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21. Three injections were made into the upper stem of each plant with a suspension of strain SS-4 (0.1 optical density or approximately 10^7 viable conidia per milliliter).
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23. Sample sizes for the second replicate were 26 plants exposed to mites, 21 controls; for the third replicate, 15 exposed, 16 controls; and for the fourth replicate, 7 exposed, 21 controls.
24. *Tetranychus turkestani* females were used to damage the cotyledons because they are more destructive than *T. urticae* [J. N. Simons, *J. Econ. Entomol.* **57**, 145 (1964)]. *Tetranychus urticae* females were used for the bioassay of the effect of wilt because they are more sensitive than *T. turkestani*. It was not possible to remove the fungus once seedlings had been inoculated; hence, this procedure does not distinguish competition from resistance against mites induced by the fungus.
25. Controls were either not injected or received three injections of distilled water into the upper stem. No differences in growth were found between these two types of controls.
26. At 28°C, *T. urticae* complete a full generation in 12 days; thus 14 days allowed at least one generation [J. R. Carey and J. W. Bradley, *Acarologia* **23**, 333 (1982)].
27. We measured stem height from the soil to the top of the plant, stem height above the cotyledon node, number of expanded true leaves, area of the true leaves, and mean internode length.
28. Mite treatment and wilting were not independent in a three-way test: $G = 13.636$, df = 1, $P < 0.005$. The frequency of wilt was not independent of replicate ($G = 44.784$, df = 3, $P < 0.005$) although the interaction between replicates, mite treatment, and wilt was not significant ($G = 6.842$, df = 3, $P > 0.05$). Even under controlled conditions, with cotton of the same variety, mites from the same laboratory cultures, and fungi of the same strain, incidence of mites and wilt varied greatly between replicates in response to subtle environmental changes [see (*22*)].
29. Mite treatment and number of plants with symptoms of chlorosis and necrosis were not independent in a three-way test: $G = 27.022$, df = 3, $P < 0.005$. Leaf symptoms were not independent of replicate but the interaction between replicates, percentage of leaf tissue with symptoms, and mite treatment was not significant.
30. For the first replicate: $t = 3.59$, df = 37, $P < 0.01$; for the second replicate: $t = 1.97$, df = 37, $P < 0.06$. One plant that had been inoculated with fungus in the first replicate and one control plant in the second replicate had no mites and were excluded from the analyses.
31. Mean stem height in centimeters above soil ± 1 SE for controls was 16.75 ± 0.48, for plants with fungus was 12.48 ± 0.44, $t = 6.52$, df = 38, $P < 0.001$. Height in centimeters above cotyledons for controls was 7.70 ± 0.46, for plants with fungus was 4.53 ± 0.38, $t = 5.35$, df = 38, $P < 0.001$. Mean internode length in centimeters for controls was 1.76 ± 0.11, for plants with fungus was 1.45 ± 0.50, $t = 0.61$, df = 38, not significant (NS). Number of leaves for controls was 4.45 ± 0.13, for plants with fungus was 4.50 ± 0.15, $t = 0.25$, df = 38, NS. Leaf area in square millimeters for controls was 936.85 ± 34.21, for plants with fungus was 681.80 ± 56.49, $t = 3.86$, df = 38, $P < 0.001$.
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Development of Two Types of Calcium Channels in Cultured Mammalian Hippocampal Neurons

YOEL YAARI,* BRIGITTE HAMON, HANS D. LUX

Calcium influx through voltage-gated membrane channels plays a crucial role in a variety of neuronal processes, including long-term potentiation and epileptogenesis in the mammalian cortex. Recent studies indicate that calcium channels in some cell types are heterogeneous. This heterogeneity has now been shown for calcium channels in mammalian cortical neurons. When dissociated embryonic hippocampal neurons from rat were grown in culture they first had only low voltage–activated, fully inactivating somatic calcium channels. These channels were metabolically stable and conducted calcium better than barium. Appearing later in conjunction with neurite outgrowth and eventually predominating in the dendrites, were high voltage–activated, slowly inactivating calcium channels. These were metabolically labile and more selective to barium than to calcium. Both types of calcium currents were reduced by classical calcium channel antagonists, but the low voltage–activated channels were more strongly blocked by the anticonvulsant drug phenytoin. These findings demonstrate the development and coexistence of two distinct types of calcium channels in mammalian cortical neurons.

