H$_2$O$_2$-Modification of Na,K-ATPase

Alterations in External Na$^+$ and K$^+$ Stimulation of K$^+$ Influx

Margaret H. Garner, William H. Garner, and Abraham Spector

Studies, at steady state, of the Na,K-ATPase dependent influx of K$^+$ into bovine lenses in organ culture are used to characterize further the H$_2$O$_2$-modification of the Na$^+$ pump. Control lenses display constants for interaction with external Na$^+$ and K$^+$ similar to those obtained for the erythrocyte. H$_2$O$_2$ treatment of the bovine lens leads to total loss of external Na$^+$ stimulation and alteration of external K$^+$ stimulation.


In human senile cataract, intracellular concentrations of Na$^+$ and K$^+$ are altered (1), the (Na,K)-ATPase is increasingly inhibited (2), the sulfur containing amino acid side chains of the lens proteins are oxidized (3), and in a certain population of cataract patients, abnormally high concentrations of H$_2$O$_2$ are found in the aqueous humor and the lens (4).

H$_2$O$_2$ inhibits the influx of $^{86}$Rb$^+$ (K$^+$) into cultured lenses. In the cultured bovine lens, H$_2$O$_2$ inhibits the influx of $^{86}$Rb$^+$ by direct modification of the (Na,K)-ATPase. The H$_2$O$_2$-modified enzyme would appear to have two binding sites for eosin maleimide and two p-nitrophenylphosphate hydrolysis sites as indicated by a twofold increase in the maximal velocity (V max). ATP hydrolysis of the H$_2$O$_2$-modified enzyme is positively cooperative, unlike the functionally unmodified enzyme for which the hydrolysis of ATP is negatively cooperative. While K$^+$ stimulation and inhibition of ATP hydrolysis is normal in the H$_2$O$_2$-modified enzyme, Na$^+$ stimulation of ATP hydrolysis is altered (K$_{50}$ Na$^+$ = 20 mM for the unmodified enzyme and 34 mM for the H$_2$O$_2$-modified enzyme).

In order to relate the observed changes in substrate hydrolysis kinetics and allosteric effector control of substrate hydrolysis to cation flux, the effect of extracellular Na$^+$ and K$^+$ upon K$^+$ influx has been studied in H$_2$O$_2$-treated and untreated, cultured bovine lenses. The results of this study confirm the earlier observations that H$_2$O$_2$ modification inhibits K$^+$ influx. The results demonstrate that, for the untreated lenses, K$^+$ influx is dependent upon both extracellular K$^+$ and Na$^+$. After H$_2$O$_2$ treatment, as demonstrated in this paper, external Na$^+$ no longer affects (Na,K)-ATPase dependent K$^+$ influx.

Materials and Methods

TC199 and a penicillin, fungizone, streptomycin solution were obtained from DIFCO (Detroit, MI). Glucose oxidase, catalase, ouabain, and choline chloride were obtained from Sigma Chemical; St. Louis, MO 63178. The $^{86}$RbCl (1.86 mCi mg$^{-1}$) was obtained from Sigma Chemical; St. Louis, MO 63178. The $^{86}$RbCl (1.86 mCi mg$^{-1}$) was obtained from Sigma Chemical; St. Louis, MO 63178. The $^{86}$RbCl (1.86 mCi mg$^{-1}$) was obtained from Sigma Chemical; St. Louis, MO 63178.

Experimental Procedure

Paired bovine eyes were processed within 4 hr of death. Lenses (1.4-1.7 g) were removed by posterior extraction. Preincubation of the lenses in bicarbonate modified TC199 medium and incubation of control lenses in the presence and absence of H$_2$O$_2$ were performed as described previously.

