M. J. Bevan, *ibid.* **325**, 192 (1987).
2. A. R. M. Townsend, F. M. Gotch, J. Davey, *Cell* **42**, 457 (1985); A. R. M. Townsend, J. Bastin, K. Gould, G. Brownlee, *Nature* **324**, 575 (1986); J. W. Yewdell *et al.*, *J. Exp. Med.* **163**, 1529 (1986).
3. J. W. Yewdell, J. R. Bennink, Y. Hosaka, *Science* **239**, 637 (1988); Y. Hosaka, F. Sasao, K. Yamanaka, J. Bennink, J. W. Yewdell, *J. Immunol.* **140**, 606 (1988); T. S. Watt and L. R. Gooding, *Nature* **283**, 74 (1980).
4. M. W. Moore, F. R. Carbone, M. J. Bevan, *Cell* **54**, 777 (1988).
5. N. A. Hosken, M. J. Bevan, F. R. Carbone, *J. Immunol.* **142**, 1079 (1989).
6. Y. Tanaka and S. S. Tevethia, *Virology* **165**, 357 (1988); J. H. Cox, J. W. Yewdell, L. C. Eisenlohr, P. R. Johnson, J. R. Bennink, *Science* **247**, 715 (1990); J. W. Yewdell and J. R. Bennink, *ibid.* **244**, 1072 (1989); J. G. Nuchtern, J. S. Bonifacino, W. E. Biddison, R. D. Klausner, *Nature* **339**, 223 (1989).
7. A. R. M. Townsend *et al.*, *Nature* **340**, 443 (1989).
8. R. DeMars *et al.*, *Proc. Nat. Acad. Sci. U.S.A.* **82**, 8183 (1985); R. D. Salter, D. N. Howell, P. Cresswell, *Immunogenetics* **21**, 235 (1985).
9. J. Alexander, J. A. Payne, R. Murray, J. A. Frelinger, P. Cresswell, *Immunogenetics* **29**, 380 (1989).
10. The JY cell line and the Jurkat cell lines transfected with HLA-A2 or H-2K^b were obtained from W. Heath and M. Irwin.
11. F. Gotch, J. Rothbard, K. Howland, A. Townsend, A. McMichael, *Nature* **326**, 881 (1987).
12. N. A. Hosken and M. J. Bevan, unpublished results.
13. L. Puddington, M. J. Bevan, J. K. Rose, L. LeFrançois *J. Virol.* **60**, 708 (1989).
14. J. E. Rothman, *Cell* **59**, 591 (1989).
15. J. Alexander, J. A. Payne, B. Shigekawa, J. A. Frelinger, P. Cresswell, *Immunogenetics*, in press.
16. The influenza matrix–specific CTL clone 219 was obtained from A. Vittielo.
17. J. M. Sheil, M. J. Bevan, L. LeFrançois, *J. Immunol.* **138**, 3654 (1987).
18. F. R. Carbone and M. J. Bevan, *J. Exp. Med.* **169**, 603 (1989).
19. We thank P. Cresswell for providing the T2 and T2K^b cell lines, W. Heath and M. Irwin for the HLA-A2 and H-2K^b expressing cell lines, A. Vittiello for CTL clone 219, J. Sheil for CTL clone 33, F. Carbone for CTL clone B3, L. LeFrançois for the VSV-N transfectant N1, and M. Moore for the OVA transfectant E.G7-OVA. N.A.H. is a recipient of an NSF Graduate Fellowship. Supported in part by NIH grant AI 19335 from the National Institute of Allergy and Infectious Diseases.

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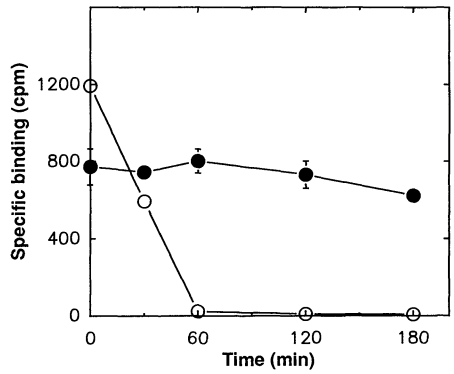

Fig. 1. Inhibition of ^{125}I–TNF-α, but not ^{125}I–IFN-γ, binding to macrophages after incubation with taxol. Thioglycollate-elicited peritoneal macrophages (CD1, 8 weeks, Charles River) were incubated with taxol (10 μ*M*) at 37°C for indicated times. The cells were washed, and ^{125}I–TNF-α (○) or ^{125}I–IFN-γ (●) binding was then measured at 4° or 25°C, respectively, for 3 hours as described (*5, 15*). Results are means ± SE from one of three similar experiments.

