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Coexpression of Cloned α_{1B}, β_{2a}, and α_2/δ Subunits Produces Non-Inactivating Calcium Currents Similar to Those Found in Bovine Chromaffin Cells

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Chromaffin cells express N-type calcium channels identified on the basis of their high sensitivity to block by ω-conotoxin GVIA (ω-CgTx GVIA). In contrast to neuronal N-type calcium currents that inactivate during long depolarizations and that require negative holding potentials to remove inactivation, many chromaffin cells exhibit N-type calcium channel currents that show little inactivation during maintained depolarizations and that exhibit no decrease in channel availability at depolarized holding potentials. N-type calcium channels are thought to be produced by combination of the pore-forming α_{1B} subunit and accessory β and α_2/δ subunits. To examine the molecular composition of the non-inactivating N-type calcium channel, we cloned the α_{1B} and accessory β (β_{1b}, β_{1c}, β_{2a}, β_{2b}, and β_{3a}) subunits found in bovine chromaffin cells. Expression of the subunits in either *Xenopus* oocytes or human embryonic kidney 293 cells produced high-threshold calcium currents that were blocked by ω-CgTx GVIA. Coexpression of bovine α_{1B} with β_{1b}, β_{1c}, β_{2b}, or β_{3a} produced currents that were holding potential dependent. In contrast, coexpression of bovine α_{1B} with β_{2a} produced holding potential-independent calcium currents that closely mimicked native non-inactivating currents, suggesting that non-inactivating N-type channels consist of bovine α_{1B}, α_2/δ, and β_{2a}.

Key words: N-type calcium channel; α_{1B} subunit; β subunits; non-inactivating calcium current; chromaffin cells; voltage-dependent calcium channel

Adrenal chromaffin cells express L-, N-, and P/Q-type calcium channels (Hans et al., 1990; Albillos et al., 1993; Artalejo et al., 1994). In many chromaffin cells, N-type calcium channels do not inactivate during long depolarizations, nor do they exhibit decreased availability at depolarized holding potentials (Artalejo et al., 1992). Similar non-inactivating N-type channels have been described in presynaptic nerve terminals (Stanley and Goping, 1991). Interestingly, a significant fraction of N-type channels in chromaffin cells exhibits robust inactivation, raising the question as to why inactivation varies so much from cell to cell.

Voltage-dependent calcium channels are composed of at least three different subunits designated α_1, α_2/δ, and β and possibly a γ subunit (Dunlap et al., 1995; Jones, 1998; Letts et al., 1998). The pore-forming α_1 subunit alone encodes a voltage-dependent calcium channel with kinetic properties different from those of native channels (Lacerda et al., 1991; Varadi et al., 1991; Stea et al., 1993). When β and α_2/δ subunits are coexpressed with a variety of cloned α_1 subunits, current amplitude is greatly augmented, and the kinetics of activation and inactivation more closely resembles the kinetics of native channels (Birnbaumer et al., 1998). Ten different calcium channel α_1 subunits have been cloned [α_{1A}–α_{1I} and α_{1S} (Jones, 1998)]. α_{1B} subunits are required to make N-type calcium channels. Four β-subunit genes have been identified [β_{1-4} (Birnbaumer et al., 1998)], each of which has different effects on calcium channel inactivation. Inactivation rates of channels produced by α_{1A} and α_{1E} are slowest with β_{2a}, fastest with β_{3a}, and intermediate with β_{1b} and β_4 (Sather et al., 1993; Olcese et al., 1994; Zhang et al., 1994; DeWaard and Campbell, 1995). Inactivation of α_{1B} currents was greatest with β_{3a} and least with β_{2a} (Patil et al., 1998). Thus expression of different accessory subunits provides a potentially important regulatory mechanism for channel inactivation.

Certain α_1-subunit regions appear to influence channel inactivation. When the IS6 and I–II loop of α_{1A} or α_{1B} were substituted for the analogous regions in α_{1E}, the chimeric channels had inactivation rates much closer to those of α_{1A} and α_{1B} than to those of α_{1E} (Zhang et al., 1994; Page et al., 1997). It was reported recently that the splice variant of α_{1A} with a valine inserted in the I–II loop inactivated more slowly and less completely than did the variant lacking this valine (Bourinet et al., 1999).

Our study was performed to determine whether the inactivation properties of chromaffin cell N-type calcium channels result from a unique α_{1B} gene or whether specific accessory subunit combinations were required to form these channels. We cloned the α_{1B} subunit and five β subunits from chromaffin cells and expressed the clones in *Xenopus* oocytes and human embryonic kidney (HEK) 293 cells. Non-inactivating currents that mimicked those found in many chromaffin cells were observed when the bovine α_{1B} clone was coexpressed with β_{2a} and α_2/δ. Channels made by coexpressing the α_{1B} clone with either β_{1b}, β_{1c} β_{2b}, or β_{3a} (and α_2/δ) showed clear inactivation and resembled the inactivating N-type calcium currents observed in some chromaffin cells.

MATERIALS AND METHODS

Amplification of bovine chromaffin cell α_{1B} cDNA. Fragments of bovine chromaffin cell α_{1B} cDNA were amplified from total chromaffin cell RNA

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by reverse transcriptase-PCR (RT-PCR) using the Superscript Preamplification System (Life Technologies, Gaithersburg, MD) and degenerate primers designed from amino acid sequences conserved in the N and C terminals of cloned human, rabbit, rat, and mouse α_{1B} subunits. A 563 bp cDNA corresponding to amino acids 65–252 of the N terminal of bovine α_{1B} and a 683 bp cDNA corresponding to amino acids 1751–1969 of the C terminal of α_{1B} were obtained.

Cloning of α_{1B} from a bovine chromaffin cell library. The bovine α_{1B} cDNAs obtained by PCR were used to screen 0.9×10^6 plaques from a random- and polydT-primed bovine chromaffin cell library in λZapII (a gift from Dr. H. Pollard, National Institutes of Health). The plaques were transferred to Hybond N+ membranes and hybridized with ^{32}P-labeled α_{1B} cDNA using standard methods (Sambrook et al., 1989). Positive plaques were picked from the library plates and purified by two successive rounds of plating and hybridization. The α_{1B} cDNA clones were excised *in vivo* from λZapII using the ExAssist helper phage (Stratagene, La Jolla, CA). The ends of each cDNA insert were manually sequenced using the T7 Sequenase version 2.0 plasmid sequencing kit. The largest cDNAs from each screening were used to rescreen the library until clones corresponding to the entire bovine α_{1B} cDNA were obtained. Ten screenings of the library yielded 44 independent α_{1B} clones ranging in size from 150 to 3600 bp.

