

**Functional Properties of CaV1.3 (α1D) L-type Ca$$^{2+}$$ Channel Splice Variants Expressed by Rat Brain and Neuroendocrine GH3 Cells*  

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Ca$$^{2+}$$ enters pituitary and pancreatic neuroendocrine cells through dihydropyridine-sensitive channels triggering hormone release. Inhibitory metabotropic receptors reduce Ca$$^{2+}$$ entry through activation of pertussis toxin-sensitive G proteins leading to activation of K$$^{+}$$ channels and voltage-sensitive inhibition of L-type channel activity. Despite the cloning and functional expression of several Ca$$^{2+}$$ channels, several involved in regulating hormone release remain unknown. Using reverse transcription-polymerase chain reaction we identified mRNAs encoding three α (α1A, α1C, and α1D), four β, and one α$$_{2}$$-δ subunit in rat pituitary GH3 cells; α1B and α1S transcripts were absent. GH3 cells express multiple alternatively spliced α1D mRNAs. Many of the α1D transcript variants encode “short” α1D (α1D-S) subunits, which have a QXXER amino acid sequence at their C termini, a motif found in all other α1 subunits that couple to opioid receptors. The other splice variants identified terminate with a longer C terminus that lacks the QXXER motif (α1D-L). We cloned and expressed the predominant α1D-S transcript variants in rat brain and GH3 cells and their α1D-L counterpart in GH3 cells. Unlike α1A channels, α1D channels exhibited current-voltage relationships similar to those of native GH3 cell Ca$$^{2+}$$ channels, but lacked voltage-dependent G protein coupling. Our data demonstrate that alternatively spliced α1D transcripts form functional Ca$$^{2+}$$ channels that exhibit voltage-dependent, G protein-independent facilitation. Furthermore, the QXXER motif, located on the C terminus of α1D-S subunit, is not sufficient to confer sensitivity to inhibitory G proteins.

All excitable cells express voltage-activated Ca$$^{2+}$$ channels fulfilling diverse cellular functions. Among other things, Ca$$^{2+}$$ influx can regulate gene expression and propagate action potentials, and it is a prerequisite for sustained neurotransmitter and hormone release (1, 2). Specialized Ca$$^{2+}$$ channels have evolved to serve these specific purposes. Ca$$^{2+}$$ channels native to excitable cells can be classified into five categories (T, L, N, P/Q, and R) on the basis of their unique biophysical and pharmacological properties. The genetic basis for the heterogeneity of Ca$$^{2+}$$ channels is apparent now that genes have been identified that encode ten α, α1A through α1I, and α1S/α1H subunits, four β subunits and three α$$_{2}$$-δ dimers. Ca$$^{2+}$$ channel α1 subunits have been grouped into three families (CaV1, 2, and 3) on the basis of their levels of amino acid sequence identity. Additional structural heterogeneity occurs through alternative splicing (5–9). Expression of recombinant homomeric α1 subunits in *Xenopus* oocytes or cell lines produces functional Ca$$^{2+}$$ channels with properties reminiscent of those of their naturally expressed counterparts. Although the α1 subunit is the principal component of the Ca$$^{2+}$$ channel, containing 24 membrane-spanning regions that form both the channel and the voltage sensor, coexpression of α1 subunits with β and α$$_{2}$$-δ subunits produces a greater current density and alters voltage dependence, current amplitude, activation, and inactivation kinetics (10–12).

High voltage-activated P/Q- and N-type Ca$$^{2+}$$ channels have been extensively studied because of their central role in controlling Ca$$^{2+}$$ entry into, and thus neurotransmitter release from, neuronal synaptic terminals (13). The activity of these channels is inhibited by the activation of metabotropic receptors that couple to pertussis toxin-sensitive G proteins. This pathway is thought to be an important means of presynaptic inhibition, allowing the feedback control of neurotransmitter release. Following metabotropic receptor activation, βγ subunits liberated from pertussis toxin-sensitive G16 protein complexes bind directly to P/Q- and N-type Ca$$^{2+}$$ channels at their QXXER amino acid motifs making them less “willing” to open when stimulated by an action potential invading the synaptic terminal (14–18). The inhibition is voltage-dependent; prolonged (19) or repetitive (20) depolarization can overcome βγ blockade.

Inhibitory metabotropic receptors can also attenuate the activity of L-type Ca$$^{2+}$$ channels in several neuroendocrine cells and some neurons (21, 22). In the growth hormone- and prolactin-secreting anterior pituitary GH3 cell line, activation of either native somatostatin and muscarinic receptors or recombinant opioid receptors leads to inhibition of L-type Ca$$^{2+}$$ channel activity (23–25). This effect can be prevented by pertussis toxin pretreatment or reversed by strong depolarization, suggesting the involvement of βγ subunits. Voltage-dependent coupling between metabotropic receptors and cloned P/Q- and N-type channels can be reconstituted in recombinant expression systems by expressing α1A or α1B subunits, respectively, with the inclusion of auxiliary β and α$$_{2}$$-δ subunits. For example, cloned μ opioid receptors couple to Ca$$^{2+}$$ channels containing α1A or α1B Ca$$^{2+}$$ channel subunits expressed in *Xenopus* oocytes (26). By contrast, expression of the α1C subunit forms L-type Ca$$^{2+}$$ channels that are insensitive to opioid receptor activation.

The prevalence of α1D subunit expression in neuroendocrine cells led to the suggestion that this may be the molecular identity of the G protein-coupled L-type channel (21, 22). Fur-
thermore, the existence of a QXXER amino acid motif in an α1D subunit splice variant led us to further hypothesize that this may be necessary for direct G protein-mediated L-type channel inhibition (27). In this study we used RT-PCR1 to identify Ca2+ channel transcripts in GH3 cells. In addition to α1A and α1C transcripts, we found numerous alternatively spliced α1D transcripts. The predicted amino acid sequences of the α1D-splice variants terminated with either a long (α1D-L) or a short (α1D-S) C terminus, the latter of which contained a QXXER amino acid motif. We used long range RT-PCR to compare the relative prevalence of alternatively spliced α1D transcripts in GH3 cells and rat brain and created cDNAs encoding α1D-L and corresponding α1D-S splice variants for electrophysiological analyses. Using these clones we examined the functional properties of several alternatively spliced α1D isoforms and determined whether the QXXER motif is sufficient to confer sensitivity of the α1D subunit to G proteins either activated directly by GTPyS or through opioid receptor activation.

