Biochemical Properties and Subcellular Distribution of the Neuronal Class E Calcium Channel α_1 Subunit

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Anti-peptide antibodies specific for the neuronal calcium channel α_{1E} subunit (anti-CNE1 and anti-CNE2) were produced to study the biochemical properties and subcellular distribution of the α_{1E} polypeptide from rat brain. Immunoblotting identified a single size form of 245–255 kDa which was a substrate for phosphorylation by cAMP-dependent protein kinase, protein kinase C, cGMP-dependent protein kinase, and calcium/calmodulin-dependent protein kinase II. Ligand-binding studies of α_{1E} indicate that it is not a high affinity receptor for the dihydropyridine isradipine or the peptide toxins ω -conotoxin GVIA or ω -conotoxin MVIIC at concentrations which elicit high affinity binding to other channel types in the same membrane preparation. The α_{1E} subunit is widely distributed in the brain with the most prominent immunocytochemical staining in deep midline structures such as caudate-putamen, thalamus, hypothalamus, amygdala, cerebellum, and a variety of nuclei in the ventral midbrain and brainstem. Staining is primarily in the cell soma but is also prominent in the dendritic field of a discrete subset of neurons including the mitral cells of the olfactory bulb and the distal dendritic branches of the cerebellar Purkinje cells. Our observations indicate that the 245–255 kDa α_{1E} subunit is localized in cell bodies, and in some cases in dendrites, of a broad range of central neurons and is potentially modulated by multiple second messenger-activated protein kinases.

[Key words: ion channel, calcium channel, phosphorylation, peptide toxin, immunocytochemistry, Purkinje cell]

Voltage-gated calcium channels regulate Ca^{2+} entry in excitable cells and control many aspects of cellular function (McCleskey, 1994). In neurons, these functions include the regulation of neurotransmitter release, modulation of firing patterns and excitability, and activation of compartment-specific signaling networks. Five types of calcium current, termed T, L, N, P, and Q, have been previously described based on physiological and pharmacological criteria (Bean, 1989; Tsien et al., 1991; Snutch and Reiner, 1992; Zhang, 1993). T-Type calcium currents activate at low voltage, inactivate quickly, and are blocked by relatively low concentrations of Ni^{2+} but not by organic or peptide calcium channel blockers. L-Type calcium currents are high-voltage-activated, slowly inactivated by sustained depolarization, and specifically blocked by dihydropyridines: N-, P-, and Q-type calcium currents are also high-voltage-activated, have variable rates of inactivation, and are preferentially blocked by the peptide neurotoxins ω -conotoxin GVIA (ω -CTx GVIA), ω -agatoxin IVA (ω -Aga IVA), and ω -conotoxin MVIIC (ω -CTx MVIIC), respectively.

Calcium channels in neurons are multisubunit complexes containing the principal, pore-forming α_1 subunits in association with auxiliary $\alpha_2\delta$ and β subunits (reviewed in Isom et al., 1994), but the major physiological and pharmacological distinctions among voltage-gated calcium channels are primarily a reflection of the genetic diversity of the α_1 subunits which are members of the structural superfamily of voltage-gated ion channels (Catterall, 1994). Five classes of α_1 subunit cDNAs have been isolated from mammalian brain (α_{1A} through α_{1E} , reviewed in Snutch and Reiner, 1992; Birnbaumer et al., 1994; Hofmann et al., 1994). A combination of functional expression and *in vitro* biochemistry indicates that α_{1C} and α_{1D} are subunits of dihydropyridine-sensitive L-type channels (Hui et al., 1991; Snutch, 1991; Dubel et al., 1992; Williams, 1992a; Hell et al., 1993a). The α_{1C} and α_{1D} subunits are localized in the cell bodies and proximal dendrites of many classes of central neurons, and calcium entry through these channels is thought to regulate biochemical processes such as protein phosphorylation and gene expression (Morgan and Curran, 1986; Ahljianian et al., 1990; Westenbroek et al., 1990; Murphy et al., 1991; Bading et al., 1993; Hell, 1993a). α_{1B} is a subunit of ω -CTx GVIA-sensitive N-type calcium channels (Dubel et al., 1992; Williams et al., 1992b; Fujita et al., 1993) which are localized in dendrites and presynaptic nerve terminals (Westenbroek et al., 1992) and can couple to neurotransmitter release in a variety of preparations (Hirning et al., 1988; Luebke et al., 1993; Takahashi and Mo-

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miyama, 1993; Turner et al., 1993; Wheeler et al., 1994). α_{1A} is a subunit of calcium channels that are resistant to ω -CTx-GVIA and sensitive to ω -CTx-MVIIIC (Mori et al., 1991; Sather et al., 1993; Zhang et al., 1993; Stea et al., 1994a). Calcium channels containing α_{1A} are located in dendrites and presynaptic nerve terminals (Westenbroek et al., 1993, 1995) and have been proposed to couple to neurotransmitter release at many synapses (Turner, 1992, 1993; Luebke et al., 1993; Takahashi and Miyama, 1993; Wheeler et al., 1994).

The most recently described member of the calcium channel family is α_{1E} (Soong et al., 1993). Functional expression of α_{1E} with auxiliary subunits in *Xenopus* oocytes produced calcium channels which are rapidly inactivated and insensitive to high affinity organic and peptide calcium channel blockers (Soong et al., 1993; Williams et al., 1994; Randall et al., 1995). Whole-cell voltage clamp and single channel recording studies of cerebellar granule cells have identified additional types of calcium currents which are resistant to dihydropyridines and ω -CTx-GVIA (R-type and G1-G3, respectively; Forti et al., 1994; Randall et al., 1995). The currents conducted by α_{1E} are similar in some respects to T-type, R-type, and G2 calcium currents (Soong et al., 1993; Forti et al., 1994; Williams et al., 1994; Randall et al., 1995), but the precise relationship between α_{1E} and native calcium currents remains to be defined.

Differential subcellular localization in cell bodies, dendrites, and nerve terminals is a critical determinant of the functional roles of neuronal calcium channels, but the biochemical properties and subcellular localization of the class E calcium channels are unknown. In this article, we report the biochemical characterization and immunocytochemical localization of calcium channels containing the α_{1E} subunit. We identify α_{1E} polypeptides of 245–255 kDa which are phosphorylated *in vitro* by multiple protein kinases and exhibit a pharmacology and subcellular localization distinct from other known calcium channels.

Materials and Methods

Materials. ³H-Isradipine (PN200-110, 80 Ci/mmol), ¹²⁵I- ω -CTx GVIA (2200 Ci/mmol), and γ -³²P ATP (3000 Ci/mmol) were purchased from New England Nuclear-Du Pont (Boston, MA); the ECL detection kit for immunoblotting from Amersham Corp. (Arlington Heights, IL); digitonin from Gallard-Schlesinger (Carle Place, NY); and protein A-Sepharose (PAS), wheat-germ agglutinin-agarose (WGA), CNBr-activated Sepharose 4B, and bovine serum albumin (BSA; IgG free) from Sigma Chemical Corp. (St. Louis, MO). Control rabbit IgG was obtained from Zymed Laboratories, Inc. (South San Francisco, CA). ¹²⁵I- ω -CTx-MVIIIC (3000 cpm/fmol) was generously supplied by Drs. A. Woppmann and G. W. Miljanich of Neurex Corp. (Menlo Park, CA). The catalytic subunit of PKA and PKC were purified by standard procedures (Kaczmarek et al., 1980; Woodgett and Hunter, 1987) and kindly provided by Drs. E. I. Rotman and B. J. Murphy, Department of Pharmacology, University of Washington, Seattle, WA 98195. Cam KII, expressed in Sf9 cells using a baculovirus expression vector and purified to homogeneity (Brickett et al., 1990), was generously supplied by Drs. D. A. Brickey and T. Soderling, Vollum Institute, Portland, OR, and PKG was purchased from Promega (Madison, WI). All other reagents were of standard biochemical quality from commercial sources. One and 2 month old Sprague-Dawley rats were obtained from Bantin and Kingman (Bellevue, WA).

