Research Results in Pharmacology

Research Results in Pharmacology 5(2): 65–70
UDC: 57.056, 615.212, 615.275.4
DOI 10.3897/rrpharmacology.5.36810

Research Article

Features of the pharmacological activity of polypeptide modulators on acid-sensitive ion channels in the experiment

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Academic editor: *Oleg Gudyrev* ♦ **Received** 5 March 2019 ♦ **Accepted** 5 May 2019 ♦ **Published** 27 June 2019

Citation: Dyachenko IA, Murashev AN (2019) Features of the pharmacological activity of polypeptide modulators on acid-sensitive ion channels in the experiment. Research Results in Pharmacology 5(2): 65–70. https://doi.org/10.3897/rrpharmacology.5.36810

Abstract

Introduction: TRPV1 receptors play a significant physiological role. To study pharmacological activity of new agonists and antagonists is important for the development of new drugs. This paper reports on the features of polypeptide antagonists of TRPV1 based on *in vivo* data.

Materials and methods: The study was performed on 250 mature white ICR male mice weighing 25–30 g. Tests were conducted to evaluate the pharmacological activity and biological properties of APHC1-3 and a hybrid polypeptide A13 in thermal pain,, inflammation and body temperature tests.

Results and discussion: APHC1-3 polypeptides showed significant antinociceptive and analgesic activity in the dose range of 0.01–0.1 mg/kg, without causing hyperthermia. A single substitution of the aspartic acid residue of APHC1 polypeptide at position 23 by transferring one asparagine residue from the cognate peptide APHC3 led to a significant change in the properties of the molecule. A new polypeptide A13 did not alter the thermal sensitivity of the mice, but showed the most significant analgesic activity in the acid-induced pain model, unlike APHC1. A13 inhibits TRPV1 and affects body temperature as a classic antagonist of this receptor.

Conclusion: Antagonistic properties of A13 became different from the properties of both initial analgesic polypeptides. Polypeptides APHC1-3 can be referred to as a new class of modulators of TRPV1, which produce a pronounced analgesic effect without hyperthermia.

Keywords

analgesic polypeptide APHC; TRPV1 receptor; animal models; temperature regulation; nociception.

Introduction

Modern strategies for searching for new potential analgesics are associated with changes in the sensitivity of nociceptors, affecting acid-sensitive ion channels. Identification of receptors and processes involved in the formation and transmission of pain signals pave the way for the use of new tools that provide more effective control of pain. One of the modern approaches to the treatment of pain is the use of highly selective agents that can specifically block receptors directly perceiving pain stimuli and/or mediators of inflammation.

The influence on the family of transient receptor potential (TRP) channels may be a new mechanism of pain relief. The target for potential modulation is TRPV1 channels (transient receptor potential vanilloid 1), also known as the capsaicin receptor, valinoid receptor, or VR1. In pathological conditions, TRPV1 is involved in inflammatory pains, neuropathic and visceral pains, as well as in inflammatory diseases, pancreatitis and migraines (Immke and Gavva 2006). TRPV1 channels are involved in the perception of exogenous risk factors, chemical irritation, mechanical impact and the effects of low temperatures, causing acute pain. It is activated by changes in the internal environment of the body, that is, inflammation, which is often accompanied by chronic pain (Levine and Alessandri-Haber 2007).

From the extract of the marine anemone *Heteractis crispa*, natural polypeptide modulators of TRPV1 – called *Analgesic Polypeptide Heteractis Crispa* (APHC) (Philyppov et al. 2012) – were isolated and characterized. A polypeptide APHC1 (Mr~6187 Da), polipeptise APHC2 (Mr~6185 Da), the amino acid sequence of which is different from that of APHC1 by a replacement of Val31 with Pro31 (Andreev et al. 2009), and APHC3 (Mr~6111 Da), which is different from APCH1 by replacing Arg18 with Pro 18 and Ala52 with Gly52 (Kozlov et al. 2009). The isolated polypeptides not only inhibit the TRPV1 receptor in model experiments in vitro (Andreev et al. 2013), but also produce a potentiating effect when using low concentrations of activating agents. The analysis of the three-dimensional structure of polypeptides APHC1 and APHC3 showed the presence of active amino acid residues modulating TRPV1 channels. Based on the data obtained, a hybrid polypeptide – A13 – was synthesized (Dyachenko et al. 2017).

