

CALCINEURIN ENHANCES L-TYPE Ca^{2+} CHANNEL ACTIVITY IN HIPPOCAMPAL NEURONS: INCREASED EFFECT WITH AGE IN CULTURE

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Abstract—The Ca^{2+} /calmodulin-dependent protein phosphatase, calcineurin, modulates a number of key Ca^{2+} signaling pathways in neurons, and has been implicated in Ca^{2+} -dependent negative feedback inactivation of *N*-methyl-D-aspartate receptors and voltage-sensitive Ca^{2+} channels. In contrast, we report here that three mechanistically disparate calcineurin inhibitors, FK-506, cyclosporin A, and the calcineurin autoinhibitory peptide, inhibited high-voltage-activated Ca^{2+} channel currents by up to 40% in cultured hippocampal neurons, suggesting that calcineurin acts to enhance Ca^{2+} currents. This effect occurred with Ba^{2+} or Ca^{2+} as charge carrier, and with or without intracellular Ca^{2+} buffered by EGTA. Ca^{2+} -dependent inactivation of Ca^{2+} channels was not affected by FK-506. The immunosuppressant, rapamycin, and the protein phosphatase 1/2A inhibitor, okadaic acid, did not decrease Ca^{2+} channel current, showing specificity for effects on calcineurin. Blockade of L-type Ca^{2+} channels with nimodipine fully negated the effect of FK-506 on Ca^{2+} channel current, while blockade of N-, and P-/Q-type Ca^{2+} channels enhanced FK-506-mediated inhibition of the remaining L-type-enriched current. FK-506 also inhibited substantially more Ca^{2+} channel current in 4-week-old vs. 2-week-old cultures, an effect paralleled by an increase in calcineurin A mRNA levels.

These studies provide the first evidence that calcineurin selectively enhances L-type Ca^{2+} channel activity in neurons. Moreover, this action appears to be increased concomitantly with the well-characterized increase in L-type Ca^{2+} channel availability in hippocampal neurons with age-in-culture. © 2002 IBRO. Published by Elsevier Science Ltd. All rights reserved.

Key words: protein phosphatase, aging, Ca^{2+} channel currents, FK-506, cyclosporin A, nimodipine, conotoxins.

In recent years it has become clear that Ca^{2+} /calmodulin-dependent protein phosphatase 2B, or calcineurin is an important modulator of a wide range of critical functions in neurons. Calcineurin plays a major role in neuronal gene regulation, and appears to exert considerable control over genes involved with Ca^{2+} signaling and homeostasis (Carafoli et al., 1999; Genazzani et al., 1999; Graef et al., 1999). Moreover, increased activation or overexpression of calcineurin impairs memory (Mansuy et al., 1998), accelerates the decay of long-term potentiation (Winder et al., 1998), and increases vulnerability to apoptosis (Asai et al., 1999; Wang et al., 1999).

In turn, calcineurin may exert negative feedback over its own activation by inactivating specific Ca^{2+} sources, including voltage-sensitive Ca^{2+} channels (VSCCs) and *N*-methyl-D-aspartate (NMDA) receptors (NMDARs). However, this regulation is somewhat controversial. Although a number of studies suggest that calcineurin is important in Ca^{2+} -dependent inactivation of VSCCs and NMDARs (Armstrong, 1989; Tong et al., 1995; Schuhmann et al., 1997; Lukyanetz et al., 1998) other reports have not reached a similar conclusion (Legendre et al., 1993; Krupp et al., 1996; Branchaw et al., 1997; Victor et al., 1997; Zeilhofer et al., 2000). One possible basis for conflicting results, at least for VSCCs, could be the differential expression of multiple VSCC types, or their association with disparate regulatory proteins in different cell types. There are at least five functional types of high-voltage-activated (HVA) VSCCs (L-, N-, P-, Q-, and R-type) in neurons (Fox et al., 1987; Bean, 1989; Fisher et al., 1990; Llinas et al., 1992; Mintz and Bean, 1993; Tsien et al., 1995) and it is well-documented that the mechanisms that regulate different VSCC types are highly selective, and moreover, can vary across cell types (Holz et al., 1986; Hescheler et al., 1987; Bean, 1989; Cox and Dunlap, 1992; Hille, 1994; Dolphin, 1995; Surmeier et al., 1995; Currie and Fox, 1997; Zamponi and Snutch, 1998; Ikeda and Dunlap, 1999). Even for a particular VSCC type, regulatory mechanisms

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Abbreviations: ANOVA, analysis of variance; $[\text{Ca}^{2+}]_i$, intracellular Ca^{2+} concentration; CN-AIP, calcineurin autoinhibitory peptide; CNQX, 6-cyano-7-nitroquinoxaline-2,3-dione disodium; CsA, cyclosporin A; DIV, days *in vitro*; EDTA, ethylene glycol-bis(2-aminoethyl-ether)-*N,N,N',N'*-tetraacetic acid; FKBP-12, FK-506-binding protein 12; HEPES, *N*-(2-hydroxyethyl)piperazine-*N'*-(2-ethanesulphonic acid); HVA, high-voltage activated; HPLC, high-performance liquid chromatography; NMDAR, *N*-methyl-D-aspartate receptor; PCR, polymerase chain reaction; RT, reverse transcription; TEA, tetraethylammonium; VSCC, voltage-sensitive Ca^{2+} channel.

may differ depending on state or environment. Regulation of the L-type VSCC by protein phosphorylation, for example, can depend on whether the full or truncated form of the α_{1C} ($Ca_v1.2$) subunit is present (Hell et al., 1993a,b, 1996), or on whether L-type VSCCs are examined in native vs. non-native cell types (Zong et al., 1995).

It is less well-recognized, however, that calcineurin may also positively regulate some ion channels, including the GABA_A receptor (Jones and Westbrook, 1997) and a renal Cl⁻ channel (Marunaka et al., 1998). Moreover, the possibility that calcineurin positively regulates VSCCs in some cell types has been raised recently. A series of preliminary studies in our laboratory revealed that the calcineurin inhibitor, FK-506, reduced rather than enhanced VSCC currents in cultured hippocampal neurons (Norris et al., 1999). A similar action of FK-506 on VSCCs also has been reported for arterial smooth muscle cells (Yasutsune et al., 1999). In addition, over-expression of calcineurin in transgenic mice enhances Ca²⁺ current density in cardiac myocytes (Yatani et al., 2001). Positive regulation of VSCCs by calcineurin in the hippocampus may be of particular interest, as calcineurin and multiple VSCC types are highly expressed in this brain structure (Fisher et al., 1990; Polli et al., 1991; Kuno et al., 1992; Llinas et al., 1992; Westenbroek et al., 1992; Hell et al., 1993a,b) and appear to be coupled functionally (Graef et al., 1999).

Here, we tested the possible positive modulation of VSCCs by calcineurin in hippocampal neurons using three distinct calcineurin inhibitors [the immunosuppressants FK-506 and cyclosporin A (CsA), and a calcineurin autoinhibitory peptide (CN-AIP)] and two negative controls (rapamycin and okadaic acid). Moreover, the effects of calcineurin inhibition on isolated VSCC subtype currents were examined to determine if hippocampal VSCC regulation by calcineurin is VSCC-type selective. Because L-type VSCC density and N-type VSCC regulation change with age in-culture (Porter et al., 1997; Blalock et al., 1999), somewhat analogously to changes with aging *in vivo* (Thibault and Landfield, 1996), we further examined the effect of age in culture on VSCC regulation by calcineurin.

EXPERIMENTAL PROCEDURES

Cell cultures

Primary hippocampal cell cultures, plated on 35 mm plastic culture dishes, were prepared from fetal pup tissue (embryonic day 18) obtained from pregnant Sprague-Dawley rats using slight modifications of the Banker and Cowan (1977) method, as previously described (Porter et al., 1997; Blalock et al., 1999; Brewer et al., 2001). Results for each condition are based on multiple dishes from at least two cultures. Generally, each rat pup provided enough tissue for two culture dishes. Most experiments were conducted on cells that were between seven and 10 days *in vitro* (DIV) in age (however, a few experiments were conducted on cells that were 13–14 DIV, see Results). For aging studies, sister cultures aged 14–17 DIV (2-week-old) and 28–31 DIV (4-week-old) were compared.

Electrophysiology

Glass electrodes. Recording pipets consisted of glass capil-

lary tubes (Drummond Scientific, Broomall, PA, USA) pulled on a horizontal micropipet puller (model P-87; Sutter Instruments, Novato, CA, USA). Whole-cell pipets were coated with polystyrene Q-dope and had a mean tip resistance of 2.16 ± 0.02 M Ω . Cell-attached patch pipets were coated with Sylgard (Dow Corning, Midland, MI, USA) and had a mean tip resistance of 2.7 ± 0.07 M Ω . All recording pipets were fire-polished immediately before recording (Corey and Stevens, 1983).

