A synthetic oligonucleotide was used to isolate mouse brain cDNA clones coding for a brain isoform of the α1 subunit of the voltage-sensitive Ca\(^{2+}\) channel. Twenty-six independent cDNA clones were isolated and sequenced. All the cDNA clones reported here showed high homology to the rat brain class C cDNA sequence (Snutch, T. P., Tomlinson, W. J., Leonard, J. P., and Gilbert, M. M. (1991) Neuron 7, 45–57). Comparison of the individual mouse brain class C (mbC) cDNA sequences indicated the presence of four regions within the α1 subunit coding sequence where alternative splicing can take place in mouse brain and raise the possibility that combinatorial arrangement of these splice variants could give rise to a heterogenous class of mbC transcripts. Northern blot analysis demonstrated that mbC mRNA sequences could be detected in highest abundance in mouse heart, at lower levels in mouse brain and spinal cord, and not at all in liver or skeletal muscle. An expression vector for one isoform of the mbC α1 subunit is functional in the transfected muscle. An expression vector for one isoform of the mouse brain α1 subunit cDNA was constructed using the human cytomegalovirus promoter to direct expression, and this expression vector was used in a novel transfection assay of primary cultures of bovine adrenal chromaffin cells. Transfection of the mbC α1 subunit expression vector increased the secretion of human growth hormone expression vector after stimulation with elevated K\(^+\) or the dihydropyridine agonist, Bay K8644. These experiments suggest that this isoform of the mbC α1 subunit is functional in the transfected chromaffin cells and that the number of Ca\(^{2+}\) channels is a limiting component in the secretion from chromaffin cells in culture.

Voltage-sensitive Ca\(^{2+}\) channels are found in the plasma membrane of many cell types where they strictly regulate the entry of extracellular calcium into the cell (Tsien et al., 1991; Miller, 1992). Influx of Ca\(^{2+}\) through these channels can regulate such diverse cellular functions as muscle contraction (Catterall, 1991a), neurotransmitter release (Miller, 1990), and gene expression (Day and Maurer, 1990; Murphy et al., 1991). Electrophysiological and pharmacological studies have demonstrated the existence of at least four different classes of voltage-sensitive Ca\(^{2+}\) channel designated as L, N, T, and P type Ca\(^{2+}\) channels (Tsien et al., 1991). Skeletal muscle contains the highest concentration of voltage-sensitive Ca\(^{2+}\) channels, and these channels are of the L type (Catterall, 1991a). Biochemical characterization of the skeletal muscle Ca\(^{2+}\) channel has shown it to consist of five individual subunits designated α1, α2, β, γ, and δ. The α1 subunit of the skeletal muscle Ca\(^{2+}\) is the largest of these five subunits and is an integral membrane protein. The α1 subunit also forms the ion-selective pore through which Ca\(^{2+}\) flows into the cell (Catterall, 1991b). The skeletal muscle α1 subunit is phosphorylated by cAMP-dependent protein kinase, and this phosphorylation alters the properties of the channel (Curtis and Catterall, 1985). Binding sites for dihydropyridine-related agonists such as Bay K8644 and antagonists such as nifedipine are also found on the α1 subunit (Nakayama et al., 1991).

The structure of neuronal Ca\(^{2+}\) channels is not as clearly understood as the skeletal channel due to their lower abundance and molecular heterogeneity. Molecular biological studies suggest the expression of at least four different genes for the α1 subunit in brain (Snutch et al., 1990), and alternative splicing has been demonstrated for two of these transcripts (Snutch et al., 1991; Starr et al., 1991). The four classes of α1 subunit in rat brain have been designated rαB, rαC, and rβD (Snutch et al., 1990). Here we report the cloning of cDNAs for isoforms of the mouse brain α1 subunit and that the expression one of these isoforms in bovine adrenal chromaffin cells facilitates secretion in a manner consistent with the production of functional Ca\(^{2+}\) channels.

