and 0.3% w/v bromophenol blue in formamide). The samples were then heated at 94°C for 4 min and loaded directly on a denaturing polyacrylamide gel. We performed two separate assays for each exon using each strand as a labeled transcript.

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Technical Comments

Sodium-Calcium Exchange

N. Leblanc and J. R. Hume (1) conclude that the intracellular calcium ion concentration ([Ca²⁺]_i) of cardiac myocytes can increase transiently, in the absence of an inward Ca²⁺ current, by the influx of Ca²⁺ through Na⁺-Ca²⁺ exchange, this influx being induced primarily by the influx of Na⁺ through tetrodotoxin (TTX)-sensitive Na⁺ channels. We suggest an alternative explanation.

Leblanc and Hume base their conclusion on their observations that TTX reduces the size of the transient rise in $[Ca^{2+}]_i$ in response to an action potential and that, in voltage-clamp experiments, inactivation of the fast Na⁺ current by a conditioning depolarization reduces the size of the transient rise in $[Ca^{2+}]_i$ in response to a subsequent depolarization. Moreover, in cells exposed to the putative Ca²⁺ channel blocker, dihydropyridine, a depolarization-induced (TTX-sensitive) Na⁺ current is associated with a transient rise in $[Ca^{2+}]_i$ only in the presence of extracellular Ca^{2+} .

Leblanc and Hume suppose that the TTX-sensitive rise in $[Ca^{2+}]_i$ arises as a result of an influx of Na⁺ through TTXsensitive Na⁺ channels, which causes a transient rise in intracellular Na⁺ concentration ([Na⁺]_i) near the inner surface of the sarcolemma that shifts the reversal potential of the Na⁺-Ca²⁺ exchanger toward negative membrane potentials. This shift in reversal potential would thereby promote a transient influx of Ca²⁺ through the exchanger that, in turn, triggers the release of Ca²⁺ from the sarcoplasmic reticulum (SR).

As pointed out by W. J. Lederer et al. (2), the idea of a TTX-sensitive Na⁺ current as the primary event poses a problem. Unless the volume into which the entering Na⁺ is distributed is restricted (at least in the short term) to a small fraction of the total cell volume, the rise in $[Na^+]_i$ would have an insignificant effect on the reversal potential of the exchanger. Even allowing for the fact that the rise in $[Na^+]_i$ need not be as high as that presumed by Lederer et al. (8 mM) to cause the necessary rise in $[Ca^{2+}]_i$ to trigger Ca^{2+} release from the SR (3), there is no escaping the need for some restriction in the space into which the incoming Na⁺ diffuses. Otherwise, the hypothesis that the influx of Na⁺ is the primary event cannot survive because Lederer et al. calculated that, without such restricted space, the rise in $[Ca^{2+}]_i$ would be an insignificant 25 μ M.

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Moreover, Lederer et al. point out that this restricted space must be accessible not only to Na⁺ channels and the Na⁺-Ca²⁺ exchanger, but to both the SR and to L-type Ca²⁺ channels that are believed to contribute to Ca²⁺-induced Ca²⁺ release from the SR. Indeed, the entire cytoplasm must be accessible to the Na⁺-Ca²⁺ exchanger, since J. H. B. Bridge et al. (5) show that all the nifedipine-sensitive inward calcium flux associated with contractures of cardiac myocytes is extruded by the exchanger.

We agree with Lederer et al. that such a space is poorly conceptualized. Its special properties are hard to reconcile with the known cellular architecture of heart cells, so much so that we question its necessity. In the squid giant axon it has been shown (6)that there are only two plasmalemmal pathways that lead to an increase in $[Ca^{2+}]_i$; one is by Na⁺-Ca²⁺ exchange and the other is through TTX-sensitive Na⁺ channels-the latter possibility not considered by Leblanc and Hume or by Lederer et al. We suggest that, unless proved otherwise, a Ca²⁺ flux through TTX-sensitive Na⁺ channels is a more likely explanation of the findings of Leblanc and Hume than a "fuzzy space" for the accumulation of Na⁺, as discussed by Lederer et al.

