13. A. M. Brown, K. S. Lee, T. Powell, *J. Physiol. (London)* **318**, 455 (1981); H. A. Fozzard, C. T. January, J. C. Makielski, *Circ. Res.* **56**, 475 (1985).
14. H. Nawrath, R. Ten Eick, T. F. MacDonald, W. Trautwein, *Circ. Res.* **40**, 408 (1977); K. S. Lee and R. W. Tsien, *Nature* **302**, 790 (1983); J. Hescheler, D. Pelzer, G. Trube, W. Trautwein, *Pfluegers Arch.* **393**, 287 (1982); N. Leblanc and J. R. Hume, *Am. J. Physiol.* **257**, C248 (1989).
15. The smaller amplitude of Na^+ currents measured under these experimental conditions might also be caused by a partial block of Na^+ currents by the high concentrations of D 600 used [J. O. Bustamante, *Pfluegers Arch.* **403**, 225 (1985)].
16. T-type Ca^{2+} chanels make only a minor contribution to total Ca^{2+} current in ventricular myocytes [B. P. Bean, *J. Gen. Physiol.* **86**, 1 (1985); R. Mitra and M. Morad, *Proc. Natl. Acad. Sci. U.S.A.* **83**, 5340 (1987); R. W. Hadley and J. R. Hume, *Circ. Res.* **62**, 97 (1988); R. Gonzalez-Rudo, J. B. Patlak, W. R. Gibbons, *Biophy. J.* **55**, 306a (1989)].
17. Adequate voltage control was also tested in experiments in which partially blocking doses of TTX were observed to reduce Na^+-current amplitudes without altering their voltage dependence.
18. L. Barcenas-Ruiz, D. J. Beuckelmann, W. G. Wier, *Science* **238**, 1720 (1987).
19. J. Kimura, A. Noma, H. Irisawa, *Nature* **319**, 596 (1986); S. Mechmann and L. Pott, *ibid.*, p. 597; J. R. Hume and A. Uehara, *J. Gen. Physiol.* **87**, 857 (1986); *ibid.*, p. 883.
20. J. R. Hume, *Am. J. Physiol.* **252**, H666 (1987); J. R. Berlin, J. R. Hume, W. J. Lederer, *J. Physiol. (London)* **407**, 128P (1988).
21. T. Akera, *Science* **198**, 569 (1977); ——— and T. M. Brody, *Trends Pharmacol. Sci.* **2**, 191 (1981).
22. M. R. Cannell, J. R. Berlin, W. J. Lederer, *Science* **238**, 1419 (1987).
23. M. Vassalle and M. Bhattacharyya, *Circ. Res.* **47**, 666 (1980); H. Nawrath, *J. Pharmacol. Exp. Ther.* **216**, 176 (1981); S.-S. Sheu and W. J. Lederer, *Circ. Res.* **57**, 578 (1985); P. Honerjager, E. Loibl, I. Steidl, G. Schonsteiner, K. Ulm, *Naunyn-Schmiedeberg's Arch. Pharmacol.* **332**, 184 (1986).
24. We thank R. D. Harvey and N. Publicover for comments on the manuscript and S. Bowen for assistance in preparing the figures. Supported by NIH grant HL30143. N.L. was supported by a fellowship provided by the Canadian Medical Research Council; J.R.H. was supported by an American Heart Association Established Investigator Award.

