Dietary cholesterol alters Na⁺/K⁺ selectivity at intracellular Na⁺/K⁺ pump sites in cardiac myocytes

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Dietary cholesterol alters Na⁺/K⁺ selectivity at intracellular Na⁺/K⁺ pump sites in cardiac myocytes. Am J Physiol Cell Physiol 286: C398–C405, 2004. First published October 1, 2003; 10.1152/ajpcell.00016.2003.—A modest diet-induced increase in serum cholesterol in rabbits increases the sensitivity of the sarcolemmal Na⁺/K⁺ pump to intracellular Na⁺, whereas a large increase in cholesterol levels decreases the sensitivity to Na⁺. To examine the mechanisms, we isolated cardiac myocytes from controls and from rabbits with diet-induced increases in serum cholesterol. The myocytes were voltage clamped with the use of patch pipettes that contained osmotically balanced solutions with Na⁺ in a concentration of 10 mM and K⁺ in concentrations ([K⁺]pip) ranging from 0 to 140 mM. There was no effect of dietary cholesterol on electrogenic Na⁺/K⁺ current (Ip) when pipette solutions were K⁺ free. A modest increase in serum cholesterol caused a [K⁺]pip-dependent increase in Ip, whereas a large increase caused a [K⁺]pip-dependent decrease in Ip. Modeling suggested that pump stimulation with a modest increase in serum cholesterol can be explained by a decrease in the microscopic association constant Kₐ describing the backward reaction E₁ + 2K⁺ → E₂(K⁺)₂, whereas pump inhibition with a large increase in serum cholesterol can be explained by an increase in Kₐ. Because hypercholesterolemia upregulates angiotensin II receptors and because angiotensin II regulates the Na⁺/K⁺ pump in cardiac myocytes in a [K⁺]pip-dependent manner, we blocked angiotensin synthesis or angiotensin II receptors in vivo in cholesterol-fed rabbits. This abolished cholesterol-induced pump inhibition. Because the ε-isotype of protein kinase C (εPKC) mediates effects of angiotensin II on the pump, we included specific εPKC-blocking peptide in patch pipette filling solutions. The peptide reversed cholesterol-induced pump inhibition.

The sensitivity of the pump to Na⁺ reflects binding to three cytosolic sites. Binding of Na⁺ at one site occurs inside the membrane dielectric in a voltage-dependent, highly selective manner. Binding at the two other sites is nonselective and occurs in competition with intracellular K⁺ ([K⁺]m) (1). The K⁺/Na⁺ antagonism exhibited by the Na⁺/K⁺ pump varies between different tissues, is particularly pronounced in the heart (32), and is subject to regulation by intracellular signal pathways (4, 5). The latter can be demonstrated experimentally as a dependence of regulation of the pump on the K⁺ concentration in patch pipette filling solutions ([K⁺]pip) used to measure Ip of voltage-clamped isolated ventricular myocytes (5). Regulation of voltage-dependent binding of Na⁺ can be demonstrated as a dependence on the test potential (Vₚ) used to voltage clamp myocytes (3, 12).

Interest in the sarcolemmal Na⁺/K⁺ pump has traditionally been focused on its role in excitation-contraction coupling and its putative role as a “receptor” for cardiac glycosides. However, strong evidence is emerging indicating that the pump has a much broader role in cell function by interacting with and modulating multiple intracellular signal pathways (35). The mechanism for the effect of dietary cholesterol supplementation on the sensitivity of the Na⁺/K⁺ pump to Na⁺ may therefore have implications for the pathogenesis and treatment of cardiovascular manifestations of hypercholesterolemia. Voltage and [K⁺]pip dependence of Ip are regulated by distinctly different intracellular signal pathways (3–5, 12) and are subject to control by hormones that can be manipulated therapeutically (12, 14, 15). The primary objective of this study was to examine the dependence of cholesterol-induced changes in Ip on [K⁺]pip and Vₚ. Because we found that cholesterol-induced changes in Ip were dependent upon [K⁺]pip and independent of Vₚ, we also examined how the changes are influenced by an in vitro experimental manipulation of a messenger pathway known to regulate K⁺/Na⁺ antagonism of the pump and an in vivo pharmacological intervention known to alter this antagonism.

