Full Paper

The M-Channel Blocker Linopirdine Is an Agonist of the Capsaicin Receptor TRPV1

Cristian Neacsu1 and Alexandru Babes1,*

1Department of Anatomy, Physiology and Biophysics, Faculty of Biology, University of Bucharest, Splaiul Independentei 91-95, Bucharest 050095, Romania

Received June 29, 2010; Accepted September 21, 2010

Abstract. Linopirdine is a well known blocker of voltage-gated potassium channels from the Kv7 (or KCNQ) family that generate the so called M current in mammalian neurons. Kv7 subunits are also expressed in pain-sensing neurons in dorsal root ganglia, in which they modulate neuronal excitability. In this study we demonstrate that linopirdine acts as an agonist of TRPV1 (transient receptor potential vanilloid type 1), another ion channel expressed in nociceptors and involved in pain signaling. Linopirdine induces increases in intracellular calcium concentration in human embryonic kidney 293 (HEK293) cells expressing TRPV1, but not TRPA1 and TRPM8 or in wild-type HEK293 cells. Linopirdine also activates an inward current in TRPV1-expressing HEK293 cells that is almost completely blocked by the selective TRPV1 antagonist capsazepine. At low concentrations linopirdine sensitizes both recombinant and native TRPV1 channels to heat, in a manner that is not prevented by the Kv7-channel opener flupirtine. Taken together, these results indicate that linopirdine exerts an excitatory action on mammalian nociceptors not only through inhibition of the M current but also through activation of the capsaicin receptor TRPV1.

Keywords: pain, sensory neuron, dorsal root ganglion (DRG), transient receptor potential (TRP) channel, KCNQ

Introduction

The Kv7 (or KCNQ, or M channels) channel family consists of five different subunits (Kv7.1 – 7.5), four of which (Kv7.2 – 7.5) are expressed in the nervous system and involved in mediating the neuronal M current (1, 2). The M current has been identified in many neuronal cell types, including hippocampal pyramidal neurons, sympathetic ganglion neurons, and dorsal root ganglion (DRG) neurons, including neurons involved in pain signaling (nociceptors) (3). Given their important role in modulating neuronal excitability, much attention has recently been devoted to the investigation of pharmacological agents with either agonistic or antagonistic action on Kv7 channels. Among the latter, one of the most widely used M-channel blockers is linopirdine [DuP996, 1,3-dihydro-1-phenyl-3,3-bis(4-pyridinylmethyl)-2H-indol-2-one]. Linopirdine inhibits native M currents in a variety of neuronal cell types (3 – 8) and also recombinant Kv7 homomers or heteromers (9 – 11), with an IC50 in the micromolar range. An even more potent M-current blocker is the linopirdine analogue XE-991 [10,10-bis(4-pyridinylmethyl)-9(10H)-anthracenone] (10, 12). Kv7 subunits are expressed in DRG neurons (including capsaicin-sensitive nociceptors) and M currents have been recorded from these cells and shown to modulate their excitability (3). Moreover, the M-channel opener retigabine was shown to reduce pain behavior in an animal model of inflammatory chronic pain, and as a consequence, Kv7 channels have been highlighted as attractive pharmacological targets for pain therapy (3). Given this involvement of Kv7 channels in acute and chronic pain signaling, we believed that it would be of interest to investigate the effect of linopirdine on an ion channel that is considered as a hallmark of nociceptive neurons, TRPV1 (transient receptor potential vanilloid subtype 1).

TRPV1 is a polymodal receptor expressed in primary sensory neurons of the DRG and trigeminal ganglia (TG), but also in other neuronal and non-neuronal tissues (13,
Linopirdine Activates TRPV1

14). TRPV1 is activated by noxious heat (with a temperature threshold of approximately 42°C), tissue acidosis, endogenous ligands such as anandamide, and also capsaicin, the active compound in chili peppers, and plays a crucial role in the development of inflammatory heat hyperalgesia (15, 16). Our results indicate that linopirdine acts as an agonist of native and recombinant TRPV1, albeit at concentrations substantially higher than those required to inhibit the M current. However, at low concentrations, close to those usually used to probe its pharmacological action on Kv7 channels, linopirdine is able to induce significant sensitization to heat in both cultured DRG neurons and TRPV1-expressing human embryonic kidney 293 (HEK293) cells, in a manner that is independent of Kv7 channels and probably reflects its direct action on TRPV1.

