

**Calcium-Dependent Desensitization of Vanilloid Receptor TRPV1:
A Mechanism Possibly Involved in Analgesia Induced by Topical
Application of Capsaicin**

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Summary

The rationale for the topical application of capsaicin and other vanilloids in the treatment of pain is that such compounds selectively excite and subsequently desensitize nociceptive neurons. This desensitization is triggered by the activation of vanilloid receptors (TRPV1), which leads to an elevation in intracellular free Ca^{2+} levels. Depending on the vanilloid concentration and duration of exposure, the Ca^{2+} influx via TRPV1 desensitizes the channels themselves, which may represent not only a feedback mechanism protecting the cell from toxic Ca^{2+} overload, but also likely contributes to the analgesic effects of capsaicin. This review summarizes the current state of knowledge concerning the mechanisms that underlie the acute capsaicin-induced Ca^{2+} -dependent desensitization of TRPV1 channels and explores to what extent they may contribute to capsaicin-induced analgesia. In view of the polymodal nature of TRPV1, we illustrate how the channels behave in their desensitized state when activated by other stimuli such as noxious heat or depolarizing voltages. We also show that the desensitized channel can be strongly reactivated by capsaicin at concentrations higher than those previously used to desensitize it. We provide a possible explanation for a high incidence of adverse effects of topical capsaicin and point to a need for more accurate clinical criteria for employing it as a reliable remedy.

Keywords: TRPV1 receptors; capsaicin; topical application; desensitization; analgesia;

Introduction

Capsaicin produces burning pain in humans when brought into contact with the corium or mucosa and, paradoxically, its topical application to the skin has been found useful in the treatment of some pain states in traditional and contemporary medicine (for reviews, see Szallasi and Blumberg 1999, Sawynok 2003, Nagy *et al.* 2004). Experimental evidence has been presented that capsaicin causes persistent functional desensitization of polymodal primary nociceptors after repeated or prolonged application (Jancso *et al.* 1967, Carpenter and Lynn 1981, Szolcsanyi 2004). This desensitization was suggested to occur rather due to physiological than to morphological alterations (McMahon *et al.* 1991), however, later convincing evidence was presented that repeated capsaicin application to the skin produces a degeneration of epidermal nerve fibers that may underlie this type of analgesia (Simone *et al.* 1998, Nolano *et al.* 1999). Although capsaicin and other vanilloids have been shown to disrupt some vital organelles and promote apoptosis in various cell types (Hail 2003 and references therein), most of the well characterized effects are mediated through the activation of a specific receptor-ion channel that is natively expressed in mammalian primary nociceptors (Caterina *et al.* 1997). This receptor was termed vanilloid receptor subtype 1 (TRPV1) because it became the founding member of the vanilloid receptor subfamily of transient receptor potential (TRP) channels, a large superfamily of nonselective cation channels that play an important role in many sensory functions (see Clapham 2003, Planells-Cases *et al.* 2005, Tominaga and Tominaga 2005, Bandell *et al.* 2007, Nilius *et al.* 2007, Pingle *et al.* 2007, Venkatachalam and Montell 2007 for recent reviews). Although the molecular identification of the TRPV1 channels has greatly facilitated understanding the mechanisms by which vanilloids increase and subsequently attenuate nociceptor activity,

there is still no general consensus about the *in vivo* role of these mechanisms in the development of vanilloid-induced analgesia. What is clear from research carried out to date is that this process is triggered by Ca^{2+} influx via TRPV1, which leads to desensitization of the channel protein complex itself and, in extreme cases, to the degeneration of TRPV1-expressing sensory neurons due to Ca^{2+} overload (Olah *et al.* 2001, Szolcsanyi 2004, Tender *et al.* 2005).

