Naloxone Is a Potential Binding Ligand and Activator of the Capsaicin Receptor TRPV1

Barbora Melkes,* Vendula Markova,* Lucie Hejnova,* Ales Marek,† and Jiří Novotný*,*

*Department of Physiology, Faculty of Science, Charles University; Prague 12800, Czech Republic; and †Institute of Organic Chemistry and Biochemistry, Czech Academy of Sciences; Prague 16000, Czech Republic.

Received September 20, 2019; accepted February 13, 2020

The transient receptor potential vanilloid-1 (TRPV1) channel is a well-known detector of noxious heat and various chemicals. There is increasing evidence for a crosstalk between TRPV1 and opioid receptors. Here we investigated the effect of the prototypical TRPV1 agonist capsaicin and selected opioid ligands on TRPV1 movement in the plasma membrane and intracellular calcium levels in HEK293 cells expressing TRPV1 tagged with cyan fluorescent protein (CFP). We observed that lateral mobility of TRPV1 increased after treatment of cells with capsaicin or naloxone (a nonselective opioid receptor antagonist) but not with DAMGO (a μ-opioid receptor agonist). Interestingly, both capsaicin and naloxone, unlike DAMGO, elicited intracellular calcium responses. The increased TRPV1 movement and calcium influx induced by capsaicin and naloxone were blocked by the TRPV1 antagonist capsazepine. The ability of naloxone to directly interact with TRPV1 was further corroborated by [3H]-naloxone binding. In conclusion, our data suggest that besides acting as an opioid receptor antagonist, naloxone may function as a potential TRPV1 agonist.

Key words naloxone; transient receptor potential vanilloid 1; receptor lateral mobility; fluorescence recovery after photobleaching; calcium

INTRODUCTION

The transient receptor potential vanilloid-1 (TRPV1) channel is a well-known detector of noxious heat. TRPV1 was initially described as a specific target of capsaicin and resiniferatoxin. However, this widely expressed channel protein proved sensitive to many other chemicals, including eicosanoids, phosphoinositide signaling lipids, lysophosphatidic acid, anandamide, protons and peptide toxins. At present, TRPV1 is considered as a polymodal sensor for noxious stimuli that may subserve quite different functions both in the nervous system and various peripheral tissues.

Since TRPV1 receptors act as cation channels permeable to Ca\(^{2+}\) ions, intracellular calcium responses can be detected after opening the channel gates. Senning and Gordon previously demonstrated that the opening of single TRPV1 channels may produce sparklets, representing localized regions of elevated Ca\(^{2+}\). These authors also noted that TRPV1 receptors diffuse laterally in the plasma membrane as they gated. Mobility of these receptors was highly variable and was affected upon channel activation by capsaicin.

There are some indications that TRPV1 may engage in crosstalk with some G protein-coupled receptors, namely μ-opioid receptors (MOR). MOR are known to play a key role in antinociception elicited by opioids. It has been shown that exposure to morphine, a prototypical opioid agonist, can modulate TRPV1 activation and induce the antinociception effects of this drug. Opioid modulation of TRPV1 responses may apparently proceed via the cAMP-dependent protein kinase A (PKA) pathway and mitogen-activated protein kinase (MAPK) signaling pathways. On the other hand, activation of TRPV1, which causes Ca\(^{2+}\) influx through these channels, was found to induce a Ca\(^{2+}\)/calmodulin-dependent translocation of G protein-coupled receptor (GPCR) kinase 5 to the nucleus, thereby blocking MOR phosphorylation, receptor internalization, and β-arrestin signaling without affecting G protein activation by MOR. Hence, the crosstalk between TRPV1 and MOR appears bidirectional.

Membrane-bound receptors and ion channels move around the cell surface. It is known that various factors can affect lateral movement of these signaling proteins in the plasma membrane, including the lipid composition of the membrane, interactions with other signaling molecules and ligand binding. Receptor dynamics (lateral diffusion) in the plasma membrane can be determined using fluorescence recovery after photobleaching (FRAP). Fluorescence photobleaching recovery measurements of GPCRs have yielded insights into the specific protein interactions that control receptor plasma membrane organization and provided further information about the desensitization process and the central role of receptor lateral mobility. We have previously reported that lateral mobility of MOR can be diversely regulated by biased μ-opioid agonists.

