Scientific paper

TRPV1 Channel as New Target for Marine Toxins: Example of Gigantoxin I, a Sea Anemone Toxin Acting Via Modulation of the PLA_2 Pathway

Eva Cuypers,[1] Steve Peigneur,[1] Sarah Debaveye,[1] Kazuo Shiomi[2] and Jan Tytgat[1,*]

[1] *Laboratory of toxicology, Campus Gasthuisberg, Onderwijs en navorsing 2, Herestraat 49, P.O. 922, B-3000 Leuven, Belgium*

[2] *Department of Food Science and Technology, Tokyo University of Marine Science and Technology, Konan-4, Minato-ku, Tokyo 108-8477, Japan*

** Corresponding author: E-mail: jan.tytgat@pharm.kuleuven.be*

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Dedicated to the memory of Professor Franc Gubenšek

Abstract

Gigantoxin I, isolated from sea anemone *Stichodactyla gigantea*, was previously described as the first epidermal growth factor (EGF)-like toxin from natural origin. In this study, we discovered the interaction between the transient receptor potential vanilloid subtype I (TRPV1) channels and gigantoxin I. The TRPV1 channel is a non-selective cation channel involved in pain sensation and is described as pharmacological target of cnidaria venom. Our results highlight the involvement of the epidermal growth factor receptor/phospholipaseA_2/arachidonic acid/lipoxygenase (EGFR/PLA_2/AA/LOX) pathway in the indirect activation of TRPV1 channels by gigantoxin I. This is the first time that this pathway is described in the indirect activation of TRPV1 channels by toxins. This knowledge not only gives insights into the possible induced effects by this new group of toxins, but also leads to a better understanding of the regulatory mechanism of TRPV1 channels themselves.

Keywords: Capsaicin receptor, Phospholipase A_2, Epidermal growth factor, Lipoxygenase, Arachidonic acid

1. Introduction

The phylum of Cnidaria represents exclusively aquatic animals such as sea anemones and jellyfish. With more than 7600 species, the Cnidaria is one of the largest phyla of marine animals. According to their morphological characters, the cnidarians are divided into 4 classes: the Anthozoa (meaning "flower animals") including corals and sea anemones, the Hydrozoa (meaning "water animals") which include for example the Portuguese man-of-war and fire corals, the Cubozoa ("cube animals") which include the box jellyfish and the Scyphozoa (meaning "bowl animals") which include the jellyfish. The name Cnidaria comes from the Greek word "cnidos" which means stinging nettle. Despite the fact that they are incredibly diverse in form, they are united because of their stinging cells called nematocysts. These stinging cells are used to capture and subdue the prey. The nematocyst is a coiled thread-like stinger. When the nematocyst is called upon to fire, the thread is uncoiled, and springs straight. The harpoon-like thread punctures through the cnidocyte wall into the prey. Most cnidarians have a toxin in their stinger that helps to immobilize the prey. The nematocyst is fired either by the tentacle touching prey or in some cases by a nerve impulse from the animal telling it to fire.[1] Most cnidarian nematocysts are not harmful to humans, as the stinger cannot penetrate sufficiently into human skin to inflict any harm. There are some jellyfish, however, which can deliver extremely painful and, in a few cases, even fatal stings to humans.

