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Structural and biophysical determinants of single $Ca_V3.1$ and $Ca_V3.2$ T-type calcium channel inhibition by N_2O

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ABSTRACT

We investigated the biophysical mechanism of inhibition of recombinant T-type calcium channels $Ca_V3.1$ and $Ca_V3.2$ by nitrous oxide (N_2O). To identify functionally important channel structures, chimeras with reciprocal exchange of the N-terminal domains I and II and C-terminal domains III and IV were examined. In whole-cell recordings N_2O significantly inhibited $Ca_V3.2$, and – less pronounced – $Ca_V3.1$. A $Ca_V3.2$-prevalent inhibition of peak currents was also detected in cell-attached multi-channel patches. In cell-attached patches containing $\leq$3 channels N_2O reduced average peak current of $Ca_V3.2$ by decreasing open probability and open time duration. Effects on $Ca_V3.1$ were smaller and mediated by a reduced fraction of sweeps containing channel activity. Without drug, single $Ca_V3.1$ channels were significantly less active than $Ca_V3.2$. Chimeras revealed that domains III and IV control basal gating properties. Domains I and II, in particular a histidine residue within $Ca_V3.2$ (H191), are responsible for the subtype-prevalent N_2O inhibition. Our study demonstrates the biophysical (open times, open probability) and structural (domains I and II) basis of action of N_2O on $Ca_V3.2$. Such a fingerprint of single channels can help identifying the molecular nature of native channels. This is exemplified by a characterization of single channels expressed in human hMTC cells as functional homologues of recombinant $Ca_V3.1$.

1. Introduction

T-type calcium channels are encoded by three genes, $Ca_V3.1$, $Ca_V3.2$ and $Ca_V3.3$ [1]. T-type calcium channels are involved in diverse physiological functions, including cardiac and neuronal pacemaking [2,3]. Stress conditions like hypoxia were shown to regulate expression of T-type calcium channels [4,5] and the relevance of these channels for certain disease states of the central and peripheral nervous system is increasingly appreciated [6,7]. More specifically, upregulation of $Ca_V3.2$ channels in dorsal root ganglion neurons emerges as a common pathophysiological mechanism of neuropathic pain of different origin [8–10].

$Ca_V3.2$ therefore could be a promising analgesic drug target [11]. Of note, several compounds have been detected to inhibit $Ca_V3.2$ in a subtype-selective manner, including Ni^{2+} [12], Zn^{2+} [13], ascorbate [14], and nitrous oxide [15]. This subtype-selective action is dependent upon a histidine residue (H191) contained within domain I on an extracellular loop of the channel which is not conserved in $Ca_V3.1$ and $Ca_V3.3$ [14,16–18].

In a previous study on the single-channel mechanism of T-type calcium channel block, we identified a dual inhibitory action of mibefradil, a non-selective T-type inhibitor [19]. In native and recombinant $Ca_V3.2$ channels, this drug reduced the fraction of depolarizing pulses causing channel openings, indicative of long-term (s) effects on channel availability. In addition, it shortened the duration of individual openings, consistent with rapid (ms) "open-channel" block.

The aim of this study was to identify the biophysical mechanism(s) of subtype-selective block of $Ca_V3.2$. A more detailed understanding of channel function in the presence of a drug should aid in the design of new therapeutically useful subtype-selective compounds. In addition, unlike standard whole-cell

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analysis, a combination of single-channel analysis and subtype-selective pharmacology may aid in defining a fingerprint of $Ca_V3.1$ and $Ca_V3.2$, and hence help to clarify the molecular identity of endogenous channels.

For practical reasons, we chose N_2O as the most promising agent to be examined. First because N_2O acts as a general anesthetic agent, we did not expect major problems for the drug to reach its site of action (presumably the extracellular face of the channel) when applied during cell-attached recording. Second, due to its volatile nature, "wash-in" and "wash-out" procedures seemed feasible under recording conditions that maintained the signal-to-noise conditions required to resolve unitary T-type currents. Finally, because this compound is a clinically useful analgesic, we felt convinced that at feasible N_2O concentrations the extent of $Ca_V3.2$ inhibition attained would be therapeutically relevant.

Thus we first tested and validated that N_2O can be applied under experimental conditions amenable to low-noise single-channel recording. Next, we examined the biophysical single-channel mechanisms of inhibition and finally focused on subtype-selectivity using recombinant human $Ca_V3.2$ and $Ca_V3.1$ channels. To address the role of channel structure regarding N_2O inhibition, we also examined two chimeric constructs with reciprocal exchange of the N-terminal (domains I and II) and C-terminal (domains III and IV) portion between $Ca_V3.1$ and $Ca_V3.2$, and a point-mutant lacking the histidine residue mentioned above (H191Q).

2. Materials and methods

2.1. Cell culture and Ca_V isoforms

Culturing of human embryonic kidney HEK-293 cells, and of hMTC, a human medullary thyroid carcinoma cell line was done as described [19]. Stably transfected HEK-293 cells were used with either human $Ca_V3.1$ (accession number, AF190860 [20]), or human $Ca_V3.2$ (accession number, AF051946 [21]) containing a pcDNA3 vector carrying a penicillin/aminoglycoside resistance. Native HEK-293 cells were transiently transfected with the same plasmids and GFP, using Superfect® or Effectene® reagent (both from Quiagen, Hilden, Germany) according to manufacturer's instructions. For another set of experiments native HEK-293 cells were transiently transfected with the $Ca_V3.2$ mutant H191Q in pcDNA3 (kindly provided by Prof. E. Perez-Reyes) using Effectene® reagent [16]. Transfection preparation was incubated for 4–24 h at 37 °C, by 6% CO_2 gassing. HEK-293 cells were grown in Dulbecco's modified Eagle's medium (Biochrom KG) supplemented with fetal bovine serum (Sigma) and G418 (0.1%; Invitrogen, Karlsruhe, Germany) in case of stably transfected HEK-cells. Cells were used 1–3 days after plating on polystyrene dishes.

