Pharmacia), and libraries were screened by standard procedures [W. D. Benton and R. W. Davis, *Science* **196**, 180 (1977)]. Plasmids in the vector Bluescript SK were prepared by the automatic excision procedure of the manufacturer (Stratagene).
7. The TB11 plasmid was digested with Bam HI and RNA was synthesized with T7 RNA polymerase following the instructions of the manufacturer (Bethesda Research Laboratories).
8. S. Longoni, M. J. Coady, T. Ikeda, K. D. Philipson, *Am. J. Physiol.* **255**, C870 (1988).
9. R. J. Jackson and N. Standart, *Cell* **62**, 15 (1990).
10. The peptide, acetyl-DDKQPLTSKEEEERR-amide, was synthesized by Multiple Peptide Services. The peptide was coupled to keyhole limpet hemocyanin with glutaraldehyde, emulsified with complete Freund's adjuvant, and injected subcutaneously at multiple sites along the back of a rabbit (about 0.4 mg of peptide per rabbit). Three weeks later, this procedure was repeated with coupled peptide and incomplete Freund's adjuvant. Rabbits were bled through the ear artery at approximately 1-month intervals thereafter. ELISA assays were performed as described [D. S. Papermaster, *Methods Enzymol* **81**, 240 (1982)].
11. M. Kozak, *J. Cell Biol.* **108**, 229 (1989).
12. D. W. Hilgemann, *Nature* **344**, 242 (1990).
13. A. M. Edelman, D. K. Blumenthal, E. G. Krebs, *Annu. Rev. Biochem* **56**, 567 (1987).
14. J. Devereux, P. Haeberli, O. Smithies, *Nucleic Acids Res.* **12**, 387 (1984).
15. G. E. Shull and J. Greeb, *J. Biol. Chem* **263**, 8646 (1988).
16. C. Sardet, A. Franchi, J. Pouysségur, *Cell* **56**, 271 (1989); K. E. Kudrycki, P. R. Newman, G. E. Shull, *J. Biol. Chem* **265**, 462 (1990).
17. G. von Heijne, *Nucleic Acids Res.* **14**, 4683 (1986).
18. G. E. Shull, A. Schwartz, J. B. Lingrel, *Nature* **316**, 691 (1985).
19. We noted that this region was cationic and resembled a calmodulin-binding peptide [K. T. O'Neil and W. F. DeGrado, *Trends Biochem. Sci.* **15**, 59 (1990)]. A peptide corresponding to residues 251 to 270 was synthesized and was found to bind calmodulin (J. T. Penniston and K. D. Philipson, unpublished observations).
20. A. Colman, in *Transcription and Translation—A Practical Approach*, D. Hames and S. Higgins, Eds. (IRL Press, Oxford, 1984), pp. 271–302.
21. F. Sanger, S. Nicklen, A. R. Coulson, *Proc. Natl. Acad. Sci. U.S.A.* **74**, 5463 (1977).
22. J. Kyte and R. F. Doolittle, *J. Mol. Biol.* **157**, 105 (1982).
23. We thank the members of the laboratory of E. Wright for many helpful discussions, D. Woo for advice on the production of antipeptide antibodies, B. Scott for the λgt11 library, R. Ward for technical assistance, and E. Wright and H. R. Kaback for commenting on the manuscript. Supported by NIH HL27821, the Laubisch Foundation, and NIH National Research Service Award training grant AHL07386.

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Voltage-Independent Calcium Release in Heart Muscle

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The Ca^{2+} that activates contraction in heart muscle is regulated as in skeletal muscle by processes that depend on voltage and intracellular Ca^{2+} and involve a positive feedback system. How the initial electrical signal is amplified in heart muscle has remained controversial, however. Analogous protein structures from skeletal muscle and heart muscle have been identified physiologically and sequenced; these include the Ca^{2+} channel of the sarcolemma and the Ca^{2+} release channel of the sarcoplasmic reticulum. Although the parallels found in cardiac and skeletal muscles have provoked valuable experiments in both tissues, separation of the effects of voltage and intracellular Ca^{2+} on sarcoplasmic reticulum Ca^{2+} release in heart muscle has been imperfect. With the use of caged Ca^{2+} and flash photolysis in voltage-clamped heart myocytes, effects of membrane potential in heart muscle cells on Ca^{2+} release from intracellular stores have been studied. Unlike the response in skeletal muscle, voltage across the sarcolemma of heart muscle does not affect the release of Ca^{2+} from the sarcoplasmic reticulum, suggesting that other regulatory processes are needed to control Ca^{2+}-induced Ca^{2+} release.