Here we set out to investigate whether treatment with selected opioid ligands of HEK293 cells expressing TRPV1 could influence the channel mobility and function. We used the FRAP technique to study the real-time dynamics of the channel in response to the TRPV1 agonist capsaicin and opioid ligands DAMGO and naloxone. The functionality of TRPV1 was monitored by measuring intracellular Ca\(^{2+}\) levels. Surprisingly, naloxone turned out to behave as potential TRPV1 agonist. Therefore, we also tested the ability of this agent to directly bind to TRPV1 by radioligand binding assay employing [3H]-naloxone.

MATERIALS AND METHODS

Materials Lipofectamine 3000 and Opti-MEM medium...
was purchased from Invitrogen (Carlsbad, CA, U.S.A.) and foetal bovine serum was from Thermo Fisher Scientific (Waltham, MA, U.S.A.). Cell Meter™ No Wash and Probencid-Free Endpoint Calcium Assay Kit was from AAT Bioquest (Sunnyvale, CA, U.S.A.). Plasmid pcDNA3.1-TRPV1-CFP was a generous gift from Dr. Leon D. Islas (National Autonomous University of Mexico). The scintillation cocktail CytoScint was from ICN Biomedicals (Irvine, CA, U.S.A.). All ligands and other chemicals were purchased from Sigma-Aldrich (St. Louis, MI, U.S.A.) and they were of the highest purity available.

\[ ^{3} \text{H}\text{-Naloxone Synthesis} \]  
\( \text{(-)-N-Propargylnoroxymorphone (6 mg, 18 \mu \text{mol}) was hydrogenated with carrier-free tri-} \]
tium gas (8.2 Ci) in 1 mL of ethyl acetate catalyzed with 18 mg of Lindlar catalyst (Sigma-Aldrich) for 75 min at 24°C with rapid stirring using a standardized protocol.\textsuperscript{12} \( ^{3} \text{H}\text{-Naloxone hydrochloride (13.7 mCi) was purified by preparative radio-} \]
HPLC (radiochemical purity >99%; specific activity 56.8 Ci mmol\textsuperscript{-1}) and dissolved in ethanol at 1 mCi/mL.

**Cell Culture and Transfection** Human embryonal kidney (HEK293) cells and HMY-1 (HEK293 stably expressing human \( \mu \)-opioid receptor tagged with yellow fluorescent protein)\textsuperscript{13} were routinely cultured in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10% foetal bovine serum (FBS) at 37°C and 5% \( \text{CO}_2 \) humidified atmosphere. The media contained 1% antibiotic–antimycotic solution (AAS) and also 800 µg/mL geneticin in the case of HMY-1. Cells were transfected with enhanced cyan fluorescent protein (CFP)-tagged TRPV1 as described previously.\textsuperscript{13} For transient transfection with the pcDNA3.1-TRPV1-CFP vector, cells were seeded on 12 well plate to 60% confluence. Lipopectamine 3000 (Invitrogen) was used for the transfections according to the manufacturer’s instructions.

**Fluorescence Recovery after Photobleaching** Cells were seeded on glass-bottom dishes and maintained in phenol red-free DMEM supplemented with 10% FBS and 1% AAS. Photobleaching experiments on living cells were performed on an inverted Zeiss LSM 880 confocal laser scanning microscope (Carl Zeiss AG, Germany) equipped with 40×/1.2 WDICIII C Apochromat objective lens and back-thinned CCD camera (Zeiss Axio Cam) as described previously.\textsuperscript{13} As a rule, 15 prebleach images were collected and, immediately after photobleaching, 300 successive postbleach images were recorded to monitor the fluorescence redistribution. The data were analysed using easyFRAP, a MATLAB platform-based tool.\textsuperscript{14}