EXPERIMENTAL PROCEDURES

 Cultures and Transfections—Control GH3 cells (ATCC, Manassas, VA) and GH3 cells were stably transfected with μ- and δ-opioid receptors (GH3MORDOR cells) as described previously (25). Both cell lines were grown in Dulbecco's modified Eagle’s medium containing 10% v/v calf serum, penicillin (100 units/ml) and streptomycin (100 μg/ml). GH3MORDOR cells were grown under positive selection conditions with hygromycin (200 μg/ml) and Geneticin (400 μg/ml). Cells were subcultured once each week and seeded into 75-cm² flasks, for maintenance, and into 35-mm diameter dishes for use in electrophysiological experiments. Human embryonic kidney (HEK) cells and Chinese hamster ovary (CHO) cells stably transfected with either HEKMOR and CHOMOR cells) or HEK293 (HEK293) cells (London, UK) were also employed. Cells were incubated in a humid atmosphere of 5% CO2 and 95% air at 37 °C.

 Patch-clamp (Axopatch 200A amplifier, Axon Instruments Inc., Foster City, CA) technique was used to record Ca2+ currents. The liquid junction potential was negligible, and so compensation was made for its cancellation. Unless otherwise stated voltage-activated Ba2+ currents were recorded from cells depolarized from the holding potential of −80 to 0 mV for 80 ms at 10-s intervals. The voltage dependence of current inhibitions induced by opioids or GTPyS (300 μM) was investigated using a double-pulse protocol: cells were depolarized from a holding potential of −80 mV to between −50 and 80 mV (10-mV increments, 95-ms duration). The pre-pulse was followed (after 10 ms at −80 mV) by a 10-ms pulse to 0 mV. The effect of a depolarizing prepulse on the current-voltage relationship was investigated by applying a 60-mV pulse for 26 ms between two depolarizing test pulses (from −80 mV to between −50 and 60 mV in 10-mV increments). Time required for β3 to reach a voltage-dependent equilibrium at −80 mV was determined by depolarizing cells from −80 to 60 mV followed by 10−140 ms (in 10-ms increments) by a test pulse to 0 mV. Currents were low-pass-filtered at 2 kHz and digitized (Digidata, Axon Instruments Inc., CA) at 10 kHz for storage on the hard drive of a Pentium PC. Tail currents were recorded using Syngard (Dow Corning Corp., Midland, MI)-coated borosilicate glass microelectrodes (Clark-Edwards Glass Co., Claremont, CA). Tail currents generated by repolarization to −80 mV following 5.8-ms depolarizations to voltages between −60 and 60 mV were filtered at 10 kHz and digitized at 200 kHz for storage on a PC hard disc. Experiments were performed at room temperature (22–24 °C).

 Curve Fitting and Statistics—Tail currents were fitted by single exponentials using pCLAMPS software (Axon Instruments Inc, CA). Tail current amplitudes were derived by extrapolating the fit to the point of hyperpolarization. There was no relationship between test pulse potential and the time constant (τ) of the tail currents. The relationship between tail current amplitude and the voltage of depolarization was fitted with a Boltzmann equation of the form,

$$I_{\text{tail}} = I_{\text{max}}[1 + \exp(-V - V_{1/2} \delta)]$$

where $I_{\text{tail}}$ is the tail current amplitude as a percentage of maximum, $V$ is the command voltage, $V_{1/2}$ is the voltage for 50% maximal activation, and $\delta$ is the slope factor.

 Concentration-response curves were fitted using the logistic equation,

$$I = I_0 + F_{\text{max}}[1 + \exp(-(V - V_{1/2} \delta))]$$

where $I$ is the Ba2+ current amplitude in the presence of a specific concentration of nimboline expressed as percent control, $F_{\text{max}}$ is the maximal percent inhibition of the current, $I_{0}$ is the concentration of nimboline that had a half-maximal effect, and $n$ is the slope.

 Data obtained from the voltage-dependent reversal of Ba2+ current inhibitions were fitted with the Boltzmann equation,

$$I = I_{\text{max}}[1 + \exp(-(V - V_{1/2} \delta))]$$

where $I$ is the Ba2+ current amplitude as a percentage of control current amplitude, $F_{\text{max}}$ is the maximum percent facilitation of the current, $V_{1/2}$ is the prepulse potential, and $S$ is the slope factor. In these experiments control amplitude was the amplitude of the current activated by stepping from −80 to 0 mV after a prepulse to −50 mV.

 Data are expressed as means ± S.E., and statistical significance was established using the Student's t test.

 Drugs Used—Persantine (from Sigma Chemical Co., St. Louis, MO) was applied to the culture medium at a final concentration of 100 ng/ml for at least 24 h before experiments. All other drugs were diluted into the extracellular solution that constantly perfused the recording chamber through gravity feed at a rate of ~5 ml/min. [d-Pen2,D-Pen5]enkephalin (DPDPE) was obtained from Peninsula Laboratories (Belmont, CA). GTPyS (Life Technologies, Inc., Gaithersburg, MD) was loaded into the recording electrode at a final concentration of 300 μM. Recordings were made −3 min after achieving the whole-cell configuration. All tissue culture reagents, including antibiotics were obtained from Life Technologies, Inc. (Gaithersburg, MD).

