Proc. Natl. Acad. Sci. USA
Vol. 87, pp. 3391–3395, May 1990
Neurobiology

Rat brain expresses a heterogeneous family of calcium channels

(cDNA cloning/hybrid arrest/dihydropyridine receptor)

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Contributed by Norman Davidson, February 21, 1990

ABSTRACT We describe the isolation and characterization of several rat brain cDNAs that are homologous to the α_1 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 mRNAs. Southern blot 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 α_1, α_2, β, and γ subunits (10–12, 30). More recently, the structure of the cardiac α_1 subunit has also been reported (13). Both the skeletal muscle and cardiac α_1-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 α_1 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 α_1-subunit cDNA directs the synthesis of similar channels in oocytes (13). Furthermore, expression of the skeletal muscle α_1-subunit cDNA in myotubes from *mdg* mice and in mouse L cells results in functional DHP-sensitive Ca currents (17, 18). Thus, the α_1 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 α_1 subunit (13).

Here we show that rat brain expresses a family of genes that are related to the α_1 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 α_1 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 (1.8×10^5 plaque-forming units) was screened with a 1.2-kilobase (kb) *Pst* I fragment (nucleotides 2813–3992) (11) of the α_1 subunit of the rabbit skeletal muscle Ca channel. After three rounds of plaque purification, the positive phage were transformed into Bluescript phage-mids by the *in vivo* excision protocol described by the supplier.

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Abbreviation: DHP, dihydropyridine.
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With respect to nomenclature, the cDNAs are described both by a clone number and by the specific pattern of RNA bands that they give on Northern blots. There are four types of RNA pattern; classes A, B, C, and D (see *Results*). Individual cDNAs are assigned a clone number with a prefix designating the RNA hybridization pattern. For example, the brain clone rbA-65 identifies rat brain clone 65, which hybridizes to brain mRNA resulting in the class A pattern.

DNA and RNA Hybridizations. For Southern blots, rat liver DNA was digested to completion with *Eco*RI or *Hin*dIII and 10 μg per lane was separated through a 0.8% agarose gel containing 89 mM Tris/89 mM boric acid/1 mM EDTA. DNA was transferred by capillary blot to nylon filters (Nytran, Schleicher & Schuell) and fixed by baking at 75°C for 75 min. Hybridization was carried out with nick-translated probes (20) at 68°C in 5X SSPE (1× SSPE = 0.18 M NaCl/10 mM phosphate, pH 7.4/1 mM EDTA)/0.3% SDS/denatured salmon sperm DNA (0.2 mg/ml). Filters were washed five times for 30 min each in 0.2× SSPE/0.1% SDS at 65°C. Probes for the various Ca-channel classes were gel-eluted *Eco*RI fragments from the Bluescript plasmids: 4.5-kb fragment of rbA-73; 3.1-kb fragment of rbB-8; 2.0-kb fragment of rbC-61; 1.3-kb fragment of rbD-40.

Northern blots were prepared with rat brain poly(A)$^+$ RNA (5 μg per lane) as described (21). Hybridizations were performed with nick-translated DNA probes at 68°C in 5× SSPE/1% SDS/2.5× Denhardt's solution (1× Denhardt's solution = 0.02% bovine serum albumin/0.02% Ficoll/0.02% polyvinylpyrrolidone)/denatured salmon sperm DNA (0.2 mg/ml). Filters were washed four times for 30 min each at 68°C in 0.2× SSPE/0.1% SDS.

DNA Synthesis and Sequencing. Oligodeoxynucleotides (oligomers) were synthesized with an Applied Biosystems DNA synthesizer (model 391). Ca-channel oligomers used for DNA sequencing and for hybrid depletion were 18 bases long (18-mers). The Na-channel sense and antisense oligomers were 19-mers and were conserved between the rat brain type I, II, IIA, and III Na-channel genes (14, 15). DNA sequencing was performed on double-stranded plasmid DNA with T7 polymerase by the modified dideoxynucleotide method (22).

