses were harvested after 18 hours' incubation. Each group contained at least four lenses. The entire experiment was repeated at least twice with similar results. Data are expressed as means ± SE.

* $P < 0.001$.

ature regarding the lens and these agents, some additional studies were undertaken. Monensin in the range of 10 nM to 10 μM decreased TPP^+ accumulation in the intact lenses in a dose-dependent manner (Fig. 4A). One micromolar monensin produced approximately a 60% decrease in TPP^+ uptake during 24-hour incubation (Fig. 4B). When lenses were incubated in TC-199 with TPP^+ for 18 hours, and monensin was then added to the medium at 1 μM, the effects on TPP^+ levels in the lens were also rather surprising (Fig. 5). In contrast to the effect of shifting lenses from TC-199 to high-K^+ medium in which the TPP^+ level in the lens decreased by nearly 50% in 5 hours, monensin elicited a much slower efflux of TPP^+—an approximately 3% decrease after 5 hours and only a 13% decrease after 9 hours (Fig. 5).

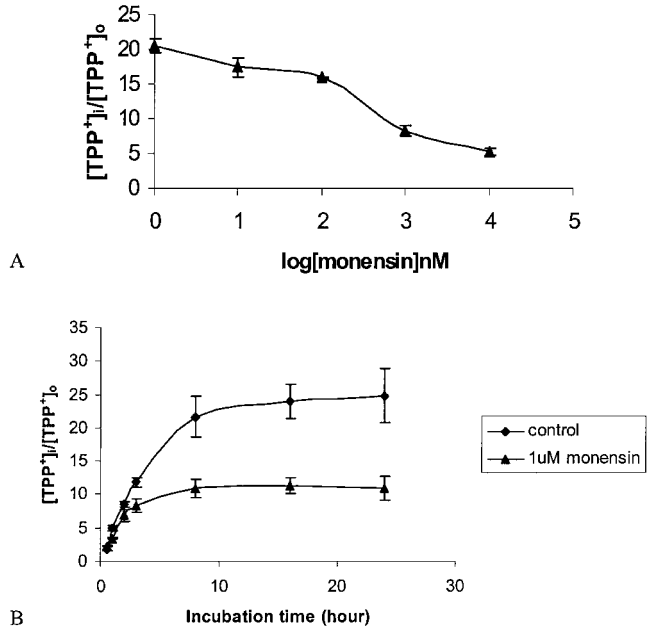

FIGURE 4. (**A**) Dose-dependent effects of monensin on TPP^+ uptake of rat lenses. Rat lenses were incubated in TC-199 medium with 10 μM TPP^+ and 10 nM to 10 μM monensin for 18 hours. (**B**) Accumulation of TPP^+ in rat lenses incubated in modified TC-199 medium with or without 1 μM monensin. TPP^+ (10 μM) was added to the media, and 1 μM monensin was added in one group at the beginning of the experiment. Data in (**A**) and (**B**) are presented as means ± SE ($n \geq 4$).

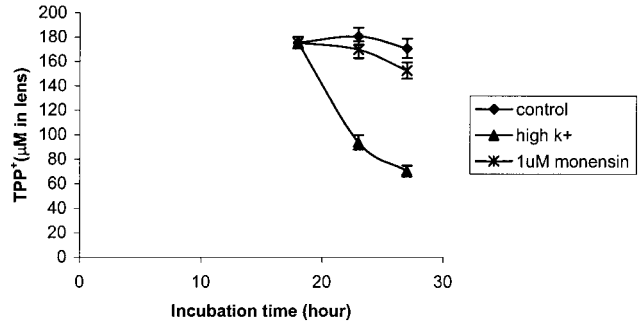

FIGURE 5. TPP^+ efflux from lenses. After an 18-hour incubation in the TC-199 medium with 10 μM TPP^+, 1 μM monensin was added into the media. One group of the lenses was transferred to high-K^+ medium with 10 μM TPP^+. [^{3}H]TPP^+ was measured after an additional 5- or 9-hour incubation. Data are presented as means ± SE ($n \geq 4$).

Lenses incubated in the presence of 1 μM of either monensin or nigericin became generally hazy within the first hour and exhibited increasing cortical cloudiness thereafter. There was a 20% to 25% increase in wet weight after 18 hours. Histologic analysis confirmed the peripheral nature of the damage, with swollen superficial fibers in the anterior cortex and extensive vacuoles and condensed nuclei in the differentiating fiber cells of the bow region (data not shown). Curiously, the distribution of TPP^+ in the lens incubated with 1 μM monensin throughout the culture period had a pattern similar to that in the control lenses, but the equilibrium level of TPP^+ in the epithelium was approximately 30% lower. Similarly, TPP^+ levels in the fiber mass were decreased to approximately the same extent.

Attempts were made to determine whether the effects of monensin were due to its ionophore activity by incubating lenses in media without sodium. Both choline chloride and NMDG were tried as substitutes for sodium, but in each case the lenses opacified within 30 minutes. This effect was presumably the result of sodium deprivation. Because changes in TPP^+ uptake caused by monensin are not evident until after at least 3 to 4 hours of incubation (Fig. 4B), the rapid deterioration of lenses in media without sodium made this approach impossible.

