Sah, K. Francis, E. M. McLachlan, P. Junankar, *Neuroscience* **54**, 157 (1993); A. H. Cornell-Bell, S. M. Finkbeiner, M. S. Cooper, S. J. Smith, *Science* **247**, 470 (1990); J. D. Lechleiter and D. E. Clapham, *Cell* **69**, 283 (1992); P. Camacho and J. D. Lechleiter, *Science* **260**, 226 (1993).
6. M. Fill and R. Coronado, *Trends Neurosci.* **11**, 453 (1988).
7. A. Minta, J. P. Y. Kao, R. Y. Tsien, *J. Biol. Chem.* **264**, 8171 (1989).
8. A. Fabiato, *J. Gen. Physiol.* **85**, 247 (1985).
9. S. C. ONeill, J. G. Mill, D. A. Eisner, *Am. J. Physiol.* **258**, C1165 (1990).
10. J. L. Sutko, K. Ito, J. L. Kenyon, *Fed. Proc.* **44**, 2984 (1985); G. Meissner, *Adv. Exp. Med. Biol.* **311**, 277 (1992).
11. G. Meissner and A. el Hashem, *Mol. Cell Biochem.* **114**, 119 (1992).
12. E. Rousseau and G. Meissner, *Am. J. Physiol.* **256**, H328 (1989).
13. E. Buck, I. Zimanyi, J. J. Abramson, I. N. Pessah, *J. Biol. Chem.* **267**, 23560 (1992); I. N. Pessah and I. Zimanyi, *Mol. Pharmacol.* **39**, 679 (1991); F. A. Lai, M. Misra, L. Xu, H. A. Smith, G. Meissner, *J. Biol. Chem.* **264**, 16776 (1989).
14. E. Rousseau, J. S. Smith, G. Meissner, *Am. J. Physiol.* **253**, C364 (1987).
15. G. Meissner, *J. Biol. Chem.* **261**, 6300 (1986).
16. H. Cheng, W. J. Lederer, M. B. Cannell, unpublished data.
17. Both the kinetics and the steady-state–equilibrium conditions of ryanodine binding are dependent on the concentration of ryanodine used. Equilibrium binding of ryanodine at very low concentrations takes several hours and is consequently difficult to achieve in experiments with single heart cells. We have taken advantage of the more rapid onset of low-dose effects when intermediate concentrations of ryanodine (100 nM) are applied to observe behavior normally associated with lower concentrations of ryanodine. The ryanodine off-rate is sufficiently low so that the binding is essentially irreversible (over the time scale of these experiments).
18. At a single wavelength
$$[Ca^{2+}] = K(F - F_{min})/(F_{max} - F)$$
where K is the dissociation constant for the indicator, F_{min} is the fluorescence in the absence of Ca^{2+}, and F_{max} is the fluorescence in the presence of saturating Ca^{2+}. For fluo-3, $F_{min} \approx 0$, so that only estimates of F_{max} and K are required to calibrate the data. F_{max} can be estimated from the rest (control) level of fluorescence because
$$F_{max} = F_{rest}(K/[Ca^{2+}]_{rest} + 1)$$
if there is no change in dye concentration or path length (a condition made more likely with the use of a confocal microscope with negligible photobleaching during the scan). Thus, dividing images by a control image will give a pseudo-ratio image in which
$$[Ca^{2+}] = KR/[(K/[Ca^{2+}]_{rest} + 1) - R]$$
where R is the fluorescence ratio and $[Ca^{2+}]_{rest}$ is the known $[Ca^{2+}]$ in the control image. Assuming K is 400 nM and the resting $[Ca^{2+}]_i$ is 100 nM, we estimate the peak $[Ca^{2+}]$ of a typical Ca^{2+} spark to be about 270 nM and a typical electrically evoked transient to be about 1.46 μM with this method. This estimate of the peak of the evoked transient is consistent with earlier estimates ratiometric dyes [M. B. Cannell, J. R. Berlin, W. J. Lederer, *Science* **238**, 1419 (1987)].
19. W. G. Wier and D. T. Yue, *J. Physiol.* (*London*) **376**, 507 (1986); K. R. Sipido and W. G. Wier, *ibid.* **435**, 605 (1991).
20. A. Tinker, A. R. G. Lindsay, A. J. Williams, *J. Gen. Physiol.* **100**, 495 (1992).
21. B. A. Block, T. Imagawa, K. P. Campbell, C. Franzini-Armstrong, *J. Cell Biol.* **107**, 2587 (1988); F. Zorzato *et al.*, *J. Biol. Chem.* **265**, 2244 (1990); A. R. Marks *et al.*, *Proc. Natl. Acad. Sci. U.S.A.* **86**, 8683 (1989); M. Inui, A. Saito, S. Fleischer, *J. Biol. Chem.* **262**, 15637 (1988); *ibid.* **263**, 10843 (1988).