In vitro studies on native and recombinant channels demonstrate that TRPV1 exhibits two types of desensitization, both of which frequently occur in conjunction (Fig. 1A and C): acute desensitization, which is a diminished response during a continuous vanilloid application, and tachyphylaxis, which is a reduction in the response to repeated applications (Docherty *et al.* 1996, Koplas *et al.* 1997, Bhawe *et al.* 2003). Over the past decade, it has become apparent that a predominant fast (acute) component of TRPV1 desensitization, in both native and overexpression contexts, critically depends on Ca^{2+} influx through the channel (Koplas *et al.* 1997, Piper *et al.* 1999, Mohapatra and Nau 2003, Numazaki *et al.* 2003, Rosenbaum *et al.* 2004, Lishko *et al.* 2007). Although it is not yet certain whether the acute desensitization and tachyphylaxis share the same cellular mechanisms (Docherty *et al.* 1996, Koplas *et al.* 1997), there is good evidence that it is the acute phase that accounts for most of the diminution of responsiveness occurring within the first few seconds after vanilloids are applied for the first time to a cell (Cholewinski *et al.* 1993, Liu and Simon 1996).

In this review, we present an update on findings concerning the mechanisms that underlie acute capsaicin-induced Ca^{2+} -dependent desensitization of the TRPV1 channel and argue that further study is needed to clarify the contribution of these mechanisms to capsaicin-induced analgesia.

No single molecular mechanism alone (such as the dephosphorylation of TRPV1) can account for the whole phenotype of capsaicin-induced Ca^{2+} -dependent desensitization

Among the Ca^{2+} -activated enzymes that were reported to modulate TRPV1 desensitization is the Ca^{2+} /calmodulin-dependent serine/threonine phosphatase 2B, calcineurin, which dephosphorylates TRPV1 receptors (Docherty *et al.* 1996, Mohapatra and Nau 2005). Conversely, phosphorylations at several consensus sites for protein kinase C (PKC), cAMP-dependent protein kinase A (PKA) and Ca^{2+} /calmodulin dependent kinase II (CaMKII) have been shown to reduce the Ca^{2+} -mediated desensitization of TRPV1. There are a number of putative phosphorylation sites at which the TRPV1 channel can be regulated: S502 and S800 have been implicated as targets of PKC-dependent phosphorylation (Vellani *et al.* 2001, Numazaki *et al.* 2002, Bhawe *et al.* 2003, Mandadi *et al.* 2004, Mandadi *et al.* 2006) and S116, T144, T370, and S502 were identified as the key sites at which PKA phosphorylation increases the open probability and reverses desensitization of the channel (Bhawe *et al.* 2002, Mohapatra and Nau 2003, Mohapatra and Nau 2005). A coincident phosphorylation of S502 and T704 by CaMKII has been proposed to be a necessary condition for the vanilloid binding capacity of TRPV1 (Jung *et al.* 2004). From these findings it was inferred that it is mostly a functional reflection of the dynamic balance between the Ca^{2+} -dependent phosphorylation and dephosphorylation of the receptor protein that accounts for the desensitization of the TRPV1 channel. Later, evidence was presented that the TRPV1 desensitization might involve a much more complex Ca^{2+} -dependent pathways. Acute Ca^{2+} -dependent TRPV1 desensitization has been shown to be accompanied by a profound change in voltage dependence (Piper *et al.* 1999, Gunthorpe *et al.* 2000), loss of capsaicin binding (Jung *et al.* 2004), and depletion of membrane phosphatidylinositol 4,5-bisphosphate (PIP_2), indicative of the involvement of Ca^{2+} -dependent PLC activity in this process. A role for ATP

was also suggested, based on the evidence that the recovery from desensitization requires a high cytoplasmic concentration of ATP to replenish PIP₂ (Liu *et al.* 2005). Mutation of the aromatic tyrosine residue Y671 within the internal pore of the TRPV1 channel greatly reduced Ca²⁺-dependent desensitization, suggesting that this residue is also involved in the structural rearrangements of the channel protein complex (Mohapatra *et al.* 2003). Although in many cases it could not be clearly distinguished which of these processes are primarily direct causes of desensitization rather than consequences of TRPV1 activation, it became clear that the dephosphorylation of TRPV1 represents only one of the possible mechanisms involved in analgesia after the topical application of capsaicin.

