The antipsychotic drug loxapine is an opener of the Na\(^+\)-activated potassium channel Slack (slo2.2).

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Abstract

Sodium-activated (K$_{Na}$) channels have been suggested to set the resting potential, modulate slow after-hyper polarizations, and control bursting behaviour or spike frequency adaptation (Bhattacharjee and Kaczmarek, 2005). One of the genes that encodes K$_{Na}$ channels is called Slack ($Kcnt1$, Slo2.2). Recent studies have found that Slack channels are highly expressed in nociceptive dorsal root ganglionic (DRG) neurons and were shown to modulate their firing frequency (Nuwer, et al., 2010). Therefore, Slack channel openers are of significant interest as putative analgesic drugs. Accordingly, we screened the ‘library of pharmacologically active compounds’ (LOPAC) on recombinant human Slack channels expressed in CHO cells, using rubidium (Rb+) efflux measurement via atomic absorption spectrometry (AAS). Riluzole at 500 µM was used as a reference agonist. The antipsychotic drug loxapine and the anthelmintic niclosamide were both found to activate Slack channels and this was confirmed using manual patch-clamp analyses (EC$_{50}$: 4.4 and 2.9 µM, respectively). Other psychotropic drugs structurally related to loxapine were also evaluated by patch-clamp but none was found to be as active as loxapine. Loxapine properties were confirmed at the single channel level on recombinant rat Slack channels. In DRG neurons, loxapine was also found to behave as an opener of native K$_{Na}$ channels and to increase the rheobase of action potential. This study identifies new K$_{Na}$ channel pharmacological tools, which will be useful for further Slack channel investigations.
Introduction

Slack channels belong to the Ca\textsuperscript{2+}-activated K\textsuperscript{+} channel family, because they contain tandem “regulators of K\textsuperscript{+} conductance” (RCK) domains (Jiang, et al., 2002). However, Slack channels and the closely related Slick (Kcnt2, Slo2.1) channel, are activated by Na\textsuperscript{+} and Cl\textsuperscript{−}, not Ca\textsuperscript{2+}. Recent mutational analyses have indicated that Na\textsuperscript{+} binding and gating occurs in RCK domain 2 (Zhang, et al., 2010). Slack channels are widely expressed throughout the rat central nervous system (CNS), including the substantia nigra (SN) but are also abundantly expressed in peripheral dorsal root ganglion (DRG) neurons (Bhattacharjee, et al., 2002; Tamsett, et al., 2009). Because Slack channels are activated by both voltage and cytosolic factors such as intracellular Na\textsuperscript{+} and Cl\textsuperscript{−}, they are ideal effectors of negative feedback during neuronal excitation. Accordingly, it has been shown in lamprey spinal cord neurons that AMPA receptor activation was linked via Na\textsuperscript{+}-transients to an increase in K\textsubscript{Na} current amplitude (Nanou, et al., 2008). Similarly tetrodotoxin (TTX) has been shown to strongly decrease the outward K\textsuperscript{+} current evoked by depolarization in rat olfactory neurons and a TTX-sensitive K\textsuperscript{+} current was eliminated by a Slack-siRNA (Budelli, et al., 2009). In locus coeruleus, the glutamate-induced post-activation inhibition was found to be mediated by AMPA/kainate receptors and bithionol-sensitive K\textsubscript{Na} currents (Zamalloa, et al., 2009). These findings suggest that Slack activation may limit abnormal neuronal activity during pathological conditions such as epilepsy or pain. With the exception of bithionol, there are few pharmacological tools available to study the functional role of K\textsubscript{Na} channels in neurons, particularly in a pathological context. In order to evaluate the therapeutic potential of K\textsubscript{Na} channel openers, we sought to identify novel lead compounds that activate Slack channels.
The screening of ion channels at medium throughput has been recently facilitated by the availability of automated patch-clamp devices. However, their use remains rather expensive when considering medium-sized libraries (i.e. greater than 10,000 compounds) and not fully appropriate when addressing compounds or channels that could give rise to background currents, which may be merged with leakage currents. Therefore, we have designed a functional assay assessing Rb⁺ efflux via atomic absorption spectrometry (AAS) in CHO cells stably expressing recombinant human Slack channels (CHO-hSlack). Indeed, this technique has already been proven rather efficient and of low cost during K⁺ channel screening (Terstappen, 2004). Riluzole, previously found to be a Slack opener (internal unpublished data), was used as a reference compound and the LOPAC 1280 has been screened as a test validation.