### Materials and Methods

**α1 Subunit cDNA Cloning and Sequencing**—Oligonucleotide A, TTTCTCATCATCTACATCATCCTCATGCTTCTTTACATGATGAACAT, based on nucleotides 3355–3401 of the rabbit skeletal muscle cDNA sequence and the complementary oligonucleotide B, ATGTTCATAGT, were synthesized (University of Michigan Biomedical Core Facility). Oligonucleotide B was extended using oligonucleotide A as template using [\(^{32}\)P]dATP and the Klenow fragment of DNA polymerase I. The extended and radiolabeled oligonucleotide B was denatured and used to screen a mouse brain cDNA library as described (Olsen and Ukler, 1991). Initially, a single cDNA clone designated as MC1 was isolated, subcloned into pTZ18U (U. S. Biochemical Corp.) and sequenced using dideoxynucleotides (Sanger et al., 1977). T7 RNA polymerase (BRL) was then used to generate a [\(^{32}\)P]-labeled RNA complementary to the MC1 cDNA sequence and this RNA was used to rescreen the mouse brain cDNA library. Ultimately the cDNA library was screened six times sequentially, and 26 independent cDNA clones were isolated and sequenced. Sequences were analyzed using the GCG Sequence Analysis software (Devereux et al., 1984).

**RNA Isolation and Northern Blot Analysis**—Total RNA was isolated from adult C57B6 mouse tissue using guanidinium isothiocyanate as described (Chirgwin et al., 1979). RNA was electrophoresed...
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Results and Discussion

Isolation of Mouse Brain cDNA Clones for the α1 Subunit of the Calcium Channel—A synthetic oligonucleotide corresponding to nucleotides 3355–3401 of the rabbit skeletal muscle was used to screen a mouse brain cDNA library in λgt11. This oligonucleotide was chosen based on the homology of this region of the skeletal muscle calcium channel α1 subunit to the α1 subunit of the sodium channel, and it was presumed that if multiple subtypes of the calcium channel α1 subunit were found in mouse brain, this probe would have a high probability of hybridizing with these isoforms. The MC1 cDNA was isolated first and used to isolate 25 other cDNA clones as shown in Fig. 1.

Composite Sequence of the Mouse Brain α1 Subunit—The individual cDNA sequences were aligned to generate the composite cDNA sequence shown in Fig. 2. The composite mBC nucleotide sequence shows that the greatest homology to the rat brain type C-I (rbC-I) which has been shown to code for an L-type α1 subunit by hybrid depletion experiments (Snutch et al., 1991). The mouse brain cDNA sequence shows 95% nucleotide sequence identity and 99% predicted amino acid sequence identity to the corresponding rbC sequences. Because of the high homology to rbC and the likelihood that this cDNA represents the mouse homologue of rbC, we have designated this family of clones the mouse brain type C (mBC) α1 subunit.

Four regions of putative alternative RNA splicing were determined from nucleotide sequence comparisons of individual mBC cDNA clones. The first region involves the coding sequence for the sixth transmembrane domain of the first repeat (amino acids 372–404). The second region of nucleotide sequence difference represents an insertion of 75 nucleotides in the mBC sequence between the codons for amino acids 463 and 464. This insertion represents an additional 25 amino acids in the intracellular loop between repeats I and II. Both of these first two splicing variants have been reported previously in comparison of smooth muscle (Biel et al., 1990) and cardiac (Mikami et al., 1989), but have not been reported to occur in brain. The third region of alternative splicing corresponds to an insertion of 9 nucleotides between the codons for amino acids 780 and 781. The last region of alternative splicing corresponds to a substitution of the codons for the third membrane spanning segment of the fourth repeat (amino acids 1277–1302). The alternate spliced forms for both region 3 and 4 have been described previously and constitute the difference between isotypes rbC-I and rbC-II. In addition, region 4 splicing has been shown to be developmentally regulated in rat heart (Diebold et al., 1992). It is of interest to note that none of the cDNAs isolated correspond to the cardiac amino terminus (Mikami et al., 1989) but only the smooth muscle variant (Biel et al., 1990). In fact, polymerase chain reaction analysis suggests that the cardiac amino-terminal sequences are not expressed in mouse brain (data not shown). Outside of the alternately spliced regions, all of

FIG. 1. Schematic diagram of mouse brain type C α1 subunit cDNA clones. Twenty-six independent mouse brain clones were isolated by sequential screening of a mouse brain λgt11 cDNA library as described under "Materials and Methods." The individual mouse brain cDNA clones (black, cross-hatched bars below) are indicated by number below the composite sequence indicated by the white bar at the top of the figure. The four homologous, internally repeated sequences are indicated by small black bars and an asterisk indicates cDNA clones with deletions with respect to overlapping cDNA clones; the dashed line indicates sequence within a cDNA that shows no homology to known α1 subunit sequences.