The prevention of acute desensitization of the TRPV1 channel by substituting Ba²⁺ for Ca²⁺ highlights a possible role for calmodulin

Structurally, like other members of the TRP channel superfamily, TRPV1 channels are homotetramers assembled around a centrally located aqueous pore (for a review, see Owsianik *et al.* 2006). Each subunit contains six transmembrane spanning domains (S1-S6) with a pore-lining P region linking the S5 and S6 domains. Although the crystal structure of the transmembrane part is not yet available for any of the TRP channels, it is generally accepted that it is analogous to the molecular architecture of the voltage-gated potassium channels: S1-S4 are putatively located on or close to the protein-lipid interface, whereas the four S5-P-S6 segments form the pore. Upon activation, Ca²⁺ permeates the TRPV1 channel pore more readily than other external cations ($P_{Ca}/P_{Na} \sim 10$, $P_{Mg}/P_{Na} \sim 5$). So, at negative membrane potentials, the driving force is favourable for a strong Ca²⁺ influx. If Ba²⁺ is used as the charge carrier instead of Ca²⁺ in whole-cell patch-clamp experiments on cells that

express TRPV1 channels, capsaicin induces responses that do not desensitize and only slightly decrease in magnitude when compared to the responses induced by capsaicin in Ca^{2+} -free extracellular solution (Fig. 1B). Although consistent effects are observed in heterologous expression systems (Gunthorpe *et al.* 2000, McNamara *et al.* 2005), earlier studies found that Ba^{2+} supports acute desensitization and tachyphylaxis of capsaicin-induced responses in native TRPV1 channels in DRG neurons isolated from rats, a result which has been interpreted by Koplas *et al.* (1997) as showing that Ba^{2+} can activate calcineurin-dependent dephosphorylation independently of calmodulin. This important calcium downstream effector was proposed to mediate TRPV1 desensitization more than a decade ago (Docherty *et al.* 1996) and this role has been debated ever since. Ba^{2+} as a surrogate for Ca^{2+} substitutes poorly at Ca^{2+} -sensitive regulatory sites and is also a poor substrate for Ca^{2+} transport mechanisms. Apparently because of its larger effective ionic radius than Ca^{2+} (1.38 Å versus 1.06 Å), Ba^{2+} also does not bind to calmodulin (CaM) (Chao *et al.* 1984). More recently, the TRPV1 channel has been shown to directly bind CaM at two sites in both a Ca^{2+} -dependent and Ca^{2+} -independent manner. One of these sites was localized in the N-terminal region (Rosenbaum and Gordon 2002, Lishko *et al.* 2007) whereas the other has been identified in the C-terminal domain (Numazaki *et al.* 2003). One of the most comprehensive models for the Ca^{2+} -induced desensitization of the TRPV1 channel has emerged from these studies (Fig. 2). The picture is built largely on recent evidence suggesting that the cytosolic ankyrin-repeat domain of TRPV1 (residues 101-364) binds ATP and that calmodulin, in a Ca^{2+} -dependent manner, competes with ATP at the same interaction site (Lishko *et al.* 2007). These two competitors have opposite effects on TRPV1 channel activity: ATP sensitizes the channel whereas Ca^{2+} -CaM inhibits it.

The apparent affinity for capsaicin is decreased in a desensitized TRPV1 channel

Acute TRPV1 desensitization depends not only on the amount of calcium influx, but also on the type of agonist. Camphor, a waxy substance used topically for its analgesic and counterirritant properties, activates TRPV1 independently of the vanilloid binding site and desensitizes it in a Ca^{2+} -independent manner (Xu *et al.* 2005). Another natural product, piperine, the pungent component of black pepper, has a higher degree of cooperativity (Hill coefficient ~ 4) and a clear propensity to cause greater desensitization of TRPV1 than capsaicin (McNamara *et al.* 2005), even in the absence of Ca^{2+} in the bath. It therefore appears that structural rearrangements in the TRPV1 channel complex include not only the putative phosphorylation sites but also several domains in or near the agonist-binding site or sites that undergo conformational changes inactivating the channel. In contrast to the rapid onset of the first capsaicin response recorded in a 2 mM Ca^{2+} -containing bath solution, the time constant characterizing the activation of the subsequent response is slower by about one order of magnitude (Fig. 1A). This might be the result of a decreased apparent affinity of desensitized TRPV1 receptors for capsaicin, which is also supported by the observation that [^3H]RTX does not bind to desensitized TRPV1 (Jung *et al.* 2004). Indeed, in the presence of 2 mM Ca^{2+} , desensitization of the recombinant TRPV1 receptor after exposure to 1 μM capsaicin can be overcome when its concentration is increased to 30 μM (Fig. 3A).