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- 3. An increase in [Na⁺], may cause an increase in [Ca²⁺], not by affecting the thermodynamics of $[Ca^{2+}]_{i}$, not by affecting the thermodynamics of Na⁺-Ca²⁺ exchange, through its reversal potential, but on the kinetics of the exchanger. As pointed out by Johnson and Kootsey (4), a relatively small increase in $[Na^+]_i$ (such as that produced by a low concentration of a cardiac glycoside) could alter the voltage-dependency of the exchanger, increasing markedly the influx of Ca^{2+} during depolarization under voltage clamp or during the plateau of an action potential.
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Response: In our report (1) we hypothesized that the influx of Ca²⁺ mediated by the Na⁺-Ca²⁺ exchange in response to Na⁺ influx through tetrodotoxin (TTX)-sensitive Na⁺ channels induces Ca²⁺ release from the sarcoplasmic reticulum (SR) in guinea pig heart myocytes. Johnson and Lemieux suggest that Ca2+ influx through voltage-dependent TTX-sensitive Na+ channels provides the Ca²⁺ trigger for release of SR Ca^{2+} , thereby minimizing the role of Na⁺- Ca^{2+} exchange. We initially considered this alternative explanation, but believe such a possibility to be unlikely.

In squid axon there can be significant Ca2+ entry through TTX-sensitive Na+ channels (2), however, most experiments were carried out in high extracellular Ca²⁺ $([Ca^{2+}]_{o})$ solutions (about 100 mM). These experiments do not, therefore, reveal the extent of Ca²⁺ influx through Na⁺ channels that might be expected in our experiments with solutions containing physiological $[Ca^{2+}]_{o}$ (2.5 mM). More recent studies of the effects of $[Ca^{2+}]_0$ on Na⁺ channel permeability in nerve (3) and cardiac preparations (4) conclude that there is little if any divalent cation permeability and that divalent cations instead block Na⁺ channels, which results in a nonlinearity of the instantaneous Na⁺ current-voltage relationship.

The hypothesis that Na⁺-Ca²⁺ exchange mediates Na⁺ current-induced Ca²⁺ release from cardiac SR is supported by experiFig. 1. Effects of Li⁺ substitution on Na⁺ cur-rent–induced Ca²⁺ transients in a guinea pig ventricular myocyte. After a conditioning protocol (ten 500-ms voltage clamp pulses from -80 to +60 mV applied at 0.2 Hz), Na⁺ current



0.2 Hz), Na⁺¹ current and associated Ca²⁺ transient were recorded during a test pulse to -50 mV from a holding potential of -80 mV in standard external solution that contained 140 mM NaCl, 5.4 mM KCl, 2.5 mM CaCl₂, 0.5 mM MgCl₂, 10 mM glucose, 5 mM Hepes (pH 7.4), and 5 µM nisoldipine to block Ca²⁺ currents (A). Another conditioning protocol was then applied to the cell and the external solution was changed to one in which Na⁺ was replaced by equimolar Li⁺. An identical test pulse was applied to the cell after it was in the Li⁺-containing solution for 5 min (B). The internal solution contained 105 mM cesium aspartate, 20 mM CsCl, 20 mM tetraethylammonium chloride, 10 mM NaCl, 5 mM adenosine triphosphate (Mg^{2+} salt), 0.5 mM MgCl₂, 0.1 mM EGTA, 0.1 mM indo-1 (pentapotassium salt), and 5 mM Hepes (pH 7.2).

ments which show that equimolar replacement of extracellular Na⁺ by Li⁺ effectively eliminates the Ca²⁺ transient (Fig. 1). Li⁺ is known to be highly permeable through Na⁺ channels, as shown by the permeability (P) ratio $P_{\text{Li}}/P_{\text{Na}} = 0.93$ (5), yet Li⁺ is a poor substitute for Na^+ in the Na^+ - Ca^{2+} exchange reaction (6).

We share the discomfort expressed by Johnson and Lemieux with the concept of a "fuzzy space" for the accumulation of Na⁺. There is, however, other evidence which suggests that in cardiac cells it may be quite real. M. Mazzanti and L. J. DeFelice (7, 8), using simultaneously cell-attached and whole-cell recording techniques, have measured the reversal potential of Na⁺ channels and Na⁺ conducting Ca²⁺ channels during action potentials in embryonic chick ventricular cells. They found the reversal potential to be significantly less than the predicted Na⁺ equilibrium potential in spontaneously beating cells, which suggests "that the Na⁺ concentration near the membrane is independent of the bulk concentration." It is interesting that such a deviation was not observed in quiescent cells.