Production and purification of peptides and antibodies. Anti-CNE1 and anti-CNE2 were produced against two highly variable regions in the intracellular loop between domains II and III of the predicted polypeptide sequence based on the rE-II cDNA clone (Soong et al., 1993), and anti-CP15 (anti-CP[1382-1400]) against the highly conserved region following the transmembrane segment IVS6 of the skeletal muscle and class C L-type α_1 subunits (Striessnig et al., 1990). The 18 residue CNE1 and CNE2 peptides were synthesized corresponding to the sequences KYQLRRHMQMSSQEALNK (predicted residues 741–756

from rE-II) and KYSASQERSLDEGVSDIG (residues 896–911), respectively. At the NH₂-termini of each peptide, the residues lysine and tyrosine were added for the purpose of cross-linking to carrier protein and radiolabeling and are not part of the channel sequence. The peptide was synthesized by the solid-phase method (Merrifield, 1963), purified by reverse-phase high-pressure liquid chromatography on a Vydac 218 TP10 column, and confirmed by amino-acid analysis. The peptides were coupled with glutaraldehyde to bovine serum albumin (Orth, 1979), dialyzed against PBS, and emulsified in the same volume of Freund's complete (initial injection) or incomplete adjuvant. Injections were done at multiple subcutaneous sites on New Zealand White rabbits at 3 week intervals. Antisera were collected, titers determined by enzyme-linked immunosorbent assay (ELISA), and antibodies purified by affinity chromatography on CNE1, CNE2, or CP15 peptide coupled to CNBr-activated Sepharose. Briefly, 4 ml of high-titer antiserum were adsorbed to the column matrix and incubated at 4°C overnight with mixing. The column was washed extensively with TBS (10 mM Tris-HCl, pH 7.4, 150 mM NaCl, pH 7.4) until the A₂₈₀ of the eluate stabilized at less than 0.05, and affinity-bound IgG was eluted with 2.8 M MgCl₂, dialyzed against TBS, and concentrated with a Centrprep-30 apparatus (Amicon, Inc., Beverly, MA).

Partial purification of brain calcium channels. All extract incubations and manipulations were carried out at 4°C or on ice to prevent proteolysis. In addition, the following protease inhibitors were included in all buffers: pepstatin A and leupeptin (each at 1 μ g/ml), and aprotinin (2 μ g/ml), phenylmethanesulfonyl fluoride (PMSF; 0.2 mM), benzamide (0.1 mg/ml), and calpain inhibitors I and II (8 μ g/ml). For each preparation, 15 rat brains were homogenized in 110 ml of 320 mM sucrose with a glass-Teflon homogenizer (12 strokes at approximately 1000 rpm). The homogenate was separated at 5000 rpm for 2 min in an SS34 rotor, and the supernatant (S1) was collected. Membranes were pelleted (P2) by centrifugation at 42,000 rpm for 1 hr in a 45 Ti rotor and solubilized in 170 ml of 1.2% digitonin, 300 mM KCl, 150 mM NaCl, 10 mM sodium phosphate buffer, pH 7.4, for 20 min. The solubilized P2 was subjected to centrifugation at 42,000 rpm for 60 min in the 45 Ti rotor to remove unsolubilized material. The supernatant (S3) was incubated for 1 hr with 20 ml of wheat-germ agglutinin-Sepharose, transferred to a column, and washed with 300 ml of 0.1% digitonin, 75 mM NaCl, 50 mM sodium phosphate, 10 mM Tris-HCl, pH 7.4, at a flow rate of 150 ml per hour. The bound glycoproteins were eluted with wash buffer containing 100 mM N-acetyl-D-glucosamine at a flow rate of 50 ml per hour. Thirty 2 ml fractions were collected, and the protein content of each fraction estimated from absorbance measurements at A₂₈₀. Peak A₂₈₀ fractions always corresponded to peak fractions for binding of PN200-110 and ω -CTx GVIA, demonstrating the partial purification of both L-type and non-L-type calcium channel complexes. Eluted peak fractions were pooled, concentrated to 10% of the initial volume by centrifugation in a Centrprep-30 apparatus (Amicon, Inc., Beverly, MA), frozen in liquid N₂, and stored at –80°C.

Immunoprecipitation and immunoblotting of calcium channels. All incubations and manipulations were carried out at 4°C or on ice and in the presence of protease inhibitors. Nonspecific sepharose-binding proteins were removed from the WGA-purified extract by incubating 0.2 ml of extract with 150 μ l of Sepharose CL-4B and 5 mg of protein A Sepharose for 30 min with mixing, followed by a brief centrifugation to remove the Sepharose matrix. Calcium channels were concentrated from the WGA-purified extract by incubating the cleared WGA extract with 10 μ g of affinity-purified anti-CNE2 antibody or control rabbit IgG antibody for 1 hr in a tilting mixer. Protein A-Sepharose [2.5 mg, prewashed three times with phosphate-buffered saline (PBS; 10 mM Na₂HPO₄, pH 7.4, 150 mM NaCl), 0.5% BSA, 0.1% digitonin] was added, and the samples were incubated an additional 2 hr with mixing. The resin was washed three times in 1 ml of 10 mM Tris-HCl, pH 7.4, 0.1% digitonin and two times in 10 mM Tris-HCl, pH 7.4, followed by extraction in 20 μ l of SDS sample buffer [125 mM Tris-HCl, pH 6.8, 2% sodium dodecyl sulfate (SDS), 2 mM EDTA, 10% sucrose, 20 mM dithiothreitol (DTT), 1 μ M pepstatin A, 2 μ g/ml leupeptin, 4 μ g/ml aprotinin, 1 μ M p-nitrophenylphosphate] for 30 min at 50–60°C with mixing every 5 min. Samples were loaded onto a 5% SDS polyacrylamide gel with a 3% stacking layer (Laemmli, 1970) and electrophoresed at 50 mA/gel for 2–3 hr. The separated proteins were transferred electrophoretically to nitrocellulose filters and blocked for 1 hr in blocking buffer consisting of 10% nonfat milk in TBS. Filters were then incubated with affinity-purified antibody (50 μ g per ml in blocking buffer) for 1 hr, washed five times for 5 min each in blocking buffer,

incubated 1 hr with protein A-horseradish peroxidase, diluted 1:1000 in blocking buffer, washed 6×10 min then 4×30 min in TBS, and developed with the ECL reagent.

