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Triphenylmethylphosphonium ($TPMP^+$) as a Probe for Peritubular Membrane Potential in the Kidney Slice (41645)

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Abstract. The present work was undertaken to assess the validity of the use of $TPMP^+$, a lipophilic cation, as a basolateral membrane-potential probe in the rabbit kidney slice. Cortical slices incubated in media containing ^{3}H-$TPMP^+$ reached an equilibrium slice/medium concentration ratio (S/M) for $TPMP^+$ after 90 min. The equilibrium S/M $TPMP^+$ was ~20 at $TPMP^+$ concentrations between 30 and 50 μM. Concentrations of $TPMP^+$ above 50 μM resulted in changes in tissue electrolytes and O_2 consumption. Anaerobic incubation of slices in the presence of iodoacetamide decreased the S/M TPMP to ~3. When medium K^+ was progressively increased, the S/M $TPMP^+$ decreased continuously. There was a linear correlation between cell water/medium concentration ratios (C/M) for $TPMP^+$ and K^+ ($r = 0.629$, $P < 0.01$). However, the C/M $TPMP^+$ was 29.0 even at C/M K^+ of 1.0, strongly suggesting the presence of a tissue binding component for $TPMP^+$. When corrections are made for the above binding, the average C/M $TPMP^+$ in slices incubated in the normal medium was ~13, equivalent to a Nernst potential of −64 mV. This value compared favorably to −54.3 mV obtained by microelectrodes in slices treated in the same fashion. It is concluded that the C/M $TPMP^+$ corrected for binding may be used as a measure of the basolateral membrane potential in the steady state.

The measurement of membrane potential in cells too small or of unfavorable cell geometry and heterogeneity for micropuncture requires the use of noninvasive probes. Two classes of probes, i.e., fluorescent indicators, e.g., thiodicarbocyanine [DiS-C_3(5)] (1), and lipophilic cations, e.g., triphenylmethylphosphonium bromide ($TPMP^+$) (2–6) have proven to be particularly useful and their use has recently been reviewed by Leader (7, 8). Despite the widespread use of $TPMP^+$ (and its analogue) in recent years, the validity of the technique has been documented in only one tissue (*Necturus* gallbladder) (8) and several single cell preparations such as neuroblastoma–glioma hybrid cells (9) and *Escherichia coli* (3). From these studies, it became clear that the usefulness of a particular probe in one preparation does not insure that it will be appropriate for another. For example, tetraphenylphosphonium (TPP^+) will adequately monitor membrane potential in neuroblastoma–glioma hybrid cell suspensions, whereas $TPMP^+$, a closely related cation, will not (9). Moreover, there is a large variation between tissues (or cells), with respect to the equilibration time, the degree of nonspecific binding, and the toxicity, when $TPMP^+$ is used as a membrane potential probe (2–9).

Recently, $TPMP^+$ was used in rabbit renal cortical slices to assess the electrochemical gradient for Na^+ ion (10). Since the Na^+ electrochemical gradient provides the putative driving force for several transported substances in the kidney, this work would be extremely important if one could be certain that $TPMP^+$ indeed monitored membrane potential. Unfortunately, such documentation has not been provided. The present work was undertaken to assess the validity of the use of $TPMP^+$ as a basolateral membrane potential probe in the rabbit kidney slice.

Methods. All experiments were carried out in the rabbit kidney-slice preparation using the technique described in detail previously (11). In brief, adult New Zealand white rabbits weighing approximately 2 kg were killed instantly by cervical dislocation and the kidneys were promptly removed and perfused through the renal artery with an ice-cold isotonic solution containing 140 mM NaCl, 10 mM KCl, and 1.5 mM $CaCl_2$. Renal cortical slices (0.4–0.5 mm thick) were prepared using a Stadie-Riggs microtome and stored in the above isotonic solution until used.

In the first series, the time course of the slice uptake of $TPMP^+$ was studied over a 120-min period. The slices (100–150 mg) were

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incubated in 4 ml of modified Cross-Taggart medium (pH 7.4) containing 140 m*M* NaCl, 10 m*M* KCl, 1.5 m*M* $CaCl_2$, 5.0 m*M* Na phosphate, and various concentrations (1–50 μM) of $TPMP^+$ (obtained as a bromide salt from ICN). A trace amount of ^3H-$TPMP^+$ (New England Nuclear) was also added to the incubation medium. All incubations were performed at 25°C in a Dubnoff metabolic shaker in a 100% O_2 atmosphere. In some experiments, the Na^+ and K^+ concentrations in the medium were changed to 10 and 140 m*M*, respectively, while in others the incubation was carried out in a 100% N_2 atmosphere. In the latter experiment the incubation medium also contained 1 m*M* iodoacetamide. The incubation time varied from 15 to 120 min.