Materials and Methods

Heterologous expression of rTRPV1 in HEK293 cells

Recombinant rat TRPV1, TRPM8, and TRPA1 [kind gifts from Dr. Michael Caterina (Department of Biological Chemistry, Johns Hopkins University School of Medicine, USA) and Dr. David Julius (Department of Physiology, University of California, USA)] were transiently transfected into HEK293 cells using the calcium phosphate precipitation method. Identification of successfully transfected cells in patch clamp experiments was carried out by co-transfection with green fluorescent protein (GFP) and visualization under fluorescent conditions. HEK293 cells were maintained in Dulbecco’s Modified Eagle’s Medium (DMEM) / Ham’s F12 Medium (1:1 mixture), supplemented with 1% penicillin/streptomycin, 10% fetal bovine serum (FBS), and 1% glutamine at 37°C and 5% CO2. After trituration the dissociated cells were plated onto Petri dishes or glass coverslips (see above) and cultured (37°C and 5% CO2) in DMEM / Ham’s F12 Medium (1:1) supplemented with 10% horse serum and gentamicin (50 μg/ml). All chemicals for cell culture were from Sigma (St. Louis, MO, USA), unless otherwise mentioned.

Calcium imaging experiments

Nonratiometric calcium imaging experiments were performed as described elsewhere (18, 19). Briefly, coverslips with attached cells were incubated for 30 min at 37°C in standard extracellular solution (see Solutions and chemicals, below) containing 2 μM Calcium Green-1 AM and 0.02% Pluronic F-127 (both from Invitrogen) and left to recover for another 30 min before recording. Temperature was controlled by local superfusion with the Peltier-based system described elsewhere (20). The temperature experienced by the cells was measured after the experiment by repeating the thermal stimulus and using a T-type thermocouple (1T-1E; Physitemp, Clifton, NJ, USA) placed where the cells had been. The stimulus consisted of a 15-s heat ramp from 32°C to 43°C. The fluorescence change in response to thermal and chemical stimuli was monitored for each cell in the recording field and was quantified as the ratio between the maximal fluorescence (ΔF/F0 or ‘amplitude’).

Patch clamp experiments

Patch clamp recordings were made in both the whole-cell and the outside-out configurations using borosilicate glass pipettes (GC150TF; Harvard Apparatus, Holliston, MA, USA), heat-polished to a resistance of 2 – 4 MΩ. Currents were recorded with an EPC-7 amplifier (HEKA Electronic Dr. Schulze GmbH, Lambrecht/Pfalz, Germany), filtered at 3 kHz and digitized with a LabMaster 160 kHz DMA interface (Scientific Solutions, Mentor, OH, USA), using software written by Dr. Gordon Reid (g.reid@ucc.ie). The experiments were carried out in calcium-free conditions to minimize TRPV1 desensitization induced by calcium entry. Data are presented as the mean ± S.E.M. Analysis (including one-way ANOVA with repeated measures and two tailed paired Student’s t-test) was performed with the Origin 6.0 software (OriginLab Corporation, Northampton, MA, USA). The χ² test was performed using Statistica 7.1 (StatSoft, Inc., Tusla, OK, USA). A value of P < 0.05 was considered statistically significant. The concentration dependence of the linopirdine-evoked current in rTRPV1-HEK293 cells (Fig. 2D) was fitted with a Hill function:
\[ y = A_1 + \frac{A_2 - A_1}{1 + \left(\frac{x}{EC_{50}}\right)^n} \]

, where \( x \) is the concentration, \( EC_{50} \) is the concentration for half-maximal activation, and \( n \) is the Hill coefficient.

