Biophysical Properties, Pharmacology, and Modulation of Human, Neuronal L-Type ($\alpha_{1D}$, Ca$_{\text{V}1.3}$) Voltage-Dependent Calcium Currents


Biophysical properties, pharmacology, and modulation of human, neuronal L-type ($\alpha_{1D}$, Ca$_{\text{V}1.3}$) voltage-dependent calcium currents. *J Neurophysiol* 85: 816–827, 2001. Voltage-dependent calcium channels (VDCCs) are multimeric complexes composed of a pore-forming $\alpha$ subunit together with several accessory subunits, including $\alpha_{1}$, $\beta$, and, in some cases, $\gamma$ subunits. A family of VDCCs known as the L-type channels are formed specifically from $\alpha_{1S}$ (skeletal muscle), $\alpha_{1C}$ (in heart and brain), $\alpha_{1D}$ (mainly in brain, heart, and endocrine tissue), and $\alpha_{1F}$ (retina). Neuroneuronal L-type currents have a significant role in the control of neurosecretion and can be inhibited by GTP-binding (G-) proteins. However, the subunit composition of the VDCCs underlying these G-protein-regulated neuronal L-type currents is unknown. To investigate the biophysical and pharmacological properties and role of G-protein modulation of $\alpha_{1D}$ calcium channels, we have examined calcium channel currents formed by the human neuronal L-type $\alpha_{1D}$ subunit, coexpressed with $\alpha_{1}$ and $\beta$, stably expressed in a human embryonic kidney (HEK) 293 cell line, using whole cell and perforated patchclamp techniques. The $\alpha_{1D}$-expressing cell line exhibited L-type currents with typical characteristics. The currents were high-voltage activated (peak at $+20$ mV in $20$ mM Ba$^{2+}$) and showed little inactivation in external Ba$^{2+}$, while displaying rapid inactivation kinetics in external Ca$^{2+}$. The L-type currents were inhibited by the 1,4-dihydropyridine (DHP) antagonists nifedipine and nicardipine and enhanced by the DHP agonist BayK S(-)-8644. However, $\alpha_{1D}$ L-type currents were not modulated by activation of a number of G-protein pathways. Activation of endogenous somatostatin receptor subtype 2 (sst2) by somatostatin-14 or activation of transiently transfected rat D2 dopamine receptors (D2$_{\text{long}}$) by quinpirole had no effect. Direct activation of G-proteins by the nonhydrolyzable GTP analogue, guanosine 5'-O-(3-thiotriphosphate) also had no effect on the $\alpha_{1D}$ currents. In contrast, in the same system, N-type currents, formed from transiently transfected $\alpha_{1B}$/GTPY, unlike the $\alpha_{1B}$ I-II loop function. These data show that the biophysical and pharmacological properties of recombinant human $\alpha_{1D}$ L-type currents are similar to $\alpha_{1C}$ currents, and these currents are also resistant to modulation by G$_{i/o}$-linked G-protein-coupled receptors.

I N T R O D U C T I O N

The L-type voltage-dependent calcium channels (VDCCs) are formed by one of four possible pore forming $\alpha$ subunits: $\alpha_{1S}$ (found in skeletal muscle) (Tanabe et al. 1987), $\alpha_{1C}$ (mainly cardiac) (Mikami et al. 1989), $\alpha_{1D}$ (in neurons and neurosecretory cells and heart) (Seino et al. 1992; Williams et al. 1992; Wyatt et al. 1997; Yaney et al. 1992), or $\alpha_{1F}$ (retinal, not yet functionally expressed) (Strom et al. 1998). The VDCC family nomenclature was recently revised by Ertel et al. (2000): $\alpha_{1S}$, $\alpha_{1C}$, $\alpha_{1D}$, and $\alpha_{1F}$ were renamed Ca$_{\text{V}1.1}$, Ca$_{\text{V}1.2}$, Ca$_{\text{V}1.3}$, and Ca$_{\text{V}1.4}$, respectively. The $\alpha_{1}$ subunits are co-assembled with the accessory subunits $\beta$, $\alpha_{1}$ (and $\gamma_{1}$ in skeletal muscle). L-type currents have been defined pharmacologically by their sensitivity to low (nM to $\mu$M) concentrations of 1,4-dihydropyridine (DHP) antagonists (e.g., nifedipine and nicardipine) and agonists [e.g., S(-)-BayK8644 (Sanguinetti and Kass 1984)]. In addition, L-type channels exhibit the following biophysical characteristics: “long-lasting” currents that show little inactivation in Ba$^{2+}$ (Nowycky et al. 1985); some selectivity for Ba$^{2+}$ over Ca$^{2+}$ and Ca$^{2+}$-dependent inactivation (Soldatov et al. 1997).

GTP-binding (G-) proteins exist as heterotrimeric complexes, composed of a $\alpha$ subunit and a $\beta\gamma$ dimer. On activation of a G-protein–coupled receptor (GPCR), the heterotrimer dissociates into free Go-GTP and $\beta\gamma$ dimers. It is these free $\beta\gamma$ subunits that are thought to be responsible for fast, membrane delimited, voltage-dependent G-protein inhibition of certain neuronal VDCCs, including $\alpha_{1A}$, $\alpha_{1B}$, and $\alpha_{1E}$ (Herlitze et al. 1996; Ikeda 1996; Page et al. 1998; for a review see Dolphin 1998). VDCCs undergoing voltage-dependent G-protein modulation display the following characteristics: a decrease in whole cell current, depolarizing shift in the current-voltage ($I-V$) relationship, and slowed activation kinetics (Bean 1989). Another characteristic is the loss of G-protein modula-
tion at large depolarizations (Bean 1989); consequently a large depolarizing prepulse immediately before a test pulse transiently removes inhibition, and the activation kinetics become faster, a phenomenon termed prepulse facilitation (Bean 1989; Elmslie et al. 1990).

Native cardiac L-type channels have long been known to exhibit G-protein-induced stimulation via Goα and a cAMP-dependent protein kinase pathway (Reuter 1983). Recently stimulation of smooth muscle L-type currents by Giβ has also been reported via a phosphinositide 3 kinase pathway (Viard et al. 1999). Inhibition via activation of Goαi, and subsequent inhibition of adenyl cyclase, is another G-protein modulatory path that regulates cardiac L-type channels (Fischmeister and Hartzell 1986). In native endocrine and neurosecretory cells and cell lines, G-protein inhibition of L-type currents has also been observed (Degtiar et al. 1997; Gilon et al. 1997; Haws et al. 1993; Hernandez-Guijo et al. 1999; Kleuss et al. 1991; Mathie et al. 1992; Tallent et al. 1996). This is thought to be involved in the inhibitory modulation of secretion. However, the subtype(s) of VDCC α1 subunit(s) involved and type of G-protein modulation observed for these L-type currents have not been fully defined (see Dolphin 1999, for review). In neuronal and neurosecretory tissue, L-type currents are formed from α1D as well as α1C subunits (Chin et al. 1992). α1D has also been shown to be expressed in heart (Hell et al. 1993; Wyatt et al. 1997). The consensus of current research suggests that L-type currents resulting from expression of neuronal (Bourinet et al. 1996; Canti et al. 1999) or cardiac (Meza and Adams 1998) α1C isoforms do not exhibit the voltage-dependent G-protein inhibition that is typical of N or P/Q currents. Nevertheless, in experiments where cloned α1C has been co-expressed with accessory subunits in Xenopus laevis oocytes (Oz et al. 1998) and HEK 293 cells (Dai et al. 1999; Kamp et al. 2000), other forms of facilitation and second-messenger-based inhibition have been observed.