In the second series, the effects of various concentrations of $TPMP^+$ on the slice uptake of $TPMP^+$, tissue water and electrolyte (Na^+, K^+) distribution, and O_2 consumption were studied. The slices obtained from each rabbit were divided into three subgroups: one for the determination of the slice uptake of $TPMP^+$, the second for the inulin space, the total tissue water content, and tissue Na^+ and K^+ content, and the third for tissue O_2 consumption. For the determination of $TPMP^+$ uptake, the slices were incubated in a manner described above for 90 min in the modified Cross-Taggart medium containing 1, 10, 30, 50, 100, or 1000 μM $TPMP^+$ (with a trace amount of ^3H-$TPMP^+$). Likewise, the inulin space was determined by incubation of the slices in the above medium containing ^{14}C-inulin (New England Nuclear).

In the third series, the effect of varying the medium K^+ concentration on the slice uptake of $TPMP^+$ was studied. The slices from each rabbit were divided into two subgroups: one for the determination of $TPMP^+$ uptake over a 90-min period and the other for the inulin space, tissue water, and K^+. For the uptake of $TPMP^+$, its medium concentration was fixed at 30 μM (plus trace amount of ^3H-$TPMP^+$). The concentrations of K^+ in the medium were increased from the usual level of 10 m*M* to 15, 20, 25, 50, or 75 m*M*. The medium osmolality was kept constant by correspondingly reducing the concentration of Na^+ such that the sum of Na^+ and K^+ was 150 m*M* in all media.

Following the incubation, the slices used for the determination of ^3H-$TPMP^+$ uptake were removed from the medium, blotted, and weighed. After the slices were dissolved in 1 *N* NaOH, the solution was neutralized with HCl and the concentration of ^3H determined using standard liquid scintillation techniques. Aliquots of the media were prepared similarly for scintillation spectrometry. Quench corrections were made using appropriate standards for each type of sample composition. Most of the uptake data are given as the slice-to-medium (S/M) ratio: the tissue concentration of $TPMP^+$ (moles per gram wet tissue) divided by that of the medium (moles per milliliter medium).

The slices incubated in the presence of ^{14}C-inulin were blotted, weighed, and dried at 95°C for 24 hr, at which time they had reached a constant weight. The dried tissues were then weighed and extracted with 0.1 *N* HNO_3 for 48 hr. The nitric acid extracts were used to determine the concentrations of Na^+ and K^+ (flame-emission photometry) and ^{14}C-inulin (liquid scintillation spectrometry, see above). Similar analyses were performed with the medium. The data from these procedures were used to calculate the tissue water content, the extracellular fluid space (S/M inulin), and intracellular Na^+ and K^+ concentrations by use of standard procedures (12).

The O_2 consumption of slices was measured with a Yellow Springs Instrument Company model 53 oxygen monitor which employed a Clark-type electrode. The data are given as the initial rate of O_2 consumption computed from the decrease in O_2 saturation of the 4-ml bathing solution over a 15-min incubation. The modified Cross-Taggart medium was used in this experiment and was saturated with O_2 prior to the start of incubation.

In the fourth series, the effect of varying the medium K^+ concentration on cell basolateral membrane potential was studied. For these studies, slices were pinned to a Celgard (Dow Corning Corp.) 184 surface and bathed in the modified Cross-Taggart medium bubbled with 100% O_2 at room temperature (23–25°C). The chamber containing the tissue was enclosed in a Faraday cage. Open-tipped microelectrodes were drawn from 1.2 mm o.d. capillary glass (Glass Co. of America) on a horizontal

puller (Narashige, Ltd., model PN-3); the resulting tips had a resistance of 20 MΩ when filled with 1 *M* KCl. Electrodes were stored in 1 *M* KCl for no more than 5 hr before use. Microelectrodes were advanced into the slice with a remote hydraulic microdrive (Narashige Ltd., model MO-8). The electrical potential difference across the basolateral cell membrane, Em, with reference to a calomel cell in the tissue bathing solution was monitored by a high-impedance (10^{15} Ω) electrometer (WP Instruments Inc., model F223B) and recorded on a chart recorder (MFE Corporation, model 1200). The criteria for successful impalement were discussed previously (13). Measurements were first made in slices bathed in Cross-Taggart medium containing 10 m*M* K^+ and then repeated in the same tissue bathed in 40 and 75 m*M* K^+; the medium osmolality was kept constant by reducing the concentration of Na^+ such that the sum of Na^+ and K^+ was 150 m*M*.

