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Role of Calcium Channel Subtypes in Calcium Transients in Hippocampal CA3 Neurons

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Multiple subtypes of voltage-gated calcium channels are differentially localized in brain neurons suggesting that they serve distinct roles in neuronal excitation and signaling. In organotypic hippocampal slice cultures, class D (L-type) calcium channels are predominantly located in the cell bodies of CA3 neurons while class B (N-type) and class A (P or Q-type) are localized in dendrites and associated presynaptic terminals with relatively low somal expression. Using specific antagonists to inhibit calcium transients recorded in CA3 neuronal cell bodies, we found that L-type calcium channels have a predominant role in somal calcium transients elicited by trains of strong stimuli applied to either the soma or the distal apical dendrite while class A calcium channels make a smaller contribution. Presynaptic class B (N-type) and class A (P- and/or Q-type) calcium channels are critical for glutamate-mediated synaptic transmission onto the dendrites of CA3 neurons. Postsynaptic class A and B calcium channels detected on the dendritic shaft by immunocytochemistry were not found to contribute substantially to somal calcium transients during repetitive stimulation of distal dendrites, but sodium channels were required for calcium transients elicited by somatic or dendritic stimulation. Our results show that the different calcium channel subtypes serve distinct roles in cellular activation and transmission of signals in CA3 neurons, consistent with their differential subcellular localization.

Calcium ions entering neurons through voltage-gated calcium channels initiate the physiological responses induced by action potentials. These include generation of calcium-dependent action potentials and spatial and temporal summation of multiple synaptic inputs in dendrites, activation of intracellular processes such as enzyme regulation and gene transcription in cell bodies, and release of neurotransmitters from nerve terminals. How are the voltage-gated calcium channels organized and regulated to carry out their roles in these diverse physiological functions?

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Voltage-gated calcium channels have been classified into at least five types (L, N, P, Q, and T) based on electrophysiological and pharmacological criteria (Bean, 1989; Llinas et al., 1989; Hess, 1990; Hillman et al., 1991; Hillyard et al., 1992; Zhang et al., 1993; Randall et al., 1995). L-, N-, P-, and Q-type channels are all high-voltage-activated requiring a strong depolarization for activation, while T-type calcium channels are low-voltage-activated. L-Type channels mediate large single channel currents with slow rates of voltage-dependent inactivation and are specifically inhibited by the dihydropyridines. N-Type channels conduct single channel currents of intermediate size with a broad range of inactivation rates and are irreversibly inhibited by ω-CTx-GVIA. P-Type channels also conduct single channel currents of intermediate size but have slow inactivation rates and are preferentially inhibited by low concentrations of ω-Aga-IVA (Mintz et al., 1992a; Usowicz et al., 1992). Finally, Q-type channels conduct intermediate single channel currents, have faster inactivation rates than P-type, and are preferentially inhibited by low concentrations of ω-CTx-MVIIC (Sather et al., 1993; Randall et al., 1995).

Calcium channels from skeletal muscle which conduct L-type currents are composed of five distinct subunits: α1, α2, β, γ, and δ (Campbell et al., 1988; Catterall et al., 1988), and neuronal L-type and N-type calcium channels have α1, α2, β, and δ subunits (Ahlijanian et al., 1990; McEnery et al., 1991; Witcher et al., 1993; Leveque et al., 1994). The α1 subunit of L-type calcium channels from skeletal and cardiac muscle can form a functional channel indicating that it contains the calcium-selective pore, gating mechanisms, and sites for the pharmacological antagonists (Mikami et al., 1989; Perez-Reyes et al., 1989). In rat brain, several classes of calcium channel α1 subunits have been defined by cDNA cloning and sequencing in neurons and are designated A, B, C, D, and E (Snutch et al., 1990; Snutch and Reiner, 1992; Soong et al., 1993; Zhang et al., 1993). The class C and D α1 subunits both conduct L-type calcium currents (Snutch et al., 1990; Hui et al., 1991; Williams et al., 1992a; Tomlinson et al., 1993), while the class B α1 subunit forms N-type calcium channels (Dubel et al., 1992; Williams et al., 1992b; Fujita et al., 1993). When expressed in *Xenopus* oocytes, the class A α1 ($\alpha 1_A$)subunit forms calcium channels that are blocked by ω-CTx MVIIC and by high concentrations of ω-Aga-IVA, and resemble Q-type (Sather et al., 1993). However, alternatively spliced forms of $\alpha 1_A$ can form calcium channels which inactivate rapidly like Q-type or slowly like P-type channels (Stea et al., 1994), and $\alpha 1_A$ is localized at sites in the cerebellum which contain mainly P-type calcium currents (e.g., Purkinje cell bodies) and at sites which contain mainly Q-type currents (e.g., granule cells and their nerve terminals) (preceding article, Wes-

tenbroek et al., 1995). These results raise the possibility that the class A $\alpha 1$ subunit is a component of calcium channels conducting both the closely related P-type and Q-type calcium currents.

Site-directed anti-peptide antibodies recognizing the different $\alpha 1$ subunits have been used to investigate the cellular and subcellular distribution of the calcium channels in rat brain. In CA3 hippocampal pyramidal cells, L-type channels containing $\alpha 1_C$ and $\alpha 1_D$ are predominantly located in the cell body and proximal dendrites (Ahlijanian et al., 1990; Westenbroek et al., 1990; Hell et al., 1993) whereas the N-type calcium channels containing $\alpha 1_B$ and the P/Q-type channels containing $\alpha 1_A$ are predominantly found along the length of the apical dendrites and in nerve terminals forming synapses upon them (Westenbroek et al., 1992, 1995). This differential localization of calcium channels implies that each channel class may contribute differentially to intracellular calcium transients and therefore to initiation of distinct physiological events, but the relationship between channel localization and physiological role has not been established. In the experiments described here, we have examined the distinct roles of calcium channel $\alpha 1$ subunits located in cell bodies, dendrites, and presynaptic terminals in the generation of calcium transients in hippocampal CA3 neurons in response to somal and dendritic stimulation using organic and peptide antagonists to block calcium channel function.

Materials and Methods

Hippocampal slice cultures. Hippocampal slice cultures were prepared from 4 d old Sprague–Dawley rats by the roller-tube method of Gähwiler (Gähwiler, 1981; Malouf et al., 1990). The slices were utilized after 10–18 d *in vitro* at which point the slices had flattened from 400 μm to 80–100 μm so that individual neurons could be viewed with phase-contrast microscopy.

Immunocytochemistry. Hippocampal slice cultures were fixed with a solution of 4% paraformaldehyde and 0.2% glutaraldehyde in 0.1 M sodium phosphate buffer (PB; pH 7.4) for 2–3 hr at 4°C. The slices were then rinsed with 10% sucrose in PB and incubated overnight at 4°C in 30% sucrose in PB. Sections (40 μm) were cut on a sliding microtome. Free-floating sections were processed for immunocytochemistry with the ABC technique at room temperature unless otherwise noted.

On day 1, tissue was sequentially incubated in: (1) 0.1 M Tris-HCl (TB, pH 7.4) for 15 min, (2) 1% sodium borohydride in TB for 30–60 min, (3) 0.1 M Tris-HCl saline (TBS, pH 7.4) for 40 min, (4) avidin blocking solution (Vector, CA) for 20 min, (5) TBS for 10 min, (6) biotin blocking solution (Vector, CA) for 20 min, (7) TBS for 25 min, (8) a solution of 1–10% normal goat serum (NGS), 0.05–0.5% Triton X-100, and 0.1% bovine serum albumin (BSA, optional) in 0.05 M TBS for 2 hr, (9) TBS for 25 min, and (10) a solution of 1–10% NGS, 0.05–0.5% Triton X-100, 0.1% BSA (optional), and affinity purified calcium channel antibody (CNA1 diluted at 1:15, CNB2 diluted at 1:30, or CND1 diluted at 1:25; Westenbroek et al., 1990; 1992, 1995) in 0.05 M TBS for 72 hr at 4°C.