**Endpoint Calcium Assay** HEK293 cells were plated in 384 well plate (8000 cells per well) and after a 24-h incubation they were transfected with plasmid DNA containing TRPV1-CFP or left untransfected (control). Cells were used for further experiments 24h after transfection. Cell Meter™ No Wash and Probencid-Free Endpoint Calcium Assay Kit (AAT Bioquest, Sunnyvale, CA, U.S.A.) was employed to detect intracellular calcium levels. Fluo-8™ AM dye solution was prepared according to the manufacturer’s instructions and mixed with 1x assay buffer. Then 25 µL of the mixture per well was added to the plate and incubated for 45 min at 37°C. After this time period, agonist dissolved in Hank’s buffer with Hepes (HHBS) buffer was added and the calcium assay was started immediately. The fluorescence intensity at Ex/Em = 490/525 nm was monitored on a fluorescence microplate reader with bottom-read fluorescence detection.

TRPV1 Binding Assay To determine the binding ability of naloxone to TRPV1 we performed a single-point radioligand binding assay with \( ^{3} \text{H}\text{-naloxone. HEK293 cells were seeded on 12-well plates and transected with a plasmid containing TRPV1-CFP construct. Both transfected and non-transfected (control) cells were incubated in serum-free DMEM medium in the presence of 100nM \( ^{3} \text{H}\text{-naloxone for 60 min at 37°C. Naloxone (100µM) was used to define non-} \)
pecific binding. In some experiments, cells were incubated in the presence of DADLE (a selective \( \delta \)-opioid receptor agonist) and/or capsazepine (a selective TRPV1 antagonist). Cells were detached by pipetting and immediately filtered on a Brandel cell harvester through Whatman GF/C filter presoaked with 0.3% polyethyleneimine, which was washed three times with 3 mL of ice-cold buffer B (50mM Tris–HCl and 1 mM MgCl\textsubscript{2}; pH 7.4). The radioactivity trapped was determined by liquid scintillation spectrometry using a Tri-Carb 2910TR counter (PerkinElmer, Inc., Waltham, MA, U.S.A.).

**Statistics** Results are presented as mean ± standard error of the mean (S.E.M.) from at least three independent experiments. Statistical significance was assessed either using Student’s two-tailed unpaired t-test or one-way ANOVA with Bonferroni correction with corresponding \( p \) values of \( *p \leq 0.05; **p \leq 0.01; ***p \leq 0.001; ****p \leq 0.0001. \)

**RESULTS**

The efficiency of transient transfection of HEK293 cells with pcDNA3.1-TRPV1-CFP construct was determined using two different fluorescence microscopy techniques, namely confocal fluorescence microscopy (Fig. 1A) and total internal reflection fluorescence of the bottom plasma membrane (Fig. 1B). Both these methods indicated clear localization of the fluorescence signal of TRPV1-CFP to the plasma membrane. There was no significant difference in fluorescence signal distribution between control cells and those affected by different ligands.

The TRPV1 agonist capsaicin, the TRPV1 antagonist capsazepine, the MOR agonist DAMGO, the MOR antagonist levallorphan and opioid antagonist naloxone were tested for their ability to affect TRPV1 movement in the plasma membrane. FRAP recovery curve analysis provided information about
the mobile fraction ($M_f$) and the apparent diffusion coefficient ($D$) characterizing the surrounding channels able to diffuse into the bleached area during the post-bleach phase of FRAP experiments. Representative fluorescence recovery curves are shown in Fig. 2A. Whereas the mobile fraction of TRPV1 determined under resting conditions significantly decreased after treatment of cells with capsaicin, DAMGO and naloxone did not exert any significant effects on the mobile fraction (Fig. 2B). The value of the apparent diffusion coefficient obtained under resting conditions ($D = 0.76 \pm 0.08 \mu m^2/s$) increased...
more than two times after treatment of cells with capsaicin ($D = 1.81 \pm 0.17 \mu m^2/\text{s}$). On the other hand, incubation of cells in the presence of DAMGO or levalloprphan did not have any effect on TRPV1 mobility. Interestingly, naloxone also markedly (about 2 times) increased the movement of TRPV1 (Fig. 2C). The observed changes in TRPV1 mobility induced by capsaicin and naloxone were blocked by the TRPV1 antagonist capsazepine (Figs. 2B, C). Capsazepine itself did not affect receptor mobility.

