Proc. Natl. Acad. Sci. USA
Vol. 85, pp. 1682–1685, March 1988
Neurobiology

Activity-dependent accumulation of calcium in Purkinje cell dendritic spines

(synapse/synaptic spine/electron probe analysis)

S. BRIAN ANDREWS*†, RICHARD D. LEAPMAN‡, DENNIS M. D. LANDIS§, AND THOMAS S. REESE*¶

*Laboratory of Neurobiology, National Institute of Neurological and Communicative Disorders and Stroke, and ‡Biomedical Engineering and Instrumentation Branch, Division of Research Resources, National Institutes of Health, Bethesda, MD 20892; §Departments of Neurology, Developmental Genetics, and Anatomy, Case Western Reserve University School of Medicine, Cleveland, OH 44106; and ¶Laboratory of Neurobiology, National Institute of Neurological and Communicative Disorders and Stroke, Marine Biological Laboratory, Woods Hole, MA 02543

Contributed by Thomas S. Reese, October 26, 1987

ABSTRACT **The calcium content of synapses of parallel fibers on Purkinje cell dendritic spines was determined by electron probe x-ray microanalysis of freeze-dried cryosections from directly frozen slices of mouse cerebellar cortex. In fresh slices frozen within 20–30 sec of excision, calcium concentrations ranging from 0.8 to 18.6 mmol/kg of dry weight were measured in cisterns of smooth endoplasmic reticulum within Purkinje cell dendritic spines. The average calcium content of spine cisterns in rapidly excised slices (6.7 ± 0.6 mmol/kg of dry weight ± SEM) was higher than the average calcium content of spine cisterns in brain slices incubated without stimulation for 1–2 hr before direct freezing (2.5 ± 0.4 mmol/kg of dry weight). Depolarization of incubated cerebellar slices by isotonic 55 mM KCl resulted in the accumulation within spine cisterns of very high amounts of calcium or isotonically substituted strontium, both derived from the extracellular fluid. These results suggest that one function of spine cisterns is to sequester free calcium that enters the spine through ligand-gated or voltage-gated channels during synaptic transmission.**

Dendritic spines are characteristic features of many classes of neurons in the central nervous system. Although the function of dendritic spines is still uncertain (1–4), several lines of evidence suggest that changes in the amount and distribution of intracellular calcium may accompany synaptic activity at spines (5). Thus, recent studies on hippocampal, striatal, and spinal cord neurons show that the *N*-methyl-D-aspartate (NMDA)-sensitive subtype of glutamate receptor (6–10)—a receptor thought to be present on several kinds of spines (11)—includes a calcium channel (12–14) and that ligand binding to such receptors results in calcium influx and an increase in free cytoplasmic calcium (15). Significant changes in the calcium concentration of dendritic spines might also be expected on the basis of voltage-dependent calcium channel activity (16).

Because spiny neurons often exhibit long-term modulation of synaptic efficacy (17), it has been suggested that spines are the anatomical locus of long-term modulation and that these effects are mediated by calcium ions (5, 14). In the cerebellum, parallel fiber synapses on Purkinje cell dendritic spines are subject to use-dependent effects specifically mediated by glutamate receptors (18) that, in this instance, are quisqualate-selective and have a conductance state similar to the large (40–50 pS), calcium-permeable state of the hippocampal receptor (14, 19). Moreover, cerebellar parallel fiber synapses are well-suited to structural studies because they can be directly frozen, undamaged and intact, without prior fixation (20). The accessibility of intact synapses to direct freezing also makes this preparation available to quantitative electron probe x-ray microanalysis (21), a method that can accurately measure the content of calcium and other elements in unfixed, directly frozen tissue at high spatial resolution.

The results of such experiments show that cisterns of smooth endoplasmic reticulum within the spines sequester high concentrations of calcium during depolarization-induced synaptic activity, and that the accumulated calcium appears to enter the spines from the extracellular space. Available data allow us to calculate that the amount of calcium sequestered in the spine cisterns is compatible with that which might be expected to enter the spine through postsynaptic channels.

