CALCIUM-DEPENDENT INACTIVATION OF NEURONAL CALCIUM CHANNEL CURRENTS IS INDEPENDENT OF CALCINEURIN

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Abstract—Dephosphorylation by the Ca^{2+} /calmodulin-dependent phosphatase calcineurin has been suggested as an important mechanism of Ca^{2+} -dependent inactivation of voltage-gated Ca^{2+} channels. We have tested whether calcineurin plays a role in the inactivation process of two types of high-voltage-activated Ca^{2+} channels (L and N type) widely expressed in the central nervous system, using the immunosuppressive drug FK506 (tacrolimus), which inhibits calcineurin after binding to intracellular FK506 binding proteins. Inactivation of L- and N-type Ca^{2+} channels was studied in a rat pituitary tumor cell line (GH_3) and chicken dorsal root ganglion neurons, respectively. With the use of antisera directed against the calcineurin subunit B and the 12,000 mol. wt binding protein, we show that both proteins are present in the cytoplasm of GH_3 cells and chicken dorsal root ganglion neurons. Ionic currents through voltage-gated Ca^{2+} channels were investigated in the perforated-patch and whole-cell configurations of the patch-clamp technique. The inactivation of L- as well as N-type Ca^{2+} currents could be well fitted with a bi-exponential function. Inactivation was largely reduced when Ba^{2+} substituted for extracellular Ca^{2+} or when the Ca^{2+} chelator EGTA was present intracellularly, indicating that both types of Ca^{2+} currents exhibited Ca^{2+} -dependent inactivation. Extracellular (perforated-patch configuration) or intracellular (whole-cell configuration) application of FK506 to inactivate calcineurin had no effect on the amplitude and time-course of Ca^{2+} channel current inactivation of either L- or N-type Ca^{2+} channels. In addition, we found that recovery from inactivation and rundown of N-type Ca^{2+} channel currents were not affected by FK506.

Our results provide direct evidence that the calcium-dependent enzyme calcineurin is not involved in the inactivation process of the two Ca^{2+} channel types which are important for neuronal functioning, such as gene expression and transmitter release. © 1999 IBRO. Published by Elsevier Science Ltd.

Key words: Ca^{2+} channel, inactivation, calcineurin, FK506, FKBP.

An increase in the intracellular concentration of free Ca^{2+} ($[\text{Ca}^{2+}]_i$) plays a pivotal role in a large variety of physiological functions, such as excitation–secretion coupling and neuronal plasticity. On the other hand, excessive increases in $[\text{Ca}^{2+}]_i$ may lead to excitotoxic cell death, which occurs, for example, during brain ischemia and epileptic seizures.¹⁶ In neurons, one important pathway that leads to an increase in $[\text{Ca}^{2+}]_i$ is the opening of voltage-gated Ca^{2+} channels in the plasma membrane. The activity of these Ca^{2+} channels is effectively regulated by G-protein-coupled receptors and by Ca^{2+} - and voltage-dependent inactivation of these ion channels.^{3,13,40} Among the different types of Ca^{2+} channels, Ca^{2+} -dependent inactivation is best documented for L-type Ca^{2+} channels.^{2,23,24,34,46} However, a Ca^{2+} -mediated component of Ca^{2+} channel inactivation has also been described for N-type channels (e.g., Ref. 25).