Recording solutions. For whole-cell recordings of isolated HVA VSCC currents, external solution contained (in mM): 111 NaCl, 5 BaCl₂, 5 CsCl, 2 MgCl₂, 10 glucose, 10 HEPES, 20 tetraethylammonium (TEA) Cl, 0.01 6-cyano-7-nitroquinoxaline-2,3-dione disodium (CNQX) and 0.001 tetrodotoxin (TTX). pH was adjusted to 7.35 using NaOH and osmolarity adjusted to 330 mOsm using sucrose. Pipette solution for whole-cell recordings contained (in mM): 145 methane sulfonic acid, 10 HEPES, 3 MgCl₂, 11 EGTA, 1 CaCl₂, 5 MgATP, 13 TEA Cl, 0.1 leupeptin. pH was adjusted to 7.35 using CsOH and osmolarity adjusted to 320 mOsm using high-performance liquid chromatography (HPLC) grade H₂O. This ratio of EGTA to Ca²⁺ buffers the intracellular Ca²⁺ concentration ([Ca²⁺]_i) at levels below resting values (e.g., at <100 nM, Bers et al., 1994). To examine whole-cell Ca²⁺, rather than Ba²⁺, currents some experiments exchanged external BaCl₂ for an equimolar amount of CaCl₂. In one subset of these experiments, the internal solution was unchanged, while in other experiments, EGTA and CaCl were omitted and MgATP was replaced with an equimolar amount of 2NaATP.

For cell-attached patch recordings of multichannel activity, the external bath solution contained (in mM): 140 K⁺ gluconate, 3 MgCl₂, 10 D-glucose, 10 EGTA, 10 HEPES. This solution, commonly used in single-Ca²⁺ channel studies, zeros the membrane and thus provides a convenient reference for setting the patch membrane potential (Fox et al., 1987; Fisher et al., 1990). pH was adjusted to 7.35 using KOH and osmolarity adjusted to 300 mOsm with HPLC grade H₂O. The pipet solution consisted of (in mM): 20 BaCl₂, 90 choline Cl, 10 TEA Cl, 10 HEPES. pH was adjusted to 7.35 using TEA-OH and osmolarity adjusted to 290 mOsm using sucrose. Prior to recording, the culture medium in each 35-mm dish was exchanged for 1.5 ml of external recording solution.

Data acquisition. Recordings were obtained according to standard patch-clamp methods (Hamill et al., 1981) using an Axopatch 200A integrating patch-clamp amplifier (Axon Instruments, Foster City, CA, USA). Data were filtered at 2 kHz and digitized at 5 kHz. Voltage commands and data acquisition were controlled by pCLAMP software (Clampex, versions 6 and 7; Axon Instruments). All experiments were carried out at room temperature.

Whole-cell studies. Prior to recording, junction potentials were nulled in the bath using the pipet offset control on the Axopatch 200A. Pipette capacitance was compensated. To estimate whole-cell membrane capacitance and pipet access resistance, a membrane current (filtered at 10 kHz, digitized at 91 kHz) was elicited at the beginning of each experiment with a 15-ms, 5-mV hyperpolarizing step from the holding potential (-70 mV). Current elicited by a 5-mV depolarizing pulse was equal in magnitude, but opposite in polarity. Due to their highly elaborate dendritic arbors, hippocampal neurons are not isopotential and exhibit capacitive current decay kinetics that are probably best fit by multiple exponential functions (Brown and Johnston, 1983; Johnston and Brown, 1983; Spruston et al., 1994). Therefore, we calculated whole-cell capacitance for each cell by integrating the area under the curve of the capacitive transient. The instantaneous peak current measured during the onset of the capacity transient was used to derive the pipet access resistance, which averaged 8.6 ± 0.14 M Ω , and was not significantly affected by the age of the cells or by the pharmacological agents used. Neurons in which the access resistance and/or the holding current changed dramatically during the course of an experiment were excluded from statistical analyses. Series resistance compensation, using the amplifier's whole-cell

correction parameters, was not performed because we, and others, have consistently found no significant differences in the shape or amplitude of activated currents before and after correction (Randall and Tsien, 1995; Porter et al., 1997; Blalock et al., 1999). For most experiments, the uncompensated error in the membrane potential during peak activation (i.e. voltage step to +10 mV) was estimated at 5–7 mV. Holding potential in all experiments was –70 mV. To generate current–voltage (I – V) relationships, cells were stepped in 10-mV increments (10-s interstep interval) from their holding potential at –70 mV, to +60 mV. Drug effects were assessed using a voltage protocol that stepped the membrane potential (for 150 ms) from its holding potential to the voltage that elicited essentially maximal/HVA VSCC current (either +10 mV when EGTA was included, or +20 mV when EGTA was excluded from the pipet solution). Unless otherwise stated, five successive current records (30-s interstep interval) were averaged to estimate maximal current for each cell. Online leak subtraction was carried out using a fractional ($P/5$) method (as described in Porter et al., 1997 and Blalock et al., 1999), which consisted of five fractionally scaled hyperpolarizing subpulses. Current density (pA/pF) was calculated by dividing the mean whole-cell current (during a voltage step) by the membrane capacitance.

Under these recording conditions, whole-cell Ca^{2+} channel currents are typically followed by a long, post-repolarization Ca^{2+} current (e.g. Campbell et al., 1996; Porter et al., 1997). The source of this current remains controversial (cf. discussion in Thibault et al., 1993; Blalock et al., 1999), but the current appears too long to be due to space clamp artifact. Because it does not appear to alter the activated current and its source is not clear, it was not analyzed in the present studies.

Data obtained from I – V curves were normalized and fit to a Boltzmann equation of the form:

$$y = \frac{I}{1 + e^{\left(\frac{V_{1/2} - V}{k}\right)}}$$

where I is the maximal current from the I – V function, V is the voltage, $V_{1/2}$ is the voltage of half-maximal activation, and k represents the steepness of the sigmoid curve. To measure inactivation of HVA Ca^{2+} currents, current amplitude sampled at 1 kHz during a 500-ms pulse was normalized to the peak current amplitude and fit with a double exponential function of the form:

$$f(x) = A_1 e^{-bx} + A_2 e^{-dx} + C$$

where $1/b = \tau_{\text{fast}}$, $1/d = \tau_{\text{slow}}$, A is the amplitude of each exponential component, and C is the steady-state asymptote. Fitted parameters were then compared across treatment groups using a z test.

Cell-attached multichannel patch studies. Seal resistance in cell-attached patch studies averaged $21.9 \pm 0.72 \text{ G}\Omega$ and was not altered by drug treatments. Patches with a seal resistance less than $15 \text{ G}\Omega$ were excluded from statistical analysis. For I – V relationships, patches were stepped in 10-mV increments (10-s interstep interval) from their holding potential to +40 mV. Maximal current in each patch was achieved by stepping the patch membrane from its holding potential of –70 mV to +10 mV. A series of 15 steps to +10 mV was used to generate an average ensemble current for each patch. In prior studies with similar methods (e.g., Thibault et al., 1993; Thibault and Landfield, 1996; Porter et al., 1997; Brewer et al., 2001), we have observed that multichannel responses from the same cell are relatively consistent, and consequently, 15 step-ensembles generate results that are highly similar to those from 30- or 45-step-based ensembles. The relatively large patches employed here, which can contain numerous channels (Thibault and Landfield, 1996; Porter et al., 1997; Brewer et al., 2001), appear to confer stochastic stability. Each depolarization step was separated by 15 s to prevent channel inactivation from confounding the ensemble current amplitude. In each patch, currents generated from a series of hyperpolarizing pulses (equal in amplitude to the depolarizing

steps) were averaged and added offline to ensemble currents to generate leak-subtracted waveforms (Fetchan version 6, Axon Instruments). Current density ($\text{pA}/\mu\text{m}^2$) for each patch was derived by dividing the average ensemble current by the patch area. The patch area, which is inversely proportional to the pipet resistance, was estimated for each patch using the equation $a = 12.6(1/R + 0.018)$, where a is the patch area and R is the pipet resistance (Sakmann and Neher, 1983).