10 October 1989; accepted 16 January 1990

The Relationship Between Charge Movements Associated with I_{Ca} and $I_{Na\text{-}Ca}$ in Cardiac Myocytes

JOHN H. B. BRIDGE,* JOHN R. SMOLLEY, KENNETH W. SPITZER

Ventricular myocytes exhibit a nifedipine-sensitive inward calcium current (I_{Ca}) and contracture when they are voltage clamped from −40 to 0 millivolt in the presence of caffeine and in the absence of extracellular sodium. However, upon repolarization they fail to relax because neither the sarcoplasmic reticulum nor the sodium-calcium exchange can reduce intracellular calcium. Sudden application of extracellular sodium during the contracture (but after repolarization) causes immediate relaxation and activates a transient inward sodium-calcium exchange current ($I_{Na\text{-}Ca}$), whose peak slightly precedes mechanical relaxation. The total charge carried by the nifedipine-sensitive I_{Ca} is twice the total charge carried by the transient inward $I_{Na\text{-}Ca}$. Assuming an exchange stoichiometry of three sodium to one calcium, these results indicate that all the calcium entering the cell during the initial depolarization is extruded by the sodium-calcium exchange.

IN THE PRESENCE OF CAFFEINE, WHICH prevents Ca^{2+} sequestration by the sarcoplasmic reticulum (SR), mechanical relaxation of mammalian ventricular muscle is dependent on extracellular Na^+ (Na_o^+) (*1–3*). This Na_o^+-dependent relaxation is also voltage sensitive (*1–2*). Similarly, in the presence of ryanodine, which interferes with SR Ca^{2+} release, the decay of elevated intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) in guinea pig cells is controlled by voltage and is associated with an inward $I_{Na\text{-}Ca}$ tail (*4*). These observations indicate that a voltage-sensitive Na^+-Ca^{2+} exchange is capable of sufficient Ca^{2+} extrusion to cause relaxation. If this exchange is electrogenic, there should be an associated current that accompanies and slightly precedes mechanical relaxation and that decays as relaxation progresses and intracellular Ca^{2+} is reduced. Here we report the isolation of a current with these characteristics.

To isolate $I_{Na\text{-}Ca}$ we used a perfusion system (*5*) that rapidly increased Na_o^+ and activated $I_{Na\text{-}Ca}$ before the exchange could significantly dissipate ionic gradients responsible for its activation. This approach has already been applied to visual rods by Hodgkin *et al.* (*6*). Guinea pig ventricular myocytes were enzymatically isolated by a method similar to that described by us (*1*) and voltage clamped with the whole-cell disrupted patch technique (7) with a discontinuous single microelectrode voltage-clamp circuit (Axoclamp 2A, Axon Instruments, Burlingame, California). Microelectrodes (2 to 6 mΩ) were filled with dialyzing solution containing 131 m*M* CsCl, 0.2 m*M* $MgCl_2$, 3.0 m*M* Mg–adenosine triphosphate (ATP), 5.5 m*M* dextrose, and 10 m*M* Hepes; they also contained 20 μ*M* EGTA and no added Ca^{2+}. The *p*H was adjusted to 7.1 with CsOH. The total Cs^+ concentration was 140 m*M* and free Mg^{2+} was estimated to be 600 μ*M*. In experiments requiring extensive intracellular Ca^{2+} buffering, the pipette solution contained 14.0 m*M* EGTA. Contraction was measured as change in cell length with a video-based edge detector (*8*). The control external solution contained 145 m*M* LiCl, 0 m*M* KCl replaced with 4.4 m*M* LiCl, 2.7 m*M* $CaCl_2$, 1.0 m*M* $MgCl_2$, 11 m*M* dextrose, 10 m*M* caffeine, and 10 m*M* Hepes-LiOH, *p*H 7.4. Na^+ replaced Li^+ in the test solution. When necessary, nifedipine was used at a concentration of 10 μ*M*.