On day 4, tissue exposed to the CNB2 and CND1 antibodies were sequentially incubated in: (1) TBS for 25 min, (2) a solution of 1–10% NGS, 0.05-0.5% Triton X-100, 0.1% BSA (optional), and biotinylated goat anti-rabbit IgG (BGAR) diluted at 1:300 in 0.05 M TBS for 2 hr, (3) TBS for 25 min, (4) ABC reagent (Vector, CA) in 0.05 M TBS for 3 hr, (5) TBS for 15 min, (6) TB for 10 min, (7) a solution of 0.005% nickel and 0.05% 3,3′-diaminobenzidine (DAB) in TB for 10 min, (8) the above solution with 0.003% H_2O_2 for 10 min, and (9) TB for 1 hr. The tissue was mounted on subbed glass slides, dehydrated, cleared in xylene, and coverslipped.

The more sensitive immunocytochemical technique, Biotin Amplification Procedure, was utilized to visualize the CNA1 immunocytochemical staining. On day 4, tissue exposed to the CNA1 antibody was treated as described in Berghorn et al. (1993) with the following modifications: (1) TBS was used as the buffer, and (2) the BGAR was diluted at 1:4000.

Control sections were processed as described above but without the primary antibody to determine the level of nonspecific staining within the hippocampal slice. In all cases, the staining patterns reported here were abolished in these control sections.

Electrophysiology. The coverglass on which the slice was grown, served as the base of a stainless steel chamber which allowed perfusion (1 ml vol; 0.5 ml/min flow rate) and temperature maintenance at 35°C (ATR-4 Adaptable Thermoregulator, Fine Science Tools, Can.). The chamber was attached to the stage of a Nikon inverted microscope (Diaphot-TMD-EF with the epifluorescence attachment) placed on a MICRO-g vibration isolation table (Technical Manufacturing Corporation, MA). The cultures were perfused with Hanks' Balanced Salt Solution (GIBCO), in mM: $CaCl_2$ 1.3, KCl 5, KH_2PO_4 0.3, $MgCl_2$ 0.5, $MgSO_4$ 0.4, NaCl 138, Na_2HPO_4 0.3, and D-glucose 5.6, with 4.16 mM $NaHCO_3$ added.

Intracellular recording of individual CA3 pyramidal neurons was made using fine tipped intracellular electrodes filled with 12 mM fura-2 pentapotassium salt (Molecular Probes, OR) in 1 M potassium acetate (pH 7.3). A Physik Instruments manipulator was used to impale the neuron. Total electrode resistance was 75–100 Ω. Neurons were filled by applying −0.5 nA current for 10 min using a Neuro Data Intracellular Recording Amplifier (model IR-283; NY), a Neuro Data Digital Stimulator (model PG 4000A), and a Tektronix Oscilloscope (model 5113; OR). All injected neurons had membrane potentials more negative than −55 mV, responded to small depolarizations with a repetitive action potential discharge, and had resting free calcium levels of 40–60 nM.

Neurons were filled to less than 50 μM fura-2 based on a comparison of the somal fluorescence with the fluorescence of calibration solutions of known path length and dye concentration. This fura-2 concentration has nominal effects on cell firing properties (Regehr and Tank, 1992). Following fura-2 injection, the intracellular electrode was removed and a concentric bipolar stimulating electrode (25 μm spacing) was placed, guided by neuronal fluorescence, at a position 5–10 μm above the soma or a position 5–10 μm above the apical dendrite 200–300 μm from the soma. The experimental stimulus train was 20 Hz for 10 sec, 100 μsec pulse duration, and 100–200 μA, delivered with a Neuro Data stimulus isolation unit (model SIU90). Neurons were stimulated at 10 min intervals as preliminary experiments demonstrated that neuronal responses were consistent in amplitude for over 80 min with this paradigm. At least two control responses were obtained for each cell. All drugs were perfused onto the slice for 10 min.

Calcium imaging. Experiments were conducted in the W. M. Keck Center for Advanced Research in Neural Signaling. A 75 W xenon lamp and a Metaltek (NC) filter wheel and shutter were separated from the microscope to prevent vibration from affecting the optical and physiological recordings. A G. W. Ellis fiberopic light scrambler (Technical Video Ltd., MA) was used to transmit the light to the microscope. Excitation, emission, and neutral density filters were from Omega Optical (VT). The objectives used were a Nikon Fluor 40/1.3 NA oil and a Nikon Fluor 40/0.85 NA. The images were intensified with a GenIIsys Image Intensifier (Dage-MTI, IN) and acquired with a Dage-MTI CCD-72 Series camera. All image acquisition was computer controlled with the Universal Imaging Corporation's (PA) IMAGE-1/FL program. Images were viewed on a Sony Trinitron color video monitor (PVM-1343MD) and printer (UP-5000) and a Javelin Electronics video monitor (model BWM9x, CA) and stored via a Panasonic optical disk drive (LF-7010). Images were acquired at 2–3 sec intervals to reduce photobleaching. Preliminary experiments verified the linearity of the fura-2 response at the camara settings utilized in these experiments. *In vitro* calibrations were performed with 1 μM fura-2 and a saline approximating intracellular conditions (100 mM KCl, 10 mM MOPS, 1 mM free Mg; pH 7.2, 35°C) with balanced EGTA (10 mM) and CaEGTA (10 mM) concentrations to yield 0 mM and 37.9 μM free $[Ca^{2+}]_i$ (Elliott and Sapolsky, 1992, 1993).

All images were corrected for background fluorescence and shading across the field of view. A background image acquired adjacent to the fura-2 loaded neuron, was subtracted from all experimental images. A shading image was acquired using 10.8 μM fura-2 to emphasize nonuniformities across the field of view. Conversion of the ratio of the fluorescent intensities at each excitation wavelength (340 λ and 380 λ) to neuronal $[Ca^{2+}]_i$ was determined through standard equations (Grynkiewicz et al., 1985). R_{min} was determined using 10.8 μM fura-2 in the intracellular saline described above with 10 mM free EGTA. R_{max} was determined with a similar solution using 10 mM CaEGTA. The fura-2 K_d of 224 nM was utilized based on the temperature and ionic strengths of the solutions.

Antagonists. Many antagonists were utilized in this investigation,

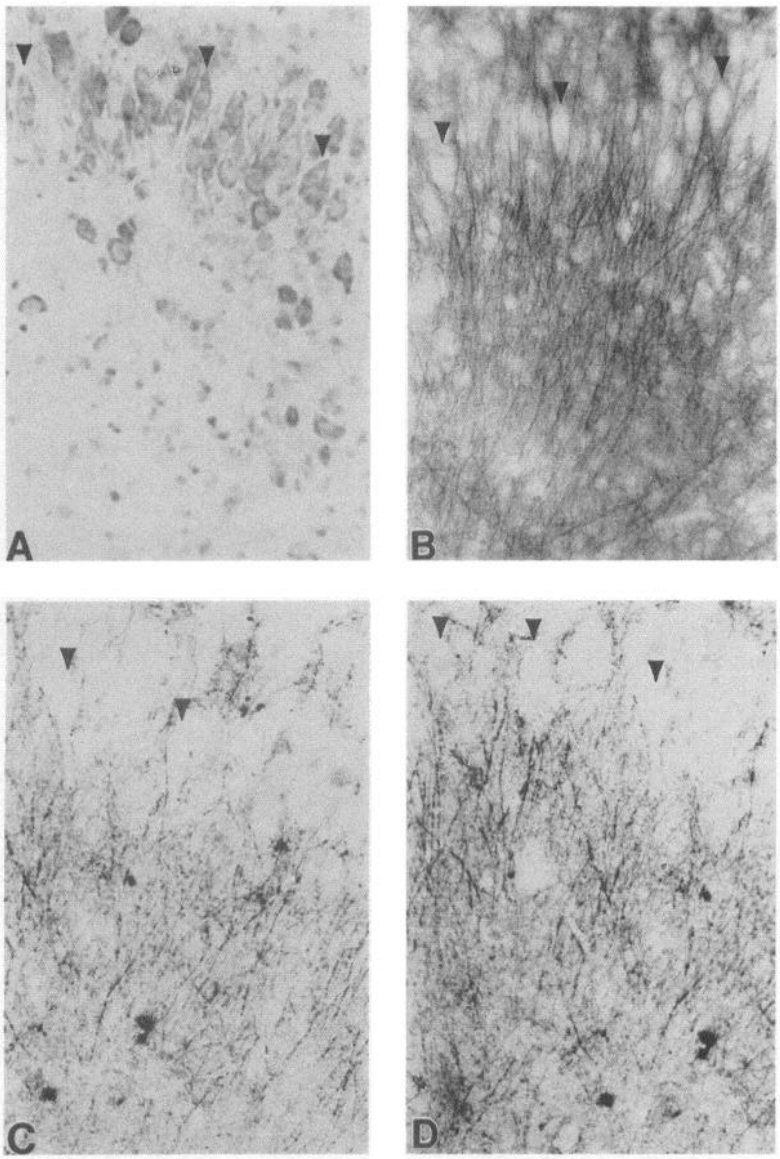

Figure 1. Differential calcium channel localization in CA3 neurons in the cultured hippocampal slice. Area CA3 of hippocampal slice cultures was stained with antibodies to the α1 subunits of class A, B, and D calcium channels. *A*, Class D (L-type) is predominantly located on the cell body and proximal apical dendrites. *B*, Class B (N-type) is predominantly located on the apical dendrites. *C* and *D*, Class A (P/Q-type) is predominantly located on the apical dendrites with a superimposed punctate distribution and a lower level of somal expression. *Arrowheads* indicate the pyramidal cell bodies. Magnification, 176×.