By using this screening approach, we now identify loxapine and niclosamide as novel Slack channel openers. Patch-clamp experiments, involving both recombinant Slack and native KNa channels were used to confirm these findings.
Methods

Drugs

Patch-clamp experiments
Riluzole, loxapine, niclosamide, bithionol and amoxapine were purchased from Sigma–Aldrich (Saint Quentin Fallavier, France).
Quetiapine, clozapine and olanzapine were purchased from Interchim.
Drugs were diluted as 25 mM stock solutions in dimethyle sulfoxide (DMSO) whose concentration did not exceed 0.08% in the final solutions used.

Atomic absorption spectrometry experiments
The reference compound riluzole hydrochloride was purchased from TOCRIS and diluted at 100 mM in DMSO whose concentration did not exceed 1% in the final solutions used.
The LOPAC was purchased from Sigma-Aldrich.
Animals

DRG neurons were isolated and cultured as previously described (Nuwer, et al., 2010). Briefly, DRG were dissected from E15 embryos of Sprague-Dawley rats. The ganglia were dissociated in 2.5 mg/ml trypsin (Invitrogen) for 40 min. Neurons were plated on poly-D-lysine (Sigma) and laminin (Invitrogen) coated coverslips, and maintained on serum-free medium containing 100 ng/ml nerve growth factor (Harlan Bioproducts). One day after dissection neurons were treated with 1 µM cytosine-D-arabinofuranoside (Sigma), an inhibitor of DNA synthesis to inhibit growth of non-neuronal cells, for 2 days. Cells were allowed to recover for 2 days prior to manipulation. All experimental protocols were approved by the University at Buffalo, Institutional Animal Care and Use Committee, and conformed to NIH guidelines.

Cell lines

A stable Chinese Hamster Ovary (CHO) cell line expressing the human Slack channel (CHO-hSlack) was generated by the Lead Identification Technologies Department of Sanofi-Aventis Research and Development (Frankfurt, Germany) from the parental Flp-In-T-Rex™ CHO host cell line. Cells were co-transfected with the human KCNT1 gene (Potassium channel family, calcium activated subfamily T member 1, accession number NM_020822) inserted into the pCDNA5-FRT-TO plasmid and with the pOG44 plasmid containing the Flp recombinase. Using a similar protocol, a CHO cell line stably expressing human MaxiK channels (encoded by the KCNMA1 gene, accession number NM_002247) together with KCNMB4 subunit (accession number NM_014505.1) has also been produced using an IRES structure (CHO-MaxiK).
CHO-hSlack, CHO-MaxiK and parental cells Flp-In-T-Rex™ cells were grown in a culture medium composed of DMEM-F12 (Dulbecco’s Modified Eagle’s Medium) (Gibco) supplemented with 10% Fetal Bovine Serum, Blasticidin, 30µg/ml, Hygromycin 300 µg/ml or Zeocin 100 µg/ml, respectively (Invitrogen) and maintained in an atmosphere of 95% air/5% CO2 at 37°C. They were dissociated for passage by using Cell Dissociation Buffer Enzyme Free PBS Based (Invitrogen).

For single channel studies we used a human embryonic kidney (HEK-293) cells that stably express rat Slack channels (Yang, et al., 2006) from Yale University. Cells were cultured on 35 mm dishes in modified low Na+ DMEM Medium containing 10% fetal bovine serum and penicillin-streptomycin (Invitrogen) and maintained in a 5% CO₂ incubator at 37°C.

**Atomic absorption spectrometry studies**

Compounds and reagents were diluted in HBSS (Hanks Balance Salt Solution) that contained (in mM) 5.4 KCl, 0.44 KH₂PO₄, 4.17 NaHCO₃, 137.9 NaCl, 0.34 Na₂HPO₄ , 5.5 Glucose and 2 hydroxyethylpiperazine-N-2-ethanesulfonic acid (HEPES), pH 7.4.

The day before the experiment, CHO-Slack cells were seeded at the density of 2x10⁴ cells/well in poly-D-lysine coated 384 well-plates and incubated for 24 hours in the culture medium containing 20 mM of Rubidium (Rb⁺) and 2 µg/ml of tetracycline (Sigma) (final volume: 40 µl/well). The day of the experiment, the cells were washed three times with HBSS, and then incubated during 20 minutes at room temperature in HBSS containing 30 µM of the tested compounds (final volume: 100 µl). Test compounds were prepared in 100% DMSO and diluted to the desired concentration in HBSS. After 20 minutes, the supernatant was pipetted and cells were lysed using Triton 1%. The rubidium contents of the cells supernatant ([Rb⁺]sup) and lysate ([Rb⁺]lys)
were determined using an ICR 8000 atomic absorption spectrometer (Aurora Biomed, Van Couver) (Gill, et al., 2003; Parihar, et al., 2003). Calibration of the instrument was performed prior each experiment, using increasing Rb⁺ concentrations.