Shared Actions of Endotoxin and Taxol on TNF Receptors and TNF Release

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Bacterial lipopolysaccharide (LPS) exerts profound effects on mammalian hosts in part by inducing macrophages to release tumor necrosis factor–α (TNF-α); the mechanisms involved are unresolved. The microtubule stabilizer taxol shared two actions of LPS on macrophages: it rapidly decreased TNF-α receptors and triggered TNF-α release. Both actions of taxol were absent in LPS-hyporesponsive C3H/HeJ mice. In recombinant inbred mice, the genes controlling responses to LPS and to taxol were closely linked. Dexamethasone blocked release of TNF-α by both stimuli but did not block the decrease in TNF-α receptors. Thus, a protein associated with microtubules may be a cellular target of LPS.

ENDOTOXIC LIPOPOLYSACCHARIDE, the major component of the outer membrane of Gram-negative bacteria, has profound effects on eukaryotic cells and mammalian organisms (*1*). Among its primary targets are macrophages, which respond to LPS by releasing inflammatory mediators, such as TNF-α, interleukin-1, interleukin-6, interferon-α, interferon-β, platelet activating factor, eicosanoids, and reactive oxygen and nitrogen intermediates (*2*). TNF-α is an endogenous mediator of septic shock (*3*). However, it is not clear to what intracellular targets LPS binds (*4*), or how LPS initiates TNF-α production or any other cellular effect. Macrophages respond to TNF-α by means of specific receptors (TNF-R) (*5*). The LPS decreases macrophage TNF-R expression so rapidly that secretion of TNF-α and its binding to TNF-R could account for only a small part of the effect (*5*). We recently observed that microtubule-depolymerizing agents also decrease TNF-R by a mechanism not involving TNF-α release (*6*). Taxol is a plant-derived antitumor drug (*7*) that binds polymerized tubulin stoichiometrically and reversibly at a site different from colchicine, podophyllotoxin, vinblastine, or guanine nucleotides and thereby inhibits depolymerization (*8*). We now show that taxol acts on macrophages to mimic two effects of LPS: to decrease TNF-R and to induce TNF-α release.

Taxol (10 μ*M*, 37°C) inhibited the binding of ^{125}I-labeled TNF-α (^{125}I–TNF-α) to macrophages by 50% within 30 min, as measured at 4°C. By 1 hour, we could detect no TNF-α binding sites (Fig. 1). Binding of interferon-γ (IFN-γ) was unaffected by taxol (Fig. 1), similar to the insensitivity of the IFN-γ receptor to LPS (*9*). A 1-hour incubation with taxol at 4°C before, or addition of taxol during, the binding step did not diminish binding of ^{125}I–TNF-α (*10*). By a limulus test, 10 μ*M* taxol contained too small an amount of LPS (<1 pg/ml) to decrease TNF-R (*5*).

To see if taxol was inducing TNF-α release, we used an antiserum (15E) against murine TNF-α (*11*). When present during incubation with taxol, 15E partially reversed the inhibitory effect of taxol on ^{125}I–TNF-α binding (Fig. 2A). This suggested that taxol triggered TNF-α release, but that binding of endogenous TNF-α was only partially responsible for decreased binding of ^{125}I–TNF-α. Induction of TNF-α by taxol was confirmed by a radioreceptor assay (*12*). Macrophage-conditioned media from the culture assayed in Fig. 1 were collected and tested for their ability to compete with ^{125}I–TNF-α for binding sites at 4°C, a condition in which the indicator cells could not respond to taxol. These conditioned media blocked binding of ^{125}I–TNF-α (Fig. 2B). Independent evidence was provided by using flow cytometry to measure the fluorescence of saponin-permeabilized murine macrophages stained with a monoclonal antibody to TNF. Staining for intracellular TNF-α was induced by both taxol and LPS (Fig. 2C).