Immunoprecipitation and phosphorylation of calcium channels. Calcium channels were concentrated on protein A-Sepharose as described in the previous section. Prior to phosphorylation the resin was washed once in 1 ml of basic phosphorylation buffer (50 mM HEPES-NaOH, pH 7.4, 10 mM $MgCl_2$, 1 mM EDTA, 0.1% digitonin, 1 μ M pepstatin). Phosphorylation reactions were performed in 50 μ l basic phosphorylation buffer with 0.5–1.0 μ g of cAMP-dependent protein kinase (PKA), protein kinase C (PKC), cGMP-dependent protein kinase (PKG), or calcium- and calmodulin-dependent protein kinase type II (Cam KII), along with 1 mM DTT, 1 μ M pepstatin A, and 0.2 μ M $\gamma^{32}P$ -ATP. The buffer was supplemented with 1.5 mM $CaCl_2$, 50 μ g of diolefin, and 2.5 mg of phosphatidylserine for PKC; 2 μ M cGMP for PKG; and 1 μ M calmodulin and 0.6 mM $CaCl_2$, without EGTA, for Cam KII. Incubations were at 32–34°C for 30 min with gentle mixing every 2 min. The samples were washed twice with 0.1% digitonin in RIA buffer (25 mM Tris-HCl, pH 7.4, 20 mM EDTA, 75 mM NaCl, 20 mM Na-pyrophosphate, 20 mM β -glycerolphosphate, 50 mM NaF, and 1 mM *p*-nitrophenylphosphate), twice with 1% Triton X-100 in RIA buffer, and once in 10 mM Tris-HCl, pH 7.4. Proteins were extracted in SDS sample buffer for 30 min at 50–60°C and electrophoresed through a 5% SDS-polyacrylamide gel. For double immunoprecipitations, the washed Sepharose pellet following phosphorylation was incubated in 30 μ l of 50 mM Tris-HCl, pH 7.4, 1% SDS, 5 mM DTT, 20 mM β -glycerolphosphate, 1 mM *p*-nitrophenylphosphate, 1 μ M pepstatin A, then diluted with 300 μ l 1% Triton X-100, 0.1% BSA, 20 mM β -glycerolphosphate, 1 mM *p*-nitrophenylphosphate, 1 μ M pepstatin A in RIA buffer and centrifuged. The supernatant was incubated with 25 μ g of affinity-purified antibody for 1.5 hr at 4°C on a tilting mixer, 2 mg of protein A-Sepharose were added, and the incubation was continued another 2.5 hr. Immunoprecipitated proteins were pelleted by centrifugation, washed three times with 1% Triton X-100 in RIA buffer, and once in 10 mM Tris-HCl, pH 7.4, extracted in SDS sample buffer, separated by electrophoresis on a 5% SDS-polyacrylamide gel, and visualized by autoradiography. For experiments with blocked antibodies, 10 μ M of each peptide was added to the affinity-purified antibody for 30 min prior to incubation with the WGA extract.

Radioligand binding studies. For 3H -PN200-110 (isradipine) binding studies, the 110 ml of S1 fraction was labeled with 5 μ l (5 μ Ci at 80 Ci/mmol) of 3H -PN200-110 at a final concentration of 0.57 nM for 1 hr on ice. The radioligand is stably bound throughout the subsequent purification steps. Ten microliters of the WGA-purified preparation (~1000 cpm) was used for purification of calcium channel subtypes using 10–20 μ g of peptide affinity-purified antibodies anti-CNE1, anti-CNE2, anti-CNC1 or control rabbit IgG. Immunoprecipitation was performed as described in the previous section. After the final wash, the antibody-bound Sepharose CL-4B matrix was transferred to vials for scintillation counting.

Total receptor-bound 3H -PN200-110 was determined by filter-binding assay. Briefly, 10 μ l of concentrated WGA column eluate was precipitated by incubation with 4 ml of ice-cold 10% polyethylene glycol (PEG, average molecular weight 8000) in 10 mM $MgCl_2$ and 10 mM Tris-HCl, pH 7.4, for 5 min and the precipitated calcium channels were collected by filtration on Whatman GF/C filters. The samples were washed four times in ice-cold PEG solution and quantified in a scintillation counter. The correction factor for loss of bound ligand in the filter-binding assay was 0.7 (Westenbroek et al., 1992).

^{125}I - ω -CTx GVIA binding was measured by incubation of 300 μ l of WGA extract containing 0.05% BSA with 3 μ l labeled toxin (0.3 μ Ci at 2200 Ci/mmol) at a final concentration of 0.45 nM for 20 min on ice. Samples were immunoprecipitated with 10–20 μ g of the peptide affinity-purified antibodies anti-CNE1, anti-CNE2, anti-CNB2, or control rabbit IgG as described above, and after washing the matrix was transferred to vials for scintillation counting. Total ^{125}I - ω -CTx GVIA binding was determined using 20 μ l of the labeled WGA extract in the filter-binding assay described above for 3H -PN200-110.

^{125}I - ω -CTx MVIIC binding was determined by addition of 200,000 cpm of labeled toxin (3000 cpm/fmol) to 50 μ l of WGA extract with 0.5% BSA to yield a final concentration of 1.3 nM ω -CTx MVIIC and incubation at 4°C for 30 min on a tilting mixer. This mixture was added to 20 μ g of peptide affinity-purified anti-CNE2, anti-CNC1, anti-CNA3, or control rabbit IgG coupled to 2 mg of protein A-Sepharose, and incubated for 5 hr at 4°C in a tilting mixer. Samples were washed quick-

ly three times in 10 mM Tris-HCl, pH 7.4, 100 mM NaCl, 0.1% digitonin, 0.5% BSA, and the pellets transferred to vials for scintillation counting.

Immunocytochemistry. Adult Sprague-Dawley rats were anesthetized with sodium pentobarbital and perfused intracardially with a solution of 4% paraformaldehyde in PB (0.1 M sodium phosphate, pH 7.4) containing 0.34% L-lysine and 0.05% sodium *m*-periodate (McLean and Nakane, 1974). The brains were immediately removed from the cranium and cryo-protected by immersion in successive solutions of 10%, 20%, and 30% (w/v) sucrose in PB at 4°C over 72 hr. Coronal and sagittal sections (40 μ m) were cut on a sliding microtome and placed in 0.1 M PB for 16–24 hr.

Free-floating sections were processed for immunofluorescence using the ABC method (Hsu et al., 1981). Tissue sections were rinsed in TB (100 mM Tris-HCl, pH 7.4) for 10 min and then TBS (100 mM Tris-HCl, pH 7.4, 150 mM NaCl) for 10 min. The tissue was then blocked in 1% avidin in TBS for 30 min, rinsed in TBS for 10 min followed by 1% biotin for 30 min, and rinsed with TBS for 30 min. Sections were incubated in affinity-purified anti-CNE1 or anti-CNE2 antibody (diluted 1:15) for 1 hr at room temperature followed by 36 hr at 4°C. All antisera were diluted in TBS containing 1% normal goat serum and 0.1% Triton X-100. The tissue sections were processed as follows at room temperature, except when otherwise stated: rinsed for 1 hr in TBS containing 0.1% Triton X-100, incubated in biotinylated anti-rabbit IgG diluted 1:300 for 1 hr at 37°C, rinsed for 1 hr in TBS containing 0.1% Triton X-100, incubated in avidin D fluorescein, diluted 1:300 for 2 hr at 37°C, rinsed in TBS for 15 min, and finally rinsed in TB for 30 min before being mounted on gelatin-coated slides and coverslipped using Vectashield. The tissue was viewed using a Bio-Rad 600 MRC confocal microscope.