Sea anemones are a rich source of protein and peptide toxins which are generally divided into three classes: hemolysins (15–21 kDa),[2–5] sodium channel toxins (3–5 kDa)[6–9] and potassium channel toxins (3.5–6.5 kDa).[10–14] However, Shiomi and Honma isolated gigantoxin I, a peptide toxin from *Stichodactyla gigantea* that has 33% homologies with mammalian epidermal growth factor (EGF)[15] (Figure 1). In accordance with these sequence homologies, including the location of the six cysteine residues, this toxin exhibits EGF activity as rounding of human epidermoid carcinoma A431 cells. Herein, a redistribution of PLC-γ1 to the plasma membrane area, in particular newly formed ruffles accompanied by increased actin polymerization, was observed.[16] Also tyrosine phosphorylation of the EGF receptor was described.[15] Interestingly, gigantoxin I is weakly lethal but potently paralytic to crabs. In spite of these known biological activities, the pharmacological target(s) of this toxin remain(s) unclear. In this study, we investigated the effect of gigantoxin I on the Transient Receptor Potential Vanilloid subtype 1 (TRPV1) channel that was previously described as pharmacological target of cnidaria (*e.g.* sea anemone) venoms and polycyclic ether toxins.[17–18] Only recently, the first sea anemone peptide, APHC1, isolated from *Heteractis crispa*, with analgesic and antagonistic activity on TRPV1 channels has been reported.[19] Unfortunately, no molecular action working mechanism has been put forward and it is currently not known if APHC1 interacts directly or indirectly with TRPV1 channels. It is only very recently that the first action mechanism of a toxin activating TRPV1 channels was revealed, showing that the outer pore domain is the targeting mechanism of a bivalent tarantula toxin.[20]

Transient receptor potential (TRP) channels constitute a superfamily of cation permeable channels. The founding member of this family was identified as a *Drosophila* gene product required for visual transduction.[21] Spontaneous mutations in the TRP-gene lead to TRP-deficient flies which are blinded by intense light.[22] This is caused by a disruption of subsequent Ca^{2+}-dependent adaptation and thus leads to a sustained Ca^{2+} entry via TRP ion channels. The name "transient receptor potential" is based on the transient (normal) rather than sustained (mutation) response to light of the flies carrying a mutant in the trp locus. Further studies on the *Drosophila melanogaster* phototransduction indicated that TRP is the target of a phosphoinositide cascade. This leads to the suggestion that phototransduction in *Drosophila* might be analogous to the general and widespread process of phosphoinositide-mediated Ca^{2+} influx in other cells.[23]

The TRPV1 channel, a ligand-gated and non-selective cation channel that is expressed in peripheral sensory neurons, is one of the key players in peripheral pain sensation (for an overview of its function and expression profi-

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                1         10        20        30        40
Gigantoxin I    DVGVACTGQYASSFCLNGGTCRYIPELGEYYCICPGDYTGHRCEQMSV
Human EGF       NSDSECPLSHDG YCLHDGVCMYIEALDKYACNCVVGYIGERCQYRDLKWWELR
Pig EGF         NSYSECPPSHDG YCLHGGVCMYIEAVDSYACNCVFGYVGERCQHRDLKWWELR
Mouse EGF       NSYPGCPSSYDG YCLNGGVCMHIESLDSYTCNCVIGYSGDRCQTRDLRWWELR
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Figure 1. Sequence homology between gigantoxin I and EGF factors

Table 1. Overview of the function and expression profiles of TRPV channels (P_{Ca}, permeability for Ca ions; P_{Na}, permeability for Na ions; TG, trigeminal ganglia; DRG, dorsal root ganglia).

gene name TRPV subfamily	P_{Ca}/P_{Na}	proposed functions / possible disease connections	highest expression
TRPV1	3.8 (heat), 9.6 (vanilloids)	detection of mild noxious heat, taste/ functional bowel disease, inflammatory bowel disease, ostheoarthritis, pancreatitis, impaired bladder contractions, impaired thermal hyperalgesia, asthma, cystitis, schizophrenia, gastroesophagal reflux	TG, DRG neurons, urinary bladder, testis, brain, respiratory tract, gastrointestinal tract
TRPV2	3	sensing thermal pain, mechanosensing/ role in cardiomyopathy, cardiac hypertrophy, interstitial fibrosis, muscular dystrophia, prostate cancer	DRG, spinal cord, brain, spleen, intestine
TRPV3	2.6	warm sensing, osmosensing/defective environmental thermosensation, hairlessness, atopic dermatitis, breast cancer, non-isulin-dependent diabetes mellitus	TG, DRG, spinal cord, brain, keratinocytes, tongue
TRPV4	6	osmosensing, warm sensing, nociception, pressure sensing/ hypotonic hyperalgesia, impaired acidic nociception, impaired thermal hyperalgesia, asthma, neuropathic pain, hypertension, cardiopathy	DRG, kidney, lung, spleen, testis, heart, liver, keratinocytes, endothelia