The existence of G-protein modulation of cloned α1D L-type VDCCs has not yet been examined. Here we have used a stable HEK 293 cell line expressing the α1D subunit, together with the human accessory subunits αδ-1 and β3a, to establish the biophysical and pharmacological properties of the expressed current and whether the resultant current shows G-protein modulation.

**METHODS**

**Materials**

The following compounds were stored at −20°C (stock concentration in mM unless stated, solvent, and source): nifedipine, NIF (3, ethanol, Sigma, St. Louis, MO); nicardipine, NIC (3, ethanol, Sigma); BayK S-(-)8644, BayK (3, ethanol, RBI, Natwick, MA); somatostatin-14, SST (0.1, deoxygenated double-distilled water, RBI, Natwick, MA); quinpirole, Quin (10, double-distilled water, RBI); forskolin (10, dimethyl sulfoxide, Sigma); genetin G-418 sulfate (100 mg/ml, double-distilled water, Life Technologies, Paisley, Scotland); Zeocin (100 mg/ml, supplied in solution, Invitrogen, Carlsbad, CA); and amphotericin-B (80 mg/ml, dimethyl sulfoxide, Sigma).

The following cDNAs were used in transient transfections: rabbit α1D (GenBank accession number D14157), rat α1D (rβIII, L15453), rat α1D (see below for details), rat β3a (Tomlinson et al. 1993), rat β3b (M88571), rat αδ-1 (neuronal splice variant, M86621), rat D2long receptor (rD2long, X17458, N5 → G), and mut-3 green fluorescent protein (GFP, U73901). All cDNAs were subcloned into the expression vector pRK5 except for the clones used in the α1Dlong transient transfection study, which were subcloned into the expression vector pMT2 (Genetic Institute, Cambridge, MA) (see Swick et al. 1992). The rat α1D (rβIII, L15453) clone has a truncated N-terminus, compared with other α1D clones. Page et al. (1998) extended this clone using a rat α1D N-terminal extension (AF057029). The resulting α1Dlong clone used in this study has homology to published mouse (L29346), human (L27745), and rabbit (X67855) α1D clones. The β1b subunit used in this study is that of Tomlinson et al. (1993). It is identical to the rat β1b clone defined in the GenBank database (X61394) except for two substitutions (R417 → S and V435 → A) and the deletion A431 (T. P. Snutch, personal communication).

**α1D Stable cell line (HEK 293 α1D)**

Standard techniques were used to transfect HEK 293 stably with human neuronal α1D (M76558), α1δ-1 (M76539) and β3a (not published); for clarity this cell line (#5D12-20) will subsequently be referred to as HEK 293 α1D. The cloning of these VDCC subunits is discussed in Williams et al. (1992). The cloning of these VDCC subunits was established by transfecting HEK293 cells using a standard Ca2+ phosphate procedure (Brust et al. 1993) with 10, 5, and 5 μg of the α1D, α1δ-1, and β3a expression constructs, respectively. The α1D subunit expression plasmid, pcDNA1α1D-RBS, does not contain an antibiotic resistance gene, whereas the αδ-1 and β3a subunit expression plasmids, pRC/CMVαδ-1 and pZeocMVβ3a, contain the neomycin and Zeocin resistance genes, respectively. Genetin G-418 sulfate (final concentration 100 μg/ml, Life Technologies) and Zeocin (final concentration 40 μg/ml, Invitrogen) were used for selection of colonies. The selection medium was added to the cells 48 h after transfection. Antibiotic-resistant colonies were transferred to 96-well plates using cloning cylinders, 2–4 wk after selection was initiated. Cell lines containing functional channels were selected with a fluo3-based calcium flux assay.

**Cell culture and transfection**

The culture medium in which the HEK 293 α1D and control HEK 293 cells were grown consisted of Dulbecco’s modified Eagle’s medium (DMEM) with 4.5-g glucose · l−1 (DMEM, Life Technologies). This was supplemented with 5% bovine calf serum (Hyclone, UT), penicillin (100 IU · ml−1) and streptomycin (100 μg · ml−1; Life Technologies) and the additional selection antibiotics for the HEK 293 α1D cell line (as described above). The cells were grown in this medium at 37°C, 5% CO2, and passaged every 2–3 days.

For transient transfection of the α1D, α1δ-1, β3a VDCC subunits and mut-3 GFP expression marker into HEK 293 cells, a mixture was made containing, respectively, 15, 5, 5, and 1 μl of the cDNAs (at a concentration of 1 μg/μl). In experiments where the rD2long was used, 5 μg of this cDNA was added; in experiments where this D2 receptor pathway was not investigated, 5 μg of blank pRK5 vector was used to give a final cDNA amount of 31 μg. The same amounts were used for the transfection of α1D, α1δ-1, β3a, and mut-3 GFP (using 5 μg of blank pMT2 to make the mixture up to a final amount of 31 μg). For transfection, 10 μl of Geneporter reagent (Genetic Therapy Systems, San Diego, CA) and 2 μl of the cDNA mix were added to each 1 ml of DMEM (no supplements) and incubated at 20°C for 1 h before addition of 1 ml to each 35-mm-diam culture dish containing approximately 2 × 106 cells. Transfected cells were then grown at 37°C for 36 h and subsequently at 28°C for 36 h. This process of 37°C/28°C incubation was also the standard procedure for the stable α1D cell line before electrophysiological experiments. In experiments on the HEK 293 α1D cells where additional transient transfection of the D2long expression was required, the cDNA mix was formed of rD2long (5 μg) and mut-3 GFP (1 μg) cDNA, and made up to 31 μg with blank pRK5, with the transfection procedure being as described above (for
$$\alpha_{1D}$$ and $$\alpha_{1b}$$. Successful transfection was determined by expression of mut3-GFP.

Electrophysiology

The internal (pipette) and external solutions and recording techniques were similar to those previously described (Campbell et al. 1995). For whole cell patch-clamp recordings, the patch pipette solution contained (in mM) 140 Cs-aspartate, 5 EGTA, 2 MgCl$_2$, 0.1 CaCl$_2$, 2 K$_2$ATP, 0.8 TrisGTP, 10 HEPES; pH 7.2, 310 mOsm with sucrose. In experiments where guanosine 5’-0-(3-thiotriphosphate) (GTP-ψS) and guanosine 5’-0-(2-thiodiphosphate) (GDP-ψS) were used, the GTP was replaced with either 100 μM GTP-ψS (Sigma) or 2 mM GDP-ψS (Boehringer, Mannheim, Germany). For perforated-patch clamp recordings the patch pipette solution contained (in mM) 100 CH$_3$O$_2$Scs, 25 CsCl, 3 MgCl$_2$, 40 HEPES; pH 7.3, and freshly supplemented (within 1 h of recording) with 240 μg/ml amphotericin-B. The external solution contained (in mM) 160 tetraethylammonium (TEA) bromide, 3 KCl, 1.0 NaHCO$_3$, 1.0 MgCl$_2$, 10 HEPES, 4 glucose, 10 or 20 BaCl$_2$ or CaCl$_2$; pH 7.4. 3, 320 mM Os with sucrose. The perfusion rate was 1–2 ml/min. Pipettes of resistance 2–5 MΩ were used. An Axon 200A or an Axopatch 1D amplifier (Axon Instruments, Foster City, CA) was used, and data were filtered at 1 kHz and digitized at 5–10 kHz using a Digidata 1200 interface (Axon Instruments). Membrane capacitance measurements were recorded from the amplifier following capacitance compensation. Analysis was performed using pClamp 6.02 (Axon Instruments) and Origin 5 (Microcal Software, Northampton, MA). Current records are shown following leak and residual capacitance current subtraction (P/4 or P/5 protocol). Data are expressed as means ± SE. Statistical analysis was performed using paired or unpaired Student’s t-test, as appropriate, where significance was defined as P < 0.05 (*) and P < 0.01 (**). Where indicated, I-V relations were fitted with a combined Boltzmann and linear fit

$$I_V = G_{max}(V - V_{rev})/([1 + \exp(-(V - V_{max})/k)])$$

where $I_V$ is the current density at voltage V, $G_{max}$ is the maximum conductance, $V_{rev}$ is the mid-point of voltage dependence of activation, $V_{max}$ is the maximum potential, and k is the slope factor.

