C101, a novel 4-amino-piperidine derivative selectively blocks N-type calcium channels

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N-type Ca2+ channels located on presynaptic nerve terminals regulate neurotransmitter release, including that from the spinal terminations of primary afferent nociceptors. Pharmacological and ion-channel gene knockdown approaches in animals have revealed N-type Ca2+ channels to be particularly attractive molecular targets for the discovery and development of new analgesic drugs. In recent years, some non-peptide small molecular N-type Ca2+ channel blockers have been reported. However, low selectivity and some side effects limit their further development. To overcome these disadvantages, some new compounds were designed and synthesized in our institute by optimizing the 4-amino-piperidine template. C101, one of these compounds, was demonstrated to block N-type Ca2+ channels with higher selectivity. It was found that C101 produced concentration-dependent inhibition on N-type Ca2+ channels expressed in Xenopus oocytes with an IC50 is 2.2±0.6 μM. The current–voltage relationship was not altered after 2-min exposure to C101. However, the steady-state inactivation relationship curve was shifted to more negative potentials for channels. Therefore, it seemed that C101 blocks the inactivated channel. C101 did not present any remarkable effects on voltage-gated potassium, sodium channels in cultured rat hippocampal neurons, and L-, P/Q-, R-type calcium channels and HERG channels expressed in Xenopus oocytes. The results suggested that C101 was a high selective blocker targeting N-type Ca2+ channels, and may have a potential to be developed as a novel analgesic agent.

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1. Introduction

Native N-type Ca2+ channels are hetero-oligomers that comprise a pore-forming α1 subunit (α1B) and at least two auxiliary subunits, β and δ, which modulate the α1 subunit function (Dubel et al., 1992). Presynaptic N-type Ca2+ channels mediate rapid Ca2+ influx into the synaptic terminal that triggers synaptic vesicle exocytosis and neurotransmitter release (Llinas et al., 1981). The N-type Ca2+ channel exhibits a number of characteristics that make it an attractive target for therapeutic intervention concerning chronic and neuropathic pain conditions (McGivern and McDonough, 2004). Within the past years, both U.S. and European regulatory agencies have approved the use of the cationic peptide Ziconotide (Prialt®) for the treatment of intractable pain. Prialt was the first N-type Ca2+ channel blocker approved for clinical use and represented the first new proven analgesic agent.

However, the intrathecal route of administration and dose-limiting adverse effects limit its use to a subpopulation of pain suffers (Miljanich 2004; McDonough et al., 2002). Drug discovery efforts during the past decade have been focused on small molecules of N-type Ca2+ channel blockers for analgesia. Examples of known Ca2+ channel antagonists included PD151307 and PD157767. Some of these calcium channel blockers have been shown to be active in animal pain models. However, these compounds presented adverse effects because of the lower selectivity to N-type Ca2+ channels. They blocked other subtypes of calcium channels, as well as sodium and potassium channels (Hu et al., 2000). To look for more potential and higher selective blockers for N-type Ca2+ channels, new compounds were designed and synthesized in our institute by optimizing the 4-amino-piperidine template. By screening these compounds, it was found that C101 (Fig. 1) could block N-type Ca2+ channels with higher efficacy. In the present experiment, the effects of C101 on calcium channels were further explored by using cellular electrophysiological technique. To evaluate the selectivity, effects of C101 on other voltage-gated channels were also observed. Our data showed that C101 presented a remarkable blocking effect on N-type Ca2+ channels with high selectivity.

2. Materials and methods

2.1. Clones

The rat cDNAs α1B (GeneBank accession no. AF055477/α1α (AF286488)/β1B (L06110), rabbit α1C (X15539), human α1A (NM 000068), rat α1E (NM 009782) and rat HERG (U04270) were kindly
provided by Dr. Lipscombe (Brown University, Providence, RI), Dr. David J. Adams (University of Queensland), Dr. Tina Brauns (Innsbruck University (IFU), Austria), Dr. Yasuo Mori, (Kyoto University) and Gail A. Robertson (University of Wisconsin-Madison), respectively.

2.2. Xenopus oocyte expression and electrophysiology

Oocytes (stages V–VI) were surgically removed from mature Xenopus laevis frogs anesthetized by immersion in 0.1% 3-aminobenzoic acid ethyl ester (MS-222). The follicular cell layer was removed by incubating oocytes in Ca2+-free solution containing (mM) 96 NaCl, 2 KCl, 1 MgCl2, and 5 HEPES (Sigma type 1) for 2 h at room temperature. Oocytes were rinsed several times, sorted and maintained at 18 °C in an ND96 storage solution containing (mM) 96 NaCl, 2 KCl, 1 CaCl2, 1 MgCl2, 5 HEPES, and 5 pyruvate plus 50 μg ml−1 gentamycin (pH 7.4) (Sutton et al., 1998; Adams et al., 2003).