2.2. Construction of Ca_V3 chimeras

For creation of the construct GGHH (composed of domains I and II of $Ca_V3.1$ and domains III and IV of $Ca_V3.2$), $Ca_V3.2$ (AF051946 modified, 7768 bp [22]) was digested by BamHI, cutting within the polylinker region and at positions 729 and 6184. $Ca_V3.1$ (AF190860, 7349 bp [20]) was cut by BamHI at positions 560, 3863 and 6858. In case of the second construct HHGG (composed of domains I and II of $Ca_V3.2$ and domains III and IV of $Ca_V3.1$), HindIII cut at a site within the linker region between domain II of $Ca_V3.2$ (AF051946 [21]) and domain III of $Ca_V3.1$ (AF027984 [23]) [16,24]. The full-length cDNA of HHGG was constructed by ligating ClaI (5′-polylinker)-PvuI (3725, $Ca_V3.2$), PvuI (3728, $Ca_V3.2$)-$HindIII^+$ (3963, $Ca_V3.2$), $HindIII^+$ (4246, $Ca_V3.1$)-KpnI (6170, $Ca_V3.1$) into pcDNA3-$Ca_V3.1$, which was opened by ClaI (5′-polylinker) and KpnI (6170, $Ca_V3.1$). Chimeric constructs were fused into the pcDNA3 plasmid and used for transient transfection. The resulting plasmids were verified by sequencing.

2.3. Reverse-transcription PCR

mRNA was isolated from hMTC cells and amplified after reverse-transcription as described [25]. In brief, subtype-selective primers for $Ca_V3.1$ (forward 5′-AGCCCCGGTTTCTTCTA-3′, reverse 5′-TGAGCGGTCGAGCACAC-3′, expected size: 397 bp) and $Ca_V3.2$ (forward 5′-CCCCGTCGCCCGTCTACTTCGTGA-3′, reverse 5′-GGTGCCGGCCCCATAGGTCTCCAT-3′, expected size of product 380 bp) were used for 35 PCR cycles (94 °C, 30 s; 60 °C, 30 s; 72 °C, 1 min), starting with an initial 15 min heating step at 95 °C and with a final elongation period of 10 min at 72 °C. The $Ca_V3.1$ and $Ca_V3.2$ amplification products were validated by direct sequencing.

2.4. Electrophysiological methods

Whole-cell Ba^{2+}-currents were elicited by depolarizing test pulses delivered at 0.3 Hz, recorded at 5 kHz and filtered at 2 kHz (−3 dB, 4-pole Bessel) using an Axopatch 200A amplifier (Axon Instruments, Union City, CA, USA). Whole-cell bath solution contained [mM]: 130 NaCl, 10 $BaCl_2$, 1 $MgCl_2$, 10 glucose and 10 HEPES, pH 7.4 (NaOH). Pipette solution was composed of [mM]: 120 CsCl, 10 $MgCl_2$, 10 HEPES, 10 EGTA, 4 Mg-ATP, pH 7.2 (CsOH). Resistance of borosilicate pipettes was 5–7 MΩ. Series resistance and capacitance values were taken directly from the amplifier. Series resistance was compensated to the maximum possible extent that still avoided current oscillation. Application of voltage commands and digitization of membrane currents were performed using Clampex 6.0 of the pClamp software (Axon Instruments, Union City, CA, USA) running on a personal computer. Cells were held at −110 mV and depolarized to respective maximum peak inward current (−30 to −10 mV) to check for current response over time. To analyze the current–voltage relationship, cells were typically held at a holding potential of −110 mV and pulsed to test potentials of −100 mV up to +50 mV, with increments of 10 mV applied every 3 s. Data were analyzed using Clampex 6.0 (Axon Instruments) and Origin 6.0 (Microcal Software, Northampton, MA, USA).

Single calcium channels were recorded in the cell-attached configuration of the patch-clamp technique. Data were filtered at 2 kHz (−3 dB, 4-pole Bessel) and acquired at 10 kHz using an Axopatch 1D amplifier (Axon Instruments). Using holding potentials (HP) of −90 mV or −50 mV, patches were depolarized to a test potential of −20 mV for 150 ms at 0.5 Hz [19]. The calculated liquid junction potential was −18 mV in cell-attached experiments (data given in this work are not corrected for this value). Experiments were performed in an external solution containing [mM]: 120 K-glutamate, 25 KCl, 2 $MgCl_2$, 10 HEPES, 2 EGTA, 1 $CaCl_2$, 1 Na_2-ATP, 10 glucose, pH 7.4 (KOH). Pipettes (borosilicate glass, 6–7 MΩ) were filled with 110 mM $BaCl_2$ and 10 mM HEPES, pH 7.4 (TEA-OH). To reduce thermal noise, pipettes were coated using Sylgard® (Dow Corning GmbH, Wiesbaden, Germany). The pClamp software (Clampex 5.5, Fetchan 6.0, and pSTAT 6.0, Axon Instruments) and Origin 6.0 (Microcal Software) were used for data acquisition and analysis. All experiments were carried out at room temperature (21–23 °C).

2.5. Single-channel analysis

Linear leak and capacity currents were digitally subtracted. Openings and closures were identified by the half-height criterion. Unitary current amplitude i was estimated visually based on ≥10 fully resolved openings per experiment under each condition. In case of $Ca_V3.1$ and $Ca_V3.2$ analysis of all-point histograms based on raw data was performed. To elucidate the putative mechanisms