The data were analyzed statistically by the Student *t* test, paired or unpaired, depending upon the design of experiments. The level of probability to denote significance was chosen as $P < 0.05$.

Results. *A. Time course of TPMP+ uptake.* The S/M $TPMP^+$ values as functions of incubation time under various experimental conditions are shown in Fig. 1. It is evident that the uptake continues over a 2-hr incubation period without reaching a steady state when the concentration of $TPMP^+$ in the medium was 15 μ*M* or below. As the medium concentration is raised above 15 μ*M*, saturation occurs as indicated by the decline in S/M. When the $TPMP^+$ concentration was raised to 30–50 μ*M*, the S/M value reached a steady state level of approximately 20 after 90 min. In several experiments, tetraphenylboron (TPB) was added to the incubation medium at 10^{-6} to 10^{-5} *M* to determine if the equilibration could be facilitated as it is in erythrocytes and lymphocytes (5). However, the time course of $TPMP^+$ uptake changed little in the presence of TPB (data not shown). Figure 1 also shows that the steady state S/M $TPMP^+$ value (obtained with 30 μ*M* $TPMP^+$ in the medium) decreased to 10 when the medium K^+ concentration was raised to 140 m*M*, in which circumstance the basolateral membrane is expected to be depolarized. Surprisingly, the S/M $TPMP^+$ value decreased fur-

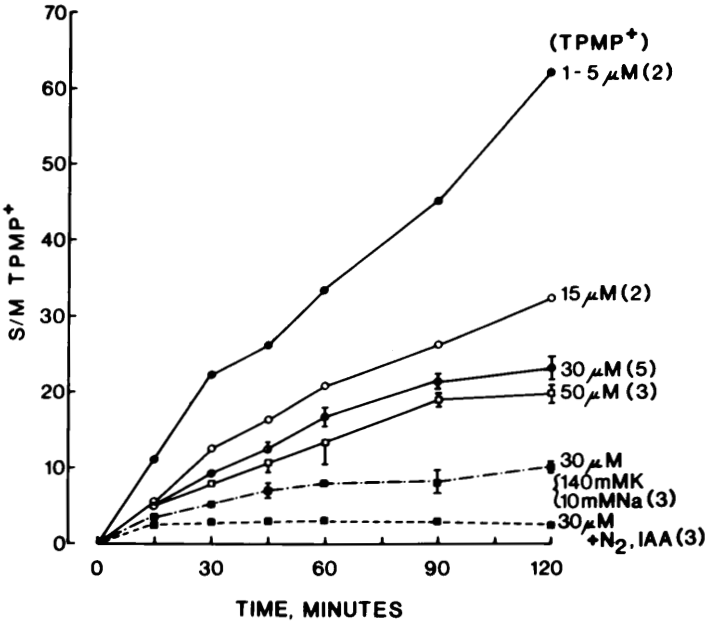

FIG. 1. The slice-to-medium concentration ratio (S/M) of $TPMP^+$ as a function of incubation time. ( ) Number of experiments. Vertical bars represent ±SE.

ther to ~3 when the cell metabolism was inhibited by iodoacetamide and N_2, apparently indicating that energy metabolism is necessary to maintain the slice in a healthy condition over the 90-min incubation period.

B. Dose-response relationship. Because the data shown in Fig. 1 indicated a concentration dependence of both the time course and the amount of uptake of $TPMP^+$, the effects of $TPMP^+$ over a wide range of concentration (1–1000 μM) on S/M $TPMP^+$ and tissue water and electrolyte distribution were examined. As shown in Fig. 2, the S/M $TPMP^+$ decreased progressively as the concentration of $TPMP^+$ increased, suggesting that the slice uptake of $TPMP^+$ is subject to saturation kinetics. Moreover, the intracellular concentrations of Na^+ and K^+ changed significantly at a $TPMP^+$ concentration of 50 μM or above. At the highest concentration of $TPMP^+$ (1000 μM) employed in this study, both the tissue water content and the inulin space changed significantly. These results suggest that $TPMP^+$ at 50 μM or above has a significant toxic effect. The actual measurement of tissue O_2 consumption (Table I) indeed showed that $TPMP^+$ at 100 μM decreased oxidative metabolism by 33%.