**Electrophysiological studies**

Whole cell patch-clamp in CHO-hSlack cell line.

For whole-cell patch-clamp experiments, CHO-hSlack or CHO-MaxiK cells were seeded on glass coverslips and incubated for 20-24h in cultivation medium containing 1µg/ml of the inducing agent doxycycline (BD Biosciences). Experimental chambers (RC-26-GLP, Warner Instruments, Hamden, USA) containing seeded coverslips, were placed on the stage of an inverted microscope (IMT2, Olympus, France) equipped with Hoffman optics (Modulation Contrast, New York, NY) and the cells viewed at a total magnification of 400 X. A gravity fed perfusion valve control system was used (VC-66CST, Warner Instruments, Hamden, USA) driven by Clampex (9.2 version, Molecular Devices, Sunnyvale, USA) connected to a 12 ways manifold ended by a glass tube (500 µm opening) placed at less than 1 mm of the recorded cells. Pipettes were pulled from thick-walled borosilicate glass capillaries (Harvard Apparatus, Edenbridge, UK) on an horizontal two-stages puller (P97, Sutter Instruments, USA) and had a resistance of 5 to 10 MΩ when filled with the pipette solution (see below). Pipettes were brought into contact with the cells with a three-dimensional piezoelectric micromanipulator (Sutter MP 285 Dipsi Industrie, Chatillon, France). Whole-cell currents were recorded with a Multiclamp 700B amplifier (Molecular Devices, Sunnyvale, USA) driven by MultiClamp2.1 software.

The standard extracellular solution contained (in mM, unless specified otherwise): 147 NaCl, 5 KCl, 2 CaCl₂, 1 MgCl₂, 10 HEPES. pH was set to 7.4 with NaOH 1 M. For Slack
current recordings, the pipette was filled with an intracellular pipette medium, containing (in mM, unless specified otherwise): 110 K-aspartate, 5 ethyleneglycol-bis-(2-aminoethyl)-tetraacetic acid (EGTA), 10 KCl, 10 NaCl, 10 Hepes, 1 MgCl₂, 1 CaCl₂. pH was set to 7.2 with KOH (1M). For MaxiK current recordings, the composition of intracellular pipette medium was (in mM, unless specified otherwise): 145 KCl, 2 MgCl₂, 10 EGTA, 5 ATP, 10 HEPES. pH was set 7.4 with KOH.

Excised-patch channel recordings
For inside-out patch-clamp recordings, the pipette solution contained (in mM, unless specified otherwise) 10 NaCl, 130 KCl, 10 HEPES, 5 EGTA, 1 MgCl₂, and 1 tetraethylammonium chloride (TEA-Cl). The bath solution contained 130 KCl, 10 NaCl, 10 HEPES, and 5 EGTA. The pH of all solutions was adjusted to 7.3 with KOH. Inside-out patches were perfused with the SmartSquirt® small volume delivery system (Automate Scientific, Berkeley, CA) using a 100 µm perfusion tip with a flow rate of 0.01mL/minute and varying concentrations of loxapine were used in the perfusion.

Action potential recordings in DRG neurons
All experiments were performed at room temperature. Whole-cell current-clamp recordings were made with a Multiclamp 700B, stored digitally using a Digidata interface (1322 series), and analyzed off-line with Clampfit software (Molecular Devices, Sunnyvale, USA). Electrode impedance was 3-5 MΩ when filled with saline containing (in mM) 124 KGluconate 2 MgCl₂, 13.2 NaCl, 1 EGTA, 10 HEPES, 4 Mg-ATP, 0.3 Na-GTP, pH 7.2. The bath solution contained (in mM) 140 NaCl, 5.4 KCl, 1 CaCl₂, 1 MgCl₂, 15.6 HEPES and 10 glucose, pH 7.4. Neurons were accepted for study only when they exhibited a resting membrane potential (RMP) more negative than -40 mV and input
resistance greater than 100 MΩ after whole-cell mode was established. Depolarizing current steps in increments of 10 pA from -10 to 200 pA (20 ms duration) were used to measure the rheobase.

**Data analysis**

Atomic absorption spectrometry studies.

The Rb⁺ efflux ratio was calculated as follows: \( \text{Rb⁺ efflux ratio} = \frac{[\text{Rb⁺}]_{\text{sup}}}{([\text{Rb⁺}]_{\text{sup}} + [\text{Rb⁺}]_{\text{lys}})} \). Dose-response experiments corresponding to eight concentrations were done in quadruplicate. The effects of compounds were expressed as a percentage of baseline (effect of 1% DMSO).

Dose-response curves and EC\(_{50}\) values were generated using a standard 4-parameter logistic nonlinear regression analysis by the mean of GraphPad Prism version 4.0 dedicated software (GraphPad Software, Inc 2003).