The aim of this paper was to study the features of pharmacological activity of new polypeptide modulators acting on acid-sensitive ion channels TRPV1 in the experiment.

Materials and methods

The studied APCH 1-3 and A13 polypeptides were produced as described earlier in (Andreev et al. 2008, Andreev et al. 2009, Andreev et al. 2010, Dyachenko et al. 2017). The experiments on the animals were conducted in accordance with the Guide for Care and Use of Laboratory Animals, after the approval by the Institutional Animal Care and Use Committee (IACUC). of the Branch of the Shemyakin-Ovchinnikov Institute of Bioorganic Chemistry of the Russian Academy of Sciences. The adult males of CD-1 (Laboratory Animal Nursery of BIBCh RAS, Pushchino, Russia) weighing 20–25 g were used. The animals had been previously acclimatized in the laboratory for at least five days. The mice were kept at room temperature (23±2 °C) with a 12-hour light-dark cycle, with food and water available *ad libitum*. The test substance or carrier was administered intravenously in accordance with the design of the study.

A statistical analysis of the data was carried out using the analysis of variance (ANOVA) followed by Tukey's test. The data are presented as an average of ± S.E.

The efficiency study was carried out in tests:

Motor activity test

Spontaneous locomotor activity was recorded for 3 min after administration of the investigated substances in a computerized device which counted photobeam interruptions (OPTO-VARIMEX (Columbus Instruments, Columbus, OH, USA) and ATM3 automatic system using Auto-Track software version 4.2).

Hot plate test

In the test, a hot plate at a temperature of 55 °C (Columbus Instruments, Columbus, OH, USA) by the reaction of jerking and/or licking the hind legs, sensitivity to thermal effects was studied.

Hypersensitivity induced by complete Freund's adjuvant

A suspended emulsion, of complete Freund's adjuvant (CFA) and saline solution (1:1), was injected into the plantar surface of the left hind leg of mice (20 µl/paw). Control mice received 20 µl of saline solution (i.p.). 24 h after CFA injection, the time of paw withdrawal in response to thermal stimulation (53 °C) was recorded.

Method to evaluate visceral pain – writhing test

Experimental groups were administered 0.6% of acetic acid in saline solution (10 ml/kg−1 intraperitoneally (i.p.)). After injection, the mice were placed in a transparent cylinder, and the number of writhes was counted for 15 minutes.

Methods to assess the effect of polypeptides on body temperature

For registration of body temperature a rectal probe MLT1404 and PowerLab software (Adinstruments Inc., Colorado Springs, CO, USA) were used. When recording the temperature, the mobility of the animals in boxes MLA5018 (ADInstruments Inc., Colorado Springs, Colorado, USA) was limited. Body temperature was recorded for 100 minutes after administration, adaptation – for 20 minutes.

The experiments were partially carried out using the equipment provided by the Center of Shred Facilities of IBCh, supported by The Russian Ministry of Education and Science, Grant RFMEFI62117X0018).

Results and discussion

A targeted study using knockout mice showed that TRPV1 receptor channels play an important role in the biological processes of a living organism: perception of thermal stimuli, development of inflammation, inflammatory thermal hyperalgesia and thermoregulation (Szallasi et al. 2007).