THE HYPOTHESIS THAT THE EFFECTS of a small amount of trigger Ca^{2+} could be amplified to provide the elevated intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) needed to activate contraction was first proposed to explain excitation-contraction (EC) coupling in skeletal muscle (*1*), although this concept of "Ca^{2+}-induced Ca^{2+} release" (CICR) is now broadly applied to excitable and nonexcitable cells (*2*–*7*). Even though CICR has a limited role in EC coupling of skeletal muscle (*8*), CICR may modulate EC coupling in skeletal muscle under specific conditions (*9*). CICR has been demonstrated in skinned heart muscle cells (*2*) and may be responsible for amplifying the effects of the Ca^{2+} influx through L-type Ca^{2+} channels in intact heart cells. Experiments in intact isolated heart muscle cells also show that their CICR can be directly activated by a photochemically produced step increase in $[Ca^{2+}]_i$ (*10*). However, it is not known if sarcolemmal membrane potential can influence CICR or directly modulate EC coupling in heart, although several reports (*11*, *12*) provide support for CICR in heart muscle: when the Ca^{2+} current (I_{Ca}) is reduced by depolarizations to the electrochemical potential of Ca^{2+} (E_{Ca}), there is no Ca^{2+} release. This is in clear contrast to findings in skeletal muscle where similar depolarizations do not result in reduction in the voltage-activated $[Ca^{2+}]_i$ transient (*13*).

One difficulty with the concept of CICR arises from the potentially large positive feedback (gain) inherent in this process. Because the CICR produces its own trigger signal as the output signal, this system would not release Ca^{2+} in a graded way, unless its gain were low. However, the experimentally observed gain of this positive feedback system was variable depending on the conditions, suggesting that the gain may be regulated (*14*). Furthermore, in heart muscle, $[Ca^{2+}]_i$ transients can be abbreviated by early repolarization (*11*), a finding not expected from CICR with a high gain. Examination of I_{Ca} showed that the flux of Ca^{2+} itself was important in triggering CICR, since replacing outside Ca^{2+}, Ca^{2+}_o, with other ions that can permeate the Ca^{2+} channels prevented CICR, as did other procedures that blocked Ca^{2+} flux through the Ca^{2+} channels (*15*). From these findings, it was concluded that the voltage dependence of Ca^{2+} release was mediated entirely by Ca^{2+} flux through Ca^{2+} channels and thus by the voltage dependence of these channels. However, additional voltage-dependent mechanisms may be involved. The voltage-dependent sodium current (I_{Na}) and the intracellular Na^+ concentration $[(Na^+]_i)$–dependent and voltage-dependent Na^+-Ca^{2+} exchanger can trigger CICR (*16*), again raising the question whether voltage itself can influence the gain of CICR in heart muscle. In order to address this point experimentally, it is necessary to activate CICR and control voltage independently. We have achieved this by activating CICR using photorelease of caged Ca^{2+} (*17*) while independently controlling membrane potential by using a patch-clamp method in the whole-cell mode.

In the absence of photorelease of Ca^{2+}, depolarization of the sarcolemmal membrane activated contraction in the normal manner (Fig. 1A), and repolarization of the cell membrane produced a small aftercontraction that reflects the I_{Ca} tail current. A twitch contraction was triggered at a holding potential of −40 mV in the same cell by photorelease of Ca^{2+} with a 230–W-s discharge through the flashlamp (Fig. 1B). The photochemically induced contraction, although similar to the contraction induced by depolarization, exhibited some expected

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differences. The delay between stimulation and onset of contraction was shorter and the initial shortening velocity was faster, presumably reflecting the more uniform and virtually instantaneous elevation of $[Ca^{2+}]_i$. The overall duration of the twitch was shorter because, in the absence of a depolarization, there was no maintained Ca^{2+} influx via I_{Ca} or Na^+-Ca^{2+} exchange. No aftercontraction was observed, as expected. An inward current transient, normally observed under these conditions, was activated by the photolytic release of Ca^{2+} (see arrow). This current arises largely from the Na^+-Ca^{2+} exchanger as indicated by its voltage dependence, Ca^{2+} and Na^+ dependence, and sensitivity to Ni^{2+} (*18*). The inward Na^+-Ca^{2+} exchange current elicited by photolysis demonstrated the virtually immediate photorelease of Ca^{2+} after a flash.