**Solutions and chemicals**

The IncMix solution for DRG incubation contained: 155 mM NaCl, 1.5 mM K2HPO4, 5.6 mM HEPES, 4.8 mM Na-HEPES, 5 mM glucose. The antibiotic gentamicin was added to 50 μg/ml. The standard extracellular solution contained 140 mM NaCl, 4 mM KCl, 2 mM CaCl2, 1 mM MgCl2, 10 mM HEPES, 4.54 mM NaOH, 5 mM glucose; pH 7.4 at 25°C. The high-KCl solution contained 94 mM NaCl, 50 mM KCl, 2 mM CaCl2, 1 mM MgCl2, 10 mM HEPES, 4.54 mM NaOH, 5 mM glucose, pH 7.4 at 25°C. The extracellular calcium-free solution used in patch-clamp experiments contained 140 mM NaCl, 4 mM KCl, 3 mM MgCl2, 10 mM HEPES, 4.54 mM NaOH, 5 mM glucose; pH 7.4 at 25°C. The pipette solution contained 135 mM KCl, 1.6 mM MgCl2, 2 mM EGTA, 2.5 mM Mg-ATP, 10 mM HEPES, with pH adjusted to 7.3 by adding NaOH.

Drugs were added from the following stock solutions: linopirdine, 200 mM in ethanol; capsaicin, 5 mM in ethanol; allyl isothiocyanate (AITC, or mustard oil), 200 mM in DMSO; (−)-menthol, 200 mM in ethanol; capsazepine, 20 mM in DMSO. All the chemicals were from Sigma. The same volume of solvent was added to the standard extracellular solution to prevent any effects induced by the vehicle.

**Results**

**Linopirdine evoked increases in intracellular calcium concentration in HEK293 cells transiently transfected with rTRPV1 but not rTRPA1 or rTRPM8**

HEK293 cells were transiently transfected with recombinant rat thermoTRP (thermally gated transient receptor potential) channels: rTRPV1, the capsaicin and heat receptor; rTRPA1, a polymodal receptor activated by noxious cold and mustard oil; and rTRPM8, an ion channel activated by mild cooling and menthol and involved in cold sensing. Linopirdine (at 200 μM) evoked substantial increases in the intracellular calcium concentration ([Ca2+]i) in cells expressing rTRPV1, which were also activated by the specific TRPV1 agonist capsaicin (300 nM) (Fig. 1A). At higher concentrations (0.5 mM), linopirdine failed to elicit any change in [Ca2+]i in wild-type HEK293 cells or in HEK293 cells expressing recombinant rTRPA1 or rTRPM8, demonstrating the specificity of linopirdine action on rTRPV1. Functional expression of rTRPA1 and rTRPM8 was verified by the activation of transfected HEK293 cells by the specific agonists allyl isothiocyanate (AITC or mustard oil, 200 μM) and (−)-menthol (200 μM), respectively (Fig. 1A). At the concentrations mentioned above, AITC, menthol, and capsaicin failed to activate wild-type HEK293 cells (Fig. 1A).

**Linopirdine at low concentrations sensitized rTRPV1-expressing HEK293 cells to heat**

Five heat ramps (from 32°C to 43°C) at 4-min interval followed by a challenge with capsaicin (300 nM) were applied to HEK293 cells transiently transfected with rTRPV1 (rTRPV1-HEK293 cells) and loaded with the fluorescent calcium indicator Calcium Green-1 AM (see Materials and Methods). Successfully transfected cells were identified by their response to capsaicin. A large proportion of capsaicin-sensitive rTRPV1-HEK293 cells were also activated by heat ramps to 43°C (60%). Capsaicin-insensitive rTRPV1-HEK293 cells were not activated by this thermal stimulus, and the same was true for wild-type HEK293 cells. In a separate set of experiments, linopirdine (10 μM) was pre-applied 1 min before and during the 4th heat stimulus, which resulted in a sensitization of the response to heat. The amplitude of the response to heat in the presence of linopirdine was 0.29 ± 0.03, compared to 0.23 ± 0.03 in control conditions, n = 27, one-way ANOVA with repeated measures, P < 0.01, resulting in a sensitization of the response of approximately 26% (Fig. 1: B, C).