Patch-clamp experiments

For whole-cell current recordings, all statistical analyses were performed on the fold after log transformation to improve the heterogeneity of variance.

The geometric mean and the 95% confidence interval are used as descriptive statistics. Depending on the objective or the study, Student’s t-test was performed in order to show a difference of treatment when compared to their own control. One-way ANOVA were performed in order to show a difference between all compounds or concentrations (Newmann Keuls test) or equivalence versus a reference compound.

EC\(_{50}\) estimation was obtained by non-linear regression using SAS V9.1 with a 4-parameter logistic model on fold values.
Results

Identification of Slack openers using Rb⁺ efflux measurements.

For these experiments, riluzole at 500 µM was used as reference agonist. This compound was previously found serendipitously to be an effective Slack opener (internal unpublished data). The signal-to-noise ratio ranged between 2 and 2.5. Out of 1280 compounds of the LOPAC, 42 compounds were identified as putative Slack channel opener (hit ratio of 3%) (Fig 1A and B). After confirmation of their lack of activity at the parental CHO cell line, their EC₅₀ values were determined. Among these were the antipsychotic drug loxapine (EC₅₀: 3 µM) and the anthelmintic drug niclosamide (EC₅₀: 0.7 µM). For comparison, riluzole increased Rb⁺ efflux with an EC₅₀ value of 97 µM (Fig. 1C).

Patch-clamp experiments

Whole cell recordings in CHO-hSlack cell line

Using a voltage step protocol (from -90 to -20 mV for 200 ms), 10 µM of loxapine, 10 µM of niclosamide and 100 µM of riluzole were found to significantly increase Slack current amplitude by 10.7 [7.1; 16.1] (p<0.0001), 3.9 [2.1;7.1] (p=0.0015) and 6.5 [3.4;12.4] (p=0.0012) folds, respectively, when measured at the end of the depolarizing voltage step (Fig 2A and B). At 10 µM, loxapine was found significantly more efficient than niclosamide (P=0.0069). In the presence of loxapine and niclosamide, Slack current
activation kinetics appeared to be accelerated while in the presence of riluzole, the
typical slow activation of Slack current was still observed. Loxapine, niclosamide and
riluzole, obviously induced an increase in steady-state inward current amplitude,
measured at holding voltage.

Using a 2s ramp protocol, depolarizing CHO-hSlack cell membrane from -120 to + 40
mV, loxapine and niclosamide at 10 µM were found to increase Slack current amplitude
in both inward and outward directions. In the presence of these two compounds, the
current-voltage relationship becomes almost linear, even in the inward direction. This
observation suggests that these compounds were able to turn Slack channel into a kind
of background potassium channel, poorly sensitive to voltage (see below loxapine and
niclosamide-induced steady-state currents at -100 mV). Considering outward currents
measured at +40 mV, loxapine and niclosamide increased their amplitude by 4.8
[3.3;6.8] (p<0.0001) and 3.2 [2.1;5] (p=0.0001) folds, respectively. Riluzole was tested at
100 µM and increased Slack current in the outward direction by 3.7 [1.3;10.2] folds
(p=0.0242) at +40 mV, being less effective at increasing inward currents (Fig 2, C and
D). Using this ramp protocol, loxapine was also found to be the most efficient of the
three compounds.

As described above, loxapine and niclosamide were found to reproducibly induce inward
Slack currents at potentials more negative than K+ equilibrium potential (EK). We used
this effect to compare the 2 compounds and build the concentration-response curves. At
a holding voltage of -100 mV, loxapine induced strong inward currents in a
concentration-dependent manner, with a calculated EC50 value of 4.4 µM [2.7;7.2 ] and a
maximal increase in current amplitude of 8.9 [8.1;9.8] folds (Fig 3A and B). Even at the
low concentration of 0.3 µM, loxapine was found to significantly increase Slack steady-
state current (p=0.001). Niclosamide was found to be of similar potency with an EC50
value of 2.9 µM [1.3; 6.5] but less efficient with a maximal current increase of 3.0 [2.2;4.1] folds (Fig 3 C and D). As shown in Figure 4C, for high concentrations (10 and 30 µM) niclosamide effect had a propensity to rapidly decrease upon time, suggesting some kind of desensitizing or open channel blocking effect that was not observed with loxapine. At every concentration tested and confirming the results described above, loxapine was found to be significantly more efficient (about 2 folds) than niclosamide (P<0.001 at 3 and 10 µM, P<0.01 at 0.3 and 1 µM and p<0.05 at 30 µM).