Steady-state inactivation data were fitted with a Boltzmann function of the form

$$I_{max}/I_{max} = 1/[1 + \exp(-(V - V_{inact})/k)]$$

where $I_{max}$ is the current normalized to maximum current, $V_{inact}$ is the mid-point of voltage dependence of inactivation, V is the conditioning potential, and k is the slope factor. Current activation was fitted with a similar Boltzmann function with $I_{max}$ replaced by $G_{max}$ (normalized conductance) and $V_{inact}$ substituted by $V_{inact}$.

The holding potential was −80 mV, unless otherwise stated. Voltages were not corrected for liquid junction potential, measured using the method described in Neher (1995), which were up to −2.7 mV in whole cell recording solutions and −5.4 mV in perforated-patch solutions.

Construction, expression, and purification of proteins

The polymerase chain reaction (PCR) was used to amplify (from full length clones) regions of cDNA encoding the calcium channel I-II loops of the rabbit $$\alpha_{1D}$$ and human $$\alpha_{1D}$$ clones (M76558). As the full length clone of the human $$\alpha_{1D}$$ was unavailable for PCR, it was necessary to perform RT-PCR from the HEK 293 $$\alpha_{1D}$$ cell line. Approximately 10$^5$ HEK 293 $$\alpha_{1D}$$ cells were lysed using the QiaShredder and the total RNA extracted using the Qiagen RNEasy kit (Qiagen, Crawley, UK). After a further phenol/chloroform extraction and precipitation the total RNA was reverse transcribed using MMLV-Reverse Transcriptase (Life Technologies, Paisley, Scotland) and random hexamer primers (Promega, Southampton, UK). PCR was performed using either $Pfu$ (Promega) or PfX (Life Technologies) high-fidelity DNA polymerase in the supplied polymerase buffers. BamHI and EcoRI restriction sites (underlined) for directional, in-frame cloning of the resulting fragments into pGEX2T (Pharmacia, St Albans, UK) were present in the primer sets as follows: $T$ Forward: 5’CTCAGATTCTTGCTAAGGAGCG3’ Reverse: 5’AGAAAATTCTGCTCATCAGTGC3’ $\alpha_{1B}$ Forward: 5’GGGATCCATTCTCAAGGAGAGG3’ Reverse: 5’GGGAATTTCGAGACATTCAC3’. Amplification was for 30 cycles before the resulting products were separated by agarose gel electrophoresis, digested with BamHI and EcoRI and subcloned into pGEX2T (Pharmacia). The resulting constructs are GST$\alpha_{1D}$I–II loop and GST$\alpha_{1B}$I–II loop, respectively. The sequences of all the fusion protein constructs were verified by cycle sequencing (Sequitherm, Epicentre laboratories, Madison, WI) or automated sequencing, before use in protein expression studies.

Expression cultures of BL21(DE3)-Codon Plus-(RII) Escherichia coli (Stratagene, Amsterdam, NL) were grown overnight at 37°C in LB medium supplemented with 34 μg/ml chloramphenicol, 50 μg/ml ampicillin, and 1% (wt/vol) glucose. The saturated cultures were diluted 10-fold in the same medium and grown for a further 2.5 h before cooling to 25°C and induction with 0.1 mM isopropyl-thio-galactopyranoside. Induced cultures were grown at 35°C for a further 2.5 h before harvesting. All further purification steps were performed at 4°C. Cells were lysed by sonication in phosphate-buffered saline, pH 7.4 (PBS: 10 mM phosphate buffer, pH 7.4, 137 mM NaCl, 2.7 mM KCl) containing 1% sarcosyl, 25 mM EDTA, 0.5 mM dithiothreitol, and protease inhibitors (1 tablet per 50 ml of lysate, Complete EDTA-free, Roche Diagnostics, Lewes, UK) followed by a 10-min incubation at 4°C, TritonX-100 was then added to a final concentration of 2% and the lysate re-sonicated and incubated at 4°C for a further 10 min. before centrifugation at 20,000 × g for 15 min at 4°C. The resulting supernatant was then applied to a 1-mL GSTrap column (Pharmacia) and the column washed with 10 column volumes of binding buffer (PBS, pH 7.4 containing 0.1% Triton X100, 20 mM EDTA, 0.5 mM dithiothreitol and 1 protease inhibitor tablet per 200 ml). Bound GST-fusion proteins were then eluted from the column with elution buffer (binding buffer, at pH 8.0, supplemented with 5 mM reduced glutathione). Glutathione was removed from the fusion protein preparations by dialysis against HBS-EP buffer (10 mM HEPES, pH 7.4, 150 mM NaCl, 3 mM EDTA, 0.005% (vol/vol) Tween 20) before samples were frozen in aliquots at −20°C.

Bovine brain G-proteins were purified to apparent homogeneity using conventional chromatographic techniques. GβY dimers were then liberated from Gα subunits in the presence of aluminum fluoride (Exner et al. 1999). Isolation and final purification of GβY was achieved using a Mono Q FPLC column (Pharmacia). GβY subunits were identified by their immunoreactivity and stored in aliquots at −80°C until required for use. A full-length $\beta_{1b}$ with C-terminal hexa-histidine tag (H6C$\beta_{1b}$) was produced by PCR (10 cycles) using $Pfu$ polymerase (Stratagene, Amsterdam), $\beta_{1b}$ in pMT2 as template and the following primers: Forward: 5’GGGAAATTCTAGGTTCCAGAAAGCG3’ Reverse: 5’GGGAAATTCTCATGATGATGATGATGGC- GGATCCAGGG3’. The resulting PCR product (approximately 1.9 k.b.) was digested with EcoRI and subcloned into the pKK233–3 vector (Amersham Pharmacia, Little Chalfont, UK). To maximize yields of the full-length H6C$\beta_{1b}$ protein the 600b.p. 3’ NcoI-EcoRI fragment of H6C$\beta_{1b}$ and the 1.3kb. 5’NcoI-NcoI fragment of $\beta_{1b}$ were subcloned into NcoI-EcoRI digested pET28b (Novagen, Nottingham, UK) to give H6C$\beta_{1b}$-pET28b. BL21(DE3)-Codon Plus-(IRL) Escherichia coli (Stratagene) were transformed with H6C$\beta_{1b}$-pET28b, and cultures were grown overnight to saturation at 37°C in LB (pH 5.5) supplemented with kanamycin (30 μg/ml), chloramphenicol (34 μg/
ml) and 1% wt/vol glucose, diluted 1:10 with the same media and grown for a further 3 h before cooling to room temperature and induction with 0.5 mM isopropylthio-β-D-galactoside (IPTG). The cultures were grown for 3 h postinduction at room temperature then harvested by centrifugation, pellets were stored at −70°C until required. *Escherichia coli* pellets containing expressed H6CB1b protein were lysed at 4°C by sonication in 20 mM phosphate buffer (pH 7.4), containing 1 protease inhibitor tablet per liter of culture pelleted. Solid NaCl was added to the lysate to a final concentration of 1 M before the lysate was cleared at 20,000 × g at 4°C for 15 min. Imidazole solution (pH 7.4) was then added to the resulting supernatant to give a final concentration of 40 mM before loading onto a nickel-primed 5 ml HiTrap Chelating column (Amersham Pharmacia) equilibrated with loading buffer (20 mM phosphate buffer, pH 7.4, 1 M NaCl, 40 mM imidazole, 0.15% wt/vol octylglucoside and 1 protease inhibitor tablet per 100 ml). The column was washed thoroughly with wash buffer (as load buffer but 70 mM imidazole) before H6C

linear gradient of 0 to 1 M NaCl in IEX buffer. Fractions containing cultures were grown for 3 h postinduction at room temperature then load buffer but 70 mM imidazole) before H6C

per 100 ml). The column was washed thoroughly with wash buffer (as load buffer but 70 mM imidazole) before H6C

1 ml SP-Sepahrose HP column (Amersham Pharmacia), the column in elution buffer (as load buffer but 200 mM imidazole).