THE ENTRY OF CALCIUM THROUGH voltage-gated membrane channels is essential for many neuronal functions (*1*). In the mammalian brain Ca^{2+} entry participates in the generation of various forms of electrical activity, such as dendritic spikes (*2*), rhythmic firing (*3*), normal and epileptiform burst discharges (*4*), as well as in the secretion of neurotransmitters and neuromodulators (*5*). The influx of Ca^{2+} may also couple neuronal activity to metabolic processes and induce long-term changes in neuronal and synaptic activity (*6*). Studies in brainstem slices (*3*) and cultured sensory neurons (*7*) have demonstrated the coexistence of two types of Ca^{2+} channels in some mammalian neurons. We have employed patch-clamp techniques (*8*) and cultured rat hippocampal neurons (*9*) to investigate whether the Ca^{2+} channels in differentiated neurons from a mammalian cortical structure are also heterogeneous (*10*), and to characterize the development and distribution of these channels during neuronal growth.

Hippocampal neurons were dissociated from 18- to 19-day-old rat embryos and maintained in culture for 4 to 6 weeks (*11*). Whole-cell membrane currents were recorded from the somatic region of the neurons (*12*). Calcium currents (I_{Ca}'s) were isolated from other voltage-dependent membrane currents by ionic substitution and addition of sodium and potassium channel blockers

Department of Neurophysiology, Max Planck Institute for Psychiatry, Am Klopferspitz 18A, 8033 Planegg-Martinsried, Federal Republic of Germany.

*To whom correspondence should be addressed at Department of Physiology, Hebrew University–Hadassah School of Medicine, P.O. Box 1172, Jerusalem 91010, Israel.