In Vivo effects of APHC1-3 and A13 in pain models

There was an *in vivo* study conducted to evaluate the pharmacological effects of polypeptide modulators APHC1, APHC2, APHC3, and A13. At the beginning of the study, the possibility of the studied polypeptides influencing the central nervous system was excluded. The evaluation was carried out in the motor activity test, since compounds capable of reducing motor activity can distort the results of behavioral tests used to test pharmacological effects, especially in pain tests. One one dosage of polypeptides, 0.1 mg/kg, was used, which was close to the effective dose of the polypeptides studied in further experiments *in vivo*. There was no change in motor activity for any of the polypeptides at a dose of 0.1 mg/kg. The tests revealed comparable results in terms of distance traveled and points. Thus, the results of the efficacy of the studied polypeptides in pain tests will not be the result of impaired motor activity or sedative effect.

Hot plate test

The hot plate test is the easiest for inhibitors of TRPV1 because TRPV1 plays an important role in the perception of exogenous temperature stimuli. The dose-effect of the studied polypeptides on changes in behavioral reactions associated with analgesic action was estimated (Fig. 1).

The studied APCH 1-3 substances significantly increased the time spent on a thermostated surface (Fig. 1), at a dose of 0.1 mg/kg. A further increase in the dose to 1 mg/kg showed no development of analgesic effect, except of APCH 3 which increased the analgesic effect, but with an increase in the dose to 10 mg/kg decreased to the control group. A13 at a dose of 0.01 mg/kg showed analgesic effect; however, a further increase in the dose had no effect on the development of analgesic effect, and was comparable with the control group.

The results showed that the investigated polypeptides exhibit a dome-shaped dose-response relationship. This effect is typical for substances whose action involves peptidergic mechanisms (McNamara et al. 2005, Wu et al. 2010). Thus, a further increase in the dose will cause no increase in the biological effect of the studied substances.

Previously, it was shown that the knockout animals exhibit analgesic activity in the hot plate test by the TRPV1 gene (Caterina et al. 2000). The results of the study of analgesic activity of polypeptides APHC1-3 and A13 in the hot plate test well coincide with the literature data and indicate the development of analgesic activity through receptors – TRPV1. The control in the study of analgesic activity was the homologue of polypeptides APHC1-3, Aprotinin, the most powerful inhibitor of serine proteases. Aprotinin showed comparable results of analgesic activity with the control group (not shown) at doses of 0.1 and 1 mg/kg. Thus, the analgesic activity of the investigated polypeptides is not the result of the ability to partially inhibit serine proteases, but the modulation of TRPV1 channels.

CFA-induced hypersensitivity

CFA-induced thermal hyperalgesia depends on the activation of TRPV1; this was shown in the TRPV1-knockout mice and using TRPV1antagonists (Davis et al. 2000). CFA-induced thermal hyperalgesia is a complex process in which various inflammatory mechanisms reduce the temperature threshold through TRPV1 and affect thermal sensitivity (Jara-Oseguera et al. 2008). In the mice, which had been administered CFA, thermal hyperalgesia was observed, which was manifested by a decreased time of the paw withdrawal in response to thermal effects. To study analgesic activity, the studied polypeptides were tested in the CFA test (Fig. 2).

All the investigated polypeptides showed a significant increase in the presence on the thermostated surface from the control group. At a dose of 0.01 mg/kg, there is a comparable activity of the studied polypeptides APHC1-3, ~21%, and A13 to ~35% relative to the control, and they are significantly different from the "parent" polypeptides APHC1 and 3. A further increase in the dose to 0.1 mg/kg showed that APHC2 and 3 did not increase their activity and remained at the same level ~22% relative to