To determine the level of nonspecific staining, control sections were incubated either without primary antibody or by blocking the primary antibody by preincubation for 6–8 hr with the corresponding peptide (20 μ M) or with a different α_{1E} peptide (20 μ M) prior to incubation of the antibody with the tissue.

Results

Identification of the α_{1E} polypeptide from rat brain

The anti-CNE1 and anti-CNE2 antibodies were raised against unique peptides from the putative cytoplasmic loop between domains II and III of α_{1E} . Affinity-purified anti-CNE1 and anti-CNE2 each immunoprecipitated polypeptides with identical molecular mass and biochemical properties and therefore were used interchangeably in our experiments (data not shown). In order to determine the size and heterogeneity of α_{1E} polypeptides from rat brain, anti-CNE antibodies were used to immunopurify the α_{1E} polypeptides from a detergent-solubilized membrane preparation by immunoblotting with anti-CNE2 (Fig. 1). Anti-CNE2 recognized a single broad protein band, which extended from 245 kDa to 255 kDa, when either anti-CNE1 (Fig. 1A, lane 1) or anti-CNE2 (Fig. 1A, lane 2) was used as the immunoprecipitating antibody. No protein band was observed when rabbit IgG was used as the immunoprecipitating antibody (Fig. 1A, lane 3). The observed molecular mass is consistent with that predicted from the rat α_{1E} cDNA sequence (252 kDa, Soong et al., 1993). The width of this protein band suggests the presence of multiple α_{1E} polypeptides differing by less than 10 kDa in apparent molecular mass. This is unlikely to be the result of partial proteolytic degradation because the entire purification procedure was carried out at 4°C or on ice with a high concentration of six protease inhibitors and a 1 hr preincubation of the extract at 37°C in the absence of protease inhibitors revealed no change in the appearance and distribution of the 245–255 kDa band (data not shown). As a further test of the specificity of the anti-CNE antibodies, immunoblotting was performed in the absence and presence of 10 μ M competing peptide (Fig. 1B). The 245–255 kDa polypeptide band was detected on immunoblots probed with anti-CNE2 antibody in the absence (Fig. 1B, lane 1) but not in the presence (Fig. 1B, lane 2) of CNE2 peptide. In contrast,

Figure 1. Immunoblotting with anti-CNE2 detects a broad protein band of 245–255 kDa. **A**, Calcium channels were enriched from solubilized brain membranes by WGA affinity chromatography and immunoprecipitated with anti-CNE1 (lane 1), anti-CNE2 (lane 2), or control rabbit IgG (lane 3). Samples were separated by SDS-polyacrylamide gel electrophoresis, immunoblotted with affinity-purified anti-CNE2, and detected with the ECL method as described in the Materials and Methods. The molecular weight indicators are α and β -spectrin (220 and 240 kDa), myosin heavy chain (205 kDa), and α_2 -macroglobulin covalently linked to Coomassie brilliant blue (191 kDa), and are shown to the right of the gel together with their respective molecular masses in kilodaltons. **B**, Channels were immunoprecipitated with anti-CNE2 as described above, and immunoblotted with anti-CNE2 in the absence (lane 1) or presence (lane 2) of 10 μM competing CNE2 peptide or with 10 μM of a nonspecific CNE1 control peptide (lane 3). Molecular mass standards are indicated by the gel as in **A** including β -galactosidase covalently linked to Coomassie brilliant blue (117 kDa).

incubation of the same blot with anti-CNE2 antibody in the presence of CNE1 peptide (Fig. 1B, lane 3) did not block recognition of the 245–255 kDa polypeptide, providing further evidence for specificity of the CNE2 peptide block. Together, these immunoblotting data identify an α_{1E} polypeptide from rat brain in the 245–255 kDa size range. The broad protein band may indicate the presence of multiple α_{1E} polypeptides of similar size.

Phosphorylation of the α_{1E} polypeptide

Voltage-gated calcium channel α_1 subunits contain consensus sites for phosphorylation by multiple second-messenger-activated protein kinases within their cytoplasmic domains, and direct phosphorylation of α_1 subunits has been shown previously to be an important modulatory pathway for voltage-gated calcium channels (Trautwein and Hescheler, 1990; Armstrong et al., 1991; Sculptoreanu et al., 1993). *In vitro* phosphorylation of α_{1E} by purified protein kinases is a first step toward functional studies of channel phosphorylation, and also serves as a method for radiolabeling α_{1E} subunits for immunochemical studies. Anti-CNE2 was used to immunoprecipitate and concentrate α_{1E} subunit polypeptides from the WGA fraction onto a protein A-Sepharose affinity matrix, and the α_{1E} subunits were then incubated with purified PKA and $\gamma\text{-}^{32}\text{P}\text{-ATP}$, resolved by SDS-PAGE, and detected by autoradiography. The anti-CNE2-immunopurified α_{1E} subunit polypeptide was substantially phosphorylated by PKA, while no phosphoprotein band was detected with control rabbit IgG (Fig. 2A, lanes 1 and 2).

α_1 subunits of voltage-gated calcium channels interact with auxiliary subunits and also may associate with other plasma membrane, vesicular, or cytoskeletal proteins. Thus, the anti-CNE2 immunoprecipitations performed under native conditions may isolate PKA substrates which are associated with α_{1E} subunit and have a similar size. In order to rule out this possibility, a double immunoprecipitation procedure was used in which

PKA-phosphorylated anti-CNE2 immunoprecipitates were denatured by heating the samples in SDS and DTT to dissociate proteins associated with α_{1E} , diluted into Triton X-100, and reprecipitated with a second antibody. When α_{1E} polypeptides were doubly immunoprecipitated with anti-CNE2, there was no change in the apparent molecular mass, distribution, or intensity of the 245–255 kDa phosphoprotein band (Fig. 2A, lane 3), indicating that the PKA substrate is α_{1E} itself rather than an associated polypeptide. A rabbit IgG double precipitation did not yield any PKA-phosphorylated polypeptides (Fig. 2A, lane 4). To confirm the identity of the 245–255 kDa polypeptide as a calcium channel α_1 subunit, we used anti-CP15 which recognizes a peptide segment conserved in all known rat voltage-dependent calcium channels and has been shown to immunoprecipitate the α_{1S} , α_{1A} , α_{1B} , α_{1C} , and α_{1D} polypeptides (Striessnig et al., 1990; Ahljianian et al., 1991; Hell et al., 1993b, 1994; Westbroek et al., 1993; Sakurai and Catterall, unpublished observations). This segment is accessible to anti-CP15 only after solubilization in Triton X-100 or other detergents which remove the $\alpha_2\delta$ subunit. Double immunoprecipitation with anti-CNE2 and anti-CP15 reveals the same 245–255 kDa phosphoprotein band, confirming its identity as a calcium channel α_1 subunit (Fig. 2A, lane 5). In order to examine the peptide specificity of the anti-CNE2 antibody in the immunoprecipitation and phosphorylation procedures, immunoprecipitations were done in the absence or presence of competing peptides. Incubation of the immunoprecipitation reaction with 2 μM CNE2 peptide effectively blocked recognition of the PKA-phosphorylated α_{1E} subunit (Fig. 2B).

