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Evidence for Coupling between Na^+ Pump Activity and TEA-sensitive K^+ Currents in *Xenopus laevis* Oocytes

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Abstract. Using the two-microelectrode voltage clamp technique in *Xenopus laevis* oocytes, we estimated Na^+-K^+-ATPase activity from the dihydroouabain-sensitive current (I_{DHO}) in the presence of increasing concentrations of tetraethylammonium (TEA^+; 0, 5, 10, 20, 40 mM), a well-known blocker of K^+ channels. The effects of TEA^+ on the total oocyte currents could be separated into two distinct parts: generation of a nonsaturating inward current increasing with negative membrane potentials (V_M) and a saturable inhibitory component affecting an outward current easily detectable at positive V_M. The nonsaturating component appears to be a barium-sensitive electrodiffusion of TEA^+ which can be described by the Goldman-Hodgkin-Katz equation, while the saturating component is consistent with the expected blocking effect of TEA^+ on K^+ channels. Interestingly, this latter component disappears when the Na^+-K^+-ATPase is inhibited by 10 μM DHO. Conversely, TEA^+ inhibits a component of I_{DHO} with a K_D of 25 ± 4 mM at +50 mV. As the TEA^+-sensitive current present in I_{DHO} reversed at −75 mV, we hypothesized that it could come from an inhibition of K^+ channels whose activity varies in parallel with the Na^+-K^+-ATPase activity. Supporting this hypothesis, the inward portion of this TEA^+-sensitive current can be completely abolished by the addition of 1 mM Ba^{2+} to the bath. This study suggests that, in *X. laevis* oocytes, a close link exists between the Na-K-ATPase activity and TEA^+-sensitive K^+ currents and indicates that, in the absence of effective K^+ channel inhibitors, I_{DHO} does not exclusively represent the Na^+-K^+-ATPase-generated current.

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Introduction

The Na^+-K^+-ATPase or Na^+ pump is an ubiquitous plasma membrane active transporter responsible for establishing Na^+ and K^+ concentration gradients in animal cells. The concentration gradients for Na^+ and K^+ are used as an energy source to maintain membrane electrical potential and to drive a variety of Na^+-coupled transporters. The kinetics of the Na^+-K^+-ATPase has been studied for a number of years (*see* [6], for a review) and detailed descriptions of specific parts of the mechanism are now becoming available [1, 2, 13, 15, 18, 23, 24, 27].

Experiments performed on cardiac cells or oocytes have recently suggested that Na^+ binding to its extracellular site occurs through an access channel experiencing a large fraction of the membrane electrical field [8, 10, 20]. The fact that the affinity of K^+ for its extracellular site is significantly decreased by a positive intracellular potential (V_M) [19, 23, 26] suggests that K^+ binding may also use the same access channel. This phenomenon is thought to be responsible for the reduction in the pump activity observed at positive V_M in *X. laevis* oocytes, which generates a characteristic negative slope in the pump *I-V* curve. The possibility of interfering with the K^+ binding process using an agent that could block the access channel was our first reason to initiate a series of experiments aimed at detecting the effect of tetraethylammonium (TEA^+) on the dihydroouabain-sensitive current (I_{DHO}) in *X. laevis* oocytes. Previous reports have indicated that TEA^+ could interfere directly with the Na^+ pump in red blood cells and muscles [21, 28].

The determination of I_{DHO} in oocytes is a convenient

way to obtain an estimate of the Na^+-K^+-ATPase activity and has often been used in the past to study the Na^+ pump current-voltage relationship (*I-V* curve) [13, 18, 19, 20, 23, 26, 27]. To be accurate, the determination of I_{DHO} requires that the inhibitor used be specific for the Na^+-K^+-ATPase and that no other ionic currents change as a consequence of pump inhibition. Although ouabain and dihydroouabain are acknowledged to be highly specific inhibitors for the Na^+ pump, K^+ activities in the unstirred layers on both sides of the membrane are bound to change following Na^+-pump inhibition, leading to changes in K^+ currents. For this reason, several investigators have used tetraethylammonium (TEA^+) and barium (Ba^{2+}) to inhibit K^+ currents while measuring I_{DHO} [19, 20, 23, 26, 27]. However, none of these inhibitors is totally effective and the possibility remains that I_{DHO} can be contaminated by some K^+ current that does not cancel out perfectly in the subtraction procedure required to obtain the ouabain-sensitive current. This is even more likely when one considers a phenomenon that has been studied in some detail in epithelial tissue: the pump-leak mechanism. It refers to the observation that whenever a change in the Na^+-pump activity is produced, a parallel change in basolateral K^+ conductance occurs [4, 14, 22]. This has been recently confirmed at the single channel level where the "*NPo*" (where *N* is the number of channels and *Po* is the open probability) of an ATP-regulated K^+ channel was indirectly modulated by pump inhibition [3, 12]. The possibility that I_{DHO} may contain a K^+ current was an additional reason to study the effect of a K^+ channel blocker on I_{DHO}.