MATERIALS AND METHODS

Treatment protocols. We fed male New Zealand White rabbits standard chow for 4 wk or the same chow supplemented with cholesterol as described previously (10). Most rabbits were given chow containing 1% cholesterol for 4 wk. A small number were given 0.3% cholesterol for 1 wk. Some rabbits given cholesterol-containing chow were treated with the angiotensin-converting enzyme (ACE)

A MODEST DIET-INDUCED INCREASE in serum cholesterol in rabbits can induce an increase in electrogenic Na⁺/K⁺ pump current (Iₚ) in cardiac myocytes. In contrast, a large increase in serum cholesterol is associated with pump inhibition to levels below those of myocytes from rabbits fed a standard diet. The changes in Iₚ can only be demonstrated when the intracellular Na⁺ concentration is near physiological levels. There is no effect of hypercholesterolemia on Iₚ measured when intracellular Na⁺ ([Na⁺]m) is at high levels expected to nearly saturate Na⁺/K⁺ pump sites (10). These findings indicate that dietary cholesterol is a determinant of the sensitivity of the sarcolemmal Na⁺/K⁺ pump to intracellular Na⁺.

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inhibitor captopril in a dose of 8 mg·kg⁻¹·24 h⁻¹ (15) or the
angiotensin (AT₁) receptor antagonist losartan in a dose of 25
mg·kg⁻¹·24 h⁻¹ (5). The drugs were given the last 8 days before
death.

Measurement of \( I_p \). Single ventricular myocytes were isolated as
described previously (15) and stored at room temperature in Krebs-
Henseleit buffer solution until used for patch-clamp studies. In some
experiments, myocytes were incubated with 10 nM angiotensin II
(ANG II) before patch-clamp studies (14). For measurement of \( I_p \) at
a fixed \( V_m \) of ~40 mV, wide-tipped (4–5 μm) patch pipettes were
filled with solutions containing (in mM) 9 Na-glutamate, 1 KH₂PO₄,
5 Na₂-hydroxyethylpiperazin-N'-2-ethanesulfonic acid (HEPES), 2
MgATP, 5 ethylene glycol-bis(β-aminoethoxy) ether)-N,N,N',N'-tetra-
acetic acid (EGTA), and 0 KCl to 140 mM. Osmotic balance of
pipette solutions was maintained with 150 to 10 mM tetrathylam-
monium chloride (TMA-Cl). The solutions were titrated to a pH of
7.05 ± 0.01 at 35°C with 1 M TMA-OH. In experiments designed to
determine the relationship between \( I_p \) and \( V_m \), we used patch pipettes
containing (in mM) 10 Na-glutamate, 1 KH₂PO₄, 5 HEPES, 5 EGTA,
2 MgATP, 60 TMA-Cl, 20 tetraethylammonium chloride, 70 CsOH,
and 50 aspartic acid. Solutions were titrated to a pH of 7.05 ± 0.01
at 35°C with 1 M HCl. Filled patch pipettes had resistances of
0.8–1.1 MΩ.

Myocytes were suspended in a tissue bath mounted on an inverted
microscope for determination of \( I_p \). The bath was perfused with
modified Tyrode’s solution which contained (in mM) 140 NaCl, 5.6
KCl, 2.16 CaCl₂, 1 MgCl₂, 0.44 NaH₂PO₄, 10 glucose, and 10
HEPES. The solution was titrated to a pH of 7.40 ± 0.01 at 35°C
with NaOH. When the whole cell configuration had been established,
we switched to a superfusate that was identical except that it was
ominally Ca²⁺ free and contained 0.2 mM CdCl₂ and 2 mM BaCl₂.
\( I_p \) was identified as the shift in holding current induced by superfusion
of 100 μM ouabain ~12 min after the whole cell configuration had
been established. \( I_p \) is reported normalized for membrane capacitance
unless otherwise indicated. Membrane currents were recorded using
the single electrode voltage-clamp mode of an Axoclamp-2B ampli-
fer before and after superfusion of

Reagents and chemicals. TMA-Cl was “purum” grade and pur-
chased from Fluka (Switzerland). All other chemicals were “analyti-
cal” grade and purchased from BDH (Australia). Cholesterol, ANG II,
and ouabain were purchased from Sigma Chemical. Captopril was
purchased from Bristol-Myers Squibb Pharmaceuticals (Australia)
and losartan was donated by Merck, Sharpe, and Dohme (Australia).
The ePKC-blocking peptide was provided by Professor Mochly-
Rosen (Stanford University School of Medicine, CA). Voltage-clamp protocols were generated with pCLAMP.