The 3-hr post-incubations, after the H$_2$O$_2$ had been destroyed, were performed in modified Tyrode's medium containing appropriate concentrations of Na$^+$ and K$^+$. Choline chloride was used to replace either Na$^+$ or K$^+$ in media which were prepared deficient in either one of the two cations. The total osmolality of these media was kept at 290 ± 5 mOsm as measured using an Osmette A osmometer (Precision Systems, Inc.; Sudbury, MA). The [Na$^+$] and [K$^+$] in the media were measured with ion specific electrodes using an Ion 85 meter (Radiometer America; West Lake, OH). Incubation in an atmosphere of 5% CO$_2$ was not necessary during the interval in which the lenses were in the Tyrode's media.

During the 3-hr, post-incubation period, $^{86}$Rb influx was measured. Five $\mu$Ci of $^{86}$Rb were added to 10 ml of...
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Fig. 1. Plots to evaluate the effect of external K⁺ (top) at Na⁺ = 135 mM and external Na⁺ (bottom) at K⁺ = 3.1 mM upon the non-ouabain sensitive influx of K⁺ into bovine lenses using ⁸⁶Rb⁺ as a tracer. Units for the influx are μmol ml⁻¹ hr⁻¹. Units for K⁺ and Na⁺ are mM. The dashed curves represent the 95% confidence limits for the regression.

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of culture medium in all cases. Counts in the medium and lenses were determined using the Cerenkov radiation technique on a 1217 Rack Beta liquid scintillation counter, LKB Instruments, Inc., Gaithersburg, MD. In experiments in which ouabain was used to inhibit (Na,K)-ATPase dependent K⁺ influx, the ouabain was added to the culture medium at the same time as the radioactive label. Effective K⁺ influx was calculated by multiplying the lens:medium (L/M) ratio by the external [K⁺], expressed as μmol ml⁻¹.

Treatment of the Results

Both Na⁺ and K⁺ interact with (Na,K)-ATPase cooperatively. Therefore, the relative velocity Vᵢ, calculated by dividing the observed (Na,K)-ATPase dependent rate of K⁺ influx by the highest observed rate of pump dependent K⁺ influx for the control lenses) was related to either Na⁺ or K⁺ as an effector using equation 1:

\[ V_r = V_i + \frac{(V_r max - V_i)/(1 + K_i/[M_j])^{n_i}}{1} \]

This general expression relates the relative velocity (Vᵢ) to a modified Hill function. The number of sites for the extracellular cation in question ([M_j] = [Na⁺] or [K⁺]) and the monovalent cation concentration for half maximal influx are indicated by n_i and K_i, respectively. V_i is included to account for residual velocity as the concentration of M_j approaches 0, while the other external monovalent cation is saturating its sites. Presumably, for all these experiments, the internal sites for both monovalent cations are saturated. When no influx is observed in the absence of the metal (M_j) ion in question, equation (1) reduces to the familiar Hill equation:

\[ V_r = \frac{V_r max}{1 + K_i/[M_j]^{n_i}} \]

Results

Experiments were first performed to compare the transport of K⁺ in the Tyrode's medium to transport in the bicarbonate modified TC199 medium used in the previous study. Lenses (10 pairs) were pre-incubated in the bicarbonate modified TC199 medium, then one lens of each pair was placed in a Tyrode's medium that contained the same amount of Na⁺ and K⁺ as the TC199 medium. After 3 h, ⁸⁶Rb influx was the same in the two media (L/M ratio (Tyrode's) = 0.31 ± 0.05, L/M ratio (TC199) = 0.32 ± 0.4).

In order to examine the (Na,K)-ATPase dependent (or active component) influx of K⁺ into the cultured bovine lenses, the (Na,K)-ATPase independent (or passive component) influx of K⁺ had to be determined at varying K⁺ and Na⁺ concentrations. Using ⁸⁶Rb as a tracer, this was accomplished by performing influx experiments in the presence of 2 mM ouabain, a specific inhibitor of (Na,K)-ATPase. The results, Figure 1, were fit using linear regression analysis. For K⁺ effects on K⁺ influx (Figure 1, top), the slope of the line was 0.104 ± 0.006, with an intercept of 0.0 and a linear correlation coefficient (R²) of 0.97. The results for Na⁺ (Fig. 1, bottom) indicate that there is no significant correlation between external [Na⁺] and ouabain insensitive K⁺ influx (multiple R² = 0.34 P > .15). For all succeeding experiments, the (Na,K)-ATPase dependent influx (or net active flux) is reported as the difference between the total measured influx and the ouabain insensitive influx as determined from Figure 1.