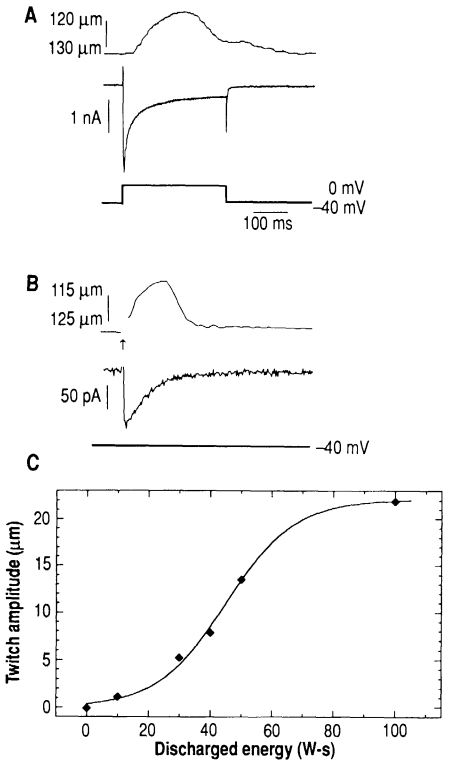

Fig. 1. Twitch contraction and associated membrane currents in cardiac myocytes isolated from guinea pigs. (**A**) Unloaded twitch contraction triggered by a voltage-clamp depolarization. Cell shortening began after a delay of ~20 ms. The twitch was followed by an aftercontraction, presumably induced by the I_{Ca} tail current. (**B**) Twitch contraction (recorded from the same cell) and inward current transient induced by photolysis of caged Ca^{2+}. At the arrow, a flash was applied (230 W-s; duration, 1 ms) resulting in intracellular photorelease of Ca^{2+} and a phasic contraction. The optical (cell-length signal) and the electrical (current) artifacts associated with the flash have been removed. The lower trace shows the corresponding transient inward current activated by intracellular Ca^{2+} at a fixed membrane potential of −40 mV. (**C**) Dependence of twitch amplitude on released Ca^{2+}. We photoreleased an increasing amount of Ca^{2+} by increasing the electrical energy discharged through the xenon-arc flashlamp from 10 to 100 W-s. The resultant twitch amplitude is plotted versus discharged energy (W-s). Resting cell length was 127 µm.

In order to compare the responses of CICR at different voltages, it must be clear that the triggering signals did not vary excessively with time, that they did in fact trigger release of Ca^{2+} from intracellular stores, and that the amount of photoreleased Ca^{2+} was small and properly simulated a triggering Ca^{2+} signal. After cells were loaded with DM-nitrophen (*19*) by dialysis from the pipette (*20*), individual cells were sequentially flashed with discharge energies ranging from 10 to 100 W-s. A small twitch was induced by a low-energy flash (10 W-s), and a near maximal twitch was produced by a 100–W-s discharge (Fig. 1C). Control experiments have shown that there is generally enough DM-nitrophen loaded into a cell to permit us to carry out ~10 flashes at 30 W-s without significant alteration of the twitch. In prolonged experiments, however, with repetitive photorelease of Ca^{2+} at low discharge energy and in the presence of blockers for the Na-Ca exchanger (see below), the resting cell length decreased while the twitch amplitude increased. This result was expected, because the principal homeostatic mechanism controlling sarcolemmal Ca^{2+} extrusion, the Na^+-Ca^{2+} exchanger (*21*), was blocked in these experiments, and a nearly constant quantity of Ca^{2+} had to be redistributed within a closed system with a decreasing amount of Ca^{2+}-buffer (DM-nitrophen).