The ability of the α_{1E} subunit to act as a substrate for other second-messenger-activated protein kinases was assessed by phosphorylating the anti-CNE2-immunoprecipitated α_{1E} subunit with each protein kinase followed by denaturation and reprecipitation with anti-CP15 to ensure that the substrate was the α_{1E} polypeptide. In addition to PKA (Fig. 2C, lane 1), the α_{1E} sub-

Figure 2. Phosphorylation of the α_{1E} subunit by protein kinases. **A**, α_{1E} subunits were immunoprecipitated with anti-CNE2 (lane 1) or control rabbit IgG (lane 2), phosphorylated with PKA, and analyzed by SDS-polyacrylamide gel electrophoresis. Double immunoprecipitations were performed with anti-CNE2 (lane 3) or control rabbit IgG (lane 4) as both first and second antibody, or anti-CNE2 followed by anti-CP15 (lane 5). Channels were phosphorylated between the first and second immunoprecipitation steps. **B**, Immunoprecipitation with anti-CNE2 was done in the absence (lane 1) or presence (lane 2) of 2 μ M competing CNE2 peptide and followed by PKA phosphorylation. **C**, Channels were immunoprecipitated with either anti-CNE2 (lanes 1, 3, 5, 7) or control rabbit IgG (lanes 2, 4, 6, 8), phosphorylated with PKA (lanes 1, 2), PKC (lanes 3, 4), PKG (lanes 5, 6), or CaM KII (lanes 7, 8), and then reimmunoprecipitated with anti-CP15. Molecular mass markers are indicated as in Figure 1.

unit is a substrate for phosphorylation by PKC, PKG, and CaM KII (Fig. 2C, lanes 3, 5, and 7). Nonimmune rabbit IgG was ineffective in precipitating α_{1E} phosphorylated by the different kinases (Fig. 2C, lanes 2, 4, 6, and 8). The distinct high molecular mass bands observed for CaM KII are likely to be nonspecific as they were observed in control rabbit IgG lanes in longer exposures (not shown). The fainter high molecular weight bands and diffuse radioactivity appearing in the other lanes probably represent incompletely denatured aggregates of α_{1E} with other polypeptides. These results demonstrate that the α_{1E} is an *in vitro* substrate for several second-messenger-activated protein kinases, and suggest the possibility that phosphorylation of α_{1E} may influence its functional properties (e.g., Stea et al., 1994b).

Binding of calcium channel blockers to α_{1E}

Selective block of voltage-gated calcium currents by various natural peptides and synthetic compounds is used as a criterion for distinguishing the different calcium current types in neurons, for characterizing cloned calcium channel α_1 subunits in expression systems, and for radiolabeling α_1 subunits during biochemical purification. Since previous work has shown that the ligand-binding profile of a purified α_1 subunit can be used to predict

the identity of the calcium channel type (Dubel et al., 1992), we have tested the binding to α_{1E} of radiolabeled ligands known to selectively block various calcium currents in neurons. ^3H -Isradipine (^3H -PN200-110) is a dihydropyridine compound which specifically binds to α_{1C} - and α_{1D} -containing L-type calcium channels. At a concentration of 0.57 nM ^3H -isradipine, anti-CNC1, an α_{1C} -specific site-directed antipeptide antibody (Hell et al., 1993a), immunoprecipitated 72% of total ^3H -isradipine binding sites while anti-CNE2 immunoprecipitated less than 2%, a value similar to that obtained with control rabbit IgG (Fig. 3A). Anti-CNB1, an α_{1B} -specific site-directed antipeptide antibody (Westenbroek et al., 1992), immunoprecipitated over 90% of total ^{125}I - ω -CTx GVIA binding sites in the WGA fraction detected by incubation with 0.45 nM of labeled toxin, but anti-CNE2 and control rabbit IgG recognized less than 5% of total ^{125}I - ω -CTx GVIA binding (Fig. 3B). ω -CTx MVIIC inhibits calcium channels containing α_{1A} subunits. Addition of 1.3 nM ^{125}I - ω -CTx MVIIC to the WGA fraction followed by immunoprecipitation with anti-CNA3, an α_{1A} -specific site-directed antipeptide antibody (Sakurai and Catterall, unpublished observations), revealed the immunoprecipitation of specific ^{125}I - ω -CTx MVIIC binding sites. In contrast, anti-CNE2 and anti-CNC1 did not immuno-

Figure 3. Radioligand binding profile of α_{1E} . **A**, Brain membranes were labeled with ^3H -isradipine, solubilized with digitonin, and calcium channels were enriched by WGA chromatography. Channels were immunoprecipitated with rabbit IgG, anti-CNC1, or anti-CNE2 and ^3H quantitated by scintillation counting. Total bound ^3H -isradipine was determined by filter-binding assay and corrected for filter efficiency as described in Materials and Methods. **B**, Calcium channels enriched by WGA chromatography were labeled with ^{125}I - ω -CTx GVIA and immunoprecipitated with control rabbit IgG, anti-CNB1, or anti-CNE2 and bound ^{125}I was quantitated by scintillation counting. Total bound ^{125}I - ω -CTx GVIA was determined by filter-binding assay and corrected for filter efficiency. **C**, Calcium channels enriched by WGA chromatography were labeled with ^{125}I - ω -CTx MVIIC and immunoprecipitated with control rabbit IgG, anti-CNA3, anti-CNC1, or anti-CNE2, and bound ^{125}I was quantitated by scintillation counting. In **A** through **C**, the values are presented as the mean $\pm$ SEM for $n = 3$ (**A**, **B**) or $n = 4$ (**C**) experiments.

precipitate significantly more than the nonspecific binding of rabbit IgG (Fig. 3C). Our results are consistent with previous studies showing that α_{1E} expressed in *Xenopus* oocytes or mammalian cells is not inhibited by dihydropyridines, ω -CTx GVIA, or ω -CTx-MVIIC (Soong et al., 1993; Williams et al., 1994). Evidently, these agents neither bind to nor inhibit class E calcium channels.

Regional and subcellular distribution of class E calcium channels

Both anti-CNE1 and anti-CNE2 antibodies were used in localization studies and gave similar patterns of staining. Throughout the rostral-caudal extent of the rat brain, there is significant labeling of the majority of neurons by the CNE antibodies. Both projection neurons and interneurons are immunoreactive for the

α_{1E} subunits recognized by the anti-CNE1 or anti-CNE2 antibodies. The most prominent staining in rostral areas was observed in deep ventral structures such as the caudate-putamen, globus pallidus, thalamus, hypothalamus, and amygdala. The density of labeling is highest in the cell soma in most brain regions, but substantial labeling of dendrites of specific neurons was also observed. To illustrate these points, selected regions and nuclei are described in greater detail below.

In all regions of the dorsal cortex, neurons throughout layers 1–6 are immunoreactive for α_{1E} with the most prominent staining observed in layers 3–5 (Fig. 4A). The highest concentration of α_{1E} immunoreactivity is in the cell bodies. Similar to α_{1C} and α_{1D} (Hell et al., 1993a), α_{1E} staining extends only into the proximal portion of the dendrites of cortical neurons and is faint compared with cell body staining.