none of which fluoresced at the same wavelengths as fura-2. All perfusion tubing was precoated with 1 mg/ml BSA and all peptide antagonist solutions included 1 mg/ml BSA to minimize nonspecific peptide binding. The diluents and concentrations utilized were: 5-amino-4-phosphonovalerate (DL-AP5, Sigma, distilled water, 100 μM), 6-cyano-7-nitroquinoxaline-2,3-dione (CNQX, RBI, distilled water, 50 μM), tetrodotoxin (TTX, Calbiochem, distilled water, 10 μM), nimodipine [gift of Miles Inc., dimethylsulfoxide (DMSO), 1 μM], isradipine (gift of Sandoz Research Institute, DMSO, 1 μM), ω-CTx-GVIA (Bachem CA, distilled water, 5 μM), ω-Aga-IVA (gift of Dr. M. K. Ahlijanian of Pfizer, distilled water, 30–500 nM), and ω-CTx-MVIIC (gift of Dr. B. Olivera, distilled water, 1.5 μM). Solutions in which DMSO was added as a diluent included 0.05% DMSO final concentration which had no effect on the measured calcium transients.

Statistics. The peak $[Ca^{2+}]_i$ response within the CA3 neuronal soma was obtained under control and experimental (drug) conditions for each cell. Data were then expressed as mean $[Ca^{2+}]_i$ peak as a percentage of the mean control $[Ca^{2+}]_i$ peak. Data were statistically analyzed by paired *t* tests.

Results

Differential calcium channel localization in CA3 neurons in organotypic cultures of hippocampal slices

Organotypic cultures of hippocampal slices are a favorable preparation for studies of the functional significance of differential calcium channel distribution in neurons because they retain much of the morphology and synaptic connectivity of the intact hippocampus yet are thin enough (80–100 μm) for direct observation of individual neurons (Gähwiler, 1981; Frotsher and Gähwiler, 1988; Dailey et al., 1994). These features facilitate the use of optical recording techniques to examine the physiology of intact hippocampal neurons. Previous experiments using a panel of site-directed anti-peptide antibodies demonstrated distinct distributions of the different calcium channel α1 subunits in adult rat hippocampal neurons (Westenbroek et al., 1990, 1992, 1995). Similar methods were used to examine the distribution of calcium channels in CA3 pyramidal neurons from cultured hippocampal slices. As in adult tissue, the class D, L-type calcium channel identified by anti-CND1 is located predominantly on the cell body with little expression along the apical dendrites (Fig. 1*A*). In contrast, the class B, N-type calcium channel identified by anti-CNB2 is located predominantly along the apical dendrites with very little somal expression (Fig. 1*B*). Higher magnification revealed a punctate distribution consistent with labeling of mossy fiber nerve terminals forming synapses

on CA3 dendrites as observed in adult hippocampus (Westenbroek et al., 1992). The class A, P/Q-type channels identified by anti-CNA1 are also primarily localized on dendrites with a substantially lower level of somal expression (Fig. 1*C,D*), and a punctate distribution is observed at higher magnification as in adult hippocampus (Westenbroek et al., 1995). Thus, the complementary localization of calcium channels in CA3 hippocampal neurons is retained in the hippocampal slice culture preparation, suggesting that this preparation would be useful for investigation of the distinct roles of these calcium channels during neuronal activation and signal transduction in intact cells.

Calcium transients in the cell bodies of CA3 neurons in slice cultures

In order to examine the response of intact hippocampal neurons to physiologically relevant stimuli, we measured calcium transients in the cell bodies of neurons in response to extracellular stimuli which elicited trains of synaptic potentials and action potentials. One CA3 neuron per slice was filled with fura-2 pentapotassium salt using a sharp microelectrode (Fig. 2*A,B*). A bipolar stimulating electrode was placed either at the cell body or 200–300 μm along the apical dendrite using the cellular fluorescence to visually guide the electrode placement. Filled cells were stimulated with a 20 Hz train of impulses for 10 sec at an intensity sufficient to produce a transient increase in somal calcium from the resting level of 40–60 nM to a peak of 100–500 nM, similar to the values observed in CA1 hippocampal neurons in acutely dissected hippocampal slices (Regehr et al., 1989; Regehr and Tank, 1992). The peak somal $[Ca^{2+}]_i$ response did not differ significantly following somal or apical stimulation (321 ± 58 nM, $n = 43$ and 407 ± 17 nM, $n = 145$, respectively, $t > 0.05$, unpaired t test). The somal $[Ca^{2+}]_i$ increases within 2 sec, reaches a maximum within 4 sec, and then rapidly declines (Fig. 2*C*). This stimulus train was applied at 10 min intervals which allowed recording of seven or eight repetitive somal $[Ca^{2+}]_i$ responses over 70–80 min.

Loss of normal resting membrane potential is a concern when studying neurons that have been impaled by a sharp microelectrode. All cells studied retained a resting membrane potential more negative than −55 mV after impalement. Several lines of evidence indicated that they maintained resting membrane potentials at least that negative throughout the experiments. (1) Resting calcium levels of 40 nM to 60 nM were recorded throughout the experiments. Calcium levels returned to the resting level of 40–60 nM repeatedly after transients as high as 500 nM. These low intracellular calcium levels are not consistent with depolarized cells because depolarization beyond −55 mV would activate L-type calcium channels yielding persistent calcium influx, high intracellular calcium, and cell deterioration. For unhealthy cells, calcium rises to greater than 1 μM and does not return to resting levels. (2) Our results (see below) show that activation of voltage-gated sodium channels is required for generation of calcium transients. Sodium channels in brain neurons are nearly completely inactivated at membrane potentials more positive than −60 mV. Thus, the robust sodium channel-dependent responses that we observed throughout the course of these experiments imply that the membrane potentials were more negative than −60 mV. (3) We consistently recorded full-size (80 mV) action potentials in response to stimulation of the cells. This also requires the activity of sodium channels and implies a membrane potential more negative than −60 mV. On the basis of these results, we conclude that the CA3 neurons we have studied in these experiments retain a resting membrane potential more negative than −60 mV and generate physiologically significant responses to synaptic and directly depolarizing stimuli.