le, refer to Table 1). TRPV1 is heat and acid sensitive and can be activated by pungent substances like capsaicin. Although capsaicin had been already known since ancient times to have an analgesic effect, the channel was only discovered in 1997 as the physiological target of capsaicin. Despite the fact that numerous analgesics are already on the market, pain treatment remains one of the major problems in the medical world. Therefore, the search for new and more effective analgesics is still going on. The discovery of TRPV1 channels started a new chapter in the research to novel generation analgesics. In order to prevent side effects, it is very important to know the mechanisms and pathways involved in TRPV1 channel functioning. Therefore, basic pharmacological research is indispensable.

Members of the TRP channel superfamily share the common structural features of six transmembrane segments (TM) which cross the cell membrane, a pore forming hydrophobic loop between the fifth and the sixth segment and both cytosolic carboxy- and aminotermini (Figure 2). Although this basic topology is common to many ion channel families, such as voltage-gated potassium channels, TRP and voltage-gated potassium channels show relatively little sequence homology. A likely common feature between voltage-gated potassium channels and TRP channels is their tetrameric structure. Recently, the 19 Å structure of TRPV1 channels was partly revealed by using electrocryomicroscopy.[24] It exhibits fourfold symmetry and comprises two distinct regions: a large open basket-like domain, likely corresponding to the cytoplasmic N- and C-terminal portions, and a more compact domain, corresponding to the transmembrane portion. Remarkably, a similar two-domain arrangement referred to as a "hanging gondola" has been observed in 3D structures of $K_v1.2$ channel. In general, this structure follows the basic structural organisation described above.

The TRPV1 channel is known as the heat transducer in sensory neurons because the channel is activated at a temperature above 42 °C. Well-known direct activators are capsaicin, the pungent substance found in hot chilli peppers, and protons. *In vivo* and *in vitro* studies reveal that inflammatory mediators like bradykinin, prostaglandine E_2 (PGE_2), extracellular ATP, glutamate and nerve growth factor can indirectly sensitize TRPV1 channels.[25–27] This is possible via various mechanisms such as increasing TRPV1 channel expression levels in the membrane,[28–29] inducing TRPV1 channel phosphorylation by protein kinases[30–31] or releasing channel inhibition by phosphatidylinositol 4,5-biphosphate (PIP_2) and thus sensitizing the channel for agonist stimulation.[30–32] In addition, these inflammatory mediators act on G-protein coupled receptors (GPCRs) or, in case of nerve growth factor, on tyrosine kinase pathways. These can induce on their turn, via activation of phospholipase C (PLC) and/or phospholipase A_2 (PLA_2), the release of arachidonic acid (AA) and lipoxygenase products of arachidonic acid (*e.g.* hydroperoxyeicosatetraenoic acid or HPETE), substances which are known as TRPV1 channel agonists.[33–34] Finally, the endogenous ligand anandamide has also been shown to activate TRPV1 channels.[35]

In our work presented here, we have set out to elucidate the pharmacological mechanism of the molecular interaction between the sea anemone peptide gigantoxin I and TRPV1 channels, and have investigated several regulating and activating pathways of TRPV1 channels. It is concluded that the interaction between the toxin and TRPV1 channels is PLA_2 pathway dependent.