Peak UV280 absorbance fractions were rapidly buffer exchanged on a Sephadex G-25 (Amersham Pharmacia) column into IEX buffer (20 mM 2-[N-morpholino]ethanesulfonic acid, pH 6.0, 1 M NaCl, 1 protease inhibitor tablet per 200 ml) supplemented with 500 mM NaCl, before dilution 1:10 with IEX buffer. The diluted sample was loaded onto a 1 ml SP-Sepharose HP column (Amersham Pharmacia), the column was washed with IEX buffer before H6C

proteins were eluted in a linear gradient of 0 to 1 M NaCl in IEX buffer. Fractions containing H6C

were identified by SDS-PAGE analysis, with Coomassie blue staining, before dialysis against storage buffer (20 mM borate, pH 8.0, 500 mM NaCl, 1 mM EDTA, 1 protease inhibitor tablet per 200 ml). H6C

prepared in this manner was found to have a half-life in excess of 60 days at 4°C as judged by SDS-PAGE and Coomassie blue staining.

Surface plasmon resonance binding assay

All assays were performed on a Biacore 2000 (Biacore AB, Uppsala, Sweden) at 25°C in HBS-EP buffer (10 mM HEPES, pH 7.4; 150 mM NaCl, 3 mM EDTA, 0.005% vol/vol polysorbate 20) unless stated otherwise. Glutathione S-transferase (GST) fusion proteins were immobilized on CMS dextran chips using an anti-GST monoclonal antibody kit according to the manufacturer’s instructions (Biacore AB). To obtain identical molar loadings of the different molecular mass fusion proteins, the following resonance unit (RU) correction factors were used during immobilization (GST-I = 1, GSTen1D-II loop = 1.52, GSTα1D-II loop = 1.57). Gβγ dimers were diluted in HBS-EP buffer before use, and Gβγ injections were performed using a flow rate of 50 μl/min for 5 min. Experiments using H6C

were performed in modified HBS-EP buffer containing 500 mM NaCl, with the same flow rate and injection time used for the Gβγ experiments.

RESULTS

α1D Sensitivity to DHPs

From electrophysiological recording of the HEK 293 α1D (α1D/α2δ-1/β3) cell line, 43% of cells were found to express calcium channel currents, and for those currents that were stable, the mean current density was −10 ± 2.1 pA/PF (mean ± SE, n = 21) in 20 mM Ba2+ at a test potential of +10 mV (and approximately half this value when recorded in 10 mM Ba2+). As expected, the α1D currents displayed sensitivity to DHP antagonists. The effects of 3 and 10 μM nifedipine are shown in the time course in Fig. 1A, and the percentage inhibition of I Ba by 3 μM nifedipine and nicardipine is shown in the bar chart in Fig. 1B. The 1,4-DHP antagonist block was also characterized by an increase in the inactivation kinetics of I Ba during the test depolarization (+10 mV, V t), which can be observed by comparing the inhibition at peak compared with the end of the 200-ms test pulse (○ and ●, respectively, in Fig. 1A, and □ and ■ in Fig. 1B). However, α1D I Ba showed very similar inhibition by nifedipine at three different holding potentials (Vh = −80, −50, and −30 mV). At Vh = −50 mV, 3 μM nifedipine inhibited α1D I Ba by 62 ± 7% (at peak) and 94 ± 3% (at the end of 200 ms pulse, n = 5); at Vh = −30 mV the inhibition was similar, being 56 ± 7% (at peak) and 91 ± 3% (at 200 ms, n = 6).

The agonist BayK S-(−)8644 (3 μM) produced a marked enhancement of the current (325 ± 25% increase, n = 5, at Vh = +10 mV in 10 mM Ba2+; 680 ± 84% increase, n = 14, at Vh = +10 mV in 20 mM Ba2+; Fig. 1C). The onset of enhancement was rapid (reaching steady-state enhancement within 1–2 min of application, Fig. 1C) and was accompanied by a characteristic hyperpolarizing shift in the I-V relationship (Fig. 1D). In some recordings BayK S-(−)8644 enhanced α1D currents also displayed the characteristic slowing of tail current deactivation (for an example, see the enhanced current trace in Fig. 6B).

**Biophysical characteristics of the α1D current**

The activation and steady-state inactivation of α1D currents are shown in Fig. 2A. Fitting the current activation gave a V50,act of +1.8 ± 1.2 mV. Examining the steady-state inactivation properties of the α1D currents showed that at test potentials up to +30 mV the α1D currents did not fully inactivate and had a relatively depolarized V50,inact of −13.4 ± 1.8 mV (n = 6). The inactivation kinetics at Vh = +10 mV were very slow in Ba2+ (see examples of control traces in Fig. 1, A, C, and D). Single exponentials were fitted to the inactivating phase during long (1,200–1,600 ms) depolarizing steps, (e.g., the white line in the example trace shown in Fig. 2B). The τinact was 439 ± 50 ms (n = 4). The inactivation kinetics with external Ca2+ rather than Ba2+ were far more rapid, shown by the overlaid example traces in Fig. 2C. Almost complete inactivation was observed during a 200-ms depolarizing pulse in Ca2+ (τinact = 44.3 ± 1.1 ms, n = 7). Additionally, the peak current in 20 mM Ca2+ was smaller than in 20 mM Ba2+ (I Ca was ~20% of I Ba). The mean I-V relationship in 20 mM Ba2+ and 20 mM Ca2+ exemplifies these differences (Fig. 2D).

α1D Currents do not show G-protein–mediated inhibition

Having established some basic biophysical and pharmacological properties of α1D currents, we then examined whether these currents displayed G-protein modulation. Initially the modulation of the α1D currents was compared with that of α1B currents (known to be G-protein modulated), transiently expressed in HEK 293 cells, by activating the endogenous somatostatin receptor subtype 2 (sst2). Application of somatostatin (SST, 100–500 nM, n = 8) had no effect on the α1D current (Fig. 3A). Using the same endogenous receptor-based signaling pathway, application of SST (500 nM) caused a rapid inhibition of α1B currents, observed in all cells tested (see Fig. 3B; mean inhibition, 40 ± 7%, n = 8).
The α1D currents are sensitive to 1,4 dihydropyridines (DHPs). A: the α1D currents show inhibition by the DHP antagonist nifedipine (NIF). Time course of currents measured at peak (○) and at the end (●) of the 200-ms test pulse. Depolarizing test pulses (V_t = +10 mV) were given every 30 s from a holding potential (V_h) of −80 mV. Application of NIF (3 and 10 µM) are denoted by the horizontal bars. The inset shows example traces taken from the time course for control (CTRL, 10 mM Ba^2⁺) and for 3 and 10 µM NIF; the test pulse protocol is above these example traces. B: bar graph depicting mean current inhibition (%) at peak (○) and end of the 200 ms test pulse (●) for nifedipine (NIF, 3 µM, n = 7) and nicardipine (NIC, 3 µM, n = 6). C: sensitivity of α1D currents to the DHP agonist S(-)-BayK8644. An example time course of measured peak current (○) recorded with step depolarizations from V_h = −80 mV to V_h = +10 mV, at 30-s intervals (see pulse protocol above inset of example traces). Application of S(-)-BayK8644 (3 µM) is denoted by the horizontal bar below time course. D: an example cell showing a family of current-voltage (I-V) traces in control (20 mM Ba^2⁺; CTRL) and during S(-)-BayK8644 (3 µM; BayK) enhancement of the current. I-V families were formed by depolarizing from −80 mV to between −40 and +60 mV in 10-mV increments, every 5 s (see pulse protocol below the CTRL family of traces). For clarity example traces are shown for currents measured by stepping to −40, −20, 0, and +20 mV only. In each condition, peak current was measured and plotted as an I-V relationship (bottom; CTRL, ●, with BayK, ●) and fitted by a modified Boltzmann equation (Eq. 1, see METHODS).