Statistical analysis. Results are expressed as means ± SE unless
indicated otherwise. Student’s t-test for unpaired data was used for
statistical comparisons. We used Dunnett’s test when the same control
group was used for more than one comparison. \( I_p/V_m \) relationships
were compared using both linear regression and a Mann-Whitney rank
sum test. \( P < 0.05 \) was regarded as significant in all comparisons.
Differential rate equations describing the kinetics of the Na⁺/K⁺
pump’s partial reactions were integrated numerically using backward
differentiation formulae within a subroutine of the Numerical Algo-
risms Group (NAG) Fortran Library. Integration yielded the enzyme
steady-state turnover number, which can be compared with the steady-
state pump current, \( I_p \). The best fit of simulations to experimental data
was determined using nonlinear regression by applying Newton’s
method, also within a subroutine of the NAG Fortran Library. The
nonlinear regression procedure involved a two-step iterative process.
First, the set of simultaneous differential rate equations describing
the enzyme’s partial reactions considered were solved numerically for
each [K⁺]pip to obtain values of the pump turnover number at each value of [K⁺]pip. The normalized reductions in enzyme turnover at
increasing values of [K⁺]pip were then compared with the experi-
mental values of the normalized current inhibition, \( I_p \). If \( I_p \) was defined as the difference between \( I_p \) recorded using K⁺-free and K⁺-containing
patch pipette solutions. The sum of the squares of the residuals between the experimental and calculated values were minimized by
varying the value of \( K_i \) alone, the microscopic association constant
for binding of K⁺ ions to the two nonspecific binding sites on the E₁
form of the pump. Each iterative variation of the value of \( K_i \) thus
required the differential rate equations to be again solved numerically
so that the residuals could be calculated.

RESULTS

Effect of an increase in serum cholesterol on the dependence
of \( I_p \) on [K⁺]pip. We gave rabbits chow containing 1% chole-
sterol for 4 wk to examine whether a decrease in \( I_p \) with a large
increase in serum cholesterol is dependent on [K⁺]pip. The dietary
cholesterol supplementation resulted in a serum cho-
lesterol of 20.6 ± 2.9 mM, a level similar to that reported
previously for an identical feeding protocol and higher than
levels in control rabbits on cholesterol-free chow (~1 mM)
(10). We measured \( I_p \) in myocytes from 12 rabbits fed standard
chow and in myocytes from 10 rabbits fed a cholesterol-
containing chow using a [K⁺]pip of 0, 35, 70, or 140 mM. We
used a different [K⁺]pip in experiments on myocytes from
the same rabbit to reduce effects of inter-rabbit variability. Figure
1 shows representative traces of membrane currents during
measurement of \( I_p \) in myocytes from control rabbits and rabbits
fed cholesterol. Experiments were included when no drift in
holding current could be identified on the digital display of the
voltage-clamp amplifier before and after superfusion of
ouabain. Holding currents were sampled five times with an
electronic cursor every 5 s before and after superfusion of

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<th>[K⁺]pip (mM)</th>
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Fig. 1. Ouabain-induced shifts in holding current (\( I_h \)) of myo-
cytes isolated from control rabbits (con) and rabbits given chow
supplemented with 1% cholesterol for 4 wk (chol). The K⁺ con-
centrations refer to patch pipette solutions ([K⁺]pip) and \( C_m \)
indicates the cell capacitance. The shift in \( I_h \) and hence
electrogenic pump current (\( I_p \)) was similar in myocytes
isolated from control rabbits and rabbits fed cholesterol when [K⁺]pip
was 0 mM, whereas a difference in \( I_h \) between myocytes from
control rabbits and cholesterol-fed rabbits was apparent when
[K⁺]pip was 70 mM.
cholesterol level was 6.2 mM for myocytes from rabbits fed cholesterol-containing chow was similar when pipettes contained K\(^+\)-free, whereas they differed when [K\(^+\)]\(_{\text{pip}}\) was 172 ± 37 mM for myocytes from rabbits with large increases in serum cholesterol, and 37 ± 6 mM for myocytes from rabbits with modest increases in serum cholesterol.

To further analyze the effect of [K\(^+\)]\(_{\text{pip}}\) in myocytes from the three groups of rabbits, we adopted the formalism and nomenclature of the Post-Albers scheme (Fig. 4), often used to describe the partial reactions in the Na\(^+\)/K\(^+\) pump cycle. A K\(^+\)-induced acceleration of the backward reaction E\(_1\) + 2K\(^+\) → E\(_2\)K\(^+\)\(_2\) may account for the [K\(^+\)]\(_{\text{pip}}\)-dependent decrease in I\(_p\). The dependence of the rate constant, \(k_{\text{obs}}\) (obtained from the literature), for this reaction on cytosolic Na\(^+\) and K\(^+\) concentrations can be described by the following equation

\[
\begin{align*}
\frac{k_{\text{obs}}}{k_{\text{max}}} &= \frac{K_1^2[K^+]^2}{(1 + K_1^2[K^+]^2 + 2K_2^2[Na]^2 + 2K_1^2K_2^2[Na]^2 + K_1^2K_2^2[Na]^3)} \\
&= \frac{k_{\text{max}}}{K_1^2[K^+]^2}
\end{align*}
\]

\(k_{\text{max}}\) represents the maximum rate for the E\(_1\) + 2K\(^+\) → E\(_2\)K\(^+\)\(_2\) transition when two sites on E\(_1\) are fully occupied by K\(^+\).

