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Antinociceptive effects of new pyrazoles compounds mediated by the ASIC-1α channel, TRPV-1 and μMOR receptors

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ABSTRACT

Pyrazoles are potent medicinal scaffolds and exhibit a wide spectrum of biological activities, such as analgesic, anti-inflammatory and antipyretic. In this paper we report on research we have performed with the aim of continuing the biological evaluation of the regio-isomeric pyrazole compounds, LQFM-020 (fluorine, para position), LQFM-021 (fluorine, meta position), and LQFM-039 (fluorine, ortho position) in models of pain induced by acidified saline, capsaicin, and formalin. We also investigated the mechanisms of action of these compounds via electrophysiological analyses using the two-electrode voltage-clamp technique and heterologous expression in Xenopus laevis oocytes. This enabled us to study different potassium channel subtypes: the ASIC-1α channel, TRPV-1, and μMOR receptors. Our results indicate that LQFM-020, LQFM-021, and LQFM-039 (15, 30 or 60 $mg.kg^{-1}$) compounds inhibited the nociceptive response induced by acidified saline in a dose-dependent manner. The dose of 30 $mg.kg^{-1}$ inhibited the nociceptive response induced by capsaicin by 53.3%, 51.4%, and 52.1%, respectively. In addition, we found that naloxone reverses the antinociceptive effect produced by the compounds in both phases of the formalin test. In electrophysiological analyses, we observed that the LQFM-020, LQFM-021, and LQFM-039 compounds did not modulate voltage-gated K + channel subtypes. In contrast, all the compounds tested inhibited the ASIC-1α channel at pH 4.5, with IC50-values of 96.1, 91.6, and 235.2 μM, respectively. All compounds also inhibited the TRPV-1 channel with IC50-values of 139.1, 212.5, and 159.1 μM, respectively. In contrast to the ASIC-1α and TRPV-1 targets, all compounds showed agonist activity on the μMOR receptor with an EC50-value of 117.4, 98.9, and 86.3 μM, respectively. We thus conclude that the ASIC-1α, TRPV-1, and μMOR channels are targets that are directly involved in the antinociceptive effect of LQFM-020, LQFM-021, and LQFM-039. Furthermore, the modifications of the fluorine positions in the phenyl analogs do not change the analgesic effect. However, LQFM-039 showed lower interaction with ASIC-1α channel.

1. Introduction

Pain is a complex phenomenon involving the transduction of noxious environmental, cognitive, and emotional stimuli processed by the brain [1]. The perception of the pain signal alerts us to dangers of various natures and triggers appropriate protective responses [2]. Normally, polymodal nociceptors can be activated by various stimuli, such as thermal, mechanical, and toxic molecules or inflammatory mediators [3]. Several receptors and ion channels are expressed in nociceptors, and these receptors/channels are vital for the detection of

Abbreviations: ASIC-1α, acid-sensing ion channels 1 alpha; TRPV-1, transient receptor potential vanilloid-1; μMOR, opioid receptor mi; NSAIDs, non-steroidal anti-Inflammatory drugs; NO, nitric oxide; cGMP, cyclic guanosine monophosphate; K_{ATP} channel, ATP-sensitive potassium channel; Kv, voltage-gated potassium channels; Shaker IR, potassium voltage-gated channel, shaker-related subfamily, member 3; hERG, potassium voltage-gated channel subfamily H member 2; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; GIRK1/GIRK2, G protein-activated inwardly rectifying K+channels 1 and 2; SEM, standard error of mean

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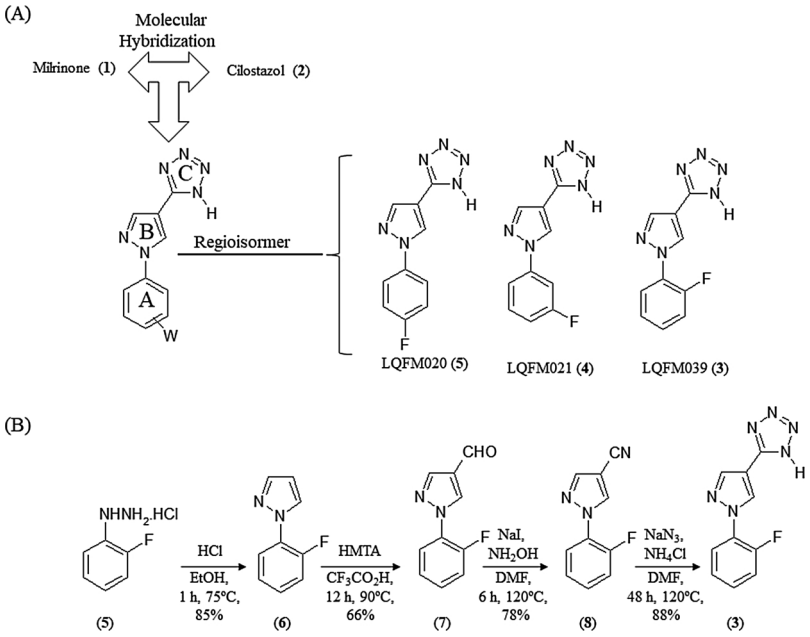

Fig. 1. (A) Structural design concept of the pyrazole compounds LQFM-020 (**5**), LQFM-021 (**4**), and LQFM-039 (**3**), from lead compounds milrinone (**1**) and cilostazol (**2**). (B) Synthetic route for the preparation of 5-(1-(2-fluorophenyl)-1*H*-pyrazol-4-yl)-1*H*-tetrazole (**3**) – LQFM-039. The molecular weight these compounds is 230.07 and the difference among these structures is the fluorine position in the phenyl ring. LQFM-020: F-*Para*; LQFM-021: F-*Meta*; LQFM-039: F-*Ortho*.

various noxious stimuli and the generation of action potentials [4].

Acid-sensing ion channels (ASICs) and the transient receptor potential vanilloid-1 (TRPV-1) have been thoroughly studied to date. The former is a proton-gated cation channel that is an important acid sensor in primary afferent fibers contributing to acid-induced nociception when an increase of protons affects peripheral tissues, and hence, leading to acidification or low pH conditions sensed by the cells in those tissues [5,6]. ASICs contribute to the excitation of primary sensory neurons and are activated by extracellular acidosis (pH from 7 to 5) in the periphery [7]. The later channel, TRPV-1, is a ligand-gated and nonselective cation channel expressed in peripheral sensory neurons. It plays a significant role in peripheral pain sensation [4]. This channel is known as the heat transducer in sensory neurons because the channel is activated at a temperature above 42 °C by acid and capsaicin. It is the pungent substance found in hot chili peppers [8].

Despite important advances in the treatment of painful conditions, opioids remain the most potent class of analgesic medications available [9]. Opioids exert their pharmacological and physiological effects through binding to their opioid receptors, generally classified in three types: μ, κ, and *δ* [10,11]. Activation of these receptors produces inhibition of adenylyl cyclase, inhibition of voltage-dependent calcium channels, and activation of an inwardly rectifying potassium channel, which all together results in analgesia [12].

Another class of therapeutic agent widely used in treatment of pain and inflammatory conditions are the non-steroidal anti-inflammatory drugs (NSAIDs) [13]. It is well known that long-term use of NSAIDs induces several negative health effects, especially involving the damaging the gastrointestinal mucosa [14]. Hence, there is still a need for better molecules synthesizing novel analgesic and anti-inflammatory compounds with reduced side effects compared with the drugs currently in use [15].

Although a considerable amount of antinociceptive drugs are available, it is important to search for new, more potent substances with less adverse effects that could contribute to our current understanding of the nociceptive signaling pathways to improve the treatment of painful conditions. Pyrazole derivatives have attracted the attention of organic chemists due to their biological and chemotherapeutic importance [16].