Different mechanisms have been proposed for Ca^{2+} -dependent inactivation of neuronal Ca^{2+} channels. One hypothesis which is still widely accepted is that inactivation of Ca^{2+} channels is mediated, at least in part, by enzymes. Dephosphorylation by the Ca^{2+} /calmodulin-dependent serine/threonine phosphatase calcineurin (CaN), also known as phosphatase 2B, has originally been proposed as the enzymatic mechanism

for inactivation of neuronal Ca^{2+} channels in both molluscan and mammalian neurons⁹ (for a review see Armstrong and Eckert⁵). Overexpression of human CaN in rodent neuroblastoma × glioma hybrid cells increased the Ca^{2+} -dependent inactivation of N-type and, to a lesser degree, L-type Ca^{2+} channels.³² A physiological significance of this phenomenon is suggested by the observation that CaN and voltage-gated Ca^{2+} channels are co-localized in dorsal root ganglion cells.³¹ Such a phosphatase-mediated mechanism of inactivation could counteract the well known up-regulation of L-type Ca^{2+} channels by phosphorylation via cyclic-AMP-dependent protein kinase.³

We have tested the possible involvement of CaN in the Ca^{2+} -dependent inactivation of L- and N-type Ca^{2+} channels in rat pituitary tumor (GH_3) cells and chicken dorsal root ganglion (DRG) neurons using the specific CaN inhibitor FK506.³⁰ This immunosuppressive drug forms an inactive tertiary complex with CaN after binding to intracellular receptor proteins called FK506 binding proteins (FKBPs). We found that CaN and at least the 12,000 mol. wt FKBP (FKBP-12) are present in GH_3 cells and in chicken DRG neurons. Application of FK506, however, had no effect on amplitudes and inactivation kinetics of Ca^{2+} channel currents in either GH_3 or chicken DRG neurons, suggesting that CaN is not involved in L- or N-type Ca^{2+} channel inactivation in these cells.

EXPERIMENTAL PROCEDURES

Cell preparation

GH_3 cells (American Type Culture Collection, Rockville, U.S.A.) were grown in 35-mm tissue culture dishes (Nunc, Wiesbaden,

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Abbreviations: $[\text{Ca}^{2+}]_i$, intracellular free Ca^{2+} concentration; CaN, calcineurin; CaN-B, B subunit of calcineurin; DRG, dorsal root ganglion; EGTA, ethyleneglycolbis(β-aminoethyl ether)-N,N,N',N'-tetra-acetate; FKBP, FK506 binding protein; FKBP-12, 12,000 mol. wt FK506 binding protein; HEPES, N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid; TBS, Tris-buffered saline.

Germany) at 37°C in a humidified 5% CO₂/95% air atmosphere in minimum essential medium supplemented with 10% fetal calf serum, 0.3% D-glucose, 2 mM L-glutamine, 25 U/ml penicillin and 25 mg/ml streptomycin.

Dissociated cultures of chicken DRG neurons were prepared as described previously.³⁹ Briefly, sensory ganglia from 10- to 12-day-old chick embryos were dissociated by gentle trituration after a 20-min incubation at 37°C in Ca²⁺-free Spinner salt solution containing 0.1% trypsin. Dissociated cells were plated on poly-D-lysine-coated glass coverslips and maintained in Eagle's basal medium supplemented with 10% horse serum, 0.3% D-glucose, 2 mM L-glutamine, 25 U/ml penicillin and 25 mg/ml streptomycin.

Immunocytochemistry and confocal microscopy

GH₃ cells were plated on poly-D-lysine-coated slides. Monolayers were fixed with 4% formaldehyde/phosphate-buffered saline for 10 min at 4°C and permeabilized with acetone (100%) at 18°C. Non-specific protein binding sites were blocked with 10% fetal calf serum for 30 min at room temperature. Cells were then incubated with the respective antibodies (dilution 1:50) overnight at 4°C. FKBP was stained with an antiserum raised against a consensus sequence of human FKBP-12; CaN was stained with a polyclonal antiserum directed against the regulatory subunit of CaN (CaN-B). After intensive washing, monolayers were incubated with fluorescein isothiocyanate-tagged goat anti-rabbit immunoglobulin G antibodies for 3 h at room temperature. Single optical sections about 400 nm thick were taken with a Biorad MRC 1000 confocal microscope attached to a Nikon Diaphot 300 equipped with an oil immersion objective lens ($\times 60$) of 1.4 numerical aperture using the 488 nm line of a krypton-argon laser.