 RT-PCR—RNA was prepared from whole rat brain and GH3 cells (passage 12) and reverse-transcribed using random hexamers or poly-dT. Specific oligonucleotides were designed for α1D subunit (α1D-L, α1D-S), β3 subunits (β3L, β3S), and the α1C subunit. GenBank accession numbers, forward and reverse primers, respectively, are as follows: α1D, M92905: 5554–5577, 6427–6450; α1C, M7516: 5957–5980, 6491–6514; α1D, M83101: 5001–5024, 5475–5500; α1D, M15453: 5892–5915, 6436–6459; α1C, U18316: 5–28, 821–844; β3, X61394: 1588–1611, 2053–2076; β3, M80505: 1642–1665, 2054–2077; β3, M83751: 1213–1236, 1498–1522; β3, M1235: 1448–1471, 1736–1759; α1D, M66251: 1055–1076, 1517–1540. The specific forward and reverse primers used to identify the α1D subunit splice gene are also listed in Table I. Primers specific to exons of the rat α1D subunit gene are also listed in Table I. Amplification was carried

1 The abbreviations used are: RT-PCR, reverse transcription-polymerase chain reaction; HEK, human embryonic kidney; GTPyS, guanosine 5′-3′-O-(thio)triphosphate; CHO, Chinese hamster ovary; DPDPE, [d-Pen2,D-Pen5]enkephalin; kb, kilobases; β-ARK, β-adrenergic receptor kinase; DOR, δ opioid receptor; MOR, μ opioid receptor.
out at 35 cycles with Ffu polymerase (Stratagene, La Jolla, CA). Each cycle was comprised of 30 s at 95 °C, 1 min at 55 °C, and 2 min at 72 °C. For the last cycle, annealing and extension times were increased to 3 and 10 min, respectively. Long-range RT-PCR amplification was carried out at 40 cycles with a Tq/Ffu polymerase mix (Stratagene, La Jolla, CA). The extension cycles were increased to 9 min. The final extension time was 15 min. Q solution (Qiagen, Valencia, CA) was added to the PCR mix to obtain a maximal yield of product. For exon-specific PCR, individual clones of plasmid DNA were used as template, and amplification was carried out at 20 cycles with Taq polymerase (Stratagene). Each cycle consisted of 30 s at 95 °C, 60 s at 55 °C, and 45 s at 72 °C.

Determination of Relative Abundance of Alternatively Spliced α1D-S(31b) Subunit Transcripts—We used either RT-PCR or a hybridization strategy to examine the number and relative abundance of α1D subunit splice variants encoded by full-length α1D transcripts in GH3 cells and rat brain. By using an oligonucleotide primer set designed to the 5'- and 3'-untranslated regions, respectively (Table I), full-length α1D tran-
scripts encoding subunits with the short C terminus were amplified by RT-PCR generating a 5.1-kb product. For the full-length transcripts encoding α1D subunits with the long C terminus (α1D-L) we used the same sense primer to the 5'-untranslated region and an antisense oligonucleotide primer directed to the coding region 3' to the C-terminal splice locus, generating a 5.2-kb product. Due to the existence of additional splicing patterns in rat brain, a second primer set encoding multiple α1D-S and α1D-L isoforms. Subcloning PCR products on masse provided a means by which to determine the abundance of each particular variant. Upon transformation into an appropriate bacterial host, individual clones represented single isoforms and analysis of each provided the splice variation of the two upstream regions of known splicing activity. A PCR-based strategy was employed to analyze individ-
ual clones by designing exon-specific primers for each exon within the sites of splicing activity (Table I). The PCR products for each clone were visualized by gel electrophoresis and representative clones for each isoform were confirmed by sequencing. Therefore, by analyzing 100 individual clones, a percent abundance for each isoform could be obtained. Alternatively, we used dot blots of individual splice variant clones and tested the presence or absence of individual exons by using γ-32P-labeled exon-specific primers. Oligonucleotides were end-labeled with γ-32PdATP using T4 polynucleotide kinase (Ambion, Austin, TX), and 1.0 × 10^6 dpm were added to the hybridization solution. Hybrid-
ization was carried out at 55 °C in 1× NaCl, 50 mM Tris (pH 7.5), 5× Denhardt's, 0.1% sodium pyrophosphate, 0.5% SDS, and 10% polyeth-
ylene glycol 8000 for a minimum of 4 h. The blots were washed three times with 0.1% sodium pyrophosphate, 0.5% SDS at room temperature and autoradiography was carried out at −80 °C.

Creating Clones of α1D Subunit Splice Variants—The long-range PCR products were digested by EcoRI generating multiple fragments with sizes of ∼3.3 kb. These fragments were ligated into pBluescript SK− (−) (Stratagene, La Jolla, CA) to generate pBlue Q.PCR(x) (where x represents a specific splice variant). A rat α1D subunit clone (Gen-Bank accession number D819101), kindly provided by Dr. Shum (Chiba University, Chiba, Japan), digested partially with BamHI and fully with XhoI, was subcloned into pcDNA3.1+ (Invitrogen, Carlsbad, CA) generating C1.0. To generate a nucleotide sequence encoding the α1D-L C terminus, RT-PCR was performed on GH4 cell RNA using amplifiers flanking DraIII and XhoI sites. The DraIII/XhoI-cut PCR product was ligated into C1.0, generating Q1.0. Both Q1.0 and C1.0 were digested with EcoRI, gel-purified, and re-ligated to remove the 3.3-kb EcoRI fragment, generating Q1.1 and C1.1. The constructs α1D-A (α1D-S) and α1D-L were generated by ligating the EcoRI fragment of pBlue Q.PCR(x) into Q1.1 and C1.1, respectively. Sequencing revealed that cDNAs generated by long-range PCR often contained nucleotide se-
quence errors that would introduce incorrect amino acids into recombi-
nant channels. Full-length α1D cDNAs were constructed from clones with sizes of 