2. Materials and Methods

2. 1. Isolation of Gigantoxin I and Determination of Amino Acid Sequence

The isolation and amino acid determination of gigantoxin I was described previously by Shiomi and Honma.[15]

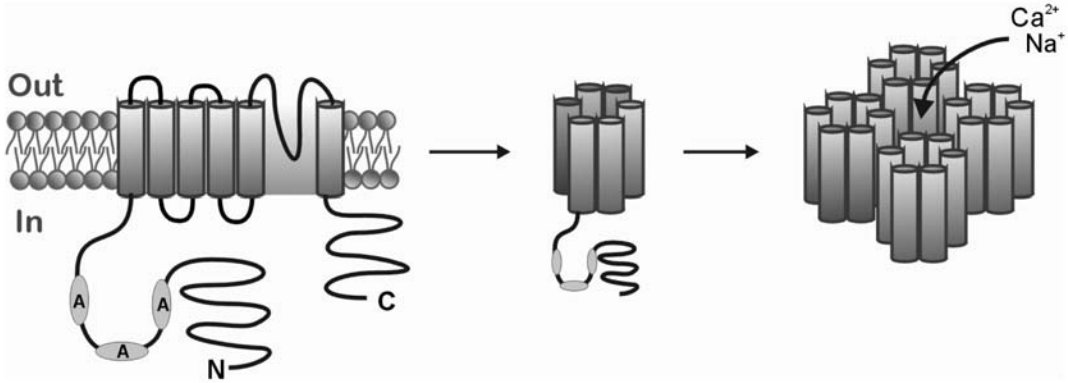

Figure 2. Basic architecture of TRP (TRPV1) channels. Tetrameric structure of the functional channel exists of four domains. One domain has six transmembrane segments and a pore forming hydrophobic loop between the fifth and the sixth segment. Ankyrin-binding domains (A) can be found in the N-terminus.

2. 2. Molecular Biology

cRNA transcripts were synthesized from XbaI-linearized VR1 cDNA templates using T7 RNA polymerase (Ambion).

Mutant S502A/S800A was made using Quick Change II site-directed mutagenesis kit (Stratagene). In a first step, mutant S502A was made using primers 5'-GGGGACTATTTCCGAG**GCA**CCGGAGA-GATCTTGTC-3' and 5'-GACAAGATCTCTCCGG**TG C**CTCGGAAATAGTCCCC-3'. Cycling parameters were set according to the manufacturer's guidelines. The mutated cDNA was transformed into XL-1 blue cells and cDNA was isolated using Wizard Miniprep kit (Promega). The construct was checked by cDNA sequencing. In a second step, mutant S800A was made starting from the S502A mutant cDNA. The following primers were used; 5'-CGTCAAGCGCACCCTG**GCC**TTCTCCCT GAGGTCAG-3' and 5'-CTGACCTCAGGGAGAA**G GC**CAGGGTGCGCTTGACG-3'. RNA was synthesized from the linearized plasmid using T7 mMessage mMachine transcription kit. A second cDNA sequencing confirmed the sequence of the full S502A/S800A construct.

Concentration of cRNA was determined by measuring the absorbance at 230, 260 and 280 nm.

2. 3. Expression in Xenopus Oocytes and Electrophysiology

Oocytes were harvested from anaesthetized female *Xenopus laevis* frogs as previously described[36] and were injected with 0.5–5 ng TRPV1 cRNA. Oocytes were incubated in ND96 solution supplemented with 50 mg/L gentamycin. Two to seven days after injection, two-electrode voltage-clamp recording was performed. Currents were measured in ND96 solution at a holding potential of –90 mV during 400 s. The recording chamber was perfused at a rate of 2 mL min^{-1} with a ND-96 solution containing (in mM) 96 NaCl, 2 KCl, 1.8 $CaCl_2$, 1 $MgCl_2$, 5 HEPES (pH 7.4). Temperature of the perfusate was kept at 22 °C and controlled using a SC-20 dual in-line heater/cooler (Warner Instruments) and pH was kept at 7.4. As previously described,[37] capsaicin (2 μM) was used as an agonist and capsazepine (10 μM) as an antagonist of TRPV1 channels. Capsaicin, capsazepine, Kt5720, U-73122, wortmannin and 5,8,11,14-eicosatetraynoic acid (ETYA) were all purchased from Sigma and dissolved in DMSO. The final dilution in ND96, used as test or incubation solution, contained maximal 0.5% DMSO. Oocytes were incubated for at least 30 minutes in Kt5720, U-73122, wortmannin or ETYA before measurements were done. All experiments were carried out on at least 3 different oocytes. Gigantoxin I was dissolved in ND96 and administered using the described perfusion system.