C. Relationship between K^+ and $TPMP^+$ distribution ratios. Because the membrane potential responds as a K^+-selective electrode to peritubular K^+ concentrations greater than 10 mM (14, 15), the marked reduction in S/M $TPMP^+$ observed in the presence of 140

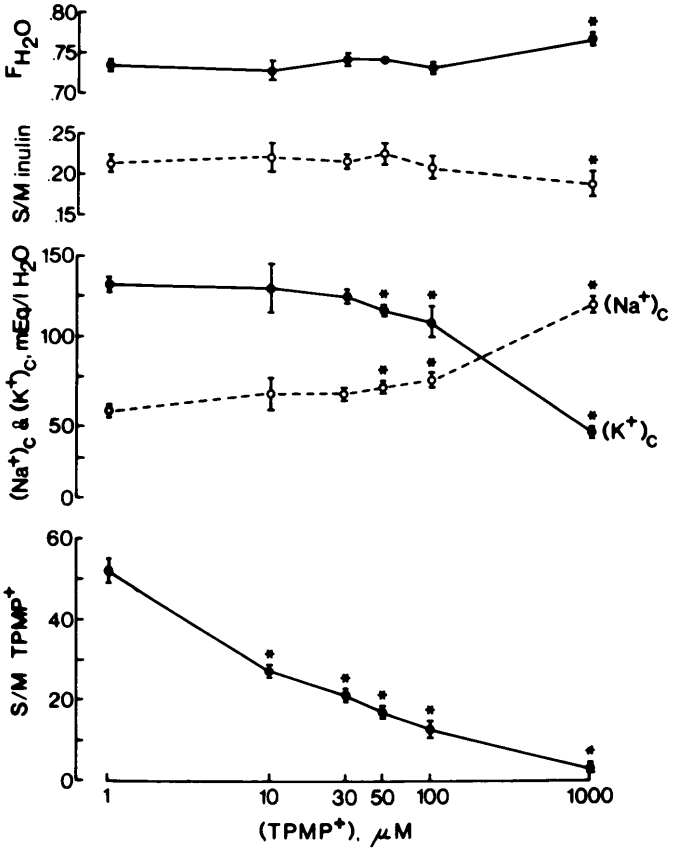

FIG. 2. The tissue water content (F_{H_2O}), inulin space (S/M inulin), intracellular Na^+ and K^+ concentrations, and slice-to-medium ratio (S/M) of $TPMP^+$ as a function of $TPMP^+$ concentration in the incubation medium. Each value was obtained at the end of 90 min incubation at 25°C, and represents mean ± SE of five experiments. *Significantly different from the corresponding value obtained at 1 μM $TPMP^+$ ($P < 0.05$, paired t test).

TABLE I. OXYGEN CONSUMPTION OF THE RENAL SLICE IN THE ABSENCE (CONTROL) AND PRESENCE (EXPERIMENTAL) OF $TPMP^+$ AT VARIOUS CONCENTRATIONS

$TPMP^+$ conc. (μM)		Oxygen consumption ($\mu l \cdot mg^{-1} \cdot hr^{-1}$)		
		Control	Experimental	Difference*
1	(5)	0.64 ± 0.13	0.62 ± 0.11	NS
10	(5)	0.71 ± 0.18	0.69 ± 0.16	NS
100	(5)	0.64 ± 0.13	0.43 ± 0.18	$P < 0.025$
100	(4)	0.57 ± 0.17	0.21 ± 0.07	$P < 0.005$

Note. Values are given as mean ± SE. ( ) Number of Experiments.
* Paired t test.

mM K^+ (and 10 mM Na^+ in the medium) (see Fig. 1) strongly suggests that the $TPMP^+$ distribution is dependent on membrane potential. Therefore, the relationship between cell-to-medium (C/M) $TPMP^+$ and K^+ concentration ratios was examined more systematically in this series. Based on the results shown in Figs. 1 and 2, the incubation was carried out for 90 min in a medium containing 30 μM $TPMP^+$ and various concentrations of Na^+ and K^+ (see Methods). The C/M $TPMP^+$ and C/M K^+ values were computed from the values of S/M $TPMP^+$, tissue water content, S/M inulin, and tissue K^+ concentration. Since preliminary studies showed that approximately 30% of the ^{3}H-$TPMP^+$ counts were lost during the drying procedure (carried out at 95°C), the S/M inulin, tissue water content and tissue K^+ were determined in separate slices obtained from the same animal (see Methods).