In the olfactory bulb, there is extensive labeling of class E calcium channels throughout most layers. The periglomerular cells which surround the glomeruli of the bulb are immunoreactive for α_{1E} . In addition, tufted cells located in the external plexiform layer, the large mitral cells in the mitral cell layer, and the numerous small interneurons, the granule cells, located in the internal granular layer are all immunoreactive for the α_1 subunit of rat brain class E calcium channels (Fig. 4B). There is also extensive labeling of dendrites in the external plexiform layer, where dendrodendritic synapses on mitral cell dendrites are located, and moderate labeling of dendrites in the internal plexiform layer (Fig. 4B).

In the caudate-putamen there is dense labeling of cell bodies, especially at locations where dendrites originate (Fig. 4C). This staining is most prevalent in medium-sized neurons (10–20 μm in diameter) which make up greater than 95% of the neurons located in the caudate-putamen. The specificity of staining is indicated by block of staining by the anti-CNE1 antibody using the CNE1 peptide (Fig. 4D) and block of staining by the anti-CNE2 antibody using the CNE2 peptide (Fig. 4E).

Neurons located in the globus pallidus are also immunoreactive for class E calcium channels. At lower magnification, immunoreactive cell bodies appear densely stained compared to the surrounding neuropil and myelinated fiber bundles (Fig. 5A). At higher magnification (Fig. 5B), both the cell bodies and the large dendritic branches of the large fusiform or triangular neurons of this region are immunoreactive for α_{1E} , as are their dendritic branches. The surrounding neuropil is also immunoreactive for class E calcium channels. The majority of this staining appears to be in dendritic processes, although a portion of it has a punctate appearance which may suggest localization within synaptic terminals.

A similar pattern of cell body, dendritic, and neuropil staining was observed throughout the thalamus (Fig. 5C). Preincubation of the anti-CNE2 antibody with the CNE1 peptide did not change the pattern of staining as compared to incubation with anti-CNE2 antibody alone (Fig. 5D). A similar result was obtained when the anti-CNE1 antibody was preincubated with the CNE2 peptide. This control plus the peptide blocks shown in Figure 4, D and E, demonstrates that the anti-CNE1 and anti-CNE2 antibodies each recognize a specific sequence within the α_{1E} subunit.

In the amygdala, there is extensive staining of class E calcium channels in the cell bodies and dendrites of neurons. This pattern of staining was especially prominent in the anterior amygdala (Fig. 5E,F), and was also observed in the other major subdivisions of the amygdala. At lower magnifications there is extensive

Figure 4. Localization of class E calcium channels in forebrain regions. Sagittal sections of adult rat brain were stained with affinity purified anti-CNE1 or anti-CNE2 antibodies using the immunofluorescence technique described in Materials and Methods. *A*, Section taken from layers IV and V of the dorsal cortex to demonstrate class E staining in cell bodies. *B*, Section taken from the olfactory bulb illustrating dense class E calcium channel staining in the mitral cells (arrows) and in their dendrites located in the external plexiform layer (*EPL*). *C*, Class E calcium channel staining in the cell body of neurons in the caudate-putamen. Control sections from caudate nucleus in which anti-CNE1 antibody is preincubated with the CNE1 peptide (*D*) or the anti-CNE2 antibody is preincubated with the CNE2 peptide (*E*). Scale bars, 50 μ m.

staining of cell bodies, dendrites and the surrounding neuropil. Regions of myelinated tracts were not stained. At higher magnifications, punctate staining is observed in the neuropil and along dendritic branches.

In the hippocampal formation, we also observed a wide distribution of neurons which are immunoreactive for class E calcium channels recognized by the anti-CNE1 and anti-CNE2 antibodies. Cell bodies of pyramidal neurons in the CA1 (Fig. 6*A*) and in the CA2/CA3 regions (Fig. 6*B*) and granule cells in the

dentate gyrus are stained with the CNE antibodies. Some interneurons in the strata oriens, radiatum, and lacunosum moleculare of the hippocampus and interneurons in the molecular layer of the dentate gyrus are also immunoreactive for α_{1E} . In all regions, there is dense immunoreactivity in neuronal cell bodies. In the CA1 region there is an apparent lack of staining in the apical and basal dendrites of the pyramidal neurons while in the CA2/CA3 region there is faint staining of the proximal portion of the apical dendrites. This is in contrast to the significant staining of

Figure 5. Distribution of class E calcium channels in the globus pallidus, thalamus, and anterior amygdala. Sagittal sections of adult brain with class E antibodies using the immunofluorescence technique described in the Materials and Methods. Low magnification (*A*) of the globus pallidus and a higher magnification (*B*) to illustrate staining of both cell bodies and dendrites of neurons located in this region. *C*, Section of class E calcium channel staining in the thalamus. *D*, Control section in which class E antibody is preincubated with CNE2 peptide. Low (*E*) and high (*F*) magnifications of the anterior amygdala region to demonstrate staining in both cell bodies and dendrites. Scale bars: *A* and *E*, 200 μm ; *B*–*D* and *F*, 100 μm .

the proximal portion of the apical dendrites with antibodies against α_{1C} and α_{1D} (Ahlijanian et al., 1990; Westenbroek, 1990; Hell et al., 1993a) and the dense staining observed along the length of the apical dendrites using antibodies against α_{1B} (Westenbroek et al., 1992). There was also occasional staining of the dendritic processes of interneurons located in the dentate gyrus.

Class E calcium channels are also present in other nuclei

throughout the rat brain. Figure 7 illustrates a few of these regions which include the subthalamic nucleus, the lateral hypothalamus, the facial nucleus, and the deep cerebellar nuclei. In all regions, there is extensive staining of the cell bodies of neurons (Fig. 7*A–D*) with variable staining of dendrites. For example, there is relatively little staining of dendrites in the subthalamic nucleus while dendritic staining is more prevalent in

Figure 6. Distribution of class E calcium channels in the hippocampus. Sagittal sections of adult brain stained with class E calcium channel antibodies as described in the Materials and Methods. *A*, Immunoreactive cell bodies of CA1 pyramidal neurons. *B*, Labeled cell bodies of pyramidal neurons in the CA2/CA3 region of the hippocampus. Scale bar, 100 μ m.

the facial nucleus. This variability of dendritic staining is observed along the length of the brainstem and appears to be nucleus dependent.

In the cerebellum, the most intense staining with the CNE antibodies is observed in the cell bodies of the principal projection neurons, the Purkinje cells. Unlike the apparent absence of staining in the dendrites of projection neurons in the dorsal cortex and the hippocampus, there is α_{1E} staining in the smaller dendritic branches of the cerebellar Purkinje neurons (Fig. 8*A,B*). However, there is a striking absence of staining in the main dendritic trunks (compare Westenbroek et al., 1995, for α_{1A}). There is relatively weak but consistent staining throughout the granular layer of the cerebellum (Fig. 8*A*) with occasionally strong staining in randomly located granule cells. Due to the dense staining of the small branches of dendrites in the molecular layer it is difficult to determine whether the interneurons located in this layer are also immunoreactive for α_{1E} . As in other brain regions, the immunocytochemical staining in the cerebel-

lum suggests that class E calcium channels are located mainly in the cell bodies of neurons and, depending on the region and cell type, are also present in the dendrites.