3. Results

Administration of 1.5 μM and 3 μM gigantoxin I on wild type TRPV1 channels induced no visible effect (n = 3) (Figure 3A). In contrast, when the toxin (1 μM) was administrated in the presence of capsaicin (2 μM), an activator of TRPV1 channels, an extra inward current was discernible (n = 10) (Figure 3B). A similar phenomenon has been observed with cnidaria venom and polycyclic ether toxins and was defined as positive allosterism.[17–18]

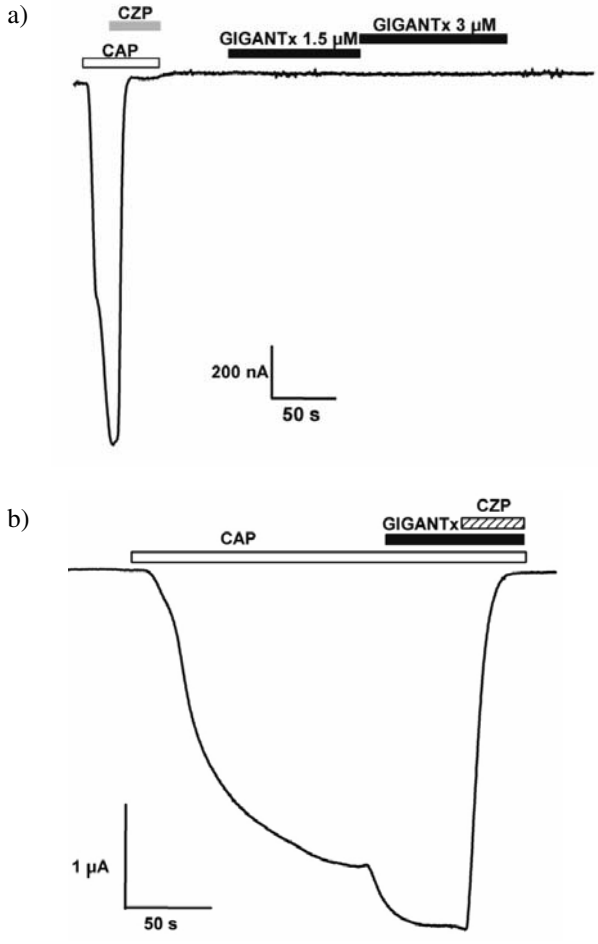

Figure 3. a) Example of TRPV1 current induced by application of 2 μM capsaicin (CAP). No current is visible after application of 1.5 and 3 μM gigantoxin I (GIGANTx). Capsazepine is used as a TRPV1 channel blocker. **b)** Example of the extra inward current visible when 1 μM GIGANTx is added together with CAP (2 μM). In (A) and (B), capsazepine (CZP, 10 μM) was used as a TRPV1 channel blocker.

In order to elucidate the mechanism behind this allosteric effect, regulatory factors of TRPV1 channels which might be involved in this effect were investigated step by step (Figure 4). The first factor we excluded was protein kinase C (PKC). This factor is known to sensitize the channel by phosphorylation on two serine residues (Ser-