MATERIALS AND METHODS

Slices of lateral cerebellum from 40- to 45-day-old NIH C57BL/6 mice were prepared for cryosectioning, as described (20, 22); excised slices were rapidly frozen within 20–30 sec of circulatory arrest or after incubation in an oxygenated physiological saline for 60–75 min (22) by pressing the uncut pial surface against a liquid helium-cooled copper block (23). For uptake experiments, strontium was isotonically substituted for calcium. Where appropriate, incubated slices were depolarized by exposure to an isotonic saline containing 55 mM KCl for 30–120 sec before freezing. Thin frozen sections, approximately 100 nm thick (hydrated), were prepared by cryoultramicrotomy at −145°C on a Reichert FC-4/Ultracut E cryomicrotome. The frozen-hydrated sections were transferred to a JEOL 100-CX or a Hitachi H700H electron microscope by means of a Gatan model 626 cryotransfer device, freeze-dried at about −110°C and recooled for viewing and microanalysis. These procedures have been described in detail (22, 24).

For plastic sections, directly frozen cerebellar slices were freeze-substituted in osmium tetroxide containing 20 mM oxalic acid in anhydrous acetone at −80°C and, without exposure to any additional heavy metal stains, the tissue was embedded in Araldite resin and sectioned on glycerol (25). This procedure is useful for comparing the morphology of plastic-embedded tissue with that of freeze-dried, cryosectioned tissues under similar low-contrast conditions and also for qualitatively localizing calcium-rich subcellular regions.

Electron probe x-ray microanalysis was done by means of a Tracor TN-5500 x-ray analysis system. The theory, instrumentation, and data reduction methods have been described, both in general (21, 26, 27) and as practiced in our laboratories (22, 28).

The publication costs of this article were defrayed in part by page charge payment. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. §1734 solely to indicate this fact.

†To whom reprint requests should be addressed at: Building 36, Room 3B-26, National Institutes of Health, Bethesda, MD 20892.

RESULTS

A dome-shaped slice of mouse cerebellar cortex can be excised by a single cut parallel to the pial surface and frozen within 30 sec of circulatory arrest (20). Numerous molecular layer synapses lie near the natural pial surface of this slice, which is thick enough to ensure that neither the presynaptic granule cells nor the postsynaptic Purkinje cell dendrites at the top of the dome have been cut. Although the exact level of synaptic activity at the instant of freezing is unknown, this preparation has nevertheless proven quite useful for high-resolution structural (20) and compositional (22) studies of the synapses between parallel fibers and Purkinje cell dendritic spines. Because the superficial 10 μm of the molecular layer can be well preserved on a millisecond time scale, this preparation also can be used to determine whether synaptic transmission promotes calcium influx in Purkinje cell spines, and whether calcium sequestration within defined cellular locations is a consequence of calcium uptake.

In the unfixed, freeze-dried cryosections used for electron probe microanalysis, synaptic junctions between parallel fibers and Purkinje cell dendritic spines were identified by their characteristic shape, the clusters of synaptic vesicles in the axon, and the membrane-limited cisterns in the spine (compare the unstained, freeze-substituted spine synapse in Fig. 1*a* to the cryosectioned synapse in Fig. 1*b*). Electron probe measurements of elemental content were validated by measuring tissue components for which independent data on elemental compositions are available. For example, the cation composition determined by microanalysis for erythrocytes in capillaries—Na = 13 ± 5, K = 260 ± 12, and Ca = −0.2 ± 1.0 mmol/kg dry wt ± SEM—was in good agreement with known values for rodent erythrocytes. Similarly, several cell types were found to have cation concentrations entirely consistent with normal cell function—e.g., the concentrations of sodium and potassium in glial cells (Na = 70 ± 18; K = 385 ± 37 mmol/kg of dry wt ± SEM) and the low calcium content in neuronal (stellate cell) nuclei (Ca = 0.4 ± 0.4 mmol/kg of dry wt ± SEM). We also confirmed and extended our previous values (22) for the calcium and potassium content of synaptic vesicles and axonal mitochondria as measured in similar cerebellar slices (Table 1). Taken as a whole, these results establish the validity of electron probe measurements of elemental content in the cerebellar slice preparation.

