Rat Brain Expresses a Heterogeneous Family of Calcium Channels

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Rat brain expresses a heterogeneous family of calcium channels
(cDNA cloning/hybrid arrest/dihydropyridine receptor)

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ABSTRACT We describe the isolation and characterization of several rat brain cDNAs that are homologous to the α1 subunit of heart and skeletal muscle dihydropyridine-sensitive Ca channels. Northern blot analysis of 32 cDNAs shows that they can be grouped into four distinct classes (A, B, C, and D), each corresponding to a distinct hybridization pattern of brain mRNA. Southern blotting and DNA sequencing suggest that each class of cDNA represents a distinct gene or gene family. In the regions sequenced, the rat brain class C and D gene products share ≈75% amino acid identity with the rabbit skeletal muscle Ca channel. In addition, the class C polypeptide is almost identical to the rabbit cardiac Ca channel (97% identity). In contrast, the rat brain class A and B cDNAs are more distantly related to dihydropyridine-sensitive Ca channels (47–64% amino acid identity) and to the brain class C and D genes (51–55% amino acid identity). To examine the functional significance of the isolated brain cDNAs, hybrid depletion experiments were performed in Xenopus oocytes. Antisense oligonucleotides against class A and B cDNAs each partially inhibited, and a class C oligonucleotide almost fully inhibited, the expression of Ba current in rat brain mRNA injected oocytes; but none of the oligonucleotides affected the expression of voltage-gated Na or K conductances. The clone characterization and sequencing results demonstrate that a number of distinct, yet related, voltage-gated Ca-channel genes are expressed in the brain. The antisense oligonucleotide experiments specifically show that one or several of the Ca-channel classes are related to the Ca channels observed in rat brain mRNA injected oocytes.

The entry of Ca into a wide variety of cell types is mediated by voltage-gated Ca channels. Calcium entry contributes to a number of physiological functions, including muscle contraction, release of neurotransmitters and hormones, and modulation of other membrane ion channels (1, 2). Pharmacological and electrophysiological evidence has demonstrated that a number of distinct Ca channels exist in neurons (for example, see refs. 3–6). These various Ca channels have been grouped into general categories according to their voltage dependence, kinetics of activation and inactivation, pharmacology, and single-channel properties. Specific cell types express a subset of Ca-channel types, suggesting a differential expression of Ca-channel genes (reviewed in ref. 7).

The exact subunit composition of neuronal Ca channels has not been determined. Biochemical analysis of the dihydropyridine (DHP) receptor/Ca channel of skeletal muscle shows that it is composed of five distinct subunits (8, 9). DNA cloning has revealed the primary structure of the skeletal muscle α1, α2, β, and γ subunits (10–12, 30). More recently, the structure of the cardiac α1 subunit has also been reported (13). Both the skeletal muscle and cardiac α1-subunit genes encode large polypeptides (212 and 242 kDa, respectively) and are structurally similar to the α subunit of Na channels (14, 15, 31). Both the Na-channel α subunit and the Ca-channel α1 subunit possess four internal repeats, each containing six putative transmembrane regions. In one of these transmembrane domains, every third residue is positively charged; this transmembrane helix is thought to contain the voltage sensor of the molecule (16).

Cardiac ventricular myocytes express mainly DHP-sensitive Ca channels with the slow inactivating properties classified as L-type, and Xenopus oocytes injected with cardiac mRNA express the same kind of channels. In agreement with this, microinjected synthetic RNA derived from the cloned cardiac α1-subunit cDNA directs the synthesis of similar channels in oocytes (13). Furthermore, expression of the skeletal muscle α1-subunit cDNA in myotubes from mdg mice and in mouse L cells results in functional DHP-sensitive Ca currents (17, 18). Thus, the α1 subunit acts both as a DHP receptor and as a voltage-sensitive ion channel. The functional role of the other Ca-channel subunits is not clear, although they may be involved in regulation of the α1 subunit (13).

Here we show that rat brain expresses a family of genes that are related to the α1 subunit of skeletal muscle and of cardiac Ca channels. That the isolated cDNAs encode Ca channels is supported by DNA sequence data and by hybrid depletion experiments using Xenopus oocytes injected with rat brain RNA. Antisense oligomers for three of the four classes of cDNA partially (and in one case almost fully) inhibit the expression of rat brain Ca channels synthesized in oocytes. These same oligomers do not affect the expression of brain K or Na channels. These results strongly suggest that at least a portion of observed Ca-channel diversity in neurons is the result of the expression of distinct α1 subunits.