The difference between mean levels of the samples defined I\(_p\) for each myocyte.

Figure 1 shows that I\(_p\) of myocytes from control rabbits and rabbits fed cholesterol-containing chow was similar when patch pipettes were K\(^+\)-free, whereas they differed when pipettes contained 70 mM K\(^+\). A summary of the I\(_p\) measured in myocytes from control rabbits and rabbits fed cholesterol at all [K\(^+\)]\(_{\text{pip}}\) used is shown in Fig. 2. The pump currents shown in Fig. 2 have been normalized to the membrane capacitance. The membrane capacitance for myocytes from control rabbits and from rabbits given cholesterol-supplemented chow were similar when pipette solutions were K\(^+\)-free. However, cholesterol supplementation induced a statistically significant increase in I\(_p\) when pipettes contained K\(^+\).

Figure 2 shows there was a decrease in I\(_p\) with an increase in [K\(^+\)]\(_{\text{pip}}\). To further characterize this [K\(^+\)]\(_{\text{pip}}\)-dependence, we measured the I\(_p\) at each [K\(^+\)]\(_{\text{pip}}\) of myocytes from the three groups of rabbits to the mean I\(_p\) obtained in the three different groups when pipette solutions were K\(^+\)-free. We then subtracted the normalized I\(_p\) at each [K\(^+\)]\(_{\text{pip}}\) from the latter to obtain \(I_p\) as a measure of the K\(^+\)-induced decrease in I\(_p\). We chose to normalize the data to avoid an implicit assumption of identity between sample means and population means of the I\(_p\) measured with K\(^+\)-free patch pipette filling solutions. The [K\(^+\)]\(_{\text{pip}}\)-I\(_p\) relationships are shown in Fig. 3. To further analyze the effect of [K\(^+\)]\(_{\text{pip}}\) in myocytes from the three groups of rabbits, we performed the formalism and nomenclature of the Post-Albers scheme (Fig. 4), often used to describe the partial reactions in the Na\(^+\)/K\(^+\) pump cycle. A K\(^+\)-induced acceleration of the backward reaction E\(_1\) + 2K\(^+\) → E\(_2\)K\(^+\)\(_2\) may account for the [K\(^+\)]\(_{\text{pip}}\)-dependent decrease in I\(_p\). The dependence of the rate constant, \(k_{\text{obs}}\) (obtained from the literature), for this reaction on cytosolic Na\(^+\) and K\(^+\) concentrations can be described by the following equation

\(k_{\text{obs}} = k_{\text{max}} \times \frac{K_1^2[K^+]^2}{(1 + K_1^2[K^+]^2 + 2K_2^2[Na]^2 + 2K_1^2K_2^2[Na]^2 + K_1^2K_2^2[Na]^3)}\)

\(k_{\text{max}}\) represents the maximum rate for the E\(_1\) + 2K\(^+\) → E\(_2\)K\(^+\)\(_2\) transition when two sites on E\(_1\) are fully occupied by K\(^+\).
Fig. 4. Simplified reaction scheme of the Na\(^+\)/K\(^+\)-ATPase, based on the Post-Albers model, showing those reactions considered to be rate-determining.

These are the ATP phosphorylation reaction \([E_1(Na^+)_3 \rightarrow E_2P(Na^+)_3]\); rate-limiting at low intracellular Na\(^+\) concentrations], the dephosphorylation reaction \([E_2P(Na^+)_3 \rightarrow E_2(K^+)_3]\); rate-limiting at low extracellular K\(^+\) concentrations] and the conformational change of unphosphorylated enzyme \([E_2(K^+)_2 \rightarrow E_1(Na^+)_3]\); rate-limiting when intracellular ATP, intracellular Na\(^+\) and extracellular K\(^+\) are all at saturating concentrations], \(k_{1obs}, k_{2obs}, k_{3obs}\) represent the rate constants that would be observed for each of these reactions at given concentrations of Na\(^+\), K\(^+\), and ATP. They are thus functions of the intracellular and extracellular Na\(^+\) and K\(^+\) concentrations and the intracellular ATP concentration. Similarly \(k_{1obs}, k_{2obs}, k_{3obs}\) represent the rate constant which would be observed for the backwards reaction \([E_1(Na^+)_3 \rightarrow E_2(K^+)_2]\) at given Na\(^+\), K\(^+\) and ATP concentrations. The maximum values of \(k_{1obs}, k_{2obs}, k_{3obs}\) and \(k_{3obs}\) under their relevant saturating concentrations used for fitting were \(70 \text{ s}^{-1}\), \(550 \text{ s}^{-1}\), \(180 \text{ s}^{-1}\), and \(312 \text{ s}^{-1}\).