Drugs and drug delivery. FK-506, CsA, rapamycin, and okadaic acid were dissolved in 100% ethanol. These drugs were then diluted $\geq 1000\times$, such that the ethanol concentration in the recording solutions did not exceed 0.1%. Higher concentrations of FK-506 and CsA ($> 20 \mu\text{M}$) typically required sonication to go into solution. CN-AIP was dissolved in dH_2O (at $1000\times$). All drugs were purchased from Calbiochem (La Jolla, CA, USA), except the FK-506-binding protein (FKBP-12), and okadaic acid, which were obtained from Sigma (St. Louis, MO, USA). For incubation studies, drugs were added to the cell culture medium 2–4 h prior to recording. Culture medium then was exchanged for drug-free recording medium immediately prior to the experiment. For intracellular application studies, drugs were dissolved in the pipet solution. In channel antagonist and aging studies, FK-506, dissolved in external recording medium, was delivered to the recording site by free diffusion from a low-resistance glass (weeper) pipet (Blalock et al., 1999). In these latter experiments, CNQX and TTX were included in the weeper pipet, but excluded from the external solution. Consequently, full diffusion from the weeper pipet could be verified by the absence of a fast Na^+ current transient and the paucity of synaptic events in the whole-cell current record.

mRNA analysis

Neuron collection. For mRNA studies, pyramidally shaped neurons from 2-week and 4-week-old sister cultures were aspirated into glass pipets (1–1.5 $\text{M}\Omega$ tip resistance), filled with 5 μl of standard whole-cell internal recording solution. For each culture dish, a single pipet was used to aspirate one neuron at a time until approximately 15 neurons were collected. With this method, the entire neuronal cell body and a portion of the neuron's proximal neurites were cleanly extracted with minimal collection of extraneous surrounding glial or neuronal processes. Pyramidally shaped neurons of the type recorded were selected for collection. For statistical analyses of mRNA data, n equaled the number of culture dishes analyzed in each age group.

Following collection, neurons were immediately pipetted into a pre-chilled microcentrifuge tube containing RNase inhibitor (1 U/ μl) and random hexamers (0.25 mM). This cellular lysate was heated for 10 min at 70°C , cooled on ice and followed by reverse transcription (RT) for cDNA synthesis. The RT was carried out in a 20- μl reaction containing $1\times$ polymerase chain reaction (PCR)II buffer (Perkin Elmer, CA, USA), 3.75 mM MgCl_2 , 0.5 mM dNTPs and 50 U MMLV reverse transcriptase (Gibco BRL). The RT reaction mixture was incubated at 42°C for 1 h followed by heating at 95°C for 10 min and then stored at -20°C until further PCR analysis.

Quantitative real-time PCR. Quantitation of relative mRNA content was performed using an ABI prism 7700 sequence detection system (Applied Biosystems, CA, USA) and the TaqMan methodology, which utilizes the $5'$ nuclease activity of Taq DNA polymerase to generate a real-time quantitative DNA assay. In brief, mRNA-specific oligonucleotide probes (TaqMan probes) containing $5'$ -fluorescent reporter and $3'$ -quencher dyes were designed and used for the extension phase of the PCR. The degradation and release of the reporter dye (i.e. FAM) results in fluorescence at 518 nm, which is monitored during the complete amplification process.

All the cDNA samples from the 2-week-old ($n = 13$) and 4-week-old ($n = 14$) groups were analyzed simultaneously by real-time PCR, with each reaction run in duplicate. The PCR solution was based on the Single Reporter real-time PCR pro-

tocol, as described in the ABI 770 system's manual. For each mRNA species, the amount of reverse transcriptase used for the PCR was optimized to ensure that the results fell within the standard curve. To normalize the amount of calcineurin and calmodulin cDNA in each RT sample, the ribosomal RNA 18S level was measured using a 100-fold dilution of the RT as the template for real-time PCR. The primers for 18S were: forward, 5'-AGTCCCTGCCCTTTGTACACA-3'; reverse, 5'-GATCCGAGGGCCTCACTAAAC-3'; and the TaqMan probe was: 6FAM-CGCCCCGTCGCTACTACCGATTGG-TAMRA (nt 1684–1752, GenBank X01117). For the calcineurin message, 1 μ l of RT was used as the template in a 50- μ l PCR reaction mixture. The primers for calcineurin were: forward, 5'-TCCCGGTGACTGGAGATGTC-3'; reverse, 5'-TTTCAC-CACCCTGTCCGTAGT-3'; the TaqMan probe was: 6FAM-CCCAAGGCGATTGATCCCAAGTTGT-TAMRA (nt 193–264, GenBank D90036). For calmodulin, 0.5 μ l of RT was used as the template in each PCR reaction and the primers were: forward, 5'-GTCTGTCTGGTCTCGGAAACC-3'; reverse, 5'-TGAATTCTGCGATCTGCTCTTC-3'; the TaqMan probe was: 6FAM-CCTTGCAGCATGGCTGACCAACTGA-TAMRA (nt 34–110, GenBank M17069).

PCR quantitative analysis

Real-time PCR quantitation was performed using the ABI 7700 system's software. At the completion of PCR (a total of 40 cycles), the amount of target message in each reaction was determined from the detection threshold cycle number (C_T), which is inversely correlated with the abundance of the message's initial level. The C_T was then converted to relative quantity by normalizing to a standard curve. A standard curve was constructed by running PCR simultaneously using 10-fold serial dilutions of a stock cDNA template containing known quantities of message, as described in the ABI 7700 system's manual.

Statistical comparisons. VSCC current records were analyzed quantitatively using Clampfit 6.0 software (Axon Instruments) and all statistical analyses were performed using Statview 5.01. In most studies, the significance of drug effects was determined by analysis of variance (ANOVA). For studies utilizing pre- and post-drug application measures, effects were determined by repeated measures ANOVA. Post hoc analyses were performed using Scheffé's *F*-test.

RESULTS

Effects of calcineurin and protein phosphatase 1 and 2A inhibition on HVA VSCC currents

In the first series of studies, we asked the question of whether calcineurin activity was necessary for HVA VSCC function in hippocampal neurons, and if so, whether a calcineurin/protein phosphatase cascade (Cohen, 1988) mediated this action. FK-506 and CsA are structurally distinct immunosuppressive agents that specifically inhibit calcineurin activity by binding to separate, endogenously expressed immunophilins. FK-506 binds to FKBP-12, while CsA binds to cyclophilin A (Snyder et al., 1998). Okadaic acid, on the other hand, is a relatively specific inhibitor of protein phosphatases 1 and 2A and exhibits little potency toward calcineurin at drug concentrations of ≤ 1 μ M (Cohen, 1991).

Hippocampal neurons were incubated for 2–4 h in medium containing either FK-506 (at three concentrations: 0.5, 5, or 50 μ M), CsA (20 μ M) or okadaic acid (1 μ M) (see Experimental procedures). These initial stud-

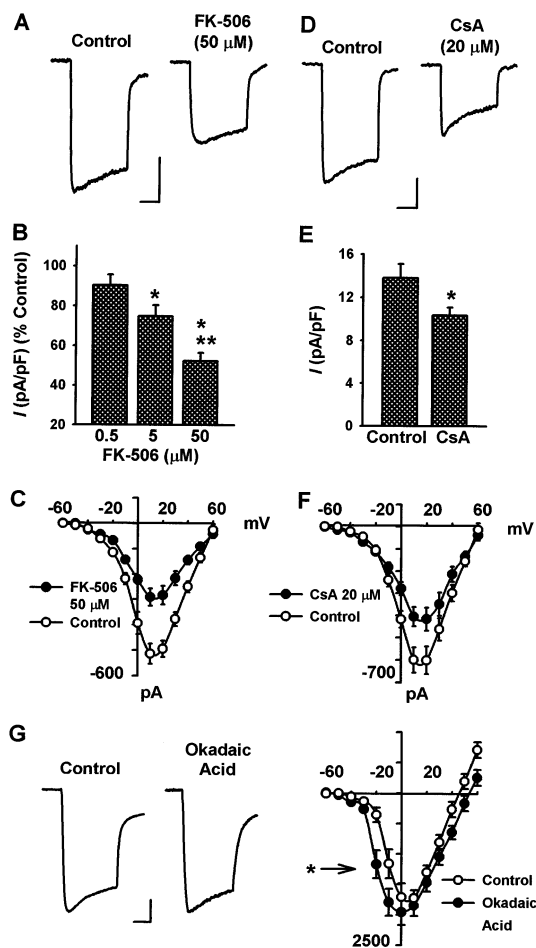


Fig. 1. FK-506 and CsA, but not okadaic acid, inhibited whole-cell HVA Ba^{2+} current. (A, D) Representative examples of averaged VSCC traces (from five successive sweeps) recorded during repeated voltage steps from -70 mV to $+10$ mV in individual vehicle control neurons, or in neurons incubated in FK-506 (50 μ M) (A) or in CsA (20 μ M) (D). (B, E) Mean \pm S.E.M. values for whole-cell current densities in neurons incubated in three concentrations of FK-506 (B), or in neurons incubated in 20 μ M CsA (E). Note that, in (B), current density at each FK-506 concentration is normalized to its respective control group. (C, F) I - V relationships recorded in FK-506 (50 μ M) and CsA (20 μ M)-treated neurons. FK-506 and CsA both reduced current density, but did not significantly alter the voltage dependence of VSCC current. (G) Left panel, representative examples of averaged VSCC traces recorded during a step to $+10$ mV in neurons incubated in 0.1% ethanol (control) or 1 μ M okadaic acid. Right panel, I - V relationships recorded in control and okadaic acid-treated neurons showing a significant leftward shift (arrow) in the okadaic acid group, in the absence of a difference in maximal current. Calibration bars are 250 pA \times 50 ms. *Represents difference ($P < 0.01$) from ethanol control, **difference ($P < 0.01$) from 0.5 and 5 μ M conditions.

