LETTERS TO THE EDITOR

Electrochemical Ion Gradients and the Na/Ca Exchange Stoichiometry

Measurements of These Gradients Are Thermodynamically Consistent with a Stoichiometric Coefficient $\geq 3$

Dear Sir:

With ion-selective microelectrodes, it is possible to measure intracellular ion activities and calculate transmembrane electrochemical potential differences. Recently, these techniques have been carefully applied to the measurement of Na and Ca ion activities in mammalian ventricular and Purkinje fibers (Sheu and Fozzard, 1982). The results show that under a wide variety of circumstances the ratio of the electrochemical gradients for Na and Ca remains remarkably constant near a value of 2.5.

It is reasonable under certain conditions to expect that this ratio represents the stoichiometric coefficient of the Na/Ca exchange. Nonetheless, we wish to point out certain difficulties inherent in the derivation of the exchange stoichiometry from such measurements. In addition, we wish to discuss Sheu and Fozzard's intriguing observation that the ratio increases to $\approx 3$ when the Na pump is inhibited.

We hypothesize that exchange may be represented by the following transmembrane reaction:

$$ n \text{Na}^+ + \text{Ca}^{2+} \rightleftharpoons n \text{Na}^{2+} + \text{Ca}^+,$$

where $n$ is a stoichiometric coefficient and "i" and "o" designate intracellular and extracellular ions, respectively. The driving force for this reaction is given by:

$$ \hat{A} = n \Delta \mu_{\text{Na}} - \Delta \mu_{\text{Ca}},$$

where $\hat{A}$ is the electrochemical affinity and $\Delta \mu$ is the transmembrane electrochemical potential difference.\(^1\) When $\hat{A} = 0$, equilibrium exists and no net transport of ions occurs via exchange. As recognized by Sheu and Fozzard, such a condition must exist for $\Delta \mu_{\text{Ca}}/\Delta \mu_{\text{Na}}$ to equal $n$.

\(^1\) We shall use the notational conventions of Sheu and Fozzard (1982), namely that positive $\Delta \mu$ is an inwardly directed electrochemical gradient. Accordingly, positive values of $\hat{A}$ imply forward progress of reaction 1.
It is unlikely that the Na/Ca exchange will be found at equilibrium in the
resting myocardial fiber. Electrophysiological data of the sort reported by Sheu
and Fozzard demonstrate that membrane potentials and ionic activities are in a
steady state. Steady state Ca activities are maintained by a variety of intracellular
Ca buffers and sequestering mechanisms, as well as plasma membrane pumps. It
would be a remarkable coincidence if all these various processes acted in concert
to yield the Ca activity required for equilibrium. It is far more likely that the
steady state Ca activity is determined by competition between myriad processes
tending to establish some nonequilibrium level, with the Na/Ca exchange oper-
ating to bring Ca activity toward an equilibrium value. As with any ion pump,
this operation of Na/Ca exchange can only occur under nonequilibrium condi-
tions.

Therefore, we raise the following question: Does a small net flux through the
exchange imply that \( \dot{A} \) is sufficiently small to ensure negligible error in the
estimate of \( n \) from the measurement of gradient ratios? Sheu and Fozzard
comment that "It seems unlikely that in resting muscle the Ca leak should not
be high enough to bias the exchange far from its equilibrium, since the \( V_{\text{max}} \)
of the Na/Ca exchange appears to be quite high (Caroni et al., 1980)." We take
issue with this position and will show that the driving force required to compen-
sate for a small Ca leak may be substantial. In doing so, we shall attempt to
clarify the relationship between the kinetic properties of an elementary system
and its rate of energy dissipation.

We assume that resting myocardial fibers do not accumulate Ca in the face of
a Ca "leak." If we agree with Sheu and Fozzard's assumption that a sarcolemmal
ATP-dependent Ca pump does not play a significant role in this preparation,
and there is no alternative means for Ca extrusion, then the passive inward leak
of Ca determines the net steady state efflux of Ca via exchange.