K\(^+\) ions. \(K_N\) and \(K_K\) represent the microscopic association constants for Na\(^+\) and K\(^+\) ions for the two nonspecific binding sites on \(E_1\), and \(K_S\) represents the association constant for the third binding site specific for Na\(^+\). A \(K_N\) of \(550 \text{ s}^{-1}\), a \(K_S\) of \(1.2\times10^5 \text{ M}^{-1}\), and a \(K_K\) of \(3.9\times10^2 \text{ M}^{-1}\) were obtained from the literature as described previously (13). It is apparent from the equation that K\(^+\)-induced activation of the backward reaction should slow down the overall forward reaction. We examined whether a change in \(K_K\) provides a reasonable explanation for the cholesterol-induced stimulation of \(I_p\). We performed numerical simulations to take into account the kinetics of the reaction \(E_1 + 2K^+ \rightarrow E_2(K^+)_2\) described by equation (1), as well as other partial reactions in the pump cycle. The other reactions taken into account were phosphorylation \([E_1(Na^+)_3 \rightarrow E_2P]\), dephosphorylation \([E_2P \rightarrow E_2(K^+)_2]\), and the \(E_2(K^+)_2 \rightarrow E_1(Na^+)_3\) transition (see Fig. 4). The simulations were fitted to the experimental data using the nonlinear regression method described under Statistical analysis (MATERIALS AND METHODS) to obtain the best-fit values of \(K_K\). The fitted values of \(I_p (I_p, sim)\) calculated in this fashion are shown in Fig. 3. The \(K_K\) values for data from control myocytes and myocytes isolated from rabbits with large and modest increases in serum cholesterol were \(91 \pm 5 \text{ M}^{-1}\), \(118 \pm 6 \text{ M}^{-1}\), and \(65 \pm 5 \text{ M}^{-1}\), respectively. These values correspond to microscopic K\(^+\) dissociation constants of \(11.0 \pm 0.6 \text{ mM}\) for control myocytes and \(8.4 \pm 0.4\) and \(15 \pm 1 \text{ mM}\) for myocytes isolated from rabbits with large and modest increases in serum cholesterol.

\(^{1}\)The term microscopic indicates that we are referring to the intrinsic affinity of both the nonspecific binding sites for Na\(^+\) and K\(^+\) ions, as opposed to the enzyme’s macroscopic association constants, for which there are two values dependent on whether one or two ions are bound (6).

\(^{2}\)A detailed description of the model will be made available on request via E-mail by R. J. Clarke (clarke@chem.usyd.edu.au) or by the corresponding author.
The effect of treatment with captopril on mean cholesterol-supplemented chow but not treated with captopril. The mean serum cholesterol was 14.9 with captopril for the last 8 days before they were killed. The chow containing 1% cholesterol for 4 wk. They were treated rabid not given captopril. Another six rabbits were given Fig. 7. The treatment induced a signi

ificant from rabbits given cholesterol-supplemented chow is shown in control rabbits fed a standard diet. Figure 6 indicates there was in pipette solutions used to voltage clamp myocytes from also examined the effect of including

free pipette solutions. The ePKC-blocking peptide induced a significant increase in $I_p$ of myocytes isolated from rabbits fed a cholesterol-supplemented diet but had no detectable effect in myocytes from rabbits fed the standard diet. *Significant difference compared with chol.

**DISCUSSION**

The dependence of the sensitivity of the Na$^+/K^+$ pump to [Na$^+$]o on dietary cholesterol (10) could arise from effects on binding of Na$^+$ at selective sites within the membrane dielectric or at nonselective sites near the cytoplasmic surface. K$^+$ in pipette solutions inhibited $I_p$ in myocytes from all groups of rabbits. The K$_{0.5}$ for $I_p$ was $\sim$39 mM in myocytes from controls. This would appear to be at odds with a value of $\sim$9 mM that we have reported previously (13), suggesting that there may be a substantial difference in K$^+$ affinity between cells that should have identical characteristics. However, the experimentally determined value for K$_{0.5}$ is not a good indication of K$^+$ affinity because the value depends on experimental conditions that affect rates of partial reactions. Extracellular solutions in our previous study were Na$^+$ free, whereas they

![Graph showing the effect of ePKC blockade on myocytes](image_url)

**Fig. 6.** Effect of ePKC blockade. Myocytes were isolated from rabbits given a diet containing 1% cholesterol for 4 wk (chol) or a standard diet (stan). Patch pipette solutions contained ePKC-blocking peptide (ε-inh) or were peptide free. The ePKC-blocking peptide induced an increase in $I_p$ of myocytes isolated from rabbits fed a cholesterol-supplemented diet but had no detectable effect in myocytes from rabbits fed the standard diet. *Significant difference compared with chol.