However, application of the D2 agonist quinpirole (300 nM), had no effect on α1D currents (n = 7, Fig. 4A), although a clear inhibitory effect was observed in 10/16 of the α1D/α2δ-1/β3 expressing cells (with a mean inhibition of 59 ± 7%, n = 10; Fig. 4B). This inhibition was greater than that produced by activation of the endogenous sst2 receptor, suggesting more efficient activation of this G-protein pathway by D2 receptors, but despite this, no inhibition of α1D currents was observed (Fig. 4C).

The GTP analogue GTP-γS can be used as a more direct way of activating G-proteins since it is nonhydrolyzable and leads to their sustained activation. Conversely, the GDP analogue GDP-βS can be used to block G-protein activation. Using these guanine nucleotide analogues, the existence of tonic modulation was examined with a prepulse (PP) protocol. Figure 5A depicts the ratio of current in the absence (no PP) or immediately following (+PP) a large depolarizing prepulse (+PP/no PP ratio) for control (CTRL, gray columns and associated example traces), with 100 µM GTP-γS (black columns...
and associated example traces) and with 2 mM GDP-βS intracellularly (white columns and associated example traces). It can be seen in both the histogram and also in the example traces relating to these +PP/no PP ratios (Fig. 5A), that there was a small degree of facilitation (+PP/no PP ratio >1) in all of the intracellular conditions. Furthermore, the activation time to 90% peak (ttf 90%) was shorter for all +PP than no PP currents (Fig. 5B). However, the magnitude of prepulse facilitation, and the activation ttf 90%, were unaltered by inclusion of GTP-γS or GDP-βS.

In comparison, in recordings made from cells transfected with α1B channels, there was no evidence of prepulse facilitation of α1B currents using control intracellular pipette solution. However, following direct activation of G-proteins with GTP-γS (Fig. 5C), there was a marked facilitation of the +PP current compared with the no PP current. Under these recording conditions, the ttf 90% was also greater in the no PP current than in the current preceded by a prepulse, whereas in control conditions this was not apparent (Fig. 5D).

G-protein modulation of calcium currents can be also identified by a decrease in the current amplitude and a depolarizing shift of the I-V relationship with intracellular GTP-γS, while opposite effects (increase in current density and hyperpolarizing shift of the I-V relationship) are seen with GDP-βS (due to removal of tonic G-protein modulation). However, in the HEK

![Diagram](http://jn.physiology.org/)

**FIG. 2.** Biophysical properties of α1D currents. A: activation and steady-state inactivation curves of α1D currents in 20 mM Ba2+. To determine the steady-state inactivation (a) a standard +10-mV test pulse for 40 ms was elicited from Vh = 80 mV, and was then preceded by a 5-s (steady-state) incremental depolarization from −70 to +30 mV every 20 s (depicted in the pulse protocol, which lies above an example family of traces for such a protocol; see inset traces). Peak currents were normalized to the current measured with no prepulse (mean ± SE; n = 6). The resulting steady-state inactivation curve was then fitted with a Boltzmann equation (Eq. 2, see METHODS). The fit gave the steady-state inactivation Vt,50,inact = −13.4 ± 1.8 mV, and k = 11.9 ± 1.7 mV. The activation (α) was calculated using the following equation Gα = Iα/(V − Vrev). Gα and Iα are the conductance and current measured at each voltage, V, in the I-V relationship pulse protocol (see Fig. 1D). The I-V relationships were fitted with a combined Boltzmann and linear fit (Eq. 1, see METHODS) to determine Vrev. The resulting Gα values were normalized to the peak Gmax for each I-V relationship, and averaged (mean ± SE; n = 10). The resulting data points were fitted with a Boltzmann equation (Eq. 2, see METHODS). The fit gave the activation Vt,α,50 = +1.8 ± 1.2 mV, and k = 7.3 ± 1.1 mV. B-D: comparison of α1D currents in 20 mM Ba2+ vs. 20 mM Ca2+. B: long test pulses (1.200–1.600 ms) were used to determine τmax in Ba2+. An example of such a fit is shown for a trace recorded in 20 mM Ba2+ with a +10-mV, 1,200-ms test pulse; this gave a τmax of 436 ms. C: example traces in Ba2+ and Ca2+ obtained by depolarizing the cells to Vh = +10 mV for 200 ms in each condition. By fitting a single exponential to the inactivation of the trace recorded in Ca2+, a τmax of 39.1 ms was obtained. D: using the same I-V pulse protocol described in Fig. 1D (depolarizing from Vh = −80 mV to between Vt = −40 and +60 mV in 10-mV increments), I-V relationships from 6 cells were measured in 20 mM Ba2+ and then in 20 mM Ca2+. Measurements for Ba2+ (peak, end of pulse, and Ca2+ (peak, end of pulse, f) were made and plotted against the Vt to give the I-V relationship.

**FIG. 3.** The lack of G-protein–coupled receptor (endogenous sst2) modulation of α1D currents. A: a time course of peak α1D currents from whole cell patch-clamp recording. Currents were evoked every 30 s from Vh = −80 mV to Vt = +10 mV (●) in α1D expressing cells in 20 mM Ba2+. Somatostatin (SST; 500 nM) and NIF (3 μM) application are denoted by the horizontal bars. No response was observed in response to SST application (100– 500 nM, n = 8). The insets show overlapping example traces observed in control (CTRL) and SST (500 nM). B: peak currents measured from whole cell patch-clamp recordings in α1D,α,δ−1/β3 expressing cells (Vh = −80 mV, Vt = +20 mV, every 30 s, ●). SST (500 nM) application inhibited the current by 40 ± 7% (n = 8). Inset shows example traces in control and during SST inhibition. C: peak currents measured during an amphotericin-B perforated patch–clamp experiment. Time course of peak α1D currents evoked in 20 mM Ba2+ by depolarizing from Vh = −80 mV to Vt = +10 mV every 30 s. Application of somatostatin (SST, 500 nM) is denoted by the hatched box area. During this time course I-V relationships were performed in CTRL, SST, and WASH conditions (denoted by “IV” during the time course). Below the time course are examples of families of traces evoked by the standard I-V pulse protocol (depolarizing from Vh = −80 mV to between −40 and +60 mV in 10-mV increments) for CTRL, SST, and WASH conditions (for clarity examples only from Vt = −40, −20, 0, and +20 mV are shown).
modulation of 1,4-DHP–sensitive currents in neuroendocrine cells have appeared (Degtiar et al. 1997; Gilon et al. 1997; Haws et al. 1993; Hernandez-Guijo et al. 1999; Kleuss et al. 1991; Tallent et al. 1996). One possible explanation is that 1,4-DHPs may be blocking non–L-type currents, and it is these currents that exhibit the G-protein modulation. Previous re-

293 α1D cell line no difference was observed in the I–V relationships across the G-protein activating conditions (control, n = 21; +GTP-γS, n = 23; +GDP-βS, n = 17; data not shown).