Construction of a full-length α_{1B} cDNA. Eight clones that spanned the entire coding region of α_{1B} were used to construct a full-length α_{1B} cDNA in the mammalian expression vector pcDNA3.1(+) (Invitrogen, Carlsbad, CA). Fragments were cut from each clone using selected restriction endonucleases and subcloned into recipient plasmids using standard molecular biology techniques. The full-length bovine α_{1B} cDNA was sequenced in its entirety by the University of Chicago Cancer Research Center Sequencing Facility using an ABI 377 automated fluorescent sequencer. Sequencing revealed a 2 bp deletion at the splice site for the +SFMG splice variant described by Lin et al. (1997). This deletion was repaired by subcloning the *Xba*I–*Bsu*36I fragment (bp 3245–3852) from a different library clone into the α_{1B} construct in pcDNA3.1(+). The sequence of bovine α_{1B} has been deposited in GenBank with the accession number AF173882.

Cloning of β_{1b}, β_{1c}, β_{2a}, β_{2b}, and β_{3a} from a bovine chromaffin cell library. A 2690 bp fragment of human β_{1b} was labeled with ^{32}P and used to screen the bovine chromaffin cell library by standard hybridization techniques (Sambrook et al., 1989). Positive plaques were picked from the library plates and purified by two more rounds of plating before *in vivo* excision of the phagemid pBSII containing each cDNA clone. This screening of the library yielded full-length clones for bovine β_{1b}, β_{1c}, and β_{3a} but no β_{2a} or β_{2b} clones. Therefore degenerate PCR primers were designed to the β_2 C-terminal amino acid sequences EAYWKAT and EWNRDVYI and used in an RT-PCR protocol to generate a 600 bp cDNA fragment of bovine β_2. This fragment was labeled with ^{32}P and used to screen the library as described above. This screening yielded clones containing all but the first 48 bp of the β_{2a}-coding region and the first 36 bp of the coding region of β_{2b}. The missing 5′ end of β_{2a} was cloned by RT-PCR from chromaffin cell RNA and ligated to the longest library clone. The missing region of the bovine β_{2b} was supplied by ligating the appropriate fragment of rabbit β_{2b} to the bovine β_{2b}. (The N-terminal amino acids 13–105 are identical between the bovine and rabbit clones. It appears likely that the first 12 amino acids may be identical as well.) All β-subunit clones were sequenced and subcloned into pcDNA3.1(+) for expression studies. The sequences of the five bovine β subunits have been deposited in GenBank with the accession numbers AF174415–AF174419.

Other cloned calcium channel subunits. Other cDNA clones used in this study were generous gifts from the following investigators: human α_2/δ (GenBank accession number M21948) and β_{1b} (M92303) from Dr. R. J. Miller (The University of Chicago), rat β_{2a} (M80545) from Dr. E. Perez-Reyes (Loyola University), and rabbit β_{2b} (X64298) from Dr. R. W. Tsien (Stanford University).

Xenopus *oocyte expression and electrophysiology.* Calcium channel proteins were expressed in *Xenopus laevis* oocytes after injection of *in vitro*-transcribed mRNA. Full-length cDNAs of each of the cloned subunits were used to generate mRNA from the T7 RNA polymerase promoter using an *in vitro* transcription kit (mMessage Machine; Ambion, Austin, TX). Oocytes were harvested from mature *Xenopus laevis* female frogs and separated from follicle cells with 2 mg/ml collagenase type IA (Sigma, St. Louis, MO). Oocytes were injected with 25 ng of both bovine α_{1B} and human α_2/δ RNA and 10 ng of various β RNAs in a total of 50 nl of DEPC-treated H_2O with a Drummond automatic microinjector. Three days after injection, oocytes were voltage-clamped at various potentials with two glass electrodes (filled with 3 M KCl and having a resistance of 0.5–2 MΩ) using an Axoclamp 2A (Axon Instruments, Foster City, CA). Oocytes were initially superfused with 96 mM NaCl, 2 mM KCl, 1 mM $MgCl_2$, and 5 mM HEPES, pH 7.6, and N-type calcium channel currents were measured in a Ba^{2+} recording solution designed to minimize the oocyte's endogenous Ca^{2+}-dependent Cl^- current [40 mM $Ba(OH)_2$, 25 mM tetraethylammonium (TEA)-OH, 25 mM NaOH, 2 mM CsOH, 5 mM HEPES, and 1 mM niflumic acid, pH adjusted to 7.5 with methanesulfonic acid]. Membrane currents were recorded on a computer using pCLAMP software (Axon Instruments). Leak currents were subtracted on-line with a P/4 protocol.

Cell culture and transfection. HEK 293 (American Type Culture Collection, Rockville, MD) cells were used for transient transfection studies and were grown in MEM and 10% calf serum supplemented with 2 mM glutamine, 100 U/ml penicillin, and 100 μg/ml streptomycin. Mammalian expression plasmids, containing the complete cDNAs for each of the calcium channel subunits, and the marker CD8 were purified on Qiagen (Hilden, Germany) columns and used to transfect HEK 293 cells. Cells were transiently transfected with cDNAs encoding the bovine α_{1B}, human α_2/δ, various β subunits, and CD8 as a reporter gene in a ratio of 15:12:5:3 using Lipofectin or Lipofectamine (both from Life Technologies). The human α_2/δ and various β cDNAs were coexpressed with α_{1B} because they have been reported to increase the expression levels of calcium channels and to normalize current kinetics (Lacerda et al., 1991; Stea et al., 1993). Immediately after the transfection, cells were replated onto polylysine-coated coverslips. Calcium currents were recorded 2–3 d after transfection. Transfected cells were detected visually by binding of anti-CD8 beads (Dynal, Great Neck, NY).

Bovine chromaffin cells were prepared and cultured as described previously (Artalejo et al., 1992). Briefly, bovine adrenal glands were digested with collagenase, and cells were purified by density gradient centrifugation. Cells were resuspended in bovine chromaffin cell media and plated onto collagen-coated glass coverslips at a density of 0.15×10^6 cells/cm^2. Cells were maintained at 37°C in an atmosphere of 92.5% air and 7.5% CO_2 and 90% humidity.