Chick embryos at embryonic day 12 were fixed in 4% formaldehyde/phosphate-buffered saline and cryosectioned at 12 μ m. Cryostat sections were mounted on poly-L-lysine-coated slides and incubated after a blocking step with 1% bovine serum albumin, 5% goat normal serum and 0.5% Triton X-100 in Tris-buffered saline (TBS) with rabbit anti-CaN or rabbit anti-FKBP-12 at a dilution of 1:100 in TBS overnight at room temperature. After buffer rinse, a Cy3-tagged goat anti-rabbit immunoglobulin G diluted 1:800 in TBS was applied for 1 h at room temperature. After a final buffer rinse, the sections were coverslipped in TBS/glycerol (1:1) at pH 8.6. Photodocumentation was done using a Leica Aristoplan epifluorescence microscope equipped with filtercube N2.1 and Kodak Tmax 400 black and white film. Control experiments included omission of the primary antiserum, application of normal rabbit serum and incubation with primary antibody pre-absorbed with the relevant antigen.

Electrophysiology

Electrophysiological experiments were performed at room temperature in acutely dissociated chicken DRG neurons and GH₃ cells 3–12 h and two to seven days after plating, respectively. Ionic currents were recorded either in the whole-cell¹⁹ or perforated-patch configuration²² of the patch-clamp technique using an EPC-7 amplifier (List Electronics, Pfungstadt, Germany). Pipettes pulled from borosilicate glass (KIMAX 51, Kimble, U.S.A.) and fire polished had resistances of 2–3 M Ω in standard recording solutions. The standard extracellular solution contained (in mM): 145 NaCl, 5 KCl, 5 CaCl₂, 2 MgCl₂, 10 HEPES, 10 D-glucose (pH 7.30), adjusted with NaOH. To record ionic currents through Ca²⁺ channels in isolation from Na⁺ currents, 1 μ M tetrodotoxin was added to the external solution. In some experiments, 5 mM CaCl₂ was substituted with 5 mM BaCl₂.

The standard internal solution contained (in mM): 120 CsCl, 20 tetraethylammonium chloride, 2 MgCl₂, 3 Na₂ATP, 0.2 Na₂GTP, 5 EGTA, 10 HEPES (pH 7.30), adjusted with CsOH. In some experiments, 1 mM EGTA was used instead of 5 mM EGTA. To obtain perforated patches, nystatin (150 μ g/ml, 0.3% dimethyl sulfoxide) was added to the pipette solution and Na₂ATP and Na₂GTP were omitted.

Materials

Antisera directed against FKBP-12 and CaN were from ABR (Golden, CO, U.S.A.). Fluorescein isothiocyanate-tagged goat anti-rabbit immunoglobulin G antibodies were from Dianova (Hamburg, Germany). Cy3-tagged goat anti-rabbit immunoglobulin G was also from Dianova. FKBP-12 was from Sigma Chemie (Deisenhofen,

Germany). FK506 (Calbiochem, Bad Soden, Germany) and nifedipine (a gift from Bayer, Leverkusen, Germany) were dissolved in dimethyl sulfoxide.

RESULTS

Expression and cellular distribution of calcineurin and FKBP-12

In GH₃ cells, both cytosol and nucleus were homogeneously stained by a polyclonal antiserum directed against FKBP-12 (Fig. 1A). In control experiments, staining was almost completely prevented by preabsorption of the primary antibody with FKBP-12. CaN immunoreactivity was detected using a polyclonal antiserum from rabbit which recognizes CaN-B. Immunoreactivity against CaN was detected in the cytosol and in the nucleus of GH₃ cells (Fig. 1C).

In DRGs of chick embryos (embryonic day 12), there was a homogeneous cytoplasmic immunostaining in a majority of neuronal cell bodies for both FKBP-12 (Fig. 1B) and CaN-B (Fig. 1D). There was also immunoreactivity in axons of dorsal roots and spinal nerves. Within the spinal cord, neuropil of both ventral and dorsal horns displayed intense immunostaining for CaN-B, but not for FKBP-12.