Hybrid Depletion Assay. Oocytes were surgically removed from anesthetized adult *Xenopus laevis* as described (23). After removal of follicular cells, stage V and VI oocytes were selected and maintained in standard oocyte saline (SOS; 100 mM NaCl/2 mM KCl/1.8 mM $CaCl_2$/1 mM $MgCl_2$/5 mM Hepes, pH 7.6) supplemented with 2.5 mM pyruvate and gentamicin (50 μg/ml). For rat brain RNA hybrid depletion, poly(A)$^+$ RNA (4 mg/ml) was heated to 65°C for 3 min, diluted with 1 vol of oligomer solution (100 mM NaCl/1.6 mg of oligomer per ml) and incubated for 10 min at 42°C. The reaction mixture was cooled on ice and 70 nl was microinjected into individual oocytes; thus, each injected oocyte contained ≈120 ng of brain RNA and ≈56 ng of oligomer.

After incubation for 2–4 days, the oocytes were tested for the expression of voltage-gated channels with a two-microelectrode voltage clamp. Microelectrodes were filled with 3 M KCl and had resistances from 0.5 to 2 MΩ. Peak Na currents were measured in SOS by stepping from −100 mV to −10 mV. Peak K currents were measured in SOS by stepping from −100 mV to +40 mV. For reasons described previously (23), Ca channels were measured as peak Ba currents at +10 mV in a modified saline containing both K- and Cl-channel inhibitors (36 mM tetraethylammonium/5 mM 4-aminopyridine/2 mM KCl/40 mM $BaCl_2$/150 μM niflumic acid/5 mM Hepes, pH 7.6). Computerized stimulation and analysis procedures utilized pCLAMP software (Axon Instruments, Foster City, CA). All currents were leak subtracted.

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 >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 α_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 ≈8.3 and ≈8.8 kb. Class B cDNAs (10 clones) hybridize to a major mRNA species of ≈10 kb. Class C cDNAs (4 clones) hybridize to two mRNAs of ≈8.0 and ≈12 kb. Finally, class D cDNAs (3 clones) hybridize to a single mRNA of ≈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 length 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

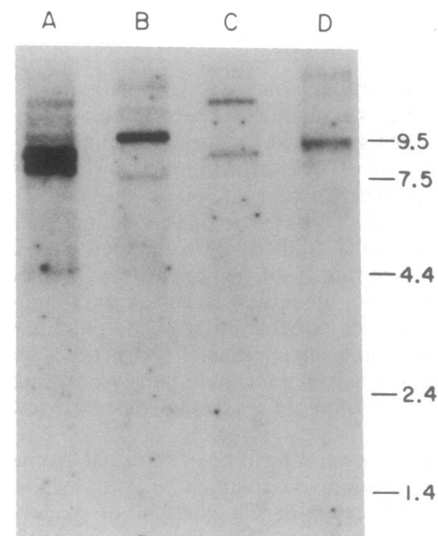

FIG. 1. Autoradiograph of hybridization of Ca-channel cDNAs to brain RNA. Brain poly(A)$^+$ RNA (5 μg per lane) was electrophoresed and hybridized. Probes were as follows: class A, rbA-63; class B, rbB-35; class C, rbC-24; class D, rbD-40. Size markers (in kb) are RNA standards. Autoradiography was for 3 days with an intensifying screen.

