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.
25. R. M. Myers and T. Maniatis, *Cold Spring Harbor Symp. Quant. Biol.* **51**, 275 (1986).
26. S. J. Baker *et al.*, *Science* **249**, 912 (1990).
27. J. Lechleiter *et al.*, *EMBO J.* **9**, 4381 (1990).
28. J. L. Bos *et al.*, *Nature* **327**, 293 (1987); K. Forrester *et al.*, *ibid.*, p. 298.
29. H. R. Bourne, D. A. Sanders, F. McCormick, *ibid.* **348**, 125 (1990).
30. M. H. Noda *et al.*, *Proc. Natl. Acad. Sci. U.S.A.* **86**, 162 (1989).
31. C. Landis *et al.*, *Nature* **340**, 692 (1989); J. Lyons *et al. Science* **249**, 655 (1990).
32. G. Tu *et al.*, *Cell* **63**, 835 (1990); G. A. Martin *et al.*, *ibid.*, p. 843; R. Ballester *et al.*, *ibid.*, p. 851.
33. W. R. Waddell and R. W. Loughry, *J. Surg. Oncol.* **24** (no. 1), 83 (1983); W. R. Waddell, G. F. Ganser, E. J. Cerise, R. W. Loughry, *Am. J. Surg.* **157** (no. 1), 175 (1989); J. Charneau *et al.*, *Gastroenterol. Clin. Biol.* **14** (no. 2), 153 (1990).
34. C. Role, L. Jelsema, J. Axelrod, *Proc. Natl. Acad. Sci. U.S.A.* **84**, 3623 (1987); Y. Kurachi *et al.*, *Nature* **337**, 555 (1989).
35. J. M. Bishop, *Science* **235**, 305 (1987); R. A. Weinberg, *Cancer Res.* **49**, 3713 (1989).
36. The authors thank J. M. Jessup, J. Willson, M. Brattain, J. Trent, K. G. Danielson, A. Giovanella, A. Gazdar, C. Paraskeva, M. Schwab, H. Lynch, and R. Whitehead for tumor cells used in this study. This work was supported by the Clayton Fund, the McAshan Fund, and NIH grants CA 09243, CA 06973, 6M 07184,and CA 35494.

22 January 1990; accepted 15 February 1990

Technical Comments

Sodium-Calcium Exchange

N. Leblanc and J. R. Hume (*1*) conclude that the intracellular calcium ion concentration ($[Ca^{2+}]_i$) of cardiac myocytes can increase transiently, in the absence of an inward Ca^{2+} current, by the influx of Ca^{2+} through Na^+-Ca^{2+} 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 Ca^{2+} 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 TTX-sensitive 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^+-Ca^{2+} exchanger toward negative membrane potentials. This shift in reversal potential would thereby promote a transient influx of Ca^{2+} through the exchanger that, in turn, triggers the release of Ca^{2+} 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 μM.

Moreover, Lederer *et al.* point out that this restricted space must be accessible not only to Na^+ channels and the Na^+-Ca^{2+} exchanger, but to both the SR and to L-type Ca^{2+} channels that are believed to contribute to Ca^{2+}-induced Ca^{2+} release from the SR. Indeed, the entire cytoplasm must be accessible to the Na^+-Ca^{2+} 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^+-Ca^{2+} 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 Ca^{2+} 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.*

EDWARD A. JOHNSON
RENALD D. LEMIEUX
National Biomedical Simulation Resource, One University Place, Suite 250, Durham, NC 27707

REFERENCES AND NOTES

1. N. Leblanc and J. R. Hume, *Science* **248**, 372 (1990).
2. W. J. Lederer *et al.*, *ibid.*, p. 283.
3. An increase in $[Na^+]_i$ may cause an increase in $[Ca^{2+}]_i$, not by affecting the thermodynamics of Na^+-Ca^{2+} 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.
4. E. A. Johnson and J. M. Kootsey, *J. Membrane Biol.* **86**, 167 (1985).
5. J. H. B. Bridge *et al.*, *Science* **248**, 376 (1990).
6. P. F. Baker, A. L. Hodgkin, E. B. Ridgway, *J. Physiol. (London)* **218**, 709 (1971); L. J. Mullins and J. Requena, *Proc. Natl. Acad. Sci. U.S.A.* **82**, 1847.

15 July 1990; accepted 17 October 1990

Response: In our report (*1*) we hypothesized that the influx of Ca^{2+} mediated by the Na^+-Ca^{2+} exchange in response to Na^+ influx through tetrodotoxin (TTX)-sensitive Na^+ channels induces Ca^{2+} release from the sarcoplasmic reticulum (SR) in guinea pig heart myocytes. Johnson and Lemieux suggest that Ca^{2+} influx through voltage-dependent TTX-sensitive Na^+ channels provides the Ca^{2+} 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 Ca^{2+} entry through TTX-sensitive Na^+ channels (*2*), however, most experiments were carried out in high extracellular Ca^{2+} ($[Ca^{2+}]_o$) solutions (about 100 mM). These experiments do not, therefore, reveal the extent of Ca^{2+} 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 $[Ca^{2+}]_o$ 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^+-Ca^{2+} exchange mediates Na^+ current–induced Ca^{2+} release from cardiac SR is supported by experi-