Effect of Neurotransmitters

It has been demonstrated recently, by using conventional electrical measurements, that acetylcholine causes a decrease in membrane conductance and induces depolarization in intact rabbit lens. This effect is blocked by atropine.[16] In our rat lens system 10 μM acetylcholine did not affect TPP^+ accumulation, whereas 50 μM caused a statistically significant decrease in TPP^+ accumulation. This effect could be completely prevented by simultaneous application of 1 mM atropine. Although the amount of acetylcholine used was much higher than physiological levels, it was in the same range as that used by Thomas et al.[16] who found maximal depolarization in cultured rabbit lenses at 10 μM. The presence of glutamate decarboxylase and GABA was demonstrated in mammalian lens.[17] Glutamic acid, which is abundant in the lens, reduces the glycation of human lens proteins.[18] Thus, it was of interest to test the effect of these compounds on TPP^+ accumulation in the lens. As can be seen in Table 2 these two compounds did not influence TPP^+ accumulation significantly.

TABLE 2. Effect of Neurotransmitters on the TPP^+ Uptake by Intact Rat Lens

Reagents	TPP^+_{in} (μM)	TPP^+ Accumulation (% of control)
Control	204 ± 12	100 ± 6
1 mM atropine	202 ± 23	99 ± 11
10 μM acetylcholine chloride	193 ± 12	95 ± 6
50 μM acetylcholine chloride	159 ± 21*	78 ± 12*
50 μM acetylcholine chloride and 1 mM atropine	192 ± 12	94 ± 6
1 mM GABA	217 ± 15	106 ± 7
1 mM L-glutamate	198 ± 6	97 ± 3

These data were from a representative experiment in which the lenses were harvested after 18 hours' incubation. Each group contained at least 4 lenses. The entire experiment was repeated at least twice with similar results. Data are expressed as means ± SE.

* $P < 0.05$.

DISCUSSION

Although the use of microelectrodes gives the most direct measure of membrane potential, limitations in the application of this method to small cells or organelles has stimulated the development of alternative, indirect methods. One such method is based on the equilibrium distribution of permeable ions across the membrane. Lipophilic ions, which have high partition coefficients between the membrane lipid phase and water and which equilibrate rapidly across membranes, are most commonly used in such systems. TPP^+ is such a lipophilic ion and has been used to quantify membrane potential in a variety of cell and organelle systems[2,19] since it was introduced to the field by Skulachev.[5] The purpose of the present study was to determine the potential utility of TPP^+ as an indicator of changes in membrane potential in intact lenses in organ culture and to characterize the accumulation of TPP^+ in such lenses.

Our results indicate that TPP^+ accumulation by the lens is biphasic, with faster uptake by the epithelial cells followed by much slower penetration into the fiber mass. Saturation of the epithelium occurred within 1 hour in the intact lens. With a culture of lens epithelium on the capsule, TPP^+ could probably fulfill the role of a quantitative indicator of membrane potential. Assessing TPP^+ accumulation into the whole lens, a $\Delta\psi$ of -75 ± 3 mV was obtained. This value is in the same range as the -69.6 mV found for rat lens using microelectrodes.[9]

Our results demonstrate that the penetration into the fiber mass is slow and requires many hours to reach steady state. This delay causes a significant obstacle in using TPP^+ accumulation as a practical method to quantify the overall membrane potential of the intact lens. TPP^+ accumulation, however, can be used as a qualitative indicator of the electrical membrane potential of the lens epithelium in intact lens systems. Results from our investigation of the effects of various ionophores and neurotransmitters provide further support for this conclusion.

The calcium ionophore A32187 has frequently been used in intact lens systems and is known to produce irreversible decreases in membrane potential as well as lens opacification.[20,21] In the current studies, these changes were shown to be accompanied by depolarization manifested by the expected abolition of TPP^+ accumulation. Ouabain, an inhibitor of NaK-ATPase, has been extensively studied in lens systems.[22,23] The failure to record ouabain-sensitive changes in TPP^+ in the lenses in this study is consistent with previous results because ouabain's effects have generally not been detected before 3 to 4 days in organ culture.[23] The maximum duration of the organ culture in this study was 30 hours.

Monensin and nigericin elicited major decreases in TPP^+ accumulation by the lens and induced opacification. To our knowledge they have not been studied previously in detail in lens organ cultures. Monensin, which exchanges Na^+ for H^+ across the plasma membranes, has been used primarily as a means of increasing intracellular pH. Bassnett[24] has demonstrated a 0.24 pH increase in cultured lens epithelial cells from embryonic chick by adding 50 μM monensin to the medium. In our whole lens studies 1 μM monensin produced a marked decrease in TPP^+ accumulation. The effect of monensin on TPP^+ accumulation was not evident until the lenses had been exposed for several hours because of the relatively slow equilibration of the lens with TPP^+. This is in contrast to similar studies in cell cultures, in which monensin-induced effects including changes in TPP^+ accumulation were seen in a few minutes, and in which the effects of monensin were abolished in sodium-free media (choline or NMDG).[2,25] Nigericin (1 μM) also markedly decreased TPP^+ accumulation in the intact rat lens. These decreases in TPP^+ accumulation are consistent with the depolarization of membrane potential that has been observed with monensin and nigericin in numerous other systems.[15,26]

In conclusion, TPP^+ accumulation can be used as an indicator of changes in membrane potential in intact lenses, but because of the long time required to reach steady state, its utility is limited. The slow accumulation of TPP^+ and its slow efflux from the lens under conditions known to depolarize membranes are consistent with a diffusion barrier in the deep cortex and nucleus of the lens.

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