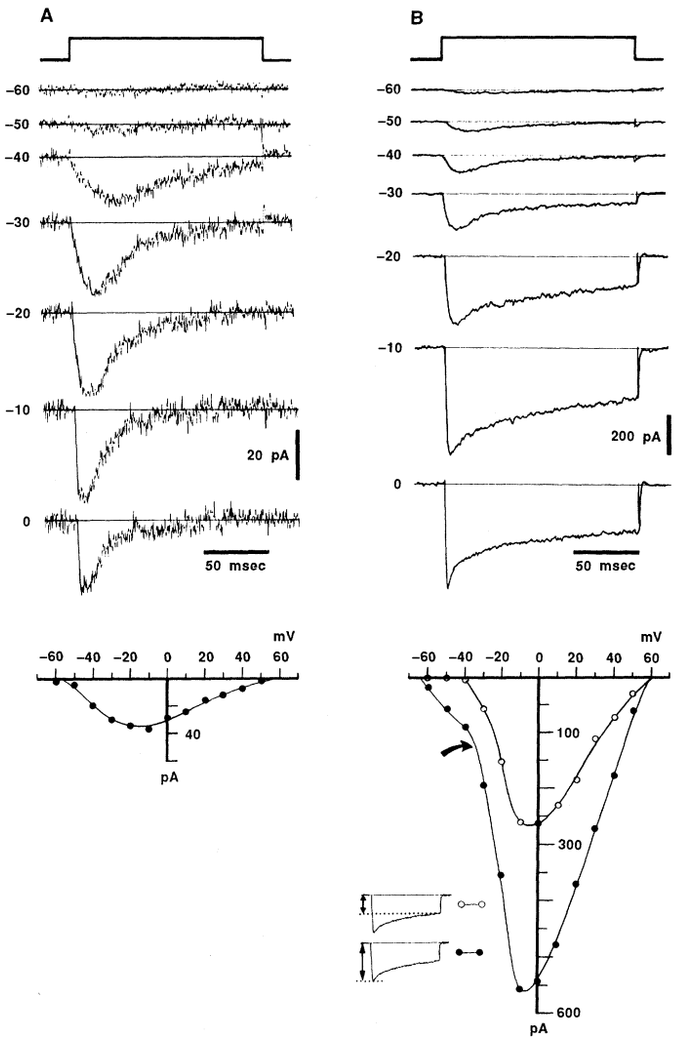

Fig. 1. Calcium currents in dissociated rat hippocampal neurons at two stages of development in culture. (**A**) I_{Ca}'s from a freshly plated cell (5 hours after plating). The cell was spherical in shape (diameter ~8 μm) and had one short (3 to 4 μm) neurite. (Top) Current responses evoked by stepping from a −90-mV holding potential to various membrane potentials (indicated on the left of each trace) for 150 msec. (Bottom) Current-voltage relation depicting the peak current intensity at each step potential. Similar observations were made in all cells (n = 27) examined in fresh cultures (3 to 5 hours after plating). (**B**) I_{Ca}'s from a neuron 2 days in culture. This neuron had several branching neurites but seems to be spatially clamped, as judged by the absence of "notches" and long "tail" currents (see Fig. 3B). (Top) Same as (A). Note the sustained current component activated positive to −30 mV. (Bottom) Current-voltage relation of peak (closed circles) and sustained I_{Ca}'s (open circles). The sustained I_{Ca} was measured at the end of a 150-msec pulse, as indicated in the inset, at which time the LVA component is presumably fully inactivated. The deflection indicated by the arrow marks the threshold membrane potential for activation of HVA I_{Ca} component. Similar current-voltage relations were observed in 32 additional neurons in 1- to 3-day-old cultures.

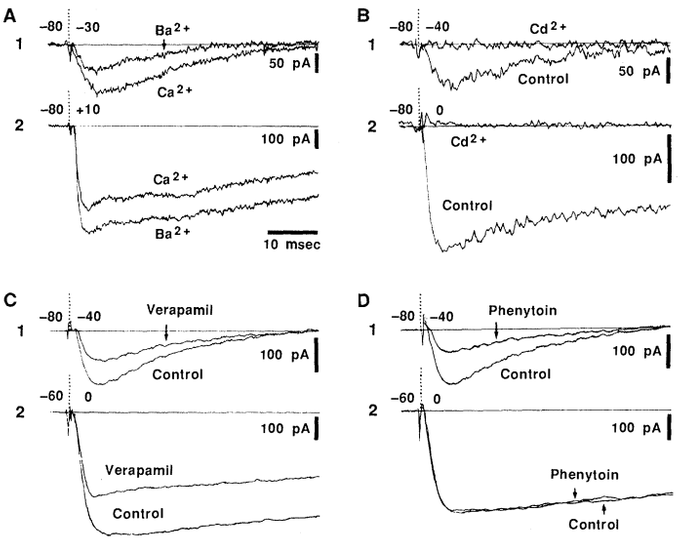

Fig. 2. Ionic selectivities and pharmacological sensitivities of two types of I_{Ca}'s in cultured rat hippocampal neurons. Depolarizing pulses were delivered at one per 15 seconds. Holding and step potentials are indicated to the left and to the right of the dashed line, respectively. Records in test solutions were taken approximately 30 seconds after solution exchange. (**A**) Differential effects of Ba^{2+} on (1) LVA and (2) HVA I_{Ca}'s. Calcium in the superfusion solution was replaced by an equimolar concentration (10 mM) of Ba^{2+}. Similar effects of Ba^{2+} were observed in all six neurons examined in this way. (**B**) Suppression of (1) LVA and (2) HVA I_{Ca}'s by 100 μM cadmium. In this neuron and another three neurons similarly examined, both LVA and HVA I_{Ca}'s were completely blocked. In another five neurons, although HVA I_{Ca} was completely suppressed by 100 μM cadmium, LVA I_{Ca}'s were reduced to 10 to 20% of the control values. (**C**) Partial suppression of both (1) LVA and (2) HVA I_{Ca}'s by 100 μM verapamil. Similar effects were observed in all eight neurons examined. (**D**) Differential effects of 100 μM phenytoin on (1) LVA and (2) HVA I_{Ca}'s. Similar differential effects were evident in all ten neurons tested with phenytoin.

to the external and internal solutions, respectively (*13*). They were further identified by their sensitivity to cadmium, a potent Ca^{2+} channel antagonist (*14*).

