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Sustained Dendritic Gradients of Ca^{2+} Induced by Excitatory Amino Acids in CA1 Hippocampal Neurons

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Spatially resolved measurements of intracellular free calcium and of the changes produced by excitatory amino acids were made in neurons isolated from adult mammalian brain. Extremely long-lasting (minutes) Ca^{2+} gradients were induced in the apical dendrites of hippocampal CA1 neurons after brief (1 to 3 seconds), local application of either glutamate or *N*-methyl-D-aspartate (NMDA). These gradients reflect the continuous flux of Ca^{2+} into the dendrite. The sustained gradients, but not the immediate transient response to the agonists, were prevented by prior treatment with the protein kinase C inhibitor sphingosine. Expression of the long-lasting Ca^{2+} gradients generally required a priming or conditioning stimulus with the excitatory agonist. The findings demonstrate a coupling between NMDA receptor activation and long-lasting intracellular Ca^{2+} elevation that could contribute to certain use-dependent modifications of synaptic responses in hippocampal CA1 neurons.

NEURONS OF THE MAMMALIAN HIPpocampus have become an important focus for the investigation of putative memory mechanisms, including long-term potentiation (LTP) of excitatory synaptic connections, kindling phenomena, and other persistent effects of conditioning paradigms (*1*). Both LTP and certain kindling phenomena show dependence on Ca^{2+} levels [Ca^{2+}] or fluxes (*2*) and Ca^{2+}-dependent enzymes (*3, 4*) for expression. Several studies have linked *N*-methyl-D-aspartate (NMDA) receptor activation with LTP induction, presumably through Ca^{2+}-dependent mechanisms (*5*). There have been no direct measurements, in hippocampus or in any other fully differentiated mammalian neurons, of the Ca^{2+} changes that result from stimulation with the putative natural excitatory transmitter glutamate or the analog NMDA, although in cultured neurons NMDA induces a large transient influx of Ca^{2+} through the receptor-operated channels (*6*).

We measured [Ca^{2+}] and the changes produced by excitatory amino acids in isolated CA1 neurons (*7*) from adult guinea pig hippocampus by using the fluorescent Ca indicator fura-2 (*8*). These completely isolated cells have been used in elucidating the membrane properties of neurons, independent of presynaptic and hormonal input (*9*). The preparation is also ideally suited to optical studies because of the freedom from absorbance and scattering by extraneous tissue. Resting [Ca^{2+}] in 120 CA1 neurons tested averaged 84 ± 3 n*M* (SEM). Neurons in the range below 100 n*M* were stable for periods of more than 30 minutes (*10*).

Figure 1 illustrates the basic finding of this study: repeated stimulation of the apical dendrite leads to localized Ca^{2+} increases of long duration. The bathing saline contained the Na^+-channel blocker tetrodotoxin (TTX) [1 μ*M*, see (*11*)]. A glutamate pulse was applied to the dendrite at two different locations. The first pulse, applied at the leftmost end of the dendrite (Fig. 1A), produced a large change in Ca^{2+} that recovered rapidly and remained stable for the next 3 minutes (Fig. 1, B through E). The second application (Fig. 1H) produced a smaller response than the first one, and there was an initial small recovery after the stimulus (Fig. 1I), but over the next 3 minutes [Ca^{2+}] progressively increased at the site of the second application, generating a gradient that tailed off to either side (Fig. 1, J and K). We emphasize that [Ca^{2+}] decreased prior to the secondary rise and the generation of the standing gradient. This secondary rise in [Ca^{2+}] occurred in 93% of the cells examined ($n = 49$). In most other experiments the successive glutamate applications were made at or near the initial site. Removing Ca^{2+} from the bathing medium abolished the standing gradients as rapidly as the solution change could be made and allowed the cell to restore low levels of Ca^{2+} (Fig. 1L). Glutamate stimuli did not produce changes in Ca^{2+}-free saline.