In the next set of experiments we assessed the effect of capsaicin, capsazepine, DAMGO, levalloprhan, morphine and naloxone on intracellular Ca$^{2+}$ levels in HEK293 cells expressing TRPV1. Capsaicin is known to mediate calcium influx through TRPV1. As expected, stimulation of cells with this TRPV1 agonist (at a concentration of 1 µM) produced a marked increase (about 100-fold) of intracellular Ca$^{2+}$ concentration. Capsazepine, DAMGO, levalloprhan and morphine (data not shown) did not have any effect, but, quite surprisingly, naloxone at a concentration of 1 µM elevated intracellular Ca$^{2+}$ level about 30 times (Fig. 3). Interestingly, naloxone displayed similar potency but lower efficacy than capsaicin. Calcium responses induced by capsaicin and naloxone were blocked by the TRPV1 antagonist capsazepine (data not shown).

In order to determine whether naloxone may directly interact with TRPV1, an investigation based on a radioligand binding assay with $[^3H]$-naloxone was conducted. In this experiment, the extent of 100 nM $[^3H]$-naloxone binding to HEK293 cells expressing TRPV1 was compared with non-transfected (control) HEK293 cells. Results of this analysis indicated a 35% increase in the number of $[^3H]$-naloxone binding sites in TRPV1-bearing cells, compared to control naïve cells (Fig. 4). The δ-opioid receptor (DOR) agonist DADLE (10 µM) was used to block the binding of $[^3H]$-naloxone to DOR that are endogenously expressed in HEK293 cells. As can be seen from the graph in Fig. 4, DADLE almost totally prevented the binding of $[^3H]$-naloxone to naïve HEK293 cells but not to TRPV1-bearing cells. The number of $[^3H]$-naloxone binding sites in HEK293/TRPV1 cells decreased by about 50% in the presence of DADLE. Interestingly, capsazepine did not significantly affect $[^3H]$-naloxone binding in HEK293 cells, but markedly reduced (by about 50%) the number of $[^3H]$-naloxone binding sites in HEK293/TRPV1 cells. The blocking effects of these two ligands were additive, indicating that capsazepine could displace $[^3H]$-naloxone from available TRPV1 and DADLE displaced $[^3H]$-naloxone from endogenously expressed DOR.

**DISCUSSION**

In the present study, we wished to examine the possible impact of selected opioid ligands on TRPV1 movement in the plasma membrane and its function. We were especially interested in uncovering what happens with TRPV1 within the span of a few minutes after channel activation with capsaicin. Beside the previously described large pool of immobile fraction of TRPV1, we detected yet another fraction of receptors that was unnoticed before. These channels displayed different behaviour and diffused very quickly in the plasma membrane. Interestingly, we were able to detect a significantly increased TRPV1 lateral movement after adding capsaicin or naloxone, albeit the latter ligand affected the channel mobility to a lesser extent. In the case of the MOR agonists DAMGO and antagonist levalloprhan, we did not found any changes either in the rate of diffusion or in the mobile fraction of TRPV1. It can be hypothesized that interactions of TRPV1 with specific protein partners may change its mobility. Different molecular complexes can apparently have different mobility. It was shown earlier that the lateral mobility of receptors in the plasma membrane can be influenced by interactions with their signaling partners.

It was recently demonstrated that TRPV1 mobility immediately (seconds) after activation of the channel by capsaicin markedly decreased. Previous studies dealing with time and spatial membrane organization of TRPV1 after binding to specific cytoskeletal proteins, microtubules and caveolin-1, revealed that there are different populations of TRPV1 in the plasma membrane. There are usually two or three different pools of membrane-associated receptors that behave differentially. For example, two major neurkinin-1 receptor populations were discerned, one showing high mobility and low lateral restriction and the other showing low mobility and high lateral restriction. In the case of TRPV1 the mobile receptor fraction represented about fifty percent of the total channel population. Interestingly, the mobile fraction rather decreased after treatment of cells with capsaicin but did not change in the presence of naloxone. As stated above, both capsaicin and naloxone increased the channel diffusion coefficient, but the effect of capsaicin was more pronounced. The distinct changes in TRPV1 mobility observed after capsaicin and naloxone treatment may indicate that these ligands affect the channel in a specific manner. These data also confirm the notion that channel activation state is an important determinant of the channel membrane dynamics.