**Linopirdine induced inward currents in HEK293 cells transiently transfected with rTRPV1**

In a total of 26 rTRPV1-HEK293 cells voltage-clamped at −60 mV, linopirdine (at concentrations ranging from 10 to 400 μM) evoked inward currents, and all 26 cells also responded to the application of capsaicin (300 nM) (Fig. 2A). The co-expression of linopirdine and capsaicin sensitivities in rTRPV1-expressing HEK293 cells was 100%. Linopirdine-evoked currents were outwardly rectifying and had a reversal potential close to 0 mV (Fig. 2B), very much like currents elicited by other TRPV1 agonists such as capsaicin and camphor (21). The selective TRPV1-channel antagonist capsazepine strongly and reversibly inhibited linopirdine-evoked inward currents in rTRPV1-HEK293 cells voltage-clamped at −60 mV (by more than 95%, from 768 ± 233 pA in control conditions to 22 ± 9 pA in the presence of capsazepine, n = 9, two tailed Student’s paired t-test, P < 0.01; Fig. 2C). The activation of TRPV1 by linopirdine was concentration-dependent, with an EC50 of 115 μM (Fig. 2D) and also occurred in excised
Linopirdine Activates TRPV1

outside-out patches (n = 3, Fig. 2E). The linopirdine-evoked current in outside-out patches excised from rTRPV1-HEK293 cells and voltage-clamped at −60 mV was strongly and reversibly inhibited by capsazepine (5 μM, Fig. 2F).

**Linopirdine induced inward currents in cultured rat DRG neurons**

Linopirdine (200 μM) and capsaicin (2 μM) were successively applied to cultured DRG neurons from the rat voltage-clamped at −60 mV. No inward current was induced by linopirdine in 7 capsaicin-insensitive DRG neurons. By contrast, linopirdine evoked small inward currents (212 ± 51 pA, range 106 to 347 pA) in 4 out of 8 capsaicin-sensitive neurons (Fig. 3A).

**Linopirdine sensitizes rat cultured dorsal root ganglion neurons to heat but has no effect on changes in [Ca2+]i induced by KCl**

In order to investigate the effects of linopirdine (10 μM) on capsaicin-sensitive (and thus TRPV1-expressing) rat DRG neurons, we used non-ratiometric calcium imaging to monitor heat-induced changes in [Ca2+]i. Control conditions consisted of the application of 5 heat ramps.
(from 32°C to 43°C) at 4-min interval, followed by the application of capsaicin (2 μM, 20 s). In another set of experiments, linopirdine (10 μM) was pre-applied 1 min before and during the fourth heat stimulus. At this sub-maximal concentration, linopirdine evoked increases in [Ca²⁺] in few cultured DRG neurons (9 of 187, 5%; all 9 linopirdine-sensitive neurons also responded to capsaicin). Heat sensitivity in cultured DRG neurons is highly correlated with capsaicin sensitivity, at least at the whole cell level (18). Of all heat-sensitive neurons ($\Delta F/F_0 > 0.1$ in response to the first heat ramp) that we encountered, the vast majority also responded to capsaicin (63 of 65, 97%). Given the agonistic action of linopirdine on TRPV1, we restricted our analysis to the subpopulation of capsaicin-sensitive neurons that did not respond to linopirdine (107/187). It should be nevertheless mentioned that linopirdine did not activate any capsaicin-insensitive neuron, nor did it sensitize the response to heat in this group of cells.

The heat response in the presence of linopirdine was
Linopirdine Activates TRPV1

strongly sensitized (by almost 100%) in capsaicin-sensitive neurons. While in control conditions there was no statistical difference between the increase in \([\text{Ca}^{2+}]_i\) induced by the 2nd, 3rd, 4th, and 5th heat stimuli \((n = 48)\), the response elicited by the heat stimulus in the presence of linopirdine was significantly increased \((\Delta \text{F}/\text{F}_0\) was increased by 90%, from 0.11 ± 0.01 to 0.21 ± 0.02, \(n = 107\), one-way ANOVA with repeated measures, \(P < 0.001\); Fig. 3: B, C). The increase in the amplitude of the response to heat was accompanied by a strong increase in the fraction of heat-responsive neurons. Thus, while in control conditions 35% \((17/48)\) of capsaicin-sensitive neurons responded to the 4th heat stimulus, in the presence of linopirdine this fraction was increased to 60% \((64/107, \chi^2\) test, \(P < 0.01)\).