Cells were clamped at a holding potential of −40 mV in the control solution that contained no Na_o^+. An initial inward I_{Ca} was activated every 10 s (Fig. 1A) by depolarizing the cell to 0 mV for 2 s. This resulted in a contracture (Fig. 1D) that persisted after the cell was repolarized. In the presence of caffeine the contraction is attributable to Ca^{2+} entry via I_{Ca}. Five hundred milliseconds after the repolarization, Na_o^+ was applied very rapidly [half-time ($t_{1/2}$) ≅ 40 ms) (*5*). The Na_o^+ application

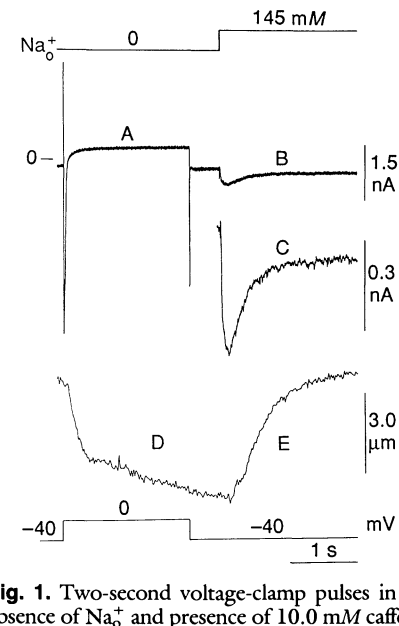

Fig. 1. Two-second voltage-clamp pulses in the absence of Na_o^+ and presence of 10.0 m*M* caffeine cause contraction. Rapid application of Na_o^+ 500 ms after repolarization causes relaxation. (**A**) I_{Ca} elicited by membrane depolarization. (**B**) The application of Na^+ produces putative transient inward $I_{Na\text{-}Ca}$. This current is displayed on an expanded scale in (**C**). (**D**) Contraction (cell shortening) activated by I_{Ca} recorded in (A). (**E**) After repolarization to −40 mV, relaxation does not occur until 500 ms after the clamp pulse when Na_o^+ is suddenly applied.

J. H. B. Bridge, Nora Eccles Harrison Cardiovascular Research and Training Institute and the Department of Medicine, University of Utah, Salt Lake City, UT 84112.
J. R. Smolley and K. W. Spitzer, Nora Eccles Harrison Cardiovascular Research and Training Institute and the Department of Physiology, University of Utah, Salt Lake City, UT 84112.

*To whom correspondence should be addressed.

lasted 6.5 s and produced a transient inward current and mechanical relaxation (Fig. 1, B, C, and E). This Na_o^+-dependent current peaked just before and decayed during relaxation. We have already inferred that Na_o^+-dependent relaxation in caffeine-treated cells is caused by Na^+-Ca^{2+} exchange (*1*). We therefore suggest that this Na_o^+-dependent current is produced by electrogenic Na^+-Ca^{2+} exchange. This cycle of contraction relaxation and solution change was, whenever possible, repeated at 10-s intervals so that an approximately steady-state relation between Ca^{2+} entry and extrusion could be measured.

To show that activation of this current requires elevated $[Ca^{2+}]_i$, we repeated the experiment with 14.0 m*M* EGTA included in the pipette solution (Fig. 2A). A clamp from −40 to 0 mV elicited an I_{Ca} but no contracture, and rapid application of Na_o^+ failed to activate putative $I_{Na\text{-}Ca}$ (Fig. 2A, left panel). We did observe a small and stable change of holding current during the Na_o^+ application for which, at present, we have no explanation. This holding current is always activated upon increasing Na_o^+ and does not require prior membrane depolarization (Fig. 2A, right panel). This result indicates that the Na_o^+-activated transient current can only be observed when $[Ca^{2+}]_i$ is elevated.