Since the analysis of Na⁺ and K⁺ dependent substrate hydrolysis suggested a change in the interaction
of Na\(^+\) with the (Na,K)-ATPase modified with H\(_2\)O\(_2\),\(^9\) the effect of H\(_2\)O\(_2\) upon Na\(^+\) stimulation of K\(^+\) influx was determined between 0 mM and 135 mM. The results are graphed in Figure 2. The curve through the experimental points for the untreated lenses was determined using equation 1. The value for \(V_0\), \(K_{50}\), and \(n\) for the Na\(^+\) stimulation of K\(^+\) influx were 0.46 \(\pm\) 0.02, 63.9 \(\pm\) 5.0, and 3.9 \(\pm\) 1.2, respectively. The multiple \(R^2\) was 0.98. The results for the H\(_2\)O\(_2\)-treated lenses, also plotted in Figure 2, indicate that Na\(^+\) no longer stimulates K\(^+\) influx. There is no significant correlation between K\(^+\) influx and [Na\(^+\)] (multiple \(R^2\) = 0.28, \(P > 0.2\)), since K\(^+\) influx remains constant at 0.06 \(\pm\) 0.01 mM hr\(^{-1}\) (\(P > 0.001\)).

Analysis of the ion-dependent ATP substrate hydrolysis\(^9\) indicated no apparent change in the interaction of the H\(_2\)O\(_2\)-modified enzyme with K\(^+\). To test if this observation was true also for K\(^+\) influx, the dependence of K\(^+\) influx on extracellular K\(^+\) was determined in modified Tyrode's medium containing 135 mM Na\(^+\). The [K\(^+\)] was varied from 0 to 26.1 mM. For the control lenses, external Na\(^+\) appeared to be necessary for maximal K\(^+\) influx, Figure 2. Therefore, an experiment was also designed to study the effect of external K\(^+\) upon K\(^+\) influx in the absence of Na\(^+\). (It should be noted that a similar experiment, of course, could not be performed for H\(_2\)O\(_2\)-treated lenses since the data, shown in Figure 2, indicated that [Na\(^+\)] has no stimulating effect upon the H\(_2\)O\(_2\)-treated lenses). The results for K\(^+\) stimulation are plotted in Figure 3. Curves through the experimental points were determined by fitting the data to equation 2. The shape of the curves for the control (●) and H\(_2\)O\(_2\) (○) (Na\(^+\) = 135 mM) indicates that K\(^+\) is a positively cooperative effector of K\(^+\) influx. The control lenses in the absence of Na\(^+\) (▲) display simple Michaelis-Menten behavior. The values for \(V_{max}\), \(K_{50}\), and \(n\) (equation 2) for K\(^+\) stimulation are collected in Table 1. The apparent \(K_{50}\), in the more physiological high Na\(^+\) containing medium for the H\(_2\)O\(_2\)-treated lenses is approximately three times greater than the \(K_{50}\) for the control lenses. The \(V_{max}\) is only 50% of the value for the control lenses. In the Na\(^+\) free medium, the \(V_{max}\) for the control lenses is

![Graph](http://iovs.arvojournals.org/pdfaccess.ashx?url=/data/journals/iovs/933126/ on 06/24/2017)