Inactivation of l-type calcium channel currents

In a first set of experiments, Ca²⁺ current inactivation was studied in GH₃ cells. These cells possess mainly L-type (and T-type) Ca²⁺ channels.³⁶ In these experiments, ionic currents through voltage-gated Ca²⁺ channels were recorded using the perforated-patch configuration of the patch-clamp technique with either Ca²⁺ or Ba²⁺ as the charge carrier. This approach was chosen since it prevents the washout of intracellular constituents which may influence Ca²⁺ channel inactivation or which may be necessary for the inhibitory effect of FK506 on CaN. Figure 2A shows averages of 10 current traces evoked by 200-ms depolarizations to 0 mV from a resting potential of -40 mV. This holding potential was chosen to record high-voltage-activated Ca²⁺ channel currents in isolation from low-voltage-activated Ca²⁺ currents. Under these experimental conditions, inward currents carried by Ca²⁺ ions had amplitudes in the range of 20 pA, inactivated by about 60% within 150 ms and were completely blocked by 100 μ M Cd²⁺ or by 10 μ M nifedipine (not shown). Inactivation was nearly absent when Ca²⁺ was substituted by Ba²⁺.

With Ca²⁺ as the charge carrier, Ca²⁺ channel currents could be well fitted to a double exponential function, with time constants $\tau_1 = 11.4 \pm 0.6$ ms and $\tau_2 = 116 \pm 12$ ms (mean $\pm$ S.E.M., $n = 10$). Extracellular application of FK506 (10 μ M, $n = 5$) had no significant effect on either amplitude or current decay kinetics (Fig. 2B, Table 1), suggesting that CaN was not involved in the inactivation of Ca²⁺ channel currents in GH₃ cells (also see Victor *et al.*⁴²).

Inactivation of N-type calcium channel currents

Ca²⁺ current inactivation was further studied in chicken DRG neurons. The high-voltage-activated Ca²⁺ channel currents in these cells belong mainly to the ω -conotoxin GVIA-sensitive N type.⁷ In these cells, conventional whole-cell patch-clamp recordings were performed, which allowed us to dialyse the cells with a known concentration of FK506 and in some experiments also with a complex of FK506 and FKBP-12. Ca²⁺ channel currents were again elicited by

Fig. 1. Immunocytochemical detection of FKBP-12 (a, b) and CaN (c, d) in GH₃ cells (a, c) and chick embryonic DRG cells (b, d). In DRGs, the majority of neurons and dorsal root axons (d, to the left) are labeled. Due to the confocal imaging process, the heterochromatin of the nucleolus of GH₃ cells stays out in black (a, c). Note granular immunostaining for FKBP in the nucleolus of GH₃ cells (a). a and c are confocal images, b and d conventional epifluorescence micrographs. Scale bars = 50 μm (a, c), 100 μm (b, d).

Fig. 2. Ca^{2+} current inactivation in GH_3 cells. Ionic currents through voltage-gated Ca^{2+} channels were recorded using the perforated-patch configuration of the patch-clamp technique with either Ca^{2+} or Ba^{2+} as the charge carrier. A and B show averages of 10 consecutively recorded current traces. (A) With 5 mM external Ca^{2+} as the charge carrier, inward currents elicited by a 200-ms voltage step from -40 to 0 mV had peak amplitudes of about 20 pA. These currents were completely blocked by application of $100 \mu\text{M}$ Cd^{2+} and exhibited pronounced inactivation during the depolarization. When Ca^{2+} was substituted by 5 mM Ba^{2+} , inactivation was largely reduced from about 60% in the presence of Ca^{2+} to less than 30%. (B) When Ca^{2+} was used as the charge carrier, inactivation kinetics could be well fitted using a double exponential function of the form $I(t) = A - B \exp(-t/15 \text{ ms}) - C \exp(-t/89 \text{ ms})$ in these experiments. External application of FK506 for 10 min had no significant effect on the time-course of inactivation.