Moreover, in recent years, the LQFM-021 pyrazole compound, 5-(1-(3-fluorophenyl)-1*H*-pyrazol-4-yl)-2*H*-tetrazole, has been reported to possess biological activities, such as vasorelaxant [17], analgesic [18], and anti-inflammatory activities [19,20]. Similar effects were reported for other analogous 5-[1-(4-fluorophenyl)-1*H*-pyrazol-4-yl]-2*H*-tetrazole [21]. In addition, this research suggested the involvement of peripheral opioid receptors and the NO/cGMP/K_{ATP} pathway involved in the antinociceptive effect displayed by these compounds.

Prompted by the variety of biological activities bestowed on these pyrazole compounds, and the fact that LQFM-021 may be a good prototype for the development of new analgesic and anti-inflammatory drugs, our research has aimed to continue with the biological evaluation of compounds LQFM-039, LQFM-020, and LQFM-021 in models of pain induced by acidified saline and capsaicin. Additionally, the mechanisms of action of these compounds were investigated in detail via electrophysiological characterization of different potassium channel subtypes, the ASIC-1α channel, and TRPV-1 and μMOR receptors using the two-electrode voltage-clamp technique.

2. Methods

2.1. Structure of the LQFMs

The compounds 5-[1-(4-fluorophenyl)-1*H*-pyrazol-4-yl]-2*H*-tetrazole (LQFM-020) (**3**), 5-(1-(3-fluorophenyl)-1*H*-pyrazol-4-yl)-2*H*-tetrazole (LQFM-021) (**4**), and 5-(1-(2-fluorophenyl)-1*H*-pyrazol-4-yl)-2*H*-tetrazole (LQFM-039) (**5**) were synthesized by "Laboratório de Química Farmacêutica Medicinal" (LQFM), Faculty of Pharmacy, Federal University of Goias, according to the synthetic route described by Martins et al. [17] and De Oliveira et al. [21], as well as Supporting Information file. The molecular weight of the compounds is 230.07 and the chemical structure is shown in Fig. 1.

2.2. Drugs and chemicals

Acetic acid (Synth, Brazil), DMSO (Sigma Chemical, USA), amiloride (Sigma-Aldrich St. Louis, USA), capsaicin (Sigma-Aldrich St. Louis, USA), capsazepine (Sigma-Aldrich St. Louis, USA), formaldehyde (Synth, Brazil), gentamycin sulfate (Sigma Aldrich St. Louis, USA), morphine sulphate (Dimorf®, Cristalia, Brazil), naloxone chloridrate (Narcan®, Cristalia, Brazil). Other chemicals were obtained from Acros Organics (Geel, Belgium) and Sigma-Aldrich (St. Louis, MO, USA)

2.3. Animals

All experiments were performed with adult male Swiss albino mice (weighing 27–32 g) obtained from the Central Animal House of UFG. The animals were kept at a controlled temperature (22° ± 2 °C) and humidity between 50–55%, under a 12-h light/dark cycle with food and water available *ad libitum*. Animal studies are reported in compliance with the ARRIVE guidelines [22,23]. Mice were group-housed with a maximum of 5 animals per cage in clear polypropylene cages (41 × 34 × 16 cm). The animals were acclimatized for seven days before the start of the experiments. For *in vivo* experiments, 8 mice per experimental group were used. A total of 208 mice were used in this study. We chose only males in order to reduce the number of animals. All experiments were performed in accordance with national and international standards (based on specific guidelines from the Brazilian Council of Animal Experimentation and the U.S. Public Health Service's Policy on Humane Care and Use of Laboratory Animals-PHS Policy), and under the ethical guidelines established for investigations of experimental pain in conscious animals [24] (Zimmermann, 1983). The study was approved by the Ethics Committee in Research of the Federal University of Goias (number 17/13).

To determine the size of each group of animals, we respected the principle of the 3 R's (replacement, reduction, and refinement). Therefore, we estimated a group size of 8 mice for each experimental group [18,25]. The number of animals and intensities of noxious stimuli used in each experiment were the minimum needed to demonstrate the consistent effects of the treatments. The animals employed in this study were used only once. Allocation concealment was performed using a randomization procedure. Behavioral evaluations were performed blindly for drug administration and experiments were carried out between 08:00 and 17:00 h. These experiments were carried out by two people: one administered treatments and injections, and the other monitored behaviour.

The analgesic activity of the pyrazole compounds were evaluated in animal models involving the following pain conditions: chemical nociception including acidified saline, capsaicin, and formalin. These tests were chosen based on the results of preliminary experiments. Male mice were used for all studies, as this species is commonly used in pharmacological and pain behavioral research and is consistent with our previous studies [18,21,26].

For electrophysiological characterization, the use of the frogs was in accordance with the license number LA1210239 with the approval of Laboratory of Toxicology & Pharmacology, University of Leuven. All animal care and experimental procedures agreed with the guidelines of the European Convention for the Protection of Vertebrate Animals used for Experimental and Other Scientific Purposes (Strasbourg, 18.III.1986). Animal studies are reported in compliance with the ARRIVE guidelines [22,23]. Oocytes were surgically removed from the ovarian tissue of *Xenopus laevis*, under conditions of anesthesia with tricaine methane-sulfonate at neutral pH and cold anesthesia. The whole animal was cooled down to 2–4 °C, and then a small part of the ovary (˜200 oocytes) was removed through a small (less than 1 cm) incision in the abdominal wall, which then was sewn with a single stitch. For each animal, the interval between surgeries was at least 3 months. A total of 12 female *X. laevis* frogs were involved in this study and were reused no more than 5 times each.

2.4. Pharmacological tests

2.4.1. Nociception induced by acidified saline or capsaicin

To verify whether ASIC and TRPV-1 channels are involved in the antinociceptive activity of the pyrazole compounds, the effect in connection with nociceptive responses elicited by specific activators of each channel/receptor was tested. In the acidified saline test, animals were treated with control (DMSO 10% (v/v), 10 mL.kg^{-1}, p.o.), LQFM-020, LQFM-021 and LQFM-039 (15, 30 or 60 mg.kg^{-1}, p.o.), 1 h before of the acidified saline injection. In the capsaicin test, animals were treated with control (DMSO 10% (v/v), 10 mL.kg^{-1}, p.o.), LQFM-020, LQFM-021 and LQFM-039 (30 mg.kg^{-1}, p.o.), 1 h before of the capsaicin injection. The positive control capsazepine (10 mg.kg^{-1}, i.p., a TRPV1 antagonist) or amiloride (100 mg.kg^{-1}, i.p., an ASIC antagonist) were administered 30 min before of the phlogistic agent injection. Then, following the treatments, mice received 20 µL intraplantar (i.pl.) injection of either capsaicin (1.6 µg.paw^{-1}) or acid saline (2% acetic acid in 0.9% saline, pH 2.0) into the plantar surface of the right hind paw. After the injection, the mice were placed into an acrylic box, and a mirror was placed under this box and were observed for 7 min (capsaicin), 20 min (acid saline) in accord with previous study [26,27]. The amount of time spent licking/biting the injected paw was recorded and considered as indicative of nociceptive response. The acidified saline test was performed in five days; The capsaicin test was performed in one day, because of the number of animals.

2.4.2. Involvement of the opioid pathway in the antinociceptive effect of pyrazoles compounds in formalin test

The possible participation of the opioid system in the antinociceptive effect of LQFM-020, LQFM-021 and LQFM-039, was evaluated by using the formalin test in which the mice were pre-treated (i.p.) with saline (10 mL.kg^{-1}) or naloxone (3 mg.kg^{-1}, i.p.) 15 min before the treatment with control, LQFM-020, LQFM-021 and LQFM-039 (30 mg.kg^{-1}, p.o.) or morphine (5 mg.kg^{-1}, s.c.). Sixty minutes after the oral treatment, or thirty minutes after the s.c. injection, the animals received 20 µL intraplantar (i.pl.) of formalin (3%, v/v) and were observed the licking time in first phase (0 to 5 min.) and second phase (15 to 30 min.), as described previously by Costa et al. [25]. In this experiment there are two controls groups, one group was pre-treated with saline and other with naloxone. In the experimental schedule, we made 10 animals a day; one animal from each group/day, performing all experiment in eight days.