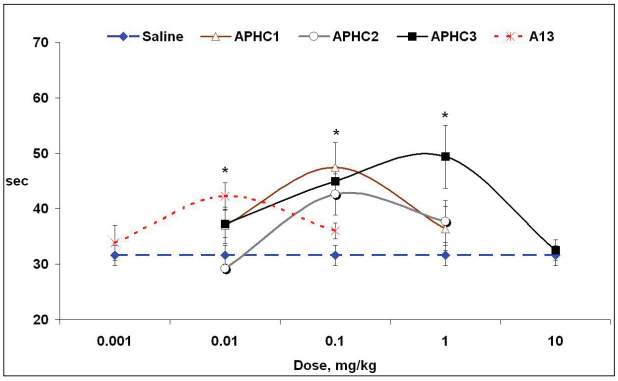

Figure 1. Time (sec) of the first reaction in the hot plate test after administration of polypeptides APHC1, 2, 3 and A13 in the hot plate test (n = 9 for each group). **Note:** * - The results are presented as the mean ± s.e.; * -p<0.05 versus saline group (ANOVA followed by Tukey's test).

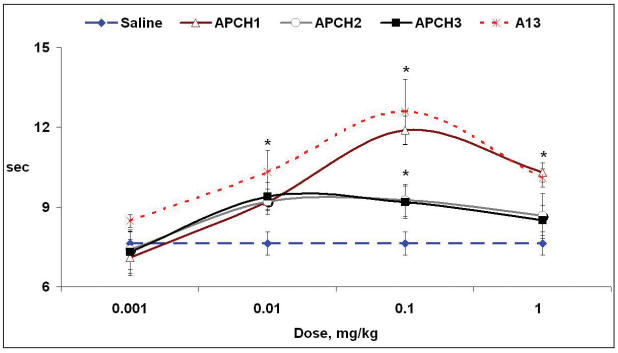

Figure 2. Time (sec) of the first reaction to CFA-induced hypersensitivity after administration of polypeptides APHC1, 2, 3 and A13 in the hot plate test (n = 9 for each group). **Note:** * - The results are presented as the mean ± s.e.; * - $p < 0.05$ versus saline group (ANOVA followed by Tukey's test).

the control. APHC1 and A13 did not differ significantly from each other and increased their activity to ~56% and ~65% relative to the control. At a dose of 1 mg/kg, all the studied polypeptides lost their pharmacological activity, polypeptides APHC2 and 3 did not differ significantly from the control, APHC1 and A13 reduced their activity by ~30% relative to the dose of 0.1 mg/kg. Hybrid polypeptide A13, consisting of APHC1 and APHC3, showed the similar activity of APHC3.

Method to evaluate visceral pain – writhing test (Acetic Acid-Induced Writhing)

Activation of TRPV1 can be triggered by low pH values. Intraperitoneal administration of acetic acid activates TRPV1 receptors, causing specific behavior (writhes) of the experimental animals, which is characterized as visceral pain (Ikeda et al. 2001, Le Bars et al. 2001, Tang et al. 2007). According to the results of the study in hot plate and CFA-induced hypersensitivity tests, further study of pharmacological activity was carried out at a dose of 0.1 mg/kg. The experimental animals were administered the investigated polypeptides 15 minutes before the injection of acetic acid. Polypeptides APHC1 and APHC2 showed a significant decrease in writhes, by ~26 and ~27% relative to the control group. APHC3 and A13 reduced the number of writhes significantly more (by ~50 and ~67% relative to the control group) and significantly differed from APHC1 and APHC2. Conventional molecules that inhibit (Tang et al. 2007) and activate (Lehto et al. 2008) pH-induced TRPV1 currents exhibit similar effects in *in vivo* experiments. They significantly reduce the number of writhes after the introduction of acetic acid. These data were confirmed by the results of the current study, APHC3 and A13 significantly reduced the number of writhes.