ies used drug incubation instead of acute application to ensure equilibrium with immunophilins, which may be limiting in brain (Kung and Halloran, 2000) and other binding sites. Incubation across multiple concentrations of FK-506, revealed a concentration-dependent reduction in whole-cell VSCC Ba^{2+} current density relative to the respective ethanol-incubated control cells ($n = 11$ – 15 control cells at each of the three FK-506 concentrations) [overall ANOVA: $F(3,75) = 17.57$, $P < 0.001$ (Fig. 1A, B)]. Post hoc analyses showed that 0.5 μ M

($n=9$) induced a small, non-significant reduction ($90 \pm 5\%$ of control, $P=0.11$), $5 \mu\text{M}$ ($n=16$) induced an $\sim 25\%$ decrease ($75 \pm 5\%$ of control, $P < 0.05$), and $50 \mu\text{M}$ ($n=13$) resulted in $\sim 50\%$ inhibition ($52 \pm 4\%$ of control, $P < 0.001$, $n=13$). Higher concentrations of FK-506 appeared to induce no further inhibition, although they were difficult to study because of the drug's lipophilicity. At none of the employed concentrations did FK-506 significantly alter the voltage dependence of VSCCs (Fig. 1C). Overall, the half-maximal activation voltage for FK-506 studies was -7 ± 0.76 mV.

CsA at $20 \mu\text{M}$ also significantly reduced VSCC current density by approximately 25% (CsA, 10.4 ± 0.7 pA/pF, $n=13$; control, 13.8 ± 1.2 pA/pF, $n=14$) [$F(1,25)=5.372$, $P < 0.05$], without altering the $I-V$ relationship (Fig. 1D–F). In contrast, cells treated with okadaic acid ($1 \mu\text{M}$) ($n=12$), did not show a significant reduction in maximal current. However, they exhibited a shift to the left in the $I-V$ curve, relative to control cells ($n=12$) (control $V_{1/2} = -11.92$ mV; okadaic acid $V_{1/2} = -20.34$ mV; $z=7.77$, $P < 0.05$; Fig. 1G), indicating that okadaic acid increased voltage sensitivity. Thus, unlike FK-506 and cyclosporin, okadaic acid tends to enhance, rather than inhibit VSCC current. These results indicate that calcineurin activity, but not protein phosphatases 1 and 2A activity, appears to be required for a significant fraction of VSCC current in hippocampal neurons.

Specificity of effects on VSCC current for inhibition of calcineurin

Although both FK-506 and CsA are considered to be specific calcineurin inhibitors, both act as immunophilin ligands and also are immunosuppressants with non-calcineurin-mediated actions (Snyder et al., 1998). In addition, CsA stabilizes mitochondrial Ca^{2+} homeostasis (Connern and Halestrap, 1994; Ankarcona et al., 1996; Friberg et al., 1998). Therefore, to confirm that the inhibition of VSCC current in Fig. 1 was specific to drug actions on calcineurin, we also examined the effects of a specific negative control, rapamycin, and a specific positive control, calcineurin-AIP on HVA Ba^{2+} currents. Rapamycin is an immunosuppressant that is similar in structure to FK-506 and competes for binding to FKBP-12 (Dumont et al., 1990). However, unlike the FK-506/FKBP-12 complex, the rapamycin/FKBP-12 complex does not bind to and inhibit calcineurin (Snyder et al., 1998). Thus, rapamycin is an advantageous agent for separating FK-506's actions on immunophilins from its actions on calcineurin. CN-AIP is similar to the C-terminal region of the calmodulin-binding domain of calcineurin (Hashimoto et al., 1990; Perrino et al., 1995) and acts as a highly specific competitive inhibitor of calcineurin A catalytic activity (Sagoo et al., 1996).

In this series of studies, agents were applied intracellularly to ensure equal cellular access of the compounds being compared. Hippocampal neurons were dialyzed, via the whole-cell recording pipet, with either $5 \mu\text{M}$ FKBP-12 alone (control, $n=29$), or FKBP-12 in combination with either FK-506 ($n=25$), rapamycin ($n=17$),

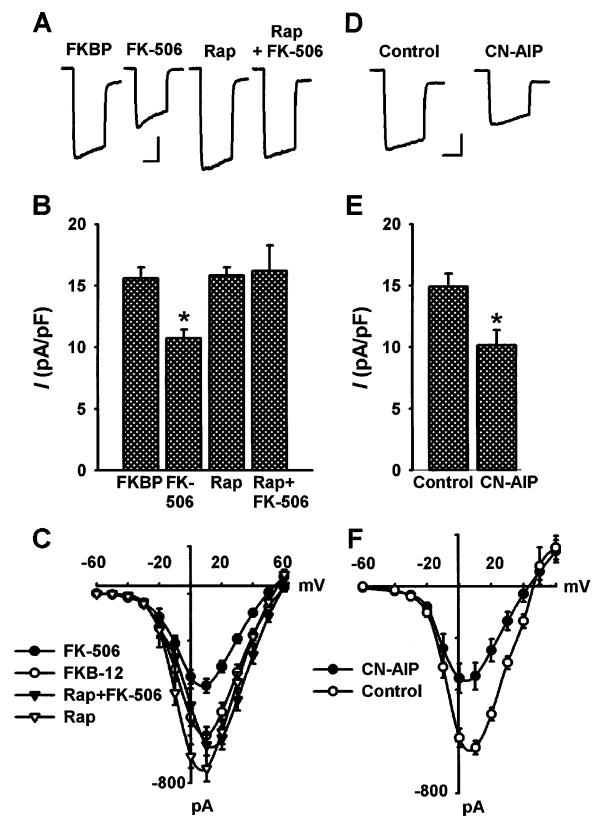


Fig. 2. Reduction of HVA Ba^{2+} current was specific to calcineurin inhibition. Whole-cell currents were recorded in neurons dialyzed with either FKBP-12 alone ($5 \mu\text{M}$), FKBP-12/FK-506 (Rap; $5 \mu\text{M}$), FKBP-12/rapamycin ($5 \mu\text{M}$), or FKBP-12/FK-506 with rapamycin (both at $5 \mu\text{M}$) (A–C), or in neurons dialyzed with CN-AIP ($100 \mu\text{M}$) (D–F). Note that in (A–C), FKBP12 is present in each condition. (A, D) Representative averaged maximal current records from neurons under the various treatment conditions. (B, E) Mean \pm S.E.M. of whole-cell current densities; and (C, F) $I-V$ relationships in each treatment condition. FK-506 and CN-AIP markedly inhibited VSCC current, but rapamycin did not and instead antagonized the effect of FK-506. Scale bar = $250 \text{ pA} \times 50 \text{ ms}$. *Difference ($P < 0.05$) from all other treatment conditions.

or FK-506 together with rapamycin ($n=7$). Thus, FKBP-12 was present in all experimental conditions. All immunophilin ligand concentrations were set at $5 \mu\text{M}$. Recordings were begun after three stable current records were obtained, at approximately 10 min after establishing the whole-cell configuration. At this point, five successive current traces generated by a 150-ms step from -70 mV to $+10$ mV (interpulse interval 30 s) were averaged. Cells treated with FK-506/FKBP-12 exhibited a significant reduction in whole-cell Ba^{2+} current density, relative to all other conditions [$F(3,73)=8.679$, $P < 0.001$] (Fig. 2A, B). Rapamycin/FKBP-12 differed only from the FK-506/FKBP-12 group ($P < 0.01$) and had no inhibitory action on VSCC current. Moreover, when rapamycin was included in the pipet with FK-506/FKBP-12, the inhibitory effects of FK-506 were blocked (post hoc comparison between the FK-506/FKBP-12 and the FK-506/FKBP-12+ rapamycin groups, $P < 0.05$). These findings suggest that rapamycin at $5 \mu\text{M}$ was able to bind FKBP-12, and compete with FK-506. They also indicate that ligand binding to FKBP-12

alone is not sufficient for VSCC inhibition. In contrast to FK-506/FKBP-12, rapamycin/FKBP-12 induced a small leftward shift in the I - V function, such that half-maximal activation occurred at -15.79 ± 0.81 mV as opposed to -10.63 ± 0.7 mV for FKBP-12 alone cells ($z=4.82$, $P<0.05$) and -11.52 ± 1.21 for FK-506/FKBP-12 cells ($z=2.92$, $P<0.05$) (Fig. 2C). Thus, rapamycin and FK-506, which differ primarily in their abilities to inhibit calcineurin, also have strikingly different effects on VSCC current. Rapamycin tended to enhance rather than inhibit VSCC current. Although it inhibits calcineurin activity through a different mechanism from FK-506, CN-AIP (100 μ M) also significantly reduced whole-cell Ba^{2+} current density [$F(1,20)=8.627$, $P<0.01$] (Fig. 2D, E). As with FK-506 and CsA, CN-AIP did not substantially alter the voltage dependence of VSCCs (control $V_{1/2}=-11.73 \pm 0.55$ mV, CN-AIP $V_{1/2}=-13.66 \pm 1.5$) (Fig. 2F). Together, the results indicate that inhibition of calcineurin is the necessary and sufficient factor in the inhibition of HVA VSCC current by these multiple agents.