2.5. Electrophysiological evaluation

2.5.1. Expression in Xenopus oocytes

For the expression of the voltage-gated potassium channels (rKv1.1, rKv1.2, hKv1.3, rKv1.4, rKv1.5, rKv1.6, Shaker IR, rKv10.1 and hERG), rASIC-1α, rTRPV-1, and µhMOR in Xenopus oocytes, the linearized plasmids were transcribed using the Ambion™ T7 or SP6 mMESSAGE-mMACHINE transcription kit (Thermofisher, Houston, TX, USA). The harvesting of stage V–VI oocytes from an anaesthetized female Xenopus laevis frog was carried out as previously described by Liman et al. [28]. Oocytes were injected with 10–50 nL of cRNA at a concentration of 1 ng.nL^{-1} using a micro-injector (Drummond Scientific, Broomall, PA, USA). The oocytes were incubated in a solution containing (in mM): 96 NaCl; 2 KCl; 1.8 $CaCl_2$, 2 $MgCl_2$,and 5 HEPES (pH 7.4), and supplemented with 50 mg/L gentamycin sulfate.

2.5.2. Electrophysiological recordings

Two-electrode voltage-clamp recordings were performed at room temperature (18–22 °C) using a GeneClamp 500 amplifier (Molecular Devices, USA) controlled by a pClamp data-acquisition system (Axon Instruments, USA). The resistances of both electrodes were kept between 0.8 and 1.5 MΩ and were filled with 3 M KCl. To eliminate the effect of a voltage drop across the bath-grounding electrode, the bath potential was actively controlled. The temperature of the perfusate was kept at 22 °C and controlled using a SC-20 dual in-line heater/cooler (Warner Instruments). The pH was kept at 7.4.

2.5.3. Potassium channels

Currents were measured in ND96 solution 1–2 days after injection. The elicited currents were filtered at 1 kHz and sampled at 500 Hz using a four-pole low-pass Bessel filter. Leak subtraction was performed using a -P/4 protocol. Kv1.1–Kv1.6 and Shaker currents were evoked by

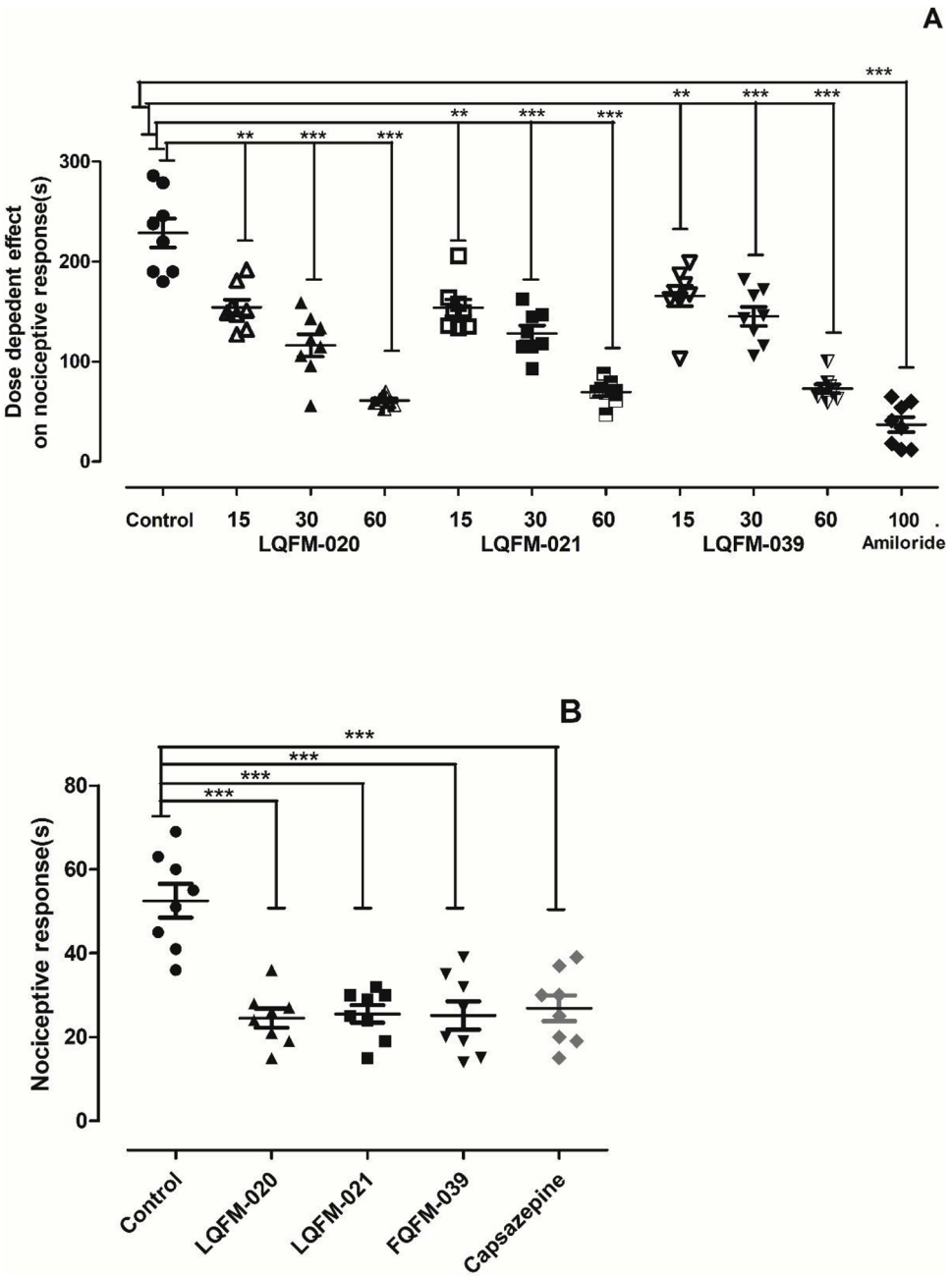

Fig. 2. Dose dependent effect of the oral administration of 15, 30, or 60 mg.kg^{-1} of the pyrazole compounds LQFM-020, LQFM-021, and LQFM-039 on nociception response caused by the intraplantar injection of acidified saline (ASIC activator, 2 A). Effect of the administration of 30 mg.kg^{-1} these pyrazole compounds in capsaicin induced-nociception (TRPV-1 activator, 2B) in mice. Grouped column scatter plot represent the mean ± SEM (n = 8). Significance levels when compared to the control group are indicated by ** P < 0.01; *** P < 0.001 (One-way ANOVA followed by *Student-Newman–Keuls* test).

500 ms depolarizations to 0 mV followed by a 500 ms pulse to -50 mV, from a holding potential of -90 mV. The elicited K_v10.1 currents were filtered at 1 kHz and sampled at 2 kHz using a four-pole low-pass Bessel filter. K_v10.1 currents were evoked by 2 s depolarizing pulses to 0 mV from a holding potential of −90 mV. Current traces of hERG channels were elicited by applying a + 40-mV prepulse for 2.5 s followed by a step to −120 mV for 2.5 s [29].

2.5.4. ASIC-1α channel

Currents were measured in ND96 solution (7.9, 7.4 and 4.5 pH) at a holding potential of –70 mV during 400 s, and the data were filtered at 20 Hz. Currents were measured 3–4 days after injection of the mRNA. A gravity-controlled fast-perfusion system (Warner Instruments) was used to ensure rapid solution exchanges. In each experiment, oocytes were initially superfused with ND96 pH 7.9 solution. Next, the superfusion was switched from ND96 pH 7.9 to ND96 pH 4.5 solution. Then, the superfusion was switched back to ND96 pH 7.9 solution. Next, the compounds LQFM-020, LQFM-021, or LQFM-039 were supplemented with ND96 (7.9 and 7.4 pH). During application of increasing concentrations of the compounds, oocytes were superfused with ND96 pH 4.5. [30,31]. For determining the IC50 values, the percentage inhibition was calculated assuming that zero current level corresponds to 100% inhibition.