Discussion

Biochemical properties of the α_{1E} subunit

The predicted molecular size of α_{1E} from the cloned cDNA is 252 kDa (Soong et al., 1993). In concordance, our immunoblotting experiments show that α_{1E} subunits purified from rat brain are 245–255 kDa in size. It remains to be determined whether the broad protein band observed in these experiments may contain multiple α_{1E} isoforms of similar size. Multiple α_{1E} isoforms from rabbit, rat, mouse, and human brain, with small differences in predicted molecular mass have been described in cDNA cloning experiments (Marubio et al., 1994; Williams et al., 1994; Soong and Snutch, unpublished observations), including COOH-terminal diversity generating two predicted size forms of 254 kDa and 246 kDa in the rabbit (Niidome et al., 1992). Microheterogeneity in the predicted primary structures of α_1 subunits, most likely generated by alternative RNA splicing, is also reported for α_{1A} , α_{1B} , α_{1C} , and α_{1D} subunits (Hui et al., 1991; Snutch et al., 1991; Starr et al., 1991; Williams et al., 1992a,b; Coppola et al., 1994; Hofmann et al., 1994). Our results with α_{1E} contrast with previous biochemical studies of rat brain α_{1B} , α_{1C} , and α_{1D} in which two size forms of the α_1 polypeptides differing by 30–50 kDa were observed due to truncation of the COOH-terminal domain (Hell et al., 1993a,b, 1994, 1995). If α_{1E} isoforms with COOH-terminal differences exist, they must be less than 10 kDa (~81 amino acids).

Phosphorylation by second-messenger-activated protein kinases is a well-established pathway for modulation of the function of neuronal calcium channels by neurotransmitters and hormones (Dolphin, 1990; Swartz, 1993; Yang and Tsien, 1993; Meriney et al., 1994), but it is not known if this regulation involves direct phosphorylation of calcium channel subunits. As previously shown for α_{1B} and α_{1C} , α_{1E} subunits are substrates for phosphorylation *in vitro* by PKA, PKC, PKG, and CaM KII (Fig. 2*C*). PKA also phosphorylates the α_{1E} subunit in intact hippocampal neurons further supporting a possible functional role for this modification (Hell et al., 1995b). In addition, stimulation of PKC in *Xenopus* oocytes expressing α_{1E} and β subunit cDNAs causes a significant increase in the whole-cell Ba^{2+} current, suggesting a direct functional phosphorylation of α_{1E} and/or β subunits by PKC (Stea et al., 1994b). However, it remains to be determined whether the phosphorylation sites for PKC, PKG, and CaM KII on α_{1E} are substrates *in vivo*, and further experiments are required to evaluate the specific effects of activated protein kinases on α_{1E} channel function.

The ligand binding profile of α_{1E} indicates that this subunit does not bind with high affinity to any of the peptide toxins or synthetic compounds used to characterize calcium currents in neurons or in heterologous systems expressing cloned α_1 cDNAs. These results are in agreement with the *Xenopus* oocyte expression studies of the rat α_{1E} subunit. Soong et al. (1993) found that I_{Ba} conducted by α_{1E} was not significantly affected by 10 μ M nifedipine or 1 μ M ω -CTx GVIA, and only partially blocked by 200 nM ω -Aga IVA. Later experiments indicated only a small (10–20%) blockade by 5 μ M ω -CTx MVIIC (Stea and Snutch, unpublished observations). Thus, these calcium channel antagonists neither specifically bind to nor block calcium channels containing α_{1E} , in agreement with the conclusion that it represents a novel type of calcium channel.

Figure 7. Staining of class E calcium channels in ventral nuclei. Sagittal sections were stained with class E calcium channel antibodies as described in Materials and Methods. *A*, Staining of cell bodies in subthalamic nucleus. *B*, Staining of dendrites and cell bodies in the lateral hypothalamus. *C*, Class E calcium channel staining in the cell bodies and dendrites of neurons in the facial nucleus. *D*, Class E immunoreactive neurons located in the deep cerebellar nuclei. Scale bars: *A–C*, 50 μm ; *D*, 100 μm .

Figure 8. Localization of class E calcium channels in the cerebellum. Sagittal sections labeled with class E calcium channels using the fluorescent method described in Materials and Methods. *A*, Section of cerebellum showing class E immunoreactivity in the molecular layer (*M*), Purkinje cell layer (*P*), and in the granular layer (*G*). *B*, Staining of class E calcium channels in the fine dendrites and at dendritic branch points (arrows) of Purkinje cells (*P*). Scale bar, 50 μm .

Broad distribution of class E calcium channels in the brain

Overall, while there is regional variation in the pattern and relative density of anti-CNE labeling, most areas of gray matter are stained above background. This suggests that there is a broad expression of the α_{1E} subunit throughout the brain. *In situ* hybridization studies in rat and mouse showed that α_{1E} transcripts are widely expressed in the CNS (Soong et al., 1993; Williams et al., 1994). Consistent with these results, we find that the CNE antibodies label calcium channels in neurons throughout the brain, including the olfactory bulb, the frontal and dorsal cerebral cortex, hippocampus, midbrain, hindbrain, and cerebellum. Large amounts of rE-II transcripts were detected in periglomerular, mitral, and granule cells of the olfactory bulb, neocortical layers II through VI, hippocampus, striatum, amygdala, hypothalamus, thalamus, and cerebellum (Soong et al., 1993). In general, our protein localization studies correspond well with these findings. However, within the thalamus, RNA expression was mainly localized to the intralaminar, parafascicular and reticular nuclei while our findings suggest that α_{1E} is localized throughout the entire thalamus. In addition, rE-II transcripts were found to be relatively high in the granule cell and Purkinje cell layers of the cerebellum. Our immunocytochemical findings suggest that α_{1E} is prominent in Purkinje cells, but is present at a substantially lower level in the granule cells of the granular layer. In mouse, abundant α_{1E} transcript levels were observed in similar regions to those investigated in the rat, although only low levels were detected in the reticular nucleus of thalamus (Williams et al., 1994). The slight differences in RNA expression between rat and mouse may be due to alternative processing of α_{1E} transcripts. For example, the NH₂-terminal sequences of rabbit, human, and mouse clones (Niidome et al., 1992; Williams et al., 1994) differ from that reported for the rat (Soong et al., 1993).

Localization of class E calcium channels in neuronal cell bodies

In contrast to the calcium channels containing α_{1A} , α_{1B} , α_{1C} , or α_{1D} subunits, class E calcium channels conduct transient calcium currents with rapid, voltage-dependent inactivation, and both activation and steady state inactivation occur at relatively negative membrane potentials (Soong et al., 1993; Williams et al., 1994). These characteristics are similar to a subset of relatively low-voltage-activated calcium channels and suggest that class E calcium channels may initiate calcium-dependent action potentials in response to excitatory postsynaptic potentials at relatively negative membrane potentials. Because they have distinct physiological properties compared to previously studied classes of brain calcium channels, the subcellular localization of class E calcium channels is of great interest. The distribution of class E calcium channels at varying levels in the cell bodies of neurons throughout the central nervous system suggests that they exert an important influence on cytosolic calcium levels and calcium-dependent electrical events in the cell bodies of many central neurons. In addition to generating a transient depolarization of the cell body in response to lower threshold stimuli, they may provide calcium entry for general cellular processes such as gene expression, regulation of cellular signaling pathways, and control of membrane excitability.