### Results and Discussion

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FIG. 2. Consensus sequence of individual mouse brain α1 subunit cDNA clones. The consensus nucleotide sequence derived from the 26 independent cDNA clones indicated in Fig. 1 is shown above with the predicted amino acid sequence below the nucleotide sequence. Transmembrane domains predicted by hydropathy plots and homology to other α1 subunit sequences are indicated by dashes between individual amino acids. The four regions where alternatively spliced forms of cDNA clones were detected are indicated by the presence of a second nucleotide and amino acid sequence below (in brackets). Where differences in the predicted amino acid sequence between the mouse brain type C and the rat brain type C sequences were detected, the rat amino acid is shown beneath the mouse sequence. Two other differences in nucleotide sequence were detected in individual cDNAs; clone 124 had a T at position -47 and clone 121 had an insert of 3 nucleotides (GAG) at position 7072.

Northern Blot Analysis of mbC Expression—Total RNA isolated from liver, skeletal muscle, heart, brain, and spinal cord were used for Northern blot analysis (Fig. 3). Hybridization with antisense MC1 32P-riboprobe showed that mbC mRNA was detected in heart, brain, and spinal cord but was not detected in liver or skeletal muscle. In all tissues...
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Fig. 3. Northern blot analysis of mbC mRNA in various tissues. Total RNA was isolated from the indicated tissues and subjected to Northern blot analysis as described under "Materials and Methods." The sizes of the hybridizing RNA species were calculated from RNA molecular weight standards which were electrophoresed on the same gel.

Fig. 4. Transient transfection of pCMV.MC1 expression vector in bovine adrenal chromaffin cells. An expression vector for the mbC cDNA was constructed as described under "Materials and Methods" in the pCMV.Neo expression vector. Chromaffin cells were cotransfected with the human growth-hormone expression vector and either the control pCMV.Neo vector or pCMV.MC1, coding for the mouse brain C type a1 subunit. Four days after transfection the cells were washed and incubated in the indicated concentrations of potassium and 2 μM (+) Bay K8644 for 15 min. Secretion of total catecholamines from the transfected cells is shown in panel A, and the secretion of growth hormone is shown in panel B. Values represent the average ± standard error of triplicate plates. Similar results were obtained in three independent experiments. There were 1-4 ng of human growth hormone and 120 nmol of catecholamines per dish.

which showed mbC transcripts, two distinct mRNAs were observed, an 8.9-kb and a 14-kb mRNA. Both of these RNAs were more abundant in heart than in brain or spinal cord, and in heart the 8.9-kb transcript appeared to predominate. In contrast, the larger 14-kb transcript was the predominant species in brain and spinal cord. The tissue distribution of the mbC mRNA is in agreement with the homology of the mbC nucleotide sequence with the rbC sequence and supports the notion that the mbC transcript is expressed in both cardiac and neural tissues.

Transient Expression of mbC1 in Bovine Adrenal Chromaffin Cells—In order to demonstrate that the mbC cDNAs code for a functional channel, an expression vector coding for the full-length mbC (Fig. 2, upper line with 25-amino-acid insert between amino acids 463 and 464) was constructed using the human cytomegalovirus promoter to direct expression of the mbC cDNA. The experimental paradigm used to detect mbC expression is analogous to that which has been used previously to detect regulation of gene transcription (Huggenvik et al., 1991). An expression vector for human growth hormone was cotransfected into chromaffin cells with either the parental pCMV.Neo vector or the mbC a1 subunit expression vector, pCMV.MC1. Primary bovine chromaffin cells have been used extensively in the study of exocytosis and have been used previously in transient expression experiments (Ross et al., 1990). Because transient transfection results in a high incidence of cotransfection of two distinct plasmids, those cells which express the transfected mbC a1 subunit also have a high probability of expressing human growth hormone. Furthermore, the transfected human growth hormone has been demonstrated previously to be targeted to the regulated secretory pathway (Moore and Kelly, 1985; Schweitzer and Kelly, 1985). Since calcium entry via calcium channels is a key step in exocytosis from chromaffin cells (Holz et al., 1991; Atlas, 1990; Burgoine, 1991), we sought to determine if expression of the mbC cDNA in chromaffin cells could alter the secretion of transfected hGH.