The sensitizing effect of linopirdine was not mediated
by closure of the M-current channels. Pre-application of the Kv7-channel activator flupirtine (10 μM) prevented neither the excitatory effect of linopirdine [4 of 40 neurons (10%) responded to linopirdine in the presence of flupirtine], nor the sensitization of heat responses in capsaicin-sensitive neurons. In the presence of flupirtine, linopirdine increased the response to heat to a similar extent as linopirdine alone (92%, from 0.12 ± 0.02 to 0.23 ± 0.04, n = 30, one-way ANOVA with repeated measures, P < 0.001; Fig. 3: B, C). Flupirtine (10 μM) alone had no effect on the response to heat in cultured rat DRG neurons (data not shown).

Finally, to investigate whether the sensitizing effect of linopirdine is limited to stimulation by heat or is a general feature of any depolarizing stimulus, we recorded changes in [Ca2+]i evoked by repeated applications of a 50 mM KCl solution (5 applications of 10 s each, at 4-min interval), followed by 2 μM capsaicin. Linopirdine was applied 1 min before and during the 4th challenge with KCl. The 50 mM-KCl solution evoked large, reproducible increases in [Ca2+]i in both capsaicin-sensitive and capsaicin-insensitive neurons (Fig. 4: A, B). In these conditions linopirdine had no significant effect on the magnitude of the response to KCl (n = 25 linopirdine-treated neurons, n = 21 control neurons; Fig. 4: A, B), which suggests that its sensitizing effect on the heat-evoked responses cannot be attributed to an overall increase in cellular excitability, as would be expected following the inhibition of a non-inactivating K+ conductance.

**Discussion**

Linopirdine was already known to have pharmacological features that were not restricted to its effect on Kv7 potassium channels. Thus, one study reported that linopirdine inhibits nicotinic acetylcholine receptors (nAChR) and GABA-induced Cl− currents in cultured sympathetic superior cervical ganglion (SCG) neurons (5). Later work demonstrated the blocking effect of linopirdine on recombinant α9α10 nAChR as well as on nAChR natively expressed in cochlear hair cells from the rat (22). The IC50 for the effect of linopirdine on nAChR was in the low micromolar range, close to the concentration at which linopirdine exerts its inhibitory action on Kv7 channels, while the inhibition of GABA_A-mediated currents occurred at higher concentrations. Work on cultured hippocampal neurons also demonstrated that linopirdine blocks Ca2+-activated K+ currents and also weakly inhibits both delayed rectifier and leak K+ currents (8). While the blocking effects of linopirdine were thus shown to lack complete selectivity, no agonistic action of this drug has been reported so far to our knowledge.

Our data shows that linopirdine induced a robust increase in [Ca2+]i in HEK293 cells transiently transfected with TRPV1 (rTRPV1-HEK293 cells), but not TRPM8 or TRPA1, and also not in wild-type untransfected

---

**Fig. 4.** Linopirdine has no effect on calcium transients evoked by 50 mM KCl in cultured rat DRG neurons. A: Upper traces: examples of responses elicited by repetitive applications of a high KCl solution (50 mM) in a capsaicin-insensitive (a) and a capsaicin-sensitive (b) DRG neuron in control conditions. Lower traces: linopirdine (Lino, 10 μM) does not sensitize the response to 50 mM KCl in a capsaicin-insensitive (c) or in a capsaicin-sensitive (d) neuron (a.u. = arbitrary fluorescence units). B: Statistical analysis of the experiments illustrated in panel A. Responses to KCl in control (open squares) and linopirdine-treated (filled circles) neurons are represented as the mean ± S.E.M.
HEK293 cells (Fig. 1A). Moreover, whole-cell linopirdine-evoked currents were recorded from the same rTRPV1-HEK293 cells voltage-clamped at −60 mV in both the whole-cell and the outside-out recording configurations (Fig. 2: A, E), indicating that this agonistic action is most likely due to a direct effect of the drug on TRPV1 channels and not mediated by release of intracellular second messengers. These currents were almost completely and reversibly inhibited by the selective TRPV1-channel antagonist capsazepine (Fig. 2: C, F). Similar to capsaicin- and camphor-evoked currents through TRPV1 (13, 21), linopirdine-induced currents in rTRPV1-HEK293 cells were outwardly rectifying and had a reversal potential close to 0 mV. The amplitude of the linopirdine-activated current was concentration-dependent, with an EC₅₀ of approximately 10 μM, demonstrating a lower potency of the drug compared to its action on Kv7 channels (Fig. 2B). Moreover, the currents activated by linopirdine were rather small compared to those evoked by 300 nM capsaicin (a concentration below the EC₅₀ for capsaicin), indicating a substantially lower efficacy of linopirdine compared to capsaicin (Fig. 2: A, D). There is no reason to believe that linopirdine effects on rTRPV1-HEK293 cells could be mediated by its action on the M current, which is not present in HEK293 cells (9, 23–25).