Stable recordings from freshly plated cells could be obtained as soon as the cells attached to the polylysine substrate (3 to 5 hours after plating). Although the cells are morphologically undifferentiated at this stage, most of them are probably neurons because the presence of glia in fresh cultures is minimal (*9*). Whole-cell I_{Ca}'s that were typical for these cells were elicited by step depolarizations from a holding membrane potential of −90 mV (Fig. 1A). They were activated at relatively low membrane potentials (−50 to −40 mV) and their current-voltage relation was smooth, attaining a maximum between −20 to −10 mV (Fig. 1A). The activation time course of these low voltage–activated (LVA) I_{Ca}'s was sigmoidal, voltage-dependent, and was faster at more positive membrane potentials (Fig. 1A). The LVA I_{Ca}'s fully inactivated during a maintained voltage step. Inactivation was similarly accelerated by more positive membrane potentials (Fig. 1A). The LVA I_{Ca}'s also exhibited a steady-state inactivation and could not be evoked at holding potentials more positive than −50 mV.

After attaching to the substrate, most cells rapidly acquired neurite extensions, which became larger and more intricate during the first week in culture. This process was associated with the appearance and gradual increase of a high voltage–activated (HVA) (between −30 to −20 mV) I_{Ca} component (Fig. 1B). After 24 to 48 hours in culture, HVA I_{Ca}'s were usually larger than their LVA counterparts. Most conspicuously, the HVA I_{Ca}'s only partially inactivated during maintained depolarizing pulses (Fig. 1B). Consequently, they could be activated from more positive holding potentials (for example, −50 mV) at which LVA I_{Ca}'s were fully inactivated.

To test the hypothesis that LVA and HVA I_{Ca}'s represent activation of two distinct populations of Ca^{2+} channels (termed LVA and HVA channels, respectively), we have compared several attributes of the two types of I_{Ca}'s.

We first tested the channels' selectivity to permeating divalent cations by replacing extracellular Ca^{2+} with an equimolar concentration of barium. This treatment markedly reduced LVA currents (Fig. 2A1), suggesting that LVA channels are less permeable to Ba^{2+}. In contrast, HVA currents were enhanced in this condition (Fig. 2A2).

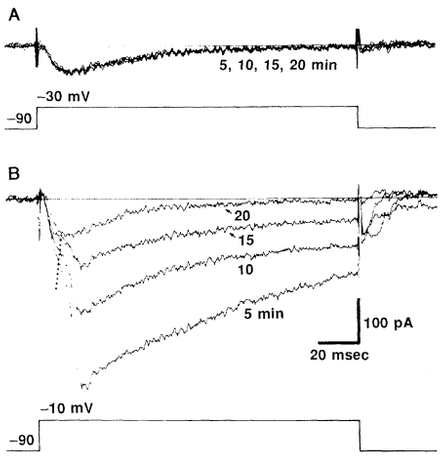

Fig. 3. Time-dependent run down of high voltage–activated calcium currents. Recordings were made in a pyramidal-like neuron possessing two main branching dendrites in a 2-week-old culture. LVA and HVA I_{Ca}'s were evoked by alternatively stepping from a −90-mV holding potential to −30 mV [(**A**) LVA only] and to −10 mV [(**B**) LVA and HVA]. Stimuli were delivered at intervals of 20 seconds. Superimposed traces are taken at various times (approximately 5, 10, 15, and 20 minutes), as indicated, after establishing the whole-cell recording. Note in (B) "notches" (marked by the dashed line) and long "tail" currents indicative of inadequate space-clamp control of the HVA I_{Ca}, which persists even when the HVA I_{Ca} runs down to 20% of its original amplitude (at 15 minutes).

Presumably Ba^{2+} passes through these HVA channels more easily than does Ca^{2+} (*1, 15*).

Interaction with Ca^{2+} channel antagonists was also examined. Both types of I_{Ca}'s were strongly suppressed by 100 μ*M* cadmium (Fig. 2B). The organic Ca^{2+} channel blocker verapamil reduced LVA and HVA I_{Ca}'s to a similar degree at 100 μ*M* (Fig. 2C). However, the anticonvulsant drug phenytoin, at a similar dose, preferentially blocked LVA I_{Ca}'s (Fig. 2D). The effects of all three agents on I_{Ca}'s were readily reversible upon washing.

Finally we examined the time-dependent "run down" of HVA I_{Ca}'s. Even without frequent stimulation, HVA I_{Ca}'s gradually declined in amplitude and largely disappeared within 15 to 20 minutes after establishing the whole-cell clamp (Fig. 3B). Presumably cytoplasmic components essential for the metabolic maintenance of HVA channels are washed out during perfusion of the cell interior by the microelectrode solution (*16*). In contrast, LVA I_{Ca}'s did not run down (Fig. 3A).