Materials and Methods

Oocytes

Mature oocytes (stage V or VI) [7] were obtained from anesthetized (3-aminobenzoic acid ethyl ester) *X. laevis* frogs. The follicular layer was removed by incubating oocytes in Barth's solution (*see below* for composition) containing collagenase (2–3 U/ml, Boehringer Mannheim, Laval, QC) for 1–2 hr and defolliculated oocytes were stored at 18°C in Barth's solution supplemented with 5% horse serum, 2.5 mM Na pyruvate, 100 U/ml penicillin and 0.1 mg/ml streptomycin. To stimulate Na^+-pump current, intracellular [Na^+] was elevated by overnight incubation in a K^+-free Barth's solution. Intracellular [Na^+] has been shown to increase to 20–40 mM using this procedure [13].

Solutions

The composition of Barth's solution used for preparing oocytes was (in mM): 88 NaCl, 3 KCl, 0.82 $MgSO_4$, 0.41 $CaCl_2$, 0.33 $Ca(NO_3)_2$ and 5 HEPES (pH = 7.6). For electrophysiological experiments, Barth's solution was modified in such a way that 40 mM NaCl was isosmotically replaced by a mixture of *N*-methyl-D-glucamine/HCl and TEACl, the TEA^+ concentration ([TEA^+]) being either 0, 5, 10, 20 or 40 mM. This low Na^+ solution will be called the Barth* solution at the indicated [TEA^+]. When 1 mM $BaCl_2$ was required, SO_4 and NO_3 were replaced by Cl in the Barth* solution.

Electrophysiology

Oocyte currents were measured with the two-microelectrode voltage clamp technique using a commercial amplifier (Oocyte Clamp model OC-725, Warner Instruments, Hamden, CT). The current and voltage microelectrodes were filled with 1 M KCl and had resistances ranging from 7 to 14 MΩ. The bath was referenced through an agar bridge containing 1 M KCl and the bath current electrode was an Ag/AgCl pellet. The voltage pulse protocol was generated using an arbitrary waveform generator (model 75, Waveteck, San Diego, CA) and consisted of 10 alternative 100 msec duration pulses separated by 300 msec intervals at the resting potential (–50 mV). The voltage range studied was from –175 to +75 mV. Currents and voltages were converted to video signals (Neuro-Corder model DR-384, Neurodata, New York, NY) and recorded on commercial video tapes. The signals were low-pass filtered (cutoff frequency of 40 Hz) and digitized at 0.5–2.0 msec per point using a data acquisition system (Computerscope, RC Electronics, Santa Barbara, CA). The current and voltage pulses were then automatically analyzed using a laboratory program taking the average of the signal between 80 and 100 msec after the onset of the pulse.

Results

Reversibility and Reproducibility of the TEA^+ Effect

We first tested the reversibility of the TEA^+ effect on the oocyte current-voltage (*I-V*) curve. As shown in Fig. 1, the effect of adding 40 mM TEA^+ (*N*-methyl-D-glucamine replacement) to the bath solution was to produce a large inward current throughout the voltage range studied. The TEA^+ effect could be completely washed out since returning to the Barth* solution after a 5 min exposure to 40 mM TEA^+ restored the oocyte *I-V* curve to its initial position. A second application of 40 mM TEA^+ produced an effect similar to the first application and it was concluded that several concentrations of TEA^+ could be accurately tested on the same oocyte as the effect appeared both reversible and reproducible.