We then investigated whether linopirdine at low concentrations was able to sensitize TRPV1 to one of its physiological agonists (heat), and for this purpose we chose a concentration of 10 μM, in the range that is often used to block M currents in neuronal tissues, including DRG (3, 4, 8, 26, 27). At this low concentration, linopirdine sensitized heat-induced increases in [Ca²⁺], in rTRPV1-HEK293 cells by 26% (Fig. 1: B, C). These changes in [Ca²⁺], evoked by heat in rTRPV1-HEK293 cells appear to be due to activation of TRPV1, as they do not occur in wild-type HEK293 cells.

The effects of linopirdine on recombinant TRPV1 should be reflected in the action of the drug on capsaicin-sensitive cultured rat DRG neurons. Linopirdine (200 μM) was able to evoke small inward currents that were restricted to the subpopulation of capsaicin-sensitive DRG neurons (Fig. 3A). Moreover, application of a low concentration of linopirdine (10 μM) resulted in a substantial sensitization (close to 100%) of the response to heat in capsaicin-sensitive neurons, that was not abolished when linopirdine was applied in the presence of the M current agonist flupirtine (10 μM) (Fig. 3: B, C), suggesting that (at least in part) this sensitization is mediated by a direct action of linopirdine on TRPV1. Moreover, if sensitization to heat were a consequence of the inhibition of the M current by linopirdine, the drug should have the same effect on the neuronal response evoked by any depolarizing stimulus strong enough to activate Kv7 channels. We have nevertheless shown that this is not the case, as linopirdine has no effect on the increase in [Ca²⁺], induced by the application of a high KCl solution (Fig. 4). Interestingly, the sensitizing effect of linopirdine was much stronger on native (90%) than on recombinant TRPV1 (26%). The reason for this difference is not clear, but may be related to the substantially higher level of expression of the channel in the heterologous expression system compared to the DRG or to the lack of an interacting protein that could increase the linopirdine sensitivity of native TRPV1. Moreover, different processes are involved in calcium transients in DRG neurons and rTRPV1-HEK293 cells, as electrical activity and activation of voltage-gated calcium channels contribute in the former case, but not the latter. However, as shown in Fig. 4, linopirdine does not appear to act by sensitizing voltage-gated calcium channels.

Taken together, our data indicate that the M-channel blocker linopirdine activates and sensitizes native and recombinant TRPV1 channels to heat. This new knowledge should lead to a more careful interpretation of the effects of linopirdine on the excitability of neurons that are known to express TRPV1, in particular primary nociceptors from the DRG or TG; while using linopirdine to probe for the involvement of the M current in pain signaling, either in vitro in DRG cultures or skin-nerve preparations or in vivo in animal models of chronic pain, its agonistic action on TRPV1 channels should also be taken into consideration for an accurate interpretation of the results.

Acknowledgments

We thank Dr. Cristian Ciobanu and Ms. Oana Toader for helpful discussions and comments on the manuscript. This project was funded by grant PNII Idei 164/2007 awarded by the Romanian Research Council (CNCSIS) to A.B. C.N. was supported by grant POSDRU 88/1.5/S/61150.

References

5 Lamas JA, Selyanko AA, Brown DA. Effects of a cognition-