Our results may be explained by assuming that, in the presence of caffeine and in the absence of an inward Na^+ gradient, sufficient Ca^{2+} may enter the cell via L-type channels to activate contraction. Because the SR cannot accumulate Ca^{2+} in the presence of caffeine, relaxation only takes place when Na_o^+ is applied and Ca^{2+} is extruded from the cell in exchange for Na_o^+. We further tested this idea by first eliciting I_{Ca} and $I_{Na\text{-}Ca}$ as described under control conditions (Fig. 2B, left panel). We then rapidly applied Na_o^+ to the cell without first eliciting I_{Ca} by membrane depolarization (Fig. 2B, right panel). Although we observed a small change in holding current, there is no Na_o^+-dependent transient inward current suggesting that initial entry of Ca_i^{2+} is required before this current can be elicited. In the same cell, application of 10 μ*M* nifedipine abolished I_{Ca} and the subsequent Na_o^+-dependent transient inward current (Fig. 2C, left panel), again suggesting that initial Ca^{2+} entry is required before the Na_o^+-dependent current can be observed. Although Ca^{2+}-channel blockers can suppress $I_{Na\text{-}Ca}$, their effect at this concentration is unlikely to explain the complete suppression of the transient $I_{Na\text{-}Ca}$ (*9*). If depolarization in the presence of nifedipine was omitted, application of Na_o^+ still produced (in the cell) a very small change in holding current (Fig. 2C, right panel). In some cells nifedipine completely abolished this change in holding current. The steady-state change in holding current (upon application of Na_o^+) could represent steady-state $I_{Na\text{-}Ca}$ that compensates steady-state Ca^{2+} leaks present under these conditions. If this leak is reduced or abolished by nifedipine, the compensatory Na^+-Ca^{2+} exchange would also be reduced. However, when $[Ca^{2+}]_i$ is 153 n*M*, which corresponds to the relaxed condition, Ehara *et al.* showed that inward $I_{Na\text{-}Ca}$ was small or not detectable (*10*), suggesting that this steady current is not forward Na^+-Ca^{2+} exchange. It is possible that before the application of Na_o^+, reverse Na^+-Ca^{2+} exchange (outward current) might be present. Suppression of this current by application of Na_o^+ would produce current change in an inward direction. The origin of this steady-state current is, nevertheless, unclear. Our results suggest that rapid application of Na_o^+ elicits $I_{Na\text{-}Ca}$, which accompanies the extrusion of Ca^{2+} that enters the cell via I_{Ca} (Fig. 2). Yau and Nakatani (*11*) have reported similar conclusions from results obtained with visual rods.

To investigate the relation between I_{Ca} and putative $I_{Na\text{-}Ca}$, we assumed that in the steady state, transient Ca^{2+} entry produced by activating I_{Ca} is matched by transient Ca^{2+} extrusion when $I_{Na\text{-}Ca}$ is activated. We further assumed that three Na^+ ions exchange with a single Ca^{2+} ion. With these assumptions the following relationship will be true (*12*):

$$\int I_{Ca}dt = 2 \int I_{Na\text{-}Ca}dt$$

To obtain the total Ca^{2+} entry, we integrated the nifedipine-sensitive Ca^{2+} current (Fig. 3B). To obtain the Ca^{2+} extrusion via Na-Ca exchange, we integrated the transient putative $I_{Na\text{-}Ca}$ (Fig. 3C). Measurements (122) on 11 cells revealed that the unweighted mean value of the integrated nifedipine-sensitive I_{Ca} was 2.08 ± 0.14 (mean ± SEM) times the integrated $I_{Na\text{-}Ca}$. We also plotted mean values of the signal-averaged integral of I_{Ca} against the signal-averaged integral of $I_{Na\text{-}Ca}$ for each cell (Fig. 3A). These values can be compared with the relationship expected between these quantities when both 3:1 and 4:1 stoichiometric exchange coefficient are assumed. With the exception of 1 point (upper right in Fig. 3A), our results are those expected of an exchange stoichiometry of 3:1 when no other processes besides electrodiffusive movement of Ca^{2+} and Na^+-Ca^{2+} exchange contribute significantly to the measured currents. Undetected reverse Na^+-Ca^{2+} exchange (during repolarization) or Ca^{2+} movement via the sarcolemmal Ca^{2+} pump