In Fig. 1, it is apparent that TEA^+ addition generates an inward current throughout the voltage range studied. While the observed effect for positive membrane potentials (V_M) is consistent with a blockade of TEA^+-sensitive K channels (the classical effect of TEA^+), the effect observed for $V_M < \sim -75$ mV is certainly not.[1] To illuminate the nature of this TEA^+ effect, different concentrations of TEA^+ (5–10–20–40 mM) were successively applied to oocytes before and after the addition of 10^{-5} M dihydroouabain (DHO). The results are shown in

[1] In Na^+-loaded oocytes, the equilibrium potential for K^+ (E_K) can be calculated to be around our experimental point of –75 mV. Intracellular Na^+ activity in these oocytes is known to increase by 20 to 70 mM [13, 19, 26] and it is likely that intracellular K^+ activity ([K_i] decreases by a similar amount. If, on average, [K_i] decreases by 40 mM, E_K must change from –88 mV in normal oocytes [5] to –75 mV in Na^+-loaded oocytes.

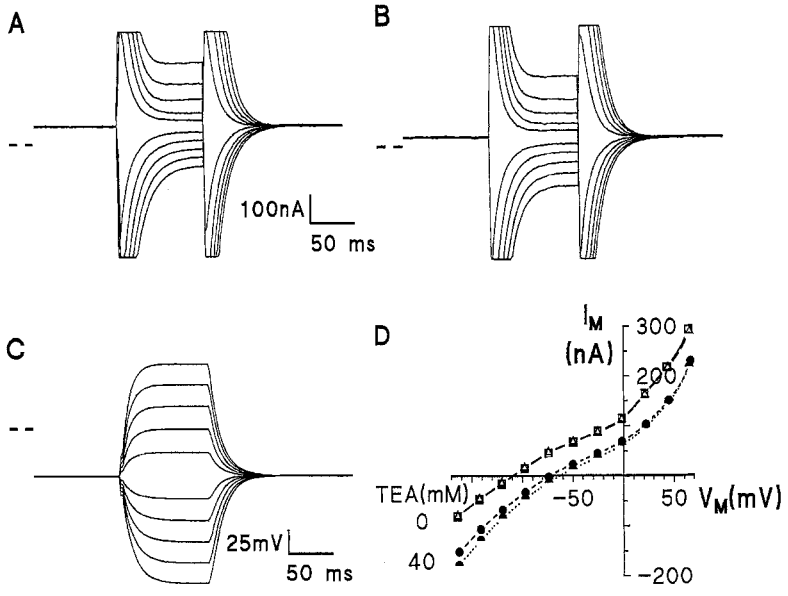

Fig. 1. Example of the reversibility of the TEA$^+$ effect on oocyte current-voltage curve. The dashed lines in *A*, *B* and *C* represent zero current/voltage levels. The experiment was performed in the sequence 0 mM TEA$^+$ (*A* and open squares in *D*), 40 mM TEA$^+$ (*B* and filled circles in *D*), 0 mM TEA$^+$ (open triangles in *D*) and 40 mM TEA$^+$ (filled semicircles in *D*). *C* shows the voltage pulse protocol used (holding potential = −50 mV).

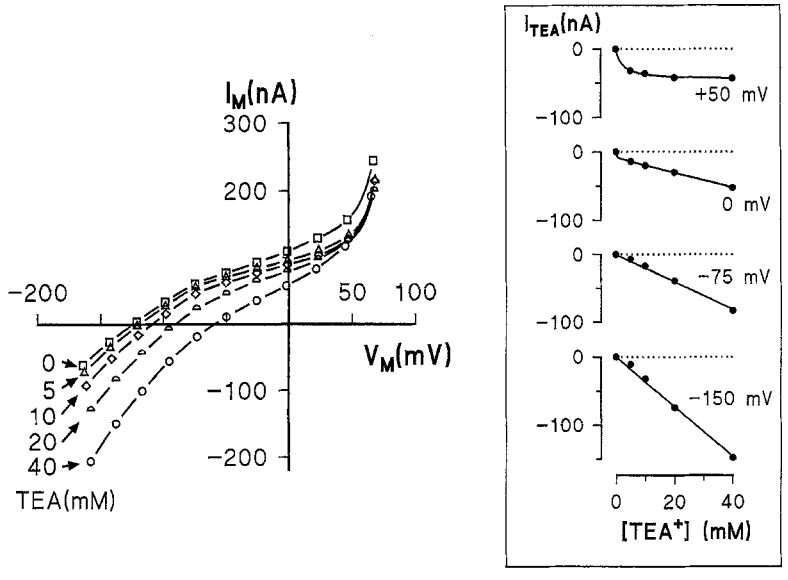

Fig. 2. Typical example of the TEA$^+$ effect on the oocyte current-voltage relationship. Inset shows the TEA$^+$-sensitive currents at selected membrane potentials as a function of TEA$^+$ concentrations.