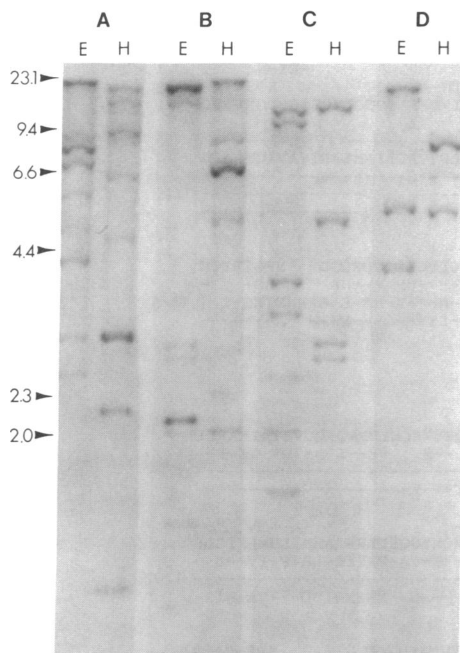

FIG. 2. Autoradiograph of hybridization of Ca-channel cDNAs to rat liver DNA. Rat liver DNA (10 μg per lane) was digested with restriction enzymes (E, *Eco*RI; H, *Hin*dIII), electrophoresed through a 0.8% agarose gel, and blotted to nitrocellulose. Hybridization and washing was carried out with ^{32}P-labeled gel-purified cDNA fragments. Probes were as follows: class A, 4.5-kb rbA-73; class B, 3.1-kb rbB-8; class C, 2.0-kb rbC-61; class D, 1.3-kb rbD-40. Size markers (in kb) are *Hin*dIII-digested phage λ DNA. Autoradiography was for 2 days with an intensifying screen.

correspond most closely to regions of the third and fourth domains of the muscle and cardiac α_1 subunits. As shown in Fig. 3, the class A, B, C, and D brain cDNAs are structurally related to each other and to the skeletal muscle and cardiac Ca-channel α_1 subunits. The proposed transmembrane segments, S1–S6, are conserved in all four classes of brain cDNA. Particularly notable is the S4 region, the putative voltage sensor of the molecule (16). Fig. 3 shows that two distinct patterns occur in the S4 regions of Ca channels isolated to date. The rabbit cardiac and the rat brain class C and D Ca channels have identical S4 segments, which are terminated by an acidic residue (glutamic acid). The skeletal muscle Ca channel is similar except for four neutral amino acid substitutions. The brain class A and B S4 segments are dissimilar to the other S4 segments in that (*i*) the last lysine and arginine are separated by only two residues, and (*ii*) the region is not terminated by a glutamic acid residue. Another region of note between the cloned Ca channels is the region separating the S3 and S4 segments. Except for the class C and rabbit cardiac genes, this region is hypervariable between the various Ca channels, with a number of deletions, insertions, and substitutions.

The sequence information can be summarized as follows. The class A and B genes (rbA-65 and rbB-10) are more highly related to each other (91% identity) than to the other Ca channels (between 47% and 64% identity). Also, rbC-61 and rbD-55 are more closely related both to each other (76% identity) and to the skeletal muscle and cardiac DHP-sensitive Ca channels than to either rbA-65 or rbB-10. An exceptionally high degree of similarity exists between rbC-61 and the cardiac DHP-sensitive Ca channel. Except for a single asparagine to serine substitution (corresponding to residue 1346 of the cardiac Ca channel), the rbC-61 gene product is identical to the cardiac Ca channel in the region shown in Fig. 3. Analysis of an additional 1600 amino-terminal amino acids (of rbC-61 and an overlapping clone, rbC-30) 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-61 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

	I_{Ba}/I_K	I_{Na}/I_K
Na sense	0.12 (±0.02)	0.96 (±0.11)
Na anti	0.13 (±0.03)	Zero I_{Na} detected
rbA-65 sense	0.14 (±0.02)	
rbA-65 anti	0.07 (±0.01)	
rbB-8 sense	0.22 (±0.03)	
rbB-8 anti	0.07 (±0.02)	
rbC-61 sense	0.13 (±0.02)	
rbC-61 anti	0.01 (±0.01)	
rbD-55 sense	0.19 (±0.01)	
rbD-55 anti	0.17 (±0.02)	

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 = 5–10) of the ratios was calculated. The antisense oligomers used were as follows: Na channel, 5′-ATACTTCTCTATCAGCTCC-3′; rbA-65, 5′-AGCACGATGGTGTTGAGA-3′; rbB-10, 5′-TCCGGAAGTTGT-TGTGTC-3′; rbC-61, 5′-TTGGGTATGTTCAGCTGG-3′; rbD-55, 5′-CATCTGCATGCCAATGAC-3′. The rbC-61 antisense oligomer corresponds to the amino acid sequence PAEHTQ in the segment separating domains IV3 and IV4 shown in Fig. 3*B*. This oligomer is not homologous to any sequences obtained to date for the class A, B, and D cDNAs.