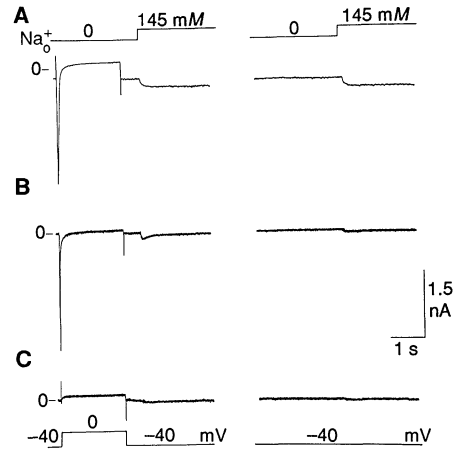

Fig. 2. (**A**) (Left panel) With 14.0 m*M* EGTA in the pipette, depolarization from −40 mV to 0 mV failed to activate contraction (not shown). Application of 145 m*M* Na_o^+ failed to activate putative transient inward $I_{Na\text{-}Ca}$, but did produce a stable change of holding current. In this example the change of holding current was particularly large. This change of holding current did not depend on prior depolarization (right panel). This inhibition of transient $I_{Na\text{-}Ca}$ by EGTA was observed in several cells. (**B**) (Left panel) I_{Ca} and putative $I_{Na\text{-}Ca}$ were activated as already described. If the cell is not depolarized, application of Na_o^+ fails to activate putative $I_{Na\text{-}Ca}$, but does cause a small charge in holding current (right panel). (**C**) (Left panel) After abolishing I_{Ca} with 10 μ*M* nifedipine, application of Na_o^+ fails to elicit putative $I_{Na\text{-}Ca}$, but does produce a small change in holding current. Again this change of holding current persists in the absence of prior depolarization (right panel).

may have contributed to the aberrant point. The average calculated Ca^{2+} entry is 60 μmol/liter of cell water available to free Ca^{2+}, which is sufficient to activate contraction (*13*). The $t_{1/2}$ for decay of the putative $I_{Na\text{-}Ca}$ is 235 ± 0.005 ms (mean ± SEM, n = 34 observations on four cells). The peak average value of I_{Ca} was 2.05 ± 0.44 nA (mean ± SEM, n = 122 observations on 11 cells) and the peak average value of $I_{Na\text{-}Ca}$ was 0.12 ± 0.027 nA (mean ± SEM, n = 122 observations on 11 cells).

We conclude that the transient inward current that is activated by Na_o^+ application (when we infer that intracellular Ca^{2+} is elevated) is $I_{Na\text{-}Ca}$. If this is correct, we have separated transient $I_{Na\text{-}Ca}$ from I_{Ca} and displayed the peak magnitude and time course of $I_{Na\text{-}Ca}$. This conclusion is strengthened by the fact that this current is absent when $[Ca^{2+}]_i$ is buffered and requires an initial entry of Ca^{2+} to elevate $[Ca^{2+}]_i$ before it can be activated. Moreover, its relationship to I_{Ca} is consistent with an Na^+-Ca^{2+} exchange stoichiometry of 3:1 (*14–17*). Although membrane conductance changes have been reported to occur with increases in $[Ca^{2+}]_i$ (*9*), it is unlikely that this Na_o^+-

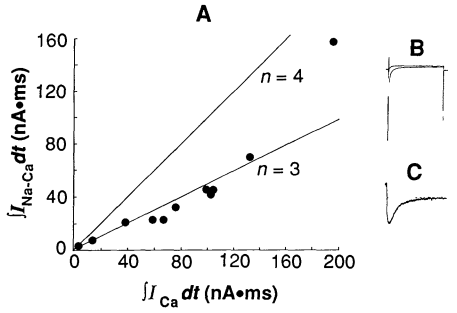

Fig. 3. (**A**) Relationship between the integral of I_{Ca} (abscissa) and the integral of $I_{Na\text{-}Ca}$ (ordinate). Each point represents measurements from a different cell. Under apparent steady-state conditions, successive trials on each cell were signal-averaged. (**B**) The integral of I_{Ca} was obtained by integrating the nifedipine sensitive current as this was assumed to reflect Ca^{2+} entry. Nifedipine (10 μM) sometimes caused a very small change in holding current (Ca^{2+} channel blockers are known to effect outward current) (*24*), which was neglected during the integration procedure. (**C**) The $I_{Na\text{-}Ca}$ was integrated for 2 s beneath the broken baseline. This typically included all the transient exchange and was assumed to reflect Ca^{2+} extrusion.