A mutant TRPV1 channel S502A/T704I in which two putative consensus sites for CaMKII are simultaneously (but not individually) replaced with nonphosphorylatable residues is insensitive to capsaicin (Jung *et al.* 2004). However, another nonphosphorylatable mutant S502A/T704A is fully functional, indicating the requirement for a specific residue at position 704 (Novakova-Tousova *et al.* 2007). Moreover, a point mutation at arginine residue R701 that, together with T704, lies within a short C-terminal juxtamembrane segment adjacent to

the TRP box, strongly affected the kinetics of capsaicin-evoked currents. As this residue constitutes a stringent CaMKII consensus site but is also predicted to contribute to the interaction with membrane PIP₂, it has been proposed that it might be involved in the slowed gating kinetics of the desensitized TRPV1 channel. The capsaicin molecule binds to the TRPV1 receptor at the channel-lipid interface between S3 and S4 and interacts with the residues around tyrosine 511, not far from the „universal“ phosphorylation site S502, positioned at the cytoplasmic loop linking S2 and S3. Based on these findings, it seems that the domains around S502 and T704 are functionally linked and might also be located relatively close to one another, jointly influencing the binding of capsaicin. T704 is critical to the activation of TRPV1 by phorbol esters (Bhave *et al.* 2003), which indicates that it might act as an allosteric site.

Functional changes in the vanilloid receptor subtype 1 channel (TRPV1) after acute desensitization

The TRPV1 channel can be activated not only by vanilloid compounds but also by protons (pH < 6.5), noxious heat (> 43 °C), phorbol esters (Premkumar and Ahern 2000, Bhave *et al.* 2003) or depolarizing voltages (Vlachova *et al.* 2003, Voets *et al.* 2004) (see Planells-Cases *et al.*, 2005; Tominaga and Tominaga, 2005; Szallasi *et al.*, 2006; Pingle *et al.*, 2007 for recent reviews). These stimuli, when applied alone, produce only submaximal activation, whereas the maximal response can only be reached by their synergistic interaction at the TRPV1 receptor channel complex (Tominaga *et al.* 1998). Such a polymodal activation property of TRPV1 certainly complicates the interpretation of functional data, since all stimuli lowering the activation energy for channel opening also lower the thermal threshold for

activation and thus may seem to act as agonists at room temperature. Therefore, in comparison to the wealth of non-TRP ion channels that are gated by either ligands or membrane voltage, the activation of TRPV1 is an extremely complex and dynamic process involving multiple, allosterically coupled and stimulus-dependent pathways.

Although the desensitization of polymodal nociceptors by the local application of capsaicin can be considered specific, the complexity of the range of known pathways that lead from TRPV1 activation to increases in intracellular Ca^{2+} concentration and channel desensitization is dazzling and they involve almost every known type of signalling molecule (Planells-Cases *et al.* 2005, Tominaga and Tominaga 2005, Lukacs *et al.* 2007, Pingle *et al.* 2007). TRPV1 is not only expressed at the plasma membrane but also resides in the endoplasmic reticulum where it forms channels that, upon binding the capsaicin that passes through the membrane, releases Ca^{2+} from internal stores (Liu *et al.* 2003, Marshall *et al.* 2003, Turner *et al.* 2003, Wisnoskey *et al.* 2003, Karai *et al.* 2004). This mechanism of activation may play an important role in Ca^{2+} signaling events localized to subcellular microdomains and may provide clues to the function of TRPV1 that we currently do not understand.