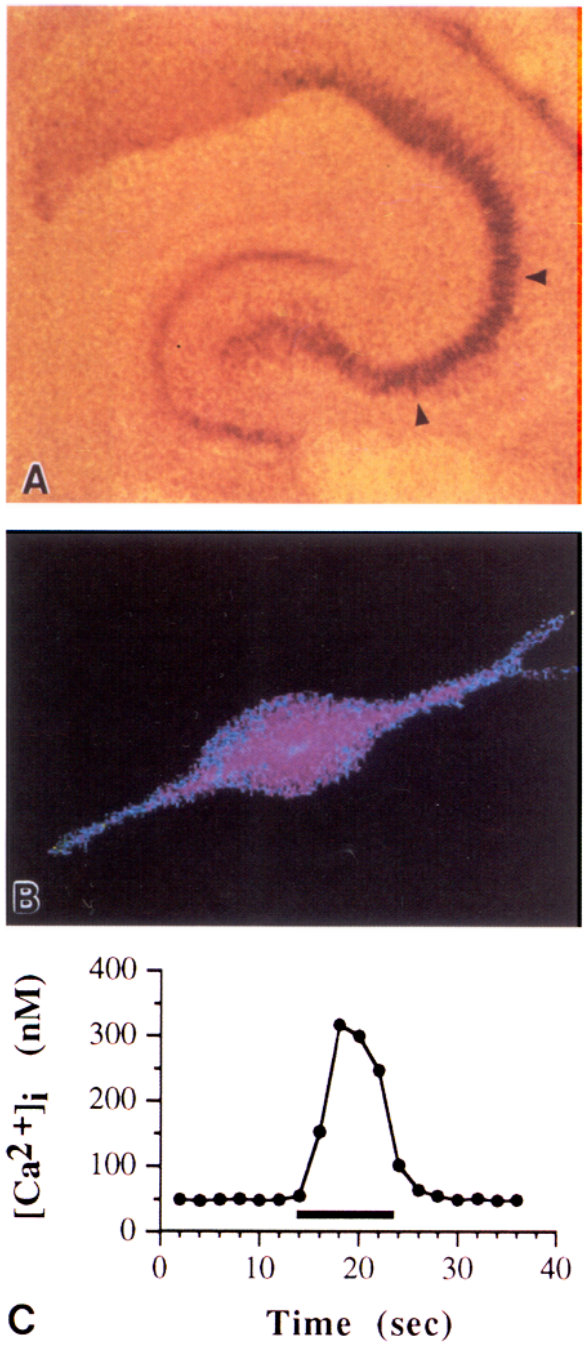

Figure 2. Physiologically induced somatic calcium transients in CA3 neurons in hippocampal slice cultures. *A*, Hippocampal slice culture stained with cresyl violet to indicate the pyramidal cell body layer. CA3 neurons utilized in this study are indicated between the two *arrowheads*. *B*, Typical CA3 neuron filled with fura-2 with a basal calcium concentration of 40–60 nM. *C*, Typical somatic calcium response to stimulation of the distal end of the apical dendrites approximately 200 μm from the cell body with a 20 Hz train (indicated by the *solid bar*). Magnification: 40× (*A*), 341× (*B*).

Somal $[Ca^{2+}]_i$ transient elicited by somal stimulation

During somal stimulation, the bipolar electrode was placed 5–10 μm above the cell body and the stimulus-induced somal

Figure 3.

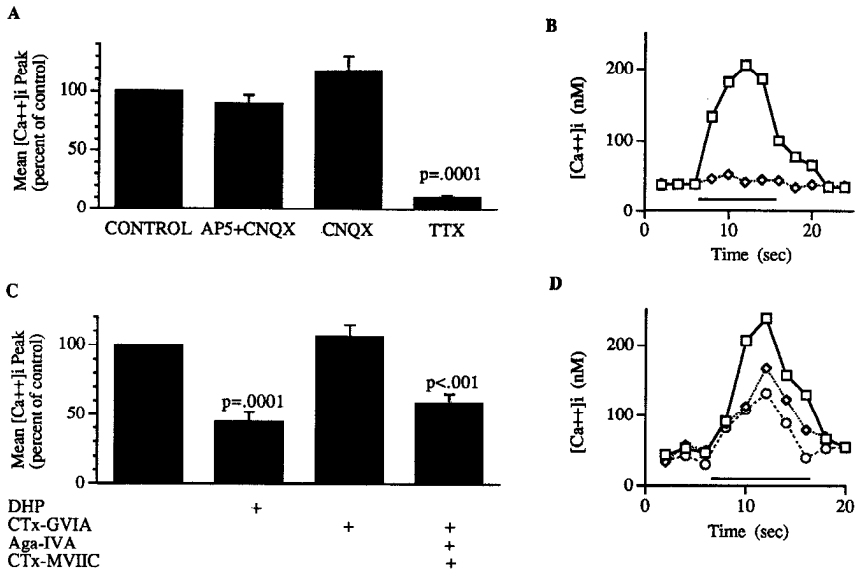

Figure 3. Class C and D L-type and class A calcium channels contribute to the somal $[Ca^{2+}]_i$ transient following somal stimulation. The somal $[Ca^{2+}]_i$ transient was measured during somal stimulation as described in Materials and Methods. *A*, Antagonists to glutamate-mediated synaptic transmission (*AP5* and *CNQX*) have no effect on the somal $[Ca^{2+}]_i$ transient whereas an antagonist to sodium channels (*TTX*) greatly reduces the $[Ca^{2+}]$ transient. *B*, Representative somal $[Ca^{2+}]_i$ transients for control (□) and TTX (◇) for experiments presented in *A*. *C*, Class C and D L-type calcium channel antagonists (*DHP*) and class A P/Q-type calcium channel antagonists (100 nM Aga IVA plus ω-CTx-MVIIC) significantly inhibit the somal $[Ca^{2+}]_i$ transient whereas the class B N-type channel antagonist (ω-CTx-GVIA) has no effect. *D*, Representative somal $[Ca^{2+}]_i$ transients for control (□), ω-CTX-GVIA, ω-Aga-IVA, and ω-CTx-MVIIC (◇), and isradipine or nimodipine (○) for experiments presented in *C*. Statistical comparisons indicated above each bar were made as follows: DHP versus none; ω-CTx-GVIA versus none; and ω-CTx-MVIIC, ω-Aga-IVA, and ω-CTx-GVIA versus ω-CTx-GVIA. Antagonist concentrations for this and subsequent figures (except Fig. 5) were 100 μM AP5, 50 μM CNQX, 10 μM TTX, 1 μM nimodipine and isradipine (L-type channel antagonists, DHP), 5 μM ω-CTx-GVIA (class B, N-type channel antagonist), and 100 nM ω-Aga-IVA plus 1.5 μM ω-CTx-MVIIC (class A, P/Q-type channel antagonists).

$[Ca^{2+}]_i$ transient, averaged over the whole cell body, was measured. The values for the peak $[Ca^{2+}]_i$ in the presence of inhibitors of glutamate receptors or calcium channels from experiments on multiple cells in separate slice cultures were averaged and compared to control values (Fig. 3). Inhibition of glutamate-mediated synaptic transmission by the NMDA and non-NMDA antagonists AP5 and CNQX had no effect on the somal $[Ca^{2+}]_i$ transient (Fig. 3*A*, $n = 8$). In contrast, inhibition of sodium channels with TTX blocked 90% of the somal $[Ca^{2+}]_i$ transient such that the remaining transient was 10 ± 2% of the control (Fig. 3*A*, $n = 5$; Fig. 3*B*). Thus, following somal stimulation, the somal $[Ca^{2+}]_i$ response results from activation of sodium and calcium channels and does not require excitatory synaptic transmission.

Immunocytochemical studies have defined distinct localizations of the different classes of calcium channel α1 subunits. In order to examine the contribution of these different α1 subunits to calcium transients, we used organic and peptide calcium channel antagonists which preferentially inhibit calcium channels containing different classes of α1 subunits. Because the specificity of the available drugs and toxins is not absolute, only a partial resolution of the contributions of different classes of calcium channel α1 subunits can be made. The dihydropyridines (DHP; nimodipine or isradipine) block L-type calcium channels containing $\alpha 1_C$ or $\alpha 1_D$ so that their contributions are measured together. ω-CTx-GVIA is specific for N-type calcium currents mediated by calcium channels containing $\alpha 1_B$ under the conditions of our experiments so that their contribution can be accurately defined. Both ω-Aga-IVA and ω-CTx-MVIIC inhibit calcium channels containing $\alpha 1_A$, and both P-type and Q-type calcium currents may be due to channels which contain this subunit (Stea et al., 1994; Westenbroek et al., 1995). Thus, calcium transients inhibited by the combination of these two toxins reflect contributions of class A calcium channels. To ensure that inhibition of class B channels by these toxins did not affect our measurements of calcium transients due to class A channels, we always applied ω-Aga-IVA and ω-CTx-MVIIC after prior application of ω-CTx-GVIA. Although P-type and Q-type calcium currents are not completely resolved by a single concentration of ω-Aga-IVA or ω-CTx-MVIIC, an estimate of their contributions to these calcium transients can be made by study of the concentration dependence of the action of ω-Aga-IVA (see below).

Antagonists of each molecular class of voltage-sensitive calcium channel had different effects on the stimulus-induced somal $[Ca^{2+}]_i$ transients. The dihydropyridine L-type channel antagonists isradipine and nimodipine inhibited 56% of the stimulus-induced somal $[Ca^{2+}]_i$ transient such that the remaining transient was 44 ± 8% of control (Fig. 3*C*, $n = 13$; Fig. 3*D*). In contrast, the N-type channel antagonist ω-CTx-GVIA had no effect on the stimulus-induced somal $[Ca^{2+}]_i$ transient (Fig. 3*C*, $n = 15$). Thus, the class C and D channels, which our immunocytochemical studies show to be preferentially localized on the neuronal soma, contribute substantially to the somal calcium transient. The class B channels, which are present in a low density in the cell soma, do not contribute detectably to the somal calcium transient.