502 and Ser-800). A TRPV1 channel mutant was made replacing both serine residues into alanines (S502A/S800A mutant). This mutant has already been described and is generally accepted as research tool in the involvement of PKC in the effect of substances on TRPV1 channels.[38] Tests of gigantoxin I on this mutated channel showed the same allosteric effect of the toxin when administered together with capsaicin (n = 4), indicating that PKC induced phosphorylation is not the main working mechanism. The second intracellular regulator we checked was protein kinase A (PKA). Because four phosphorylation sites on the TRPV1 channel are known for PKA (serine-116, threonine-144, threonine-370 and serine-502), we have chosen to use the potent and selective PKA inhibitor, Kt5720. This pharmacological tool is widely used when investigating the role of PKA in the modulation of ion channels.[39] Control tests were carried out in order to check the activity of Kt5720. Indeed, the capsaicin induced current after 30 min incubation in Kt5720 (3 μM) was significantly decreased, indicating that the PKA dependent recovery from desensitization was prevented (data not shown).[40] Measurements on Kt5720 incubated oocytes showed the same allosteric effect of the toxin together with capsaicin (n = 3), indicating that PKA is most probably not involved in the toxin effect. A third factor that might be involved in the described action of gigantoxin I is PIP_2. It has been shown that mutations that weaken the TRPV1-PIP_2 interaction induce a reduction of thresholds for chemical and physical stimuli.[41] Therefore, it can be postulated that gigantoxin I reduces the threshold to capsaicin by promoting the hydrolysis of PIP_2 by phospholypase C (PLC) into diacylglycerol (DAG) and inositol (1,4,5)-triphosphate (IP_3). As a consequence, more TRPV1 channels will be activated by the same capsaicin concentration, leading to a greater inward current. In order to test this hypothesis, 3 μM U-73122, a PLC and PLA_2 inhibitor, was added to the incubation solution as described before.[42–43] Control experiments were carried out. Herein, desensitization of TRPV1 channels to capsaicin was clearly inhibited by U-73122 (data not shown).When gigantoxin I was tested on