22. We calculated the opening rate of the SR Ca^{2+}-release channel as follows: The number of RyRs per adult rat heart cell is estimated to be about 1.0×10^6 per cell on the basis of data given by Bers and co-workers (*27*). This estimate arises from the number of dihydropyridine receptors (DHPRs) per surface area (14.8 per square micrometer), the surface-to-volume ratio (0.6 μm^2 per cubic micrometer), the volume of our cells ($\approx 2.0 \times 10^4$ μm^3), and the number of RyRs per DHPR (around seven) (*27*). (Surface area includes the transverse-tubular membranes.) If a cardiac myocyte contains g ryanodine receptors and if we survey a fraction m of the entire cell with a raster scan once over a period of n seconds observing p sparks and the spark is observable for q seconds, then the opening rate r is given by
$$r = p/(gmnq/n) = p/(gmq)$$
assuming that a spark corresponds to a single-channel opening (where the fraction q/n compensates for missed events). A similar approach can be used for a line-scan image, where m should be calculated with respect to the geometry scanned. Taking both lens resolution and the spatial spreading of sparks into account, each scan surveys a volume V_s of approximately 2 μm by 2 μm multiplied by the length of the line scanned. Thus, $m = V_s/V_{cell}$. For a typical heart cell with dimensions of 10 by 20 by 100 μm, V_{cell} is 2×10^4 μm^3. Because the lifetime of a spark is much longer than the time taken to scan a line (2 to 4 ms), compensation for missed events is not needed. Hence, $r = p/(gmn)$. Here, the estimate of r was $1.10 \times 10^{-4} \pm 0.22 \times 10^{-4}$ s^{-1} (mean ± SEM; $n = 13$) based on the line-scan image data. Estimates of r by whole-cell imaging were similar [$r = 1.23 \times 10^{-4} \pm 0.17 \times 10^{-4}$ (mean ± SEM; $n = 18$)]. A change in $[Ca^{2+}]_i$ of >50 nM is needed to recognize a spark, and openings less than about 2 ms would probably be missed.
23. M. Valdeolmillos, S. C. ONeill, G. L. Smith, D. A. Eisner, *Pfluegers Arch.* **413**, 676 (1989); J. R. Berlin, M. B. Cannell, W. J. Lederer, *Circ. Res.* **65**, 115 (1989); *Am. J. Physiol.* **253**, H1540 (1987); T. Takamatsu and W. G. Wier, *Cell Calcium* **11**, 111 (1990); R. S. Kass, W. J. Lederer, R. W. Tsien, R. Weingart, *J. Physiol.* (*London*) **281**, 187 (1978); P. Arlock and B. G. Katzung, *ibid.* **360**, 105 (1985); P. Lipp and L. Pott, *ibid.* **397**, 601 (1988).
24. W. G. Wier, M. B. Cannell, J. R. Berlin, E. Marban, W. J. Lederer, *Science* **235**, 325 (1987).
25. M. B. Cannell, R. D. Vaughan-Jones, W. D. Lederer, *Fed. Proc.* **44**, 2964 (1985); E. Marban and W. G. Wier, *Circ. Res.* **56**, 133 (1985); W. G. Wier, D. T. Yue, E. Marban, *Fed. Proc.* **44**, 2989 (1985).
26. J. S. K. Sham, L. Cleemann, M. Morad, *Science* **255**, 850 (1992); J. R. Hume, P. C. Levesque, N. Leblanc, *ibid.* **251**, 1370 (1991); W. J. Lederer, E. Niggli, R. W. Hadley, *ibid.*, p. 1371; P. C. Levesque, N. Leblanc, J. R. Hume, *Ann. N.Y. Acad. Sci.* **639**, 386 (1991); N. Leblanc and J. R. Hume, *Science* **248**, 372 (1990); W. J. Lederer, E. Niggli, R. W. Hadley, *ibid.*, p. 283.
27. W. Y. W. Lew, L. V. Hryshko, D. M. Bers, *Circ. Res.* **69**, 1139 (1991); D. M. Bers and V. M. Stiffel, *Am. J. Physiol. Cell Physiol.* **264**, 1587 (1993).
28. R. Mitra and M. Morad, *Am. J. Physiol.* **249**, H1056 (1985).
29. Supported by NIH, the University of Maryland Graduate Assistantship program, and the British Heart Foundation. We thank R. Hadley, M. Kirby, T. Rogers, R. Bloch, and H. Valdivia for valuable discussion of the material presented here and for comments on the manuscript.