In 29% of the 34 neurons tested for glutamate response, two stimuli were required before the long-lasting response was expressed. Once the long-lasting response was established, [Ca^{2+}] almost never recovered spontaneously to their initial values during the usual experimental time course of 20 to 25 minutes. When the response was allowed to persist for ten or more minutes, [Ca^{2+}] in the soma and dendrite gradually increased to 300 to 500 n*M*. At this stage of the response the dendritic Ca^{2+} gradient was gone and the nuclear region of the soma showed higher levels than other parts of the cell. In 32% of the neurons, the extended response was elicited after the first application of glutamate; however, most of these cells already showed a small Ca^{2+} gradient, dendrite higher than soma. The rest of the cells showed the extended response only after three or more applications. Spacing between stimuli was a necessary part of the

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protocol. For example, a 4-second glutamate application was generally insufficient to produce a sustained gradient, whereas two 2-second applications separated by an interval of 1 minute or more were generally sufficient. Maintained gradients of Ca^{2+} were also observed in cells where the free acid form of fura-2 was injected, implying that these are cytoplasmic gradients.

Electrical recordings, made with the use of the whole-cell patch electrode technique, showed that glutamate applications of the magnitude used in the optical studies gave depolarizations of 10 to 20 mV in the presence of 1 μM TTX. The depolarizations generally recovered within a few hundred milliseconds after the iontophoretic current was turned off. On rare occasions, after a large glutamate stimulus, a persistent depolarization of 2 to 3 mV lasting for 30 to 60 seconds was seen; however the whole-cell recording technique dialyzes the cell interior, crippling intracellular messenger systems.

Iontophoretic application of NMDA in Mg^{2+}-free saline produced responses similar to those of glutamate in normal saline ($n = 15$), with the important exception that the maintained response was elicited with the first stimulus in a higher percentage of the cells (67% versus 32% for glutamate). We do not know whether to ascribe this difference to greater efficacy of NMDA or to impaired Ca^{2+} regulation in low Mg^{2+} saline. However, we found that resting $[Ca^{2+}]$ in Mg^{2+}-free saline (88 ± 5 mM, $n = 23$) did not differ significantly from those observed in normal saline (83 ± 5 nM, $n = 64$). Figure 2 shows two examples of cells stimulated with NMDA at the apical dendrite. An iontophoretic application (2 seconds, 200 nA) caused a transient increase in Ca^{2+}, and there was an appreciable dendrite-soma gradient (Fig. 2A). Initial levels were quickly reestablished, but during the next 6 minutes the level of Ca^{2+} in the dendrite slowly crept up while $[Ca^{2+}]$ in the soma and proximal dendrite remained relatively unchanged. A second application of NMDA that was three times as large as the first application produced a response from which the cell did not recover. However, even in this case there was an initial, partial recovery during the first 30 seconds after the stimulus.

In a second cell (Fig. 2B), three NMDA stimuli were applied to the same distal location on the cell in more rapid succession than in the preceding example. The onset of the maintained response occurred soon after application of the first stimulus but was localized to the application site. After the second stimulus, the maintained response spread to the intermediate dendritic location, as evidenced by the development of a gradient between the intermediate and proximal sites. The third stimulus caused a further increase in $[Ca^{2+}]$ and of the gradient between the distal and proximal dendrite.

The responses to NMDA, both the immediate Ca^{2+} increase and the delayed rise, were blocked by the addition of 20 μM 2-amino-5-phosphonovaleric-acid (APV) to the bath (three of three cells). The response to NMDA was also significantly reduced in saline with normal Mg^{2+} levels (1.2 mM). Three attempts failed to reverse a standing gradient once it was induced, either by the addition of APV to the bathing solution or by the restoration of Mg^{2+}. The effects of adding γ-aminobutyric acid (GABA) after stimulation were examined in five cells. In three cells, standing gradients of Ca^{2+} that existed for at least 2 minutes were reduced and reached levels that were within 30% above initial values. In the other two cells, GABA slowed the rate of further development of the gradient.