RESULTS

Opioid Receptors Couple to ω-Agatoxin IVa-resistant Ca2+ Channels in GH3MORDOR Cells—Ca2+ enters GH3 cells through dihydropyridine-sensitive channels that can be inhibited by the activation of pertussis toxin-sensitive G proteins (24, 25, 29). Taken together with similar studies in pancreatic cell lines and neurons, these observations indicate the exist-
ence of G protein-sensitive L-type Ca2+ channels (21). How-
ever, because G/δ proteins inhibit P/Q- and N-type channels (18), we re-examined the possibility that GH3 cells may express either of these Ca2+ channel subtypes (Figs. 1 and 2). The P/Q-type channel antagonist ω-agatoxin IVa (10 nM) had no discernible effect on Ba2+ currents activated by depolarizing GH3 cells stably expressing μ and δ opioid receptors (GH3MORDOR cells) (Fig. 1A). We used RT-PCR to examine whether GH3 cells express transcripts encoding the α1A sub-
unit, the principle component of P/Q-type channels (Fig. 1B). Transcripts encoding α1A subunits can be alternatively spliced giving rise to α1A-α and α1A-β subunits that may represent P- and Q-type Ca2+ channels, respectively (6). Low concentrations of ω-agatoxin IVa inhibit recombinant Ca2+ channels contain-
ing α1A-α subunits, whereas higher concentrations are required to inhibit channels formed by α1A-β subunits. We identified mRNA encoding the α1A-α subunit in GH3 cells using RT-PCR analysis with a primer (see "Experimental Procedures") selective for this splice variant (Fig. 1B). In view of the presence of mRNA encoding the α1A-α subunit, we examined a potential role for ω-agatoxin IVa-sensitive channels in G protein-mediated inhibition of Ca2+ channel activity in GH3MORDOR cells. Despite an inhibition of Ca2+ channel activity induced by ω-agatoxin IVa (500 nM), the δ opioid receptor-selective agonist DPDPE (100 nM) continued to inhibit Ba2+ currents. Therefore, opioid receptors couple to ω-agatoxin-resistant Ca2+ channels in GH3MORDOR cells (Fig. 1C). In later experiments we ex-
amined the properties of recombinant α1A subunits providing further evidence for a lack of P/Q-type channels in GH3 cells (Fig. 7).

Having ruled out a role for P/Q Ca2+ channels in the G protein modulation of Ba2+ currents recorded from GH3MORDOR cells, we turned our attention to N-type channels. Electrophysiological studies demonstrate that GH3 cells lack functional N-type Ca2+ channels (24). Furthermore, Lievano and colleagues (30) were unable to detect the α1H transcript in these cells using the ribonuclease protection assay. We confirmed lack of mRNA encoding the α1H subunit in these cells using RT-PCR (Fig. 2).

GH3 Cells Contain Multiple α1 Subunit Transcripts—We synthesized cDNAs from RNA extracted from GH3 cells, whole rat brain and rat skeletal muscle, using PCR to determine the complement of transcripts encoding Ca2+ channel α1 subunits and accessory β and δ subunits (Fig. 2). We did not examine whether GH3 cells express transcripts encoding T-type Ca2+ channels, because several studies have demonstrated their resistance to G protein modulation (31, 32). Furthermore, Ba2+ currents recorded from GH3 cells lack a consistent T-type channel contribution (24). Specific forward and reverse oligonucleo-

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tide primers were designed for five cloned high voltage-activated α subunits (α1B, α1A, β subunits (β1-4)), and an α2δ subunit (see “Experimental Procedures”). Sequencing of the bands excised from gels positively identified mRNAs encoding α1C and α1D subunits (confirming a previous report (30)) and α2-δ subunits in GH3 cells (Fig. 2). Because α1B, α1E, or α1S transcripts were absent and α1A channels do not contribute to the effects of DPDPE (Fig. 1), G protein-coupled inhibition of Ca2+ channels in GH3 cells appears to occur through regulation of α1C and/or α1D channel activity. We considered the α1C subunit to be an unlikely candidate because of its inability to couple to opioid receptors (26). Therefore, we focused our attention on the α1D subunit believing this to be the most likely candidate for the G protein-sensitive L-type Ca2+ channel expressed by GH3 cells.

Multiple α1D Splice Variants in GH3 Cells—Multiple splice variants of the α1D Ca2+ channel subunits exist in various cell types (7, 28, 33). In rat tissue, splicing causes variations in amino acid sequences located at three distinct loci: 1) between domains I and II, 2) between the S2 and S4 regions of domain IV, and 3) in the C-terminal region (Fig. 3). We used RT-PCR with oligonucleotide primer sets (Table I) spanning each of the three regions to investigate the presence or absence of splice variants in GH3 cells (see “Experimental Procedures”). Gel extraction of candidate bands and subsequent sequencing confirmed multiple splice variants at all three splicing loci, resulting in numerous combinatorial α1D subtype possibilities (Fig. 3). Interestingly, analysis of the amino acid sequences in the C termini of α1D subunits indicated the presence of a truncated isoform containing the Gln-X-X-Glu-Arg (QXXER) motif, which has been observed previously in rat brain (33). We refer to α1D subunit variants that contain this truncated C terminus as α1D-S subunits. The QXXER motif is absent from the longer alternatively spliced α1D-L isoforms (Fig. 3). The fact that the QXXER sequence is present in α1A, α1B, and α1E subunits, which can all couple to inhibitory G proteins (15, 16), led to our hypothesis that this motif is sufficient to confer G protein sensitivity to recombinant channels formed by expressing
Fig. 3. Summary of the αD subunit splice variants detected in GH3 cells.
Three separate coding regions of the αD Ca²⁺ channel subunit were investigated for the presence of splice variants by RT-PCR. Exons 10–13 correspond to the intracellular loop between domain I and II. Exons 30–33 correspond to a region between S1 and S5 of domain IV. Exons 40–49 correspond to the cytoplasmic tail. These three regions are indicated by α, β, and γ, respectively. The identity of each PCR product was confirmed by sequencing. *, this splice fragment (c2) contains the putative G protein β/subunit-binding motif, whereas the other fragment, c1, does not.