Figs. 2 and 3 for a typical experiment. Before pump inhibition (Fig. 2), the effect of TEA$^+$ appeared strictly proportional to the TEA$^+$ concentration ([TEA$^+$]) for negative V_M while it became progressively saturable at positive V_M. This is shown better in the insets of Fig. 2 where the TEA$^+$-sensitive current (I_{TEA^+} = current with 0 [TEA$^+$] minus current with different [TEA$^+$]s) is plotted as a function of [TEA$^+$] for selected V_Ms. At −75 mV, I_{TEA^+} is strictly diffusive and the average TEA$^+$-sensitive current (n = 8) can be accounted for by the Goldman-Hodgkin-Katz equation (GHK) using a single permeability constant of 8.8×10^{-8} cm/sec (assuming an apparent membrane area of 4.5 mm^2 per oocyte as predicted for a spherical oocyte of 1.2 mm in diameter). For more positive V_M, as the importance of the TEA$^+$ electrodiffusion decreases, a saturable component of I_{TEA^+}, can be detected which is consistent with inhibition of some outward current (a K$^+$ current, for example). For positive V_M, the TEA$^+$ dissociation constant for blocking this current component was approximately 5 mM.

When the same experiment was performed in the presence of 10 μM DHO (*see* Fig. 3), the saturable component of I_{TEA^+} disappeared leaving a simple nonsaturable effect of TEA$^+$. This component can be accurately fitted by the GHK equation as a function of [TEA$^+$] (*see* Fig. 3 inset) or as a function of V_M (*see* Fig. 4) using a permeability constant of 8.9×10^{-8} cm/sec, identical to the permeability constant found at a V_M of −75 mV in the absence of DHO.

The fact that the effects of TEA$^+$ were different in

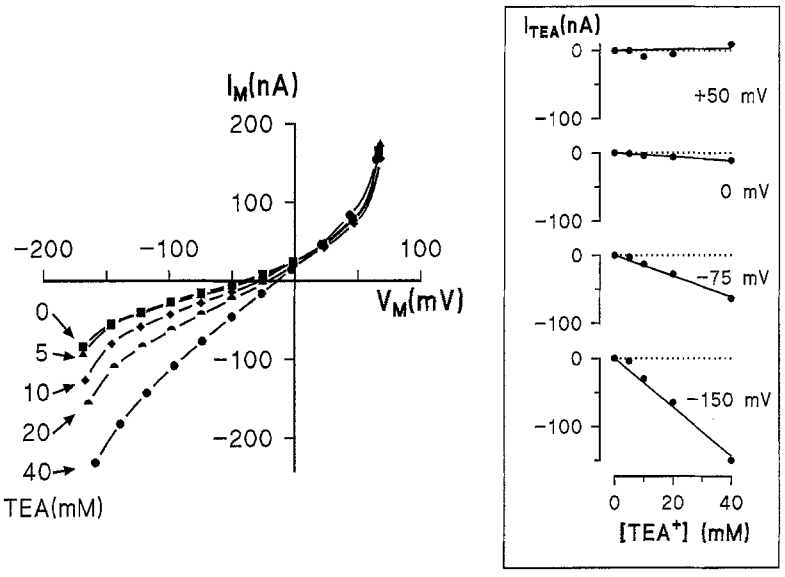

Fig. 3. Typical example of the TEA^+ effect on the oocyte current-voltage relationship in the presence of 10 μM DHO. Inset shows the TEA^+-sensitive currents at selected membrane potentials as a function of TEA^+ concentrations.