In order to evaluate if the Slack opening properties of loxapine were linked to its typical tricyclic structure, the effects of structurally related antipsychotic or antidepressant drugs on steady-state Slack current were evaluated at 10 µM (olanzapine, quetiapine, clozapine, amoxapine). As shown in Fig 4, amoxapine and to a lesser extent quetiapine were the most efficient, inducing increases in Slack steady-state current amplitude of 3 and 2.2 folds, respectively. Clozapine and olanzapine were almost ineffective, suggesting that Slack channel activation is not a general property of tricyclic antipsychotic drugs.

Whole-cell recordings in CHO-MaxiK cell line.

Bithionol has been previously shown to be an opener of MaxiK channels (Yang et al., 2006) it was therefore of interest to compare loxapine and bithionol effects on MaxiK currents, evoked by depolarizing CHO-MaxiK cell from a holding voltage of -85 mV to 20 mV during 100ms (F = 0.1 Hz). Both compounds were superfused at 10 µM. As expected, bithionol behaved as a powerful and reversible MaxiK channel opener, increasing current amplitude by 11.5 [6.3;21] folds (p<0.0001). In contrast, loxapine was inactive (1.05 [0.96;1.15] fold) (Figure 5 A and B).
Effect of loxapine on recombinant rat Slack and native KNa channels expressed in HEK cells and cultured DRG neurons, respectively.

We evaluated the effects of loxapine directly on recombinant rat Slack channels by conducting excised inside-out patch-recordings from a rat Slack stable cell line (Yang 2006). We found that direct application of loxapine to patches clearly caused an increase in Slack channel activity that reversed after wash (n=11, p<0.01) (Fig. 6 A, B and C). We also found a time-dependent increase in channel activity during outside-out patch recordings (n=4, p<0.05 at t=3 min) (Fig. 6 D). The time-dependent effects suggest that loxapine does not act at the pore of the channel but likely acts at the RCK domains internally and affecting the gating of Slack. We also tested loxapine on native KNa channels recorded from cultured DRG neurons (Fig.7A and B). Embryonic DRG neurons express both Slack (Nuwer, et al., 2010) and Slick channel subunits (unpublished observations). In excised patch recordings, native KNa channels were also activated by loxapine in a reversible manner (n=4).

Effect of loxapine on neuronal excitability

We tested the threshold of action potential generation in neurons incubated with 10 μM loxapine. We found that loxapine caused a significant increase in (n=13, p<0.05) rheobase compared to neurons not treated DMSO only (Fig. 7 C and D). In addition, the resting potential of loxapine treated neurons was -57.9 +/- 2.8 mV vs control -51.9 +/- 3 mV (P<0.05). These data support the specific effects of loxapine on native KNa channels and suggest that loxapine can directly affect the excitability of neurons.

Discussion
To our knowledge, this is the first report of a screening assay development in a medium throughput format, targeting Slack channels. This assay, based on AAS, was performed under physiological intracellular [Na+] concentrations. It has been well demonstrated for several decades that radioactive Rb+ could be used as an analog for K+ in flux-based assays. However the use of radioactivity is a limitation for high capacity screening. More recently, AAS methods have been developed for measuring non radioactive Rb+ flux through several K+ channels including Kv1.3 (Gill, et al., 2007) KCNQ2/3 (Scott, et al., 2003), MaxiK (Parihar, et al., 2003) and hERG (Chaudhary, et al., 2006) channels. These results further confirm that Rb+ efflux measurement by AAS represents a reliable, safe and low-cost alternative for K+ channel screening purposes (Parihar, et al., 2003;Gill, et al., 2007;Terstappen, 2004). EC50's values determined using AAS and patch-clamp were in good keeping for loxapine (3 and 4.4 µM, respectively) but showed some discrepancies for niclosamide (0.7 and 2.9 µM, respectively). Similar findings have already been described for KCNQ2 openers, potencies varying from 2 to 10 folds, greater affinities being found by AAS as compared with patch-clamp data (Wang, et al., 2004).

This screening campaign using the LOPAC has led to the identification of loxapine, a first generation antipsychotic drug still prescribed, as an efficient opener of Slack channels. This new property has been confirmed on human and rat recombinant Slack channels and native KNa channels in DRG neurons. Slack studies have been hampered by the lack of efficient and selective pharmacological tools: at the present time, only bithionol, an anthelmintic veterinary drug, has been used as a reference Slack opener. However, this compound has also been reported to be an activator of MaxiK channels (Yang, et al., 2006). This property has not been found for loxapine, which should facilitate the functional study of native Slack channels in preparations expressing
both Slack and MaxiK channels. Among the two other Slack openers that we have identified (i.e. niclosamide and riluzole) it is noteworthy that niclosamide is another anthelmintic, still on the market. Slack-like channels are well expressed in nematodes such as *C. elegans* and this species is classically used for anthelmintic drugs screening (Kaewintajuk, et al., 2010; Wei, et al., 1996). It cannot be ruled out that the Slack channel opening properties of bithionol and niclosamide could be part of their anti-parasitic properties. This could be addressed by studying the toxicity of these 2 molecules in *C. elegans* mutants previously described to have strongly decreased Slo-2 mediated currents. (Yuan, et al., 2003). Clinically, the possible effects of niclosamide or bithionol on CNS excitability via Slack channels activation will not be relevant because these compounds are poorly absorbed and do not cross the blood brain barrier (Bagheri, et al., 2004; Dagorn, 1982).