| Table I | Primer sequences for PCR |
|---------|--------------------------|
| Primer Direction | Primer sequence | Starting at base |
| α1D Forward 5'-TACTGGCCTGGATACCCAGGCA-3' | 1067 |
| α1D Reverse 5'-ACAGGAGCTAGTGAGCTGAGG-3' | 1738 |
| α1D Forward 5'-TGGAAAGAACGGCCGGAGG-3' | 1849 |
| α1D Reverse 5'-AGCTTCGCGGAGCTTGGTTC-3' | 2074 |
| α1D Forward 5'-AGAGTTGAGATATCTGGATG-3' | 4213 |
| α1D Forward 5'-GAGACGGTTTGACCTCTATCGG-3' | 4338 |
| α1D Forward 5'-GGTACCGTCGTTGATATTGCTAT-3' | 394 |
| α1D Forward 5'-GGACGAGATATCTGGATG-3' | 4400 |
| α1D Forward 5'-GAGACGGTTTGACCTCTATCGG-3' | 4411 |
| α1D Forward 5'-GGTCCTCGTGATGTTGTCGAT-3' | 4597 |
| α1D Forward 5'-GGTACCGTCGTTGATATTGCTAT-3' | 5549 |
| α1D Forward 5'-GGTACCGTCGTTGATATTGCTAT-3' | 5549 |
| α1D Forward 5'-GGTACCGTCGTTGATATTGCTAT-3' | 5549 |

All starting positions of primers are based on rat αD subunit sequence (GenBank™ accession number D38101) except accession M57682 and accession M57970. For exon-specific PCR, α1D(S)- and α1D(L)-forward primers were used with α1C, and α1D(L)-reverse primers, respectively. For C-terminal splice analysis α1D-S forward primer was used with α1D(S)- and α1D(L)-reverse primers. Amplification of full-length α1D(S) as well as α1D(L) cDNAs were used for amplification of full-length α1D(S) clones (long-range PCR).

cDNAs encoding α1D(S) subunit variants.

Relative Abundance of Alternatively Spliced α1D Isoforms—We used RT-PCR with exon-specific primers (Table I) and a hybridization strategy to compare the number of α1D(S) and α1D(L) subunit mRNA variants caused by the combinatorial possibilities of the three known splicing hotspots depicted in Fig. 3 (see “Experimental Procedures”). The process was performed first using RNA isolated from GH3 cells in which α1D(S) and α1D(L) variants were examined and subsequently, for α1D(S) variants specifically, using rat whole brain RNA (Fig. 5). Bands containing a mixture of α1D(S) and α1D(L) cDNAs encoding α1D(S) or α1D(L) subunits were excised from agarose gels (Fig. 4A). These cDNAs encoding α1D(S) or α1D(L) cDNAs were isolated by using a method called pBluescript allowing transformation of bacteria. We picked 100 colonies of bacteria transformed with the vector containing pBluescript fragments of either α1D(S) from GH3 cells or rat brain, or α1D(L) from GH3 cells. We then employed PCR or exon-specific hybridization with specific oligonucleotides (Table I) to identify exons introduced by alternative splicing into cDNAs isolated from individual bacterial colonies (Fig. 4B).

Expression of Recombinant α1D Subunits—We made three full-length cDNAs encoding α1D(S)(31a), α1D(S)(31b), and α1D(L)(31b) subunits. The α1D(S)(31a) and α1D(S)(31b) cDNAs encode the most abundant α1D(S) subunit variants detected in the GH3 cells and rat brain, respectively (Fig. 5). These subunits differ in the variety of alternatively spliced exon 31 (variants 3 and 4, respectively, in Table II). The α1D(L)(31b) subunit sequence differs from the α1D(S)(31b) subunit sequence at the C terminus (see Fig. 3).

We examined the functional properties of the α1D(S)(31a), α1D(S)(31b), and α1D(L)(31b) subunit variants by transiently introducing their cDNAs with accompanying β2a, α2-δ, and δ opioid receptors (HEK293 and CHO cells, respectively). The properties of the expressed channels were compared with recombinant channels formed by the expression of cDNAs encoding α1A, β2a, and α2-δ subunits (Fig. 6). Currents were recorded using the whole-cell patch-clamp technique with Ba²⁺ as the charge carrier (see “Experimental Procedures”). The voltage dependence of current activation was investigated by depolarizing cells from −80 mV to between −50 and 60 mV (10-mV increments) for 80 ms. Ba²⁺ currents had a threshold of activation of around −45 mV and peaked at 0 mV for α1D(S)(31a), α1D(S)(31b), and α1D(L)(31b) channels (Fig. 6B). These values were similar to those reported for Ba²⁺ currents recorded from GH3 cells under the same conditions (22). By contrast, currents recorded from cells expressing α1A subunits had significantly higher voltages at which threshold of activation and peak current amplitudes occurred (−30 and 10 mV, respectively, Fig. 6B). There was no significant differences between the peak current densities for the α1D(S)(31a), α1D(S)(31b), and α1D(L)(31b) channels, which were 75 ± 29 picoamperes (pA/pF) (n = 5), 71 ± 12 pA/pF (n = 5), and 49 ± 5 pA/pF (n = 3), respectively.

A failure of Ba²⁺ currents mediated by α1D channels to reverse (presumably due to low Ca²⁺ permeability) prevented accurate determinations of voltages for half-maximal activation by fitting the current-voltage relationships in Fig. 6B. Therefore, we performed tail current analysis to determine the voltage required for half-maximal channel activation (V½) for currents mediated by α1D(S)(31a) and α1D(S)(31b) subunits. Tail currents were well fitted by single exponentials with time constants of 0.2 ms in both cases (Fig. 6C). The relationships between tail current amplitude and voltage for α1D(S)(31a) and α1D(S)(31b) subunits were similar. Fitting the data points with a Boltzmann function (see “Experimental Procedures”) yielded...
Fig. 4. Isolation and identification of α_{1D} transcripts expressed by GH_{3} cells and rat brain. A, amplification of full-length α_{1D-S} transcripts containing the putative Q predominantly binding domain (denoted by \( +QXXER \)) and partial-length α_{1D-L} transcripts (denoted by \( -QXXER \)). B, PCR with exon-specific amplimers was used to analyze α_{1D} clones in GH_{3} cells and rat brain. Numbers above each lane denote exons recognized by each primer set. Shown are representatives from a GH_{3} cell (top) and a rat brain (bottom) α_{1D-S} clone.