Pretreatment of cells with 10 μM D-sphingosine (Sigma), a protein kinase C inhibitor (*12*), had no significant influence on the resting $[Ca^{2+}]$ or the primary response to glutamate but abolished or greatly reduced the development of the secondary Ca^{2+} response. A series of six stimuli were delivered to a neuron that had been exposed to sphingosine for 20 minutes (Fig. 2C). This cell showed an initial soma-dendritic Ca^{2+} gradient; had there been no pretreatment, it would have been expected to develop a long-lasting response after the initial stimulus. Instead, the recoveries from the first five stimuli were to $[Ca^{2+}]$ below the starting values (2-minute data point). After the sixth stimulus (30 minutes after isolation of the cell) the cell failed to recover; however, the failure was characterized by a uniform rise of Ca^{2+} which could reasonably reflect saturation of Ca^{2+} buffers and rundown of Ca^{2+} transport capacity of the cell membrane. In none of the eight cells given glutamate stimulus after pretreatment did we observe an extended response after two or three stimuli. There was no significant difference in the response to first stimuli between treated and untreated cells.

We also examined $[Ca^{2+}]$ changes in response to a flow of 50 mM K^+ saline across the cell body and dendrites from a small pipette (*13*). In TTX saline this stimulus caused depolarizations of 20 mV that persisted for the duration of the exposure to high K^+ and decayed within a second after the flow was terminated. Transient dendrite-soma Ca^{2+} gradients on the order of 100 nM were established by this stimulus; however, the gradients collapsed within 5 to 10 seconds after K^+ stimulation and $[Ca^{2+}]$ generally recovered to the original values within 45 to 60 seconds. Thus, there do not seem to be any diffusion barriers in the dendrite that would promote the existence of gradients for the long periods shown in Figs. 1 and 2.

In a situation without diffusion barriers, standing gradients of Ca^{2+} imply the existence of a continued Ca^{2+} source at the stimulated region of the dendrite that is balanced by energy-coupled efflux and storage at the soma and proximal dendrite. The rapid abolition of this gradient in Ca^{2+}-free medium, and the estimate that internal stores would not have the capacity to maintain such a gradient (*14*), leads to the conclusion that there is a maintained Ca^{2+} influx through the membrane. Since the largest Ca^{2+} increase consistently occurred at the site of the transmitter application, it appears that the sustained Ca^{2+} influx cannot be caused simply by depolarization or sustained Ca^{2+} action potential generation. We suggest that stimulation by the agonist sets up conditions in which more Ca^{2+}-permeable channels are open after the stimulation than before or that the same channels are open, on a statistical basis, a greater percentage of the time. We infer from the ability of GABA to reduce or at least stabilize the persisting Ca^{2+} gradients that these channels are voltage-dependent, since GABA hyperpolarizes the cells. Because the immediate agonist-induced response always showed at least partial recovery, it does not appear that the secondary Ca^{2+} rise is due to flux through the transmitter-gated channels, although this hypothesis is difficult to test directly.

The most likely mechanism for our observations is that a population of Ca^{2+}-carrying channels is altered by a C kinase–mediated process or other process that is interrupted by sphingosine. Enhancement of voltage-gated Ca^{2+} fluxes due to either cyclic adenosine monophosphate– or C kinase–mediated phosphorylation has been reported in neurons and heart cells (*15*). In some cases the increases result from alterations in the kinetics of active channels, and in *Aplysia* bag neurons from the recruitment of dormant or "covert" Ca^{2+} channels. The latter response appears to be controlled by one of the C kinases. Either type of mechanism could produce our observations.