30 July 1993; accepted 1 October 1993

Microdomains with High Ca^{2+} Close to IP_3-Sensitive Channels That Are Sensed by Neighboring Mitochondria

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Microdomains of high intracellular calcium ion concentration, $[Ca^{2+}]_i$, have been hypothesized to occur in living cells exposed to stimuli that generate inositol 1,4,5-trisphosphate (IP_3). Mitochondrially targeted recombinant aequorin was used to show that IP_3-induced Ca^{2+} mobilization from intracellular stores caused increases of mitochondrial Ca^{2+} concentration, $[Ca^{2+}]_m$, the speed and amplitude of which are not accounted for by the relatively small increases in mean $[Ca^{2+}]_i$. A similar response was obtained by the addition of IP_3 to permeabilized cells but not by perfusion of cells with Ca^{2+} at concentrations similar to those measured in intact cells. It is concluded that in vivo, domains of high $[Ca^{2+}]_i$ are transiently generated close to IP_3-gated channels and sensed by nearby mitochondria; this may provide an efficient mechanism for optimizing mitochondrial activity upon cell stimulation.

Changes in $[Ca^{2+}]_i$ modulate a variety of cellular functions, from secretion to contraction, enzyme activation, and cell cycle regulation (*1*). Imaging of $[Ca^{2+}]_i$ in living cells (*2*, *3*) has revealed not only the heterogeneity of responses within the same cell population but also complex spatiotemporal patterns in the changes of $[Ca^{2+}]_i$ evoked by receptor stimulation (*1*). Even in nonexcitable cells, increases in $[Ca^{2+}]_i$ are often composed of series of asynchronous rapid oscillations or waves (*4*) or of large local increases, often far away from the site of receptor activation (*5*). However, with few exceptions (Ca^{2+}-activated ion channels) (*6*), it is still unknown whether cells have systems capable of sensing and translating large but localized increases in $[Ca^{2+}]_i$ into metabolic responses. Obvious candidates for detecting regional changes of $[Ca^{2+}]_i$ would be enzymes or organelles that

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are sensitive to Ca^{2+} concentrations higher than those normally occurring in the cytosol. Mitochondria have a low-affinity, high-capacity Ca^{2+} uptake mechanism, which is of paramount importance for the modulation of mitochondrial metabolism (*7*). We have developed a technique to monitor the changes in $[Ca^{2+}]$ that occur within the mitochondrial matrix of intact cells. This is accomplished by transfection of cells with a chimeric complementary DNA (cDNA) encoding the presequence of a mitochondrial enzyme (subunit VIII of cytochrome c oxidase) fused to the Ca^{2+}-sensitive photoprotein aequorin (*8*).

We used a HeLa cell clone that stably expressed mitochondrial aequorin (*9*). Addition of histamine, an agonist coupled to IP_3 generation, induced a large increase in $[Ca^{2+}]_i$ (Fig. 1) (*10*): changes in $[Ca^{2+}]_i$ were measured with fura-2 in three representative cells (Fig. 1A) or in a population of the same clone (Fig. 1B) (*11*). At the same time that $[Ca^{2+}]_i$ changed, a large and rapid increase in $[Ca^{2+}]_m$ was observed (Fig. 1C). The average peak of $[Ca^{2+}]_i$, in both the population and the single cells, was ≈0.6 μM, whereas $[Ca^{2+}]_m$ increased to approximately 3 μM. After the peak, $[Ca^{2+}]_i$ decreased to a steady state two to three times the basal level, whereas $[Ca^{2+}]_m$ returned to a value indistinguishable from the resting level. Because of the low accuracy of aequorin at values below 300 to 400 nM, we cannot exclude the possibility that a small increase in $[Ca^{2+}]_m$ is indeed maintained during the plateau phase. The quick dissipation of the mitochondrial transient probably results from the activation of the mitochondrial H^+/Ca^{2+} antiporter (*12*). The high rate of mitochondrial Ca^{2+} accumulation was unexpected because mitochondrial Ca^{2+} uptake (investigated in isolated mitochondrial fractions) is slow when the surrounding $[Ca^{2+}]$ is set to submicromolar values (*7*), similar to those measured in the cytosol of histamine-stimulated HeLa cells. This discrepancy was confirmed in aequorin-transfected HeLa cells permeabilized with digitonin, a detergent that makes large holes in the plasma membrane but leaves organelles largely intact (*13*). The rate of increase in $[Ca^{2+}]_m$ was very slow when the Ca^{2+} concentration in the incubation medium ($[Ca^{2+}]_0$) was below 2 μM and increased to values similar to those observed in intact histamine-stimulated cells only if $[Ca^{2+}]_0$ was at least 5 μM. Three possible explanations of this result were considered: (i) fura-2 greatly underestimates the real increases in $[Ca^{2+}]_i$ caused by receptor stimulation; (ii) permeabilization with digitonin results in the loss of a cytosolic factor that increases the affinity of the mitochondrial uptake system for Ca^{2+}; or (iii) mitochondria are exposed to local Ca^{2+} concentrations much higher than those averaged over the entire cytoplasm (*14*).