From these results we conclude that LVA and HVA I_{Ca}'s are conducted by two separate populations of Ca^{2+} channels. The question arises whether the differential development of the two channel types in time with respect to neurite outgrowth is also expressed in their somatodendritic distribution. When evoking HVA I_{Ca}'s in neurons with extensive dendritic arborizations, we commonly observed "notch" currents during the depolarizing voltage step, and large and prolonged "tail" currents thereafter (Fig. 3B), indicative of inadequate space clamp control (*17*). In the same neurons, LVA I_{Ca}'s were adequately clamped (Fig. 3A). These data are consistent with a predominant somatic distribution of LVA channels and a more profuse dendritic distribution of HVA channels. However, a considerable overlap of the two distributions cannot be excluded. Indeed, comparable LVA and HVA Ca^{2+} channels coexist in somata of cultured mammalian (*7, 18*) and avian (*18, 19*) sensory neurons, where LVA channels are observed in isolation in embryonic stages before there is significant neurite outgrowth (*20*).

Our findings show that two distinct types of Ca^{2+} channels develop in hippocampal neurons during growth and differentiation in culture. We have also confirmed their presence in well-differentiated hippocampal neurons (4 to 6 weeks in culture) of the pyramidal-like, stellate-like, and morphologically atypical classes. This suggests that LVA and HVA Ca^{2+} channels may play a functional role in several types of mammalian cortical neurons. LVA channels may be involved in near-threshold membrane phenomena. For example, they may speed up depolarization to threshold after neuronal hyperpolarization (*3*). The blockade of these Ca^{2+} channels by the anticonvulsant drug phenytoin is thus consistent with its depressant action on repetitive neuronal firing and may contribute thereby to its efficacy in suppressing seizure discharge (*21*). In contrast, HVA channels would be activated during the generation of action potentials. Because they are widely distributed and slowly inactivating, this may cause the intracellular Ca^{2+} concentration to rise substantially (*22*), thereby coupling neuronal discharge with various membrane (*3, 23*) and metabolic events (*6*).

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11. Hippocampal cultures were prepared by conventional techniques (*9*). The tissue from 10 to 14 dissected hippocampi was mechanically dissociated in incubation medium by trituration through siliconized Pasteur pipettes with narrowed orifices. The cells were plated at a density of 6×10^5 cells per dish on polylysine-coated culture plates (32-mm diameter), each containing 2 ml of incubation medium (Eagle's basal medium enriched with 2 m*M* glutamine, 10% glucose, and 10% horse serum). The plates were kept in a humified incubator at 37°C. Medium was replaced twice a week. Glia proliferation was suppressed by adding β-cytosine arabinoside (10^{-6}*M*) on the fourth day for 48 hours.
12. Whole-cell recordings were made with low resistance (3 to 5 Mohm) patch microelectrodes. Capacitative currents were reduced by an analog circuit. Signals were stored on a frequency modulated tape recorder at 5 kHz and digitized off line at 7 to 12 kHz by a 12-bit, analog-to-digital converter and analyzed by an LSI 11/23 minicomputer. Leakage and residual capacitative currents were minimized by subtracting appropriately scaled current responses to 30 to 40 mV hyperpolarizing pulses from the holding potential.
13. The standard composition of the extracellular solution was 120 m*M* choline chloride, 10 m*M* $CaCl_2$, 2 m*M* $MgCl_2$, 10 m*M* Hepes, and 3×10^{-5}*M* tetrodotoxin. For testing of different solutions, a multibarreled superfusion pipette [W. Boll and H. D. Lux, *Neurosci. Lett.* **56**, 335 (1985)] was positioned about 50 to 100 μm from the cell. During the recording period the cell was superfused continuously with either the standard or test solution. Microelectrodes were filled with a solution composed of 130 m*M* CsCl, 20 m*M* tetraethylammonium chloride, 2 m*M* $MgCl_2$, 10 m*M* EGTA, and 10 m*M* Hepes. All solutions were adjusted to *p*H 7.3 (with CsOH) and an osmolarity of 300 mos*M* (with glucose). Experiments were performed at room temperature.
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