Calcium imaging experiments in cells transfected with the TRPV1 channel demonstrate that noxious heat ($>43^\circ\text{C}$) induces rapid and reversible cytosolic calcium transients. In contrast, the capsaicin-evoked increase in intracellular Ca^{2+} concentration decays slowly and is maintained over a period of minutes (Fig. 3B). An increase in temperature from 25 to 40°C together with the application of capsaicin, a strong sensitizing stimulus for TRPV1, does not lead to enhanced Ca^{2+} influx when compared to the first capsaicin response. This seems to be in contrast to the synergy between heat and capsaicin observed under patch-clamp conditions (Fig. 3Dg). Although these experimental results have to be interpreted cautiously, for at least the above reasons, the Ca^{2+} accumulation clearly

corresponds to the period of desensitization of the membrane current responses observed in cells clamped at a negative membrane potential (-70 mV).

We and others have shown that various activators can cross-desensitize the TRPV1 channel (Tominaga *et al.* 1998, Vlachova *et al.* 2001). The capsaicin-induced Ca^{2+} -dependent desensitization of the TRPV1 channel is accompanied by a decreased responsiveness to voltage and heat (Fig. 3C,D). In this case, the maximum amplitudes induced by simultaneous applications of heat together with capsaicin are even greater than the first maximal response to capsaicin obtained at room temperature (Fig. 3Db and g), indicating that the activation capacity of desensitized channels is not significantly reduced. This consistent finding of the strong synergy between capsaicin and heat might provide a possible explanation for the adverse reactions observed in around one third of patients suffering from musculoskeletal or neuropathic pain, in which the local administration of capsaicin results in adverse events or even in an increase of pain above tolerable levels (Mason *et al.* 2004).

Conclusion

Both the published data and our own results on the recombinant TRPV1 receptor suggest that multiple mechanisms are involved in the analgesia induced by topical application of capsaicin. We are still in the process of understanding them better in order to increase our ability to treat pain in cases when other means are unsatisfactory or potentially hazardous. Although the topical application of capsaicin remains a promising alternative for the treatment of patients who suffer from local pain that cannot be adequately treated by routine analgesics, the mechanisms involved in the reduction of the pain will need to be investigated further. Better characterization of the pain states suitable for such a treatment will also need to be an area of study to avoid adverse reactions and ensure a reliable remedy. The next challenge will

be to understand the role of the TRPV1 channel in the process of nociceptor desensitization that occurs during topical capsaicin application and exploit this information for therapeutic ends in chronic neuropathic pain states such as diabetic neuropathy, postherpetic neuralgia, arthritis, musculoskeletal pain or inflammatory bowel disease. A clear understanding of the pathogenesis at the molecular and cellular levels of nociceptor function will be a significant step towards the development of more effective, better targeted and more specific pain therapies in the future.

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Figure Legends

Fig. 1. Effects of extracellular Ca^{2+} on acute desensitization of capsaicin-activated currents in HEK293T cells transfected with rat TRPV1. **(A)** Whole-cell current responses to consecutive applications of 1 μM capsaicin induced in a nominally Ca^{2+} -free extracellular solution (*a* and *b*) and in standard bath solution containing 1 mM Ca^{2+} (*c* and *d*). The methods used in this study are described in detail in Novakova-Tousova *et al.* (2007). Briefly, human embryonic kidney (HEK) 293T cells, transiently transfected with recombinant plasmid cDNA encoding wild-type rat TRPV1, were used for whole-cell recordings using a conventional patch-clamp technique. The cells were superfused with an extracellular control solution (ECS) (mM): NaCl, 160; KCl, 2.5; CaCl_2 , 1; MgCl_2 , 2; HEPES, 10; glucose 10. The intracellular pipette solution contained (mM): Cs-gluconate, 125; CsCl, 15; EGTA, 5; HEPES, 10; CaCl_2 , 0.5; MgATP, 2; pH was adjusted to 7.3 with CsOH. Holding potential was -70 mV. Capsaicin was applied for the durations indicated by the bars above the records. Dashed lines indicate zero current level. **(B)** Barium cannot substitute for Ca^{2+} in acute capsaicin-induced desensitization of the TRPV1 channel. Capsaicin (1 μM) was applied either in Ca^{2+} -free solution to which 1 mM EGTA was added (*a*, *c*) or in the bath solution in which Ba^{2+} (2 mM) was substituted for Ca^{2+} (*b*). **(C)** Whole-cell membrane currents from another TRPV1-HEK293T cell. Capsaicin was applied in Ca^{2+} -free solution to which 1 mM EGTA was added to chelate residual Ca^{2+} (0 Ca^{2+}) or in solution containing 2 mM Ca^{2+} .