The dependence of twitch amplitude on flash magnitude must reflect various nonlinear features, including the apparent gain of CICR (*14*), the $[Ca^{2+}]_i$-tension relationship (*22*), the force-length relationship (*23*), and the small consumption of DM-nitrophen. The twitch amplitude was not steeply dependent on flash energy, consistent with relatively low gain CICR. To demonstrate that the low gain of CICR did not arise simply from a direct activation of the contractile proteins by the photoreleased Ca^{2+}, we repeatedly stimulated twitches as phasic sarcoplasmic reticulum (SR) Ca^{2+} release was increasingly blocked by the application of 10 µM ryanodine. Low-energy flashes (30 W-s) were applied every 30 s with the cell at a holding potential of −40 mV. Approximately 7% of the twitch remained after release was blocked by ryanodine, indicating that about 93% was due to CICR (Fig. 2). When a high-energy flash (230 W-s) was applied in the presence of ryanodine, direct activation of the twitch was still possible, supporting our observation that depletion of caged Ca^{2+} did not lead to any significant reduction of twitch.

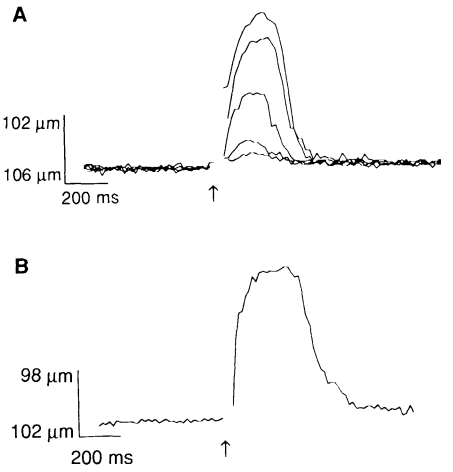

Fig. 2. Effect of ryanodine on twitch contractions triggered by photorelease of Ca^{2+}. (**A**) Twitch contractions were induced by low-energy flashes (30 W-s). After the control contraction (upper tracing), ryanodine (10 µM) was added to the superfusion solution. Flashes were applied every 30 s. (**B**) The recontrol with a discharged energy of 230 W-s elicited a full twitch. The arrow marks the time of the flash.

To investigate the effect of membrane potential on CICR, we established a cellular holding potential of −40 mV to remove the influence of the I_{Na}. The Na^+-Ca^{2+} exchanger and Ca^{2+} influx through I_{Ca} was blocked with 5 mM Ni^{2+} (*24*) or by removal of extracellular Ca^{2+}. Neither procedure alters the dihydropyridine (DHP)-sensitive gating charge (*25*) that may sense membrane voltage for the release channel (*26–28*). Using isolated guinea pig cells loaded with DM-nitrophen, we applied low-energy flashes (30 W-s) at different potentials in the same heart cell (Fig. 3). Cells were flashed on depolarization to +100 mV (near E_{Ca}), to 0 mV (near the maximum of the peak I_{Ca}-voltage curve), and on hyperpolarization to −100 mV (negative to the normal resting potential). When Ca^{2+} jumps were imposed simultaneously with the voltage step, almost identical twitches were produced at every membrane potential. It is also apparent from gating current experiments (*27, 28*) examining the voltage sensors in cardiac muscle that there is a time-dependent immobilization of DHP-sensitive Ca^{2+} channel gating charge in heart muscle (*27*). Since these DHP receptors act as the charge sensors for Ca^{2+} release in skeletal muscle (*13*) and perhaps in heart (*14, 26*), we repeated the above experiment at two other times during the voltage pulse. Twitches induced by photorelease at the same three potentials but 500 ms after depolarization or at the time of repolarization back to −40 mV were also virtually identical. We have found similar results in 14 heart cells (111 twitches) in the

presence of extracellular Ca^{2+}, using Ni^{2+} to block I_{Ca} and the Na^+-Ca^{2+} exchanger, and in four cells (18 twitches) in the absence of extracellular Ca^{2+}.

Sarcolemmal voltage appears to exert no effect on CICR under the conditions of these experiments (Fig. 3B). Thus, our findings clearly suggest that Ca^{2+} release from the SR depends simply on a phasic elevation of triggering Ca^{2+} (*2*) and not on voltage. These findings do not rule out the possibility that, under other conditions (such as Ca^{2+} overload of the SR or elevated $[Na^+]_i$), voltage may influence CICR directly (*29*).