dependent current is a nonspecific current that is activated when $[Ca^{2+}]_i$ is elevated, since the available evidence indicates that nonspecific currents are just as effectively carried by Li^+, which we used as a replacement ion (*18, 19*). It is only when Na_o^+ is added in the presence of elevated $[Ca^{2+}]_i$ that $I_{Na\text{-}Ca}$ can be activated. Our integral of $I_{Na\text{-}Ca}$ was obtained by subtracting the stable current that was present after the transient exchange when the cell had mechanically relaxed. Whatever the origin of this stable current, it is unlikely to contain measurable inward $I_{Na\text{-}Ca}$ at resting values of $[Ca^{2+}]_i$ (that is, $\leq$153 nM) (*10*). Provided this stable current does not change significantly with $[Ca^{2+}]_i$, our integral should provide a reliable estimate of Ca^{2+} extrusion via Na^+-Ca^{2+} exchange. Our approach suggests that transient Ca^{2+} entry (I_{Ca}) and exit ($I_{Na\text{-}Ca}$) can be separated. This approach does not, however, allow us to separate steady-state Ca^{2+} entry (leak) from steady-state extrusion by the exchanger although, as we have already mentioned, some results suggest that the latter may be small (*10*).

Our results suggest that rapid perfusion methods may be used to separate I_{Ca} and transient $I_{Na\text{-}Ca}$ in ventricular cells. They also support earlier suggestions that prolonged Ca^{2+} current measurements in the presence of an inwardly directed Na^+ gradient (when $[Ca^{2+}]_i$ is elevated) will be contaminated by contributions from $I_{Na\text{-}Ca}$ (*20–23*). In addition to its importance in understanding transsarcolemmal Ca^{2+} extrusion, a clear separation of I_{Ca} and $I_{Na\text{-}Ca}$ is likely to be of value in any attempt to investigate possible "triggers" for Ca^{2+}-induced Ca^{2+} release that might underlie excitation contraction coupling in heart (*24*).

REFERENCES AND NOTES

1. J. H. B. Bridge, K. W. Spitzer, P. R. Ershler, *Science* **241**, 823 (1988).
2. D. Bers and J. Bridge, *Circ. Res.* **65**, 334 (1989).
3. T. Kitazawa, *J. Physiol. (London)* **402**, 703 (1988).
4. L. Barcenas-Ruiz, D. J. Beuckelmann, W. G. Wier, *Science* **238**, 1720 (1987).
5. K. Spitzer and J. Bridge, *Am. J. Physiol.* **256**, C441 (1989).
6. A. Hodgkin, P. McNaughton, B. Nunn, *J. Physiol. (London)* **358**, 447 (1985).
7. O. Hamill, A. Marty, E. Neher, B. Sakmann, F. Sigworth, *Pfleugers Arch.* **395**, 6 (1981).
8. B. Steadman, K. Moore, K. Spitzer, J. Bridge, *IEEE Trans. Biomed. Eng.* **35**, 264 (1988).
9. J. Kimura, S. Miyaumae, A. Noma, *J. Physiol. (London)* **384**, 199 (1987).
10. T. Ehara *et al.*, *ibid.* **410**, 227 (1989).
11. K.-W. Yau and K. Nakatani, *Nature* **311**, 661 (1984).
12. D. Noble, in *Excitation and Regulation of Contraction*, R. Nathan, Ed. (Academic Press, New York, 1986), p. 171.
13. A. Fabiato, *Am. J. Physiol.* **245**, C1 (1983).
14. B. Pitts, *J. Biol. Chem.* **254**, 6232 (1979).
15. J. Reeves and C. Hale, *ibid.* **259**, 7733 (1984).
16. J. H. B. Bridge and J. B. Bassingthwaighte, *Science* **219**, 178 (1983).
17. S. Wakabayashi and K. Goshima, *Biochem. Biophys. Acta* **642**, 158 (1981).
18. G. Yellen, *Nature* **296** (1982).
19. T. Ehara, A. Noma, K. Ono, *J. Physiol. (London)* **403**, 117 (1988).
20. L. Mullins, *Am. J. Physiol.* **236**, C103 (1976).
21. M. Mitchell, T. Powell, D. Terrar, V. Twist, *Br. J. Pharmacol.* **81**, 543 (1984).
22. D. Eisner and W. Lederer, *Am. J. Physiol.* **248**, C189 (1985).
23. J. Hume, *ibid.* **252**, H666 (1987).
24. B. London and J. Kreuger, *J. Gen. Physiol.* **88**, 4 (1986).
25. Supported by National Institutes of Health grants HL42357, HL34288, and HL42873 and by an award from the American Heart Association (Montana Affiliate); the Nora Eccles Treadwell Foundation and the Richard A. and Nora Eccles Harrison Fund for Cardiovascular Research.