Electrophysiology on HEK 293 and chromaffin cells. Cells were voltage-clamped in the whole-cell configuration of the patch-clamp technique (Hamill et al., 1981) with a List model L/M-EPC7 patch clamp. Gigaohm seals were obtained in an extracellular Tyrode's solution (130 mM NaCl, 20 mM glucose, 10 mM HEPES, 1 mM $MgCl_2$, 2 mM KCl, and 5 or 10 mM $CaCl_2$, pH adjusted to 7.3 with NaOH). Ionic currents were measured in an extracellular TEA-Ba^{2+} solution (140 mM TEA-Cl, 10 mM glucose, 10 mM HEPES, and 5 or 10 mM $BaCl_2$, pH adjusted to 7.3 with TEA-OH). For chromaffin cell recordings, 1 μM tetrodotoxin and 1 μM nisoldipine were added to the TEA-Ba^{2+} solution. Electrodes were pulled from glass capillary tubes (Drummond, Broomall, PA), coated with Sylgard (Dow Corning, Midland, MI), and fire-polished to a final resistance of ~2.0 MΩ when filled with a CsCl-based internal solution (110 mM CsCl, 10 mM EGTA, 40 mM HEPES, 5 mM $MgCl_2$, 0.3 mM GTP, and 2 mM ATP, pH adjusted to 7.3 with CsOH, or 110 mM CsCl, 10 mM EGTA, 20 mM HEPES, 4 mM $MgCl_2$, 0.3 mM GTP, 6 mM ATP, and 14 mM creatine phosphate, pH adjusted to 7.3 with CsOH). ω-Conotoxin GVIA (Alomone Labs, Jerusalem, Israel) was diluted in extracellular recording solution and added to the bath at a final concentration of 1 μM.

Ionic currents were activated by step depolarizations of 20–200 msec duration from various holding potentials. For chromaffin cell recording, the cells were continuously perfused with recording solution at a rate of 2–3 ml/min by gravity flow. Leak currents were generated by averaging hyperpolarizing steps. All data presented here are leak and capacitance subtracted. Series resistance was partially compensated (>60%) using the series resistance compensation circuit of the patch clamp.

Data analysis. For *I–V* curve calculations, the peak current from each cell was recorded, and groups were pooled to calculate the average and SEM. For steady-state inactivation curves, currents from each cell at each holding potential were normalized to the peak current at the most hyperpolarized holding potential. The normalized data were averaged across cells and fit to single Boltzmann functions.

RESULTS

We have described previously a biophysically distinct N-type calcium channel found in bovine chromaffin cells (Artalejo et al., 1992). This "atypical" N-type calcium channel does not exhibit voltage-dependent inactivation while retaining other defining

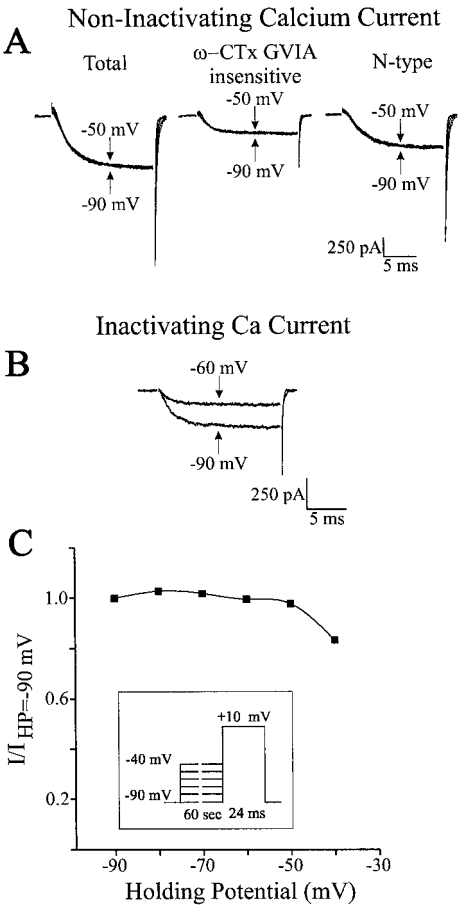

Figure 1. Some chromaffin cell N-type calcium channel currents observed in isolation are insensitive to changes in the holding potential. *A*, The strategy used to isolate N-type calcium channel currents in chromaffin cells is shown. Leak-subtracted calcium current traces were obtained by depolarizing a chromaffin cell to +10 mV for 24 msec from holding potentials in the range of −90 to −50 mV. Each holding potential was maintained for 60 sec before eliciting the test depolarization. *Left*, *Total* represents the total current obtained in the absence of any calcium channel antagonist. *Middle*, *ω-CTx GVIA-insensitive* shows calcium currents elicited after application of 1 μM ω-CgTx GVIA. *Right*, *N-type* shows currents after subtraction of ω-CgTx GVIA-insensitive currents from the total currents. For each condition there are five superimposed current records, one from each holding potential in the range of −90 to −50 mV (10 mV steps). *B*, Isolated N-type current from a different cell that exhibited strong holding potential dependence is shown. More than one-half of the current was inactivated when the holding potential was changed from −90 to −60 mV. *C*, Peak N-type current amplitude as a function of holding potential (*HP*) is shown. The peak current at each *HP* was normalized by dividing by the current observed at an *HP* of −90 mV. *Inset*, The voltage-clamp protocol.

characteristics of N-type channels such as ω-conotoxin GVIA (ω-CgTx GVIA) sensitivity. In addition, a large subset of chromaffin cells exhibits N-type calcium channel currents that show robust inactivation and thus resemble neuronal N-type channels. Examples of both varieties of chromaffin cell N-type calcium channels are shown in Figure 1. The method used to isolate N-type calcium currents from whole-cell calcium currents is illustrated in Figure 1*A*. The whole-cell calcium currents in Figure 1*A* labeled *Total* consist only of N- and P/Q-type currents because the specific L-type calcium channel blocker nisoldipine was included in the extracellular medium. Whole-cell currents were elicited by test depolarizations to +10 mV from holding potentials in the range of −90 to −50 mV. Note that current *traces* from five holding potentials are superimposed in each part of Figure 1*A*, indicating that the calcium currents in this cell are not sensitive to changes in the holding potential. Addition of 1 μM ω-CgTx GVIA to the extracellular medium blocked approximately two-thirds of the current at each of the holding potentials. Five superimposed traces illustrating the P/Q-type calcium currents remaining after ω-CgTx GVIA application are plotted in Figure 1*A*, *middle* (labeled *ω-CTx GVIA insensitive*). Subtraction of the ω-CgTx GVIA-insensitive current from the total current yields the ω-CgTx GVIA-sensitive N-type calcium current in isolation (Fig. 1*A*, labeled *N-type*). The currents from this cell were stable for >30 min and exhibited little rundown. Although many chromaffin cells exhibit calcium currents that are holding potential insensitive, other cells exhibit strong inactivation of currents. Figure 1*B* shows N-type current from a different cell, isolated in a similar manner, that showed a strong holding potential dependence; over one-half of the current was inactivated by a change in holding potential in the range of −90 to −60 mV. Figure 1*C* shows a plot of peak N-type current elicited at each holding potential, using the data from the non-inactivating cell shown in Figure 1*A, right*. Each current has been normalized to the current observed at the −90 mV holding potential. The data demonstrate that the N-type channels in this cell are not sensitive to changes in holding potential. Channel availability was not tested at very depolarized potentials (more than −40 mV) because these potentials activate Ca^{2+} currents in chromaffin cells. N-type Ca^{2+} current inactivation appears to involve a component of Ca^{2+}-dependent inactivation (Cox and Dunlap, 1994), which would complicate our studies of voltage-dependent inactivation.