2.5.5. TRPV-1 receptor

Four days after injection, currents were measured in ND96 solution at a holding potential of –70 mV during 400 s. The recording chamber was perfused at a rate of 2 mL/min with a ND-96 solution (pH 7.4).

Capsaicin (2 μM) was used as an agonist and capsazepine (10 μM) as an antagonist of TRPV-1. All experiments were carried out on at least three different oocytes. LQFM-020, LQFM-021, and LQFM-039 were dissolved in ND96 and administered using the perfusion system [32]. For determining the IC50 values, the percentage inhibition was calculated assuming that zero current level corresponds to 100% inhibition.

2.5.6. Opioid μMOR receptor

Four days after injection, two electrode voltage-clamp recordings were performed. Currents were measured in ND96 solution at a holding potential of –90 mV during 400 s. At the beginning and conclusion of each experiment, oocytes were superfused with the ND-96 solution. During application of increasing concentrations of the compounds, oocytes were superfused with a high-potassium (HK) solution, with the following composition (in Mm): KCl 96, $NaCl_2$, $MgCl_2$ 1, $CaCl_2$ 1.8, HEPES 5, and pH 7.5. In HK solution, the K^+ equilibrium potential is close to 0 mV and enables K^+ inward currents to flow through inwardly rectifying K^+ channels at negative holding potentials. A gravity-controlled fast-perfusion system (Warner Instruments) was used to ensure rapid solution exchanges. In each experiment, oocytes were clamped at a holding potential of −70 mV and superfused with ND96 solution. Next, the superfusion was switched from ND96 to HK solution. Subsequently, morphine (opioid receptor agonist – 1 μM) or defined concentrations of LQFM-020, LQFM-021, and LQFM-039 were applied, always in the presence of HK solution. Each concentration was applied for as long as needed to achieve a steady-state GIRK1/GIRK2 current activation. Each ligand concentration was washed out by superfusing with HK solution. At the end of each experiment, the oocyte was superfused with HK solution containing 300 μM $BaCl_2$ causing the block of the net GIRK1/GIRK2-gated inward current. Finally, the superfusion was switched back to ND96 solution to confirm complete reversibility [11,33]. The percentage-activated current was calculated using the equation: percentage activation = activated current amplitude control current amplitude × 100–100. Zero percent was taken as the control-current level. Current percentages were then used for the calculation of concentration–response curves using the Hill equation: $I = I_{max}/[1 + (EC_{50}/A)^{nH}]$, where I represents the current percentage, Imax the maximal current percentage, EC_{50} the concentration of the agonist that evokes the half-maximal response, A the concentration of agonist, and nH the Hill coefficient.

2.6. Statistical analysis

The nociception data were analyzed statistically by one-way ANOVA, if indicated significance, and a Student-Newman–Keuls test was performed post-hoc. All the results are expressed as the mean ± SEM. Electrophysiological data were representing at least three independent experiments ($n > 3$) and are presented as mean ± SEM. Current percentages were used for the calculation of the relative EC_{50} and IC_{50} value using the Hill equation. All statistical analyses were carried out using Graph Pad Prism version 5.00. Values of $P \leq 0.05$ were considered significant.

3. Results

3.1. Effect of the pyrazole compounds on nociception induced by acidified saline and capsaicin

The results in Fig. 2 shown the effect of LQFM-020, LQFM-021, and LQFM-039 for the acidified saline and capsaicin tests. The data indicate that when compared to the control group (228.6 ± 14.4) all treatments reduced the nociceptive response in a dosage-dependent manner, induced by i.pl. injection of acidified saline. The compound LQFM-020 (15, 30 and 60 mg.kg-1) had an inhibition of 40.6% ($P \leq 0.01$), 40.1% ($P \leq 0.001$), and 73.2% ($P \leq 0.001$), respectively. LQFM-021 (15, 30 and 60 mg.kg-1) had an inhibition of 32.6% ($P \leq 0.01$), 43.9% ($P \leq 0.001$), and 69.5% ($P \leq 0.001$) respectively. LQFM-039 (15, 30 and 60 mg.kg-1) had an inhibition of 27.5% ($P \leq 0.01$), 36.4% ($P \leq 0.01$), and 68.1% ($P \leq 0.001$) respectively. The positive control amiloride 100 $mg.kg^{-1}$ was able to reduce the nociceptive response by 83.8% ($P \leq 0.001$), (Fig. 2A). In capsaicin-induced nociception, we also observed that LQFM-020, LQFM-021, and LQFM-039 reduce the nociceptive response by 53.3% ($P \leq 0.001$), 51.4% ($P \leq 0.001$), 52.1% ($P \leq 0.001$), respectively, as compared to the control group (52.50 ± 4.03). The positive control capsazepine 10 $mg.kg^{-1}$ was able to reduce the nociceptive response by 48.8% ($P \leq 0.001$) (Fig. 2B).

3.2. Involvement of the opioidergic system in the antinociceptive effect of pyrazole compounds in the formalin test

The administration of naloxone (nonselective antagonist at opioid receptors, 3 $mg.kg^{-1}$, i.p.), given 15 min prior to formalin injection, affects the antinociceptive activity produced by LQFM-020, LQFM-021, and LQFM-039 in both phases of the formalin test. A similar effect was observed with morphine (positive control). The saline-control group showed response nociceptive of 60.63 ± 2.98 in the first phase and 184.6 ± 6.14 in the second phase. The naloxone-control group showed the response nociceptive of 71.13 ± 2.97 in the first phase and 193.5 ± 7.20 in the second phase. The administration of naloxone, at the dose tested, did not affect the nociceptive response in the both phases the formalin test, when compare with saline-control (Fig. 3A–B).

3.3. Effect of the pyrazole compounds on the ASIC-1α channel

The effect of LQFM-020, LQFM-021, and LQFM-039 compounds on the ASIC-1α channel expressed in Xenopus oocytes is shown in Fig. 4. At pH 7.9, these compounds did not modulate the current. Interestingly, the three compounds blocked the current through the ASIC-1α channel at pH 4.5 (Fig. 4A, C, E, and G). The LQFM-020 and LQFM-021 compounds showed similar relative IC_{50}-values of 96.12 and 91.65 μM, respectively. The compound LQFM-039 showed a relative IC_{50}-value of 235.2 μM. Dose-response relationships and IC_{50}-values are represented in Fig. 4B, D, F, and H, respectively.

3.4. Effect of the pyrazole compounds on the TRPV-1 receptor

The effect of LQFM-020, LQFM-021, and LQFM-039 on the TRPV-1 receptor

expressed in Xenopus oocytes is shown in Fig. 5. Neither compounds showed agonist activity on this channel. On the other hand, all the compounds blocked the current through TRPV-1, when stimulated by the capsaicin agonist, both when the channel was closed or already previously opened by capsaicin (Fig. 5A, C, and E). The LQFM-020, LQFM-021, and LQFM-039 compounds showed relative IC_{50}-values of 139.1, 212.5, and 159.7 μM, respectively. Dose-response relationships and IC_{50}-values are represented in Fig. 5 B, D, and F, respectively.

3.5. Effect of the pyrazole compounds on the μMOR receptor

The μMOR receptor was co-expressed with GIRK1/GIRK2 channels (these are inward-rectifying K channels) and RGS4 (these are cytosolic regulators of G-protein signaling). In our study, we examined the effect of LQFM-020, LQFM-021, and LQFM-039 (Fig. 6) on the opioid receptor, called hMOR. Representative current traces of agonist-gated currents evoked in oocytes expressing hMOR by LQFM-020 (Fig. 6A), LQFM-021 (Fig. 6C), and LQFM-039 (Fig. 6E), with relative EC_{50}-values of 117.4, 98.97, and 86.32 μM, respectively. Dose-response relationships and relative EC_{50}-values of these compounds are shown in Fig. 6B, D, and F, respectively.