Fig. 5. Percent abundance of α_{1D} isoforms in rat brain and GH_{3} cells. A total of 100 individual clones for rat brain α_{1D-S} (bars with lines), GH_{3} cell α_{1D-S} (checkered bars), and α_{1D-L} (solid bars) isoforms were analyzed by PCR or hybridization to determine the percent abundance of each splice variant. The α_{1D-S} variants 3 (α_{1D-S(31a)}) and 4 (α_{1D-S(31b)}) are the most prevalent in GH_{3} cells and brain, respectively. The numbering scheme for α_{1D} subunit splice variants is provided in Table II.

TABLE II

Summary of α_{1D-S} variants in GH_{3} cells

PCR with exon-specific primers revealed multiple variants terminating with exon 41a, i.e. α_{1D-S}. Numbers in the column represent the nomenclature assigned to each type of variant. Exons are shown in the top row.

V_{1/2} values of −2.9 and −3.9 mV for α_{1D-S(31a)} and α_{1D-S(31b)} subunits, respectively (Fig. 6D).

The dihydropyridine sensitivity of Ba^{2+} currents recorded from cells expressing the α_{1D-S(31b)} subunit was similar to that of Ba^{2+} currents recorded from GH_{3} cells (24), whereas currents recorded from cells expressing the α_{1A} subunit were significantly less sensitive to nimodipine (Fig. 7). A previous study demonstrated that nimodipine caused a more potent inhibition of Ca^{2+} channels when GH_{3} cells were depolarized from a holding potential of −40 mV rather than one of −80 mV (24).
Alternatively Spliced α_{1D} Subunits

FIG. 6. Current-voltage relationship of recombinant α_{1D} Ca^{2+} channels. Cells were transiently transfected with cDNAs encoding α_{1D} β_{2γ} and α_{2δ} subunits. A, superimposed currents activated by depolarizing cells expressing α_{1D-S(3a)} subunits to between −50 and 60 mV from −80 mV. B, mean normalized I–V curves for α_{1A} (△, n = 9), α_{1D-S(3a)} (●, n = 6), α_{1D-S(3b)} (●, n = 4), and α_{1A-L(3a)} Channels (■, n = 6). Currents were normalized to peak amplitude 15 ms after the onset of test pulse. C, a CHO cell expressing α_{1D-S(3b)} subunits was depolarized from −100 mV to −30, 10, and 50 mV in the top, middle, and bottom traces, respectively. A single-exponential fit, shown superimposed on each current trace, was used to determine the time constant of deactivation (τ = 0.2 ms) and tail current amplitude. D, a plot of the tail current amplitude (expressed as percent maximum tail current) against voltage was generated for α_{1D-S(3a)} (○) and α_{1D-S(3b)} (●) subunits. Data points were fitted using the Boltzmann equation (see “Experimental Procedures”).

FIG. 7. Nimodipine sensitivity of α_{1A} and α_{1D} channels. Concentration-response relationship for the inhibition of α_{1A} (△, n = 6) and α_{1D-S(3b)} (●, n = 5) Ca^{2+} channels by nimodipine. Cells were held at −40 mV, and currents were recorded in the presence of 30 mM Ba^{2+} to minimize voltage-dependent inactivation. The concentration-response relationship of α_{1D-S(3b)} Ca^{2+} channels for inhibition by nimodipine was shifted to the right when cells were held at −80 mV (○, n = 5). Curves were generated by a logistic equation (see “Experimental Procedures”).

Likewise, the nimodipine inhibition of currents mediated by α_{1D-S(3b)} subunits was potent in cells held at −40 mV (IC_{50} = 0.25 ± 0.02 μM). Nimodipine inhibited Ba^{2+} currents recorded from cells held at −80 mV with an IC_{50} of 1.0 ± 0.2 μM. Recombinant α_{1A} channels were inhibited by nimodipine with an IC_{50} of 49 ± 1 μM in cells voltage-clamped at −40 mV. Taken together, these data support our assertion that, despite the presence of detectable levels of mRNA encoding the α_{1A} subunit in GH_{3} cells (Fig. 1), their functional channels are predominantly L-type.

G Protein Sensitivity of Recombinant Ca^{2+} Channels—Cloned μ and δ opioid receptors couple to L-type Ca^{2+} channels in GH_{3}MORDOR cells through activation of inhibitory G_{i/o} proteins (25). We hypothesized that the G protein-sensitive Ca^{2+} channel component is comprised of an α_{1D} subunit. Furthermore, we anticipated that the QXXER motif within specific α_{1D-δ} subunits was necessary for βγ binding and therefore channel inhibition. Here we examined whether the QXXER motif is sufficient to confer opioid receptor coupling or G protein sensitivity to recombinant channels formed by the α_{1D-δ} subunit coexpressed in HEKDOR cells with β_{2γ} and α_{2δ} subunits.