To discriminate among these possibilities, we dissociated the IP_3-induced Ca^{2+} release from intracellular stores from the concomitant Ca^{2+} influx across the plasmalemma. For this purpose, the cells were

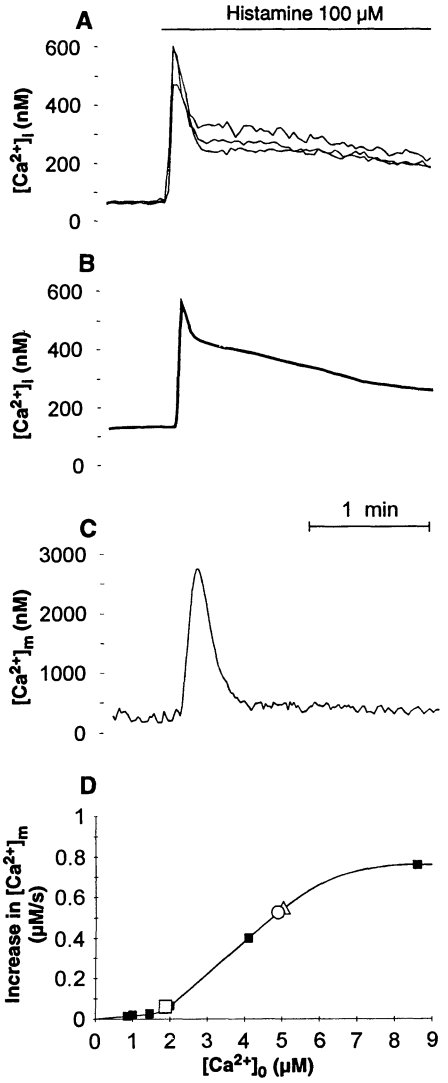

Fig. 1. Changes of $[Ca^{2+}]_i$ and $[Ca^{2+}]_m$ in intact and permeabilized cells. The cells were trypsinized, plated on glass cover slips, left in culture for 2 days, and loaded with fura-2 and coelenterazine (*9, 11*). (**A**) Measurement of $[Ca^{2+}]_i$ in single cells. Medium was modified Krebs-Ringer buffer (KRB): 125 mM NaCl, 5 mM KCl, 1 mM Na_3PO_4, 1 mM $MgSO_4$, 1 mM $CaCl_2$, 5.5 mM glucose, 20 mM Hepes (pH 7.4 at 37°C). The three traces refer to $[Ca^{2+}]_i$ measured with fura-2 in representative single cells. Where indicated, histamine (100 μM) was added to the medium. Individual cells (80) were analyzed in three separate experiments. In 90% of them the peak $[Ca^{2+}]_i$ increases were within the range of the three cells presented here, and 10% showed lower $[Ca^{2+}]_i$ increases. This and the following experiments are representative of at least three different trials. (**B**) Measurement of $[Ca^{2+}]_i$ in a monolayer of cells. Other conditions as in (A). (**C**) Measurement of $[Ca^{2+}]_m$ in a monolayer of cells. Where indicated, the cells were perfused with KRB containing histamine (100 μM). (**D**) Measurement of $[Ca^{2+}]_m$ in permeabilized cells. Medium (buffer a) contained the following: 140 mM KCl, 10 mM NaCl, 1 mM K_3PO_4, 5.5 mM glucose, 2 mM $MgSO_4$, 1 mM ATP, 2 mM sodium succinate, 20 mM Hepes (pH 7.05 at 37°C), supplemented with 50 μM EGTA (free $[Ca^{2+}] < 10^{-8}$ M). After permeabilization with digitonin (100 μM), the monolayer of cells was perfused with the same medium, but with Ca^{2+} buffered at different concentrations with EGTA. The data points of the curve (■) refer to the initial values of $[Ca^{2+}]_m$ increase at the different $[Ca^{2+}]_0$. The data of the initial rates of $[Ca^{2+}]_m$ increase in intact cells stimulated with histamine are fitted into the curve. The values are the means of five independent experiments: (△) histamine in KRB; (○) histamine in Ca^{2+}-free, EGTA-containing KRB; and (□) Ca^{2+} readdition in the presence of histamine. Other conditions as in (C).

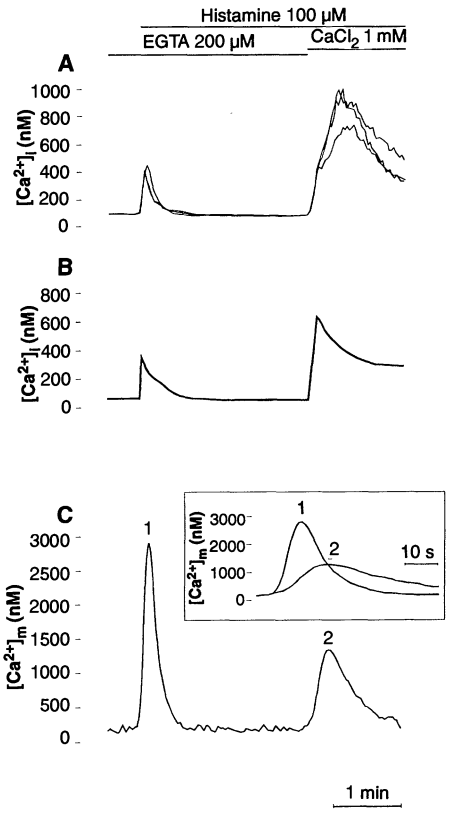

Fig. 2. Effects on $[Ca^{2+}]_m$ of histamine-induced Ca^{2+} mobilization and influx. (**A**) Measurement of $[Ca^{2+}]_i$ in three representative single cells. (**B**) Measurement of $[Ca^{2+}]_i$ in a monolayer of cells. (**C**) Measurement of $[Ca^{2+}]_m$ in a monolayer of cells. In the inset, the $[Ca^{2+}]_m$ peaks (1, mobilization; 2, influx) are compared on an expanded time scale. Conditions as in Fig. 1, except that the initial medium did not contain $CaCl_2$. Where indicated, the medium was supplemented with EGTA (200 μM), histamine (100 μM), and $CaCl_2$ (1 mM).