To test if responses to LPS and taxol share a genetic basis, we examined LPS-hyporesponsive C3H/HeJ mice, whose gene defect has been mapped to the fourth chromosome (LPS^d) (*13*). TNF-R expression on C3H/HeJ macrophages was unaffected by taxol (10 μ*M*), whereas on macrophages from the closely related LPS-responsive strain C3H/HeN (LPS^n) TNF-R surface expression was decreased by 50% with ~0.15 μ*M* taxol and by >95% with 1 μ*M* taxol (Fig. 3). In contrast, exogenous TNF-α decreased

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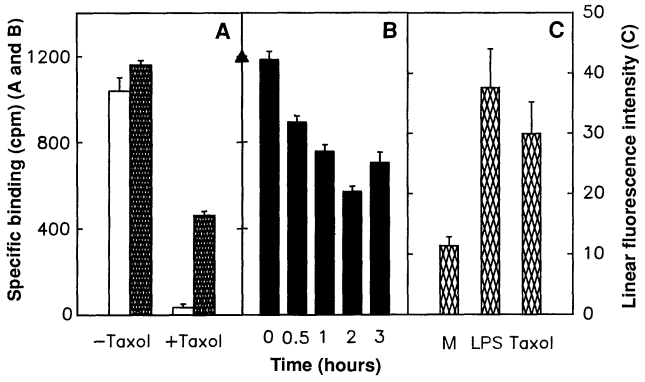

Fig. 2. Taxol induces TNF-α release. (**A**) Antiserum for murine TNF-α prevented TNF-R decrease by taxol. Macrophages were treated with taxol (10 μ*M*) (37°C, 1 hour) in the presence of preimmune serum (open bar) or antiserum 15E (1:640) (hatched bar) (*11*), then assayed for TNF-α binding. (**B**) Competition of ^{125}I–TNF-α binding at 4°C by conditioned media from taxol-treated macrophages. Supernates from Fig. 1 were used as the medium in which to measure ^{125}I–TNF-α binding to untreated macrophages at 4°C. Abscissa indicates the duration of taxol treatment before the conditioned media were collected. Control (▲) shows binding in the absence of taxol. (**C**) Induction of TNF-α in macrophages treated with LPS (10 ng/ml) or taxol (10 μ*M*) for 1 hour at 37°C. M indicates medium alone. The cells were washed and stained with hamster monoclonal antibody against murine TNF-α (*16*) and fluorescein-conjugated second antibody in the presence of 0.04% saponin; then 10,000 cells per condition were analyzed by a FACScan flow microfluorometer (Becton Dickinson). Background fluorescence was assayed by omitting the first antibody and was subtracted. Results are means of mean linear fluorescence channel ± SE of triplicates from one of four experiments.

Table 1. Comparison of responses to LPS and taxol in BXH recombinant inbred strains. Thioglycollate-elicited peritoneal macrophages in 24-well culture plates (10^6 cells per well) were first incubated with taxol (10 μ*M*) or LSP (1 ng/ml) or medium alone for 1 hour at 37°C. Results are means of triplicates from one of four similar experiments.

Mouse strains	^{125}I–TNF-α binding (cpm)		
	Medium	LPS	Taxol
CD1	1922	126	293
C3H/HeJ	2963	2743	2628
BXH-2	1157	1038	1053
BXH-3	1717	1479	1553
BXH-6	2075	2243	2376
BXH-7	1665	1768	1570
BXH-9	1333	1597	1418
BXH-10	1916	26	68
BXH-11	2428	2254	2253
BXH-14	2664	90	4
BXH-19	2063	76	25

the expression of TNF-R on C3H/HeJ macrophages (*10*). Thus, the inability of C3H/HeJ macrophages to decrease TNF-R in response to taxol did not reflect a generalized inability to decrease TNF-R. Taxol was equally efficient at blocking proliferation of C3H/HeN and C3H/HeJ bone marrow cells in vitro (*10*). The mitotic spindle apparatus in C3H/HeJ mice, therefore, appears to be inhibited normally by taxol.