depolarizing voltage steps from -40 to 0 mV for several hundred milliseconds. With 5 mM external Ca^{2+} as the charge carrier, currents had amplitudes of 1–2 nA. To test for the Ca^{2+} dependence of inactivation, the neurons were dialysed with different concentrations of the Ca^{2+} chelator EGTA (1 and 5 mM). With 1 mM EGTA, Ca^{2+} currents inactivated again in a bi-exponential manner, with time constants $\tau_1 = 39 \pm 6$ ms and $\tau_2 = 400 \pm 39$ ms ($n = 10$). When 5 mM EGTA was used, time constants increased significantly to $\tau_1 = 145 \pm 29$ ms and $\tau_2 = 936 \pm 163$ ms ($n = 5$), respectively. FK506 ($10 \mu\text{M}$, internally applied) again had no significant effect on Ca^{2+} current inactivation under these conditions ($\tau_1 = 48 \pm 5.3$ ms and $\tau_2 = 474 \pm 43$ ms, $n = 9$; Fig. 3, Table 1). Similar results were obtained when the cells were perfused with the complex of FK506 and FKBP-12 ($1 \mu\text{M}$).

Recovery from inactivation

Although the involvement of CaN in the inactivation process of Ca^{2+} channels during a depolarizing voltage step appears unlikely from our experiments, this enzyme may still influence the ratio of phosphorylated and unphosphorylated channels on a longer time scale. We have therefore tested whether FK506 speeds up the recovery of Ca^{2+} channel currents from inactivation. In these experiments, Ca^{2+}

Fig. 3. Ca^{2+} current inactivation in chicken DRG cells. Ca^{2+} currents evoked from a holding potential of -40 mV to 0 mV for 1 s are shown from three different cells with similar Ca^{2+} current amplitudes. With 1 mM EGTA, Ca^{2+} currents exhibited pronounced inactivation (1). When FK 506 ($10 \mu\text{M}$) was added to the internal solution, the inactivation time-course was very similar (2), whereas increasing the intracellular EGTA concentration to 5 mM strongly reduced Ca^{2+} current inactivation (3).

currents were elicited at increasing intervals (20 ms to 25 s) after a depolarizing prepulse to 0 mV for 2 s (Fig. 4A). With 5 mM EGTA in the internal solution, Ca^{2+} channel currents inactivated by about 85% within 2 s. Recovery from inactivation occurred with a double-exponential time-course, with time constants of about 600 ms and 10 s. The maximum recovery obtained was different in control cells and cells treated with FK506 (86% and 63%, respectively). Despite this difference, neither the slow nor the fast time constant of the Ca^{2+} current recovery were significantly changed when FK506 ($10 \mu\text{M}$) was added to the internal solution. Time constants of recovery were, on average, 660 ms (control, $n = 4$) versus 570 ms (FK506, $n = 5$), and 8.93 s versus 10.8 s for the fast and slow processes (Fig. 4B). Similar results were obtained when FK506 was applied extracellularly (not shown).

Rundown

A second phenomenon possibly related to phosphorylation is the so-called rundown or washout of high-voltage-activated Ca^{2+} channels.^{6,12,15,27} Since the gradual loss of Ca^{2+} channel current amplitude could be slowed down or reversed by addition of ATP, Mg^{2+} , cyclic-AMP or the catalytic subunit of protein kinase A to the intracellular medium, a loss of phosphorylation has been suggested as the underlying mechanism of rundown. We have investigated whether CaN is involved in this phenomenon in chick DRG neurons. Cells were dialysed with $\text{Ca}^{2+}/\text{Ca}^{2+}$ -EGTA buffers with different free Ca^{2+} concentrations. The time-course of Ca^{2+} current rundown was faster with $[\text{Ca}^{2+}]_i$ of about 200 nM as compared to virtually Ca^{2+} -free solutions (Fig. 5), suggesting that a Ca^{2+} -dependent enzyme like the Ca^{2+} -dependent phosphatase CaN or a Ca^{2+} -dependent protease could be involved. By adding FK506, we have tested a possible role of CaN in the rundown process. Intracellular application of FK506 ($10 \mu\text{M}$) did not prevent the rundown, but instead slightly facilitated the loss of the Ca^{2+} current amplitudes.