13 October 1989; accepted 8 March 1990

Erythropoietin Retards DNA Breakdown and Prevents Programmed Death in Erythroid Progenitor Cells

MARK J. KOURY AND MAURICE C. BONDURANT

The mechanism by which erythropoietin controls mammalian erythrocyte production is unknown. Labeling experiments in vitro with [^{3}H]thymidine demonstrated DNA cleavage in erythroid progenitor cells that was accompanied by DNA repair and synthesis. Erythropoietin reduced DNA cleavage by a factor of 2.6. In the absence of erythropoietin, erythroid progenitor cells accumulated DNA cleavage fragments characteristic of those found in programmed cell death (apoptosis) by 2 to 4 hours and began dying by 16 hours. In the presence of erythropoietin, the progenitor cells survived and differentiated into reticulocytes. Thus, apoptosis is a major component of normal erythropoiesis, and erythropoietin controls erythrocyte production by retarding DNA breakdown and preventing apoptosis in erythroid progenitor cells.

ERYTHROPOIETIN (EPO), A GLYCOPROTEIN produced in the mammalian kidney and liver, controls erythrocyte production by acting directly on erythroid progenitor cells in the hematopoietic organs. Possible modes of Epo action include stimulation of mitosis, induction of a program of terminal differentiation, or maintenance of cellular viability. Eaves *et al.* (*1*) speculated that Epo maintains the viability of late-stage erythroid progenitors. Studies with purified, late-stage erythroid progenitor cells indicate that they require Epo for survival, notwithstanding any effects on mitosis or differentiation (*2, 3*). The Epo-dependent period extends from at least the colony-forming unit–erythroid (CFU-E) stage (*4*) to the stage at which hemoglobin synthesis begins (*2*).

To study the mechanism of Epo action, we used a homogeneous population of erythroid progenitor cells in the Epo-dependent period of development; the cells were isolated from spleens of mice infected with the anemia-inducing strain of Friend leukemia virus (FVA cells) (*5*). The Epo dependence and Epo-mediated differentiation of FVA cells in vitro were similar to those of CFU-E from normal, uninfected mice (*2, 5*). In earlier work, we observed no difference in DNA synthesis rates, but we did find a differential DNA breakdown in FVA cells cultured with Epo and those cultured without Epo (*2*). FVA cells cultured without Epo contained broken DNA, which was detected by 8 hours and was a prominent component by 16 hours. Because DNA breakage in Epo-deprived cells was observed only after 8 hours of culture, no conclusions could be drawn as to whether DNA break-

Division of Hematology, Vanderbilt University Medical Center and Veterans Administration Medical Center, Nashville, TN 37232.