We have attempted to determine the molecular basis of the inactivating or non-inactivating N-type calcium channels found in bovine chromaffin cells by cloning the α_{1B} subunit and a variety of accessory β subunits from a bovine chromaffin cell cDNA library and then characterizing the calcium currents resulting from the expression of the cloned subunits. The cDNA library was screened as described in Materials and Methods, and a full-length bovine α_{1B} cDNA was assembled from eight overlapping library clones. The full-length clone was sequenced, and the deduced amino acid sequence for the bovine chromaffin cell α_{1B} subunit was aligned with reported sequences for human α_{1B} (Williams et al., 1992a), rabbit α_{1B} (Fujita et al., 1993), rat α_{1B} (Dubel et al., 1992), and mouse α_{1B} (Coppola et al., 1994), and a consensus sequence was generated (Fig. 2). The bovine α_{1B} was most similar to the human α_{1B} with 93% of the deduced amino acid residues being identical. Identities with the α_{1B} subunits cloned from mouse, rat, and rabbit ranged from 89 to 91%. The N terminal and the four putative transmembrane domains of α_{1B} were most highly conserved with 98–99% of the deduced amino acids being identical to the consensus. There was significantly more variation among species in the C terminal and the putative intracellular loop between transmembrane domains II and III with 89–93% of the C-terminal residues for each species being identical to the consensus sequence and 83–84% of the II–III loop residues agreeing with the consensus.

We also compared the bovine α_{1B} sequence with the reported splice variants for cloned α_{1B}. All of the three library clones containing the C terminal of bovine α_{1B} were similar to the longer splice form described by Williams et al. (1992a) for human α_{1B}. None of the four clones covering the II–III loop region of bovine α_{1B} contained the 22 amino acid insert found in mouse α_{1B}