Ca2+ channel proteins were expressed in Xenopus oocytes after injection of in vitro-transcribed mRNAs. cRNA was synthesized in vitro from linearized template cDNA using a Promega MMessage mMachine kit. For co-expression studies, cRNAs encoding for the α1b, α1a, α1C, α11δ/α12β3′β1b subunits were injected at a 1:1:1 concentration ratio, respectively (Zamponi and Snutch, 1998). The oocytes were injected with 25 ng of each Ca2+ channel subunit cRNA as indicated in a total of 46 nl DEPC-treated H2O using a Nanoliter 2000 microinjector (WPI, USA). Injected cells were maintained in ND96 solution at 18 °C for 10 days prior to experiments. Before recording, oocytes were injected with 46 nl of a 50 mM BAPTA solution to reduce activation of the endogenous Ca2+-activated Cl− current (Pan and Lipscombe, 2000). N-type Ca2+ channel currents were recorded with the two-microelectrode voltage-clamp technique (Axoclamp2B amplifier, Axon Instruments, USA). Depolarization-activated Ba2+ currents were evoked from a holding potential of −100 mV by test voltages generated using pCLAMP 9.0 software and a Digidata 1322 series interface (Axon Instruments). Capacitative and leak currents were subtracted on-line using a P/4 pulse protocol. Oocytes were continuously perfused with recording solution at a flow rate of 2 ml/min at room temperature (Canti et al., 2000).

For hippocampal cells Na+, K+ channels recording, the extracellular solution for whole-cell recording contained (in mM): 140 NaCl, 5 KCl, 1 MgCl2, 2 CaCl2, 10 Dextrose, 10 HEPES, with NaOH adjusted to a pH of 7.4 and osmolarity was adjusted to 320 mOsm with sucrose. The intracellular pipette solution contained (in mM): 140 KCl, 10 HEPES, 10 EGTA, 2 Adenosine triphosphate, and the pH was adjusted to 7.2 by NaOH.

2.5. Data analysis

The dose–response curve was fitted by Origin (OriginLab, Northampton, MA, U.S.A.) software with Hill equation. For steady-state inactivation curve, currents from each cell at each holding potential were normalized to the peak current at the most hyperpolarized holding potential. The normalized data were averaged across cells and fit to single Boltzmann functions. Origin 7.5 software was used to perform nonlinear fit of data. SPSS 12.0 software (SPSS Inc., Chicago, IL, U.S.A.) was used for statistical analysis. All data were expressed as mean±S.D. and P<0.05 indicated statistically significant.

3. Results

3.1. Concentration-dependent inhibition of C101 on Ica

The cDNAs encoding for calcium channel α1b/α1δ/β3′β1b subunits were transiently expressed in oocytes. In this experiment, 5 mM Ba2+ was used as charge carrier and the inward currents induced by depolarization could be completely depressed by selective blocker of N-type Ca2+ channels (α-conotoxin MVIIA 1 μM, data were not shown). The effect of C101 on Ica was observed by depolarizing the membrane potential from the holding potential of −100 mV to +20 mV in the presence of C101. The concentration-dependent blocking effect of C101 on N-type Ca2+ channels was shown in Fig. 2. The semilog concentration–response curve was fitted with the Hill equation, \( I/I_{\text{max}} = 1/(1 + (A/K_{50})^n) \). The concentration of C101 that induced a half-maximal current block (IC50) was 2.2 ± 0.6 μM. The blockage of C101 could be fully reversed by washing for 5 min with a normal extracellular solution.
3.2. Inhibitory effect of C101 on I-V relationship of IBa

To identify whether the inhibition of IBa caused by C101 was voltage-dependent, the I-V relationships in the presence or absence of C101 was studied. α1B/α2δ/β1b subunits generated recombinant N-type Ca2+ channel currents. Current-voltage (I-V) relationship was obtained by step depolarization between −80 mV and +100 mV in 10 mV increment. After 2-min exposure to 10 μM C101, the amplitude of IBa was reduced at most potential levels that elicited currents. The threshold of activation and the reversal potential of IBa were not altered. The potential at which maximum current was elicited was also not altered by C101. Voltage steps positive to +100 mV elicited outward currents, and the currents were also affected by C101. This outward current was carried by internal K+ flowing outward through N-type Ca2+ channels, and blocked by ω-conotoxin MVIIA (data were not shown). The results suggested that C101 blocks the current at all voltages. Apparently, block by C101 was voltage-independent, so that currents were blocked almost completely for all voltages (Fig. 3).