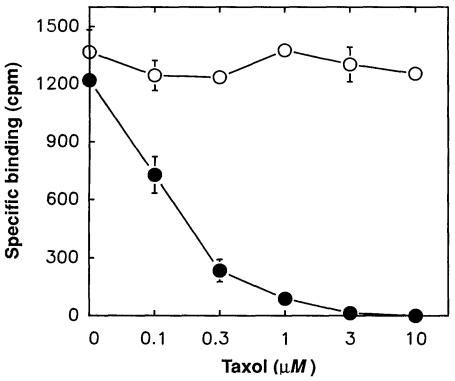

Fig. 3. Differential responses to taxol by macrophages from C3H/HeN (●) (Charles River) and C3H/HeJ mice (○) (Jackson Laboratory). Macrophages were incubated with indicated concentrations of taxol at 37°C for 1 hour, then assayed for TNF-α binding. Results are means ± SE from one of six similar experiments.

We next examined a panel of recombinant inbred strains (BXH) from the F_2 generation of the cross between C57BL/6J (LPS high responder) and C3H/HeJ (LPS low responder) (*13*). Individual BXH strains were characterized as LPS high or low responders on the basis of the mitogenic responses of their splenocytes (*13*). All nine strains tested were concordant for the presence or absence of decrease of TNF-R in response to the two stimuli, LPS and taxol (Table 1). Thus, the genes controlling responses of TNF-R to LPS and taxol are closely linked.

TNF-α induction by LPS in macrophages from C3H/HeJ mice is blocked transcriptionally and translationally (*14*). Dexamethasone induces similar defects in cells from endotoxin-sensitive mice (*14*). We compared the effects of dexamethasone on decrease of TNF-R (Fig. 4A) and induction of TNF-α (Fig. 4B) in endotoxin-sensitive macrophages. Dexamethasone (1 μ*M*) blocked macrophage release of TNF-α in response either to LPS or to taxol; their conditioned media competed poorly with ^{125}I–TNF-α for binding to indicator macrophages at 4°C (Fig. 4B). In contrast, dexamethasone had no effect on the LPS- or taxol-induced decrease in TNF-R (Fig. 4A). Thus, induction of endogenous TNF-α was not the main reason why LPS (*5*) and taxol decreased TNF-R.

In conclusion, taxol, a drug whose best known action is to stabilize tubulin polymers, mimics at least two related but independent actions of LPS on macrophages: decrease of TNF-R surface expression and induction of TNF-α release. The gene controlling these responses to taxol is closely linked to the gene controlling responsiveness to LPS. Thus, an intracellular target affected by taxol might be involved in actions of LPS on macrophages and other cells. Because the relevant target of taxol does not seem to be a key component of the mitotic spindle, this target is probably not tubulin, but it may be a microtubule-associated protein whose binding to tubulin is affected by taxol. Further experiments are necessary to determine which intracellular molecules LPS and taxol affect in common. This information may lead to new insights into the actions of LPS on mammalian cells and the participation of the cytosekleton in responses of cells to their environment.

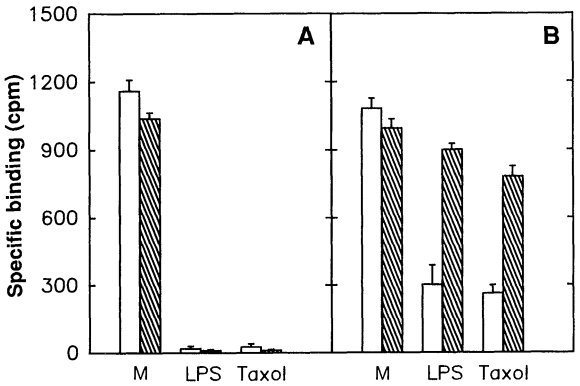

Fig. 4. Effect of dexamethasone on LPS- and taxol-induced (**A**) TNF-R decrease and (**B**) TNF-α release. LPS (10 ng/ml) or taxol (10 μ*M*) was added to macrophages that had been treated overnight with (shaded bar) or without (open bar) dexamethasone (1 μ*M*). After 2 hours at 37°C, supernatants were removed, and in (A) TNF-α binding was measured at 4°C. In (B), the LPS- and taxol-conditioned media from (A) were used as the binding buffer in the ^{125}I–TNF-α binding assay of untreated macrophages. Results are means ± SE for triplicates. M, medium alone.