We used ω-Aga-IVA (100 nM) and ω-CTx-MVIIC (1.5 μM) to estimate the component of the intracellular calcium transients that was due to class A calcium channels. With class B channels

Figure 4.

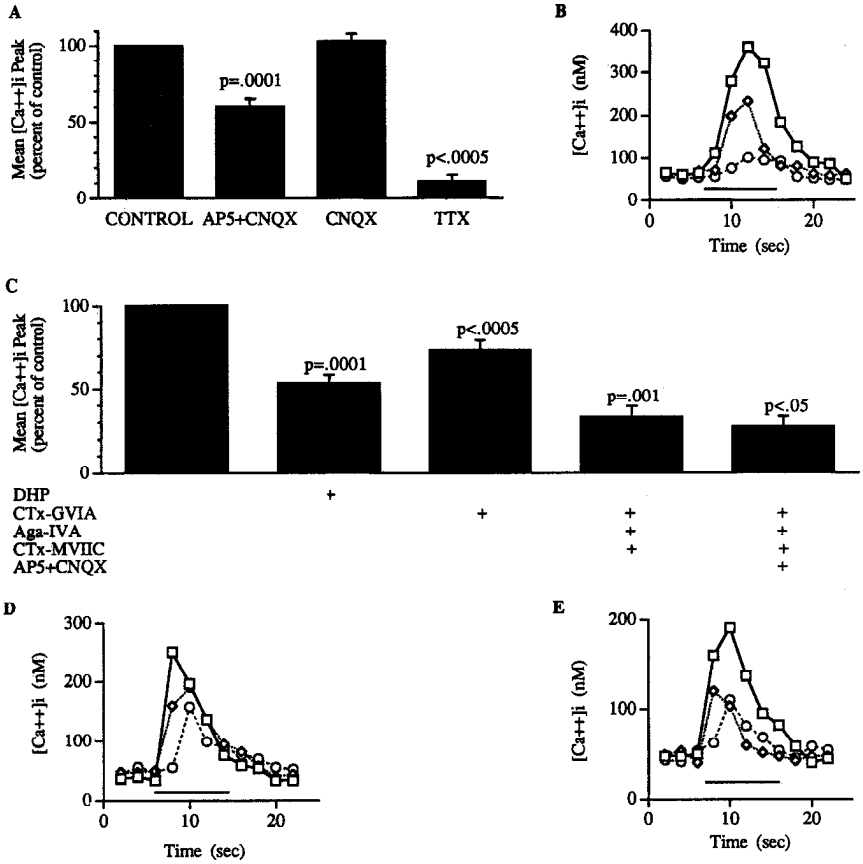

Figure 4. Class C and D L-type, class B N-type, and class A P/Q-type channels contribute to the somal $[Ca^{2+}]_i$ transient following dendritic stimulation. The somal $[Ca^{2+}]_i$ transient was measured during apical dendritic stimulation. *A*, In contrast to that following direct somal stimulation, antagonists to glutamate-mediated synaptic transmission (*AP5* and *CNQX*) inhibit the somatic $[Ca^{2+}]_i$ transient. Inhibition of only the non-NMDA subtype of glutamate receptors (*CNQX*) has no effect on the $[Ca^{2+}]_i$ transient. Similar to that following somal stimulation, an antagonist of sodium channels (*TTX*) greatly reduces the somal $[Ca^{2+}]_i$ transient. *B*, Representative somal $[Ca^{2+}]_i$ transients for control (□), AP5 + CNQX (◇), and TTX (○) for experiments presented in *A*. *C*, The indicated antagonists of class A-D calcium channels all significantly inhibit the somal $[Ca^{2+}]_i$ transient. Antagonists to glutamate-mediated synaptic transmission (*AP5* and *CNQX*) caused a small but significant reduction in the somal $[Ca^{2+}]_i$ transient even in the presence of antagonists of the class A and B calcium channels. *D*, Representative somal $[Ca^{2+}]_i$ transients for control (□), ω-CTx-GVIA (◇), and DHP (○), from the experiments presented in *C*. *E*, Representative somal $[Ca^{2+}]_i$ transients for ω-CTx-GVIA (□); ω-CTX-GVIA, ω-Aga-IVA, and ω-CTx-MVIIC (◇); and ω-CTX-GVIA, ω-Aga-IVA, ω-CTx-MVIIC, and AP5 + CNQX (○) from experiments presented in *C*. Statistical comparisons indicated above each bar were made as follows: DHP versus none; ω-CTx-GVIA versus none; ω-CTx-MVIIC, ω-Aga-IVA, and ω-CTx-GVIA versus ω-CTx-GVIA; and ω-CTx-MVIIC, ω-Aga-IVA, ω-CTx-GVIA, and AP5 + CNQX versus ω-Aga-IVA, ω-CTx-GVIA, and ω-CTx-MVIIC.

blocked by ω-CTx-GVIA, treatment with ω-Aga-IVA followed by ω-CTx-MVIIC further reduced the stimulus-induced somal $[Ca^{2+}]_i$ transient to 59 ± 7% of the control (Fig. 3*C*, $n = 6$; Fig. 3*D*). Thus, calcium channels containing $\alpha 1_A$ accounted for 41% of the peak calcium transient. Our immunocytochemical studies indicate that class A α1 subunits are present at an intermediate density on the cell soma relative to the class C and D L-type channels and the class B N-type channels. Consistent with this localization, their inhibition causes an intermediate reduction in the somal calcium transient.

Somal $[Ca^{2+}]_i$ transients elicited by apical dendritic stimulation

During dendritic stimulation, the bipolar electrode was placed 5–10 μm above the distal apical dendrite at a point 200–300 μm from the cell body, stimuli were applied that elicited a train of action potentials as recorded with an intracellular electrode in the cell body (not shown), and stimulus-induced somal $[Ca^{2+}]_i$ transients were measured. In contrast to our results with somal stimulation, inhibition of glutamate-mediated synaptic transmission with the antagonists AP5 and CNQX reduced the somal $[Ca^{2+}]_i$ response to apical dendritic stimulation to 60 ± 5% of the control transient (Fig. 4*A*, $n = 62$; Fig. 4*B*). This reduction was primarily due to inhibition of NMDA receptors since CNQX, which inhibits non-NMDA receptors, had no effect on the somal $[Ca^{2+}]_i$ transient when tested alone (Fig. 4*A*, $n = 3$). Similar to our results following somal stimulation, sodium channel inhibition with TTX blocked 89% of the dendritic stimulus-induced somal $[Ca^{2+}]_i$ transient such that the remaining transient was 11 ± 4% of the control transient (Fig. 4*A*, $n = 4$; Fig. 4*B*). Thus, following dendritic stimulation, the somal $[Ca^{2+}]_i$ response results from both direct dendritic stimulation and glutamate-mediated synaptic stimulation, and both stimuli require sodium channel activation to elicit a somal calcium transient.

The contribution of the different classes of voltage-sensitive calcium channels to the somal $[Ca^{2+}]_i$ transient following den-

dritic stimulation differed from that following somal stimulation (Fig. 4*C–E*). Dihydropyridine L-type channel antagonists reduced the dendritic stimulus-induced somal $[Ca^{2+}]_i$ transient to 53 ± 5% of the control transient (n = 10), comparable to their effect on somal stimulation. ω-CTx-GVIA inhibited the somal $[Ca^{2+}]_i$ transient by 27% such that the remaining transient was 73 ± 6% of the control transient (n = 20). These results show that the class B, C, and D calcium channels are all required for somal calcium transients when they are elicited by a distal dendritic stimulation. Addition of ω-Aga-IVA (100 nM) followed by ω-CTx-MVIIC (1.5 μM) further reduced the somal $[Ca^{2+}]_i$ transient to 33 ± 7% of the control transient (n = 9). Thus, the two antagonists of class A calcium channels inhibit 40% of the somal $[Ca^{2+}]_i$ transient following apical dendritic stimulation.