Electron probe measurements of elemental content within spine cisterns revealed a mean calcium concentration of 6.7 ± 0.6 mmol/kg of dry weight with individual values ranging up to 18.6 mmol/kg; in contrast, the calcium concentration of the cytoplasmic matrix of the spines was 1.3 ± 0.5 mmol/kg of dry weight (Table 1). However, the range of values for the concentration of calcium within the cisterns was not normally distributed (Fig. 2). The level of synaptic activity in the freshly excised cerebellar slices is difficult to predict and to measure; thus, variations in calcium content might possibly reflect variations in the activity of spine synapses during excision and handling of the slice.

To obtain tissue with more defined synaptic activity, cerebellar slices were incubated for 1–2 hr in an oxygenated physiological saline solution before rapid freezing. The calcium content of the spine cisterns from these unstimulated slices was 2.5 ± 0.4 mmol/kg of dry weight, ranging from undetectable to 7.1 mmol/kg (Table 1, Fig. 2). The range of

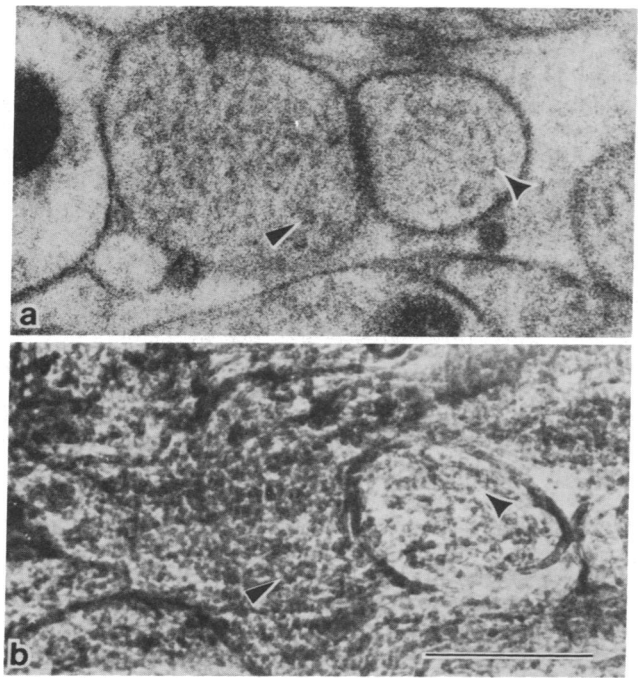

FIG. 1. Electron micrographs of a freeze-substituted, plastic-embedded section (*a*) and a thin, freeze-dried cryosection (*b*) from the molecular layer of directly frozen slices of freshly excised cerebellum. Both micrographs illustrate intact parallel fiber synapses on Purkinje cell spines in which specific small membrane-bound organelles, such as synaptic vesicles (straight arrowheads, *Left*) and membrane-limited spine cisterns (curved arrowheads, *Right*), can be identified, even though differential contrast is low because the tissue is totally unstained (*b*) or stained only with the osmium tetroxide used as primary fixative (*a*). Micrograph *b* was taken in the conventional transmission mode at a nominal temperature of −145°C with an accumulated dose of <100 e/A^2. Bar = 0.5 μm.