As shown in Fig. 4A, the secretion of catecholamines from cells cotransfected with the growth hormone expression vector (pXGH5) and either the parental pCMV.Neo expression vector or the mbC a1 subunit expression vector, pCMV.MC1, was measured after incubation with the calcium channel agonist Bay K8644 and moderately elevated K+*. Measurement of catecholamine secretion measures the secretion from all cells and thus since less than 1% of cells are transfected, no effect of mbC a1 subunit transcription should be seen on catecholamine secretion. As expected, catecholamine release was small and similar in dishes with or without the a1 subunit expression vector. In cells transfected with the pCMV.Neo control expression vector, hGH release, which specifically measures secretion from only the transfected cells, paralleled to catecholamine release (Fig. 4B). This is consistent with transiently expressed hGH being stored in secretory vesicles which are similar if not identical to catecholamine-containing vesicles (chromaffin granules). In contrast, hGH secretion from cells cotransfected with the plasmid for the a1 subunit showed a much greater secretory response to elevated K+ and Bay K8644 than did hGH secretion from cells cotransfected with pCMV.Neo. Maximal depolarization-induced catecholamine secretion is usually attained at 56 mM K+ and it typically releases 10-20% of total cellular content (Holz et al., 1982). In these experiments, suboptimal K+ (20 mM) stimulated GH secretion in the presence of the a1 subunit vector to the same degree that 56 mM K+ normally stimulates cate-
cholamine secretion in non-transfected cells. Bay K8644 in the absence of elevated K+ also induced significant secretion in cells cotransfected with the α1 subunit. In the absence of co-transfected α1 subunit, Bay K8644 does not stimulate hGH or catecholamine secretion in non-depolarized cells. The secretion induced by the combination of 20 mM KCl and 1 μM Bay K8644 was Ca2+-dependent from the pCMV.MC1 transfected cells (Fig. 5). For example, secretion in the presence of 20 mM KCl and 1 μM Bay K8644 was reduced from 31.9 ± 0.62% to 3.1 ± 0.5% of total content upon removal of Ca2+ (in the presence of 1 mM EGTA) in cells with the α1 subunit. Similarly, the Ca2+ channel antagonist D-600 inhibited secretion induced by the combination of 20 mM KCl and 1 μM Bay K8644 by 65–75% in transfected cells. In summary, overexpression of the mbC α1 subunit sensitizes calcium-dependent secretion to both depolarization and the effects of the dihydropyridine agonist, Bay K8644. Since α1 subunits alone do not efficiently form functional channels (Mori et al., 1991), it is quite likely that the transfected α1 subunit may be recruiting other subunits of the endogenous calcium channels in the formation of the Bay K8644-sensitive channels.

These experiments are significant for several reasons. First, these experiments demonstrate that the transfected mbC α1 subunit cDNAs in oocytes have demonstrated calcium influx (Mikami et al., 1989; Snutch et al., 1990), this report is the first to demonstrate functional expression using secretion. Secondly, these results suggest that the mbC protein is responsive to activation by Bay K8644. This is consistent with expression experiments with other isoforms of the cardiac α1 subunit in oocytes, which suggest that this isoform is L-type and responsive to dihydropyridines. Finally, these experiments suggest that Ca2+ influx through voltage-sensitive calcium channels is rate-limiting for secretion from chromaffin cells and that altering the character or the number of calcium channels can alter the rate of secretion. The mbC α1 subunit expression vector and the chromaffin cell transfection system should be useful in the detailed analysis of α1 subunit function as well as the role of this protein in regulated secretion.