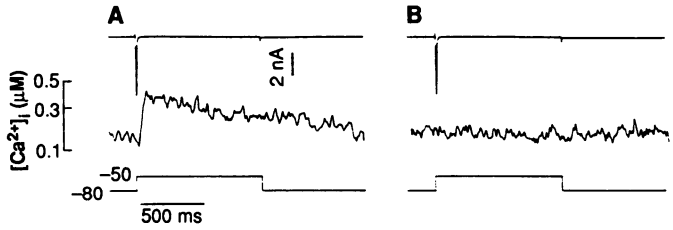

Fig. 1. Effects of Li^+ substitution on Na^+ current–induced Ca^{2+} transients in a guinea pig ventricular myocyte. After a conditioning protocol (ten 500-ms voltage clamp pulses from −80 to +60 mV applied at 0.2 Hz), Na^+ current and associated Ca^{2+} transient were recorded during a test pulse to −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 $CaCl_2$, 0.5 mM $MgCl_2$, 10 mM glucose, 5 mM Hepes (pH 7.4), and 5 μM nisoldipine to block Ca^{2+} 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 $MgCl_2$, 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 Ca^{2+} transient (Fig. 1). Li^+ is known to be highly permeable through Na^+ channels, as shown by the permeability (*P*) ratio P_{Li}/P_{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 Ca^{2+} 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.

JOSEPH R. HUME
PAUL C. LEVESQUE
Department of Physiology, University of Nevada School of Medicine, Reno, NV 89557–0046
NORMAND LEBLANC
Department of Physiology, University of Manitoba, Winnipeg, Canada R2H 2A6

REFERENCES
1. N. Leblanc and J. R. Hume, *Science* **248**, 372 (1990).
2. P. F. Baker, A. L. Hodgkin, E. B. Ridgway, *J. Physiol. (London)* **218**, 709 (1971); H. Meves, *Philos. Trans. R. Soc. London Ser. B Biol. Sci.* **270**, 377 (1975).
3. D. Yamamoto, J. Z. Yeh, T. Narahashi, *Biophys. J.* **45**, 337 (1984).
4. M. F. Sheets, B. E. Scanley, D. A. Hanck, J. C. Makielski, H. A. Fozzard, *ibid.* **52**, 13 (1987).
5. B. Hille, *J. Gen. Physiol.* **59**, 637 (1972).
6. P. F. Baker, *Prog. Biophys. Mol. Biol.* **24**, 177 (1972).
7. M. Mazzanti and L. J. DeFelice, *Biophys. J.* **51**, 115 (1987).
8. ———, *ibid.* **52**, 95 (1987).

2 August 1990; accepted 17 October 1990

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^+-Ca^{2+} exchange mechanism. In a Perspective (*2*), we noted that for this to occur intracellular Na^+ ($[Na^+]_i$), which activates the Na^+-Ca^{2+} 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^+-Ca^{2+} 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) Ca^{2+} entry through the Na^+-channel and (ii) Na^+-dependent alterations of the voltage-dependence of the Na^+-Ca^{2+} exchanger. These hypothesis seem unlikely for several reasons.

Ca^{2+} 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 Ca^{2+} on Na^+ current in guinea pig heart cells, the same experimental preparation used by Leblanc and Hume. With elevated extracellular Ca^{2+} (20 mM), he estimated that "the flux of Ca^{2+} 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 Ca^{2+} 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 Ca^{2+} on these currents, and an Eyring rate theory permeation model (*5*). It is possible to estimate the peak Ca^{2+} 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 Ca^{2+} current through the Na^+ channels is no more than 25 pA, which is in basic agreement with Nilius' upper-limit estimate. The effect of this Ca^{2+} 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 Ca^{2+} channels. Thus the integrated Ca^{2+} 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 voltage-dependence of the Na^+-Ca^{2+} 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^+-Ca^{2+} exchange. Y. Miura and J. Kimura (*3*) show that the voltage dependence of the Na^+-Ca^{2+} 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 $[Na^+]_i$ to activate the Na^+-Ca^{2+} exchange and thereby modulate Na^+-Ca^{2+} exchange.

W. JONATHAN LEDERER
ERNST NIGGLI
ROBERT W. HADLEY
Department of Physiology, University of Maryland School of Medicine, Baltimore, MD 21201

REFERENCES
1. N. Leblanc and J. R. Hume, *Science* **248**, 372 (1990).
2. W. J. Lederer, E. Niggli, R. W. Hadley, *ibid.*, p. 283.
3. Y. Miura and J. Kimura, *J. Gen. Physiol.* **93**, 1129 (1989).
4. B. Nilius, *J. Physiol. (London)* **399**, 537 (1988).
5. B. Hille, *J. Gen. Physiol.* **56**, 535 (1975).
6. P. F. Baker, A. L. Hodgkin, E. B. Ridgway, *J. Physiol. (London)* **218**, 709 (1971).
7. J. R. Hume, P. C. Levesque, N. Leblanc, *Science* **251**, 1370 (1991).

1 August 1990; accepted 17 October 1990

Response

JOSEPH R. HUME, PAUL C. LEVESQUE and NORMAND LEBLANC

Science **251** (4999), 1370-1371.
DOI: 10.1126/science.251.4999.1370-a