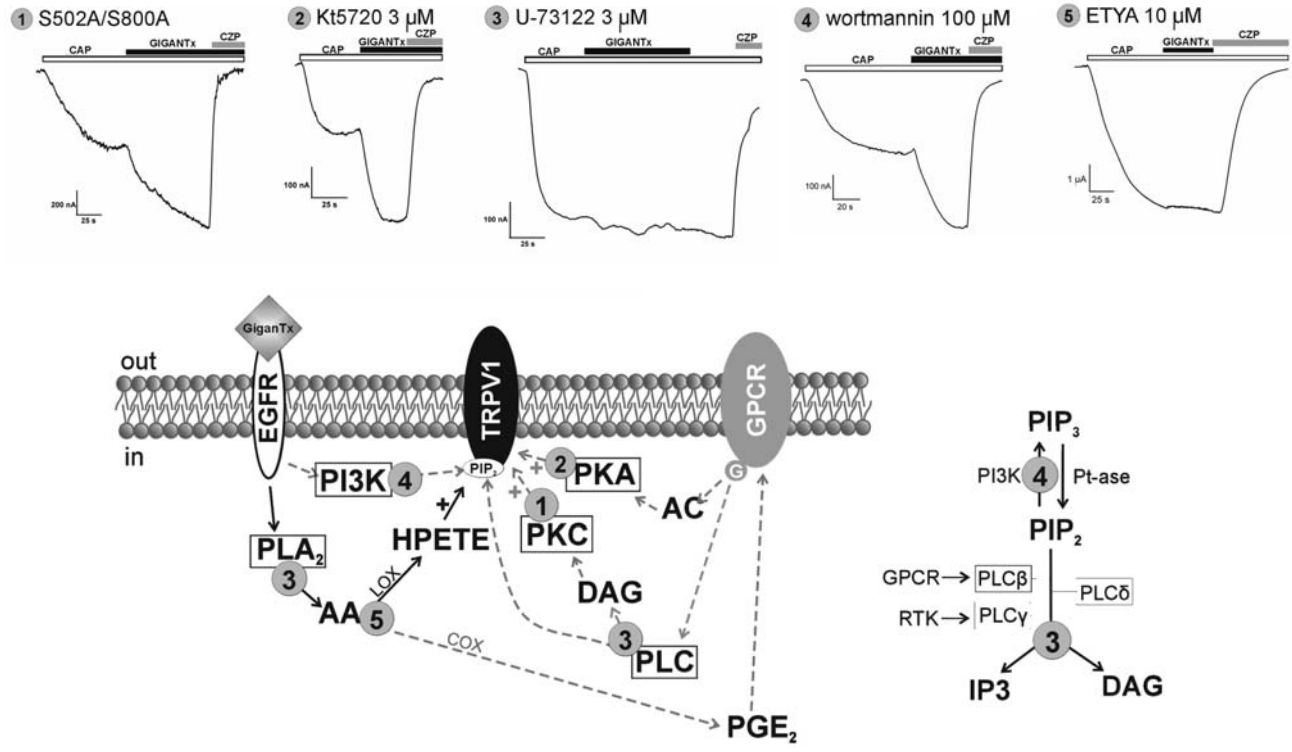

Figure 4. The involvement of different intracellular pathways in the gigantoxin I induced effect on TRPV1 channel is checked. Numbers indicated in the pathway figures refer to tests excluding specific pathways. The corresponding currents are shown.
1. Gigantoxin I effect is tested on S502A/S800A mutants in order to check the involvement of PLC. The allosteric effect is still visible, indicating no mayor involvement of this pathway. **2.** Kt5720 is used as PKA inhibitor. Current show visible effect of the toxin, indicating no mayor effect of PKA in the induction of the gigantoxin I effect. **3.** U-73122, used as non-specific PLC and PLA_2 inhibitor, blocked the allosteric effect of gigantoxin I. **4.** Wortmannin incubation, leading to a higher PIP2 concentration via the block of PI3K, does not influence the gigantoxin I effect on TRPV1 channels. **5.** ETYA, a non-specific COX and LOX inhibitor, blocked the allosteric effect. The pathway that appears to be involved in the described effect is indicated with black arrows.
TRPV1, transient receptor vanilloid subtype 1; CAP, capsaicin; CZP, capsazepin; PIP3, phosphatidylinositol triphospate; AC, adenylate cyclase; GIGANTx, gigantoxin I; EGFR, epidermal growth factor receptor; GPCR, G-protein coupled receptor; PLA_2, phospholipase A2; AA, arachidonic acid; COX, cyclooxygenase; LOX, lipoxygenase; PIP2, phosphatidylinositol biphosphate; HPETE, hydroperoxyeicosatetraenoic acid; PKC, protein kinase C; PKA, protein kinase A; PLC, phospholipase C; DAG, diacylglycerol; IP_3, inositol (1,4,5)-triphosphate; PGE_2, Prostaglandine E2.

the incubated oocytes, no further effect was visible (n = 5). This test leaves two open possibilities: the toxin decreases the PIP_2 concentration or PLA_2 is involved in the induction of the effect. Therefore, wortmannin (100 μM), an inhibitor of phosphoinositide 3-kinases (PI3K) was added to the incubation solution. PI3K phosphorylates PIP_2 into PIP_3 and thus decreases the PIP_2 concentration. Indeed, previous articles describe the depletion of PIP_2 in pre-incubated cells with high concentrations of wortmannin.[34–44] Interestingly, in our test the toxin still showed an allosteric effect with capsaicin (n = 6). As a corollary, the only remaining possibility is a PLA_2 involvement. The involvement of this pathway was checked by adding ETYA, a non-specific inhibitor of cyclooxygenases (COX) and lipoxygenases (LOX). Both compounds are connected to the PLA_2 pathway (Figure 4). In this test, no effect was visible confirming the involvement of PLA_2 pathway in the effect of gigantoxin I on TRPV1 channels (n = 3).

Since voltage-gated potassium and sodium channels are considered as the prime targets of sea anemone peptides, the effect of 1 μM gigantoxin I was also tested on a large collection of ion channels, including: K_v 1.1, K_v 1.2, K_v 1.3, K_v 1.4, K_v 1.5, K_v 1.6, *Shaker*IR, h*ERG*, Na_v 1.1, Na_v 1.2, Na_v 1.4, Na_v 1.5, Na_v 1.6, Na_v 1.7, Na_v 1.8 and $DmNa_v1$. Here, no effect on these subtypes was found (data not shown), indicating a certain selectivity of gigantoxin I.