FK-506 inhibits maximal VSCC current amplitude, but does not affect Ca^{2+} -dependent inactivation

In the previous experiments, Ba^{2+} rather than Ca^{2+} was used as the charge carrier, and the internal $[Ca^{2+}]$ was buffered to the low nanomolar range by the Ca^{2+} chelator EGTA. As such, these conditions may underestimate or confound the role of a Ca^{2+} /calmodulin-dependent enzyme, such as calcineurin, in VSCC regulation. However, although maximal catalytic activity of calcineurin requires a substantial elevation in cytosolic Ca^{2+} (e.g. to the micromolar range), calcineurin can still exhibit significant phosphatase activity in the presence of low Ca^{2+} and/or EGTA (Stewart et al., 1982; Liu et al., 1991; Stemmer and Klee, 1994). Nonetheless, it seems important to determine whether calcineurin regulation of VSCCs is dependent on the charge carrier used, or on whether internal Ca^{2+} is buffered at a low level. In the following experiments, therefore, external BaCl was replaced with an equimolar amount of CaCl. In Fig. 3A, the pipet solution still contained 11 mM EGTA, while the experiments in Fig. 3B, C were conducted with no EGTA in the pipet. As described above for Fig. 2, cells were dialyzed with either FKBP-12 alone (5 μ M) or FKBP-12 along with FK-506 (both at 5 μ M) and maximal VSCC current was measured. As shown in Fig. 3A, replacing external Ba^{2+} with Ca^{2+} did not appear to impair FK-506's capacity to inhibit VSCC current. Similar to the results in Fig. 2, maximal current amplitude recorded from cells dialyzed with FK-506/FKBP-12 ($n=10$) was significantly reduced compared to those dialyzed with FKBP-12 alone ($n=12$) [7.75 ± 0.64 vs. 10.33 ± 0.78 pA/pF, $F(1,20)=6.2$, $P<0.05$].

Fig. 3B shows that removal of EGTA from the pipet solution also did not prevent FK-506 from inhibiting Ca^{2+} current through VSCCs [FK-506/FKBP-12 = 13.35 ± 1 pA/pF, $n=12$; FKBP-12 alone = 16.97 ± 1.27 pA/pF, $n=14$; $F(1,24)=4.8$, $P<0.05$]. The exclusion of EGTA from the pipet solution also gave rise

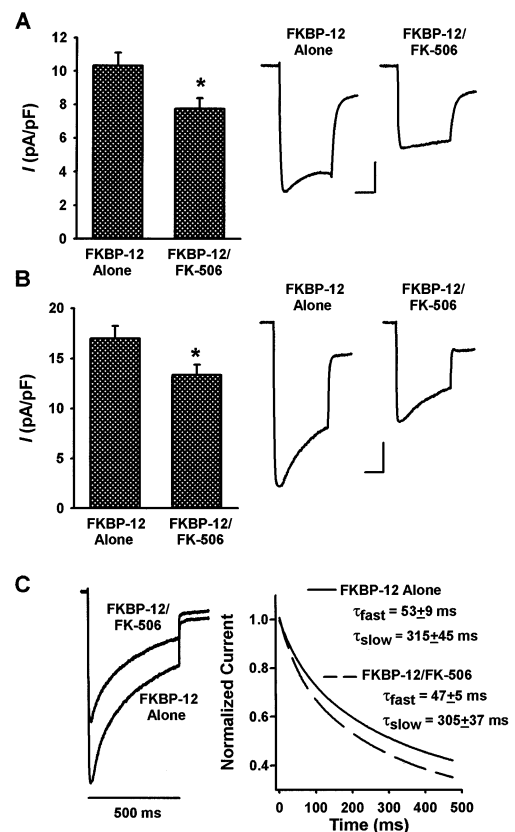


Fig. 3. FK-506 reduced HVA Ca^{2+} currents, but did not alter VSCC inactivation. Extracellular Ba^{2+} was replaced with equimolar Ca^{2+} and neurons were dialyzed with either FKBP-12 alone (5 μ M) or FKBP-12/FK506 (5 μ M) as described in Fig. 2. FK-506 significantly reduced HVA Ca^{2+} currents whether 11 mM EGTA was included (A) or excluded (B) from the pipet solution. Representative averaged maximal current records from neurons under the various treatment conditions are illustrated in the right panels. Scale bar = 250 pA \times 50 ms. (C) Left panel, overlays of averaged maximal Ca^{2+} currents during a 500-ms pulse in neurons without EGTA in the pipet solution. Under these conditions, marked inactivation of VSCC currents was observed. The rate of inactivation for the FKBP-12 alone and FKBP-12/FK-506-treated groups could be fit using a double exponential function (right panel). However, unlike VSCC current amplitude, neither the fast or slow component of VSCC inactivation was significantly altered by FK-506. *Difference ($P<0.05$) from control.

to VSCC currents that exhibited substantial inactivation. That is, with EGTA present in the pipet solution (Fig. 3A), Ca^{2+} current dropped by only $8 \pm 2\%$ from its peak amplitude to its minimum amplitude at the end of the 150-ms voltage step. However, cells recorded without EGTA in the pipet showed a much greater reduction ($34 \pm 3\%$) in VSCC current during the voltage step [$F(1,21)=63.9$, $P<0.001$], indicating considerable Ca^{2+} -dependent inactivation in EGTA-free cells.

To examine whether FK-506 altered Ca^{2+} -dependent inactivation, a 500-ms depolarizing pulse to +20 mV was applied to cells in the absence of internal EGTA (Fig. 3C). The time course of VSCC inactivation could be well-fit using a double exponential function (see Experimental procedures). The results showed that, in contrast to its effects on maximal VSCC amplitude, FK-506 did not significantly alter the rate of VSCC inac-

tivation (Fig. 3C, right panel). However, both the slow and the fast time constants were slightly reduced in the FK-506/FKBP-12 group. Although inactivation kinetics in dendritically ramified neurons can be confounded by multiple compartments (Brown and Johnston, 1983; Johnston and Brown, 1983; Spruston et al., 1994), the absence of an effect on either exponential function indicates that, in hippocampal neurons, FK-506 can reduce whole-cell Ca^{2+} , as well as Ba^{2+} currents, without appreciably altering the inactivation kinetics of HVA VSCCs. Interestingly, cells without EGTA exhibited larger Ca^{2+} currents relative to cells in which Ca^{2+} was buffered [$F(1,24) = 18.42$, $P < 0.001$] (Fig. 3B vs. Fig. 3A). Although the fraction of current inhibited by FK-506 in these two conditions was similar, the results are at least consistent with the possibility that elevating intracellular Ca^{2+} can, under some circumstances, enhance Ca^{2+} channel activity, perhaps via a calcineurin mechanism.

FK-506 inhibits VSCC activity in multichannel cell-attached patches

As noted, the whole-cell recording configuration introduces problems that may confound measures of VSCC activity. For example, hippocampal neurons exhibit extensive dendritic arbors and may be subject to space clamping errors (Brown and Johnston, 1983; Johnston and Brown, 1983; Spruston et al., 1994). In addition, cytosolic components, including calcineurin, immunophilins, kinases, or other phosphatases critical for regulating VSCCs may be lost when cells are dialyzed with the whole-cell pipet recording solution. Analysis of single-channel-level activity in the cell-attached patch-clamp configuration circumvents space clamp problems and avoids membrane rupture, thereby preserving the integrity of cytosolic constituents. Therefore, to test whether FK-506-mediated VSCC inhibition was influenced by the whole-cell recording configuration, VSCC activity also was examined in cell-attached patches in hippocampal neurons after FK-506 treatment.