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REFERENCES

Atlas, D. (1990) Cyto. Cell 31, 129-139
Biel, M., Ruth, P., Hulinn, R., Stuhmer, W., Flockerzi, V., and Hofmann, F. (1990) FEBS Lett. 269, 409-412
Borggreve, R. D. (1981) Biochem. Biophys. Acta 1071, 174-202
Catterall, W. A. (1991a) Cell 64, 871-874
Catterall, W. A. (1991b) Science 253, 1499-1500
Chang, J. M., Przybylea, A. E., MacDonald, J. R., and Rutter, W. J. (1979) Biochemistry 18, 5294-5299
Curtis, B. M., and Catterall, W. A. (1986) Proc. Natl. Acad. Sci. U.S.A. 82, 2528-2532
Day, R. N., and Maurer, R. A. (1990) Mol. Endocrinol. 4, 736-742
Devereux, J., Haefeli, P., and Smithies, O. (1984) Nucleic Acids Res. 12, 387-393
Diebold, R. J., Koch, W. J., Ellinor, P. T., Wang, J.-J., Muthuchamy, M., Wiesner, D. F., and Schwartz, A. (1992) Proc. Natl. Acad. Sci. U.S.A. 89, 1497-1501
Holz, R. W., Senter, R. A., and Frye, R. A. (1982) J. Neurochem. 39, 635-646
Holz, R. W., Sensenbrenn, J., and Bittner, M. (1991) Annu. N.Y. Acad. Sci. 655, 382-392
Huguenin, J. L., Collard, M. W., Stocko, E. R., Seasholtz, A. F., and Uhler, M. D. (1991) Mol. Endocrinol. 5, 921-930
Mikami, A., Imoto, K., Tanabe, T., Niidome, T., Mori, Y., Takeshima, H., Narumijia, S., and Numa, S. (1988) Nature 340, 230-233
Miller, R. J. (1986) J. Biol. Chem. 261, 1485-1490
Moore, H. P. H., and Kelly, R. R. (1980) J. Biol. Chem. 101, 1773-1781
Mori, Y., Friedrich, T., Kim, M., Mikami, A., Nakai, J., Ruth, R., Bosse, E., Hofmann, F., Flockerzi, V., Furushi, T., Mikoshita, K., Imoto, K., Tanabe, T., and Numa, S. (1991) Nature 350, 398-402
Murphy, T. H., Worley, P. F., and Baraban, J. M. (1991) Neuron 7, 625-635
Nakayama, H., Taki, M., Striessing, J., Grossmann, H., Catterall, W. A., and Kanazawa, Y. (1991) Proc. Natl. Acad. Sci. U.S.A. 88, 3905-3907
Olsen, S. R., and Uhler, M. D. (1991) J. Biol. Chem. 266, 11158-11162
Ross, M. E., Ehringer, M. J., Hyman, S. E., Carroll, J. M., Mucke, L., Comb, M., Reis, D. J., Joh, T. H., and Goodman, H. M. (1990) J. Neurosci. 10, 529-530
Sanger, R., Rubenstein, J. L., and Nicolas, J. F. (1986) EMBO J. 5, 3114-3124
Sanger, F., Nicklen, S., and Coulson, A. R. (1977) Proc. Natl. Acad. Sci. U.S.A. 74, 5463-5467
Schweizer, E. S., and Kelly, R. R. (1985) J. Cell Biol. 101, 657-670
Selden, R. F., Howe, K. B., Rowe, M. E., Goodman, H. M., and Moore, D. D. (1986) Mol. Cell. Biol. 6, 3173-3179
Snutch, T. P., Leonard, J. P., Gilbert, M. M., Lester, H. A., and Davidson, N. (1990) Proc. Natl. Acad. Sci. U.S.A. 87, 3391-3395
Snutch, T. P., Tomlinson, W. J., Leonard, J. P., and Gilbert, M. M. (1991) Neuron 7, 45-57
Starr, T. V. B., Prystay, W., and Snutch, T. P. (1991) Proc. Natl. Acad. Sci. U.S.A. 88, 5621-5625
Terbush, D. R., and Holz, R. W. (1990) J. Biol. Chem. 265, 21179-21184
Tsien, R. W., Ellinor, P. T., and Horne, W. A. (1991) Trends Pharmacol. Sci. 12, 349-354
Waymire, J. C., Bennett, W. F., Boehme, R., Hanteins, L., Gilmore-Waymire, K., and J. Haycock. (1983) J. Neurosci. Methods 7, 29-531