In addition, we are the first to demonstrate that riluzole, a compound prescribed for patients suffering from amyotrophic lateral sclerosis (RILUTEK®), opens Slack channels in a concentration range similar to that found active at TRAAK or TREK-1 channels (Duprat, et al., 2000). A recent study (Budelli, et al., 2009) has shown that riluzole at 20 µM was able to inhibit K_Na currents attributed to Slack channels in cultured olfactory neurons. As suggested by the authors, riluzole effects can probably be attributed to the blockade of the late component of Na⁺ currents. These results are not in opposition with those we report on, obtained at higher riluzole concentrations. Given the rather high estimated EC₅₀ value we found (97 µM in AAS, not assessed with patch-clamp), it is unlikely that this new property of riluzole may be involved in its therapeutic properties. However, it should be taken into account when considering pharmacological *in vitro* or *in vivo* studies performed with high riluzole concentrations, such as those needed to activate TRAAK or TREK-1 channels (Duprat, et al., 2000). As an example, a
recent study has shown that riluzole (100-500 µM) locally applied in vivo onto injured DRG neurons in a rat model of allodynia, decreased the spontaneous firing rate of A-fibers. This effect was attributed to the inhibition of non-inactivating Na⁺ current but at such high concentrations, an increase in K⁺Na currents cannot be ruled out (Xie, et al., 2011).

When trying to correlate in vitro data to plasma concentrations achieved in patients and clinical effects, loxapine appears to be a rather complex drug. First the steady-state plasma levels of loxapine vary (about 9 folds reported) in individuals treated chronically with the same oral dosage (Cooper, et al., 1979). Furthermore, loxapine is extensively metabolized by cytochrome P450 to amoxapine, 7-hydroxy-loxapine (7-OH-loxapine) and 8-hydroxy-loxapine which are further metabolized to 7-hydroxy-amoxapine (7-OH-amoxapine) and 8-hydroxy-amoxapine (Wong, et al., 2012; Heel, et al., 1978). In the very recent study by Wong et al, conducted in the rat, it is shown that 4 hours after administration of 1mg of loxapine, amoxapine, 7-OH-amoxapine and 7-OH-loxapine plasma concentrations are about 15-20 times higher than that of loxapine. In the brain, 7-OH-loxapine was found to accumulate achieving concentrations as high as 124 ng/g in the striatum, while loxapine, amoxapine and 7-OH-amoxapine concentrations were less than 5 ng/g. Despite interspecies variability in loxapine metabolism (Bun, et al., 2003), the antipsychotic effects of this drug in humans are also more correlated with the plasma levels of its hydroxylated and hydroxylated desmethyl metabolites rather than to the very low parent drug concentration (Simpson, et al., 1978). Nonetheless, the maximal plasma concentration following an oral administration of 50 mg of loxapine is about 30 ng/mL (9.10⁻⁸ M). This is two order of magnitude lower than the EC₅₀ value we have calculated (EC₅₀ = 4.4 µM). However, a significant increase in Slack current amplitude was observed for concentrations as low as 0.3 µM and C_max values as high as 0.4 µM (135
µg/L) have been reported shortly after nasal administration of 10 mg of loxapine (Spyker, et al., 2010). Altogether, these data strongly suggest that the direct Slack activation by the unmetabolized form of loxapine during chronic treatment is clinically unlikely. However, it would be of high value to evaluate the Slack modulating properties of loxapine and amoxapine hydroxylated derivatives. Considering side-effects, loxapine is known to be among the tricyclic antipsychotics most prone to induce extrapyramidal syndrome (EPS) as shown by the very high rate of anti Parkinson drug co-prescription associated with loxapine use (Yang, et al., 2007). Moreover, the study conducted by Yang et al., confirmed previously published studies, supporting a quantitative correlation between the dosage administered and the EPS incidence rate (Ereshefsky, 1999). EPS involves the midbrain dopaminergic structure the Substantia Nigra (SN) which has been shown to abundantly express Slack channels. Because these channels are located at dopaminergic (DA) neurons, Slack activation would be expected to decrease SN neuron firing and DA release. This effect would potentiate the post-synaptic blockade of dopaminergic receptors and exacerbate EPS. Again, additional experiments directly addressing the concentration-dependent effect of loxapine and amoxapine metabolites on recombinant Slack channels and native KNa channels would be necessary to test this hypothesis. Very recently, a study using cultured neurons derived from schizophrenic patients, has shown the unique and unexplained positive effect of loxapine (10 µM) on neuronal connectivity, as compared with structurally related other antipsychotic drugs (Brennand, et al., 2011). It would be of interest to do the same experiments in the presence of a Slack antagonist or in Slack knockdown studies, in order to evaluate the possible involvement of Slack activation in this process.