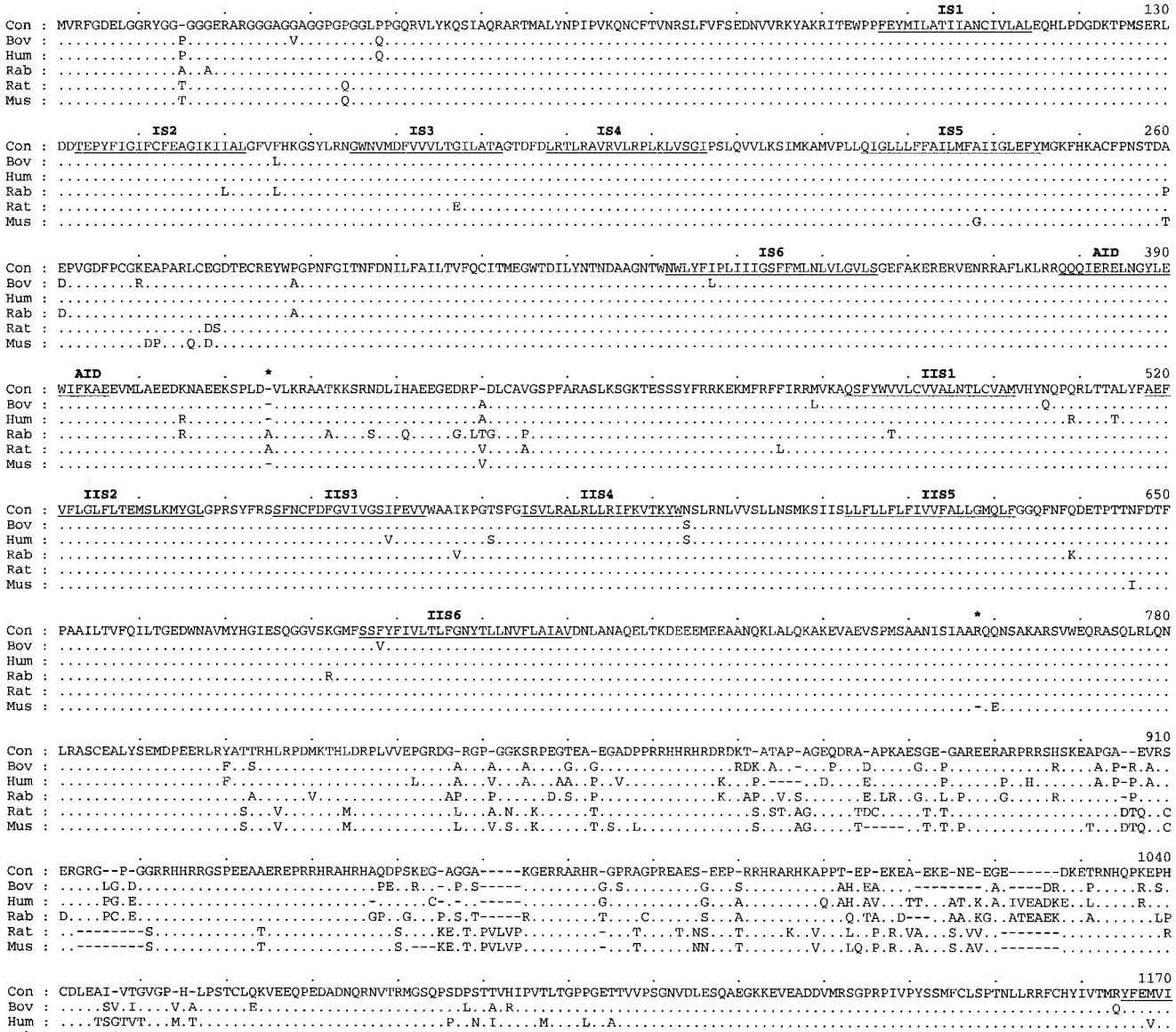

Figure 2. Alignment of the deduced amino acid sequence of bovine chromaffin cell α_{1B} with those from human, rabbit, rat, and mouse α_{1B}. The consensus sequence (*Con*) calculated from this alignment is shown on the *top line,* and only differences from the consensus are shown for the other sequences. The putative transmembrane domains and the α_1 interaction domain (*AID*) are *underlined* and *labeled* in *bold* above the consensus sequence. The splice variants identified here and by Lin et al. (1997) are indicated by *. The GenBank accession numbers and the sources of the sequences are as follows: *Bov,* bovine chromaffin cell (AF173882) (this study); *Hum,* human neuroblastoma (M94172) (Williams et al., 1992a); *Mus,* mouse neuroblastoma (U04999) (Coppola et al., 1994); *Rab,* rabbit brain (D14157) (Fujita et al., 1993); and *Rat,* rat brain (M92905) (Dubel et al., 1992). (*Figure 2 continues*.)

(Coppola et al., 1994), but interestingly a different splice variant was observed at this same splice site. Two of the four library clones and the full-length bovine α_{1B} construct contained an arginine residue at this splice site (Fig. 2, R757), and the other two library clones did not. Three sites for alternative splicing of rat α_{1B} have been described as follows: ±A(415), ±SFMG (1242–1245), and ±ET (1574–1575) (Lin et al., 1997). None of three library clones coded for A, and none of three library clones coded for SFMG, but we found one library clone that was +ET and one that was −ET. The full-length expressed bovine α_{1B} was −A, +R, −SFMG, and −ET. The IS6 region, which has been suggested to be important in N-type channel inactivation (Zhang et al., 1994), contained a single amino acid substitution, leucine in bovine α_{1B} rather than isoleucine.

Five accessory β subunits (β_{1b}, β_{1c}, β_{2a}, β_{2b}, and β_{3a}) were cloned from the chromaffin cell cDNA library. The bovine β_{1b},

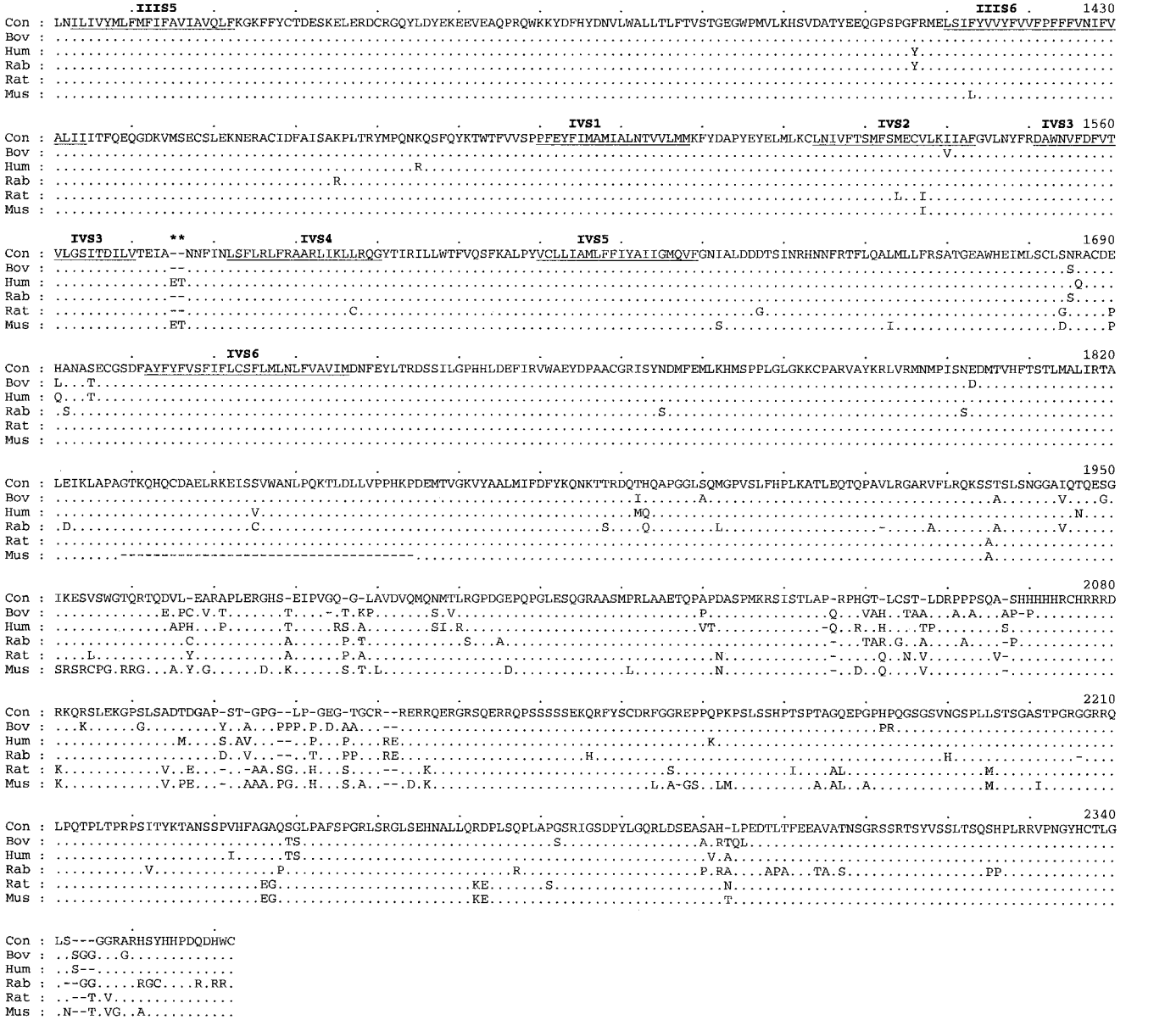

Figure 2 continued.

$\beta_{1c,}$ and β_{3a} subunits were recovered from the library as full-length clones and were sequenced in their entirety. The predicted amino acid sequences of these three β subunits were 97–98% identical with those of human clones (Powers et al., 1992; Williams et al., 1992b; Collin et al., 1994). The initial screen of the chromaffin cell cDNA library yielded several clones corresponding to the region common to β_{2a} and β_{2b}, but no clones contained the complete and unique N terminals of β_{2a} or β_{2b}. Therefore the N terminal of β_{2a} was cloned from chromaffin cell RNA by RT-PCR and ligated to a library clone to produce full-length β_{2a}. Overall the bovine β_{2a} subunit was 94% identical to human β_{2a}, but almost all of the differences were confined to the common C terminal shared by β_{2a} and β_{2b}. This region was only 82% identical to human β_{2a}. The full-length β_{2b} was obtained by ligating the incomplete β_{2b} library clone with a cDNA fragment from rabbit β_{2b} that coded for the first 12 missing amino acids. No human β_{2b} clone has yet been reported.