3.3. Effect of C101 on voltage-dependence of steady-state of inactivation curve of IBa channels

Voltage-dependent inactivation was studied using a conventional double-pulse protocol. Inactivation was induced by 5-s potential displacements (conditioning pulse) from −100 mV to 40 mV with increments of 20 mV immediately before 200-ms test pulse from −100 mV to 20 mV. When the conditioning potential was changed from −100 mV to 40 mV, an increasing proportion of channels became inactivated and the amplitude of IBa evoked by the test pulse would be decreased. Data points were fitted with a smooth curve derived from the Boltzmann equation

$$\frac{I}{I_{\text{max}}} = \frac{1}{1 + \exp \left( \frac{V_m - V_{1/2}}{k} \right)}$$

where $V_m$ was prepulse potential, $V_{1/2}$ was half-inactivation potential. The currents were normalized using the largest current from control recorded after conditioning prepulses from −100 mV to 20 mV. The data were fitted with Boltzmann function and the half-inactivation potentials were $V_{1/2} = -54.5 \pm 5.2$ mV ($n=6$), $V_{1/2} = -61.7 \pm 6.3$ mV ($n=6$) for control and 2 min after 2.5 μM C101 exposure, respectively (Fig. 4). The one-factor ANOVA showed significant difference between the effect of the C101 vs. control group ($P<0.05$).

3.4. Effect of C101 on recombinant N-type Ca2+ channels expressed in oocytes

It is now accepted that high-voltage-activated Ca2+ channel types are expressed as complex of the main subunit α1 and the auxiliary subunits α2δ, β, and γ (Bleakman et al., 1995). It has been shown that different Ca2+ channel antagonists bind with high affinity to α2δ or α1B subunit (Field et al., 2006; Mould et al., 2004). Here, we observe the effect of C101 on recombinant N-type Ca2+ channels expressed in oocytes (Fig. 5).
Whole-cell currents, generated by the recombinant N-type Ca\(^{2+}\) of four different combinations of subunits (\(\alpha_{1B}, \alpha_{1B}/\alpha_{2}, \alpha_{1B}/\beta_{1b}\) and \(\alpha_{1B}/\alpha_{2}/\beta_{1b}\)) were elicited by step depolarization to +100 mV from a holding potential of −100 mV. The activation of P/Q-type Ca\(^{2+}\) was reversed after wash-out for 5 min with normal extracellular solution (n = 7).

3.5 Selectivity of C101 on voltage-sensitive Ca\(^{2+}\) channels subtype

3.5.1 Effect of C101 on voltage-sensitive L-type Ca\(^{2+}\) channels transiently expressed in Xenopus oocytes

L-type calcium channel currents were elicited by 300-ms step depolarization to +60 mV from a holding potential of −100 mV. The compounds were applied for 2 min while the currents were induced. C101 (100 μM) did not present any visible effect on L-type calcium channel currents (Fig. 6).

3.5.2 Effect of C101 on voltage-sensitive P/Q-type Ca\(^{2+}\) channels transiently expressed in Xenopus oocytes

P/Q-type Ca\(^{2+}\) channels were elicited by 300-ms step depolarization to +60 mV from a holding potential of −100 mV. The compounds were applied for 2 min while the currents were induced. C101 (100 μM) did not present any visible effect on P/Q-type Ca\(^{2+}\) channel currents (Fig. 7).

3.5.3 Effect of C101 on voltage-sensitive R-type Ca\(^{2+}\) channels transiently expressed in Xenopus oocytes

R-type Ca\(^{2+}\) channels were elicited by 300-ms step depolarization to +60 mV from a holding potential of −100 mV. The compounds were applied for 2 min while the currents were induced. C101 (100 μM) did not present any visible effect on the inward R-type Ca\(^{2+}\) channel currents (Fig. 8).