Methods to assess the effect of polypeptides on body temperature

TRPV1 is involved in thermoregulation, and almost all its known agonists and antagonists change core body temperature (Garami et al. 2010, Romanovsky et al. 2009). Hyperthermia is a critical side effect of TRPV1 antagonists, so the effect of polypeptides on body temperature was evaluated. As a control group, the antagonist of TRPV1, AMG9810 (30 mg/kg) and Aprotinin (0.1 mg/kg), was used, which is an inhibitor of serine proteases and acts as a homologue of the studied substances. Introduction of saline solution, which was used as solvent of AMG9810 (10% DMSO in normal saline), did not change the body temperature of the experimental animals. The introduction of AMG9810 (as the main antagonist of TRPV1) contributed to an increase in the body temperature of mice by 1.6 °C, as previously reported (Gavva et al. 2007). Serine proteinase inhibitor, polypeptide Aprotinin, caused an increase in body temperature (0.4–0.5 °C), which was not significantly different from the control group. APHC1 and 3 caused a decrease in body temperature of the experimental animals. APHC1 resulted in a more rapid effect of lowering the temperature by -0.8 °C within 30 minutes after injection. The reduced temperature remained throughout the period of observation of the animals in the experiment. APHC3 caused a slow decrease in body temperature, reaching -0.6 °C from the 60th minute after administration. The results obtained were not significantly different from the control group. APHC2 did not cause changes in body temperature. A13 at a dose of 0.1 mg/kg increased body temperature by 2.3±0.2 °C from the 15th minute after administration. A hyperthermal response to A13 administration was observed during 90 min of temperature registration (not shown) and was similar to the effect from low-molecular antagonist AMG9810. Antagonists potentiating pH-induced activation of TRPV1 either reduce or do not change body temperature (Lehto et al. 2008, Romanovsky et al. 2009).

Antagonists interacting with the intracellular TRPV1 channel exhibit a hyperthermal effect if they are able to inhibit pH-induced TRPV1 currents (Honore et al. 2009, Lehto et al. 2008). The hyperthermal reaction that occurs when an antagonist is administered is that the antagonist's ability to inhibit the permanently activated abdominal channels of TRPV1 provokes a cold defence response (Gavva et al. 2007, Steiner et al. 2007). The factors maintaining the channels in the activated state have not yet been identified. PH modulation was proposed as one of the most likely factors (Garami et al. 2010).

Thus, the investigated polypeptide A13 obviously inhibits TRPV1 and affects body temperature as a classic antagonist of this receptor. Consequently, the antagonistic properties of A13 became different from the properties of both initial analgesic peptides.

Conclusion

The results of the study showed that partial inhibition of TRPV1 *in vivo* may be more useful than its complete inhibition. APHC1-3 polypeptides showed significant antinociceptive and analgesic activity *in vivo* at low doses (0.01–0.1 mg/kg). Despite the partial inhibition of TRPV1, polypeptides APHC1-3 significantly reduced pain response both in the tests directly related to the functions of TRPV1 (hot plate, CFA-induced hypersensitivity), and in general models of pain (acetic acid). Unlike most TRPV1 antagonists provoking severe hyperthermia *in vivo*, APHC1-3 caused a moderate decrease in body temperature. Therefore, polypeptides APHC1-3 can be attributed to a new class of modulators of TRPV1, which exhibit pronounced analgesic properties without hyperthermia.

A single substitution of the aspartic acid residue of the polypeptide APHC1 at position 23, by transferring one asparagine residue from the cognate peptide APHC3 led to a significant change in the properties of the molecule. The new polypeptide A13 did not alter the thermal sensitivity of mice, but showed the most significant analgesic activity in the acid-induced pain model, unlike APHC1.

While APHC1 and 3 peptides reduced the body temperature of the experimental animals, showing the properties of incomplete antagonists of TRPV1. Hybrid A13 raised the body temperature of mice, like most non-peptide antagonists of TRPV1, blocking all types of activation of this receptor. Thus, the analgesic properties of APHC1 were enhanced by transferring a single residue from the cognate peptide APHC3. Replacement of a single amino acid (D23 → N23) in APHC1 resulted in analgesic properties inherent in APHC3, and the emergence of a new hyperthermic actions, uncharacteristic for both of the original polypeptides.

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