stimulated with histamine in the absence of external Ca^{2+}, thereby inducing only the release of stored Ca^{2+}. Once the stores were emptied, we initiated Ca^{2+} influx by adding external Ca^{2+} in the continuous presence of the agonist. If hypotheses (i), (ii), or both were correct, mitochondrial Ca^{2+} accumulation should depend on $[Ca^{2+}]_i$, regardless of the origin of the increase. On the contrary, if mitochondria sense local changes of $[Ca^{2+}]_i$, close to the organelle, $[Ca^{2+}]_i$ increases of similar amplitude, but originating from a different source, could induce different rates of mitochondrial Ca^{2+} uptake. When analyzed in single cells (Fig. 2A) or in populations of cells (Fig. 2B), the increase in $[Ca^{2+}]_i$ induced by histamine in Ca^{2+}-free medium (due to release from intracellular stores) was somewhat smaller and faster (400 nM; 2 to 3 s to peak) than the subsequent increase in $[Ca^{2+}]_i$, which resulted from Ca^{2+} influx (≈800 nM, 10 to 12 s to peak).

The changes in $[Ca^{2+}]_m$ induced by histamine under the same conditions were different. The increase in $[Ca^{2+}]_m$ due to release from stores (histamine in Ca^{2+}-free medium) was hardly distinguishable from that in control cells (that is, from both stores and influx), both in kinetics and amplitude (compare Figs. 1C and 2C), whereas that induced by Ca^{2+} influx was smaller and slower (Fig. 2C, inset). The rates of increase in $[Ca^{2+}]_m$ were compared with those obtained in permeabilized cells exposed to different $[Ca^{2+}]_0$ (Fig. 1D) (*15*). An apparent extramitochondrial concentration of Ca^{2+} was extrapolated, which corresponded to 1.5 to 2 μM in the case of Ca^{2+} influx and 5 to 6 μM in the case of Ca^{2+} mobilization. Taken together, these data demonstrate that the same average increases in $[Ca^{2+}]_i$ caused by intracellular Ca^{2+} mobilization or influx across the plasma membrane have different effects on the uptake of Ca^{2+} into mitochondria in situ. Mobilization of Ca^{2+} caused an increase in $[Ca^{2+}]_m$ more than one order of magnitude faster than that predicted on the basis of the amplitude of the mean rise in $[Ca^{2+}]_i$ (*16*). Similar results were also obtained with other agonists coupled to IP_3 generation, such as carbachol and adenosine triphosphate (ATP) (*17*).

The simplest interpretation of these results is that mitochondria close to the Ca^{2+} release sites are exposed to local concentrations of Ca^{2+} much higher than those measured in the bulk of the cytoplasm. This hypothesis correlates with electron microscopy data from various cell types, which commonly show a close proximity between mitochondria and the endoplasmic reticulum (ER). In particular, in Purkinje neurons, which express stacked ER cisternae highly enriched in IP_3 receptors, mitochondria often appear adjacent to, or even in physical contact with, such structures (*18*). In various cell types (for example, hepatocytes and pancreatic β cells) the $[Ca^{2+}]_i$ increases caused by receptor stimulation consist of numerous brief oscillations, whose amplitude hardly exceeds micromolar concentrations (*4*). Given the low affinity of their Ca^{2+} uptake system, if mitochondria were exposed to the mean $[Ca^{2+}]_i$, the Ca^{2+} concentration in the matrix would not change appreciably even during the $[Ca^{2+}]_i$ spikes. On the contrary, it appears that the position of mitochondria may allow them to respond to localized $[Ca^{2+}]_i$ increases with a fast rise in $[Ca^{2+}]_m$. The rapid dissipation of the local gradients by simple diffusion ensures a decrease in the rate of mitochondrial Ca^{2+} uptake and thus prevents excessive Ca^{2+} accumulation and mitochondrial damage. The increase in $[Ca^{2+}]_m$ might result in a transient activation of the matrix dehydrogenases (*7*).

A final point needs to be stressed, relative to the values of $[Ca^{2+}]_m$ and of the surrounding $[Ca^{2+}]_i$. The observed proximity to Ca^{2+} release sites is most likely not shared by all mitochondria or could even be merely stochastic. If this is the case, the $[Ca^{2+}]_m$ transients and the $[Ca^{2+}]_i$ increases of the microdomains sensed by the responding mitochondria are even larger than our estimates.