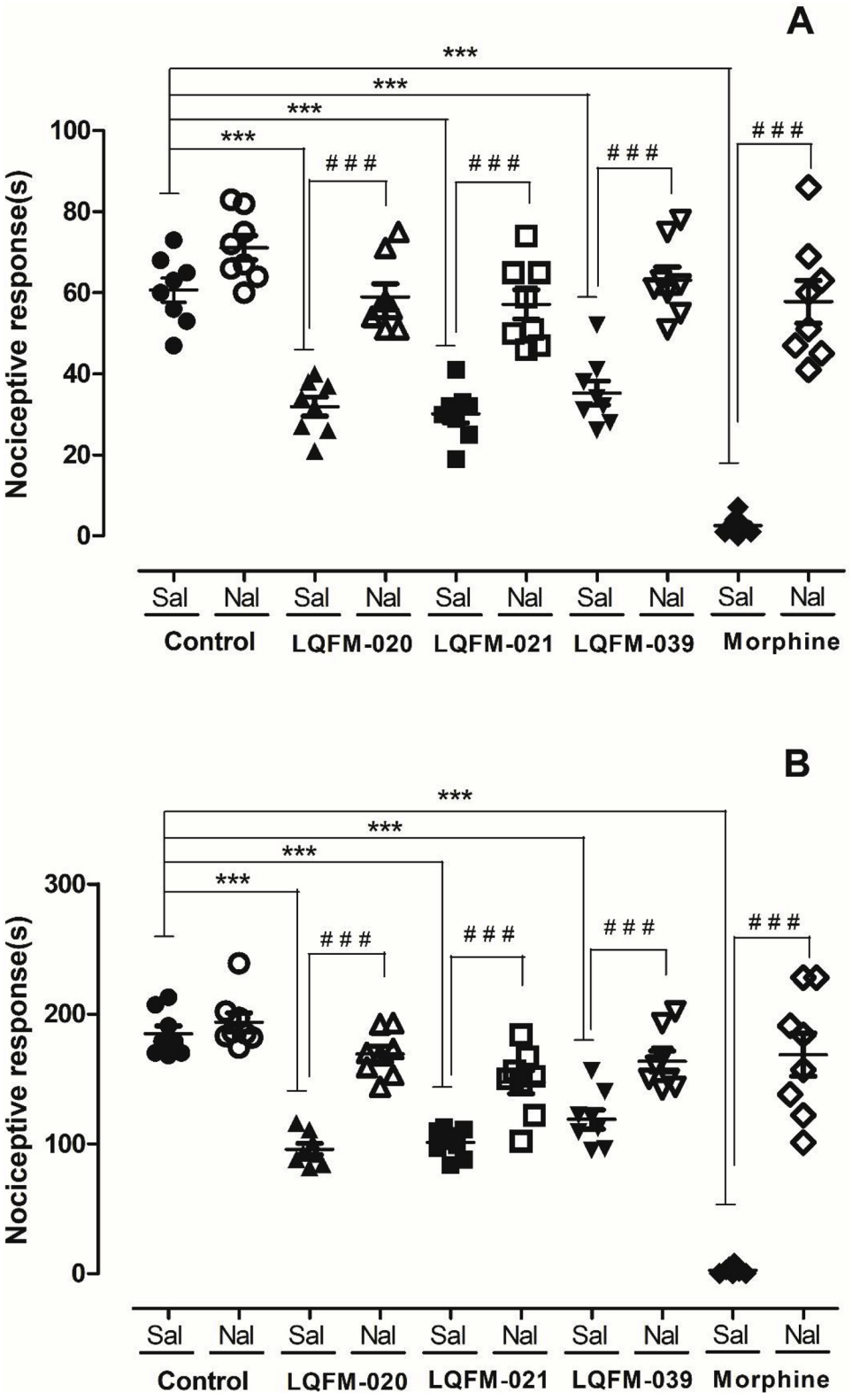

Fig. 3. Effect of the oral administration of 30 mg.kg^{-1} of the pyrazole compounds LQFM-020, LQFM-021, and LQFM-039 on the nociception response caused by the intraplantar injection of formalin. (3 A) represents the first phase (0–5 min) and (3B) represents the second phase. Grouped column scatter plot represent mean ± SEM of pain reaction time, in seconds (n = 8). *** $P < 0.001$ (compared to control group, pre-treated with saline) and ### $P \leq 0.001$ (compared to respectively treated group) according to One-way ANOVA followed by post-hoc *Student-Newman–Keuls* test. Abbreviations: Nal: Naloxone; Sal: Saline.

3.6. Effect of the pyrazole compounds on Potassium Channels

LQFM-020, LQFM-021, and LQFM-039 were tested for their activity against 9-voltage-gated potassium channel isoforms. The compounds, tested at a concentration of 50 μM, were not able to inhibit or activate the potassium currents generated through the mammalian Kv1.1, Kv1.2, Kv1.3, Kv.1.4, Kv1.5, Kv1.6, Kv10.1, *Shaker*, and hERG potassium channel subtypes (Fig. 7).