We also assessed intracellular calcium levels in TRPV1-bearing HEK293 cells after adding selected ligands. It is well

---

**Fig. 4. Binding of Naloxone to Naïve HEK293 Cells and HEK293 Cells Expressing TRPV1**

Single-point radioligand binding assay was conducted using 100nM $[^3H]$-naloxone. HEK293 cells were transiently transfected with TRPV1-CFP construct (HEK293/TRPV1). The ability of selective ligands to displace $[^3H]$-naloxone binding to DOR and TRPV1 was assessed in the presence of 10µM DAMGO (D) and 10µM capsazepine (CPZ), respectively. Data are means ± S.E.M. from three independent experiments performed in triplicates ($**p \leq 0.01; ***p \leq 0.001$ vs. naïve untreated HEK293 cells; $\dagger\dagger p \leq 0.01$ vs. untreated HEK293/TRPV1 cells; $\dagger\dagger\dagger p \leq 0.001$ vs. D; $\ddagger\ddagger p \leq 0.01$ vs. CPZ).
known that capsaicin causes the opening of TRPV1 channel and massive Ca$^{2+}$ influx.\textsuperscript{59} We observed that naloxone, besides capsaicin, could markedly increase intracellular Ca$^{2+}$ concentration. By contrast, DAMGO, levallorphan and morphine did not affect intracellular Ca$^{2+}$ level. Importantly, it is known that different compounds that interact with TRPV1 may cause Ca$^{2+}$ influx through TRPV1 to different extents.\textsuperscript{20} The ability of naloxone to bind to Ca$^{2+}$ influx through TRPV1 suggests that this ligand may directly interact with the channel. The ability of naloxone to bind to TRPV1 was confirmed by radio-ligand binding. These findings support the notion that naloxone is able to bind to and promote TRPV1 activity.

CONCLUSION

Altogether, we observed that the selective MOR agonist DAMGO and antagonist levallorphan influenced neither TRPV1 mobility nor intracellular Ca$^{2+}$ levels. By contrast, the opioid antagonist naloxone markedly increased the rate of TRPV1 diffusion in the plasma membrane and promoted Ca$^{2+}$ influx through these channels, albeit not to the same extent as the TRPV1 agonist capsaicin. These findings imply that naloxone may function as a potential TRPV1 agonist. The presumed naloxone’s ability to directly interact with TRPV1 was confirmed by its binding to the channel. This is the first study to show that naloxone may directly affect the function of TRPV1. The potential of naloxone to interfere with TRPV1 signaling should be taken into consideration when using this drug in future experiments, as well as when evaluating the molecular mechanism of its therapeutic effects.

Acknowledgments This work was supported by the Charles University, project GA UK No. 668216 and institutional project SVV-260434/2019 and cofinanced by the European Regional Development Fund and the state budget of the Czech Republic (Project No. CZ.1.05/4.1.00/16.0347 and CZ.2.16/3.1.00/21515). Synthesis of [3H]-naloxone was supported by the Czech Academy of Sciences (research program RVO: 6138963).

Conflict of Interest The authors declare no conflict of interest.