Separation of the contributions of P-type and Q-type calcium currents to somal $[Ca^{2+}]_i$ transients

P-Type channels in Purkinje cells and other central neurons are preferentially inhibited by a low concentration of ω-Aga-IVA (EC_{50} = 2 nM, Mintz et al., 1992a,b) while Q-type channels are inhibited by higher concentrations in cerebellar granule cells or *Xenopus* oocytes (Sather et al., 1993; Randall et al., 1995). In the experiments presented in Figure 3, addition of ω-Aga-IVA (100 nM) in the presence of ω-CTx-GVIA reduced the stimulus-induced somal $[Ca^{2+}]_i$ transient only slightly to 93 ± 5% of the control (data not shown). These results suggest that most of the class A calcium channels which contribute to the somal calcium transient have relatively low affinity for ω-Aga-IVA, consistent with the properties of Q-type channels as defined in previous work (Sather et al., 1993; Randall et al., 1995). In the experiments of Figure 4, addition of ω-Aga-IVA (100 nM) in the presence of ω-CTx-GVIA reduced the somal $[Ca^{2+}]_i$ transient elicited by distal dendritic stiimulation by 20% (n = 5, $p < 0.01$, data not shown). To investigate in more detail whether this component of the $[Ca^{2+}]_i$ transient was due to P-type or Q-type calcium channels or both, we tested the effects of increasing concentrations of ω-Aga-IVA on the dendritic stimulus-induced somal $[Ca^{2+}]_i$ transient (Fig. 5). The threshold concentration of ω-Aga-IVA necessary to reduce the somal $[Ca^{2+}]_i$ transient was between 30 nM and 100 nM, indicating that there was only a small contribution of P-type channels (EC_{50} = 2 nM) to this $[Ca^{2+}]_i$ transient. The EC_{50} for the inhibition of the $[Ca^{2+}]_i$ transients was between 100 nM and 150 nM. This is most consistent with the conclusion that the primary effect of ω-Aga-IVA on calcium transients elicited by apical dendritic stimulation reflects inhibition of Q-type calcium channels, since these channels are inhibited half-maximally by 100–200 nM ω-Aga-IVA when recorded from cerebellar granule cells or from *Xenopus* oocytes expressing $\alpha 1_A$ cDNA (Sather et al., 1993; Randall et al., 1995).

Presynaptic and postsynaptic actions of calcium channels in somal calcium transients elicited by apical dendritic stimulation

The apical dendritic stimulation of somal $[Ca^{2+}]_i$ transients has two components: direct dendritic stimulation and glutamate-mediated synaptic stimulation due to evoked release of glutamate from nerve terminals in the stimulus field. Therefore, the inhibition of the stimulus-induced somal $[Ca^{2+}]_i$ transient by the calcium channel antagonists could result either from the inhibition of presynaptic glutamate release and/or the inhibition of postsynaptic apical dendritic calcium channels. To determine which classes of calcium channels contribute to the somal $[Ca^{2+}]_i$ transient resulting from direct dendritic stimulation, we tested whether the calcium channel antagonists were still effective at reducing the somal $[Ca^{2+}]_i$ transient in the presence of AP5 and CNQX to block synaptic transmission (Fig. 6). The L-type channel antagonists isradipine or nimodipine inhibited the somal $[Ca^{2+}]_i$ transient by about 40% such that the remaining transient was 61 ± 12% (n = 14) of the control transient, which was comparable to the level of inhibition observed without inhibition of synaptic transmission. These results indicate that the class C and D L-type calcium channels contribute directly to somal $[Ca^{2+}]_i$ transients and do not contribute significantly to glutamate-mediated synaptic transmission under these conditions. In contrast, ω-CTx-GVIA was no longer effective at inhibiting the somal $[Ca^{2+}]_i$ transient (n = 16), indicating that the entire effect of the N-type channel antagonist on calcium transients elicited by apical dendritic stimulation was due to inhibition of glutamatergic synaptic transmission. Evidently, class B N-type calcium channels in the dendritic shaft do not contribute significantly to the the activation of somal $[Ca^{2+}]_i$ transients elicited by distal dendritic stimulation, but do contribute significantly to glutamatergic transmission at the excitatory synapses on the distal CA3 dendrites.

Surprisingly, ω-Aga-IVA (100 nM) increased the somal $[Ca^{2+}]_i$ transient elicited by apical dendritic stimulation to 198 ± 31% of the control transient after block of excitatory transmission (Fig. 6, n = 12). It is likely that this increase reflects block of tonic release of the inhibitory transmitter GABA by ω-Aga-IVA, thus allowing more efficient activation of the cell (see Discussion). Further addition of ω-CTx-MVIIC had no effect on the somal $[Ca^{2+}]_i$ transient (n = 5). These results indicate

Figure 5.

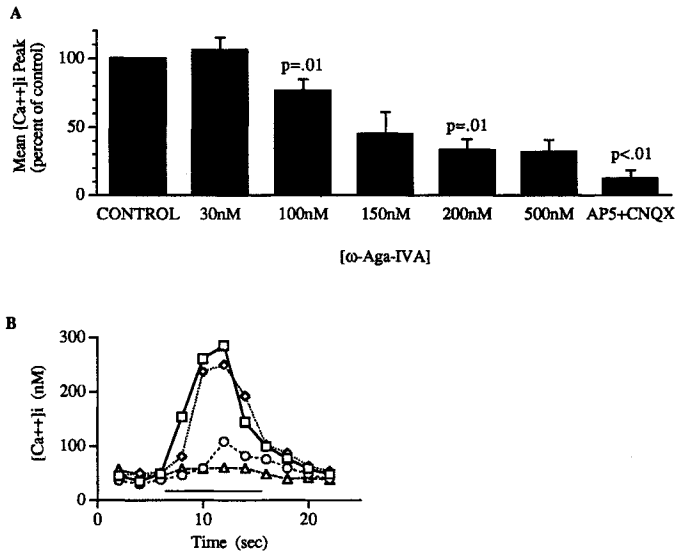

Figure 5. Effects of increasing concentrations of ω-Aga-IVA on the somal $[Ca^{2+}]_i$ transient elicited by distal dendritic stimulation. *A*, The somal $[Ca^{2+}]_i$ transient was measured during apical dendritic stimulation in the presence of the indicated concentrations of ω-Aga-IVA or in the presence of 500 nM ω-Aga-IVA and AP5 + CNQX. *B*, Representative somal $[Ca^{2+}]_i$ transients for control (□), 100 nM ω-Aga-IVA (◇), 200 nM ω-Aga-IVA (○), and 500 nM ω-Aga-IVA and AP5 + CNQX (△) from experiments presented in *A*. Statistical comparisons indicated above each bar were made as follows: 30 nM versus control; 100 nM versus 30 nM; 150 nM versus 100 nM; 200 nM versus 150 nM; 500 nM versus 200 nM; and AP5+CNQX and 500 nM ω-Aga-IVA versus 500 nM ω-Aga-IVA.

Figure 6.

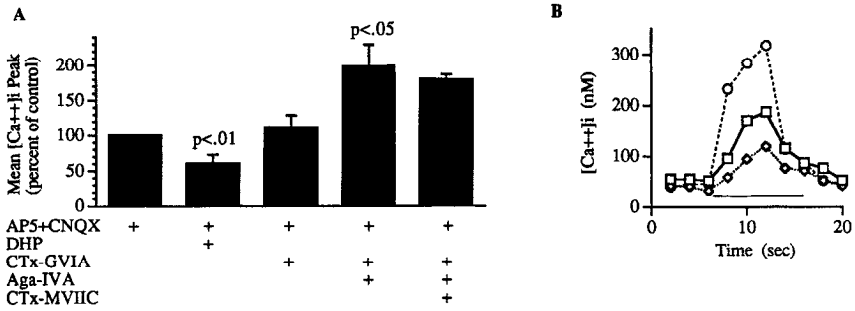

Figure 6. Class B N-type and class A P/Q-type calcium channels contribute to glutamate-mediated synaptic transmission. *A*, Glutamate-mediated synaptic transmission was inhibited and the effects of each calcium channel antagonist on the remaining somal $[Ca^{2+}]_i$ transient were then measured during apical dendritic stimulation. Data are expressed as the mean $[Ca^{2+}]_i$ peak as a percentage of the response obtained with AP-5 and CNQX present. The inhibitory effects of the class A channel antagonists (ω-Aga-IVA and ω-CTx-MVIIC) and the class B channel antagonist (ω-CTx-GVIA) on the somal $[Ca^{2+}]_i$ transient were occluded in the absence of glutamate-mediated synaptic transmission. ω-Aga-IVA increased the somal $[Ca^{2+}]_i$ transient. In contrast, DHPs effectively reduced the somal $[Ca^{2+}]_i$ transient even in the absence of glutamate-mediated synaptic transmission. *B*, Representative somal $[Ca^{2+}]_i$ transients for AP5 + CNQX (□), AP5 + CNQX and DHP (◇), and AP5 + CNQX, ω-CTx-GVIA, and ω-Aga-IVA (○) from the experiments presented in *A*. Statistical comparisons indicated above each bar were made as follows with AP5 + CNQX in all samples: DHP versus none; ω-CTx-GVIA versus none; ω-Aga-IVA and ω-CTx-GVIA versus ω-CTx-GVIA; and ω-CTx-MVIIC, ω-Aga-IVA, and ω-CTx-GVIA versus ω-Aga-IVA and ω-CTx-GVIA.