DISCUSSION

Several studies have clearly demonstrated that at least L-type Ca^{2+} channels, both in neurons and in the heart, are regulated via phosphorylation,^{2,4,5} which affects both the number of available ion channels and the channel open time.^{34,41,45} Many investigators have proposed that the

Fig. 4. Recovery from Ca^{2+} channel inactivation in chicken DRG neurons. (A) Recovery from Ca^{2+} channel inactivation was tested after a depolarizing prepulse from -40 to 0 mV for 2 s by short (15 ms) depolarizations to 0 mV after time intervals of 0.020 , 0.145 , 0.600 , 2.66 and 7.67 s. The line represents a double-exponential fit ($\tau_1 = 322$ ms and $\tau_2 = 6.6$ s) of the peak Ca^{2+} current amplitudes recorded during the recovery process. (B) Percentage of recovered Ca^{2+} current amplitude versus logarithmic time (means $\pm$ S.E.M.). The lines represent double-exponential fits to the data points. (●) Control, $f(t) = 85.9$ pA $- 23.3$ pA $\exp(-t/66$ ms) $- 62.7$ pA $\exp(-t/8930$ ms), $n = 4$; (■) internal FK506 (1 μ M), $f(t) = 62.2$ pA $- 17.2$ pA $\exp(-t/0.570$ s) $- 46.7$ pA $\exp(-t/10.8$ s), $n = 5$.

Table 1. Time constants (mean $\pm$ S.E.M.) of Ca^{2+} channel current inactivation in dorsal root ganglion and GH $_3$ cells under different experimental conditions

	τ_{fast} (ms)	τ_{slow} (ms)
GH $_3$ cells (perforated-patch configuration)		
Control	11.4 ± 0.6	116 ± 12
FK506 (10 μ M)	12.7 ± 0.7	85.0 ± 5.8
DRG cells (whole-cell configuration)		
EGTA (5 mM)	145 ± 29	936 ± 163
EGTA (1 mM)	39 ± 6	400 ± 39
EGTA (1 mM)/FK506 (10 μ M)	48 ± 5.3	474 ± 43

inactivation of L-type Ca^{2+} channels might be due to a loss of the number of phosphorylated channels and a strong candidate for an enzyme that could mediate this process is the Ca^{2+} -calmodulin-dependent phosphatase CaN.²⁶ Similar mechanisms have been proposed for N-type Ca^{2+} channels.^{1,18,20,21,33,44}

Within the CNS, CaN is abundantly expressed¹⁰ and constitutes about 1% of the total brain protein.²⁶ Inhibition of CaN by FK506 and cyclosporin A has been reported to increase the spontaneous neuronal firing rate observed in rat cortices.⁴³

Fig. 5. Rundown of Ca^{2+} channels in a chicken DRG neuron. (A) Ca^{2+} currents were evoked by 50 -ms depolarizations to 0 mV every 10 s. Every third current trace is shown. This neuron was perfused with a Ca^{2+} buffer containing 3 mM Ca^{2+} /5 mM EGTA ($[\text{Ca}^{2+}]_i \approx 200$ nM at pH 7.30). Ca^{2+} current amplitudes were reduced by 80% within 3 min of whole-cell recording. (B) The time-course of the rundown process of Ca^{2+} currents. Cells were perfused either with virtually Ca^{2+} -free internal solution (5 mM EGTA/ 0 Ca^{2+} , circles) or with 200 nM free Ca^{2+} (5 mM EGTA/ 3 mM Ca^{2+} , rectangles). Open and filled symbols represent recordings in the presence or absence of external FK506 (1 μ M), respectively (means $\pm$ S.E.M., $n = 3-6$).