REFERENCES

1) Szallasi A, Blumberg PM. Characterization of vanilloid receptors in the dorsal horn of pig spinal cord. Brain Res., 547, 335–338 (1991).
2) Vriens J, Appendino G, Nilius B. Pharmacology of vanilloid transient receptor potential cation channels. Mol. Pharmacol., 75, 126–129 (2009).
3) Gerton M, Hazan A, Priel A. Animal toxins providing insights into TRPV1 activation mechanism. Toxins (Basel), 9, 326 (2017).
4) Senning EN, Gordon SL. Activity and Ca$^{2+}$ regulate the mobility of TRPV1 channels in the plasma membrane of sensory neurons. eLife, 4, e03819 (2015).
5) Pasternak GW. Molecular insights into mu opioid pharmacology: from the clinic to the bench. Clin. J. Pain, 26 (Suppl. 10), S3–S9 (2010).
6) Bao Y, Gao Y, Yang L, Kong X, Yu J, Hou W, Hua B. The mechanism of μ-opioid receptor (MOR)-TRPV1 crosstalk in TRPV1 activation involves morphine anti-nociception, tolerance and dependence. Channels (Austin), 9, 235–243 (2015).
7) Scherer PC, Zaeccor NW, Neumann NM, Vasavada C, Barrow R, Ewald AJ, Rao F, Sumner CJ, Snyder SH. TRPV1 is a physiological regulator of μ-opioid receptors. Proc. Natl. Acad. Sci. U.S.A., 114, 13561–13566 (2017).
8) Agula B, Sinaan M, Laporte SA. Study of G protein-coupled receptor/β-arrestin interactions within endosomes using FRAP. Methods Mol. Biol., 756, 371–380 (2011).
9) Carayon K, Moulédous L, Combedazou A, Mazères S, Haanappel E, Salome L, Mollereau C. Heterologous regulation of Mu-opioid (MOP) receptor mobility in the membrane of SH-SYSY cells. J. Biol. Chem., 289, 28697–28706 (2014).
10) Moravcova R, Melkes B, Novotny J. TRH receptor mobility in the plasma membrane is strongly affected by agonist binding and by interaction with some cognate signaling proteins. J. Recept. Signal Transduct. Res., 38, 20–26 (2018).
11) Melkes B, Hejnova L, Novotny J. Biased μ-opioid receptor agonists diversely regulate lateral mobility and functional coupling of the receptor to its cognate G proteins. Naunyn Schmiedebergs Arch. Pharmacol., 389, 1289–1300 (2016).
12) Filer CN, Ahern DG, Fazio R, Seguin RJ. Reduction of the N-propargyl group with triuim—general procedure for the preparation of N-[2,3-3H]allyl opiate ligands at high specific activity. J. Org. Chem., 46, 4968–4970 (1981).
13) De-la-Rosa V, Rangel-Yescas GE, Ladrón-de-Guevara E, Rosenbaum T, Islas LD. Coarse architecture of the transient receptor potential vanilloid 1 (TRPV1) ion channel determined by fluorescence resonance energy transfer. J. Biol. Chem., 288, 29506–29517 (2013).
14) Rapsomaniki MA, Kotsantis P, Symeonidou I-E, Giakoumakis N-N, Taraviras S, Lygerou Z. easyFRAP: an interactive, easy-to-use tool for qualitative and quantitative analysis of FRAP data. Bioinformatics, 28, 1800–1801 (2012).
15) Thurner P, Gsandtner I, Kudlacek O, Choquet D, Nanoff C, Freissmuth M, Zezula J. A two-state model for the diffusion of the A2A adenosine receptor in hippocampal neurons: agonist-induced switch to slow mobility is modified by synapse-associated protein 102 (SAP102). J. Biol. Chem., 289, 9263–9274 (2014).
16) Storti B, Dr Rienzo C, Cardarelli F, Bizzarri R, Beltram F. Unveiling TRPV1 spatio-temporal organization in live cell membranes. PLOS ONE, e0116900 (2015).
17) Storti B, Bizzarri R, Cardarelli F, Beltram F. Intact microtubules preserve transient receptor potential vanilloid 1 (TRPV1) functionality through receptor binding. J. Biol. Chem., 287, 7803–7811 (2012).
18) Veya L, Piguet J, Vogel H. Single molecule imaging decipher the relation between mobility and signaling of a prototypical G protein-coupled receptor in living cells. J. Biol. Chem., 290, 27732–27735 (2015).
19) Caterina MJ, Schumacher MA, Tominaga M, Rosen TA, Levine JD, Julius D. The capsaicin receptor: a heat-activated ion channel in the pain pathway. Nature, 389, 816–824 (1997).
20) Töth A, Wang Y, Kedei N, Tran R, Pearce LV, Kang SU, Jin MK, Choi HK, Lee J, Blumberg PM. Different vanilloid agonists cause different patterns of calcium response in CHO cells heterologously expressing rat TRPV1. Life Sci., 76, 2921–2932 (2005).