An obvious prediction of our model is that, provided that the anatomical relationships between mitochondria and the Ca^{2+} release sites are maintained intact, Ca^{2+} release from stores induced by IP_3 should cause rapid mitochondrial Ca^{2+} accumulation even in permeabilized cells. We incubated HeLa cells in Ca^{2+}-free medium containing EGTA, treated them with digitonin, and then perfused them with medium buffered at 1.3 μM Ca^{2+} (Fig. 3). A slow increase of $[Ca^{2+}]_m$ was observed under these conditions. After 2 min, the perfusing medium was changed to medium containing EGTA (free $Ca^{2+} < 10^{-8}$ M) and IP_3 was added. Addition of IP_3 caused a rapid increase of $[Ca^{2+}]_m$, similar to that induced by a receptor agonist in intact cells. The effect of IP_3 was observed only if the intracellular Ca^{2+} stores were full and was abolished by the collapse of mitochondrial membrane potential (*19*).

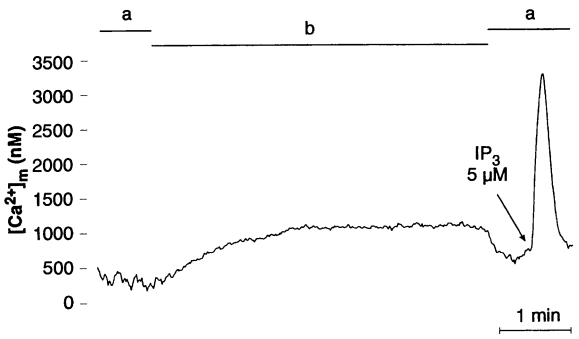

Fig. 3. Effects of IP_3 on $[Ca^{2+}]_m$ in permeabilized cells. The cells were first perfused with buffer a (Fig. 1). Where indicated, the permeabilized cells were perfused with buffer b (same as with a, but free Ca^{2+} buffered at 1.3 μM with EGTA), to allow refilling of the stores. Before IP_3 addition, the cells were returned to buffer a. Where indicated, 5 μM IP_3 was added to the perfusion buffer.

The existence of microdomains of high $[Ca^{2+}]_i$ in subplasmalemmal regions, close to the mouth of Ca^{2+} channels has been postulated (*20*) and supported by experimental evidence (*6*, *21*). Localized regions of high $[Ca^{2+}]_i$ due to Ca^{2+} release from stores have also been described (*22*), and we have observed them in histamine-stimulated HeLa cells; their functional significance, however, is still unclear. Our data confirm the existence of regions of high local $[Ca^{2+}]_i$ close to IP_3-gated channels and suggest that this microheterogeneity of $[Ca^{2+}]_i$ may provide an efficient way for inducing the accumulation of Ca^{2+} within the mitochondrial matrix [well above the concentrations necessary for the modulation of key enzymes (*7*)], even during rapid modest increases of mean $[Ca^{2+}]_i$.

REFERENCES AND NOTES

1. M. J. Berridge and R. F. Irvine, *Nature* **341**, 197 (1989); J. Meldolesi, L. Madeddu, T. Pozzan, *Biochim. Biophys. Acta* **1055**, 130 (1990); C. W. Taylor and I. C. B. Marshall, *Trends Biochem. Sci.* **17**, 403 (1992).
2. R. Y. Tsien, T. Pozzan, T. J. Rink, *Nature* **295**, 68 (1982); G. Grynkiewicz, M. Poenie, R. Y. Tsien, *J. Biol. Chem.* **260**, 3440 (1985).
3. R. Y. Tsien, *Annu. Rev. Neurosci.* **12**, 227 (1989).
4. R. Jacob, *Biochim. Biophys. Acta* **1052**, 427 (1990); R. W. Tsien and R. Y. Tsien, *Annu. Rev. Cell Biol.* **6**, 715 (1990).
5. E. C. Toescu, A. M. Lawrie, O. H. Petersen, D. V. Gallacher, *EMBO J.* **11**, 1623 (1992); M. Poenie, R. Y. Tsien, A. M. Schmitt-Verhulst, *ibid.* **6**, 2223 (1987).
6. L. S. Satin, W. F. Hopkins, S. Fatherazi, D. L. Cook, *J. Membr. Biol.* **112**, 213 (1989); Y. V. Osipchuk, M. Wakui, D. I. Yula, D. V. Gallacher, O. H. Petersen, *EMBO J.* **9**, 697 (1990); W. M. Roberts, R. A. Jacobs, A. J. Hudspeth, *J. Neurosci.* **10**, 3554 (1990).
7. D. Pietrobon, F. Di Virgilio, T. Pozzan, *Eur. J. Biochem.* **193**, 599 (1990); J. G. McCormack, A. P. Halestrap, R. M. Denton, *Physiol. Rev.* **70**, 391 (1990); R. M. Denton and J. G. McCormack, *Annu. Rev. Physiol.* **52**, 451 (1990).
8. R. Rizzuto, A. W. M. Simpson, M. Brini, T. Pozzan, *Nature* **358**, 325 (1992); R. Rizzuto, M. Brini, T. Pozzan, *Methods Cell Biol.*, in press.
9. The chimeric cDNA was constructed and the stably transfected clones were isolated and grown as described (*8*). All experiments shown were done with a specific clone (# 75), strongly positive by Northern (RNA) analysis of aequorin