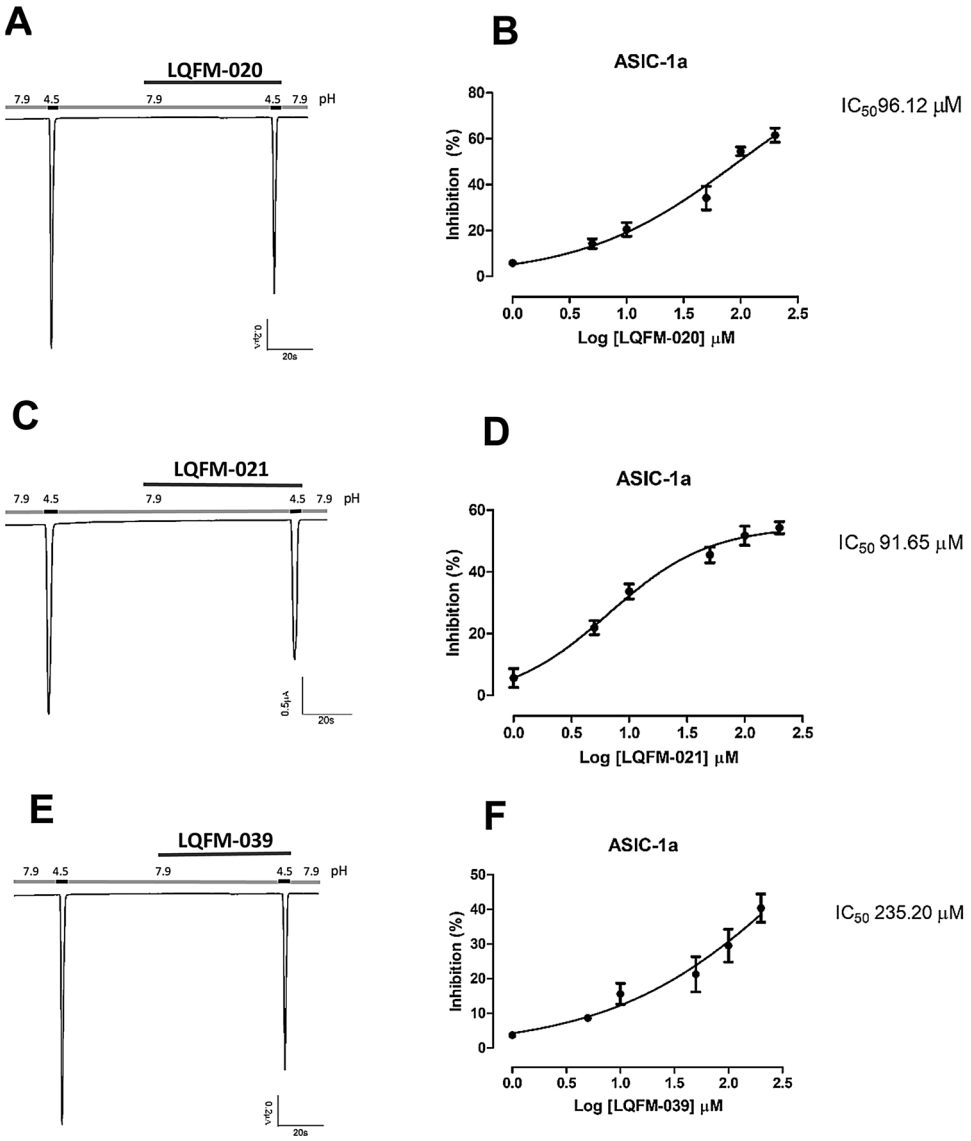

Fig. 4. Activity profile of the pyrazole compounds LQFM-020, LQFM-021, and LQFM-039 on the ASIC-1α Channel. ASIC1a currents evoked by a rapid extracellular pH change from 7.9 to 4.5. (A, C and E). Whole-cell current traces in control (pH 4.5) and pyrazole compounds (in pH 7.9 and 4.5) conditions are shown. (B, D, and F) Concentration–response curves for LQFM-020, LQFM-021, and LQFM-039, respectively on the ASIC-1α channel. All data represent the average of at least three independent experiments ($n \geq 3$) and are presented as mean ± SEM.

4. Discussion

Pain and inflammatory conditions are significant health problem, and there is a need for new drugs that provide safe and effective pain management [34,35]. In this context, the search for new compounds that could be applied in the therapy of painful conditions is important. In the present study, we greatly extended our knowledge of previous data in the literature on the biological effects of some pyrazole compounds studied by our own group [17–21].

The present study demonstrates, for the first time, that the analgesic effect of the compounds (LQFM-020, LQFM-021, and LQFM-039) may be associated, at least in part, with their ability to directly reduce the activation of nociceptors by blocking the current of ASIC-1α channels and of TRPV-1 receptors. Furthermore, an agonist activity on the μMOR receptor was observed. Alongside with the identification of these molecular targets, we have also demonstrated that these compounds show analgesia in pain models induced by different chemical agents.

These pyrazole compounds were originally designed and synthesized through molecular hybridization of the milrinone and cilostazol, resulting in three compounds with fluorophenyl and tetrazole moieties appended to a pyrazole ring. These compounds differ structurally by modifications (*para*, *meta*, and *ortho*) of the fluorine positions in the phenyl ring (i.e., they are isomers). The modifications of the fluorine position maintained analgesic activity of the pyrazole derivatives; however, LQFM-039 has a lower affinity for the ASIC-1α channel. Thus, it is interesting to observe that there are isoform-specific modifications that can be exploited and utilized as potent therapeutic agents in future.

The literature has demonstrated ample evidence showing that TRP and ASIC channels play a key role in the detection of noxious stimuli [27,36], and ASIC and TRPV-1 have complementary roles in the proton sensitivity of sensory neurons [37]. As such, the development of blockers of these ion channels may be of clinical interest for the control of pain states.

This study demonstrated that LQFM-020, LQFM-021, and LQFM-039 inhibit the nociceptive response induced by i.pl. injection of capsaicin and acidified saline, which activate TRPV-1 and ASIC channels, respectively. Importantly, the nociceptive responses induced by acidified saline and capsaicin also were inhibited by treatment with

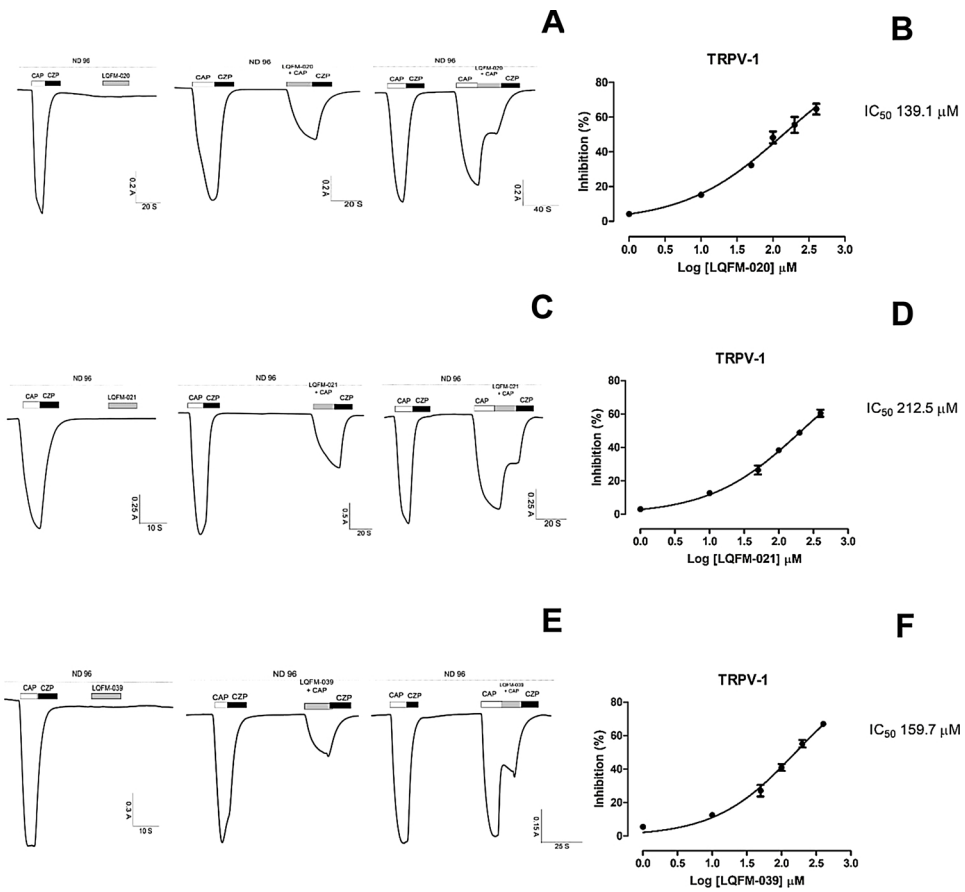

Fig. 5. Activity profile of the pyrazole compounds LQFM-020, LQFM-021, and LQFM-039 on the TRPV-1 receptor. The result of one representative experiment is shown (A, C and E). The lack of effect of pyrazole compounds in the absence of capsaicin; the antagonistic effect of LQFM-020, LQFM-021, or LQFM-039 (100 μM) co-applied with CAP (2 μM); CZP (10 μM). (B, D, and F) Concentration–response curve for LQFM-020, LQFM-021, and LQFM-039, respectively on the TRPV1 receptor. All data represent the average of at least three independent experiments (n ≥ 3) and are presented as mean ± SEM. CAP: capsaicin (2 μM); CZP: capsazepine.

amiloride and capsazepine, which are blockers of ASIC and TRPV-1 channels. These data are agreement with other studies reported in the literature [27,38] that showed the antinociceptive effect of LQFM-020 and LQFM-021, in another model of pain [18,21].

Our research group has previously characterized LQFM-021 as an analgesic opioid drug, since its analgesic activity, in the formalin test, was prevented by the administration of naloxone, a nonselective opioid antagonist [18]. On that basis, we decided to investigate and discern whether other pyrazole compounds would act through the same mechanism.