REFERENCES AND NOTES

1. L. B. Hinshaw, Ed., *Handbook of Endotoxin: Pathophysiology of Endotoxin* (Elsevier, Amsterdam, 1984), vol. 2.

2. C. F. Nathan, *J. Clin. Invest.* **79**, 319 (1987); J. Bauer *et al.*, *Blood* **72**, 1134 (1988); K. Matsushima *et al.*, *J. Exp. Med.* **167**, 1883 (1988).
3. B. Beutler, I. W. Milsark, A. C. Cerami, *Science* **229**, 869 (1985).
4. S. D. Wright, *J. Exp. Med.* **164**, 1876 (1986); M.-G. Lei and D. S. Morrison, *J. Immunol.* **141**, 996 (1988); R. Y. Hampton *et al.*, *J. Biol. Chem.* **263**, 14802 (1988); P. S. Tobias, K. Soldau, R. J. Ulevitch, *ibid.* **264**, 10867 (1989).
5. A. Ding, E. Sanchez, S. Srimal, C. F. Nathan, *J. Biol. Chem.* **264**, 3924 (1989).
6. A. Ding, F. Porteu, E. Sanchez, C. F. Nathan, *J. Exp. Med.* **171**, 715 (1990).
7. M. C. Wani, H. L. Taylor, M. E. Wall, P. Coggon, A. T. McPhail, *J. Am. Chem. Soc.* **93**, 2325 (1971).
8. P. B. Schiff, J. Fant, S. B. Horwitz, *Nature* **277**, 665 (1979); P. B. Schiff and S. B. Horwitz, *Biochemistry* **20**, 3247 (1981).
9. A. Celada and R. D. Schreiber, *J. Immunol.* **139**, 147 (1987).
10. A. Ding and C. F. Nathan, unpublished data.
11. Rabbit antiserum against murine TNF-α, lot 15E B1 070886, neutralizing titer ~6.4×10^5 units per milliliter (Genentech).
12. B. A. Beutler, I. W. Milsark, A. Cerami, *J. Immunol.* **135**, 3972 (1985).
13. J. Watson, R. Riblet, B. A. Taylor, *ibid.* **118**, 2088 (1977). BXH mice were from Jackson Laboratory, Bar Harbor, ME.
14. B. Beutler, N. Krochin, I. W. Milsark, C. Luedke, A. Cerami, *Science* **232**, 977 (1986).
15. Human recombinant TNF-α (4.1×10^7 units per milligram of protein) and murine recombinant IFN-γ (5.2×10^7 units per milligram of protein) were from Genentech. Taxol was provided by the Drug Synthesis and Chemistry Branch, Developmental Therapeutics Program, Division of Cancer Treatment, National Cancer Institute, Bethesda, MD.
16. K. C. F. Sheehan, N. H. Ruddle, R. D. Schreiber, *J. Immunol.* **142**, 3884 (1989).
17. Supported by National Cancer Institute grant CA-43610.

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Sodium Current–Induced Release of Calcium from Cardiac Sarcoplasmic Reticulum

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The role of sodium-calcium exchange at the sarcolemma in the release of calcium from cardiac sarcoplasmic reticulum was investigated in voltage-clamped, isolated cardiac myocytes. In the absence of calcium entry through voltage-dependent calcium channels, membrane depolarization elicited release of calcium from ryanodine-sensitive internal stores. This process was dependent on sodium entry through tetrodotoxin-sensitive sodium channels. Calcium release under these conditions was also dependent on extracellular calcium concentration, suggesting a calcium-induced trigger release mechanism that involves calcium entry into the cell by sodium-calcium exchange. This sodium current–induced calcium release mechanism may explain, in part, the positive inotropic effects of cardiac glycosides and the negative inotropic effects of a variety of antiarrhythmic drugs that interact with cardiac sodium channels. In response to a transient rise of intracellular sodium, sodium-calcium exchange may promote calcium entry into cardiac cells and trigger sarcoplasmic calcium release during physiologic action potentials.