α1D Currents are also resistant to G-protein modulation in the presence of a 1,4-DHP agonist

In a recent study by Hernandez-Guijo et al. (1999), a form of voltage-independent G-protein modulation was observed in rat chromaffin cell L-type currents. Inhibition was observed during perfusion of a cocktail of BayK S-(−)8644, by a combination of a number of receptor agonists including ATP, opioids with or without the additional inclusion of catecholamines. In Fig. 6, we investigated whether there was any G-protein modulation of the α1D currents during BayK S-(−)8644 perfusion. The α1D expressing cells were also transiently transfected with rD2long receptors, and after enhancement of the α1D current by BayK S-(−)8644 (3 μM), a cocktail of BayK S-(−)8644 (3 μM), SST (500 nM), and quinpirole (300 nM) was applied. No effect was observed of this cocktail of drugs (n = 5, Figs. 6, A and B).

Selectivity of 1,4-DHP antagonists for L-type currents

Despite the lack of G-protein modulation of expressed α1D channel currents in HEK 293 cells, several reports showing the

FIG. 4. The lack of G-protein–coupled receptor (transiently expressed rD2, R) modulation of α1D currents: whole cell recordings. A: α1D expressing cells were co-transfected with rD2long receptor and GFP (expression marker); application of the D2 agonist quinpirole (Quin, 300 nM) had no effect on current (n = 7; ●): test pulses (from Vh = −80 mV to Vt = +10 mV) were given every 15 s. Traces during Quin and control (CTRL) conditions are shown in the inset (overlapping). B: transient expression of α1D/α2B/β3, and co-expression rD2long receptor: time course of measured peak current (same pulse protocol as in A, except Vt = +20 mV, ■): application of Quin (300 nM) inhibited IaCa in 10/16 cells tested. Example traces during CTRL and Quin application are shown in the inset. C: bar graph showing %inhibition (mean ± SE) for SST application on α1D and α1B currents (1st 2 columns), and for Quin on α1D and α1B currents (additionally co-transfected with rD2long receptor: 3rd and 4th columns, respectively).

FIG. 5. Lack of G-protein modulation of α1D via GTP-γS and GDP-βS. The following shading is used in all histograms: control intracellular (CTRL, gray), with 100 μM GTP-γS (+GTP-γS, black) and with 2 mM GDP-βS (+GDP-βS, white). A and C: using the pulse protocol depicted in the top right, in which the test pulse was applied either preceded (+PP) or not (no PP) by a 100-ms prepulse to +120 mV. The measurements of (+PP/no PP) ratio were measured in CTRL, +GTP-γS, and +GDP-βS for cells expressing α1D (A) and α1B (C). The +PP/no PP ratios were calculated by measuring the values of IaCa at 20 ms after the start of the test pulse. Figures below columns denote the numbers of experiments. Statistical significance was determined by using an unpaired Student’s t-test (**P < 0.01) between CTRL and experimental conditions. Example traces for no PP and +PP in α1D with CTRL, +GTP-γS and +GDP-βS intracellular conditions are shown at the top of A and in α1B with CTRL and +GTP-γS intracellular conditions at the top of C. B and D: using the same cells used for the +PP/no PP determination in A and C, the ttp 90% was measured for both sets of currents. The ttp 90% values were measured by determining the maximum current amplitude, and measuring the time at which the current reached 90% of its maximum amplitude. Statistical significance was determined using a paired Student’s t-test between the ttp90% for no PP (−) and + PP (+) currents, for each of the conditions (*P < 0.05, **P < 0.01).
search has shown that the selectivity of DHP antagonists for L-type channels may not be as absolute as previously thought (Diochot et al. 1995; Furukawa et al. 1999). To further examine this possibility of \( \alpha_{1D} \) channels providing a G-protein–modulated, 1,4-DHP–sensitive current, we investigated currents resulting from transient expression of \( \alpha_{1E} \) long/\( \alpha_{2,\delta,1}/\beta_{1b} \). It was observed that these \( \alpha_{1E} \) currents were inhibited by both nifedipine and nicardipine (10 \( \muM \)), although the onset of inhibition is slower than for inhibition of \( \alpha_{1D} \) currents (data not shown). The % inhibition was compared at the peak of the current and at the end of the 200-ms depolarizing test pulse. For nifedipine, there was 13 \( \pm \) 4% inhibition of the peak current and 32 \( \pm \) 9% inhibition at 200 ms (\( n = 9 \)). For nicardipine, there was 63 \( \pm \) 5% inhibition of the peak current and 87 \( \pm \) 7% inhibition at 200 ms (\( n = 3 \)). Thus the increased inactivation that was associated with 1,4-DHP inhibition of \( \alpha_{1D} \) currents is also apparent for these \( \alpha_{1E} \) currents.

\( \beta \) and VDCC \( \beta \) subunit binding to \( \alpha_{1} \) I–II loops expressed as GST fusion proteins

To examine biochemically the basis for the lack of G-protein modulation of the \( \alpha_{1D} \) VDCC, the cytoplasmic loops between transmembrane domains I–II of the human \( \alpha_{1D} \) and rabbit \( \alpha_{1B} \) clones used in this study were expressed in *Escherichia coli* as GST fusion proteins and purified as described in METHODS. The purified fusion proteins are shown after separation on 12.5% SDS-PAGE gels (Fig. 7A). The proteins were bound via the GST moiety to the Biacore 2000 CM5 sensor chip, as described, and GST itself was used as a control. Purified bovine brain GB\( \beta \gamma \) subunits were then applied to the sensor chip surface at a rate of 50 \( \mu l/min, for 5 \) min. The sensogram traces are shown in Fig. 7B, for three concentrations (10, 25, and 50 nM) of GB\( \beta \gamma \) exposed to the \( \alpha_{1B} \) I–II loop, and a single concentration of GB\( \beta \gamma \) (100 nM) exposed to the \( \alpha_{1D} \) I–II loop. In contrast to the data for the \( \alpha_{1B} \) I–II loop, which showed concentration-dependent binding of GB\( \beta \gamma \), no binding of GB\( \beta \gamma \) was observed to the \( \alpha_{1D} \) I–II loop. A similar lack of binding was observed for up to 4 \( \mu M \) GB\( \beta \gamma \) exposed to a fusion protein of the I–II loop from a rat pancreatic \( \alpha_{1D} \) clone (D38101, results not shown).

Kinetic analysis was performed for the lowest concentration of GB\( \beta \gamma \) (10 nM) binding to the \( \alpha_{1B} \) I–II loop. Single exponential fits were made to the binding and dissociation phases of the sensogram (Fig. 7B). The observed on-rate \( k_{\text{on(obs)}} \) for GB\( \beta \gamma \) binding was 0.0121 s\(^{-1}\), and the off-rate \( k_{\text{off}} \) after termination of GB\( \beta \gamma \) perfusion was 0.0104 s\(^{-1}\). Assuming a unimolecular reaction in which

\[
K_D = k_{\text{off}}/k_{\text{on}}
\]

the \( K_D \) for GB\( \beta \gamma \) binding was calculated to be 62.2 nM. This is very similar to the \( K_D \) determined previously for GB\( \beta \gamma \) binding to parts of the \( \alpha_{1A} \) I–II loop (De Waard et al. 1997).

As a positive control for the integrity of the GST fusion proteins, the ability of purified H6C\( \beta_{1b} \) (Fig. 7A) to bind to the same I–II loops was examined. Both \( \alpha_{1D} \) and \( \alpha_{1B} \) I–II loops bound H6C\( \beta_{1b} \) reversibly (Fig. 7C), with the \( \alpha_{1D} \) I–II loop demonstrating a higher binding affinity with a \( K_D \) of 10 nM compared with 21 nM for \( \alpha_{1B} \), determined as above, using the Biacore kinetic analysis software with 1:1 interaction.