EXPERIMENTAL PROCEDURES

Isolation of Rat Brain Ca-Channel cDNAs. RNA was isolated from the brains of 16-day-old rats by a modification of the lithium chloride/urea procedure (19). A size-selected rat brain cDNA library was constructed into the phage vector Lambda ZapII (Stratagene). A portion of the unamplified library (1.8 × 109 plaque-forming units) was screened with a 1.2-kilobase (kb) Pst I fragment (nucleotides 2813–3992) (11) of the α1 subunit of the rabbit skeletal muscle Ca channel. After three rounds of plaque purification, the positive phage were transferred into Bluescript phage-mids by the in vivo excision protocol described by the supplier.

Abbreviation: DHP, dihydropyridine.

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RESULTS

Isolation of Rat Brain Ca-Channel cDNAs. Our previous studies have shown that the majority of the Ca-channel activity induced in *Xenopus* oocytes by rat brain mRNA is encoded by mRNAs \( \geq 6 \) kb (24). To enrich for large mRNAs, a rat brain cDNA library was constructed from size-fractionated cDNA > 4 kb. Utilizing a portion of the rabbit muscle Ca-channel \( \alpha_1 \)-subunit gene as a probe, an initial screen resulted in the purification of 47 rat brain cDNAs. The average size of the cDNAs was 5.3 kb. Utilizing the brain cDNAs as probes we have recently isolated an additional 70 brain cDNA clones (T.P.S., unpublished results).

**Ca Channels Are a Multigene Family.** To characterize the brain cDNAs, Northern blots to brain mRNA were prepared. Of the 32 cDNAs assayed, each hybridized to one of four distinct RNA banding patterns (Fig. 1). Class A cDNAs (15 clones) hybridize to two major mRNAs of almost equal intensity of \( \approx 8.3 \) and \( \approx 8.8 \) kb. Class B cDNAs (10 clones) hybridize to a major mRNA species of \( \approx 10 \) kb. Class C cDNAs (4 clones) hybridize to two mRNAs of \( \approx 8.0 \) and \( \approx 12 \) kb. Finally, class D cDNAs (3 clones) hybridize to a single mRNA of \( \approx 9.5 \) kb. All of the brain cDNAs hybridize to mRNAs large enough to encode polypeptides of the sizes reported for the skeletal muscle and cardiac Ca channels (1873 and 2171 amino acids, respectively). We note that the doublet of high molecular weight RNAs that hybridize to the class C cDNAs is similar to that observed by hybridization of the cardiac Ca-channel gene to brain RNA (13). To determine the molecular nature of the different classes of brain clones, we hybridized a radiolabeled probe from each class of cDNA to blots of rat genomic DNA. Under the high-stringency washing conditions used, members of the four classes all hybridize to unique patterns of genomic DNA fragments (Fig. 2), suggesting that each of the four classes of cDNA is encoded by a distinct gene or gene family. We find that cDNAs assigned to a specific class by Northern blot analysis also show a similar banding pattern to genomic DNA (i.e., rbA-73 and rbA-65), confirming that at least four different Ca-channel genes are expressed in brain.

**Primary Structure of Brain Ca Channels.** The derived amino acid sequence of a member of each of the brain Ca-channel classes is shown in Fig. 3. The partial sequences...
terminal amino acids (of rbc-C61 and an overlapping clone, rbc-C30) shows that the brain class C peptide is 97% identical to the rabbit cardiac peptide over the region compared (data not shown).

**Hybrid Depletion.** Injection of rat brain RNA into *Xenopus* oocytes results in the expression of a high-threshold, partially inactivating Ba current that is insensitive to DHPs and to ω-conotoxin (23–25). A number of studies have demonstrated that *Xenopus* oocytes possess an endogenous RNase H activity that, in the presence of antisense oligonucleotides, acts to block expression of exogenous mRNAs (26, 27). To examine the functional significance of the various brain Ca-channel cDNAs, we synthesized sense and antisense oligomers (18-mers), hybridized them briefly to rat brain mRNA, and injected the mixtures into oocytes. After 2–4 days, the oocytes were tested for the expression of functional voltage-gated Ca channels (measured as a Ba current). For controls, we took advantage of the fact that rat brain mRNA also induces the synthesis of Na channels and a delayed rectifier-type K channel (28).