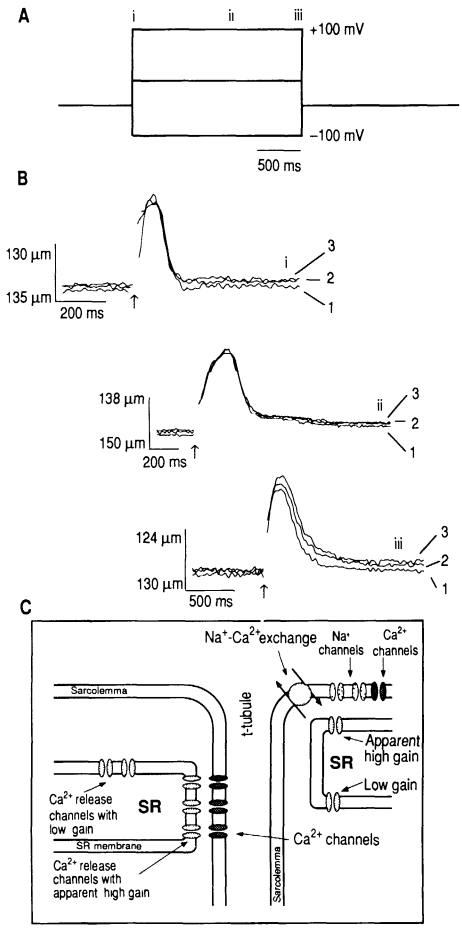

Fig. 3. Voltage dependence of CICR. (**A**) Schematic diagram of the voltage-clamp protocol used. From a holding potential of −40 mV, the membrane voltage was changed to −100 mV, 0 mV, or +100 mV for 2 s. Flashes were triggered at (i), (ii), or (iii). (**B**) Twitch contractions triggered by photorelease of Ca^{2+} at different membrane potentials. Photorelease of Ca^{2+} was activated at the same time as the voltage change (i), in the steady-state 500 ms after the voltage step (ii), or upon returning to the holding potential (iii). Superimposed contractions are shown for each series. The flash was activated at membrane potentials of −100 mV (trace 1), 0 mV (trace 2), and +100 mV (trace 3). Ni^{2+} (5 mM) was added to the superfusion solution to block the Na^+-Ca^{2+} exchanger and the Ca^{2+} inward current. Series (ii) and (iii) were performed at 22°C with flash energies of 30 W-s series, (i) at 31°C with flash energies of 25 W-s. (**C**) Proposed interaction of Ca^{2+} channels, Na^+ channels, and Na^+-Ca^{2+} exchange with the Ca^{2+} release channels of the SR. A close association in a space with restricted diffusion yields release channels with an apparent high gain (positive feedback) because they are exposed to a high $[Ca^{2+}]$, during Ca^{2+} influx and release, whereas release channels farther away from the sarcolemma exhibit low gain.

Although we do not know the exact distribution of DM-nitrophen in the cytosol, flash photolysis of caged Ca^{2+} produces an elevation of trigger $[Ca^{2+}]_i$ in our cells that is unusually uniform, unlike the elevation of trigger $[Ca^{2+}]_i$ that occurs normally in heart muscle with depolarization. Depolarization activates I_{Ca} and I_{Na} (hence, Na^{2+}-Ca^{2+} exchange) (*16*) and thereby initially elevates trigger $[Ca^{2+}]_i$ close to the sarcolemmal (including t-tubular) membrane to a greater extent and more rapidly than it does deeper in the cell. We suggest that this short-lived spatial inhomogeneity of the triggering $[Ca^{2+}]_i$ is important in vivo because it can differentially activate (*2*) and possibly inactivate (*30*) CICR in different regions of each cell, enabling the SR to release Ca^{2+} in a graded way and not in an "all-or-nothing" fashion. Such $[Ca^{2+}]_i$ inhomogeneities, which occur in diverse cell types (*3–6*), including heart (*16, 31–34*), may explain the apparent shifts in gain in the CICR system in heart (*11, 14*). If physiologically significant spatial gradients of $[Ca^{2+}]_i$ exist during excitation (*35*), then many functionally discrete CICR elements (such as the many Ca^{2+} release channels in the SR) could have the same low gain that respond quite differently to a single stimulus. The apparent difference in gain would arise because release channels may be exposed to different $[Ca^{2+}]_i$ during the Ca^{2+} transient. Thus, our results suggesting that the gain of the cellular CICR channel is relatively low are consistent with previous observations suggesting voltage dependence of gain (*11*), the voltage dependence being mediated by changes of $[Ca^{2+}]_i$ in a "fuzzy" (restricted) space (*35*). High local trigger-Ca^{2+} (such as near the sarcolemma) may thus induce significant local release (and result in apparent high gain), whereas a lower spatially averaged $[Ca^{2+}]_i$ would trigger less release from the other CICR elements (Fig. 3). Thus, if short-lived spatial gradients for $[Ca^{2+}]_i$ occur, relatively low gain CICR elements may be adequate to account for the apparent discrepancies in gain, the subthreshold CICR (*36*), and waves of propagated elevated $[Ca^{2+}]_i$ due to CICR that have been noted in hepatocytes (*3*), in astrocytes (*6*), and in Ca^{2+}-overloaded cardiac myocytes (*32–34*), but not in unloaded myocytes (*37*). However, the results do not rule out the possibility that a small fraction of release sites exhibits voltage dependence of the gain. Furthermore, the gain of CICR may be modulated by physiologically important processes (such as phosphorylation), cellular conditions (such as pH and SR Ca^{2+} load), and natural or applied chemicals [such as inositol phosphates, caffeine (*9*), ryanodine, DHPs (*38*), and general anesthetics (*39*)]. Here we have shown that a relatively low-gain CICR system exists in heart muscle and that this system is not directly modulated by membrane voltage, under the conditions of our experiments. The presence of low gain positive feedback and the existence of short-lived temporal gradients of $[Ca^{2+}]_i$ are probably also relevant to our understanding of the normal physiologic control and modulation of CICR.