4. Discussion

Gigantoxin I, the first isolated EGF-like toxin of natural origin, was previously described to have crab toxicity with weakly lethal effect. Nevertheless, the physiological target of this toxin remained unclear. In this study, an allosteric effect on TRPV1 channels was shown. Remarkably, this effect corresponds with the previously described effect of cnidaria venom, indicating that EGF-like toxins might be the link between the cnidaria venom and TRPV1 channels. Because this new information may lead to the development of new analgesic treatments in cnidaria intoxication, it is important to find out the exact action mechanism of this TRPV1 channel modulating toxin. This study shows the involvement of the intracellular PLA_2 dependent pathway. Keeping in mind that gigantoxin I possesses 33% homology with the mammalian EFG factor (see Figure 1), we hypothesize that gigantoxin I acts as an EGF factor by stimulating a tyrosine kinase activity of the EGF receptor and thus the calcium-dependent PLA_2 activity. As a consequence, PLA_2 releases AA which is oxidized by LOX generating metabolites such as HPETE which in turn act as TRPV1 channel agonist. Prostaglandine E2 (PGE_2), another AA metabolism product generated via COX, can act on G-protein coupled receptors (GPCR), inducing PKA and PKC activity and thus sensitizing TRPV1 channels. Since we ruled out the participation of PKA or PKC in this study, a role of the PLA_2 dependent pathway involving GPCRs is unlikely. Therefore, we can conclude that PLA_2/AA/LOX is most probably the main pathway that is involved in the gigantoxin I induced effect on TRPV1 channels. An important remaining question is why TRPV1 channels first need to be activated before visible effect is induced by gigantoxin I. It is known that EGF requires concomitant elevation of intracellular calcium for PLA_2 activity.[45] The same conditions may be needed for the activation of PLA_2 when gigantoxin I is bound to EGF receptor. Therefore, the opening of TRPV1 channels by an agonist (*e.g.* capsaicin) inducing inward calcium current might be needed for the activation of the intracellular cascade leading to the described TRPV1 effect.

In conclusion, this study describes the physiological target of gigantoxin I and the involved intracellular EGFR/PLA_2/AA/LOX pathway in the action mechanism of the toxin. This is the first time that this pathway is described in the indirect activation of TRPV1 channels by toxins. This pathway and its coupling to TRPV1 channel activation can serve as a model in further research on toxins and ion channels including the APHC1 toxin mentioned before. This new knowledge also leads to a better understanding of the action mechanism of TRPV1 channels themselves. Since gigantoxin I is a link between the previously described painful cnidaria envenomation symptoms and the effect on TRPV1 channel, these new insights may lead to the development of new analgesic treatments in cnidaria intoxications.

5. Acknowledgements

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Povzetek

Gigantoksin I, izoliran iz morske vetrnice *Stichodactyla gigantea*, je prvi toksin naravnega izvora strukturno podoben epidermalnemu rastnemu faktorju (EGF). V pričujoči raziskavi smo odkrili interakcijo med gigantoksinom I in vaniloidnim kanalčkom podvrste I s prehodnim receptorskim potencialom (TRPV1). TRPV1 je neselektivni kationski kanalček, udele en pri zaznavanju bolečine in opisan kot farmakološka tarča knidarijskih strupov. Naši rezultati odkrivajo vpletenost signalne poti EGF receptor/fosfolipaza A_2/arahidonska kislina/lipooksigenaza (EGFR/PLA_2/AA/LOX) v posredno aktivacijo kanalčkov TRPV1 z gigantoksinom I. Vpletenost te signalne poti v posredno aktivacijo kanalčkov TRPV1 z gigantoksinom I je opisana prvič. Naši rezultati ne odkrivajo le vpogleda v potencialne učinke, ki jih lahko izzove ta nova skupina toksinov, temveč omogočajo tudi boljše razumevanje mehanizma delovanja kanalčkov TRPV1 samih.