![Graph showing the effect of captopril, losartan, and angiotensin II (angII) on myocytes](image_url)

**Fig. 7.** Effect of captopril, losartan, and angiotensin II (angII). Myocytes were isolated from rabbits given both cholesterol and captopril were exposed to angiotensin II in vitro. Results shown in columns indicated by stan and chol are also shown in Fig. 5. *Significant difference compared with chol; †significant difference compared with chol + cap.

The mean serum cholesterol was 25.1 ± 2.9 mM. This was not significantly different from the mean level of myocytes from rabbits given cholesterol-containing chow but no losartan. The mean levels of $I_p$ measured in myocytes from the two groups of rabbits treated with losartan are included in Fig. 7. They were similar to the levels recorded in myocytes from rabbits given standard diet and captopril.

The effect of ANG II receptor blockade implicates ANG II in the cholesterol-induced inhibition of $I_p$. To examine this further, we isolated myocytes from rabbits who were given cholesterol-containing chow and who were treated with captopril. The myocytes were incubated with 10 nM ANG II for 45 min after isolation and then resuspended in ANG II-free solutions until $I_p$ was measured. Details of this protocol have been described previously (14). The mean $I_p$ of myocytes from four rabbits is included in Fig. 7. ANG II induced a decrease in mean $I_p$ to a level characteristic of myocytes isolated from rabbits given the same cholesterol-supplemented chow but not captopril.
contained 140 mM Na\(^+\) in the present study. This is a likely cause for the difference between \(K_{0.5}\) because Na\(^+\) stimulates the forward rate-determining \(E_2(K^+)_2 \rightarrow E_1 + 2K^+\) conformational transition by binding to the E2 state (17). We used a model to evaluate the expected effect of extracellular Na\(^+\). The model should be considered a tool to examine if our data is consistent with the large amount of already published information on the kinetics of the Na\(^+\)/K\(^+\) pump cycle. However, it does not allow a firm testing of a specific hypothesis based on the data in the present study.

With a Na\(^+\)-induced increase in the \(E_2(K^+)_2 \rightarrow E_1 + 2K^+\) conformational transition, an increase in [K\(^+\)]\(_{off}\) is expected to be needed to accelerate the rate of the backward reaction, \(E_1 + 2K^+ \rightarrow E_2(K^+)_2\) and observe inhibition of \(I_p\). The effect of extracellular Na\(^+\) on the observed rate constant, \(k_{obs}\), for the \(E_2(K^+)_2 \rightarrow E_1 + 2K^+\) transition can be described (17) by the following equation

\[
k_{obs} = \frac{k_1 + k_3K_N[Na^+]_o + k_4K_A[ATP] + k_5K_N[Na^+]_o[ATP]}{(1 + K_N[Na^+]_o)(1 + K_A[ATP])}
\]

(2)

where \(k_1, k_3, k_4, k_5\), and \(k_i\) are the rate constants for the \(E_2(K^+)_2 \rightarrow E_1 + 2K^+\) transition when neither extracellular Na\(^+\) nor intracellular ATP are bound, when only extracellular Na\(^+\) is bound, when only intracellular ATP is bound, and when both extracellular Na\(^+\) and intracellular ATP are bound, respectively. \(K_N\) represents the association constant of extracellular Na\(^+\) to the E2 state, and \(K_A\) is the association constant of ATP to its low-affinity binding site. The values of the various parameters contained in Eq. 2 used for fitting were based on previously published measurements, i.e., \(k_1 = 2.3 \times 10^{-2} \text{s}^{-1}\) (1), \(k_3 = 0.8 \text{s}^{-1}\) (7), \(k_4 = 11 \text{s}^{-1}\) (17), \(k_5 = 54 \text{s}^{-1}\) (17), \(K_N = 32 \text{M}^{-1}\) (17) and \(K_A = 1.4 \times 10^4 \text{M}^{-1}\) (7).

When we took the extracellular Na\(^+\) concentration of 140 mM into account in the fitting procedure by using Eq. 2, we derived a \(K_N\) of 91 (±5) \text{M}^{-1}. A value of \(K_N\) of 100 \text{M}^{-1}, obtained from the literature (32), gave a good fit in the previous study performed using Na\(^+\)-free extracellular solutions (13). We conclude that there is good agreement between the microscopic association constants derived from data in two independent studies performed under very different experimental conditions, provided the specific conditions are taken into account in the analysis of the data. The findings in these studies on intact cardiac myocytes are also consistent with the kinetics determined by others in isolated cardiac sarcosomal Na\(^+\)/K\(^+\) ATPase (32).