Net Ca flux through exchange is given by:

\[
J_{\text{Ca}}^{\text{exch}} = J_{\text{Ca-o}}^{\text{i}} - J_{\text{Ca-i}}^{\text{o}}
\]

where positive values of \( J \) represent net influx and \( j \) represents unidirectional
flux. It is not possible to predict an exact relationship between \( \dot{A} \) and the resulting
net flux. However, the following relationship\(^a\) has been applied to the Na/K
pump (Chapman et al., 1979):

\[
\dot{A} = RT \ln \frac{J_{\text{i-o}}}{J_{\text{o-i}}}
\]

The proper application of this equation is restricted to reactions that consist of
a series of elementary steps, each of which is amenable to  
transition state theory
with all of its assumptions. While Na/Ca exchange may or may not be such a

\(^a\) Boudart (1976) has shown that this relationship applies to a series of elementary reactions if
the average stoichiometric number for all elementary steps in the reaction is equal to 1. Under
other circumstances, \( \dot{A} = \dot{X}RT \ln \frac{J_{\text{i-o}}}{J_{\text{o-i}}}, \) where \( \dot{X} \) is the average stoichiometric number for
all steps in the reaction. Thus, to the extent that \( \dot{X} > 1, \) we underestimate the rate of energy
dissipation for any given flux ratio in subsequent calculations and thereby present a "best case."
reactions, the use of this equation in the following analysis illustrates a crucial point in the simplest possible case. Substituting from Eq. 3 yields:

$$\dot{A} = RT \ln \left( 1 - \frac{J_{\text{exch}}}{J_{\text{f-o}}} \right) = -RT \ln \left( 1 + \frac{J_{\text{exch}}}{J_{\text{f-o}}} \right).$$

(5)

It follows from Eq. 5 that if $J_{\text{exch}} = 0$, then $\dot{A} = 0$ and the system is at equilibrium. Similarly, if $|J_{\text{exch}}|$ is small compared with the unidirectional fluxes, $\dot{A}$ is small and may be negligible. In all other cases, the assumption that $\dot{A} = 0$ is significantly in error. In fact, for any finite value of $J_{\text{exch}}$, it is seen that as either of the unidirectional fluxes approaches zero, the driving force becomes infinite. Clearly, a small Ca leak, and therefore a small $J_{\text{exch}}$, does not ensure that the system will be close enough to equilibrium to justify the assumption that $\dot{A}$ is negligible. Knowing that the $V_{\text{max}}$ for Na/Ca exchange is “high” provides little additional insight.

Given these considerations, it is of particular interest to note the effect of ouabain on the $\Delta \mu_{\text{Ca}}/\Delta \mu_{\text{Na}}$ ratio; namely, that the value of this ratio increases to $\sim 3$ (Sheu and Fozzard, 1982, p. 341). This finding may have several explanations. As suggested by Sheu and Fozzard, intracellular sequestration of Ca at high levels of $a_{\text{Ca}}$ might retard the collapse of the Ca gradient with respect to the Na gradient. Alternatively, however, we could hypothesize that the true exchange ratio is 3.0 and that lesser values represent the exchange in circumstances other than equilibrium. Let us consider two hypothetical cases.

Case I: The true exchange ratio is 2.5. In the unpoisoned preparation, the ratio $\Delta \mu_{\text{Ca}}/\Delta \mu_{\text{Na}}$ reflects the true value of $n$ and hence $\dot{A} = 0$. Maintenance of the low steady state $a_{\text{Ca}}$ is accomplished through Na/Ca exchange, but with a driving force immeasurably different from zero.

Application of ouabain causes $J_{\text{pump}}^{\text{Na}}$ to become zero and allows a slow increase in $a_{\text{Na}}$ with respect to $a_{\text{Ca}}$. The ratio $\Delta \mu_{\text{Ca}}/\Delta \mu_{\text{Na}}$ is then calculated to be close to 3.0. Since now $\Delta \mu_{\text{Ca}}/\Delta \mu_{\text{Na}} > n$, then $\dot{A} < 0$ and exchange is proceeding in a Na efflux mode. By implication, exchange is unable, despite an appropriately directed driving force, to re-establish an equilibrium because of (a) competition from passive Na influx or (b) intracellular Ca sequestration.

Case II: The true exchange ratio is 3.0. In the unpoisoned preparation, $\Delta \mu_{\text{Ca}}/\Delta \mu_{\text{Na}} < n$. Therefore, $\dot{A} > 0$ and exchange is proceeding in a Ca efflux mode. In this case, exchange is unable, despite an appropriately directed driving force, to establish an equilibrium because of (a) competition from the passive Ca influx or (b) competition from uninhibited Na pump activity.