This finding could be explained by the observation that FK506 and cyclosporin A increase both resting and 4-aminopyridine-stimulated intrasynaptosomal Ca^{2+} concentrations, and facilitate the Ca^{2+} -dependent release of the excitatory neurotransmitter L-glutamate from rat brain synaptosomes.³⁸ These two findings suggest a presynaptic site of action and support a role for CaN also in the regulation of N- and P-type Ca^{2+} channels, which trigger the release of neurotransmitters.¹⁴ Such an interaction of CaN with N-type Ca^{2+} channels is further supported by an immunocytochemical study, which demonstrates the co-localization of CaN with Ca^{2+} channels in DRG neurons.³¹

Previous studies, which have used the immunosuppressive drugs FK506 and cyclosporin A, have provided evidence both for and against an involvement of CaN in the Ca^{2+} -dependent inactivation of Ca^{2+} channels. In a recent study,⁴² no

experimental evidence was obtained that supported an involvement of CaN. The significance of this negative result, however, critically depends on the presence of the intracellular binding proteins, i.e. FKBP and cyclophilins, in the cytoplasm, and on the ability of these proteins to interact with CaN. In a developmental study, Polli *et al.*³⁵ have suggested that, in the developing rat brain, CaN immunoreactivity is not detectable until postnatal day 4 and developmental changes in neuronal FKBP expression cannot be ruled out. Our study clearly shows that CaN and FKBP-12 are expressed in embryonic chick DRG neurons and undifferentiated GH₃ cells. Positive evidence for the involvement of CaN has recently been provided by Lukyanetz *et al.*³² in a rodent neuroblastoma × glioma cell line, but it is not clear whether this process is of physiological significance in differentiated neurons. A possible mechanism that might explain these divergent results could be that the susceptibility of the Ca²⁺ channel proteins to modulation by CaN depends on the developmental stage, e.g., on the occurrence of different splice variants of L-³⁷ or N-type^{28,29} Ca²⁺ channels or of different combinations of α and β Ca²⁺ channel subunits.⁸

In the present paper, we provide further evidence that CaN is not involved in the inactivation process of L-type Ca²⁺ channels in GH₃ cells and of N-type channels in chicken DRG neurons, although both cell types have CaN and FKBP-12 in their cytoplasm. To exclude the possibility that FKBP might be washed out from the cytoplasm, we have

performed perforated-patch recordings in GH₃ cells which prevent such a washout of macromolecules. In chicken DRG neurons, we have used conventional whole-cell recordings, which allowed us to perfuse the cells with a known concentration of FK506 and also with the complex of FK506 and FKBP. Our results therefore provide direct evidence against a significant contribution of CaN to the Ca²⁺-dependent inhibition of L- and N-type channels. An alternative pathway that may account for the Ca²⁺-dependent inactivation of Ca²⁺ channels is the direct interaction of Ca²⁺ ions with the channel protein.^{34,46} Such a direct binding of Ca²⁺ ions has been proposed by Gutnick *et al.*¹⁷ for molluscan Ca²⁺ channels. de Leon *et al.*¹¹ and Imredy and Yue²³ have recently provided evidence for such a direct effect on native and artificially expressed cardiac L-type Ca²⁺ channels, and similar mechanisms may also exist in neuronal L- and N-type Ca²⁺ channels. In summary, our results provide strong evidence against the involvement of CaN in the inactivation process of L- and N-type Ca²⁺ channels, and support the dominant role of direct binding of Ca²⁺ ions to the channels.

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