Inhibition of Transiently Expressed Low- and High-Voltage-Activated Calcium Channels by Trivalent Metal Cations

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Abstract. Calcium channels are important regulators of neuronal excitability and contribute to transmitter release, calcium dependent gene expression, and oscillatory behavior in many cell types. Under physiological conditions, native low-voltage (T-type)- and high-voltage-activated (HVA) currents are potently inhibited by trivalent cations. However, the presence of multiple calcium channel isoforms has hampered our ability to unequivocally assess the effects of trivalent cations on channel activity. Here, we describe the actions of nine trivalent metal ions on transiently expressed α1G (Cav3.1) T-type calcium channels cloned from human brain. In 2 mM external barium solution, yttrium most potently inhibited α1G current \( IC_{50} = 28 \text{ nM} \), followed by erbium > gadolinium = cerium > holmium > ytterbium > neodymium > lanthanum > scandium. With the exception of scandium, blocking affinity was loosely correlated with decreasing ionic radius. A detailed characterization of yttrium block revealed a 25-fold decrease in blocking affinity when the external concentration of charge carrier was increased from 2 mM to 20 mM. In 20 mM barium, yttrium also effectively inhibited various types of cloned HVA channels indicating that this ion is a nonselective blocker. For all calcium channels examined, yttrium preferentially inhibited inward over outward current, but block was otherwise voltage independent. In addition to peak current inhibition, P/Q- and L-type channels underwent a unique speeding of the macroscopic time course of inactivation. Whereas peak current block of α1A channels was highly sensitive to the external charge carrier concentration, the inactivation effects mediated by yttrium were not, suggesting that the two effects are due to distinct mechanisms. Moreover, the speeding effect was greatly attenuated by manipulations that slowed the inactivation kinetics of the channels. Thus, our evidence suggests that yttrium effects are mediated by two distinct events: peak current block likely occurring by occlusion of the pore, and kinetic speeding arising from yttrium interactions with the channel that alter the state of the inactivation gate.

Key words: Voltage gated calcium channel — Low voltage activated — High voltage activated — Trivalent metal cation — Lanthanide — HEK cells

Introduction

Calcium influx through voltage-gated calcium channels mediates a number of unique functions in the central nervous system, including pacemaker activity, generation of low-threshold spikes, and neurotransmitter release (e.g., Deschenes, Roy & Steriade, 1982; Bal & McCormick, 1993; McCleskey, 1994; Magee & Johnston, 1995; Kim et al., 2001). Calcium channels are important pharmacological targets in the treatment of pain and certain forms of epilepsy (Tsakiridou et al., 1995; van Luijtelaar et al., 2000; Beedle & Zamponi, 2001; Kim et al., 2001) and have therefore been subjected to intense study. One area that has received considerable interest is their inhibition by divalent and trivalent metal ions, not only because nickel ions were once considered to be selective T-type calcium channel inhibitors, but also because these ions have a defined ionic radius and therefore constitute excellent structural probes of the permeation pathway. However, unequivocal interpretation of the data has been hampered by the presence of multiple calcium channel isoforms (e.g., Narahashi, Tsunoo & Yoshii, 1987; Akaile et al., 1989a; Magee & Johnston, 1995), and in the literature, a wide range of responses of native currents to divalent cations has been described. For example, reported \( IC_{50} \) values for

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nickel block of native calcium channels range from 47 to 600 μM (Narahashi et al., 1987; Akaike et al., 1989a; Akaike, Kostyuk & Osipchuk, 1989b; Todorovic & Lingle, 1998). Similarly, the reported potency of lanthanum (La^{3+}) inhibition ranges from IC_{50} values of 8 nM for block of high-voltage activated current in aortic smooth muscle cells (Akaike et al., 1989a) to 1.5 μM for block of low-voltage activated current in neuroblastoma cells (Narahashi et al., 1987). In addition, trivalent metal cation (M^{3+}) block has been reported to be both voltage dependent by some groups (Lansman, Hess & Tsein, 1986; Lansman, 1990; Block, Stacey & Jones, 1998) and voltage independent by others (Boland, Brown & Dingledine, 1991; Mlinar & Enyeart, 1993).

The cloning of calcium channel α1 subunits such as the T-type calcium channel family, α1G (Perez-Reyes et al., 1998; Cribbs et al., 2000; Monteil et al., 2000a), α1H (Cribbs et al., 1998), and α1L (Lee et al., 1999a; Monteil et al., 2000b); the N-type channel, α1B (Dubel et al., 1992; Williams et al., 1992a); the P/Q-type channel, α1A (Mori et al., 1991; Stea et al., 1994; Westenbroek et al., 1995); the R-type channel, α1E (Zhang et al., 1993); and the L-type channels, α1C (Miki et al., 1989; Hui et al., 1991), α1D (Snutch et al., 1991; Williams et al., 1992b), α1F (Bech-Hansen et al., 1998), and α1S (Ellis et al., 1988), has allowed detailed functional studies on these channel types in isolation. For divalent ions such as nickel, Lee and coauthors showed that α1H channels are much more potent inhibited than those formed by α1G (Lee et al., 1999b). However, little information about block of cloned calcium channels by trivalent cations has been reported to date.