At least two other factors, residual transmitter and long-term closing of K^+ channels, could possibly cause maintained Ca^{2+} gradients, but we regard them as unlikely. Residual transmitter action is ruled out because of the lack of effect of washing, and of blockers applied after stimulation, and the effect of sphingosine. Although there is evidence that phorbol esters and, by implication, C kinase activation produce a depression of K^+ current in hippocampal neurons

(*3, 16*), this mechanism is inadequate. The isolated CA1 neurons are electrically compact (*9*). Therefore, dendritic Ca^{2+} channels could not be appreciably more activated than somatic ones as a result of a depolarization produced by K^+ channel closing alone. Moreover, large soma-dendrite gradients did not exist during K^+ depolarizations of long duration (*13*). In regard to the use of phorbol esters, brain protein kinase C is an enzyme family (*17*), and it is unclear which members are affected by activators or what might be the different effects of multiple activation as opposed to selective activation of one member.

In the isolated neurons we see large sustained Ca^{2+} gradients after stimulation. These gradients need not be present in situ, where inhibitory inputs, the GABA and monoamine systems, tend to hold membrane voltage at negative levels. There is also a great loss of dendritic membrane, rendering the input resistance of the cells exceedingly high and making it possible for small inward currents to produce appreciable depolarizations. In fact, the resting potentials of the isolated cells are somewhat lower than in the slice preparation. Thus it is reasonable to speculate that in the isolated cells conditions favor the influx of Ca^{2+} through the modified, voltage-dependent channels. In the physiological setting these modified channels might not be open at resting voltages, but with depolarizing stimulus, as from synaptic input, they would open readily, resulting in enhanced Ca^{2+} influx during dendritic spikes (*18*) or graded depolarizations. This could account qualitatively for some of the criteria used to define the LTP measured in CA1 neurons; that is, decreased latency of firing, larger postsynaptic response, or enhanced Ca^{2+} fluxes in kindling experiments, although one must also recognize the probable importance of presynaptic mechanisms (*19*).