The δ opioid receptor-selective agonist DPDPE (100 nM) caused a voltage-dependent inhibition of α_{1A} channel activity recorded from transiently transfected HEKDOR cells (Fig. 8A). By contrast, δ receptor activation had no effect on the activity of either α_{1D-S(3a)} or α_{1D-L(3b)} channels expressed in HEKDOR cells demonstrating that the QXXER motif is not sufficient to confer opioid receptor coupling (Fig. 8B). We transiently transfected cDNAs encoding the Ca^{2+} channel subunits into CHO cells stably expressing δ receptors (CHODOR cells) to examine whether a different expression environment may enable coupling. Once again, DPDPE (100 nM) inhibited α_{1A} channel activity but had no effect on the activity of either of the two α_{1D} splice variants tested (in each case n = 4). We investigated whether direct activation of G proteins would cause a voltage-dependent inhibition of recombinant channels formed by α_{1D-δ} subunits. Depolarization of CHO cells expressing either α_{1D-S(3a)} or α_{1D-L(3b)} subunits with GTPγS (300 μM) in the electrode solution did not significantly alter the level of Ba^{2+} current facilitation seen with the double-pulse protocol in the absence of GTPγS (F_{max} = 34.8 ± 1.7%, F_{50} = −5.2 ± 3.1 mV versus F_{max} = 30.8 ± 2.3%, F_{50} = 0.9 ± 4.3 mV and F_{max} = 35.8 ± 0.79%, F_{50} = −11.2 ± 1.3 mV versus F_{max} = 24.5 ± 3.92%, F_{50} = −19.9 ± 1.93 mV, respectively, Fig. 8A and B). We repeated these experiments by expressing α_{1D-S(3b)} subunits in CHO cells to determine whether G protein coupling occurs in the exon 31 variant that predominates in GH_{3} cells. GTPγS had no significant effect on basal Ba^{2+} current facilitation compared with control (F_{max} = 33.7 ± 2.7%, F_{50} = 1.6 ± 4.4 mV versus F_{max} = 24.5 ± 1.3%, F_{50} = −7.9 ± 3.0 mV, Fig. 8C). Similar experiments also indicated a lack of G protein coupling
when α1D-S(31b) or α1D-L(31b) subunits were expressed in HEK-DOR cells (n = 6 and 4, respectively). Because opioid receptor coupling to Ca^{2+} channels is thought to be mediated by G_{i/o} (34), CHO, CHODOR, and HEK-DOR cells were transiently transfected with G_{i/o} or G_{oB} cDNAs to determine whether the lack of coupling is due to inadequate levels of these G proteins. Neither the overexpression of G_{i/o} (n = 5 and 4 for HEK-DOR and CHODOR, respectively) nor G_{oB} (n = 3 and 5 for HEK-DOR and CHO) resulted in G protein-mediated inhibition of α1D-S(31b) channels by GTP·γ·S. Syntaxin-1A may play a permissive role in G_{i/o}-mediated inhibition of N-type Ca^{2+} channels (35). We examined whether this may also be the case for α1D-S channels. No Ba^{2+} current inhibition was observed in the presence of GTP·γ·S (n = 3) in CHO cells transiently transfected with cDNAs encoding syntaxin-1A and α1D-S(31b), β_{2α2}, and δ2-5. These data demonstrate that the QXER motif is not sufficient to confer sensitivity of α1D-S(31a) or α1D-S(31b) subunits to activated G proteins.

Voltage-dependent Facilitation of α1D Channel Activity—Depolarizing pre-pulses caused voltage-dependent facilitation of Ba^{2+} currents mediated by all three recombinant α1D channel variants (Fig. 9). We investigated the effect of pre-depolarization on the current-voltage relationship of α1D-S(31b) Ca^{2+} channels. An increase in peak current and a transient leftward shift in the current-voltage relationship occurred after application of a 60-mV depolarizing pre-pulse (I_{peak} = 938 pA versus I_{peak} = 1097 pA for pre- and post-pulse, respectively; Fig. 10A). Current amplitude was unaffected at voltages greater than 30 mV. We also examined the time required for recovery from voltage-dependent facilitation in cells expressing α1D-S(31b) channels (Fig. 10B). A single-exponential fit of the decline in current amplitude following a 60-mV pre-pulse yielded the time constant for recovery from facilitation (τ = 58.6 ± 10.5 ms). The rapid time course of recovery indicates that this event is unlikely to involve protein phosphorylation. This time course is similar to those reported previously for voltage-dependent reversal of G protein inhibition of α1A and α1B channels (36). However, G protein activation through inclusion of GTP·γ·S in the recording electrode had no effect on the observed facilitation of α1D-S(31b) channels (Fig. 9A). We examined further whether the facilitation of α1D channel activity was caused by reversal of constitutive G protein coupling. The reversal of G_{i/o} protein-mediated inhibition of Ca^{2+} channel activity is known to depend on the concentration of activated G proteins (37). However, pretreatment of cells with pertussis toxin (100 ng/ml) 24 h prior to recording, a procedure that should significantly reduce G_{i/o} activation, had no significant effect on the time course for the recovery from facilitation (τ = 51.5 ± 5.3 ms).
Alternatively Spliced α₁D Subunits

FIG. 10. Voltage-dependent facilitation of α₁D channels. A, voltage-dependent facilitation of currents was investigated by comparing the current-voltage relationship before and after a depolarization to 60 mV. Left, superimposed Ba²⁺ currents recorded from a CHO cell expressing α₁D-S(31b). Right, graph showing the relationship between current amplitude and voltage before (●) and after (○) a depolarization to 60 mV. B, left, superimposed currents mediated by α₁D-S(31b) channels during a prepulse to 60 mV and in response to a test pulse to 0 mV 10–140 ms following the prepulse. Right, a graph of the time required for recovery from voltage-dependent facilitation. The peak current amplitude was measured and expressed relative to the peak amplitude of the maximally facilitated current, i.e., the first current (I1) after the depolarizing pulse. Curves are exponential fits to the data. Neither pre-treatment with pertussis toxin (100 ng/ml, ○) nor co-expression of the β₂-ARK minigene (●) caused a significant change in the time required for recovery from voltage-dependent facilitation when compared with control (●). Data points are averages of at least four determinations.

Furthermore, transfection of cells with cDNA encoding the β-adrenergic receptor kinase (β₂-ARK) minigene peptide thought to inhibit Gβγ from binding to its effectors (38) had no significant effect on the time constant of recovery from facilitation (τ = 69.8 ± 19.5 ms). Our data therefore suggest that the observed voltage-dependent facilitation of α₁D-S(31b) channels is independent of G protein activity.