REFERENCES AND NOTES

1. M. Endo, *Physiol Rev* **57**, 71 (1977).
2. A. Fabiato, *Am. J. Physiol* **245**, C1 (1983).
3. N. M. Woods, K. S. R. Cuthbertson, P. H. Cobbold, *Cell Calcium* **8**, 79 (1987).
4. T. K. Gosh, J. M. Mullaney, F. I. Tarazi, D. L. Gill, *Nature* **340**, 236 (1989).
5. T. Kawanishi, L. M. Blank, A. T. Harootunian, M. T. Smith, R. Y. Tsien, *J Biol Chem.* **264**, 12859 (1989).
6. A. H. Cornell-Bell, S. M. Finkbeiner, M. S. Cooper, S. J. Smith, *Science* **247**, 470 (1990).
7. S. Fleischer and M. Inui, *Annu. Rev. Biophys Biophys Chem* **18**, 333 (1989).
8. S. M. Baylor and S. Hollingworth, *J Physiol (London)* **403**, 151 (1988).
9. B. L. Simon, M. G. Klein, M. F. Schneider, *Biophys. J.* **55**, 793 (1989).
10. M. Valdeolmillos, S. C. O. O'Neill, G. L. Smith, D. A. Eisner, *Pfluegers Arch.* **413**, 676 (1989); M. Näbauer and M. Morad, *Am J Physiol* **258**, C189 (1990).
11. M. B. Cannell, J. R. Berlin, W. J. Lederer, *Science* **238**, 1419 (1987).
12. D. J. Beuckelmann and W. G. Wier, *J Physiol. (London)* **405**, 233 (1988).
13. E. Ríos and G. Pizarro, *News Physiol Sci.* **3**, 223 (1988).
14. W. J. Lederer, M. B. Cannell, N. M. Cohen, J. R. Berlin, *Mol. Cell. Biochem* **89**, 115 (1989).
15. M. Näbauer, G. Callewaert, L. Cleemann, M. Morad, *Science* **244**, 800 (1989).
16. N. Leblanc and J. R. Hume, *ibid.* **248**, 372 (1990).
17. Light from the flash lamp was collected by an ellipsoidal mirror, reflected off a dichroic mirror (330 to 410 nm), and focused on the aperture of a liquid light-guide. We imaged the output aperture of the light-guide onto the preparation in an epi-illumination arrangement using a planoconvex condenser lens and the microscope objective (Zeiss Neofluar 63×, numerical aperture, 1.25). Wavelength selection was enhanced by two additional dichroic mirrors (310 to 390 nm) between the condenser lens and the microscope objective. Upon photolysis, DM-nitrophen increases its equilibrium dissociation constant for Ca^{2+} from 5 nM to 3 mM (at pH 7.0 and in the absence of Mg^{2+}) and releases Ca^{2+} with a time constant of less than 200 µs. If the buffer is weakly loaded with Ca^{2+}, the rapid release of Ca^{2+} is followed by reequilibration with the remaining DM-nitrophen, and a phasic Ca^{2+} pulse is produced, as opposed to a stepwise increase in Ca^{2+}. J. H. Kaplan, *Annu Rev. Physiol* **52**, 897 (1990).
18. E. Niggli and W. J. Lederer, unpublished observation.
19. J. H. Kaplan and G. C. R. Ellis-Davies, *Proc. Natl Acad. Sci. U.S.A.* **85**, 6571 (1988).
20. Cardiac myocytes were isolated from guinea pig ventricles by enzymatic digestion. Hearts were perfused with a Ca^{2+}-free solution (5 min), followed by