expression. We reconstituted the functional chemiluminescent protein by incubating the intact cells with 2.5 μM coelenterazine for 2 to 3 hours before an experiment (*8*); cells were also incubated with fura-2/AM for the last 30 min before an experiment (*11*). After reconstitution, the monolayer of HeLa cells, grown on a small glass cover slip (13 mm in diameter), was washed with fresh medium and introduced into the perfusion chamber; light emission was measured with a luminometer [P. H. Cobbold and J. A. C. Lee in *Cellular Calcium, A Practical Approach*, J. G. McCormack and P. H. Cobbold, Eds. (IRL Press, Oxford, 1991), pp. 55–81]. The perfusion chamber was maintained at 37°C by a water jacket. To obtain a rapid and synchronous equilibration of medium in the chamber, during the changes of medium the flow rate was increased from 2.5 to 6 ml/min. The time necessary for the new medium to reach the perfusion chamber was 12 ± 1 s ($n = 5$), and 50% equilibration was obtained in 2 s. The light emission from aequorin was transformed into $[Ca^{2+}]_m$ by means of a program provided by R. Cuthbertson, assuming an intramitochondrial Mg^{2+} concentration of 1 mM [G. A. Rutter, N. J. Osbaldeston, J. G. McCormack, R. M. Denton, *Biochem. J.* **271**, 627 (1990)]. In each experiment we obtained the final discharge of unconsumed aequorin, necessary for calibration, by exposing the cells to a solution of 10 mM $CaCl_2$.

10. M. D. Bootman, M. J. Berridge, C. W. Taylor, *J. Physiol.* **450**, 163 (1992).
11. Fura-2 loading was done as described [A. Malgaroli, D. Milani, J. Meldolesi, T. Pozzan, *J. Cell Biol.* **105**, 2145 (1988)]. Two types of cover slips were used. One, 20 mm long and 8 mm wide, was held in place in the cuvette of a multiwavelength fluorimeter maintained at 37°C (Cairn Research Ltd., Sittingbourne, Kent, UK, excitation at 350 and 380 nm, emission at 500 nm), as described [F. Di Virgilio, B. C. Meyer, S. Greenberg, S. C. Silverstein, *J. Cell Biol.* **106**, 657 (1988)]. In this type of experiment the signal represents the average of a few thousand cells illuminated by the incident beam. At the end of each experiment, ionomycin (2 μM) and then Mn^{2+} (1 mM) were added to obtain the background signal to be subtracted before calculating $[Ca^{2+}]_i$. The second type of cover slip, diameter 24 mm, was placed in a chamber held at 37°C with a thermostat (Medical System Corporation, Greenvale, NY) on the stage of an inverted fluorescent microscope (Zeiss Axiovert 100TV) equipped with the imaging apparatus (Analytical Imaging Concepts, Atlanta, GA) for fura-2 measurement. The $[Ca^{2+}]_i$ images (1 image per second) were calculated off-line after subtraction of the background (obtained as above) from each single image. The absolute values of $[Ca^{2+}]_i$ were calculated as described [G. Grynkiewicz, M. Poenie, R. Y. Tsien, *J. Biol. Chem.* **260**, 3440 (1985); A. Malgaroli, D. Milani, J. Meldolesi, T. Pozzan, *J. Cell Biol.* **105**, 2145 (1988)].
12. R. Rizzuto *et al.*, in preparation; R. Rizzuto, P. Bernardi, M. Favaron, G. F. Azzone, *Biochem. J.* **246**, 271 (1987).
13. Permeabilization with digitonin, a detergent preferentially directed toward membranes containing cholesterol, has been used to study the function of mitochondria in situ. Digitonin treatment of HeLa or endothelial cells results in complete release of the cytosolic marker enzyme lactic dehydrogenase, whereas the mitochondrial marker enzymes citrate synthase or glutamate dehydrogenase and recombinant chimeric aequorin remain associated with sedimented cells.
14. Similar values of $[Ca^{2+}]_i$ are measured with fura-2 and with injected aequorin in hepatocytes [N. M. Woods, K. S. R. Cuthbertson, P. H. Cobbold, *Nature* **319**, 600 (1986); T. A. Rooney, E. J. Sass, A. P. Thomas, *J. Biol. Chem.* **264**, 17131 (1989)]. In cells transfected with cytosolic aequorin, the peak $[Ca^{2+}]_i$ measured with this method closely agrees with that measured with fura-2 (R. Rizzuto *et al.*, unpublished data).
15. The concentration of Ca^{2+} in the buffers was calculated as described [A. Fabiato, in *Cellular Calcium, A Practical Approach*, J. G. McCormack and P. H. Cobbold, Eds. (IRL Press, Oxford, 1991), pp. 159–176]. The calculated concentrations of free Ca^{2+} in the medium were always compared with the values measured directly with fura-2 in an aliquot of the same buffers. If a discrepancy was noticed, the values measured with fura-2 were considered correct.
16. A discrepancy between the increases in $[Ca^{2+}]_i$ in intact cells measured with fura-2 and the calculated extramitochondrial $[Ca^{2+}]$ is observed also in the case of Ca^{2+} influx. The difference (approximately a factor of 2) may be ascribed to the loss of a cytosolic factor, perhaps spermine [J. G. McCormack, H. M. Brown, N. J. Dawes, *Biochim. Biophys. Acta* **973**, 420 (1989)].
17. R. Rizzuto *et al.*, unpublished data.
18. T. Satoh *et al.*, *J. Cell Biol.* **111**, 615 (1990); K. Takei *et al.*, *J. Neurosci.* **12**, 489 (1992).
19. If, after permeabilization, ATP was omitted, the response to IP_3 was reduced and eventually disappeared. Marginal increases of $[Ca^{2+}]_m$ were observed if the uncoupler of mitochondrial respiration *p*-(trifluoro-methoxy)phenylhydrazone (FCCP) was included in the buffer before the addition of IP_3. Substitution of EGTA with an equivalent concentration of the faster Ca^{2+} chelator BAPTA did not change the effect of IP_3 on $[Ca^{2+}]_m$. Conversely, when high concentrations of either chelator were used (for example, 500 μM), the IP_3-induced $[Ca^{2+}]_m$ transient was virtually abolished.
20. J. E. Chad and R. Eckert, *Biophys. J.* **45**, 993 (1984); S. M. Simon and R. Llinás, *ibid.* **48**, 485 (1985); G. J. Augustine and E. Neher, *Curr. Opin. Neurobiol.* **2**, 302 (1992).
21. R. Llinás, M. Sugimori, R. B. Silver, *Science* **256**, 677 (1992).
22. A. J. O'Sullivan *et al.*, *EMBO J.* **8**, 401 (1989); T. A. Rooney, E. J. Sass, A. P. Thomas, *J. Biol. Chem.* **265**, 10792 (1990).
23. Supported by grants from the Italian Research Council (CNR) "Biotechnology" and "Oncology," from "Telethon," from the Italian Association for Cancer Research (AIRC), and from the "AIDS Project" of the Italian Health Ministry to T.P. We thank Y. Kishi for the gift of coelenterazine; Y. Sakaki for the aequorin cDNA; P. H. Cobbold and R. Cuthbertson for the data analysis software and for advice on the construction of the aequorin detection system; G. Ronconi and M. Santato for technical assistance; C. Bastianutto and L. Pasti for carrying out some of the experiments; J. Meldolesi, C. Montecucco, G. Schiavo, and P. Volpe for critically reading the manuscript; R. Bruzzone for discussion; and G. F. Azzone for encouragement and support.