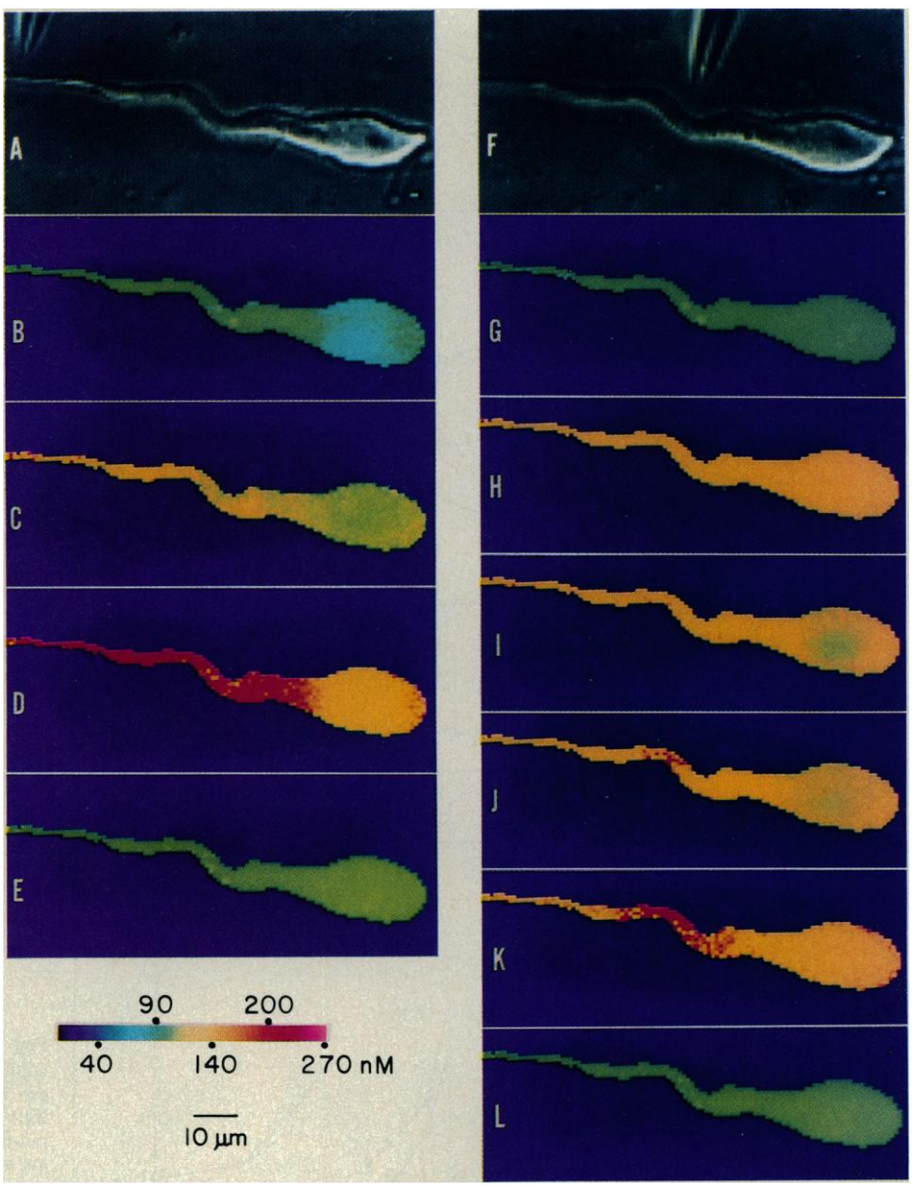
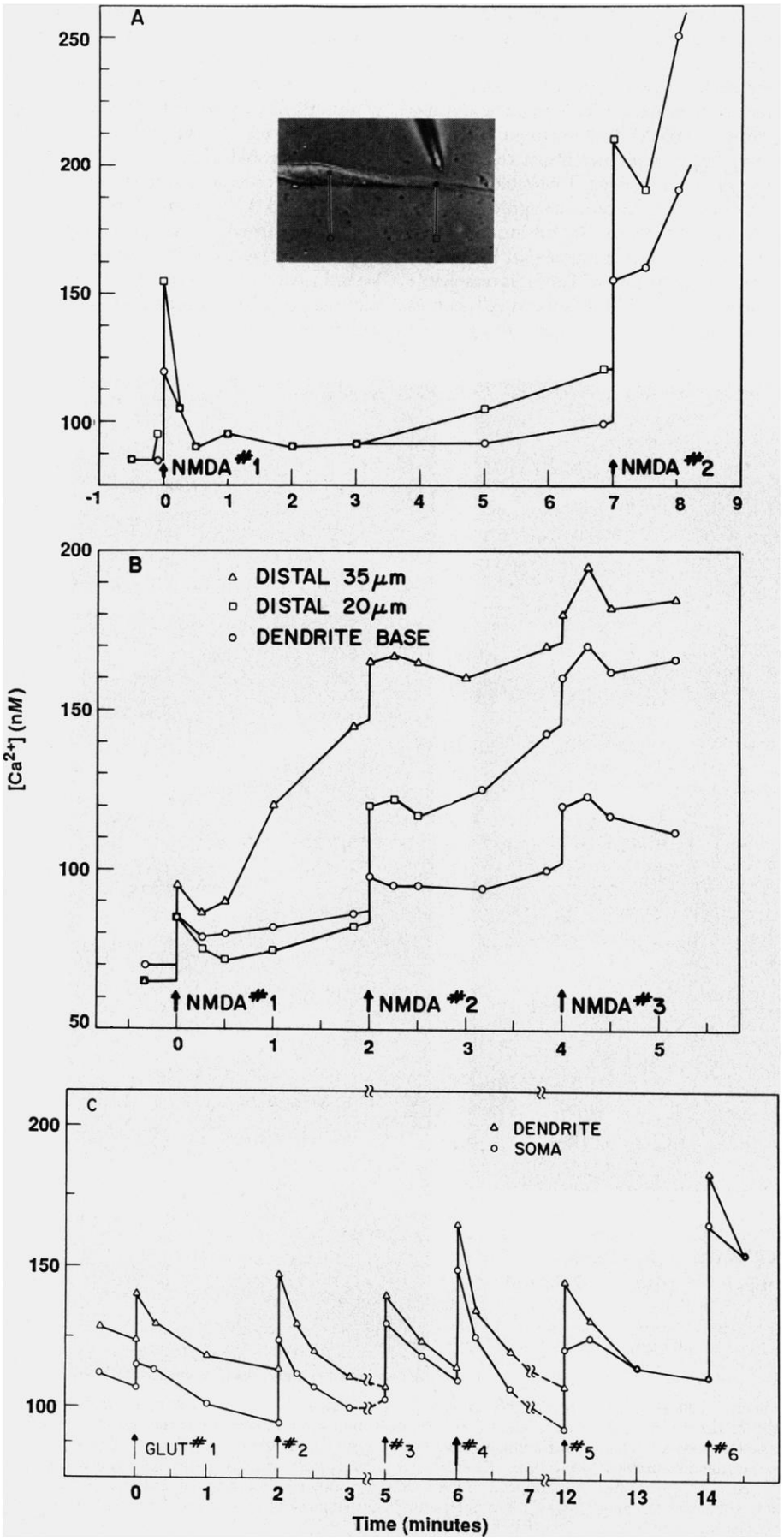