**DISCUSSION**

*L-type current characteristics exhibited by expression of the human neuronal \( \alpha_{1D} \) clone*

There are a number of key characteristics shown by the calcium channel currents expressed by the HEK 293 \( \alpha_{1D} \) cells that are acknowledged as being traits of L-type currents. Sensitivity to the DHP antagonists (nifedipine and nicardipine) and an agonist [BayK S-(–)8644] were observed. The degree of inhibition and enhancement are comparable with other studies investigating the pharmacology of expressed cloned L-type channels (Tomlinson et al. 1993; Williams et al. 1992). In addition, the increased inactivation observed during DHP antagonist application that has been reported previously for native cardiac L-type channels (Lee and Tsien 1983) was also apparent for the \( \alpha_{1D} \) currents. This effect of DHP antagonists on the inactivation kinetics was recently investigated by Handrock et al. (1999), who suggested that it is due to a second DHP binding site. However, caution must be taken when using the characteristics of antagonism by DHPs, since, as was observed by the application of nifedipine and nicardipine to cells transiently expressing \( \alpha_{1E} \) channels in this study, \( \alpha_{1E} \) channels also exhibit inhibition by DHP antagonists (Stephens et al. 1997), including the characteristic increase in inactivation (compare peak inhibition with that at 200 ms into the depolarizing prepulse). More selective pharmacological definition of L-type over \( \alpha_{1E} \) or other non-L-type currents might be obtained by using low micromolar concentrations of nifedipine (rather than...
the more promiscuous nicardipine; an effect also observed in oocytes) (Furukawa et al. 1999). However, in the present study, 10 μM nifedipine was required to completely inhibit α1D currents. Enhancement by BayK S-(−)8644 remains a defining characteristic of L-type currents, since α1B currents have previously been shown to be insensitive to BayK S-(−)8644 (Stephens et al. 1997).

Many of the biophysical characteristics expected of L-type currents are also observed for the α1D currents. The I-V relationship in Fig. 2D shows that the currents activate at about −20 mV and with the peak at approximately 1 mV; see Fig. 2A) is very similar to expressed cardiac (Pérez-Garcia et al. 1995) and neuronal (Tomlinson et al. 1993) α1C L-type channel currents. The steady-state inactivation (V50,inact of −13.9 mV; see Fig. 2A), is also comparable to expressed cardiac α1C L-type currents (Lacinova et al. 1995). The inactivation kinetics are also typical of "long-lasting" neuronal L-type currents (Nowycky et al. 1985). In 20 mM Ba2+, very little inactivation of α1D currents was observed, while rapid and striking calcium-dependent inactivation was observed in 20 mM Ca2+ (see Fig. 2, B–D).

Another characteristic of the α1D currents that correlates well with other studies of expressed α1C channels (Dai et al. 1999; Kamp et al. 2000) is the small but reproducible facilitation following a large depolarizing prepulse (see Fig. 5). Such attributes are often indicative of G-protein modulation; however, for the α1D current this effect was independent of G-protein modulation, as it was similar in the presence of GTP-γS and GDP-βS.

As yet there are no biophysical characteristics or pharmacological tools that can differentiate between currents resulting from either native or expressed α1C and α1D channels. Previous research has shown that the block by DHP antagonists is voltage dependent, with greater inhibition being observed when the holding potential is more depolarized (Sanguinetti and Kass 1984). However, for the α1D currents no such voltage dependence of block by DHP antagonists was observed, with similar block occurring (at both peak current and 200 ms) at all holding potentials examined. Another aspect that may prove to be different is the τ inact of α1D currents in Ba2+. In a previous study examining the τ inact of α1C when co-expressed with β3 in Xenopus laevis oocytes (Soldatov et al. 1997), slower rates of

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**FIG. 7.** Lack of Gβγ binding and binding of voltage-dependent calcium channel (VDCC) β subunit to α1D I–II loop. A: silver-stained SDS gel (12.5%) of the proteins used in the surface plasmon resonance binding assays. Approximately 0.5 μg was loaded of the following proteins: GST, GSTα1D-I–II loop and GSTα1D-I–II loop, and VDCC β1b subunit, as indicated. The positions of molecular mass markers (Sigma) are shown for comparison in the outside lanes. B: Biacore 2000 sensorgrams. Approximately 4 fmol of each fusion protein was immobilized on an individual flow cell of a CMS dextran sensor chip via an anti-GST monoclonal antibody according to the manufacturer’s instructions. This corresponds to approximately 100 reference units (R.U.) of GST, 157 R.U. of GST α1D-I–II loop, and 152 R.U. of GSTα1D-I–II loop. Gβγ dimers were diluted to the concentrations stated, in HBS-EP buffer before use and injected over all flow cells at a flow rate of 50 μl/min for 5 min. The resulting sensorgrams from the flow cell containing GST were subtracted from those containing the GSTα1D-I–II loop (solid line) and GSTα1D-I–II loop (dashed line) as a correction for bulk refractive index changes during Gβγ perfusion and for nonspecific binding of the Gβγ analyte to the GST moieties of the fusion proteins. C: Biacore 2000 sensorgrams for β1b binding to the α1B and α1D-I–II loop GST fusion proteins immobilized as in B. β1b was applied at 10 nM to the α1D-I–II loop and 100 nM to the α1B-I–II loop in HBS-EP buffer containing 300 nM NaCl, at 50 μl/min for 5 min. The data were subjected to the same subtraction procedure as described in B. Solid line, α1B I–II loop; dashed line, α1D I–II loop.
inactivation were observed (~1,300 ms) than seen here for \( \alpha_{1D} \) currents. Nevertheless, care must be taken in interpreting such results since expression system (oocyte vs. HEK 293) and specific accessory subunit composition (particularly \( \beta \) subunits) will have marked effects on the inactivation properties.

**Lack of G-protein modulation of \( \alpha_{1D} \) currents**

The preceding biophysical and pharmacological characterization clearly defined the \( \alpha_{1D} \) currents as being L-type in nature. We then investigated the possibility of G-protein modulation of this L-type current. G-protein modulation was examined either by activation of the endogenous sst2 receptors or by transient expression of another GPCR, the rD2long receptor. However, no modulation was observed of \( \alpha_{1D} \) currents via either pathway. To ensure that the G-protein pathways were intact and capable of coupling to calcium channels in the HEK 293 cells, both the endogenous sst2 and the transiently expressed exogenous rD2long receptors were stimulated via their respective agonists in HEK 293 cells expressing \( \alpha_{1B} \) currents, which have been shown to be G-protein modulated by many groups (for review, see Dolphin 1998). These positive controls showed obvious G-protein modulation, confirming that modulation is possible by these pathways. Furthermore, the modulation of the \( \alpha_{1D} \) current was also investigated during application of BayK \( S(-)8644 \), since G-protein–mediated inhibition of L-type currents had been observed in native rat chromaffin cells, during enhancement by BayK \( S(-)8644 \) (Hernandez-Guijo et al. 1999). However, a combination of BayK \( S(-)8644 \), SST, and quinipride did not reveal inhibitory G-protein modulation of BayK \( S(-)8644 \)–enhanced \( \alpha_{1D} \) currents co-expressed with rD2long (see Fig. 6).

Another method to examine G-protein modulation is to use the nonhydrolyzable GTP and GDP analogues GTP-\( \gamma \)S and GDP-\( \beta \)S. The advantage of using these guanine nucleotide analogues is that they act directly on all G-proteins, producing sustained activation (in the case of GTP-\( \gamma \)S) or blockade of activation (GDP-\( \beta \)S) (Dolphin and Scott 1987). Using a standard large depolarizing (+120 mV) prepulse protocol to detect G-protein modulation, no GTP-\( \gamma \)S–dependent effect was observed on \( \alpha_{1D} \) currents, yet the \( \alpha_{1B} \) currents do exhibit marked tonic G-protein modulation in these conditions.