IN MAMMALIAN HEART MUSCLE, THE release of Ca^{2+} from the sarcoplasmic reticulum (SR) is important in the activation of contraction. In contrast to skeletal muscle, where Ca^{2+} release is directly controlled by membrane voltage through a charge-coupled release mechanism (*1*), Ca^{2+} release in the heart is generally believed to involve a Ca^{2+}-induced release mechanism (*2*). Sarcolemmal Ca^{2+} channels are thought to provide the primary source of Ca^{2+} for both the trigger for release of Ca^{2+} as well as the loading of Ca^{2+} into the SR (*3*). In studies (*4*) that have used Ca^{2+}-sensitive dyes to monitor SR Ca^{2+} release, it has not been possible to show Ca^{2+} release in the absence of Ca^{2+} entry through voltage-dependent Ca^{2+} channels. This had led to the suggestion (*5*) that Ca^{2+} influx through sarcolemmal Ca^{2+} channels is required for coupling membrane depolarization to SR Ca^{2+} release in heart muscle. However, intracellular Na^+ concentration is well known to be another important determinant of myocardial contractile strength (*6*). For example, Na^+ is implicated in the inotropic mechanism of action of digitalis glycosides, in which a rise of intracellular Na^+ is thought to augment Ca^{2+} entry into heart cells by promoting reverse-mode activity of the Na^+-Ca^{2+} exchanger (*7*). Although there is substantial evidence that Na^+-Ca^{2+} exchange is an important transport system for extruding Ca^{2+} (forward-mode activity) from cardiac cells (*8*), there is controversy whether this system promotes sarcolemmal Ca^{2+} entry during the action potentials and thereby contributes to excitation-contraction coupling under physiological conditions (*9*). These considerations prompted us to re-examine whether Ca^{2+} influx through voltage-dependent Ca^{2+} channels is mandatory for cardiac SR Ca^{2+} release. Our experiments show that Ca^{2+} influx through voltage-dependent Ca^{2+} channels is not a prerequisite for SR Ca^{2+} release. After pharmacological blockade of myocardial Ca^{2+} channels, SR Ca^{2+} release can be elicited in response to activation of tetrodotoxin (TTX)-sensitive Na^+ currents in an extracellular Ca^{2+}–dependent manner.

In isolated guinea pig myocytes, internally dialyzed with the Ca^{2+} indicator indo-1 (potassium salt) (*10*), action potentials elicited under current-clamp conditions were accompanied by a rapid increase in intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) (Fig. 1A). The onset of this rise of $[Ca^{2+}]_i$ was delayed by approximately 15 to 30 ms after the upstroke of the action potential. During the action potential plateau, $[Ca^{2+}]_i$ either continued to slowly rise, was sustained, or slowly declined. Repolarization of the action potential resulted in the return of Ca^{2+} toward previous resting values. To test if Na^+ entry through voltage-dependent Na^+ channels might contribute to excitation-contraction coupling during the action potential, we examined the effects of the Na^+ channel antagonist TTX on the action potential Ca^{2+} transient. Exposure of cells to 5 μ*M* TTX significantly reduced the size of the Ca^{2+} transient (Fig. 1, A_1 and A_2). In this cell, TTX produced little, if any, change in the action potential plateau or duration, which makes it unlikely that the change observed in the Ca^{2+} transient was caused by diminished Ca^{2+} entry through Ca^{2+} channels during the plateau. The ability of TTX to reduce the amplitude of the Ca^{2+} transient is consistent with its known negative inotropic effects in the heart (*11*).

In other experiments, we voltage-clamped isolated myocytes (*12*) and directly examined the possibility that activation of Na^+ currents might contribute to the Na^+ transients recorded. Membrane holding potential was varied to examine the possible role of myocardial Na^+ currents (Fig. 1B). From a holding potential of −70 mV (near the cell's resting membrane potential), a 500-ms test pulse to 0 mV elicited an inward current that activated quickly and inactivated in two phases (Fig. 1, B_1). This inward current reflects the activation of two components: fast TTX-sensitive Na^+ channels and dihydropyridine-sensitive Ca^{2+} channels. During the test pulse, $[Ca^{2+}]_i$ rose rapidly from a resting value to reach a plateau slightly higher than 1 μ*M*. On return to the holding potential, $[Ca^{2+}]_i$ returned to a resting level, as observed in current-clamp experiments during the last phase of action potential repolarization. Na^+ currents were voltage-

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