The cloned α_{1B} and β subunits were expressed both in HEK 293 cells and in *Xenopus* oocytes (Fig. 3). Although the experiments could be done more efficiently in oocytes where virtually every injected cell exhibited large currents, HEK 293 cells were also used in certain experiments to exclude possible effects of the endogenous β_{3xo} subunit in oocytes (Tareilus et al., 1997). HEK 293 cells transiently transfected with mammalian expression plasmids containing bovine α_{1B} and human α_2/δ and β_{1b} subunits expressed a high-threshold calcium current (Fig. 3*A*) that was blocked by 1 μM ω-CgTx GVIA (Fig. 3*B*). Sham-transfected HEK 293 cells did not have any observable calcium currents (data not shown). Oocytes were injected with *in vitro*-transcribed mRNAs for each subunit, and current recordings were done 2–3 d later. Oocytes expressing all three calcium channel subunits exhibited a large inward Ba^{2+} current that was not present in uninjected oocytes. Small outward currents (<50 nA) were occasionally seen in oocytes injected with only the α_2/δ and α_{1B} subunits (data not shown). Figure 3*C* shows data from an oocyte injected with mRNA for bovine α_{1B} and human α_2/δ and β_{1b}. The

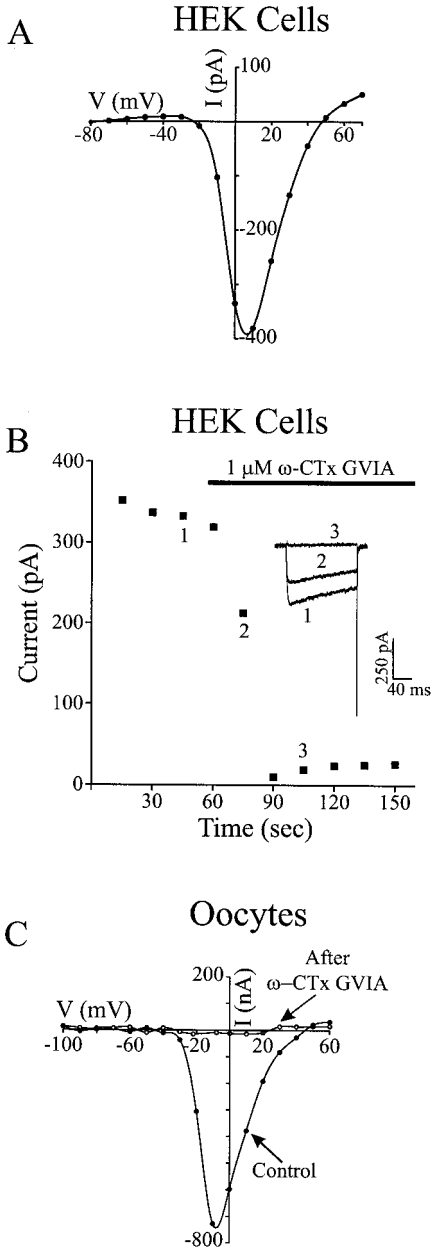

Figure 3. Coexpression of bovine α_{1B}, human β_{1b}, and α_2/δ in HEK 293 cells and *Xenopus* oocytes. *A*, Peak currents in HEK 293 cells plotted as a function of voltage. Transfected cells were depolarized for 200 msec to a variety of test potentials from an HP of −80 mV; peak current was measured and then plotted as a function of test potential. *B*, Peak currents plotted as a function of time. The HEK 293 cell was depolarized to +10 mV every 15 sec from an HP of −80 mV. ω-CgTx GVIA was added to the bath as indicated by the *horizontal bar*. Toxin application produced >95% inhibition of the calcium current. *C*, Peak currents in oocytes expressing α_{1B}, β_{1b}, and α_2/δ plotted as a function of voltage. Oocytes were depolarized for 200 msec to a variety of test potentials from an HP of −120 mV. The experiment was repeated after the addition of 1 μM ω-CgTx GVIA.

high-threshold currents observed were blocked by ω-CgTx GVIA. Thus in both oocytes and HEK 293 cells the bovine α_{1B} subunit when coexpressed with a β and an α_2/δ subunit appears to make N-type calcium channels.

We next investigated the inactivation properties of the cloned α_{1B} subunit expressed with α_2/δ and a variety of β subunits.

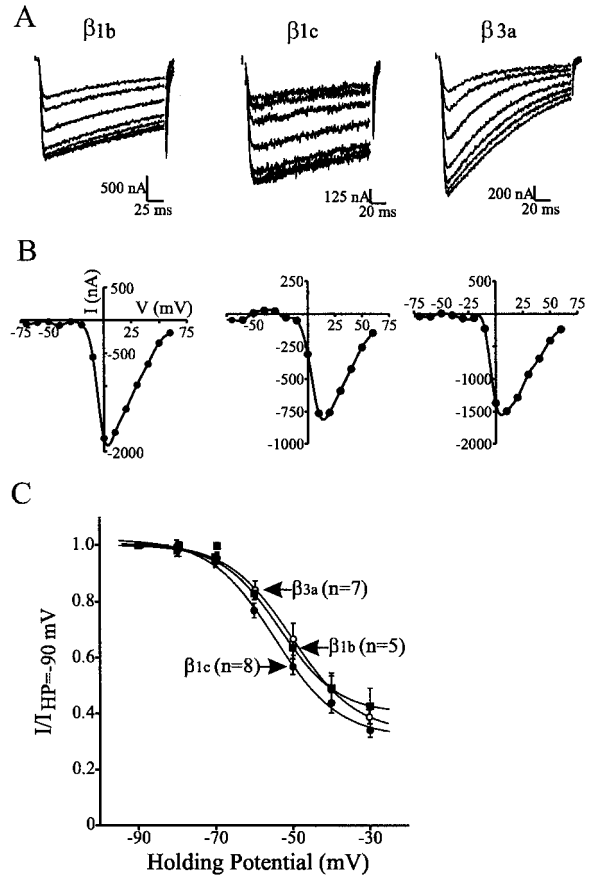

Figure 4. Coexpression of bovine α_{1B} with bovine β_{1b}, β_{1c}, or β_{3a} and α_2/δ in *Xenopus* oocytes produced inactivating calcium currents. *A*, Families of current *traces* obtained by depolarizing oocytes for 200 msec to +10 mV from holding potentials in the range of −90 to −30 mV. Each holding potential was maintained for 60 sec before the test depolarization. *B*, Current–voltage relationship for each of the cells in *A*. The oocytes were depolarized for 200 msec to a variety of test potentials from an HP of −100 mV. *C*, Mean normalized peak current as a function of holding potential for groups of cells expressing the β subunit indicated. The *number* of cells in each group is indicated in *parentheses*.