DISCUSSION

In this study we identified mRNAs encoding three α₁C (α₁A, α₁C, and α₁D), four β, and α₂δ-δ Ca²⁺ channel subunits in GH₃ cells. The transcripts encoding α₁A and α₁B subunits were not present. Despite detectable levels of α₁A-b subunit mRNA, which would be expected to encode Q-type Ca²⁺ channels, Ba²⁺ currents recorded from GH₃MORDOR cells are almost abolished by the dihydropyridine nifedipine (24). Furthermore, ω-agatoxin IVa (500 nM) at a sufficient concentration to block α₁A-b channels (6) did not affect the inhibition of Ba²⁺ currents induced by δ opioid receptor activation. Together, these observations suggest that P/Q-type channels do not contribute to the opioid receptor-sensitive Ca²⁺ channel population in GH₃MORDOR cells. The highest concentration of ω-agatoxin IVa (500 nM) tested did cause an inhibition of Ba²⁺ current amplitude. There are two possible explanations for this observation. Functional Q-type Ca²⁺ channels that are unable to couple to opioid receptors may exist in GH₃MORDOR cells. Alternatively ω-agatoxin IVa (500 nM) may inhibit a subset of L-type Ca²⁺ channels (perhaps α₁D) that do not couple to opioid receptors. Further experiments will be required to distinguish between these two possibilities. Taken together these data support the hypothesis that opioid receptors can couple to dihydropyridine-sensitive L-type Ca²⁺ channels in GH₃MORDOR cells. However, the fact that μ and δ receptor activation inhibits Ca²⁺ channel activity in GH₃MORDOR cells by <20% on average suggests that not all of their L-type channels are G protein-regulated (25).

Both α₁C and α₁D subunits may participate in the L-type Ca²⁺ channel activity of GH₃ cells. However, recombinant μ receptors failed to couple to α₁C subunits when both were expressed in Xenopus oocytes (26). Therefore, we hypothesized that α₁D subunits couple to opioid receptors in GH₃MORDOR cells (25). RT-PCR analysis revealed three loci within the α₁D gene at which alternative splicing occurs in GH₃ cells. Transcripts encoding α₁D subunits are also alternatively spliced at these sites in human and rat brain, hamster insulin-secreting cells, and chicken cochlea (7, 33, 39, 40). The observation that alternative splicing occurs at the C terminus of the gene producing transcripts of differing lengths was of particular interest; the shorter transcript (α₁D-S) (33) ends with a sequence that encodes an amino acid motif (QXXER) found in all other Ca²⁺ channel α₁ subunits (α₁A, α₁D, and α₁K) that couple to G proteins. This motif is also present in other effectors, including adenyl cyclase 2, G protein-activated inward rectifying K⁺ channels, and β-adrenergic receptor kinase (β₂-ARK), which all couple in a membrane-delimited fashion to inhibitory metabotropic receptors (16). Several lines of evidence support the hypothesis that βγ subunits mediate the inhibitory actions of G protein-coupled receptors on Ca²⁺ channels, and inhibition is thought to occur subsequent to the binding of the βγ subunit to the QXXER domain (15, 18, 37).

In view of their putative role in G protein signaling, we used long range RT-PCR to identify the alternatively spliced transcripts encoding α₁D subunits found in GH₃ cells and rat whole brain. The relative abundances of α₁D-S, and α₁D-L, variants in GH₃ cells were determined by analyzing 100 transformed bacterial clones for each. Similarly, the number of α₁D-S variants was examined in whole rat brain. In retrospect, the large number of isoforms detected was not surprising considering the presence of multiple hotspots of splice variation. However, the number of observed variants was larger in GH₃ cells than in brain. The additional splicing may be a characteristic of GH₃ cells.
Alternatively Spliced α1D Subunits

cells. Alternatively, analyzing 100 clones in the whole brain might not be sufficient to detect the presence of specific variants, which may only occur in small subpopulations of neurons. Nonetheless, analysis of the transcripts in the whole brain confirmed that the majority of splicing activity was not exclusive to GH3 cells.

To date, there have been relatively few studies demonstrating functional expression of α1D Ca\(^{2+}\) channel variants (41–44). Analysis of the biophysical properties of the three α1D variants expressed in our study revealed that they have a similar current-voltage relationship to native α1D currents of the cochlear inner hair cells, activation threshold occurred at around ~45 mV, and currents peaked near 0 mV (45). By contrast, α1C channels expressed with β2a and α1C-δ subunits had a higher threshold of activation and peaked at 20 mV (46).

Voltage-dependent inhibition by dihydropyridine antagonists distinguishes L-type channels from all other Ca\(^{2+}\) channels (47). Nimodipine blocked distribution on cell bodies and proximal dendrites in the hippocampus and many other brain regions (52, 53), where they may participate in the activity-dependent initiation of gene expression that is inhibited by dihydropyridines (54, 55). Furthermore, their voltage-dependent facilitation may also enable activity-dependent enhancement of Ca\(^{2+}\) influx contributing to long-term potentiation and depression (56–58).

Our results suggest that the QXXER motif is not sufficient to confer G protein sensitivity to α1D channels. Recent reports suggest that, in addition to the two proposed G\(\beta_{7}\) sites in the intracellular loop between domains I and II, domain I and N- and C-terminal regions of α1D and α1E Ca\(^{2+}\) channels may be important for G protein coupling (36, 59, 60). Similarly, multiple regions may be required for G protein-mediated inhibition of α1D channels to occur. Thus other α1D splice variants may be responsible for the G protein-sensitive L-type Ca\(^{2+}\) channels in GH3 cells. Alternatively, the required signaling molecules for G protein-mediated inhibition of L-type Ca\(^{2+}\) channels present in GH3 cells may not be present in HEK or CHO cells.

There are few studies of functional α1D channels, and this is among the first to examine multiple alternatively spliced isoforms. We have shown that alternatively spliced α1D subunits form functional L-type channels that resemble those of GH3 cells in their voltage dependence of activation and nifedipine sensitivity.

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