14 May 1993; accepted 30 August 1993

Alterations of a Zinc Finger–Encoding Gene, *BCL-6*, in Diffuse Large-Cell Lymphoma

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The molecular pathogenesis of diffuse large-cell lymphoma (DLCL), the most frequent and clinically relevant type of lymphoma, is unknown. A gene was cloned from chromosomal translocations affecting band 3q27, which are common in DLCL. This gene, *BCL-6*, codes for a 79-kilodalton protein that is homologous with zinc finger–transcription factors. In 33 percent (13 of 39) of DLCL samples, but not in other types of lymphoid malignancies, the *BCL-6* gene is truncated within its 5′ noncoding sequences, suggesting that its expression is deregulated. Thus, *BCL-6* may be a proto-oncogene specifically involved in the pathogenesis of DLCL.

The molecular analysis of specific chromosomal translocations has improved our understanding of the pathogenesis of non-Hodgkin lymphoma (NHL), a heterogeneous group of B cell and (less frequently) T cell malignancies (*1*, *2*). The (14;18) chromosomal translocation, which causes the deregulated expression of the anti-apoptosis gene *BCL-2*, plays a critical role in the development of follicular lymphoma (FL) (*3*), which accounts for 20 to 30% of all NHL diagnoses (*4*). Burkitt's lymphoma (BL) and mantle-cell lymphoma, two relatively rare NHL types, are characterized by chromosomal translocations that cause the deregulated expression of the cell-cycle progression genes *MYC* and *BCL-1*/cyclin D1, respectively (*5*, *6*).

Relatively little is known about the molecular pathogenesis of DLCL, the most frequent and most lethal human lymphoma (*4*). It accounts for ~40% of initial NHL diagnoses and is often the final stage of progression of FL (*4*). A small percentage of DLCLs display *MYC* rearrangements (*7*) and 20 to 30% display alterations of *BCL-2*, reflecting the tumor's derivation from FL (*8*). However, no consistent molecular alteration has been identified that is specific for DLCL.

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