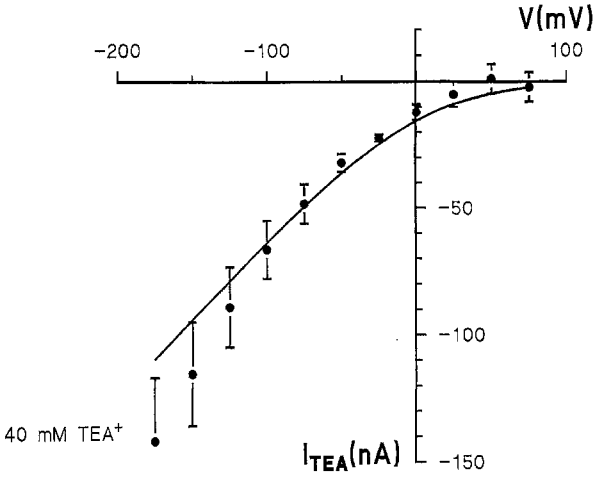

Fig. 4. Fit of the Goldman-Hodgkin-Katz equation to the TEA-sensitive current observed during pump inhibition. The averaged inward current ($n = 8$) produced by 40 mM TEA^+ addition in the presence of 10 μM DHO (an example of which is given in Fig. 3) was fitted using a permeability coefficient of 8.9×10^{-8} cm/sec (oocyte area = 0.045 cm^2).

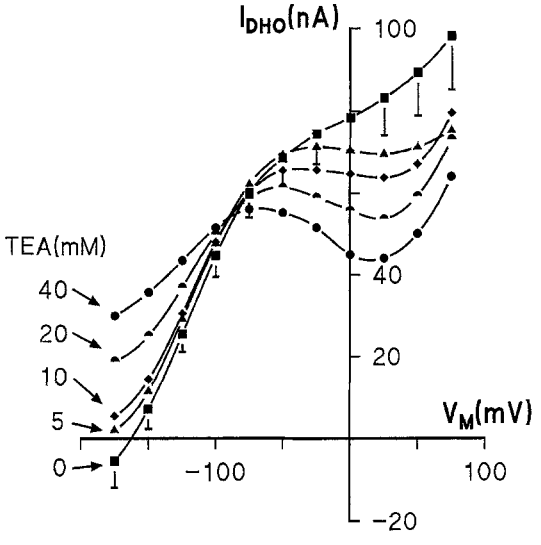

Fig. 5. Averaged dihydroouabain-sensitive currents in the presence of TEA^+ concentrations varying from 0 to 40 mM. Experiments were performed on eight oocytes where every TEA^+ concentration could be tested. SE bars are shown only in the absence of TEA^+ for reasons of clarity.

the absence and in the presence of DHO indicates that part of the TEA^+ effects were somehow linked to the Na^+-K^+-ATPase activity. By subtracting the data of Fig. 3 from the data of Fig. 2, one can calculate the I_{DHO}-V_M curves at different [TEA^+]s. I_{DHO} values averaged from eight different oocytes are plotted against V_M in Fig. 5. It can be seen that TEA^+ has a profound effect on I_{DHO} and that this effect reverses around −75 mV, indicative of an inhibitory effect of TEA^+ on a K^+ current. Taken directly from the average currents of Fig. 5, the effects of TEA^+ on I_{DHO} at different membrane potentials are represented in Fig. 6. Dissociation constants (K_D) and maximal TEA^+-sensitive currents (I_{MAX}) were calculated using a one-site ligand binding equation for the membrane potential range where the effects of TEA^+ were saturable. SE on the averaged TEA^+-sensitive currents were used to obtain weighting coefficients (=1/SE) in the curve-fitting procedure (FigP version 6.0, Biosoft, Ferguson, MO) and error bars for K_D and I_{MAX} represent the SE corresponding to the square root of the variance on the estimated parameters. K_D appeared to be voltage sensitive and, at +50 mV, averaged 25 ± 4 mM.