The preferential staining of α_{1E} in cell somas is similar to the α_{1C} and α_{1D} subunits of L-type calcium channels (Ahlijanian et al., 1990; Westenbroek et al., 1990; Hell et al., 1993a). Similar to α_{1D} , α_{1E} is distributed relatively smoothly over the cell surface

in contrast to the patchy distribution of α_{1C} . The distinct physiological properties of class E channels may be important in initiating calcium-dependent action potentials in cell bodies and may provide a distinct pattern of calcium entry that is important in some aspects of neuronal regulation. The distribution of α_{1E} staining in the cell bodies also contrasts with our immunocytochemical studies using anti-peptide antibodies to the α_{1A} and α_{1B} subunits (Westenbroek et al., 1992, 1993, 1995) which exhibit little or no immunostaining on the cell soma of most neurons.

The staining of the cell body of most neurons by the CNE antibodies appears to fill the soma rather than just outline its periphery. This pattern is more noticeable for α_{1E} than for other calcium channel classes, and is observed with both the anti-CNE1 and anti-CNE2 antibodies and in both peroxidase-antiperoxidase and fluorescent visualization methods. In part, this staining pattern probably reflects the intracellular location of the epitopes against which the CNE antibodies were prepared. In addition, it is likely that pools of α_{1E} undergoing biosynthesis and processing in the endoplasmic reticulum and Golgi apparatus are also prominently stained and contribute to the intracellular staining. There may be a larger intracellular pool of α_{1E} precursors than for the other non-L-type calcium channel α_1 subunits that we have studied. Direct identification of such an intracellular pool of α_{1E} and characterization of its role as a biosynthetic intermediate will require electron microscopic analysis of its localization and pulse-labeling studies of its metabolic fate.

The staining for α_{1E} in Purkinje neurons contrasts with electrophysiological studies which identify P-type current as the predominant calcium current in this cell (Mintz et al., 1992; Usowicz et al., 1992). However, these measurements were made in the cell body and large dendrites, while the most prominent immunostaining we observe is in the fine distal dendrites. These electrophysiological studies would not have detected calcium currents in the distal dendrites. Much of the cell body immunostaining that we observe with the anti-CNE antibodies is intracellular, so it is difficult to correlate with calcium current measurements. The development of selective blocker for class E calcium channels is needed to quantitate their contribution to the calcium current in Purkinje cell bodies more accurately.

Localization of class E calcium channels in dendrites

Class E calcium channels are also strikingly localized in the dendrites of a distinct subset of CNS neurons. The distribution of α_{1E} immunoreactivity in dendrites appears to be more complex than for other channel types we have studied since dendrites are labeled in only certain nuclei and cell types. For example, in the hippocampus and the dorsal cortex there is barely detectable labeling of α_{1E} in proximal dendrites while in other regions, such as the olfactory bulb, the amygdala, and the cerebellum, there is intense α_{1E} staining along the distal portions of the dendrites. This contrasts with our previous immunocytochemical studies in which high-voltage-activated channels were localized along the entire length of dendrites of most neurons using anti-peptide antibodies to α_{1B} (Westenbroek et al., 1992) or α_{1A} (Westenbroek et al., 1993, 1995).

Both low-voltage activated, transient calcium channels and high voltage-activated calcium channels are thought to be involved in the generation of calcium-dependent action potentials that are conducted along the length of dendrites (Llinas and Sugimori, 1979). The dendritic arbor of the cerebellar Purkinje neurons is an important site of calcium entry and electrogenesis in these neurons (Llinas and Sugimori, 1980; Tank et al., 1988).

Recently, whole-cell patch-clamp recording combined with real-time confocal laser microscopy have revealed that stimulation of parallel fibers in the cerebellum results in a rise in intracellular calcium within the fine branches of the dendrites of Purkinje cells (Eilers et al., 1994). Other studies in the cerebellum suggest that long-term depression of synaptic transmission is initiated by climbing fiber activation of voltage-gated calcium channels located on the dendrites of Purkinje neurons and by the subsequent rise in intracellular calcium postsynaptically (Konnerth et al., 1992; Ito et al., 1982). These responses were blocked by the AMPA-specific glutamate receptor blocker CNQX, and voltage clamp of the Purkinje cells was shown to reduce or eliminate the calcium signals. These findings suggested that these calcium signals arise from depolarization of the distal dendrites by activation of AMPA-specific glutamate receptors resulting in opening of voltage-gated calcium channels. Our immunocytochemical studies suggest that these may be the class E calcium channels which we observed specifically localized at high density in the distal branches of the Purkinje cell dendrites.

We also observed an increased density of class E immunoreactive calcium channels at branch points in Purkinje cell dendrites. A similar pattern of dendritic staining at branch points was observed with our antibody to α_{1B} (Westenbroek et al., 1992) and by Fortier et al. (1991) using an anti- ω -CTx GVIA antibody. This high concentration of calcium channels at dendritic branch points may contribute to the generation and conduction of calcium-dependent action potentials at these sites. In addition, ryanodine-sensitive and inositol triphosphate-activated intracellular calcium release channels appear to be more abundant at the main branch points of the Purkinje cell dendritic arbor (Ellisman et al., 1990; Walton et al., 1991) suggesting that the dendritic branch points play a vital role in the integration of dendritic signals which are calcium-dependent.

Correlation between α_{1E} and native calcium channels

Electrophysiological analyses of rat α_{1E} expressed in *Xenopus* oocytes, together with our ligand binding and immunolocalization data, demonstrate that α_{1E} exhibits distinct functional and pharmacological properties from L, N, P, and Q-type channels. α_{1E} has been proposed to be a "non-classic" low-voltage-activated calcium channel based on its relatively low threshold for voltage activation, fast kinetics of activation and inactivation, hyperpolarization-dependent repriming, high-affinity Ni^{2+} blockade (Soong et al., 1993), and permeation properties favoring Ca^{2+} over Ba^{2+} as a charge carrier (Bourinet et al., 1994). However, expression of structurally related α_1 subunits from marine ray (Ellinor et al., 1993), rabbit (Wakamori et al., 1993), and human (Williams et al., 1994) have shown that the voltage dependence of activation of α_{1E} -containing calcium channels can vary over a range of membrane potentials between those characteristic of low-voltage-activated and high-voltage-activated channels. While there are uncertainties inherent in comparing data from different expression protocols using different coexpressed auxiliary subunits, there are several previously described calcium currents with properties similar to those of the expressed α_{1E} cDNA, including the R-type whole-cell calcium current of cerebellar granule neurons (Zhang et al., 1993), the G2 single-channel current in cerebellar granule cells (Forti et al., 1994), calcium transients in the distal dendrites of cerebellar Purkinje cells, as discussed above, and a novel transient calcium current in inhibitory neurons of the thalamic reticular nucleus (Huguenard and Prince, 1992). Our immunolocalization studies are con-

sistent with a role for class E calcium channels in the transient calcium currents in the distal dendrites of cerebellar Purkinje cells and in thalamic reticular neurons, since we observe a substantial level of immunostaining for α_{1E} in those locations. Staining of the cerebellar granule cells by the CNE antibodies is clearly detectable, but is less prominent than staining of many other classes of neurons. The R-type current represents approximately 15% of total calcium current in cerebellar granule neurons (Zhang et al., 1993). A strict quantitative correlation between immunostaining and current density is not expected because multiple calcium channels may contribute to the R-type current as presently defined, and alternative splicing may produce class E calcium channels that are not recognized by our two antibodies. Therefore, the R-type whole-cell calcium current or the G2-type single-channel current in cerebellar granule neurons may also be due to class E calcium channels.

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