The experiments performed in DRG neurons confirmed that loxapine can activate native KNa channels. Our demonstration that loxapine can affect excitability of DRG
neurons could be important in terms of pain therapeutics. Increasing $K_{Na}$ channel activity should decrease DRG excitability and decrease pain signalling in nociceptive fibers. Although Slack channels are trafficked from the DRG membrane during inflammatory pain signalling (Nuwer, et al., 2010), it is not known if the same process occurs during other painful pathological situation such as neuropathic pain. Nonetheless, activating residual $K_{Na}$ channels remaining at the membrane may still offer a way to decrease DRG hyperexcitability and therefore our studies provide the basis for assessing the putative analgesic properties of Slack channel openers.

This paper describes for the first time the Slack activating properties of three marketed molecules: niclosamide, riluzole and loxapine, found by screening the LOPAC using AAS. The efficiency of loxapine has been confirmed at both human and rat recombinant Slack channels and rat $K_{Na}$ channels in DRG neurons. In keeping with the proposed role of Slack channel regulation of DRG neuron excitability, loxapine was found to hyperpolarize the resting potential and increase the threshold for action potential firing, suggesting that novel analgesics could be developed by Slack channel targeted drugs. Though the relevance of the effect of loxapine on Slack at the clinically achieved dosages is undetermined, the drug can be a valuable tool in characterizing the physiological role of the channel.
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Ref Type: Hearing


Legends for figures

Figure 1: Medium throughput screening at recombinant Slack channels using atomic absorption spectrometry. (A) and (B): Bargraph representation of typical results obtained in a 384 well plate format, showing Rb⁺ concentration variation in the supernatant in response to LOPAC compounds tested at 30 μM and the identification of loxapine (A) and niclosamide (B). The effect of riluzole at 500 μM (used as a reference Slack opener) can be seen at the second column level.

(C) Effect of increasing concentrations of loxapine, niclosamide and riluzole on Rb⁺ flux in CHO-hSlack. Data are expressed as % of baseline. Data points and bars represent mean values ± SEM (n=4).

Figure 2: Loxapine, niclosamide and riluzole are Slack channels openers. (A) Original whole-cell current traces showing Slack current time course evoked in CHO-hSlack cells, by depolarizing steps from -90 to -20 mV during 200ms, under control condition or after superfusion of loxapine, niclosamide or riluzole. Note the inward current (black arrow) evoked by loxapine (the dotted line shows the control leakage current). (B) Using the same protocol as described in (A), bargraph plot of slack current amplitude variations expressed in fold increase. Data points are geometric mean value ± 95% confidence interval. * symbol: Student’s t-test versus constant, † symbol: One way ANOVA/Newman Keuls test, comparison between treatment groups. (C) Effect of loxapine, niclosamide and riluzole on the time course of Slack currents evoked by a ramp protocol, depolarizing the membrane from -120 to +40 mV in 2s. Note the linear aspect of the current voltage relationship under superfusion with loxapine or niclosamide.
at 10 µM and the strong inward currents elicited by these compounds, as already shown above. In contrast, riluzole at 100 µM only weakly modified the current-voltage relationship and induced moderate inward currents. (D) Using the same protocol as described in (C) bargraph plot of Slack current amplitude variations expressed in fold increase.

Figure 3: Concentration-dependent effects of loxapine and niclosamide. (A and B) Typical recordings showing the concentration-dependent effect of loxapine (A) and niclosamide (B) on steady-state inward Slack currents evoked at a holding voltage of -100 mV. Slack steady-state current amplitude was close to zero in control condition (dotted line) and strongly increased by the two compounds. (C and D) Concentration-response curves for loxapine (C) and niclosamide (D). For each compound, data points indicate the mean ± SEM of steady-state current amplitudes (n = 3 to 7 cells), normalized to currents evoked by 10 µM of the compound. Curves are the best fit of data point with the following single site equation: \( y = \frac{\text{Max} \times C^n}{(C^n + \text{EC}_{50}^n)} \), where C is the concentration, Max the maximal effect, and n the hill coefficient. The EC_{50} value is given with a 95% confidence interval.