3.6 Effect of C101 on the voltage-sensitive sodium and potassium channels in cultured hippocampal neurons

The voltage-gated whole-cell currents were evoked by depolarizing the membrane potential from −70 mV to +40 mV in 10 mV increments. The activated inward Na\(^+\) channels (I\(_{\text{Na}}\)) were blocked by 1 μM tetrodotoxin completely and reversibly. The outward currents consisted of rapidly inactivating outward potassium currents (I\(_{\text{K}(A)}\)) and delayed rectifier potassium currents (I\(_{\text{K}(DR)}\)) which were fully and reversibly blocked by 1 μM 4-AP and 10 mM tetraethylammonium chloride, respectively (data were not shown). After 2-min application of 100 μM C101, the amplitudes of I\(_{\text{Na}}\), I\(_{\text{K}(A)}\) and I\(_{\text{K}(DR)}\) were not affected. The observation indicated that C101 had no effect on I\(_{\text{Na}}\), I\(_{\text{K}(A)}\) and I\(_{\text{K}(DR)}\) (Fig. 9).

3.7 Effect of C101 on HERG channels transiently expressed in Xenopus oocytes

To record HERG channels expressed in oocytes, the cell was held at −70 mV and depolarized to voltages between −50 and +30 mV for 4 s, and then clamped to −70 mV for 4 s. The compounds were applied for

Fig. 6. Effect of C101 on L-type Ca\(^{2+}\) channel currents (\(\alpha_{1B}/\alpha_{2}/\beta_{1b}\)) expressed in oocytes. There was no effect found on L-type Ca\(^{2+}\) channels (P > 0.05, n = 10) while C101 100 μM was applied. I–V curve relationship also shows no inhibitory effect of C101 on L-type Ca\(^{2+}\) channels (n = 10).

Fig. 7. Effect of C101 on the voltage-gated P/Q-type Ca\(^{2+}\) channels (\(\alpha_{1B}/\beta_{1b}\)) expressed in oocytes. (A) There was no effect found on P/Q-type Ca\(^{2+}\) channels (P > 0.05, n = 10) while C101 100 μM was applied. (B) I–V curve relationship shows no inhibitory effect of C101 (100 μM) on P/Q-type Ca\(^{2+}\) channel currents expressed in oocytes (n = 10).

Fig. 8. Effect of C101 on the voltage-gated R-type Ca\(^{2+}\) channel currents (\(\alpha_{1B}/\alpha_{2}/\beta_{1b}\)) expressed in oocytes. (A) There was no effect found on R-type Ca\(^{2+}\) channels (P > 0.05, n = 10) while C101 100 μM was applied. (B) I–V curve relationship shows no inhibitory effect of C101 (100 μM) on R-type Ca\(^{2+}\) channel current expressed in oocytes (n = 10).

Fig. 9. Effect of C101 on voltage-gated potassium and sodium channels in primary cultured hippocampal neurons. (A) There was no effect found on the whole-cell currents while C101 100 μM was applied (P > 0.05, n = 10). (B, C) I–V curve relationship also shows no inhibitory effect of C101 on I\(_{\text{Na}}\) and I\(_{\text{K}(A)}\) (n = 10).
4. Discussion

Considerable research in the last decade has focused on the therapeutic potential of the N-type Ca^{2+} channel blockers as a new class of analgesic agents. As the first N-type Ca^{2+} channel blocker to be tested in humans, Ziconotide has been shown both non-clinically and clinically to be an effective analgesic. The intrathecal route of its use to a subpopulation of pain sufferers (Bell et al., 2004). This is clinically to be an effective analgesic. The intrathecal route of pain suffers (Bell et al., 2004). This is clinically to be an effective analgesic. The intrathecal route of treatment for drug failure in preclinical safety trials (Sanguinetti and Tristani-Firouzi, 2006). The results had shown that C101 had no effect on HERG channels expressed in oocytes. In future research, we are going to find out whether C101 has any selective effect on the different subclasses of the voltage-gated channels, such as T-type or other types of channels.

N-type Ca^{2+} channels are highly concentrated in both dorsal root ganglia cell bodies and also in the synaptic terminals where they are made in the dorsal horn of the spinal cord (Meir et al., 1999). The crucial role of the N-type Ca^{2+} channels in nociception is also confirmed by the evidence that mice lacking the N-type Ca^{2+} channel gene have higher pain thresholds compared to wild type. In the spinal cord, N-type Ca^{2+} channels control the release of the neurotransmitters substance P, CGPR, and glutamate, and reduction in N-type channel-mediated neurotransmitter release in the spinal cord almost certainly explains the antinociceptive effects of Ziconotide. As mentioned previously, C101 did not inhibit I_{K(A)}, I_{K(DR)}, L-, P/Q-, R-type Ca^{2+} channels and HERG channel at the concentrations (100 μM) where its inhibition on N-type Ca^{2+} channel currents occurs. These characteristics also indicate that C101 small molecular non-steroid blockers of N-type Ca^{2+} may have advantages in the clinical field as an analgesic candidate.

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