Table 1. Potassium and calcium concentrations in synapses of cerebellar cortex

	No. of analysis	Elemental concentration, mmol/kg of dry wt	
		K^+	Ca^{2+}
Freshly excised slice			
Presynaptic			
Synaptic vesicle cluster	55	365 ± 9	1.3 ± 0.2
Axonal mitochondria	15	280 ± 18	2.9 ± 1.0
Postsynaptic			
Spine cistern*	40	401 ± 14	6.7 ± 0.6
Spine cytoplasm	17	364 ± 24	1.3 ± 0.5
Incubated slice			
Presynaptic			
Synaptic vesicle cluster	23	356 ± 15	2.4 ± 0.7
Axonal mitochondria	8	321 ± 22	2.0 ± 0.6
Postsynaptic			
Spine cistern	32	474 ± 22	2.5 ± 0.4

Elemental concentrations ± SEM are derived from characteristic x-ray/continuum ratios using point-probe analyses. To achieve stated precision, enough x-ray counts were collected over 200–500 sec for each element at each point so that statistical errors for microanalysis were much smaller than (for K^+) or similar to (for Ca^{2+}) the biological variability. Therefore, errors quoted for Ca^{2+} are combined statistical and biological errors. Analyzed compartments are defined as follows: synaptic vesicle cluster, group of 2–4 vesicles including intervening cytoplasm; axonal mitochondria, central inner matrix of single mitochondria located adjacent to vesicle-rich presynaptic varicosities; spine cistern, individual cistern of endoplasmic reticulum, either in transverse or cross-section, but laterally equal to or larger than the electron probe; and spine cytoplasm, organelle-free areas of the matrix of dendritic spines. The first two compartments are fully described and illustrated in ref. 22. Data are from four animals.

*Concentration histogram for Ca^{2+} in fresh spine cisterns was not normally distributed (see Fig. 2); one possible explanation is discussed in the text.

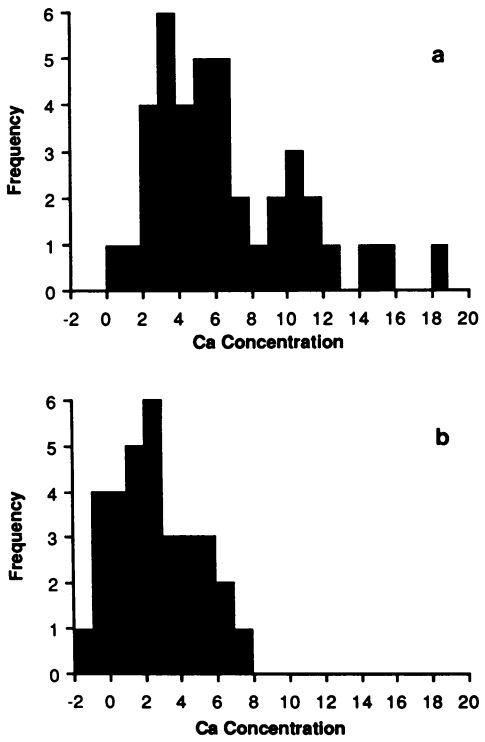

FIG. 2. Histogram of the distributions of calcium concentrations (mmol/kg of dry wt) within Purkinje spine cisterns from directly frozen cerebellar cortex, as determined by electron-probe microanalysis of freeze-dried cryosections. (*a*) Spine cisterns from a freshly frozen cerebellar slice. Mean of the data set is 6.69 ± 4.06 (SD), but data are not normally distributed; this distribution may be partly due to a subpopulation of spines that did not take up any calcium. (*b*) Spine cisterns from a cerebellar slice that had been incubated in physiological saline without depolarization before freezing. Data are normally distributed with a mean of 2.51 ± 2.37 (SD).

these values was normally distributed (Fig. 2) and overlapped the low end of the concentration distribution observed in freshly excised slices. A plausible interpretation of this result is that synaptic activity in the unstimulated, incubated slices was uniformly low. Thus, the overlap in the distributions of calcium concentration in spine cisterns could reflect the presence of inactive spine synapses in both freshly excised and unstimulated, incubated slices. In this instance, the mean value of 6.7 ± 0.6 mmol/kg of dry weight for calcium in spine cisterns from freshly excised slices should represent a minimum estimate of the calcium accumulation in recently active cisterns.