**Cholesterol and the Na\(^+\)/K\(^+\) pump.** The kinetic model we used assumes competition between two of the intracellularly bound Na\(^+\) ions and two K\(^+\) ions. With such competition, membrane cholesterol might be expected to affect the affinity for both ligands. Cholesterol had an effect on the apparent K\(^+\) affinity, whereas the apparent Na\(^+\) affinity was unaffected. This selective K\(^+\)-dependent effect of cholesterol supplementation might indicate that Na\(^+\) and K\(^+\) are not binding to the same site. Alternatively, the ions may bind to the same site, but cholesterol may affect the rate of the \(E_1 + 2K^+ \rightarrow E_2(K^+)_2\) transition in the presence of K\(^+\) and have no effect on the \(E_1 \rightarrow E_2\) transition in the absence of K\(^+\), i.e., there may be some intrinsic difference in the conformation of the nonspecific ion binding sites when Na\(^+\) and K\(^+\) ions are bound.

The dependence of cholesterol-induced changes in \(I_p\) on [K\(^+\)]\(_{off}\) was consistent with changes in \(K_N\) and, hence, in the rate of the backward \(E_1 + 2K^+ \rightarrow E_2(K^+)_2\) reaction. This, in turn, should shift the equilibrium of the \(E_2(K^+)_2 \rightarrow E_1 + 2K^+\) reaction. Although the model was fitted to the data assuming there is a change in the K\(^+\) affinity alone, it cannot be excluded that other reactions in the pump cycle could be affected by dietary cholesterol supplementation. Any change that shifts the E1/E2 equilibrium could result in an apparent change in the K\(^+\) affinity, even in the absence of a change in the intrinsic binding characteristics of either ligand to the E1 form. The E1/E2 distribution of Na\(^+\)/K\(^+\) ATPase reconstituted in lipid vesicles has been reported to be cholesterol dependent in two studies (8, 38). However, interpretation of this is difficult because there was a discrepancy between the studies in the direction of the shift in E1/E2 equilibrium. The discrepancy was attributed to differences in preparations used and experimental conditions as discussed by Cornelius (8).

We found that modest and large increases in serum cholesterol were associated with opposite effects on \(I_p\). On the surface, this seems in good agreement with previous studies on isolated membrane fragments by Yeagle et al. (37). In vitro cholesterol stimulated Na\(^+\)/K\(^+\) ATPase activity at low concentrations and inhibited activity at high concentrations. Because there could be no effect on complex messenger pathways in these studies, one might invoke a direct effect of cholesterol on one or more partial reactions of the enzyme. However, these effects of in vitro exposure to cholesterol should not be invoked to explain the biphasic effect of dietary cholesterol supplementation in our study. Yeagle et al. (37) used experimental conditions expected to maximally stimulate ATPase activity. Because we have previously found that dietary cholesterol has no effect on \(I_p\) of cardiac myocytes when experimental conditions are expected to induce nearly maximal pump stimulation (10), it is probably not valid to directly compare our results with those of Yeagle et al. (37).

Effects of in vivo dietary cholesterol supplementation in this study are inevitably much more complex than in vitro manipulation of cholesterol in artificial membrane systems. In vivo dietary cholesterol may be absorbed into the bulk of the myocyte membrane, into membranes of the T-tubular system, and into sarcolemmal membrane caveolae. Dietary cholesterol may also affect lipid-dependent hormones, membrane receptors, and intracellular messenger pathways that regulate the Na\(^+\)/K\(^+\) pump.

Caveolae are lipid-rich microdomains with a high density of membrane receptors, signaling molecules, including protein kinases (9), and Na\(^+\)/K\(^+\) pumps (22). It has been suggested that cholesterol contributes to regulation of cardiac caveolae Na\(^+\)/K\(^+\) pumps involving cholesterol-induced changes in caveolae messenger molecules and membrane receptors (22). ANG II receptors and the protein kinase C family of isozymes are of particular interest because of their sensitivity to dietary fat and their role in regulating the Na\(^+\)/K\(^+\) pump. Hypercholesterolemia in humans is associated with large increases in membrane-associated PKC activity in blood cells (26), and hypercholesterolemia induced in animals causes an increase in activity of PKC in vascular smooth muscle (25, 30) and skeletal muscle (29). It also causes an increase in the density of ANG II receptors (24, 36) and activity of ACE (16, 23), and
caveolar cholesterol facilitates ANG II receptor-mediated signal transmission (33).