Fig. 1. (**A**) CA1 neuron and microelectrode, at the left of the field, positioned for iontophoretic application of glutamate to the dendrite. (**B**) Resting $[Ca^{2+}]$ in the cell before placement of the electrode. (**C**) $[Ca^{2+}]$ in the cell after placement of the glutamate-containing microelectrode. Small leak of glutamate produced a local increase in $[Ca^{2+}]$. (**D**) $[Ca^{2+}]$ at the end of a 1-second iontophoretic application of glutamate (200 nA). Large $[Ca^{2+}]$ increase in the dendrite (to 230 n*M*) and smaller perturbation in the soma, resulted in a substantial intracellular Ca^{2+} gradient (~100 n*M*). The electrode was immediately removed from the vicinity of the neuron. (**E**) Ratio image 1 minute after stimulus. (**F**) Iontophoresis electrode at second stimulus location. (**G**) $[Ca^{2+}]$ distribution 3 minutes after the measurement of (E) and before placement of the electrode in (F). (**H**) $[Ca^{2+}]$ just after second stimulus. Smaller response reflects perhaps less glutamate reaching the dendrite, differences in receptor density, or faster dispersion of Ca^{2+} in this thicker section. (**I**) $[Ca^{2+}]$ 15 seconds after the stimulus and removal of electrode. (**J**) $[Ca^{2+}]$ distribution 1.5 minutes after the stimulus. $[Ca^{2+}]$ is beginning to show secondary rise in the vicinity of the second stimulus location. (**K**) $[Ca^{2+}]$ 3 minutes after stimulus. Secondary rise is now well developed, and the dendrite shows a bidirectional gradient of Ca^{2+}. (**L**) $[Ca^{2+}]$ 2 minutes after beginning a slow wash in Ca^{2+}-free saline containing 2 m*M* EGTA.



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7. Cells were isolated by the methods of A. Kay and R. Wong [*J. Neurosci. Methods* **16**, 227 (1986)]. After tituration in Dulbecco's minimum essential medium containing 4 μ*M* fura-2 acetoxymethyl ester (Molecular Probes), they settled onto the bottom of a temperature-controlled (30° to 32°C) measuring chamber. Loading, trapping, and deesterification of the indicator were completed within 20 minutes, as judged by stable fluorescence ratios and the manipulation of $[Ca^{2+}]$ with the Ca^{2+} iontophore ionomycin. After this period, perfusion with Hepes-buffered saline was started. A number of neurons were injected with the free acid form of fura-2 as a control for internal compartmentalization. Indicator concentrations were between 100 and 300 μ*M*, estimated as described elsewhere [see J. A. Connor *et al.*, *J. Neurosci.* **7**, 1384 (1987)]. Excitatory amino acids were applied by iontophoresis from microelectrodes (tip diameter ~0.5 μm) containing 10 m*M* agonist in solution, *p*H 7.0; high K^+ was applied by pressure ejection from micropipettes (tip diameter 2 to 4 μm) containing buffered saline with 50 m*M* KCl. The ratio imaging apparatus has been described elsewhere (*8*). Free calcium concentration was calcu-