To determine the source of the accumulated calcium, cerebellar slices were incubated in a medium in which calcium was replaced by strontium. Comparison of electron microprobe data from slices frozen with and without brief intervals of depolarization (55 mM KCl) before freezing showed strontium accumulation, to the virtual exclusion of calcium, within spine cisterns from depolarized slices only.[‖] This finding indicates an extracellular origin for most, if not all, of the divalent ions that become concentrated in cisterns after depolarization.

DISCUSSION

From these experiments, we conclude that: (*i*) Purkinje cell spines contain membrane-limited cisterns that accumulate calcium avidly; (*ii*) synaptic activity results in calcium accumulation in spine cisterns; and (*iii*) the calcium taken up into spine cisterns comes from the extracellular space. These results offer direct evidence that cisterns, which appear to represent endoplasmic reticulum in dendritic spines (29), accumulate calcium under physiologically relevant conditions. This finding agrees well with much previous evidence on the calcium-accumulating and -releasing activities of endoplasmic reticulum in several cell types (30) and is also consistent with previous cytochemical studies of calcium binding in spine cisterns (31, 32).

We presume that natural activity, as well as the activity evoked by surgical manipulation of freshly excised slices, produces variation in the activity of individual molecular layer synapses, as manifested by the wide range of calcium content in individual spine cisterns. In a second type of experiment we incubated the slices in physiological saline before freezing them; spine cisterns from these slices showed a lower mean and less variation in their calcium content, presumably because these cisterns were now more uniformly quiescent. To test this interpretation and to determine the source of the increased calcium, we also examined cerebellar slices that had been incubated in a strontium-containing solution and then frozen with and without brief intervals of depolarization induced by 55 mM potassium. Virtually all cisterns in these depolarized slices had accumulated strontium—a result supporting the idea that synaptic activity is accompanied by accumulation in spine cisterns of divalent cations from the extracellular space.

Because the measurement of elemental composition by electron probe microanalysis is quantitative, we can ask whether the amount of calcium accumulated by spine cisterns in cerebellar synapses is comparable to the amount that might be expected to have entered through postsynaptic ion channels. The dry-weight calcium concentrations can be converted to concentrations per liter of wet tissue, either by estimating the dry mass (typically 20–25 g/100 g of wet wt) on the basis of continuum x-ray counts, or by directly calculating the concentrations in mmol/liter by comparison with erythrocytes of known concentrations in the same cryosection (28). This calculation shows that the level of total calcium in the spine cisterns of freshly excised synapses is three times that of undepolarized synapses from incubated slices (1.5 as compared with 0.5 mmol/liter of wet tissue). Measurements of surface area ratios in directly frozen, freeze-substituted spines show that the cisterns occupy ≈10% (8.9 ± 0.9%, n = 43; excluding the spine neck) of the spine volume, corresponding to a cisternal volume of 0.002 μm^3 per spine. We can accordingly estimate that cisterns in freshly excised slices contain an average of 1800 calcium ions, which is 1200 ions more than the average content of cisterns in unstimulated, incubated slices. This estimate may prove conservative to the extent that our sample includes unactivated, low-calcium containing spines.

We assume that synaptic activity during dissection and mounting of the freshly excised slices results in glutamate (11) release from some fraction of the parallel fiber terminals. Transmitter binding to postsynaptic receptors should depolarize the spines by a sodium current; this binding may also promote calcium influx, although this relationship is not certain because the calcium conductance of transmitter-gated channels in Purkinje cell spines is not yet known. Nevertheless, considerable information about the glutamate receptor complex is now available, including the conductance and mean open time of the large conductance state (9, 14, 19), the Ca^{2+}/Na^{+} permeability ratio of this state (33), and the number and distribution of putative receptors in the active zone of cerebellar (34) and hippocampal (11) spine synapses. [The latter estimate is based on the assumption that the number of particles associated with the extracellular half of freeze-fractured postsynaptic membranes (34) reflects

[‖] Strontium loading also occurred in damaged synapses (defined by their low K^{+}/Na^{+} ratios), some of which were always present in incubated slices.

the number of glutamate receptor sites.] Because in mature Purkinje cells only a small fraction—we estimate perhaps 10%—of the quisqualate-selective glutamate receptors (18) are likely to open to the large-conductance state (19), the calcium flux during synaptic activity should be on the order of thousands of ions per millisecond. It is therefore plausible that the amount of calcium measured in spine cisterns by analytical electron microscopy entered the spine as part of a transient, transmitter-induced membrane current.