Figure 4*A* shows data from three different oocytes expressing bovine α_{1B} and human α_2/δ in addition to the β subunit indicated in the figure. The families of current records were obtained by depolarizing cells for 200 msec to +10 mV from a variety of holding potentials in the range of −90 to −30 mV. Each holding potential was maintained for 60 sec before the test depolarization. Expression of either β_{1b}, β_{1c}, or β_{3a} produced currents that were strongly dependent on holding potential. Figure 4*B* plots current–voltage relationships for each of the cells shown in Figure 4*A*. The cells were depolarized to a variety of test potentials from an HP of −100 mV; peak current was measured and then plotted as a function of test potential. Figure 4*C* graphs voltage-dependent inactivation curves made by averaging data from groups of cells expressing different β subunits. The curves were constructed by normalizing the currents; the currents observed at each holding potential were divided by the current recorded at −90 mV. Note that the currents generated by the channels containing any of the three β subunits used in this experiment produced strong inactivation.

In contrast, expression of rat β_{2a} with α_{1B} produced currents that were virtually holding potential independent. Figure 5*A*

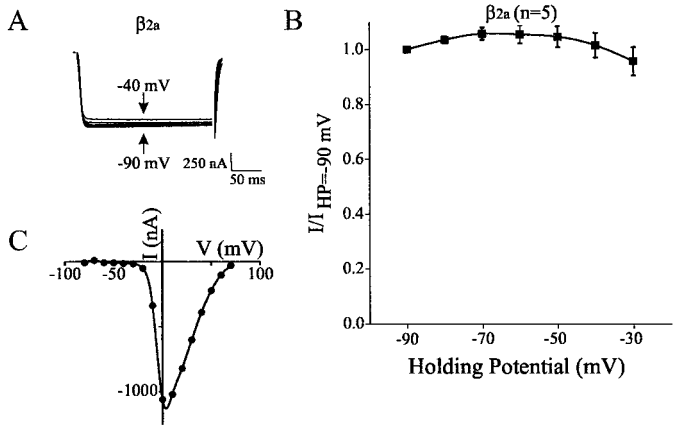

Figure 5. Coexpression of bovine α_{1B} with rat β_{2a} in *Xenopus* oocytes produced non-inactivating calcium currents. *A*, Family of current *traces* obtained by depolarizing an oocyte for 200 msec to +10 mV from holding potentials in the range of −90 to −40 mV. Each holding potential was maintained for 60 sec before the test depolarization. *B*, Mean normalized peak current as a function of holding potential for eight cells expressing the bovine α_{1B} and rat β_{2a} subunits. *C*, Current–voltage relationship for the cell in *A*. The oocyte was depolarized for 200 msec to a variety of test potentials from an HP of −100 mV.

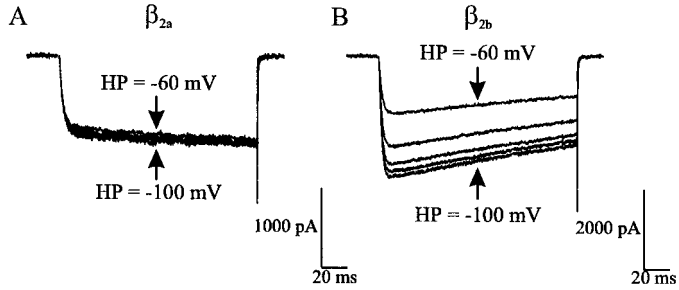

Figure 6. Coexpression of bovine α_{1B} and human α_2/δ with bovine β_{2a} but not β_{2b} produced a non-inactivating calcium channel current in HEK 293 cells. *A*, Family of current *traces* obtained by depolarizing an HEK 293 cell expressing bovine α_{1B} and β_{2a} along with human α_2/δ for 140 msec to +10 mV from holding potentials in the range of −100 to −60 mV. Each holding potential was maintained for 60 sec before the test depolarization. *B*, Family of current *traces* obtained by depolarizing an HEK 293 cell expressing bovine α_{1B} and β_{2b} along with human α_2/δ. The voltage-clamp protocol is described in *A*.

shows a family of current records obtained by depolarizing an oocyte for 200 msec to +10 mV from a variety of holding potentials in the range of −90 to −40 mV. Each holding potential was maintained for 60 sec before the test depolarization. Figure 5*B* plots the current–voltage relationship for this cell. The cell was depolarized to a variety of test potentials from an HP of −90 mV; peak current was measured and then plotted as a function of test potential. Figure 5*C* plots the voltage-dependent inactivation curve obtained by averaging data from five oocytes expressing bovine α_{1B} and rat β_{2a} and illustrates the holding potential independence of N-type calcium channels constructed with β_{2a} subunits. Similar results were obtained when the human α_2/δ subunit was expressed along with α_{1B} and rat β_{2a} (data not shown).

Bovine β_{2a} differs significantly from rat β_{2a} primarily in the C terminal where only 81% of the predicted amino acids are identical. Coexpression of bovine β_{2a} along with α_{1B} and α_2/δ produced non-inactivating N-type calcium channel current (Fig. 6*A*). In contrast, coexpression of bovine β_{2b} along with α_{1B} and α_2/δ produced a robustly inactivating N-type calcium channel current (Fig. 6*B*). These results suggest that the C terminal is not likely to contribute to the unique properties of β_{2a} because similar results were obtained with both rat and bovine β_{2a}. The N terminal of β_{2a} is, however, a good candidate for mediating the non-inactivating calcium currents seen with β_{2a}, because β_{2a} differs from β_{2b} only in its short N terminal.