As shown in Fig. 3, there is a significant correlation between C/M $TPMP^+$ and C/M K^+ ($r = 0.629$, $P < 0.01$), with the slope of 1.16 which is not significantly different from 1.0. It is, however, important to note that the C/M $TPMP^+$ value at C/M K^+ of 1.0 is 29. Because the membrane should be virtually depolarized at C/M K^+ of 1.0, the latter C/M $TPMP^+$ value most likely represents intracellular binding of $TPMP^+$ independent of the membrane potential (8). The significant linear correlation between C/M K^+ and C/M $TPMP^+$ suggests that the change in C/M $TPMP^+$ is in fact due to a change in potential and not to a K^+ effect on $TPMP^+$ binding. Furthermore when a constant C/M $TPMP^+$ attributable to binding (i.e., 29.0) is subtracted, no change in slope is observed. Therefore, the experimentally determined C/M $TPMP^+$ value must be corrected for this binding (by subtracting 29.0) before it is used to calculate the membrane potential.

D. Comparison of the membrane potential determined by $TPMP^+$ with that determined using microelectrodes. Table II shows the membrane potentials (E_m) determined using microelectrodes and membrane potentials computed from $TPMP^+$ distribution. Slices were incubated for 2 hr with TPMP in Cross-

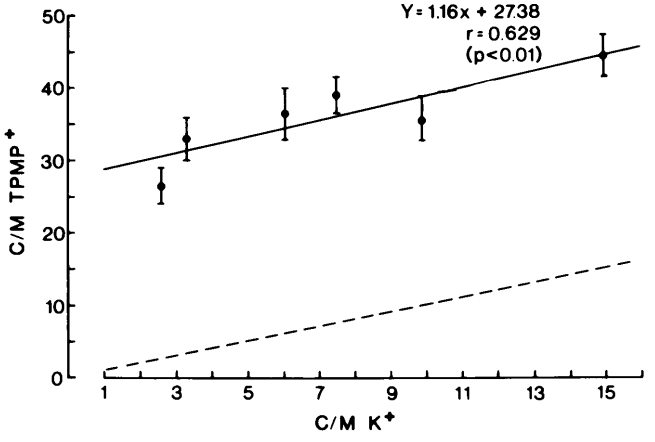

FIG. 3. The cell-to-medium concentration ratio (C/M) of $TPMP^+$ versus C/M K^+. Each point represents mean ± SE of three experiments. The regression equation: $Y = 1.16x + 27.38$, $r = 0.629$ ($P < 0.01$). The broken line indicates the identity line.

TABLE II. DIRECT MEASUREMENT OF MEMBRANE POTENTIAL (EM) IN RABBIT KIDNEY CORTICAL SLICES

$[K^+]_M$ (meq/l)	$[K^+]_C$* (meq/l)	E_m (mV) Measured	E_m (mV) Calculated**
9 ± 0	118 ± 10	−54 ± 2	−64
34 ± 5	126 ± 7	−35 ± 1	−25
66 ± 1	135 ± 5	−15 ± 2	− 9

Note. Values represent the mean ± SE of three animals. From two to eight successful impalements were made in each slice.

* $[K^+]_M$ and E_m were measured in the same slice. $[K^+]_C$ was determined in different slices incubated in the same manner.

** 58 log (C/M)TPMP+, see text for the calculation of C/M TPMP+ correcting for binding.

Subscripts M and C refer to medium and cell water, respectively.

Taggart media containing nominal K^+ concentrations of 10, 40, and 75 m*M*. The C/M K^+ was determined from measured $[K^+]$ in the media and in the slice. C/M K^+ values were 13, 4, and 2. From Fig. 3 the corresponding C/M TPMP values, corrected for binding by substracting 29, were 13, 3, and 1.5 equivalent to a Nernst potential of −64, −25, and −9 mV (Table II). The membrane potential determined using microelectrodes in slices treated in the same manner were 54, 35, and 15 mV.