If the effect of TEA^+ on I_{DHO} is through some K^+

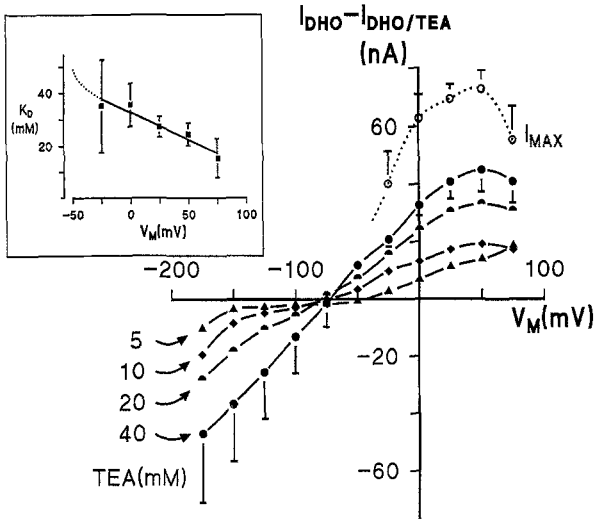

Fig. 6. Averaged TEA^+-sensitive currents present in I_{DHO}. SE bars are shown only in the presence of 40 mM TEA^+ for reasons of clarity. The TEA^+ dissociation constant (K_D) and the maximal TEA^+-sensitive currents (I_{MAX}) were calculated between −25 and +75 mV where the effect of TEA^+ appeared saturable.

channels which are somehow dependent on the Na^+ pump activity, it should also be affected by Ba^{2+}, a voltage-dependent inhibitor of many K^+ channel families. The effect of TEA^+ was therefore tested on a series of four oocytes bathed in a saline solution (identical to the Barth* solution but replacing NO_3^-, PO_4^- and SO_4^- with Cl^-) containing 1 mM Ba^{2+}. The total oocyte *I-V* relationships are illustrated in Fig. 7. The most striking effect of Ba^{2+} was to completely remove the inward diffusion of TEA^+ (as seen in Fig. 2), whereas the saturable component remained present. As this saturable component was also DHO sensitive, TEA^+ has no effect on oocyte currents in the presence of 10 μM DHO and 1 mM Ba^{2+}.

Average I_{DHO}-V_M currents measured in the presence of 1 mM Ba^{2+} are plotted in Fig. 8. While TEA^+ still had its effect on I_{DHO} for V_M values more positive than −75 mV, the TEA^+ effect at more negative potentials disappeared. This is a typical voltage-dependent effect of Ba^{2+} on K^+ currents; a low concentration of Ba^{2+} is only effective in blocking inward K^+ currents. This supports our contention that the effect of TEA^+ on I_{DHO} is through inhibition of K^+ channels whose activity is closely related to the Na^+ pump activity. In the presence of Ba^{2+}, the TEA^+ dissociation constant for blocking this K^+ current is around 20 mM and appears slightly voltage sensitive in the voltage range (from −50 to +75 mV) where the TEA^+-sensitive currents are large enough to be accurately fitted with the Michaelis-Menten equation. Using the maximal TEA^+-sensitive current given by the Michaelis-Menten equation, the shape of the I_{DHO}-V_M curve that would be obtained with a saturating [TEA^+] could be extrapolated (Fig. 8).

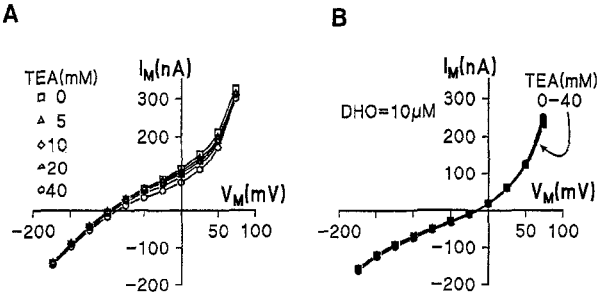

Fig. 7. Typical example of the effect of TEA^+ in the presence of 1 mM Ba^{2+}. *A* depicts the TEA^+ effect in the presence of an active Na^+ pump while *B* is in the presence of 10 μM DHO.