that the reduction of calcium transients by ω-CTx-MVIIC following apical dendritic stimulation is also due primarily to inhibition of glutamatergic synaptic transmission rather than inhibition of class A calcium channels in the dendritic shaft. Thus, neither class A nor class B calcxium channels located in the dendritic shaft are required for activation of somal $[Ca^{2+}]_i$ transients in response to distal dendritic stimulation, but both class A and class B calcium channels are required for normal synaptic transmission at excitatory synapses on CA3 neurons.

Very little glutamate-mediated synaptic transmission remained after application of ω-CTx-GVIA and ω-CTx-MVIIC, as the addition of AP5 and CNQX at this point decreased the somal $[Ca^{2+}]_i$ transient only slightly such that the remaining transient was reduced from 32 ± 7% to 27 ± 6% ($p < 0.05$) of the original control transient (Fig. 4*C*, $n = 9$; Fig. 4*E*). Following the addition of 500 nM ω-Aga-IVA to block all class A channels, AP5 and CNQX still effectively decreased the somal $[Ca^{2+}]_i$ transient such that the remaining transient was reduced from 32 ± 9% to 12 ± 6% of the control transient (Fig. 5). The remaining glutamatergic transmission under these conditions is likely to be due primarily to class B N-type channels which remain active in 500 nM ω-Aga-IVA. These results provide further support for the conclusion that both class A and class B calcium channels are critically involved in glutamate-mediated synaptic transmission as their inhibition by calcium channel antagonists occludes the effects of AP5 and CNQX on calcium transients elicited by stimulation of apical dendrites. In contrast, the class C and D L-type calcium channels do not appear to serve a major role in glutamate-mediated excitatory synaptic transmission in CA3 neurons in the cultured hippocampal slice. Antagonists of L-type calcium channels are approximately equally effective in blocking somal calcium transients whether synaptic transmission is blocked or not.

Discussion

Differential localization of calcium channel subtypes

In adult hippocampus, the calcium channel subtypes encoded by the class A through E α1 subunit genes are differentially localized as assessed by immunocytochemical studies with monoclonal and site-directed anti-peptide antibodies (Ahlijanian et al., 1990; Westenbroek et al., 1990, 1992, 1995; Hell et al., 1993). The class C and class D L-type channels are localized predominantly on the cell bodies and proximal dendrites of pyramidal neurons, although patches of class C channels can also be detected on medial dendrites of CA3 and dentate granule neurons (Hell et al., 1993). In contrast, class B N-type and class A P/Q-type channels are generally localized at low density along the dendrites of hippocampal pyramidal neurons with superimposed areas of high density, punctate staining that appear to be associated with presynaptic terminals. Anti-CNA1 may recognize P-type and Q-type channels as this antibody strongly binds to both cerebellar Purkinje cells which are rich in P-type channels and granule cells and their terminals which are rich in Q-type channels (Westenbroek et al., 1995).

Determination of the functional roles of these differentially localized calcium channels requires development of an *in vitro* experimental system in which calcium transients can be measured in minimally perturbed neurons which retain the morphology, synaptic connectivity, and physiological properties characteristic of the hippocampus *in vivo*. In this article, we show that the *in vitro* hippocampal slice culture is an appropriate experimental system for this analysis. Differential localization of class D L-type, class B N-type, and class A P/Q-type calcium channel subtypes is observed in postnatal rat hippocampal slice cultures, as in adult neurons, enabling direct comparisons between calcium channel localization and function. On the basis of these results, the experiments described here were designed to test the functional implications of the differential localization of these calcium channel subtypes and to examine whether they serve distinct roles in neuronal activation and signal transduction.

Intracellular calcium transients as a measure of neuronal calcium channel function

The functional properties of different types of calcium channels have been studied extensively using whole cell voltage-clamp procedures applied to dissociated neurons to measure barium currents in response to prolonged depolarizations of the cells

(reviewed in Bean, 1989; Hess, 1990; Zhang et al., 1993). These studies give a clear picture of the ionic currents conducted by distinct calcium channel types in the cell bodies of central neurons. In order to examine the roles of different classes of calcium channels in regulation of cytosolic calcium levels in intact neurons, we have measured intracellular calcium transients in the cell bodies of CA3 neurons in cultured hippocampal slices in response to trains of brief stimuli which elicit synaptic activation and neuronal action potentials. The somal calcium transients that we measure are the mechanism of coupling of cell surface electrical signals to intracellular events within the cell body and therefore represent a physiologically relevant index of calcium channel function. Our results using this approach complement previous studies of calcium channel function in dissociated preparations and reveal distinct functional roles of calcium channels in different cellular compartments.

Class C and D L-type and class A P/Q-type calcium channels are required for somal $[Ca^{2+}]_i$ transients elicited by somal stimulation

Upon direct somal stimulation, the class C and D L-type and the class A P/Q-type, but not class B N-type, calcium channels mediate calcium entry into the CA3 neuronal soma. Antagonists of L-type channels inhibit 56% of the somal $[Ca^{2+}]_i$ transient. Antagonists of class A P/Q-type channels inhibit 41% of the somal $[Ca^{2+}]_i$ transient. ω-Aga-IVA had little or no effect on directly elicited somal calcium transients at 100 nM, a concentration which is relatively specific for P-type versus Q-type calcium channels, suggesting that most class A calcium channels participating in generation of these calcium transients in CA3 cell bodies have the pharmacological characteristics of Q-type channels. ω-CTx-GVIA had no effect on the somal $[Ca^{2+}]_i$ transient indicating no important contribution from class B N-type calcium channels.

Our results indicate that the concentration of class C and D L-type calcium channels in cell bodies allows them to play a primary role in cell body calcium transients, while the primary localization of class B N-type calcium channels in dendrites and nerve terminals prevents them from exerting a major influence on directly elicited cell body calcium transients. Class A calcium channels containing $\alpha 1_A$ also contribute significantly to $[Ca^{2+}]_i$ transients in cell bodies, as assessed by inhibition by ω-CTx-MVIIC, consistent with the intermediate somal expression of this α1 subunit. Glutamate receptors do not appear to mediate somal calcium influx following direct somal stimulation of CA3 pyramidal neurons as glutamate receptor antagonists were ineffective at reducing the somal $[Ca^{2+}]_i$ transients. The cell bodies of CA1 hippocampal neurons are also unresponsive to glutamate receptor antagonists (Alford et al., 1993).

The presence of class C and D L-type and class A P/Q-type calcium channels on neuronal cell bodies and their primary role in generation of cell body $[Ca^{2+}]_i$ transients indicates their probable involvement in calcium-dependent events such as the regulation of enzyme activity and gene expression. L-Type channels have been shown to participate in both of these calcium-dependent events in other cell preparations (e.g., Kennedy, 1989; Murphy et al., 1991).

Class B N-type and class A P/Q-type calcium channels participate in presynaptic release of glutamate

Antagonists of N-type calcium channels containing $\alpha 1_B$ and P/Q-type calcium channels containing $\alpha 1_A$ significantly inhibit somal calcium transients elicited by apical dendritic stimulation. However, their inhibition is completely occluded following the blockade of excitatory synaptic transmission with AP5 and CNQX. These results demonstrate an essential role of calcium channels containing $\alpha 1_A$ and $\alpha 1_B$ in synaptic transmission onto CA3 neurons in cultured hippocampal slices. These results correlate well with the localization of these two calcium channel types in punctate clusters in presynaptic nerve terminals at synapses on both CA1 and CA3 pyramidal neurons (Westenbroek et al., 1992, 1995).