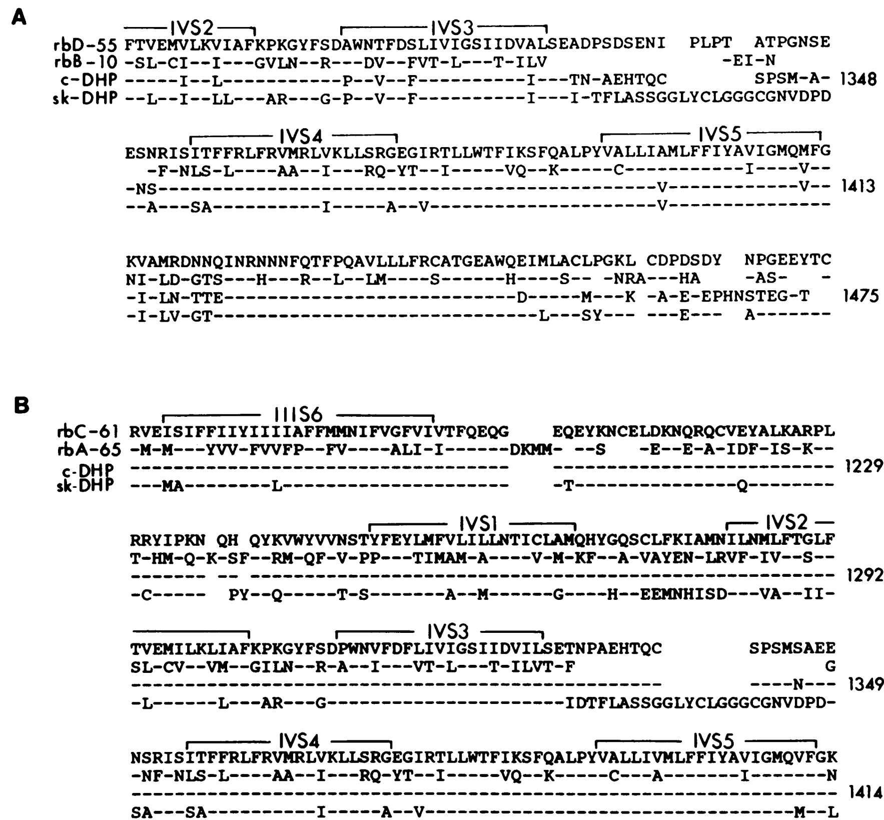

FIG. 3. Comparison of amino acid sequences (single-letter code) of cloned Ca channels. The deduced amino acid sequences of portions of the four brain classes and of the cardiac and skeletal muscle α_1 subunits were aligned. The amino acid sequence of rbD-55 is shown on the top line of *A*, while the 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 (IIIS6–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.

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 α_1-subunit genes in the rabbit genome. By the criteria of Northern blot, Southern blot, and deduced amino acid sequence, we find that at least four distinct α_1-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 molecular 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 α_1-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.

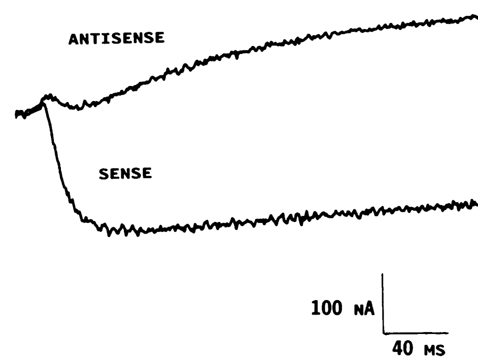

FIG. 4. Hybrid depletion of rat brain Ba current in *Xenopus* oocytes. Rat brain poly(A)$^+$ RNA was hybridized with sense or antisense oligomers for rbC-61, the mixture was coinjected into *Xenopus* oocytes, and currents were studied as described. The traces represent voltage-clamp currents measured 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 electrophysiologial 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 α_1 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.

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