DISCUSSION

In an attempt to identify the molecular basis for the non-inactivating N-type calcium channels observed in bovine chromaffin cells (Fig. 1) (Artalejo et al., 1992), we cloned the α_{1B}, β_{1b}, β_{1c}, β_{2a}, β_{2b}, and β_{3a} calcium channel subunits from a chromaffin cell library and expressed these clones in both HEK 293 cells and *Xenopus* oocytes. In both systems expression of the cloned α_{1B} and β_{2a} along with human α_2/δ yielded N-type calcium currents that resembled the non-inactivating N-type currents in bovine chromaffin cells. However, expression of the α_{1B} and α_2/δ with β_{1b}, β_{1c}, β_{2b}, or β_{3a} yielded calcium currents that showed distinct inactivation during prolonged depolarizations as well as decreased availability from depolarized holding potentials. These results suggest that the β_{2a} subunit is a key determinant of the non-inactivating properties of the N-type calcium channel in chromaffin cells. Previous studies with cloned α_{1A}, α_{1B}, and α_{1E} have shown that inactivation rates of these calcium channels depended on which β subunit was coexpressed; inactivation was slowest with β_2, fastest with β_3, and intermediate with β_1 and β_4 (Ellinor et al., 1993; Sather et al., 1993; Olcese et al., 1994; Zhang et al., 1994; DeWaard and Campbell, 1995; Parent et al., 1997; Patil et al., 1998). In addition coexpression of β_{2a} has been found to shift the voltage-dependent inactivation curves obtained with α_{1E} to more depolarized potentials and to result in incomplete steady-state inactivation (Sather et al., 1993; Olcese et al., 1994; Parent et al., 1997). However, steady-state voltage-dependent inactivation of α_{1A} and α_{1E} was never totally abolished even with the β_{2a} subunit as was seen in this study that used bovine α_{1B} and either the bovine or rat β_{2a}. A recent study using single-cell RT-PCR linked β_{2a} expression to a slowly inactivating Q-type current in neostriatal neurons, whereas β_{1b} expression was linked to fast-inactivating Q-type currents in cortical neurons (Mermelstein et al., 1999).

Several previous studies have suggested that the N terminal of the β subunit is an important region regulating channel inactivation (Olcese et al., 1994; Cens et al., 1999). The different inactivation rates that we observed with bovine β_{2a} and β_{2b} (Fig. 6) support this hypothesis because β_{2a} and β_{2b} differ from each other only in their short N terminal. Palmitoylation of the two cysteines in the N terminal of β_{2a} has been shown to be essential for certain modulatory effects of β_{2a} on α_{1C} and α_{1E} (Chien et al., 1996; Qin et al., 1998). The C terminals of bovine β_{2a} and β_{2b} were markedly more different from this region of the human, rabbit, rat, and mouse β_{2a} or β_{2b} than these were from each other. To determine whether these C-terminal differences were functionally important, we also expressed the rat β_{2a} (in which 19% of the amino acids in the C terminal differ from those in bovine β_{2a}) with the bovine α_{1B} and found that this combination of subunits also yielded non-inactivating N-type calcium channels. Thus it appears that the differences in the C terminal of β_{2a} are not functionally important in terms of regulating calcium channel inactivation.

Despite the apparent importance of the β_{2a} subunit in regulat-

ing the rate of inactivation of N-type calcium channels, a role for the α_{1B} subunit itself cannot be excluded. Both the IS6 transmembrane domain and the adjacent I–II cytoplasmic loop have been implicated previously in the rate of inactivation exhibited by cloned α_{1A} and α_{1B} subunits (Zhang et al., 1994; Page et al., 1997). Bovine α_{1B} differs from the consensus in only one position within the IS6 region and in two positions in the I–II loop region, but one of these is the absence of alanine 415. The presence or absence of alanine 415 is a known splice variant in α_{1B} (Lin et al., 1997) that corresponds to the recently described splice variant ±valine 421 in α_{1A} (Bourinet et al., 1999). The presence of valine 421 confers much slower inactivation on α_{1A}. None of the three library clones corresponding to the alanine 415 region that were isolated from the bovine chromaffin cell library contained alanine 415. Another splice variant of α_{1B} [+A, −SFMG, and +ET (Lin et al., 1997)] was originally suggested to have slower inactivation kinetics than +A, +SFMG, and −ET, but more recent work has suggested that the differences between these splice variants are primarily in the rates of activation and depend primarily on +ET (Lin et al., 1999). Our expressed α_{1B} was −A, −SFMG, and −ET and thus did not correspond exactly to either of these forms. However, two of the four library clones covering the ±ET region did have +ET. Work is in progress to incorporate this splice variant into full-length α_{1B}. In discussing the various splice variants of α_{1B}, it should also be noted that we do not know the exact splice variant composition of the native α_{1B} in chromaffin cells. The clones we used to construct the full-length α_{1B} may not be the ones normally spliced together, a limitation that is true of all full-length α_1 subunits that have been constructed from more than one cDNA library clone. RT-PCR of smaller portions of α_{1B} could easily determine which of the individual known splice variants of α_{1B} (e.g., ±A415 or ±ET1575) are expressed in chromaffin cells, but it still would not answer the question of which combinations of splice variants make up a single mRNA.

In addition to modifications to inactivation made by different β subunits or by changes to the α_1 subunits described above, inactivation of N-type Ca^{2+} channels can be altered by interactions with synaptic vesicle docking and fusion machinery. Coexpression of syntaxin 1A with N-type channels in *Xenopus* oocytes dramatically decreased channel availability because of the stabilization of channel inactivation (Bezprozvanny et al., 1995). Interestingly, we observed no such changes in inactivation when we coexpressed syntaxin 1A with α_{1B}, β_{2a}, and α_2/δ (our unpublished observations), which suggests that syntaxin 1A alters inactivation exclusively in N-type channels that exhibit inactivation.

N-type calcium channels, which are widely distributed throughout the nervous system, have been unambiguously linked to a variety of important physiological processes including synaptic transmission (Takahashi and Momiyama, 1993; Turner et al., 1993; Wheeler et al., 1994). In secretory cells, like chromaffin cells, activation of N-type calcium channels by themselves can trigger catecholamine secretion (Artalejo et al., 1994). It seems likely that non-inactivating N-type calcium channels would operate more effectively during periods of intense stimulation, which would lessen the availability of inactivating channels thereby diminishing Ca^{2+} influx. In addition to expressing inactivating or non-inactivating N-type calcium channels, chromaffin cells also expressed inactivating or non-inactivating P/Q-type calcium channels (our unpublished observation), which should also have dramatic effects on Ca^{2+} influx. Thus, the changes in channel inactivation properties because of alterations in β-subunit channel composition that are described in this study may have important functional consequences for a wide variety of Ca^{2+}-dependent processes including catecholamine secretion.

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