Figure 4: Loxapine effect on Slack steady-state current: comparison with structurally related antipsychotic drugs. (A) Typical current trace showing the effect of loxapine, quetiapine, amoxapine, clozapine, and olanzapine (all compound at 10 µM) on steady-state Slack current recorded at -100 mV. (B) Bargraph plot of Slack current amplitude variation induced by each compound. Data points (n= 5 to 12) are geometric mean value ± 95% confidence interval.
For this experiment, the analysis of the fold (control/compounds) on different treatment groups was performed after log transformation to verify their equivalence. For each treatment compared to loxapine group, equivalence will be declared when the 90% confidence interval of the relative difference between means will be included in the acceptance interval [-0.15; 0.15]. Since 90% CI of the relative differences between treatments and loxapine are not included in the acceptance zone, equivalence could not be claimed between loxapine and the related compounds evaluated.

Figure 5: Bithionol but not loxapine activates human MaxiK currents.
(A) MaxiK current traces showing the strong effect of bithionol and the lack of effect of loxapine. Currents were induced by depolarizing steps from a holding voltage of –85 mV to +20 mV. (B) Similar experiment and result where MaxiK current amplitude has been plotted as a function of time. (C) Bargraph plot of MaxiK current amplitude variations expressed in fold increase. Data points are geometric mean value ± 95% confidence interval. * Symbol: Student’s t-test versus constant, † symbol: Student’s t-test : bithionol versus loxapine

Figure 6: Loxapine activates rat Slack channels
(A) Representative traces of excised inside-out patches of Slack channels from HEK cells stably expressing Slack were recorded before and after loxapine application. Patches were held at 0 mV and stepped to 80 mV for 500 ms. (B) loxapine affected the open probability and not the unitary conductance of Slack channels (C) NP(O) values were normalized to the NP(O) before drug application. (n=11). Error bars represent S.E.M. *,** denote p<0.01 unpaired t-test (D) Channel activity measured in outside-out patch recordings. Again, NP(O) values were normalized to the NP(O) before drug
application. Channel activation became significant 3 minutes after initiation of drug perfusion (n=4, * p<0.05 unpaired t-test) Error bars represent S.E.M.

Figure 7: Loxapine activates native K\textsubscript{Na} channels and reduces neuronal excitability

Representative traces of excised inside-out patches of K\textsubscript{Na} channels in DRG neurons were recorded before and after loxapine application. Patches were held at 0 mV and stepped to 80 mV for 2 s. Ten sweeps are shown superimposed on each other. Pipette solutions contained 1 mM TEA and patches were always perfused with Na\textsuperscript{+} to verify Na\textsuperscript{+}-dependence (B) NPO after Na\textsuperscript{+} and loxapine addition. Data was normalized to control (n=4*,**, p<0.05 unpaired t-test). Error bars represent S.E.M. (C) Representative action potential recordings from neurons incubated in loxapine-free or 10 µM loxapine containing solutions. Depolarizing current steps in increments of 10 pA from -10 to 200 pA (20 ms duration) (D) Loxapine significantly increased rheobase versus DMSO control (n=13, * p<0.05 unpaired t-test).
Figure 1

A

Loxapine

[B+]

Column

B

Niclosamide

[Rb+]

Row

C

% Baseline

[C] M

- Niclosamide EC$_{50}$ = 0.7 μM
- Loxapine EC$_{50}$ = 3.5 μM
- Riluzole EC$_{50}$ = 97 μM
Figure 3

A. Loxapine (μM)

B. Niclosamide (μM)

C. EC<sub>50</sub> = 4.4 μM [2.7; 7.2]

D. EC<sub>50</sub> = 2.9 μM [1.3; 6.5]
Figure 4

A

B

Fold increase

0
1
2
3
4
5
6
7
8
9
10

Loxapine  Quetiapine  Amoxapine  Clozapine  Olanzapine

n=8  n=12  n=11  n=5  n=2

0.1 nA
50 s
Figure 6

A

B

Control

10 μM Loxapine

C

Inside-Out Patch

D

Outside-Out Patch

20 pA

50 ms

1 μM Loxapine

5 μM Loxapine

10 μM Loxapine

Wash

NP(O)

Control 20 Na Na Wash 10 μM Lox Lox Wash

NP(O)

Control 1 min. 2 min. 3 min.

* **
Figure 7

A

Control 10 μM Loxapine

20 μM Loxapine

Wash

B

NP(0)

Control 20 Na Na Wash 10 μM Lox Lox Wash

C

Control 10 μM Loxapine

D

AP Threshold Current (μA)

Control 10 μm Loxapine

*