Among the several experimental models for acute pain study, the formalin test is the most useful tool to verify whether a compound has antinociceptive activity. Injection of formalin into the hind paw induces a biphasic pain response, neurogenic and inflammatory phases [39]. Since the pyrazole compounds could inhibit both phases, it is possible to infer that it is able to inhibit the direct activation of nociceptors, as well as to reduce the release of inflammatory mediators. Similar results were reported by Florentino et al. [18] and De Oliveira et al. [21]. In addition, we observed that the pretreatment with naloxone (a non-selective antagonist of opioid receptor) reversed the antinociceptive response of LQFM-020, LQFM-021, and LQFM-039, completely in the first phase, and partly in the second phase of the formalin test. This suggests that the antinociceptive action of these pyrazole compounds is tightly involved with the opioid receptor and its modulation. When compare with saline-control group, the administration of naloxone, per se, did not increase the nociceptive response in both phases of the formalin test. But, as expected, the pretreatment with naloxone reversed the antinociceptive effect of the classical agonist opioid, morphine.

Although the tested compounds show an antinociceptive effect mediated by the opioid system, oral administration of LQFM-021 did not produce an antinociceptive effect in the tail-flick and hot-plate tests. These results suggest that this compound does not act on the central nervous system and that the opioid effect observed is most likely mediated by peripheral opioid receptors. Our results are in accordance with a study that showed that the opioid-induced antinociceptive effect of pyrazole compounds may also be mediated by the activation of opioid receptors located outside the central nervous system [35].

The next step in this work was to evaluate the possible mechanism responsible for the compounds-induced antinociception by the two-electrode voltage-clamp technique using heterologous expression of targets in *Xenopus laevis* oocyte. This is a powerful method and a well-known tool for investigating the functions and regulation of ion-channel proteins and receptors [40]. It therefore provides a useful tool for studying the mechanism of action of new drugs in development. Firstly, we observed the effect of the compounds on a cell-viability test for electrophysiological measurements. In this assay, none of the compounds studied showed toxic effects that would impair electrophysiological assessments or indicate false positive results in the other tests. This data was relevant for the study's follow-up (data in supplementary material). In agreement with the results of Martins et al. [17] and De Oliverira et al. [21], our research showed that LQFM-021 and LQFM-020 have low cytotoxicity, and the compounds also are well tolerated when administered orally.

All compounds tested could block the ASIC1α channels at pH 4.5. The compounds LQFM-020 and LQFM-021 showed similar relative IC_{50}-values (91.65 and 96.12 μM, respectively), suggesting that *meta* and *para* fluorine position in the phenyl ring does not interfere with the interaction this channel. On the other hand, LQFM-039 *(ortho* fluorine) showed a relative IC_{50}-value to inhibit the ASIC1α (235.5 μM) that is higher than other pyrazole compounds, suggesting the importance of the location of the fluorine substitution.

The literature reports that therapeutic concentrations of NSAIDs, such as ibuprofen, aspirin®, and diclofenac also directly inhibit ASIC-1a

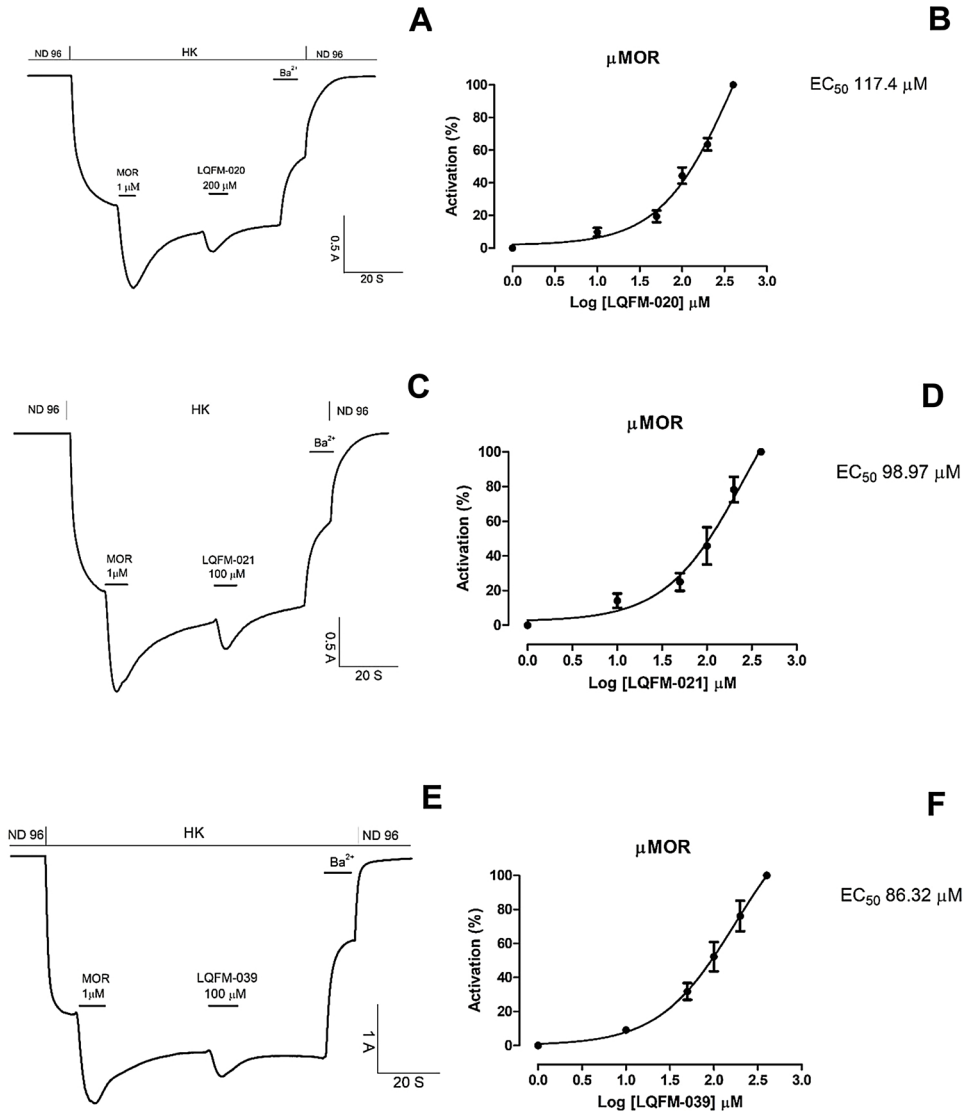

Fig. 6. Activity profile of the pyrazole compounds LQFM-020, LQFM-021, and LQFM-039 on the μMOR receptor. The result of one representative experiment is shown (A, C, and E). Whole-cell current traces for MOR with LQFM-020, LQFM-021, or LQFM-039 conditions are shown (A, C, and E). (B, D, and F) Concentration–response curve for LQFM-020, LQFM-021, and LQFM-039, respectively, on the μMOR receptor. All data represent the average of at least three independent experiments (n ≥ 3) and are presented as mean ± SEM. HK: High potassium solution; MOR: Morphine; Ba^{2+}: Barium chloride.

with IC_{50}-values between 92–350 μM [41]. In addition, amiloride has been the first blocker known and inhibits ASIC channels in the micromolar concentration range (IC_{50}-value ranging between 5 and 100 μM) [42,43]. Thus, our results are in agreement with literature describing other inhibitors. Such an inhibition could be especially relevant in nociceptors where ASIC-1α is co-expressed with TRPV-1 [44,45]. TRPV-1 shares the property of H^+ permeability with ASIC-1a, and TRPV-1 activation could control ASIC-1a activity in nociceptors [46].

Several natural and synthetic compounds, as well as endogenous ligands, have been identified for TRPV-1 [47]. The literature shows more than 1.000 natural and synthetic compounds acting on TRPV-1 as either an activator or blocker [48]. TRPV-1 is a multimodal sensor: its sensitization by many pain pathways and the effects of inflammatory mediators (protons, bradykinin, ATP, prostaglandins, lipoxygenases, glutamate, and nerve-growth factor) on TRPV-1 have been extensively studied [49–52]. Some studies have shown that TRPV-1 allows protons to enter the cell in an acidic environment [7,53,54]. The conductance of H^+ through TRPV-1 maybe result in intracellular acidification, which in turn may act on membrane channels that are sensitive to changes in intracellular pH, such as, certain two-pore domain K^+ channels [7].