an enzyme solution [collagenase (0.5 mg/ml) and protease (0 1 mg/ml), 5 min]. The experimental superfusion solution contained. NaCl, 145 mM; KCl, 4 mM; $CaCl_2$, 1 mM; CsCl, 1 mM; $BaCl_2$, 0.5 mM; Hepes, 10 mM, glucose, 10 mM; pH 7.4. $NiCl_2$ (5 mM) was added to this solution to block the Ca^{2+} channels and the Na^+-Ca^{2+} exchanger. Another solution designed to prevent Ca^{2+} influx into the cells was nominally Ca^{2+}-free, and NaCl was replaced by LiCl. The pipette filling solution had the following composition. CsCl, 120 mM; Hepes, 20 mM; tetraethylammonium, 20 mM; DM-nitrophen, 2 mM; $CaCl_2$, 0.5 mM; potassium adenosine triphosphate, 5 mM; pH 7.2. Most experiments were carried out at 21° to 23°C, some at 31° to 32°C. The gigaohm-seal technique in the whole-cell configuration was used to control the membrane voltage and to equilibrate the cytosol with the pipette filling solution. Cells were held at a holding potential of −40 mV during the loading procedure (8 to 12 min). Electrodes were pulled from borosilicate glass and had a series resistance between 0.8 and 1.5 megohms. Cell length was measured with a video-dimension analyzer (60 cell-length measurements per second)

21. D. M. Bers and J. H. Bridge, *Circ. Res.* **65**, 334 (1989); J. H. Bridge, J. R. Smolley, K. W. Spitzer, *Science* **248**, 376 (1990); L. M. Crespo, C. J. Grantham, M. B. Cannell, *Nature* **345**, 618 (1990).
22. S. M. Harrison and D. M. Bers, *Am. J. Physiol* **258**, C274 (1990).
23. E. Niggli and W. J. Lederer, *Biophys J.* **57**, 552a (1990).
24. J. Kimura, S. Miyamae, A. Noma, *J Physiol. (London)* **384**, 199 (1987).
25. R. W. Hadley and W. J. Lederer, unpublished observation.
26. N. M. Cohen and W. J. Lederer, *J. Physiol. (London)* **406**, 115 (1988).
27. R. W. Hadley and W. J. Lederer, *ibid* **415**, 601 (1989).
28. B. P. Bean and E. Ríos, *J Gen. Physiol.* **94**, 65 (1989).
29. J. R. Berlin, M. B. Cannell, W. J. Lederer, *Am. J. Physiol.* **253**, H1540 (1987).
30. A. Fabiato, *J. Gen Physiol.* **85**, 247 (1985).
31. D. T Yue and W. G. Wier, *Biophys J.* **48**, 533 (1985).
32. J. R. Berlin, M. B. Cannel, W. J. Lederer, *Circ. Res* **65**, 115 (1989).
33. T. Takamatsu and W. G. Wier, *FASEB J* **4**, 1519 (1990).
34. P. H. Backx, P. P. deTombe, J. H. K. van Deen, B. J. M. Mulder, H E. D. J. terKeurs, *J. Gen Physiol.* **93**, 963 (1989).
35. W. J. Lederer *et al.*, *Science* **248**, 283 (1990).
36. H. A. Spurgeon, A. Talo, E. G. Lakatta, M. D. Stern, *Biophys J* **57**, 176a (1990).
37. S. C. O'Neill, J. G. Mill, D. A. Eisner, *Am. J Physiol* **258**, C1165 (1990).
38. L. V. Hryshko, V. M. Stiffel, D. M. Bers, *Biophys. J.* **57**, 167a (1990).
39. D. M. Wheeler, R. T. Rice, R. G. Hansford, E. G. Lakatta, *Anesthesiology* **69**, 578 (1988).
40. We thank J. H. Kaplan for his gift of DM-nitrophen and his counsel. Thanks also to B. E. Alger, M. B. Cannell, and R. W. Hadley for discussions. Supported by a fellowship from the Swiss National Science Foundation (87-30 BE) to E.N. and NIH grants HL36974 and HL25675 to W.J.L.