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Response: N. Leblanc and J. R. Hume (1) suggest that sodium influx through Na⁺ channels contributes to excitation-contraction (EC) coupling in heart muscle by activating calcium entry that is mediated by the sarcolemmal Na⁺-Ca²⁺ exchange mechanism. In a Perspective (2), we noted that for this to occur intracellular Na^+ ([Na^+]_i), which activates the Na⁺-Ca²⁺ exchanger (3), would have to be significantly elevated. We hypothesized that if Na⁺ diffusion were restricted [because of a subsarcolemmal "fuzzy" space (2)], then Na⁺ could rise sufficiently to activate the Na⁺-Ca²⁺ exchanger, as suggested by Leblanc and Hume. In their comment, E. A. Johnson and R. D. Lemieux note that two additional hypotheses should be considered: (i) Ca²⁺ entry through the Na⁺-channel and (ii) Na⁺-dependent alterations of the voltagedependence of the Na⁺-Ca²⁺ exchanger. These hypothesis seem unlikely for several reasons.

Ca²⁺ flux through Na⁺ channels has been estimated experimentally in several ways and found to be small. Recently, B. Nilius (4) carried out single-channel measurements of the actions of Ca²⁺ on Na⁺ current in guinea pig heart cells, the same experimental preparation used by Leblanc and Hume. With elevated extracellular Ca²⁺ (20 mM), he estimated that "the flux of Ca²⁺ ions through the Na⁺ channel is less than 10^{-3} pA . . ." with Na⁺ current through the Na⁺ channel at -20 mV being less than 1 pA. This would be consistent with a permeability (P) ratio P_{Ca}/P_{Na} of less than 0.003. In the experimental conditions of Leblanc and Hume (1), if peak Na⁺ current were 50 nA, then Nilius would have found that the peak Ca²⁺ current through Na⁺ channels would

be less than 7 pA. Nilius' estimate comes from direct measurements of currents through single Na⁺ channels, the actions of Ca²⁺ on these currents, and an Eyring rate theory permeation model (5). It is possible to estimate the peak Ca²⁺ current through Na⁺ channels (where $P_{Ca}/P_{Na} = 0.01$) using the data of Baker et al. (6) which is cited by Johnson and Lemieux. This estimate suggests that peak Ca²⁺ current through the Na⁺ channels is no more than 25 pA, which is in basic agreement with Nilius' upperlimit estimate. The effect of this Ca²⁺ influx on EC coupling would be even less than this small value indicates (particularly in the absence of "fuzzy" space), since the Na⁺ channels inactivate much faster (about ten times) than Ca²⁺ channels. Thus the integrated Ca²⁺ influx though Na⁺ channels would be only a small fraction of what is normally carried by the Ca^{2+} current. Recent experiments by Hume, Levesque, and Leblanc (7) indicate no $[Ca^{2+}]_i$ transient associated with depolarization when Li⁺ replaces Na⁺ in the extracellular solution, which confirms the earlier findings of Leblanc and Hume (1)

The second hypothesis suggested by Johnson and Lemieux is that the voltagedependence of the Na⁺-Ca²⁺ exchanger may change with minimally altered [Na⁺]_i. Thus, at positive potentials, a small elevation of Na⁺ would be sufficient to enhance the kinetics of Na⁺-Ca²⁺ exchange. Y. Miura and J. Kimura (3) show that the voltage dependence of the Na⁺-Ca²⁺ exchanger positive to -50 mV is largely unchanged over a range of intracellular Na⁺ from 4.7 mM to 18.8 mM [(3), figure 3D].

In conclusion, the possibility of a "fuzzy space" just under the sarcolemmal membrane appears to remain an attractive hypothesis to explain how Na⁺ influx by means of Na⁺ current can elevate [Na⁺]; to activate the Na⁺-Ca²⁺ exchange and thereby modulate Na⁺-Ca²⁺ exchange.

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