The role of TRPV1 in pain can be counteracted both by true antagonists as well as by desensitizing TRPV-1 agonists [7,48]. Consistent with this, our data also show that LQFM-020, LQFM-021, and LQFM-039 have no agonist activity on TRPV-1 receptors. However, in contrast, they possess similar and significant inhibitory effects on TRPV-1 when expressed in Xenopus oocytes. These results corroborate with the analgesic effect observed in a capsaicin-induced pain model.

One of the relevant observations in our study was the pretreatment with naloxone that reversed the analgesic effected the compounds which were tested. Corroborating herewith, our electrophysiological evaluations on the μMOR receptor has shown that LQFM-020, LQFM-021, and LQFM-039 possess agonist activity on this receptor. Opioid analgesics that target the μMOR-opioid receptor, such as morphine, remain powerful analgesics for pain relief. The inhibitory modulation of the opioid system is a point critical for of pain transmission [55]. The three pyrazole compounds tested showed similar EC_{50}-values, which suggests that the modifications (*para*, *meta*, and *ortho*) of the fluorine position in the phenyl ring does not play a role in modifying the receptor.

In this paper, we showed that pyrazole compounds can modulate

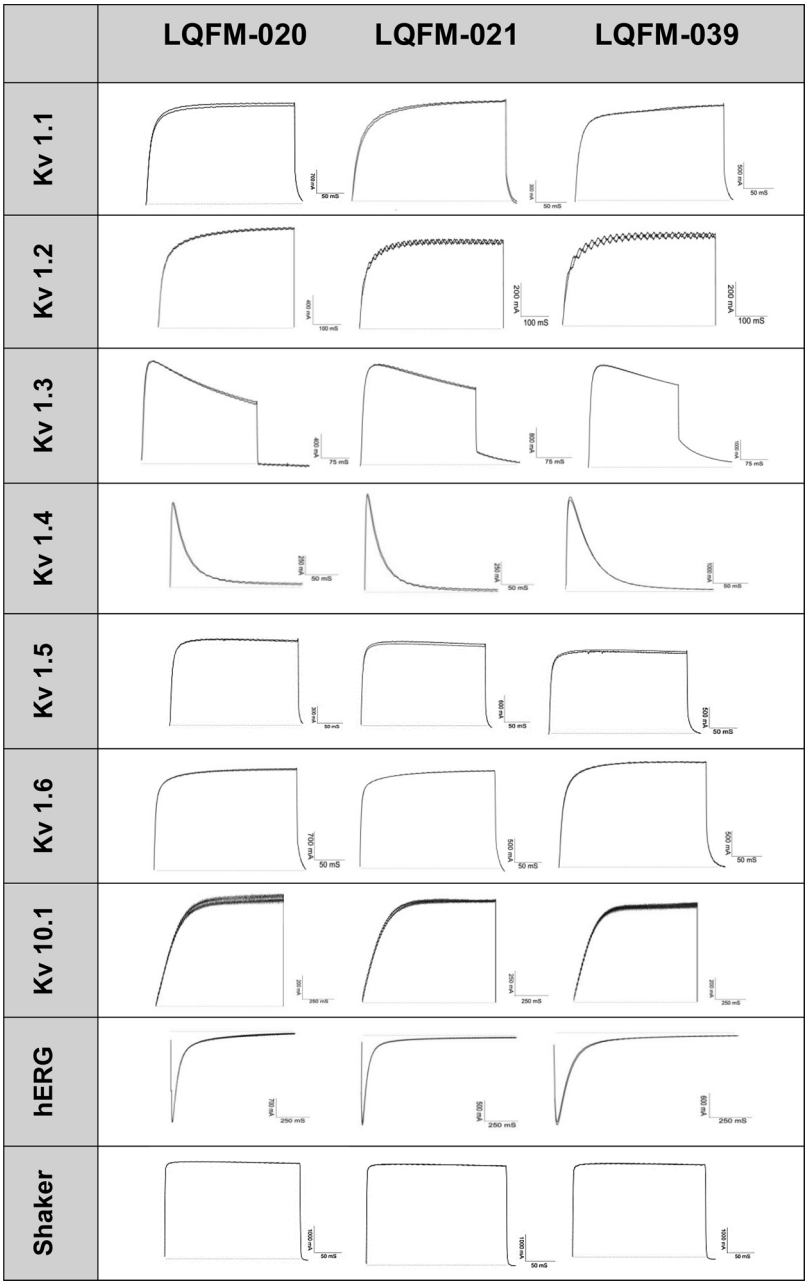

Fig. 7. Activity profile of the pyrazole compounds LQFM-020, LQFM-021, and LQFM-039 on the voltage-gated potassium channels. This figure shows that the dose of 50 μM the compounds tested no change in the ionic currents of the Kv1.1, Kv1.2, Kv1.3, Kv.1.4, Kv1.5, Kv1.6, Kv10.1, *Shaker*, and hERG potassium channel subtype.

the activity of functionally active ASIC channels in a way similar to that demonstrated by the literature that classical NSAIDs can modulate the activity of functionally active ASIC homomers [41]. Importantly, all the tested compounds in this study, which comprise the pyrazole group, have very simple chemical structures. This structure differs of the amiloride and other known modulators of acid-sensing ion channels. However, the exact mechanism and sites of the binding of the pyrazole compounds to inhibit ASICs and TRPV1 channels and to activate MOR receptor require further research. The compounds can have a directly ligand-binding inhibition effect, or an indirect effect. *in vivo* experiment, we observed different effects and more experiments need to be performed to arrive at the exact mechanism.

Recent studies have shown that voltage-gated potassium channels (Kv) are potential therapeutic targets for several types of pain, such as neuropathic and inflammatory pain [56,57]. Although previous results suggest a possible involvement of ATP-sensitive K^+ channels in peripheral antinociception induced by LQFM-021 [18], and a vasorelaxant effect by LQFM-021 and LQFM-020 [17,21], our results show that (up to 50 μM) neither compounds LQFM-020, LQFM-021, and LQFM-039 changed the current carried through the different isoforms of voltage-gated potassium channels (Kv1.1–Kv1.6, *Shaker*, Kv10.1, and hERG), suggest that these compounds do not directly modulate the activity potassium channels. But we cannot rule out an interaction in an indirect way or the action on ATP-sensitive K^+ channels.

5. Conclusion

Our findings not only confirm literature evidence, but also significantly extend its insights in connection with the antinociceptive profile of pyrazole compounds. In addition to antinociceptive effects previously demonstrated, this study demonstrates for the first time that LQFM-020, LQFM-021, and LQFM-039 are capable of modulating the

activation of nociceptors with the molecular targets being the ASIC-1α channel, TRPV1, and μMOR receptors. Together, it is reasonable to propose that these pyrazole compounds might be of potential interest in the development of new clinically relevant drugs for the management of different pain states.

Author contributions

Iziara F. Florentino designed and carried the pharmacological analyses, electrophysiological analyses and drafted the manuscript. Daiany P.B. Silva and Carina Sofia Cardoso designed and carried the pharmacological analyses. Ricardo Menegatti (Head) conceived the study, designed and carried synthesis of the LQFMs. Flávio S. de Carvalho and Luciano M. Lião carried the synthesis of the LQFMs. Paulo M. Pinto carried the electrophysiological analyses. Steve Peigneur carried the electrophysiological analyses and drafted the manuscript. Elson A. Costa (Head) and Jan Tytgat (Head) and supervised the research project, carried the pharmacological analyses, electrophysiological analyses and drafted the manuscript. All authors have commented on the initial and final drafts of the manuscript and are responsible for the approval of the final version of the manuscript in all aspects.

Conflict of interest statement

The authors declare that they have no conflicts of interest.

Acknowledgments

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