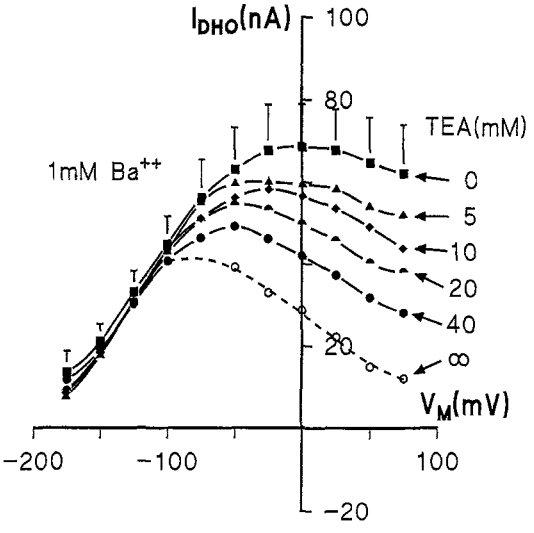

Fig. 8. Average dihydroouabain-sensitive current in the presence of 1 mM Ba^{2+} and TEA^+ concentrations ranging from 0 to 40 mM. Experiments were performed on four oocytes where every TEA^+ concentration could be tested. SE bars are shown only in the absence of TEA^+ for reasons of clarity. Open circles represent the expected currents to be seen in the presence of a saturating concentration of TEA^+, based on the voltage-dependent affinity of TEA^+ for blocking the putative K^+ current (K_D varying from 20 mM at positive membrane potentials to 35 mM at −25 mV).

Discussion

TEA^+ Effects Observed on Oocyte Currents

In Fig. 9, we propose a model for the oocyte that explains the main features of the TEA^+ effects which will be further discussed below. The most important electrophysiological effect of TEA^+ observed in the present study is a simple electrodiffusion through Ba^{2+}-sensitive channels (which are probably K^+ selective). In addition to these channels, TEA^+ can block a different class of channels closely associated to the Na^+ pump activity. These channels were identified as K^+ channels since their reversal potential is −75 mV and they are Ba^{2+} sensitive

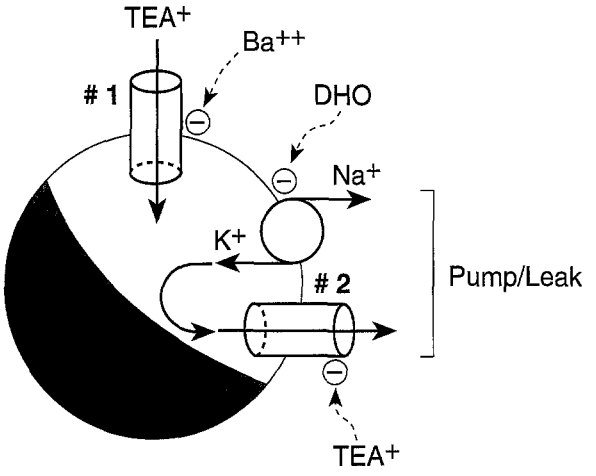

Fig. 9. Summary of the currents involved in the TEA^+ effects on I_{DHO} in *X. laevis* oocytes. The proposed model includes Ba^{2+}-sensitive TEA^+ diffusion and the TEA^+-sensitive K^+ channel whose activity is linked to the Na^+ pump activity.

in a voltage-dependent manner. The two types of channels involved in the TEA^+ effects are thought to be distinct since electrodiffusion of TEA^+ remains intact in the presence of DHO while the activity of the Na^+-pump-related channel becomes undetectable.

Contamination of I_{DHO} with a K^+ Current

Since 1986 [13], several laboratories have taken advantage of *X. laevis* oocytes to study Na^+-K^+-ATPase activity in conditions where V_M can be accurately controlled. Much attention was paid to the fact that Na^+ pump inhibition is expected to decrease the $[K^+]$ gradient in the immediate vicinity of the membrane, which would change the K^+ current at the same time as the Na^+ pump current. The effect of such an artifact would be to shift the *I-V* relationship of the K^+ current toward more positive V_M values during Na^+ pump inhibition, which would produce an extra outward current (of varying amplitude) in I_{DHO} at every V_M including the reversal potential for K^+. The data presented in this study show something completely different and, at first sight, unexpected: the effect of TEA^+ on the dihydroouabain-sensitive current indicates that a K^+ current is included in the calculation of I_{DHO} when it is measured in the absence of efficient K^+ channel blockers. This contaminating current reverses around the expected E_K which is incompatible with effects due to K^+ accumulation/depletion in extra- and intracellular unstirred layers upon Na^+ pump inhibition. For the same reason (reversal at E_K), a direct effect of TEA^+ on the Na^+-K^+-ATPase is unlikely. For example, if TEA^+ interfered with K^+ binding to the Na^+ pump, I_{DHO} would be reduced across the entire voltage range studied. As the TEA^+-sensitive current in I_{DHO} is also Ba^{2+}-sensitive (for a given voltage range) it is very likely that it comes from a K^+ channel whose activity "follows" Na^+-K^+-ATPase activity.