Fig. 2. (**A**) Ca^{2+} changes resulting from NMDA application. Inset shows positions of the iontophoresis electrode and the measurement sites. The first stimulus, applied as a 2-second, 200-nA pulse, produced a short-lived gradient. The second stimulus, applied as three 1-second pulses in rapid succession, produced a large, long-lasting response. (**B**) $[Ca^{2+}]$ in a second neuron given three identical stimuli (1 second, 200 nA). A sustained Ca^{2+} gradient developed after partial recovery from the first stimulus. (**C**) Effects of multiple large stimulations with glutamate in a third neuron treated with sphingosine. Stimuli 2 through 6 were delivered as three 1-second pulses instead of as a single pulse.

lated from the formula:

$$[Ca^{2+}] = K_D(F_0/F_s)(R - R_{min})/(R_{max} - R)$$

where the dissociation constant $K_D = 225 \times 10^{-9} M$, $F_0/F_s = 7.5$, $R_{min} = 0.47$, $R_{max} = 11.7$, and R is the ratio of the measured fluorescence excited by 340- and 380-nm light. Numerical values reported are an average of at least 32 adjacent pixels. Exposure time for each picture was 250 or 500 msec. Cells were exposed to ultraviolet light only during collection periods, less than 30 seconds for any one cell. Bleaching was less than 2%. Data acquisition for a 140 × 240 pixel image required approximately 1.5 seconds and was always begun at the end of a stimulus pulse. Because of the long acquisition time, we underestimate the peak amplitude of the Ca^{2+} changes resulting from the application of agonists.

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10. In a separate, control series of experiments, 19 cell preparations from six animals were observed for periods of 1 to 1.5 hours after dissociation. The 96 neurons in this group had a mean unstimulated $[Ca^{2+}]$ of 80 n*M*. Of these neurons, 70% held their $[Ca^{2+}]$ constant to within 15% during 30 to 40 minutes of observations by microscopy. The rest underwent much larger increases during the observation period, but fewer than 5% of the neurons showed rapid, spontaneous Ca^{2+} increases.
11. In experiments without TTX, the initial application of agonist nearly always caused a runaway response; the cells went to conditions where internal $[Ca^{2+}]$ exceeded 300 n*M* in a period of several minutes. The magnitude of the primary Ca^{2+} change was also systematically larger than the responses shown in Fig. 1. This instability is not surprising given that the isolated cells have been stripped of all inhibitory inputs. Bath application of GABA greatly dampened the responses to glutamate.
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13. High K^+ saline was applied with the center of the flow pattern on the soma. Experiments were designed to maximize the dendrite-soma gradients of Ca^{2+} by making the influx period brief. That is, even if one assumes a uniform influx of Ca^{2+} in the soma and dendrite, one expects a larger transient change in the dendrite than in the soma from surface to volume considerations. This difference decreased with longer stimuli as the cytoplasmic buffers saturated in the soma. Where the high K^+ stimulus was applied for more than 10 seconds, the dendritic soma Ca^{2+} difference was never more than 20 to 30 n*M* at a high mean level.
14. Measured gradients of Ca^{2+} have been used to compute the associated flux down the dendrite given that there are no longitudinal barriers. The three fluxes of importance, free Ca (Ca^{2+}), Ca bound to indicator (CaI), and Ca bound to mobile cell buffers (CaB), were computed from the integrated form of Fick's law using the following parameters: the diffusion coefficient $D_{Ca} = 6 \times 10^{-6}$ cm^2/sec; mean dendrite diameter = 4 μ*m*; fura-2 concentration = 300 μ*M*; $K_D = 225$ n*M*; diffusion coefficient of CaI = 2×10^{-6}; intrinsic buffer concentration = 250 μ*M* with $K_D = 1$ μ*M*; Ca^{2+} gradient of 250 to 150 n*M* over 20-μm distance. The three components sum to approximately 1 pA, and from this we make two observations. First, this flux could be supplied by a small number of channels. Second, it is unlikely that internal stores could supply the Ca^{2+} required to maintain the gradient. A current of 1 pA over a 5-minute period involves the movement of 1.5×10^{-15} mol of $[Ca^{2+}]$ from the distal 10 to 15 μm of the dendrite in our preparations (~50 fl volume). Even if all of this volume were internal store, Ca^{2+} would have to be 30 m*M*, an enormous level, in order to support the gradient. Furthermore, this store would be inaccessible to fura-2 since the indicator reports submicromolar $[Ca^{2+}]$ in unstimulated dendrites.
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Highly Cooperative Opening of Calcium Channels by Inositol 1,4,5-Trisphosphate