Discussion. TPMP+ has been successfully used to determine the membrane potential in many single cell preparations (2–6) in which it equilibrates rapidly (especially when TPB was added to the medium). Although several workers have also used TPMP+ in various epithelia, such as the toad urinary bladder, the *Necturus* gallbladder (7, 8), and the rabbit kidney slice (10), the basic characteristics of TPMP+ distribution in these tissues has not been systematically documented.

Leader (8) has discussed in detail the problem associated with the use of chemical probes of membrane potential particularly in regard to the heterogeneity of the tissue in which they are used. Several considerations suggest that the use of TPMP+ for measurements of basolateral membrane potential may be appropriate despite obvious heterogeneity. First, little accumulation of TPMP+ should occur across the luminal membranes since they are partially collapsed and functionally occluded (16), therefore only basolateral membrane potential is being monitored. Second, although undoubtedly there is a small contribution from distal convoluted tubules and collecting ducts in the slice, this should not influence the results since there is no reason to believe the basolateral potential is different in these regions. In this regard, at least in guinea pig kidney, 97% of the slice is proximal convoluted tubule (16). Finally, there is the possibility that the TPMP+ distribution might be affected by the basolateral rheogenic pump of distal tubular epithelia. Proverbio and Whittembury (15), however, could only see the activity during transients and reported that at steady state E_m closely approximates E_K. Thus it seems reasonable, at least in the steady state, to expect that the rheogenic pump component of the potential would be small.

The results obtained in the present work indicate that TPMP+ may be used to estimate the basolateral membrane potential under certain conditions in the kidney slice. Calculation of the membrane potential from C/M TPMP+, corrected for binding, gives values which compare favorably to those determined with microelectrodes (Table II) under similar conditions. However, the usefulness of TPMP+ as a membrane-potential probe is severely limited by the fact that (i) it takes at least 90 min to reach a steady state (with or without TPB in the medium) (Fig. 1), (ii) TPMP+ binds to tissue as indicated by the concentration dependence of S/M TPMP+ (Fig. 2) and the large value of C/M TPMP+ at C/M K^+ of 1.0 (Fig. 3), and (iii) TPMP+ inhibits metabolism at a concentration of 50 μM or above (Fig. 2 and Table 1). These findings indicate that TPMP+ is entirely unsuitable for dynamic experiments. Although the C/M TPMP+ corrected for binding appears to give a reasonable estimate of membrane potential, this should also be done with considerable caution, since S/M TPMP+ determined in slices treated with iodoacetamide and N_2 was markedly lower than that determined in the presence of 140 m*M* K^+ (Fig. 1). As the membrane potential must be extremely low under these conditions, the above results suggest that the tissue binding of TPMP+ is dependent on metabolism. If so, it is very difficult to determine the correction factor for binding in experiments in which the cell metabolism is partially altered.

The nature of this membrane potential in-

sensitive binding is not known at present, although this phenomenon has been observed in human neutrophils and dogfish erythrocytes (7) and human erythrocytes and lymphocytes (5); on the other hand, no evidence for such a binding was found in the *Necturus* gallbladder and toad urinary bladder (8).

The results obtained from the present work cast a serious doubt about the validity of the membrane potential calculated by Podevin *et al.* (10) in the rabbit kidney slice. In some experiments, the incubation time was only 35 min, much shorter than the 90 min required to reach a steady state (Fig. 1); moreover, they have not corrected for nonspecific binding and for the possible loss of ^{3}H-TPMP$^+$ activity during desiccation at 60°C. Therefore, the computation of the electrochemical gradient based on the above measurements could lead to an erroneous conclusion.

Based on the present work, it is evident that TPMP$^+$ may be used to monitor membrane potential in the kidney slice with the following restrictions: (i) the TPMP$^+$ concentration must be approximately 30 μM, (ii) incubation time must be at least 90 min, (iii) the intracellular TPMP$^+$ concentration using the values of intracellular and extracellular water content determined in other slices from the same kidney must be calculated, and (iv) a correction for the membrane potential insensitive TPMP$^+$ binding must be determined by raising the medium K^+ concentration to depolarize the membrane. Unfortunately, however, there is no acceptable method for using TPMP$^+$ in experiments of short duration.

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