To minimize the effects of variable expression normally observed in RNA injected oocytes, the results are presented as ratios of $I_{Na}/I_K$ or $I_{Ba}/I_K$ from each oocyte ($n = 5–10$). Table 1 shows that coinjection of Na-channel sense oligomer and brain RNA results in the expression of functional Na channels, whereas no Na-channel activity is detected after coinjection of an antisense Na-channel oligomer. Thus, 56 ng of antisense oligomer is sufficient to block 100% of the Na-channel activity in brain mRNA injected oocytes. As compared to a sense oligomer, coinjection of an antisense oligomer against rba-65 results in the inhibition of ~50% of the rat brain Ba current. An antisense oligomer from rbb-10 blocks ~68% of the Ba current, while an antisense oligomer against rbc-C61 inhibits >90% of the brain Ba current (see also Fig. 4). The injection of an antisense rbd-55 oligomer had no significant effect on the rat brain Ba current. These oocytes showed no detectable endogenous $I_{Ba}$. None of the Ca-channel sense or antisense oligomers had an appreciable effect on the expression of brain K or Na channels. That the class A, B, and C antisense oligomers suppress only the expression of Ca channels in brain RNA injected oocytes.

### Table 1. *Xenopus* oocyte hybrid depletion

<table>
<thead>
<tr>
<th></th>
<th>$I_{Ba}/I_K$</th>
<th>$I_{Na}/I_K$</th>
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<tr>
<td>Na sense</td>
<td>0.12 (±0.02)</td>
<td>0.96 (±0.11)</td>
</tr>
<tr>
<td>Na anti</td>
<td>0.13 (±0.03)</td>
<td>Zero $I_{Na}$ detected</td>
</tr>
<tr>
<td>rba-65 sense</td>
<td>0.14 (±0.02)</td>
<td></td>
</tr>
<tr>
<td>rba-65 anti</td>
<td>0.07 (±0.01)</td>
<td></td>
</tr>
<tr>
<td>rbb-8 sense</td>
<td>0.22 (±0.03)</td>
<td></td>
</tr>
<tr>
<td>rbb-8 anti</td>
<td>0.07 (±0.02)</td>
<td></td>
</tr>
<tr>
<td>rbc-61 sense</td>
<td>0.13 (±0.02)</td>
<td></td>
</tr>
<tr>
<td>rbc-61 anti</td>
<td>0.01 (±0.01)</td>
<td></td>
</tr>
<tr>
<td>rbd-55 sense</td>
<td>0.19 (±0.01)</td>
<td></td>
</tr>
<tr>
<td>rbd-55 anti</td>
<td>0.17 (±0.02)</td>
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Peak Na currents were measured at −10 mV in SOS without gentamicin and pyruvate. Peak K currents were measured in the same solution at +40 mV. Peak Ba currents were measured at +10 mV in modified saline. All currents were leak subtracted and the ratio of either Na/K or Ba/K was determined. The SEM ($n = 5–18$) of the ratios was calculated. The antisense oligomers used were as follows: Na channel, 5'-ATACCTCTCTATCACGTC-3'; rba-65, 5'-AGCCAGATTTGTTGGA-3'; rbb-10, 5'-TCCGGAAGTTGT-3'; rbc-61, 5'-TGGGATATGCTAGCTG-3'; rbd-55, 5'-CATCCGTATGCCAATGC-3'. The rbc-C61 antisense oligomer corresponds to the amino acid sequence PAEHTQ in the region separating domains IV3 and IV4 shown in Fig. 3B. This oligomer is not homologous to any sequences obtained to date for the class A, B, and D cDNAs.
strongly suggests that these cDNAs encode brain Ca
channels, rather than channels for other cations.

**DISCUSSION**

Using low-stringency hybridization with a skeletal muscle
probe, Ellis et al. (11) suggested the existence of multiple
Ca-channel α1-subunit genes in the rabbit genome. By the
criteria of Northern blot, Southern blot, and deduced amino
acid sequences, we find that at least four distinct α1-subunit
genes are expressed in rat brain alone. The partial amino acid
sequence derived from DNA sequencing of the class A and
class B cDNAs shows that they share between 47% and 64%
identity with the skeletal muscle and cardiac DHP-sensitive,
L-type Ca channels (10, 13). The class C and class D
polypeptides are more closely related to the rabbit cardiac
and skeletal L-type channels than to the rat brain class A and
class B Ca channels. One of the rat brain genes, rbC-61,
shows ≈97% amino acid identity to the rabbit cardiac L-type
channel in the region sequenced so far. That the putative
voltage-sensor region (S4; Fig. 3) of the brain class A and B
channels is distinct from that of the brain C and D channels
suggests the possibility that these molecules have different
voltage-dependent gating properties (16, 29).