To enhance access of FK-506 to channels in the patch, cells were incubated in FK-506 (50 μM) for at least 2 h. Neurons exposed to FK-506 ($n = 12$) exhibited a significant decrease in cell-attached VSCC activity ($\sim 45\%$) relative to control patches ($n = 8$) [$F(1,18) = 9.64$, $P < 0.01$] (Fig. 4A, B), with no change in the voltage dependence of VSCCs (data not shown). Thus, inhibition of VSCCs by FK-506 was not limited to the whole-cell recording configuration. Moreover, the magnitude of inhibition was relatively similar in both whole-cell (Fig. 1) and cell-attached patch recording modes (Fig. 4).

Isolation of specific VSCC types

Because saturating concentrations of calcineurin inhibitors did not block all HVA VSCC current, we tested whether calcineurin's apparent enabling or enhancing action might be selective for only some VSCC types. HVA VSCCs in neurons comprise at least five identified functional types, L-, N-, P-, Q- and R-type (Fox et al.,

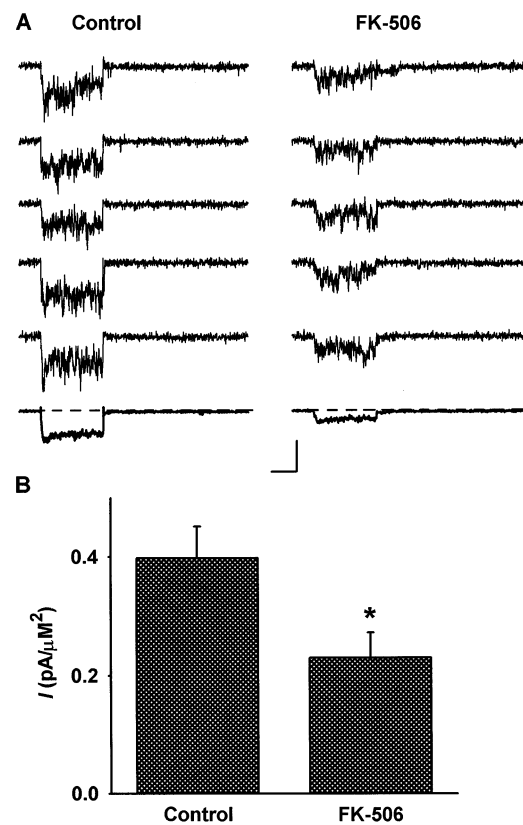


Fig. 4. FK-506 reduced HVA Ba^{2+} current activity in multichannel cell-attached patches. (A) Representative waveforms of five current traces, and the average ensemble current (lowest trace) generated from 15-step depolarizations in multichannel cell-attached patches from vehicle control (left panel) and FK-506-treated (right panel) cells. (B) Similar to whole-cell HVA Ba^{2+} , multichannel patch current was also significantly inhibited by FK-506. Scale bar = 4 pA \times 50 ms. *Difference ($P < 0.01$) from ethanol-treated control.

1987; Bean, 1989; Fisher et al., 1990; Llinas et al., 1992; Mintz and Bean, 1993; Tsien et al., 1995) generally corresponding to different α_1 subunits encoded by specific genes (Birnbaumer et al., 1994; Catterall, 1995). The L-type VSCCs are blocked highly selectively by dihydropyridines, such as nimodipine (Fox et al., 1987; Fisher et al., 1990), whereas N-type and P/Q-type channels are fully and selectively blocked by snail toxins (GVIA and MVIIC conotoxins, respectively) (Mintz and Bean, 1993; Randall and Tsien, 1995; McDonough et al., 1996). Using these specific channel antagonists to isolate VSCC current components, we tested whether any antagonists occlude the effects of FK-506. In these experiments, whole-cell maximal Ba^{2+} current records were obtained every 30 s and specific VSCC antagonist drugs and FK-506 were applied locally to neurons via diffusion from a 'weeper' pipet (see Experimental procedures). Drug application was begun after at least three stable current traces were recorded, which was usually between 5 and 10 min after establishing the whole-cell configuration. Current records for the analysis of drug effects were obtained at the end of a drug application, when current amplitudes were stable. Ten minutes of ethanol vehicle alone ($n = 9$) induced no inhibition or

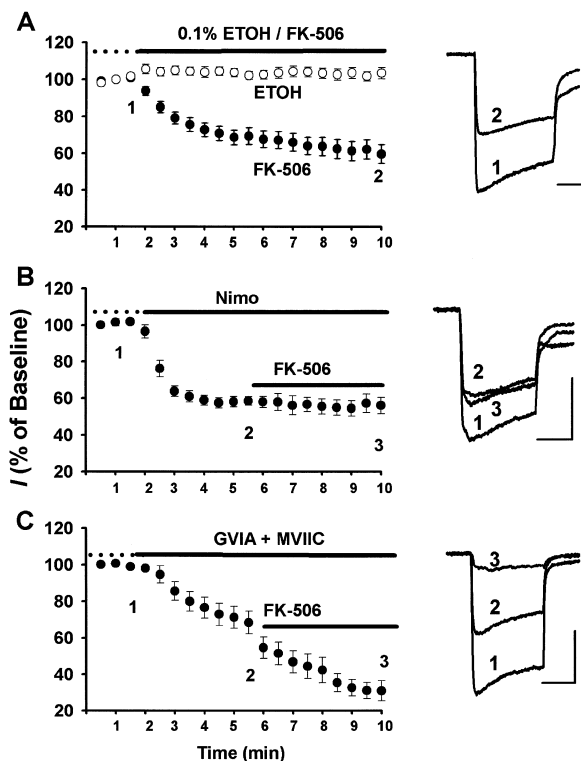


Fig. 5. FK-506 selectively inhibited L-type VSCC current. (A–C) Left panels. Time course plots of mean (\pm S.E.M.), normalized VSCC currents of neurons exposed to acute external perfusion of 0.1% ethanol alone (A, open circles), 50 μ M FK-506 after 0.1% ethanol (A, filled circles), FK-506 following application of the L-VSCC antagonist nimodipine (Nimo; 10 μ M) (B), or FK-506 following application of the N and P/Q type antagonists, conotoxins GVIA (1 μ M) and MVIIC (3 μ M) (C). The dashed line in each time course plot represents a baseline period in which 0.1% ethanol was delivered to neurons. In the right panels are overlays of representative whole-cell VSCC currents recorded from individual neurons at the points indicated in the corresponding time plots. Note that VSCC inhibition by FK-506 was most effective when L-type VSCC current was enriched during GVIA/MVIIC application (C). In contrast, inhibition by FK-506 was absent after L-type VSCC blockade by nimodipine (B). Scale bar = 250 pA \times 50 ms.

rundown of VSCC current (Fig. 5A, open circles). FK-506 (50 μ M) was delivered after 3–5 min application of either 0.1% ethanol alone (Fig. 5A, filled circles), nimodipine (10 μ M) (Fig. 5B), or a solution containing both ω -conotoxins, MVIIC (3 μ M) and GVIA (1 μ M) (Fig. 5C). The occurrence and/or the extent of VSCC inhibition by FK-506 was highly dependent on the components of VSCC current available during FK-506 application [$F(3,31) = 36.7$, $P < 0.001$]. When delivered after 0.1% ethanol, FK-506 induced a slowly developing (usually within 2–3 min) inhibition of VSCC activity, that reached maximal, asymptotic levels by 6–7 min (current remaining after FK-506: $62 \pm 3\%$ of prior ethanol baseline, $n = 16$, $P < 0.001$) (Fig. 5A, filled circles). This FK-506 effect was not reversible upon wash for up to 10 min (data not shown). In contrast, prior inhibition of the L-type fraction of VSCC current with a saturating concentration of nimodipine ($n = 6$) completely occluded further inhibition by FK-506 (current remaining after FK-506: $94 \pm 6\%$ of prior nimodipine baseline, $P > 0.05$) (Fig. 5B).

Conversely, when VSCC current was first inhibited by the N- and P/Q-type VSCC blockers, MVIIC and GVIA ($n = 4$), such that the remaining HVA current comprised L-type and R-type components (Randall and Tsien, 1995; McDonough et al., 1996), FK-506 induced a significantly larger fractional reduction in the remaining L-type-enriched current (current remaining after FK-506: $35 \pm 2\%$ of prior conotoxin baseline, $P < 0.01$, Fig. 5C). Thus, L-type VSCC activity was required for the inhibitory effect of FK-506 on VSCC current (Fig. 5B), and, in L-type channel-enriched HVA VSCC current, FK-506 blocked proportionally more VSCC current (Fig. 5C). Consequently, the L-type VSCC appears to be both necessary and sufficient for the enabling/enhancing action of calcineurin on Ca^{2+} current.