We found that in vivo ACE inhibition with captopril and ANG II receptor blockade with losartan or in vitro blockade of PKC reversed cholesterol-induced Na\(^+/\)K\(^+\) pump inhibition. We have previously found that treatment with captopril or losartan induces an increase in \(I_p\) measured using patch pipettes that contain K\(^+\), whereas there is no effect of such treatment on \(I_p\) measured when pipettes are K\(^+\) free (5). In vitro exposure of cardiac myocytes to ANG II or activation of PKC induces a decrease in \(I_p\) that depends on [K\(_{\text{pip}}\)] in an identical manner (5). These findings indicate a similarity in the changes of Na\(^+/\)K\(^+\) pump functional characteristics that are induced by cholesterol, ANG II receptor activation, and PKC-mediated cellular messenger pathways.

We examined the effect of blockade of \(\varepsilon\)PKC because this isoform of PKC is activated by lipid accumulation in muscle (20) and because it mediates regulation of Na\(^+/\)K\(^+\) pump by ANG II in cardiac myocytes (4). The \(\varepsilon\)-isoform of PKC is particularly resistant to degradation (20), consistent with the persistence of pump inhibition we measured hours after myocyte isolation in this study. Activation of \(\varepsilon\)PKC in cardiac myocytes involves translocation from the cytosol to anchoring proteins in the lipid-rich caveolar microdomains (28). Binding and unbinding from anchoring proteins for \(\varepsilon\)PKC in cardiac myocytes occurs with half-times in the minute domain (at 20°C) (27). Reversal of effects should therefore be possible with blockade of anchoring proteins on the time scale (~12 min) that we used in this study. In agreement with this pump, inhibition induced by a large increase in serum cholesterol was abolished when we included \(\varepsilon\)PKC-blocking peptide in pipette solutions to prevent binding to the anchoring proteins and, hence, prevent activation of the kinase. ACE inhibition and ANG II receptor blockade in vivo had a similar effect, a finding in agreement with the effect of ANG II to induce activation of \(\varepsilon\)PKC and [K\(^+\)]\(_{\text{pip}}\)-dependent Na\(^+/\)K\(^+\) pump inhibition in cardiac myocytes (4). Taken together, the data suggest that altered signaling plays a role in the cholesterol-induced pump inhibition.

The differential effects of modest and large increases in serum cholesterol on \(I_p\) may have arisen from effects on increases in the cholesterol content in different membrane microdomains, and, effects of cholesterol mediated by different mechanisms may have opposing effects with a relative importance dependent upon the serum cholesterol levels. For example, \(\varepsilon\)PKC translocates to the Z-lines in cardiac myocytes rather than to a diffuse distribution throughout the sarcolemmal membrane (27). An increase in membrane cholesterol in some parts of the membrane may have stimulated the Na\(^+/\)K\(^+\) pump. However, this may have been counterbalanced by inhibition of pump units in the sarcolemma near the translocated \(\varepsilon\)PKC. Firm support for such speculations would require an accurate and representative determination of the cholesterol content in all relevant membrane microdomains. This would be very difficult. Isolation procedures for sarcolemmal membranes are plagued by very low, often nonrepresentative recovery of membrane fractions (11).

Lipid-related disease process and the Na\(^+/\)K\(^+\) pump. The transmembrane electrochemical potential gradient for Na\(^+\) provides the electrochemical energy for a variety of ion co- and countertransport processes. It is widely recognized that the Na\(^+/\)K\(^+\) pump as a consequence is a key determinant of cardiac contraction and rhythm and of vascular tone. However, the pump also has a much more broadly based role in cell function. For example, it participates in pathways regulating cellular energy metabolism (18), and it may have an important role in the pathogenesis of the “metabolic syndrome” of insulin resistance, hypertension, and dyslipidemia (21).

Dyslipidemia, hormonal and cellular messengers, and membrane transport are usually studied as distinctly separate entities in the pathogenesis of disease. It may be useful for our understanding of disease states and their treatments to view the pump as an integral component, or at least a modifier, of the complex hormonal and intracellular messenger pathways that regulate cell function. The pharmacological intervention we used in this study, ACE inhibition and ANG II receptor blockade, are useful in lipid-related disorders (31, 39). However, the mechanism for these beneficial effects of the drugs is poorly understood. It seems reasonable to speculate that drug-induced reversal of Na\(^+/\)K\(^+\) pump inhibition may play a role.

GRANTS

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REFERENCES

14. Hool LC, Gray DF, Robinson BG, and Rasmussen HH. Angiotensin-converting enzyme inhibitors regulate the Na\(^+/\)K\(^+\) pump via effects on...