**Table 1.** Fitted values for K\(^+\) stimulation of K\(^+\) influx\(^*\)

<table>
<thead>
<tr>
<th></th>
<th>Control lens [Na(^+)] = 0</th>
<th>Control lens [Na(^+)] = 135 mM</th>
<th>H(_2)O(_2) lens [Na(^+)] = 135 mM</th>
</tr>
</thead>
<tbody>
<tr>
<td>(V_{max})</td>
<td>0.57 (\pm) 0.06</td>
<td>0.89 (\pm) 0.04</td>
<td>0.47 (\pm) 0.01</td>
</tr>
<tr>
<td>(K_{50})</td>
<td>0.73 (\pm) 0.18</td>
<td>1.54 (\pm) 0.15</td>
<td>4.28 (\pm) 0.12</td>
</tr>
<tr>
<td>(n)</td>
<td>1.14 (\pm) 0.18</td>
<td>2.76 (\pm) 0.59</td>
<td>2.97 (\pm) 0.23</td>
</tr>
<tr>
<td>(R^2)</td>
<td>0.998</td>
<td>0.993</td>
<td>0.998</td>
</tr>
</tbody>
</table>

\(^*\) See text for description of terms.

\(^\dagger\) Units for \(K_{50}\) are mM.
approximately 50% of that for the control lenses in the high Na+ -containing media.

Discussion

Since Na+ and K+ control of Na,K-ATPase is complex, the monovalent cation sites of the unmodified enzyme must be described if H2O2's dramatic alteration of pump dependent K+ influx is to be fully understood. This goal is best accomplished using the dimer relaxation mechanism of Swann and Albers, Figure 4.11 The mechanism includes a single binding site for ATP between two functional subunits, two different conformations (R and T), and three sets of monovalent cation binding sites (A, B, and C). At each set of binding sites, either Na+ or K+ can bind. Each set of monovalent cation binding sites is easily distinguished from the others by its affinity for K+.11,13 As demonstrated in Table 2, the A, B, and C sites have low (K50 > 100 mM), moderate (K50 > 1 mM), and high (K50 < 1 mM) affinity, respectively, for K+. The B and C sites are on the extracellular membrane surface; the A sites are on the intracellular surface.14,15 The A and B sites are present on the free enzyme. K+ binding to the A and B sites is demonstrable in the phosphatase reaction in the absence of ATP and Na+.9,11,13 The C sites are demonstrable only after a conformational change which occurs either by formation of the phosphoenzyme or binding of K+ or Na+ plus K+ to the A and B sites (Fig. 4).11 The C sites are demonstrable only after a conformational change which occurs either by formation of the phosphoenzyme or binding of K+ or Na+ plus K+ to the A and B sites (Fig. 4).11

In the transport experiments with the control bovine lenses, sites with moderate affinity for K+ (K50 = 0.73 mM), the C sites were observed (Fig. 3). When Na+ was removed from the incubation medium, sites with high affinity for K+ (K50 = 0.73 mM), the C sites were observed. In the absence of extracellular Na+, binding of K+ at the B sites does not occur. Binding of external Na+ to the C sites (K50 = 64 mM, Fig. 2) appears to be necessary for maximal K+ influx. Previously, it was demonstrated that Na+ binding to these sites is necessary for Na+ efflux from red blood cells.16 K+ and Na+ binding to the A sites and Na+ binding to the B sites were not observed in the K+ influx experiments. Presumably, these sites will become apparent in future studies to elucidate intracellular monovalent cation control of Na+ and K+ transport in bovine lenses.

After H2O2-modification, binding of Na+ or K+ to the C sites no longer stimulates K+ influx. External Na+ control is totally lost. There is no difference in K+ binding at 3.1 mM K+ in the presence or absence of Na+, indicating a loss of K+ binding at the C sites. After H2O2-modification, external K+ binding at the B sites still stimulates K+ influx. This value is similar to the value obtained for the bovine brain enzyme and similar to the value obtained for K+ stimulation of the phosphatase reaction for (Na,K)-ATPase isolated from lenses incubated in the presence and absence of H2O2.9,11 Although Vmax for H2O2-treated lenses is 47% of the Vmax for the control lenses, at physiological concentrations of external K+ (2-5 mM), the H2O2-treated lens (Na,K)-ATPase pumps at 5 to 25% of the control enzyme. At similar external K+ concentrations, the control enzyme is pumping at 50-90% of its maximal velocity.

Key words: lens, H2O2, Na,K-ATPase, transport
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References