Age-dependent effects of FK-506 on VSCC current

Brain aging has been associated with an increase in the density of available L-type VSCCs and L-VSCC current in hippocampal neurons (Landfield et al., 1989; Disterhoft et al., 1993; Thibault and Landfield, 1996). In an apparently analogous manner, L-VSCC currents and L-type channel density undergo an age-dependent increase in cultured hippocampal neurons, albeit over a much shorter time course. A major increase in L-VSCC current occurs between 1 and 4 weeks in culture (Porter et al., 1997; Blalock et al., 1999). During this same time frame, the inhibitory regulation of N-type and P/Q type VSCCs by the membrane-delimited G-protein-coupled pathway (Holz et al., 1986; Hescheler et al., 1987; Bean, 1989; Cox and Dunlap, 1992; Hille, 1994; Dolphin, 1995; Currie and Fox, 1997; Ikeda and Dunlap, 1999) decreases substantially (Blalock et al., 1999). Therefore, it seemed of considerable interest to determine whether the selective regulation of L-VSCCs by calcineurin also changes with weeks in culture. To answer this question, neurons from 2-week- and 4-week-old sister cultures were perfused with FK-506, under conditions similar to those outlined for studies in Fig. 5. However, rather than using a saturating 50- μ M concentration of FK-506, which appears to block all L-type VSCC activity (Fig. 5A, B), and consequently might obscure differences in the sensitivity or content of calcineurin, cells in the present experiment were perfused with 5 μ M FK-506. Although a significant overall reduction in whole-cell Ba^{2+} current was found across both age groups after FK-506 application [$F(1,14) = 28.6$, $P < 0.001$], the extent of current modulation (measured as percent of baseline) was significantly greater ($P < 0.05$) in 4-week-old (current remaining after FK-506: $73 \pm 7\%$, $n = 8$), relative to 2-week-old cells (current remaining after FK-506: $90 \pm 7\%$, $n = 8$) (Fig. 6A, B). In neither age condition did whole-cell current amplitude change by the end of a 10-min application of 0.1% ethanol vehicle alone (2 weeks, $99 \pm 4\%$, $n = 8$; 4 weeks, $102 \pm 7\%$, $n = 6$, data not shown). Extracellular application of 5 μ M FK-506 did not reduce HVA current as much in younger cells in the present experiment as in the study shown in Fig. 1. This may be related to the extended incubation used in the Fig. 1 study or to normal variability among different

cultures. Nevertheless, the present experiment compared sister cultures and the results reveal a greater FK-506 effect as a function of age in culture. Thus, as L-type VSCC density increases, the calcineurin-dependent component of total HVA VSCC current appears to increase concomitantly. However, the possibility of enhanced sensitivity of calcineurin to FK-506 cannot be ruled out by the present data.

Relative expression of calcineurin mRNA is elevated in older neurons

Hippocampal aging, across months *in vivo* (Herman et al., 1998; Chen et al., 2000) or weeks in culture (Porter et al., 1997), also is associated with an increase in mRNA for the $\text{Ca}_v1.3$ (α_{1D}) pore-forming subunit of the L-type VSCC. Because one explanation for the greater effect of 5 μM FK-506 on VSCC current could be an up-regulation of calcineurin concomitantly with the L-type VSCC increase, we tested whether mRNA levels for the catalytic subunit of calcineurin (calcineurin A) also increase with age in culture. Neurons were collected individually from culture dishes (15 neurons/dish) of 2-week- and 4-week-old cells. mRNA for calcineurin was amplified and quantified using real-time PCR (see Experimental procedures). Semiquantitative estimates (normalized to 18S mRNA) of calcineurin mRNA levels in the 4-week-group ($n=14$) were more than 2-fold greater than those of their 2-week-old counterparts ($n=13$) [$F(1,25)=5.36$, $P<0.05$] (Fig. 6C). However, no significant differences in normalized mRNA content for calmodulin were observed (data not shown), indicating that age-related changes in calcineurin expression may be relatively selective.

DISCUSSION

Channel type-specific enhancing action of calcineurin in hippocampus

As discussed, a number of previous studies have found evidence that calcineurin mediates aspects of negative feedback regulation of VSCCs (Armstrong, 1989; Schuhmann et al., 1997; Lukyanetz et al., 1998; Burley and Sihra, 2000), in particular, of N-type VSCCs (Zhu and Yakel, 1997; Lukyanetz et al., 1998). Moreover, in some cells, D_2 dopamine receptors apparently inhibit L-type VSCCs via a calcineurin-mediated pathway (Hernandez-Lopez et al., 2000). Conversely, the present studies provide the first evidence, to our knowledge, of a positive regulatory mechanism of calcineurin on VSCCs in neurons. No indication of an action of calcineurin on Ca^{2+} -dependent inactivation was seen in this preparation (Fig. 3). Thus, this finding appears to extend the class of channel type-specific VSCC regulatory mechanisms (e.g., Bean, 1989; Hille, 1994; Dolphin, 1995; Surmeier et al., 1995; Zamponi and Snutch, 1998; Ikeda and Dunlap, 1999) to include an enhancing/enabling action of calcineurin on L-type VSCCs.

In the present work, a series of disparately acting cal-

calcineurin inhibitors, phosphatase inhibitors, and immunophilin ligands were compared to show that the common feature among agents that suppressed VSCC current was calcineurin inhibition. That is, these studies found strong evidence that calcineurin inhibition is both necessary (e.g. the similar FKBP-12 immunophilin ligand, rapamycin, which does not inhibit calcineurin, did not inhibit VSCC current) and sufficient (CN-AIP, which inhibits calcineurin but does not bind to immunophilins, also inhibited VSCC current) for mediating inhibition of VSCC current (Fig. 2). Consistent with these results, the PPI/PP2A inhibitor, okadaic acid, did not suppress VSCC current (Fig. 1). Finally, the effect of calcineurin inhibition did not depend on the conditions of the whole-cell recording configuration, as VSCC inhibition was of relatively similar magnitude in the cell-attached patch configuration (Fig. 4). Together, the results indicate clearly that inhibition of VSCCs by these agents specifically required calcineurin inhibition.

The enhancing effect of calcineurin also was targeted selectively to one type of VSCC, the L-type. The functional availability of L-type VSCCs appeared to be both necessary (i.e., nimodipine, the specific L-type antagonist, blocked all action of FK-506 on VSCCs) and sufficient (in the absence of N-, and P/Q-type VSCC activity, the relative FK-506 effect on L-type current was considerably greater) for the full manifestation of FK-506-mediated inhibition of VSCC currents in hippocampal neurons. Thus, the L-type VSCC was the only VSCC that was dependent on some degree of calcineurin phosphatase activity. Moreover, calcineurin-inhibition did not shift VSCC voltage dependence but instead appeared to block fully the activation of L-VSCCs. These data imply that calcineurin action is essential for enabling L-type VSCC availability in hippocampal neurons. However, it is also possible that, in addition to enabling availability, calcineurin enhances L-type VSCC activity through other processes (e.g. increased open probability).

Magnitude of the L-type current

Previous studies have found that L-type currents account for ~ 20 – 50% of somal VSCC currents in brain neurons (Fisher et al., 1990; Mintz and Bean, 1993; Randall and Tsien, 1995; Blalock et al., 1999) (although the conductance of L-type VSCCs, in particular, may vary depending on whether Ca^{2+} or Ba^{2+} is the charge carrier (Church and Stanley, 1996). The L-type component of HVA Ba^{2+} current increases with age in culture from $\sim 30\%$ in younger (1–2-week-old) to nearly 50% of current in older (3–4-week-old) hippocampal cultured neurons (Porter et al., 1997; Blalock et al., 1999). However, there is significant variability across cultures, such that comparisons across ages are generally carried out in sister cultures (see Experimental procedures). In the present study, a slightly larger L-type component appeared to be present in younger cells than in earlier studies. This is indicated by the $>40\%$ of current blocked by nimodipine in younger neurons (Fig. 5B), which was similar to the $\sim 38\%$ blocked by 50 μM FK-506 under similar application conditions (Fig. 5A).

Moreover, in one experiment of the present studies, 50 μM FK-506 suppressed $\sim 50\%$ of VSCC current in younger neurons (Fig. 1). Conceivably, the prolonged incubation with FK-506 in the Fig. 1 study could have resulted in inhibition of a small residual amount of L-type current not readily blocked by acute application of nimodipine or FK-506. Alternatively, the differences between these studies could be due to normal variability across cultures.