Upon application of ouabain, the ratio $\Delta \mu_{\text{Ca}}/\Delta \mu_{\text{Na}}$ increases to 3.0. Three possible explanations for this exist. First, the exchange may be at equilibrium so that $\dot{A} = 0$. Since Na/Ca exchange cannot operate as a pump under these conditions, the gradient ratio must be stabilized by other processes. This is unlikely, as explained above. Second, the ratio may have stabilized at a value slightly greater than 3.0, but immeasurably so. Third, the ratio may continue to increase with time to values substantially greater than 3.0.
We find case II to be entirely consistent with the data of Sheu and Fozzard. Case I, on the other hand, presents several problems. First, as we have discussed above, case I is dependent on the assumption that a low intracellular Ca activity may be maintained by exchange without a measurable driving force. Without explicit information as to the relationship between driving force and exchange flux magnitude, this assumption is unwarranted.

Second, although intracellular sequestration of Ca when $a_{Ca}$ is 491 nM might account for a rise of the $\Delta \mu_{Ca}/\Delta \mu_{Na}$ ratio to 3.0 in case I, the data show no evidence for such an effect for $a_{Ca}$ from 113 to 332 nM (Sheu and Fozzard, 1982; Tables II and III). Furthermore, this phenomenon would be observable in Ca activity records as a decline in the rate of rise of $a_{Ca}$ as it approached 491 nM (Sheu and Fozzard, 1982, Fig. 12 and p. 341).

Third, kinetic data provided by other investigators require a stoichiometric coefficient of $\geq 3$ (Barry and Pitts, 1979; Bridge and Bassingthwaighte, 1983; Reeves and Hale, 1984). These kinetic data may be reconciled with thermodynamic data only by case II.

Finally, Sheu and Fozzard describe their steady state data (Fig. 13) with the following least-squares fit:

$$\Delta \mu_{Ca} = 2.44 \Delta \mu_{Na} + 11.$$

This implies that $\tilde{A} = -11.0$ mV, from which it follows that exchange is producing net Ca influx at a time when, by hypothesis, Ca efflux is required to maintain the steady state Ca activity.

It is noteworthy that the data of Sheu and Fozzard are adequately explained by values of $n > 2.5$, and that in no way do they compel us to consider transport "slippage" or any such form of variable stoichiometry. Furthermore, the data are consistent with the hypothesized role of exchange in maintaining low intracellular Ca activity. If we hypothesize that $n = 3$, we may calculate from Sheu and Fozzard's data (see Figs. 6 and 7) under conditions of low external Ca (0.36 mM) that the passive Ca leak is driven by a $\Delta \mu_{Ca}$ of 395 mV and the value of $3\Delta \mu_{Na} - \Delta \mu_{Ca}$ is $\sim 55$ mV. When extracellular Ca is high (4.8 mM), the passive Ca leak is driven by a $\Delta \mu_{Ca}$ of 423 mV and the value of $3\Delta \mu_{Na} - \Delta \mu_{Ca}$ is 82 mV. This is reasonable and simply indicates that if a greater demand is placed on the Na/Ca exchange, a greater driving force (and more free energy dissipation) is required to meet that demand. The calculated driving forces represent the free energy dissipation required to keep the Na/Ca exchange extruding Ca at a rate sufficient to counter the passive leaks.

As we have noted, one expects $\Delta \mu_{Na}$ to decline in value more rapidly than $\Delta \mu_{Ca}$ following Na pump inhibition because of the combined effects of a greater membrane permeability for Na and intracellular buffering of Ca ion. Whether as a consequence the gradient ratio rises to a value greater than the stoichiometric coefficient or instead stabilizes at this value (as considered above in case 2) is of considerable interest. As $\Delta \mu_{Na}$ collapses to the point where $\Delta \mu_{Ca}/\Delta \mu_{Na} > n$, Na/Ca exchange becomes subject to electrochemical forces that cause it to reverse direction. Provided that it is not kinetically restrained from doing so, it will extrude Na from the cell and allow the entry of Ca. This is consistent with the findings of Dietmer and Ellis (1978), who showed that the regulation of $a_{Na}$...
following Na pump inhibition was probably due to Na/Ca exchange operating in a Na efflux mode.

If the exchange stoichiometry is 3 and the value of $\Delta \mu_{\text{Ca}}/\Delta \mu_{\text{Na}}$ stabilizes at a value just slightly above 3, then, by implication, exchange is capable of Na efflux sufficient to keep the gradient ratio constant. This must occur with a driving force that is close, but not equal, to zero. This is to say that reverse motion of the exchange under these conditions does not require the dissipation of appreciable quantities of energy. Using a mechanical analogy, it is virtually frictionless. Such an exchange might be well suited to bring Ca into myocardial cells with ease during energetically favorable periods of the cardiac cycle. This idea has already been developed in some detail (Mullins, 1979).