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Restoration of Inactivation in Mutants of *Shaker* Potassium Channels by a Peptide Derived from ShB

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Site-directed mutagenesis experiments have suggested a model for the inactivation mechanism of *Shaker* potassium channels from *Drosophila melanogaster*. In this model, the first 20 amino acids form a cytoplasmic domain that interacts with the open channel to cause inactivation. The model was tested by the internal application of a synthetic peptide, with the sequence of the first 20 residues of the ShB alternatively spliced variant, to noninactivating mutant channels expressed in *Xenopus* oocytes. The peptide restored inactivation in a concentration-dependent manner. Like normal inactivation, peptide-induced inactivation was not noticeably voltage-dependent. Trypsin-treated peptide and peptides with sequences derived from the first 20 residues of noninactivating mutants did not restore inactivation. These results support the proposal that inactivation occurs by a cytoplasmic domain that occludes the ion-conducting pore of the channel.

DELETIONS AND POINT MUTATIONS near the amino terminal of the ShB potassium channels of *D. melanogaster* dramatically show inactivation (*1*). These data plus the lack of voltage dependence of the inactivation rates and the ability of internal proteolytic agents to modify inactivation suggest a mechanism of inactivation similar to the ball and chain model originally proposed by Armstrong and Bezanilla (*2*) for voltage-gated Na^+ channels. In ShB channels, the first 20 amino acids in the NH_2-terminus are proposed to form a structural domain that interacts with part of the open channel to cause inactivation. This structural domain, or "ball" region, is connected to the rest of the protein by a "chain" sequence of 60 or more amino acids that tether the inactivation ball near its receptor. According to this model of inactivation, the putative ball region should be able to interact with the rest of the channel and produce inactivation even when it is not covalently attached to the rest of the channel protein. We tested this hypothesis by examining the effects of a peptide corresponding to the ball region (first 20 amino acids of ShB: MAAVAGLYGLGEDRQHRKKQ) (*3*) on the gating of ShB channels expressed in *Xenopus* oocytes. The experiments were performed with mutant ShB channels that contain, near their NH_2-terminus, a large deletion that effectively removes fast inactivation (*4*).

ShBΔ6-46 is a 41–amino acid deletion mutant of ShB that does not inactivate with a rapid time course (*1*). Application of the ShB peptide to the cytoplasmic side of ShBΔ6-46 channels accelerates their inactivation rate (Fig. 1). We tested the effects of different concentrations of peptide on macroscopic currents in inside-out patches elicited by voltage steps to 0 mV and +50 mV. The peptide-induced inactivation occurs rapidly and can be readily reversed when peptide-free solution is perfused into the bath, indicating that the peptide only weakly associates with the channel. At each voltage, the rate of the macroscopic inactivation is dependent on the peptide concentration and increases with increasing concentrations of peptide, as expected for a simple bimolecular reaction. In addition, the macroscopic inactivation rate at a given peptide concentration is dependent on the voltage. At the more positive voltages, where the channels activate more rapidly, peptide-induced inactivation occurs more rapidly. However the rate of the inactivation transition induced by peptide observed in single-channel recording is independent of voltage. These results indicate that the inactivation produced by the peptide is coupled to activation, as is the case for the normal inactivation process. Application of the ShB peptide to the extracellular side did not have any effect on the currents recorded from ShBΔ6-46 channels.

The rate of recovery from peptide-induced inactivation, however, appears to be slower than that from normal fast inactivation. During repeated voltage pulses at a frequency where ShB currents completely recover in the interval between pulses, the currents in the presence of peptide tend to decrease in amplitude progressively because the channels accumulate in the peptide-blocked state. This difference in recovery rates probably arises because the synthetic peptide has a greater binding affinity for the channel than does the normal NH_2-terminal ball domain. This difference may result from the absence of covalently linked residues, which normally destabilize the inactivated state. Alternatively, the peptide could bind to additional sites on the channel, which could cause slow inactivation. In either case, the slower recovery rate from peptide-in-

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