Calcineurin sensitivity to inhibitors and $[\text{Ca}^{2+}]_i$

Studies with calcineurin inhibitors in neuronal tissues (e.g., Tong et al., 1995; Lukyanetz et al., 1998; Zeilhofer et al., 2000) often have employed drug concentrations that are high relative to the IC_{50} found in *in vitro* biochemical studies (Liu et al., 1991) or peripheral cell assays (Kung and Halloran, 2000). The relatively high effective concentrations of FK-506 in the present study (Fig. 1) also exceeds those seen in *in vitro* and peripheral assays. This discrepancy may relate to the recent observation that, contrary to the general assumptions, immunophilins in many tissues, particularly brain, are limiting for calcineurin inhibition by FK-506 and CsA (Kung and Halloran, 2000). This limitation might somehow result in an altered IC_{50} (e.g., immunophilins may be less accessible in neurons). Alternatively, there may be endogenous competitors or multiple compartments of calcineurin, with varying accessibility.

Maximal catalytic activity of calcineurin requires a large rise in $[\text{Ca}^{2+}]_i$ (to the μM range) and subsequent binding to Ca^{2+} /calmodulin (Stemmer and Klee, 1994). However, many of the present studies were performed in neurons in which Ba^{2+} was the charge carrier and/or $[\text{Ca}^{2+}]_i$ was buffered by EGTA to near-resting (< 100 nM) concentrations (Bers et al., 1994). Thus, $[\text{Ca}^{2+}]_i$ presumably did not rise substantially during current influx. Nevertheless, calcineurin also shows some phosphatase activity at resting or low $[\text{Ca}^{2+}]_i$ levels, stimulated by Ca^{2+} bound to the calcineurin B subunit of the calcineurin holoenzyme. This activity is seen in the absence of calmodulin or in the presence of EGTA (Stewart et al., 1982; Liu et al., 1991; Stemmer and Klee, 1994). Therefore, it can be concluded that relatively low levels of calcineurin activity at near-resting $[\text{Ca}^{2+}]_i$ can mediate the enabling action of calcineurin on L-type VSCCs. However, EGTA is a relatively slow Ca^{2+} buffer and the possibility that intracellular Ca^{2+} release and/or capacitive Ca^{2+} entry increased calcineurin activity above anticipated levels cannot be ruled out. Finally, the preliminary evidence that Ca^{2+} currents were larger when $[\text{Ca}^{2+}]_i$ was not buffered (Fig. 3A,B) raises the intriguing possibility that, while low or basal levels of $[\text{Ca}^{2+}]_i$ may be able to maintain the basic enabling effect of calcineurin on L-type VSCCs, elevating $[\text{Ca}^{2+}]_i$, and in turn, increasing calcineurin activity, may recruit previously unavailable L-type VSCCs or enhance channel open probability. Clearly additional studies will be required to clarify the Ca^{2+} dependence of calcineurin's actions on L-type VSCCs in hippocampal neurons.

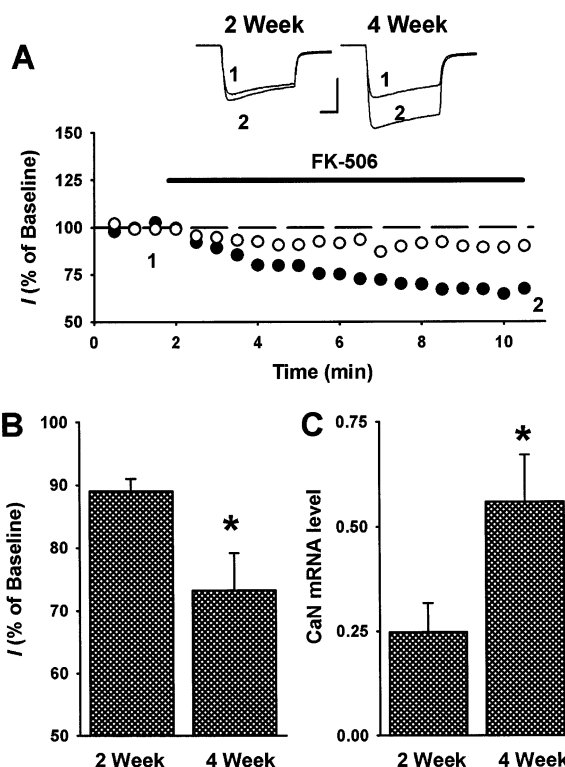


Fig. 6. Effects of age in culture on VSCC inhibition by FK-506 and calcineurin mRNA level. (A) Representative time plots of normalized VSCC currents from a representative 2-week (open circles) and a representative 4-week (filled circles) old neuron during acute application of FK-506 (5 μM). (Inset) Overlays of representative VSCC current traces from individual 2-week and 4-week-old neurons recorded at the points indicated in the time plot. (B) Mean \pm S.E.M. data showing that the fraction of current reduction (percent of baseline) induced by FK-506 was substantially greater in the 4-week-old group. (C) Concomitantly, relative calcineurin A (CaN) mRNA content exhibited a more than 2-fold increase from 2 to 4 weeks in culture. Scale bar = 500 pA \times 50 ms. *Difference ($P < 0.05$) from the 2-week group.

Channel enablement by dephosphorylation

Higher phosphorylation/dephosphorylation ratios appear to positively modulate ion channel and ionotropic receptor functions under many conditions (Armstrong, 1989; Artalejo et al., 1992; Catterall, 1995; Dolphin, 1996). However, phosphorylation at some sites also can decrease ion channel activity. For example, protein kinase G-dependent phosphorylation of the L-type α_{1C} subunit at position Ser (533) inhibits L-VSCC activity (Jiang et al., 2000). Moreover, calcineurin has been found to enhance the activity of other ion channel types (Jones and Westbrook, 1997; Marunaka et al., 1998). Conceivably, therefore, calcineurin activity might enable L-type VSCCs by dephosphorylating channel sites at which phosphorylation during channel opening contributes to inactivation. This could imply a rapid and continuous modulation of L-type VSCC availability that might require a close physical juxtaposition and/or complexing of calcineurin and L-VSCCs. Interestingly, there is some evidence that calcineurin is localized on neuronal membranes in close

association with VSCCs (Lukyanetz, 1997). Moreover, there is increasing evidence of physical and functional coupling of protein phosphatases with intracellular Ca^{2+} channels (Cameron et al., 1995) or L-type Ca^{2+} channels (Davare et al., 2000). However, the possibility that calcineurin acts on L-type VSCCs indirectly through some other enzyme pathway obviously cannot be ruled out.

Age-dependent changes and functional implications

The present studies found that VSCC current was more sensitive to inhibition by 5 μM FK-506 in older than in younger cultured neurons (Fig. 6A, B). This might reflect an increase in calcineurin content, potentially as a required coordinated up-regulation with an increasing availability of L-type channels (Porter et al., 1997; Blalock et al., 1999). The relative age-related increase in calcineurin A mRNA content (Fig. 6C) is at least preliminarily consistent with this possibility. On the other hand, the results could also be accounted for by a heightened calcineurin sensitivity to FK-506 and/or an age-dependent increase in immunophilin abundance, among other possibilities.

Interestingly, this is the third VSCC-type-specific process found to change with age in long-term hippocampal culture. The other two include an increase in L-type VSCC density (Porter et al., 1997), and a substantial decline in membrane-delimited, G-protein-coupled inhibition of non-L-type VSCCs (Blalock et al., 1999). Thus, with age in culture, multiple changes in VSCC-type-specific regulation occur that seem likely to increase Ca^{2+} influx.

Although these VSCC alterations in culture may well

reflect basic developmental processes (e.g., (Turrigiano et al., 1994), there also is some evidence that they may be, in part, analogous to brain aging processes. That is, changes in VSCC activity have been found consistently in models of brain aging and likely contribute to age-related alterations in Ca^{2+} -dependent processes such as neuronal excitability, plasticity, and memory (cf., reviews, Khachaturian, 1989; Landfield et al., 1989; Disterhoft et al., 1993; Foster and Norris, 1997; Thibault et al., 1998; Verkhratsky and Toescu, 1998; Griffith et al., 2000). Further, growing evidence indicates that calcineurin function may be increased with brain aging (Norris et al., 1998; Foster et al., 2001) and, in preliminary studies, FK-506 inhibited VSCC current in hippocampal slice neurons of aged rats (Norris et al., 2001).

Additional studies will of course be required to determine whether similar aging changes in calcineurin content or sensitivity occur during aging *in vivo*. However, regardless of whether calcineurin regulation changes with brain aging, the present studies suggest that calcineurin plays a major enabling/enhancing role in normal and pathological processes mediated by L-type VSCC activity. As a corollary, modulation of calcineurin may provide a basis for developing new therapeutic strategies aimed at regulating L-type VSCC activity and L-type VSCC-dependent disorders.

Acknowledgements—This work was supported in part by Grants from the National Institute on Aging (AG04542, AG10836) and Training Grants (AG00242 and 1F32 AG05903). We wish to thank Elsie Barr, Janice Staton, and Veronique Thibault for important technical assistance, and Kelley Secrest and Judy Hower for excellent assistance with the manuscript.

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(Accepted 13 November 2001)