In addition to calcium entering via ligand-gated channels, there is also the possibility that synaptic activity opens voltage-dependent calcium channels on the spine or main dendrite (16). Such channels are present on the dendritic membrane (35) and may also be on the spine membrane (36), although in either instance the exact types of calcium channels (37) are not known.

It has been estimated that the peak ionized internal calcium concentration during synaptic activity is about 5 μM (38). Only 50 *free* calcium ions would be required to reach this level in the volume of a Purkinje cell dendritic spine. Our estimates of calcium flux and measurements of the resulting calcium content in spine cisterns suggest that far more than 50 calcium ions entered the dendritic spine during synaptic activity and that much of the new calcium enters the spine cisterns within a few seconds of stimulation. This rapid calcium accumulation appears to be a concrete example of *sequestration*; if the spine cisterns only take up calcium when cytoplasmic free calcium exceeds a certain threshold, then this system might more precisely be described as a calcium *buffer*. The latter possibility is especially interesting in view of the likelihood that neurons have some mechanism for regulating free cytoplasmic calcium in the 10^{-6} M range (38).

Our results show that most of the additional divalent cations accumulated in spine cisterns are of extracellular origin, but they do not exclude a contribution from intracellular sources. Though calcium-releasing cisterns exist in neurons (38), we were unable to detect in spines any synaptic cisterns in which the calcium content *decreased* upon stimulation. However, physiologically significant calcium release from some cisterns could have resulted in a depletion too small for detection by microanalysis. Nonetheless, our results do imply that calcium, acting via a transmembrane current that causes an increase in free calcium, may function as an intracellular messenger in activated dendritic spines. This in turn lends support to the hypothesis, for which several mechanisms have been proposed (39–43), that local changes in calcium concentration might regulate plasticity and potentiation in spine-bearing neurons.

We are indebted to M. O'Connell for excellent technical assistance, and to J. Murphy for his photographic work. We also thank C. E. Fiori and C. R. Swyt for valuable discussions. This work was supported in part by National Institutes of Health Grant NS 23641 (D.M.D.L.).