The lack of G-protein modulation of this \( \alpha_{1D} \) clone is corroborated by the lack of G\( \beta \)\( \gamma \) binding to a GST fusion protein of the \( \alpha_{1D} \) I–II loop, whereas in parallel experiments, reversible binding of G\( \beta \)\( \gamma \) was observed to the \( \alpha_{1B} \) I–II loop. In contrast, both the \( \alpha_{1D} \) and \( \alpha_{1B} \) I–II loops reversibly bound purified \( \beta_{1B} \) subunit, indicating that they are probably folded in a native conformation (see Fig. 7C). While the I–II loop is not the only region of the \( \alpha_{1B} \) calcium channel that is involved in its G-protein regulation (Canti et al. 1999; Page et al. 1998; Zhang et al. 1996), nevertheless it is certainly one of the key sequences contributing to the inhibition of neuronal calcium channels (De Waard et al. 1997).

G\( \gamma \)–protein modulation of L-type currents was also investigated by activation of the G\( \gamma \)-adenyl cyclase pathway with forskolin. No effect of forskolin was observed (\( n = 4 \), data not shown). Protein kinase A (PKA) modulation of channels has been shown to require A-kinase anchoring proteins (AKAPs) (Johnson et al. 1997). The presence of AKAPs was not examined in this study, although they are likely to be present since they are found in tsA-201 cells which are an HEK 293–derived cell line (Johnson et al. 1997).

From these results, the \( \alpha_{1D} \) L-type clone used in the present study does not appear to be the molecular counterpart of the native neuronal and endocrine L-type channels that have been shown to exhibit G-protein modulation in several neuroendocrine preparations (Degtiar et al. 1997; Gilon et al. 1997; Haws et al. 1993; Hernandez-Guijo et al. 1999; Kleuss et al. 1991; Tallent et al. 1996).

**Source of G-protein–modulated neuroendocrine L-type current?**

Since we have shown that the \( \alpha_{1D}/\alpha_{1E}-1/\beta_{3a} \) currents do not exhibit inhibitory G-protein modulation, what is the molecular counterpart of the native L-type current in neuroendocrine cells that do exhibit G-protein modulation? L-type currents are generally identified by their sensitivity to DHP antagonists; however, we previously demonstrated DHP antagonist block of a rat \( \alpha_{1E} \) isoform, rbEII (Stephens et al. 1997). Because this isoform has a 50 amino acid truncation of the N-terminus compared with \( \alpha_{1E} \) clones from other species (Page et al. 1998), and may therefore not represent a native isoform in rat brain (Schramm et al. 1999), we have now confirmed and extended the finding of DHP sensitivity of \( \alpha_{1E} \) currents, using \( \alpha_{1Elong} \), an isoform whose extended N-terminus is homologous to the cloned human (L27745), rabbit (X67855), and mouse (L29346) (Williams et al. 1994) \( \alpha_{1E} \) sequences. The partial DHP sensitivity (particularly to nicardipine) of \( \alpha_{1E} \) currents observed, as well as the DHP sensitivity of other cloned non–L-type currents observed recently (Furukawa et al. 1999) suggests the caveat that some studies apparently demonstrating G-protein modulation of “L-type” currents (according to their sensitivity to DHP antagonists) may need to be reviewed. However, this uncertainty over identification of L-type currents by DHP antagonist sensitivity may only be relevant to a few studies, and the significant bank of evidence for G-protein modulation of neuroendocrine L-type channels will be unaffected, particularly those studies in which L-type currents have been defined by \( S(-) \)-BayK8644 enhancement (Hernandez-Guijo et al. 1999).

Additional \( \alpha_{1D} \) isoforms have been cloned from pancreatic \( \beta \)-cells in rat (Ihara et al. 1995) and hamster (Yaney et al. 1992). There is little functional expression data available for these clones. Expression of \( \alpha_{1D} \) clones appears to be problematic with relatively low current density yields (even in the clone used in this study, a low percentage of cells exhibited stable currents), a problem that has hindered research in this area and may indicate that the full-length clones currently available are not naturally occurring splice variants. A number of sequences within the \( \alpha_{1A}, \alpha_{1B}, \alpha_{1E} \) and \( \beta_{1E} \) VDCC subunits have been shown to be important for G\( \beta \)\( \gamma \) binding and modulation of the channel. These important \( \alpha_{1} \) VDCC subunit sequences include the intracellular loop between domains I and II (De Waard et al. 1997), a region within the N-terminus (Canti et al. 1999; Page et al. 1998) and the C-terminus (Qin et al. 1997; Zhang et al. 1996). Two particularly relevant motifs present in the I–II loop (QQIER) (Zamponi et al. 1997) and the N-terminus (YKQ-SIAQRART) (Canti et al. 1999) of \( \alpha_{1E} \) sequences. The partial)}
ronal α1D clone used in this study, most elements in putative regions pertinent to G-protein modulation are homologous to each other. This suggests that these additional published α1D clones may also be predicted to exhibit no G-protein modulation. Indeed, similar results have been obtained regarding the lack of inhibitory G-protein modulation of another α1D clone (Yaney et al. 1992) (A. Scholze, T. D. Plant, A. C. Dolphin, and B. Nürnberg, unpublished results). However, the α1D sequences show least conservation in the C-terminal tails, with complexity. Among these combinations of possibility that the C-terminus of alternative BEAN BP. Neurotransmitter inhibition of neuronal calcium currents by changes 80262. N. Torrey Pines Rd., #160, La Jolla, CA 92037; A. Nesterova, Dept. of Columbia University, New York, NY 10032; P. F. Brust, Ambryx Inc., 11099 and Dohme collaborative Ph.D. studentship. This work was supported by the Medical Research Council (MRC), Wellcome Trust and Royal Society. D. C. Bell was funded by a MRC/Merck, Sharp acknowledged. Strange (Reading, UK); T. Hughes (Yale, New Haven, CT); and Genetics Columbia, Vancouver, Canada); Y. Mori (Seriken, Okazaki, Japan); P. G. Perez-Reyes (University of Virginia); T. Snutch (University of British 1D clone used here) or short C-terminus isoforms (as in Yaney et al. 1992) providing scope for the possibility that the C-terminus of alternative α1D splice variants may provide a means of G-protein modulation of certain splice variants.

As further progress is made in the elucidation of neuroendocrine L-type channels, it is becoming clear that a sophisticated level of complexity is likely to exist. For example, in the GH3 (a rat pituitary derived) cell line alone, several mRNA transcripts encoding splice variants of the α1D subunit have been reported (Safa et al. 1998). Further complexity of these channels will be added due to the differing combinations of accessory subunits. Although β3 appears to be a significant accessory subunit associated with neuronal L-type channels (Pichler et al. 1997), nevertheless, β3 is also prominently associated with neuronal L-type channels, and β1b and β2a are also associated with a small proportion of the channels (Pichler et al. 1997). Between β subunit isoforms there are also splice variants (for reviews see Birnbaumer et al. 1998; Castellano and Perez-Reyes 1994) that add to the channel subunit complexity. Among these combinations of α1D splice variants and accessory subunits, there may be a sub-set that do exhibit the G-protein modulation observed in native neuroendocrine cells and derived cell lines. Alternatively, an as yet undiscovered accessory protein may be required for coupling of the neuronal L-type channels to G-protein inhibitory pathways, or modulation may involve Ca2+-dependent mechanisms (Mathie et al. 1992), a process not investigated in the present study.

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