Using transient transfection of tsA-201 cells, we can express identified calcium channels and conduct voltage-clamp studies under identical conditions. This is an ideal system in which to examine the actions of trivalent cations and to determine the usefulness of these ions as probes of the channel pore structure. Thus, we undertook a detailed study to clarify trivalent cation blocking effects on cloned calcium channels. We have cloned an α1G channel construct from human brain tissues, transiently expressed it and other rat calcium channel cDNAs, and examined their inhibition by trivalent metal ions. Our data show that all trivalent metal ions tested, with the exception of scandium, are potent inhibitors of T-type channels with affinities in the submicromolar range, and that blocking affinity is loosely correlated with ionic radius. Whereas peak current inhibition was relatively nonselective among all types of calcium channels examined, P/Q-type and L-type channels underwent a speeding of inactivation kinetics that might be indicative of the presence of a second binding site outside of the pore region that is coupled to the inactivation machinery.

**Materials and Methods**

**Calcium Channel Constructs**

α1G. Four different human α1G constructs were cloned and combined to create a full-length α1G channel. Fragment I was obtained by filter hybridization screening, according to manufacturer’s protocol, of a human cerebellum library (Clontech) in λgt11. Similarly, fragment IV was retrieved from a human fetal brain library (gift from Dr. S. Kaneko, Kyoto University) by alternately screening with two radioactive probes. These probes for screening were created by PCR with the following primers: fragment I, sense 5’-TCATCGTACCTGCAAGGGAATGCTGGAAG and antisense 5’-GGTTCTGGCGTGCTGGGAGACAG (designated from GenBankTM AF126965 sequence); fragment II, sense 5’TGGCCCTCAGGACCAAGAGGC TGAGTGG, antisense 5’-CAATTGCAAGGGAGGAGGAGGAGG (designated from GenBankTM AF029228 and AF029229 EST sequences). Probes were radio labelled with α-32P dCTP using T7 DNA polymerase (Pharmacia). Both clones were removed from lambda phase and inserted into pBluescript KS (Stratagene). Fragments II and III were obtained by RT-PCR of human neuroblastoma cell RNA (gift from Dr. M. Kelly, Dalhouse University) and inserted into pCR-Blunt vector from Invitrogen (primers: fragment II, sense 5’-TCATCGTACCTGCAAGGGAATGCTGGAAG, antisense 5’-TCGCGTCGACACGGATGCTGGAAG, antisense 5’-TCGCGTCGACACGGATGCTGGAAG, antisense 5’-TCGCGTCGACACGGATGCTGGAAG, antisense 5’-TCGCGTCGACACGGATGCTGGAAG). Both sense and antisense strands of all clones were sequenced by ABI PRISM standard automated sequencing with BigDye. Then fragments were joined sequentially using Sall (clones I and II), BglII (clones II and III), and BstEII (clones III and IV) restriction sites and inserted into a modified PMT2 mammalian expression vector, via NotI and KpnI (NEB), for transfection.

cDNA constructs encoding α1A, α1B, α1C and α1D, plus ancillary β and δ subunits were kindly provided by Dr. Terry Snutch.

CecCCC: In this ultra-slowly inactivating L-type channel mutant, a region encompassing the first two thirds of the domain I-II linker was replaced with corresponding α1A sequence. The creation of this construct and its biophysical evaluation have been described previously by our laboratory (see Stutz & Zamponi, 2001).

**Cell Culture and Transient Transfection**

HEK tsA-201 cells were grown to 85% confluence at 37°C (5% CO₂) in Dulbecco’s modified Eagle’s medium (DMEM) containing 10% fetal bovine serum, 200 U/ml penicillin, and 0.2 mg/ml streptomycin (Life Technologies, Inc.). Cells were dissociated with trypsin (0.25%)-EDTA (1 mm) before plating at 8% confluence on glass coverslips. Calcium channel (6 μg) and green fluorescent protein marker (2 μg) DNA were transfected into cells with calcium phosphate. For T-type expression, only human α1G and green fluorescent protein DNA were necessary for expression; however, for all other calcium channels, the rat α1 subunit + α2-δ + β1 (or, if explicitly stated, β2) + green fluorescent protein channel protein were transfected. Cells were transferred to 28°C the day following transfection and stained for 1 or 2 days before recording.

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1Abbreviations: cerium, Ce^{3+}; erbium, Er^{3+}; gadolinium, Gd^{3+}; holmium; Ho^{3+}; lanthanum, La^{3+}; neodymium, Nd^{3+}; scandium, Sc^{3+}; trivalent metal cation, M^{3+}; ytterbium, Yb^{3+}; yttrium, Y^{3+}.