1. Swindell, N. V. (1981) *Trends Neurosci.* **4,** 240–241.
2. Gray, E. G. (1982) *Trends Neurosci.* **5,** 5–6.
3. Crick, F. (1982) *Trends Neurosci.* **5,** 44–46.
4. Rall, W. (1974) in *Cellular Mechanisms Subserving Changes in Neuronal Activity*, eds. Woody, C., Brown, K., Crow, T. & Knipsel, J. (Brain Information Service, University of California, Los Angeles), pp. 13–21.
5. Smith, S. J. (1987) *Trends Neurosci.* **10,** 142–144.
6. Harris, E. W., Ganong, A. H. & Cotman, C. W. (1984) *Brain Res.* **323,** 132–137.
7. Morris, R. G., Anderson, E., Lynch, G. S. & Baudry, M. (1986) *Nature (London)* **319,** 774–776.
8. Collingridge, G. L., Kehl, S. J. & McLennan, H. (1983) *J. Physiol. (London)* **334,** 33–46.
9. Nowak, L., Bregestovski, P., Ascher, P., Herbet, A. & Prochiantz, A. (1984) *Nature (London)* **307,** 462–465.
10. Mayer, M. L., Westbrook, G. L. & Guthrie, P. B. (1984) *Nature (London)* **309,** 261–263.
11. Harris, K. M. & Landis, D. M. D. (1986) *Neuroscience* **19,** 857–872.
12. Asher, P. & Nowak, L. (1986) *J. Physiol. (London)* **377,** 35P.
13. Mayer, M. L. & Westbrook, G. L. (1985) *Proc. Soc. Neurosci.* **11,** 785 (abstr.).
14. Jahr, C. E. & Stevens, C. F. (1987) *Nature (London)* **325,** 522–525.
15. MacDermott, A. B., Mayer, M. L., Westbrook, G. L., Smith, S. J. & Barker, J. L. (1986) *Nature (London)* **321,** 519–522.
16. Gamble, E. & Koch, C. (1987) *Science* **236,** 1311–1315.
17. Bliss, T. V. P. & Lomo, T. J. (1973) *J. Physiol. (London)* **232,** 331–356.
18. Kano, M. & Kato, M. (1987) *Nature (London)* **325,** 276–279.
19. Cull-Candy, S. G. & Usowicz, M. M. (1987) *Nature (London)* **325,** 525–528.
20. Landis, D. M. D. & Reese, T. S. (1983) *J. Cell Biol.* **97,** 1169–1178.
21. Hall, T. A. & Gupta, B. L. (1983) *Q. Rev. Biophys.* **16,** 279–330.
22. Andrews, S. B., Leapman, R. D., Landis, D. M. D. & Reese, T. S. (1987) *Proc. Natl. Acad. Sci. USA* **84,** 1713–1717.
23. Heuser, J. E., Reese, T. S., Dennis, M. J., Jan, Y., Jan, L. & Evans, L. (1979) *J. Cell Biol.* **81,** 275–300.
24. Andrews, S. B. & Reese, T. S. (1986) *Ann. N.Y. Acad. Sci.* **483,** 284–294.
25. Ornberg, R. L. & Reese, T. S. (1980) *Fed. Proc. Fed. Am. Soc. Exp. Biol.* **39,** 2802–2808.
26. Shuman, H., Somlyo, A. V. & Somlyo, A. P. (1976) *Ultramicroscopy* **1,** 317–339.
27. Kitazawa, T., Shuman, H. & Somlyo, A. P. (1983) *Ultramicroscopy* **11,** 251–262.
28. Andrews, S. B., Mazurkiewicz, J. E. & Kirk, R. G. (1983) *J. Cell Biol.* **96,** 1389–1399.
29. Spacek, J. (1985) *Anat. Embryol.* **171,** 235–243.
30. Somlyo, A. P., Bond, M. & Somlyo, A. V. (1985) *Nature (London)* **314,** 622–625.
31. Burgoyne, R. D., Gray, E. G. & Barron, J. (1983) *J. Anat.* **136,** 634–635.
32. Fifkova, E., Markham, J. A. & Delay, R. J. (1983) *Brain Res.* **266,** 163–168.
33. Mayer, M. L. & Westbrook, G. L. (1987) *J. Physiol. (London)* **394,** 501–527.
34. Landis, D. M. D. & Reese, T. S. (1974) *J. Comp. Neurol.* **155,** 93–126.
35. Llinas, R. & Sugimori, M. (1980) *J. Physiol. (London)* **305,** 297–213.
36. Siekevitz, P., Carlin, R. & Wu, K. (1985) *Proc. Soc. Neurosci.* **11,** 646 (abstr.).
37. Nowycky, M. C., Fox, A. P. & Tsien, R. W. (1985) *Nature (London)* **316,** 440–443.
38. McBurney, R. N. & Neering, I. R. (1987) *Trends Neurosci.* **10,** 164–169.
39. Shepherd, G. M., Brayton, R. K., Miller, J. P., Segev, I., Rinzel, J. & Rall, W. (1985) *Proc. Natl. Acad. Sci. USA* **82,** 2192–2195.
40. Miller, S. G. & Kennedy, M. B. (1986) *Cell* **44,** 861–870.
41. Lynch, G. & Baudry, M. (1984) *Science* **224,** 1057–1063.
42. Akers, R. F. & Routtenberg, A. (1985) *Brain Res.* **334,** 147–151.
43. Acosta-Urquidi, J., Alkon, D. L. & Neary, J. T. (1984) *Science* **224,** 1254–1257.