In 1988, Schweigert et al [23] measured I_{DHO} in the presence of 5 mM Ba^{2+} or 20 mM TEA^+ to check if I_{DHO} was contaminated by passive K^+ currents. They concluded that the general shape of the I_{DHO}-V_M curve was not affected by these K^+ channel blockers. Conversely, DHO did not affect other electrogenic transport mechanisms as long as the Na^+ pump was already inhibited using a K^+-free bathing solution. While we agree on the latter conclusion (DHO effect on K^+ current is not direct but through Na^+ pump inhibition), our data contradict their first conclusion. As recognized by these authors [23], seasonal variations in the shape of the I_{DHO}-V_M curve (maximal value of I_{DHO} and in its position on the voltage axis) were observed. Because of this, we feel that any accurate comparison between I_{DHO}-V_M curves needs to be done on a paired basis and that the comparison between I_{DHO} measured in the presence of TEA^+ [23] and previously published measurements [13] is irrelevant. In addition, although it was not studied systematically, seasonal variations on the TEA^+ effects were also observed, especially for TEA^+ diffusion which can vary over a wide range. It is possible that similar variations exist in the expression of the K^+ channel linked to the Na^+-pump activity, explaining why the effect of TEA^+ remained undetected in preceding studies.

Pump-coupled K^+ Channels and the Coupling Mechanism

The channel coupled to the Na^+ pump activity has a dissociation constant for TEA^+ around 20 mM and this constant appears to increase as V_M is made more negative. TEA^+ is known to block a variety of voltage-dependent K^+ channels in excitable tissues with apparent dissociation constants varying from 0.4 mM in the node of Ranvier [11] to 12 mM in molluscan neuron [9, 16, 25]. Interestingly, in *Rana esculenta* oocytes, a voltage-dependent K^+ current involved in maintaining membrane potential was described as being weakly TEA^+ sensitive since 50 mM did not inhibit it completely [17]. Not enough is known yet about the K^+ channels of *X. laevis* oocytes [5] to be able to speculate on the identity of the K^+ channel functionally linked to the Na^+ pump.

Neither is anything known about the mechanism coupling the Na^+ pump activity to the K^+ current in *X. laevis* oocytes. In other tissues, it has been shown that K^+ channels could be regulated as a function of Na^+ pump activity through changes in some internal effector such as pH, Ca^{2+}, ATP, etc. [4, 14, 21, 22]. It could also be due to selective internalization of specific K^+ channels during pump inhibition or, alternatively, to the presence of certain pump conformations acting as K^+ channels.

It is not known if the K^+ channel involved in the present experiment is either pH or Ca^{2+} sensitive, but ATP-sensitive K^+ channels do not seem to be present in defolliculated oocytes as there are no effects caused by K^+ channel openers [28] nor are there any effects of sulfonylurea (*unpublished observation*). Again, identification of the coupling mechanism involved in the oocyte pump-leak mechanism must await a better microscopic (patch clamp level) knowledge of the K^+ channels present on the oocyte plasma membrane.

Estimation of the Real *I-V* Curve of the Na Pump

The conclusion supported by the present study is that TEA^+ does not affect the Na^+ pump itself but inhibits a K^+ current closely associated to it. Knowing the dissociation constant and the maximal current blocked by TEA^+, it is possible to predict the shape of the real Na^+ pump *I-V* curve. This prediction is depicted by the dotted line in Fig. 8. Clearly, using 20 mM TEA^+, as has been done in the past, does not appear sufficient to completely eliminate the K^+ current that downregulates with the Na^+ pump activity. From Fig. 8, it can be concluded that the absence of efficient K^+ channel blockers leads to underestimation of the negative slope in the pump *I-V* relationship. This effect may have important consequences on the conclusions previously reached using detailed kinetic analysis of the voltage-dependent properties of the Na^+ pump.

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