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The kinetics of calcium release by inositol 1,4,5-trisphosphate (IP_3) in permeabilized rat basophilic leukemia cells were studied to obtain insight into the molecular mechanism of action of this intracellular messenger of the phosphoinositide cascade. Calcium release from intracellular storage sites was monitored with fura-2, a fluorescent indicator. The dependence of the rate of calcium release on the concentration of added IP_3 in the 4 to 40 n*M* range showed that channel opening requires the binding of at least three molecules of IP_3. Channel opening occurred in the absence of added adenosine triphosphate, indicating that IP_3 acts directly on the channel or on a protein that gates it. The channels were opened by IP_3 in less than 4 seconds. The highly cooperative opening of calcium channels by nanomolar concentrations of IP_3 enables cells to detect and amplify very small changes in the concentration of this messenger in response to hormonal, sensory, and growth control stimuli.

THE PHOSPHOINOSITIDE CASCADE plays a central role in the transduction of many hormonal, sensory, and growth control stimuli (*1*). Hydrolysis of phosphatidylinositol 4,5-bisphosphate by a receptor-triggered phospholipase C generates two intracellular messengers, 1,4,5-trisphosphate (IP_3) and diacylglycerol. IP_3 raises the cytosolic level of calcium ion by releasing it from the endoplasmic reticulum (*2*). High-affinity binding sites for IP_3 have been detected in permeabilized cells (*3*) and microsomal membrane preparations (*4*). The effectiveness of IP_3 in releasing Ca^{2+} in the absence of adenosine triphosphate (ATP) or other high-potential phosphoryl donors has suggested that IP_3 directly activates a Ca^{2+}-selective channel (*5*).

We have investigated the kinetics of IP_3-induced Ca^{2+} release from intracellular storage pools in permeabilized rat basophilic leukemia (RBL) cells (*6*) to gain insight into the molecular mechanism of action of IP_3. The antigen-mediated cross-linking of immunoglobulin E (IgE)–receptor complexes on the surface of RBL cells leads to the formation of IP_3 (*7*). The consequent rise in the cytosolic Ca^{2+} (*8*) contributes to the exocytic release of histamine and other mediators. RBL cells from the 2H3 subline (*9*) were harvested (*10*), washed by centrifugation, and resuspended in a buffered salt solution without added divalent cations (135 m*M* NaCl, 5 m*M* KCl, and 20 m*M* Hepes, *p*H 7.4). After three washes, 2×10^6 cells per milliliter were resuspended in an ice-cold, high ionic strength KCl buffer (140 m*M* KCl, and 30 m*M* Hepes, *p*H 7.4) that contained 1.5 μ*M* fura-2 (Molecular Probes), a fluorescent Ca^{2+} indicator (*11*). Buffers containing fura-2 and all inositides were passed through a chelating column (diphenylenediaminepentacetic acid coupled to ω-aminobutyl agarose) to lower the total Ca^{2+} concentration and that of heavy metal ions to less than 100 n*M*. The plasma membrane of the RBL cells was permeabilized by incubating them in KCl

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