Previous studies have shown that calcium channels inhibited by ω-CTx-GVIA and by ω-CTx-MVIIC or high concentrations of ω-Aga-IVA are important for synaptic transmission from the Schaffer collaterals of the CA3 neurons to the CA1 pyramidal neurons (Dutar et al., 1989; Luebke et al., 1993; Takahashi and Momiyama, 1993; Wheeler et al., 1994) and for transmission from the mossy fibers of the dentate granule cells to the proximal dendrites of CA3 pyramidal neurons (Kamiya et al., 1988). Our results show that class B N-type and class A P/Q-type calcium channels are also important for synaptic transmission onto the distal dendrites of CA3 neurons in organotypic hippocampal slice cultures. Together with previous studies, our results support the conclusion that class B N-type and class A P/Q-type calcium channels are important in synaptic transmission at a broad range of synapses on central neurons.

In contrast to the results with antagonists of N-type and P/Q-type calcium channels, we have not observed a significant contribution of L-type calcium channels to glutamate-mediated synaptic transmission. Antagonists of L-type calcium channels reduce somal calcium transients induced by apical dendritic stimulation substantially in the presence or absence of blockers of excitatory transmission. It is likely that these inhibitory effects are due to block of the L-type calcium channels in the proximal dendrites and cell body which are directly responsible for calcium entry into the cell soma. This conclusion is consistent with immunocytochemical results showing that the L-type channel α1 subunits are primarily localized in the cell body and proximal apical dendrites.

Luebke et al. (1993) reported that 100 nM ω-Aga-IVA enhanced postsynaptic responses to applied AMPA in CA1 neurons. Similarly, we found that 100 nM ω-Aga-IVA potentiated the somal $[Ca^{2+}]_i$ transient in CA3 neurons following inhibition of glutamate-mediated synaptic transmission. In our study, we inhibited AMPA receptor function with CNQX excluding a major direct effect of ω-Aga-IVA on AMPA-sensitive glutamate receptors. Instead, P-type channels may be involved in release of other neurotransmitters in the hippocampal slice cultures which alter the $[Ca^{2+}]_i$ transient elicited by distal dendritic stimulation. For example, P-type channels may initiate GABA release from inhibitory interneurons within the hippocampal slice cultures such that 100 nM ω-Aga-IVA, which inhibits P-type calcium currents relatively specifically, causes a relief of inhibitory input to the CA3 neurons and thereby potentiates their $[Ca^{2+}]_i$ transients elicited by direct depolarization while excitatory transmission is blocked. These inhibitory effects are apparently small since they do not appear to affect somal calcium transients elicited by distal dendritic stimulation when excitatory neurotransmission is intact (Fig. 5). Nevertheless, these effects of low concentrations of ω-Aga-IVA in the presence of AP5 and CNQX point to an important caveat for interpretation of pharmacological studies in complex preparations like the hippocampal slice. It is possible that the pharmacological agents we used

in these studies have undetected effects on interneurons which alter ongoing synaptic transmission onto CA3 neurons and indirectly influence their responses to distal dendritic stimuli. Because these indirect effects were only revealed when excitatory transmission was blocked, it seems unlikely that they are large enough to affect the main conclusions of this study. However, indirect effects of the calcium channel blockers on synaptic transmission from interneurons may well have influenced the quantitative aspects of the results.

High-voltage-activated dendritic calcium channels are not required for activation of cell body calcium transients by distal dendritic stimulation

Calcium-dependent action potentials can be recorded in the dendrites of many central neurons including cerebellar Purkinje cells (Llinas and Sugimori, 1979) and hippocampal pyramidal neurons (Wong et al., 1979). Therefore, it has been anticipated that the calcium-dependent action potentials would be essential for transmission of synaptic responses along the dendrites to the cell body, at least in long dendrites like those of the CA3 neurons studied here (Llinas and Sugimori, 1979). In contrast to this expectation, inhibition of the class A and B calcium channels that have been shown to be located on dendrites in immunocytochemical studies did not alter somal $[Ca^{2+}]_i$ transients following dendritic stimulation. Thus, the effects of antagonists of both class A and class B calcium channels on somal $[Ca^{2+}]_i$ transients elicited by distal dendritic stimuli were completely occluded by inhibition of excitatory glutamatergic transmission indicating that only the presynaptic class A and class B calcium channels were involved. Since block of excitatory transmission would make the activation of the postsynaptic CA3 neurons even more sensitive to inhibition of postsynaptic dendritic calcium channels, our experiments should have detected contributions of the high-voltage-activated dendritic channels if they were significant. We conclude that high-voltage-activated dendritic calcium channels do not have a major role in transmitting synaptic input to the cell body when strong, synchronous stimuli are applied to the distal dendrites as in our experiments.

In neocortical neurons, low-voltage-activated dendritic calcium channels generate a $[Ca^{2+}]_i$ transient, in response to a single subthreshold dendritic EPSP, that may propagate toward the soma (Markram and Sakmann, 1994). Although we have not resolved a role for high-voltage-activated dendritic calcium channels in transmission of synaptic stimuli to the cell body in these experiments, we cannot rule out an essential role for them in the response of the CA3 neurons to individual or nonsynchronous synaptic stimuli or to low intensity synaptic stimuli as are likely to occur in many circumstances *in vivo*. Dendritic calcium-dependent action potentials may allow summation of such subthreshold synaptic stimuli to reach the level of depolarization required for action potential generation or to modulate the frequency of action potential generation. The dendritic calcium channels may also function to increase local calcium in the dendrites initiating local intracellular regulatory events. These are likely to include activation of calcium-dependent protein kinases resulting in protein phosphorylation and cellular regulation.

Role of sodium channels in mediation of CA3 neuronal calcium transients

We found that sodium channel inhibition blocks 90% of the somal $[Ca^{2+}]_i$ transient following either apical dendritic or direct somal stimulation. Evidently, sodium channels are essential for initiation of neuronal calcium transients following trains of strong stimuli in CA3 neurons in the organotypic hippocampal slice as was previously demonstrated for CA1 neurons (Jaffe et al., 1992; Miyakawa et al., 1992; Regehr and Tank, 1992). We interpret these results in light of the recent findings of Stuart and Sakmann (1994) on sodium-dependent action potentials in dendrites. They recorded sodium-dependent action potentials blocked by low concentrations of tetrodotoxin in the distal apical dendrites of cortical pyramidal cells in acute slice preparations. These action potentials were initiated in the axon initial segment and propagated in the retrograde direction into the dendrites. They were elicited by synaptic stimuli as well as by direct depolarization of the dendrites. These results suggest that cortical neurons are sufficiently electrically compact that strong stimuli to the distal dendrites can elicit an action potential in the axon initial segment by electrotonic conduction. By analogy, we hypothesize that our apical dendritic stimuli elicit sodium-dependent action potentials initiated in the axon initial segment, and these action potentials in turn are responsible for the cell body calcium transients that we have recorded. Thus, at least in response to strong trains of stimuli as used here, activation of sodium channels in the axon initial segment apparently is critical for generation of a cell body calcium transient in response to both direct stimulation of the cell body and indirect stimulation of the distal dendrites.

Distinct roles for the calcium channel classes

Based upon the differential subcellular localization of the calcium channel subtypes within the neuron, it was predicted (Ahlijanian et al., 1990; Westenbroek et al., 1990, 1992, 1995) that each channel subtype would serve a distinct role in neuronal signal transduction. We have shown this to be true for the CA3 hippocampal neuron. Following somal stimulation, only those channels whose $\alpha 1$ subunit was found to be localized to the cell body (class A, C, and D, but not class B), contributed significantly to the somal $[Ca^{2+}]_i$ transient and did so in a manner related to their relative abundance on the cell body. These channels may regulate such somal processes as enzyme activation and gene induction. Following apical dendritic stimulation, only those channels whose $\alpha 1$ subunit was found to be localized to synaptic structures on the medial-distal apical dendrites (class A and B, but not class C and D), contributed to glutamate-mediated synaptic transmission. Further information on the role of each calcium channel subtype in signal transduction in neurons will come from determination of the contribution of each calcium channel subtype to the $[Ca^{2+}]_i$ transients recorded in the medial-distal apical dendrite. The value of the approach utilized in this study is that direct comparisons can be made between calcium channel subtype localization and function in the same intact neuron. This approach may also be useful for determination of the contribution of these channels to calcium transients in dendrites.

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