We have demonstrated that at least four major classes of
neuronal Ca channel exist and that each class is encoded by
at least one gene. We have also found a number of cDNAs
that hybridize to brain RNA to give identical banding patterns
but upon further study prove to have unique coding
sequences (data not shown). For example, the brain expresses
two distinct class C transcripts (rbC-61 and rbC-48) and two
distinct class D transcripts (rbD-55 and rbD-40). The molecu-
lar nature of these subclasses of Ca channel has not been
determined. How many distinct Ca-channel transcripts are
expressed in brain? While we have direct evidence for the six
α1-subunit transcripts described, it is likely that even more
exist. For example, the existence of multiple class A-related
Ca channels is suggested by the fact that class A cDNAs
hybridize to two major RNAs of ≈8.3 and ≈8.8 kb (Fig. 1)
and that restriction enzyme digest analysis of several class A
cDNAs shows a significant amount of heterogeneity (data not
shown). We estimate that a minimum of eight different Ca-channel transcripts are expressed in rat brain.

**FIG. 3.** Comparison of amino acid sequences (single-letter code) of cloned Ca channels. The deduced amino acid sequences of portions of the four brain classes and of the cardiac and skeletal muscle α1 subunits were aligned. The amino acid sequence of rbD-55 is shown on the top line of A, while the sequence for rbC-61 is shown on the top line of B. Residues of other Ca channels that are identical to the top lines in A and B are indicated by dashes. Gaps are indicated by spaces. The putative transmembrane segments (IIS6–IVS5) are indicated by brackets above the sequences. (A) Comparison of the brain class D (rbD-55), class B (rbB-10), rabbit cardiac (c-DHP), and rabbit skeletal muscle (sk-DHP) sequences. (B) Comparison of the brain class C (rbC-61), class A (rbA-65), rabbit cardiac (c-DHP), and skeletal muscle (sk-DHP) sequences.

**FIG. 4.** Hybrid depletion of rat brain Ca current in *Xenopus* oocytes. Rat brain poly(A)+ RNA was hybridized with sense or antisense oligomers for rbC-61, the mixture was coinjected into *Xenopus* oocytes, and currents were studied as described. The traces represent voltage-clamp currents measured by stepping from a holding potential of −80 mV to a test potential of +10 mV.
Xenopus oocytes provide a convenient assay system for the functional significance of cDNAs thought to encode channels or receptors (28). The Ca channels expressed when brain mRNA is injected into oocytes are partially inactivating, DHP and ω-conotoxin insensitive (23–25). Our results show that the expression of these channels is almost fully blocked by the class C antisense oligomer. This oligomer (see Fig. 3 and Table 1 legend) is not closely related to any sequence obtained thus far for any of the class A, B, or D cDNAs. We take this as strong evidence that the class C gene or genes encode a significant component of brain Ca channels expressible in oocytes. However, because there are two or more distinct class C transcripts, we do not know at present what fraction of the brain RNA-induced signal is due to the particular transcript cloned as rbc-61. The antisense oligomers rbA-65 and rbb-10 are 83% and 78%, respectively, identical to the corresponding rbc-61 cDNA sequence. Their partial suppression (50% and 68%, respectively) of brain injected Ba currents may be due to this sequence homology. The rbd-55 cDNA antisense oligomer does not block expression of Ba currents in brain RNA injected oocytes at all, although this clone is moderately expressed in brain RNA by the criterion of Northern blotting. The significance of the lack of any hybrid depletion effect due to the D clone is at present unknown.

Several types of Ca channels have been observed by electrophysiological studies of peripheral and central neurons (3–6). For presently unknown reasons, using rat brain mRNA we detect the expression in oocytes of only a single class of channel that fits none of the available descriptions perfectly (23–25). The hybrid depletion results suggest that the class C gene is most closely related to this channel type.

That different populations of mammalian neurons express specific types of Ca channel presumably reflects the diverse roles that Ca channels play in mediating transmitter release and in regulating excitability (7). While several possible molecular mechanisms could generate neuronal Ca-channel diversity, our results suggest that the expression of distinct Ca-channel α1 subunits is a significant source of this diversity.

We are grateful to Steve Dubel for technical assistance and to Ellis et al. (11) for providing the rabbit Ca-channel probe. We also thank Mike White for the suggestion of using niflumic acid to block the oocyte Cl current. This work was supported by grants from the Medical Research Council of Canada (T.P.S.) and National Institutes of Health Grants NS-26432 (J.P.L.), GM-10991 (N.D.), and GM-29836 (H.A.L.). During the earlier phases of this work, T.P.S. was supported by an American Heart Association Fellowship, Greater Los Angeles Affiliate, at the California Institute of Technology.