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Na/Ca Exchange in the Intact Cardiac Cell

Dear Sir:

In reply to the letter of Axelsen and Bridge, we shall address three points:

(a) Is the Na/Ca exchange system at equilibrium in a nonstimulated cardiac muscle cell?

(b) What is the stoichiometry of Na/Ca exchange?

(c) In which direction is the Na/Ca exchange system operating, pumping Ca^{2+} out or in?

Na/Ca exchange is unlikely to be at equilibrium. As stated in our article (Sheu and Fozzard, 1982), "Leakage of Ca^{2+} into the cell requires that Na/Ca exchange operate in its Ca^{2+} efflux mode, so that the steady level (or intracellular Ca^{2+}) is determined by a balance between exchange and leak, analogous to the Na/K pump-leak balance." Bridge and Axelsen are right in pointing out that the degree to which exchange is displaced from equilibrium by a Ca^{2+} leak is unknown, and it could be substantial. Without detailed knowledge of the magnitude of the leak and the kinetics of the Na/Ca exchange system, no quantitative estimate of the displacement can be made. However, the assumption that the exchange system was near equilibrium enabled us to predict with fair accuracy the changes in intracellular Ca^{2+} in response to a variety of conditions.

While there is much evidence in favor of a 3Na^{+}:1Ca^{2+} stoichiometry, caution must be used in interpreting the data. Almost all the experimental maneuvers used to obtain this number are known to affect processes other than Na/Ca exchange. In our study, we measured the levels of intracellular Na^{+} and Ca^{2+} in intact cells under a variety of conditions, where both levels changed considerably. The ratio between the electrochemical energy gradients established in these cells consistently showed a value substantially less than 3. This gradient ratio is the same as the molecular transport coupling ratio only if the Na/Ca exchange is at equilibrium. As we have already pointed out, equilibrium is quite unlikely. If the intact cell is not near Na/Ca exchange equilibrium, then our measurements cannot be used to estimate the molecular stoichiometry without detailed information about the molecular reactions.

Deitmer and Ellis (1978) have suggested that when the Na/K pump is inhibited and intracellular Na^{+} is increased, the Na/Ca exchange system can pump Na^{+} out of the cell by the use of the energy in the Ca^{2+} electrochemical gradient. The reverse mode of the Na/Ca exchange system could fortuitously cause the gradient ratio to increase, since (a) depression of Na^{+} efflux by inhibiting the Na/K pump would reduce the Na^{+} electrochemical gradient perceived by the Na/Ca exchange system, and (b) augmentation of Ca^{2+} efflux by stimulating other Ca^{2+} pumping systems would increase the Ca^{2+} electrochemical gradient perceived by Na/Ca exchange. This may be why we saw a gradual rise in the calculated value of n after the Na/K pump was inhibited (Fig. 1). In the presence of an active Na/K pump, Na/Ca exchange seems to operate in a "forward" mode
LETTERS TO THE EDITOR

(Na⁺ influx and Ca²⁺ efflux), with a gradient ratio of ~2.5. Conversely, during blockade of the Na/K pump, Na/Ca exchange seems to operate in a "reverse" mode (Na⁺ efflux and Ca²⁺ influx), with a gradient ratio of ~3. The thermodynamic argument suggests that 2.5 is a lower limit and 3 is an upper limit for the stoichiometry of Na/Ca exchange. Further examination of the question of stoichiometry must await new experiments.

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FIGURE 1. Trajectory of changes in the Ca²⁺ electrochemical gradient (2(VCa - Vm)) as a function of the Na⁺ electrochemical gradient (VNa - Vm) after exposure of ventricular muscle to 5 μM ouabain. The figure shows the results of five experiments in which simultaneous measurements of intracellular Na⁺ activity and intracellular Ca²⁺ activity were measured. Each symbol type represents a different muscle. The rightmost points are the control values and the connected points are the subsequent values at 3-min intervals, until the impalements were lost. The dashed lines are the theoretical relationships between the equilibrium electrochemical gradients for Na-Ca exchange with coupling ratios of 2, 3, and 4. Under control conditions, the muscles had apparent coupling ratios of ~2.6. The apparent coupling ratio tended to rise as intracellular Na⁺ increased after exposure of the muscles to ouabain.
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