Fig. 2. Tentative model of TRPV1 capsaicin-induced Ca^{2+} -dependent desensitization. The cytosolic ankyrin-repeat domain of TRPV1 (residues 101-364) binds ATP and calmodulin in a Ca^{2+} -dependent manner competes with ATP at the same interaction site (Lishko *et al.* 2007). These two competitors have opposite effects on TRPV1 channel activity: ATP sensitizes the channel whereas the Ca^{2+} -CaM complex inhibits it. A short C-terminal juxtamembrane segment adjacent to the TRP box (most likely around R701) is predicted to contribute to the interaction with membrane phosphatidylinositol 4,5-bisphosphate (PIP_2) and might be involved in the slowed gating kinetics of the desensitized TRPV1 channel. The capsaicin molecule binds to the TRPV1 receptor at the channel-lipid interface between S3 and S4. Ca^{2+} -dependent activation of phospholipase C (PLC) results in the hydrolysis of localized PIP_2 to 1,4,5-trisphosphate and diacylglycerol (DAG), leading to inactivation of the TRPV1 channel. Acute desensitization could alter the Ca^{2+} -dependent allosteric coupling between the capsaicin binding site and the PIP_2 -sensitive gating apparatus of the TRPV1 channel. A high concentration of intracellular ATP is required for PIP_2 resynthesis by sequential phosphorylation of its precursor phosphatidylinositol by phosphatidylinositol 4-kinases (PI4K) and phosphatidylinositol 4-phosphate 5-kinases (PIP5K).

Fig. 3. (A) Capsaicin applied at high concentration (30 μM) reactivates TRPV1 channels that had previously been desensitized to 1 μM capsaicin in Ca^{2+} -containing bath solution (2 mM). (B) Changes in intracellular calcium concentration induced by consequent applications of a heat ramp (20-48°C in 30 s), 1 μM capsaicin and a temperature ramp (20-42°C) applied in the presence of capsaicin, monitored using ratiometric Ca^{2+} -imaging in the presence of 2 mM Ca^{2+} ($n = 6$ cells). For microfluorometric Ca^{2+} measurements, TRPV1-expressing HEK293T

cells were incubated in extracellular solution containing 2 μM FURA-2 AM for 45 min at 37 °C. (C, D, E) The effects of acute Ca^{2+} -dependent capsaicin-induced desensitization on the gating of TRPV1 by other modalities. (C) Time course of TRPV1-mediated whole-cell currents induced by depolarizing voltage steps recorded at 25°C in extracellular control solution (holding potential -70 mV, voltage steps from -140 to +140 mV, increment +20 mV) before (open circles) and after (filled circles) desensitization evoked by 1 μM capsaicin. Current-voltage relationship for currents obtained before and after acute desensitization such as shown in the inset. (D) Whole-cell current responses to consecutive applications of a heat ramp (from 25°C to 48°C in 3 s) (a) and capsaicin (1 μM and 30 μM) (b-e) in a Ca^{2+} -containing bath solution. Consecutive currents recorded in response to heat ramps applied in a standard bath solution (f), in the presence of 1 μM (g) and 30 μM (h) capsaicin. Current-temperature relationships for currents shown in (a, f-h) demonstrate a strong potentiating effect of capsaicin on the heat-induced currents.

Fig. 1

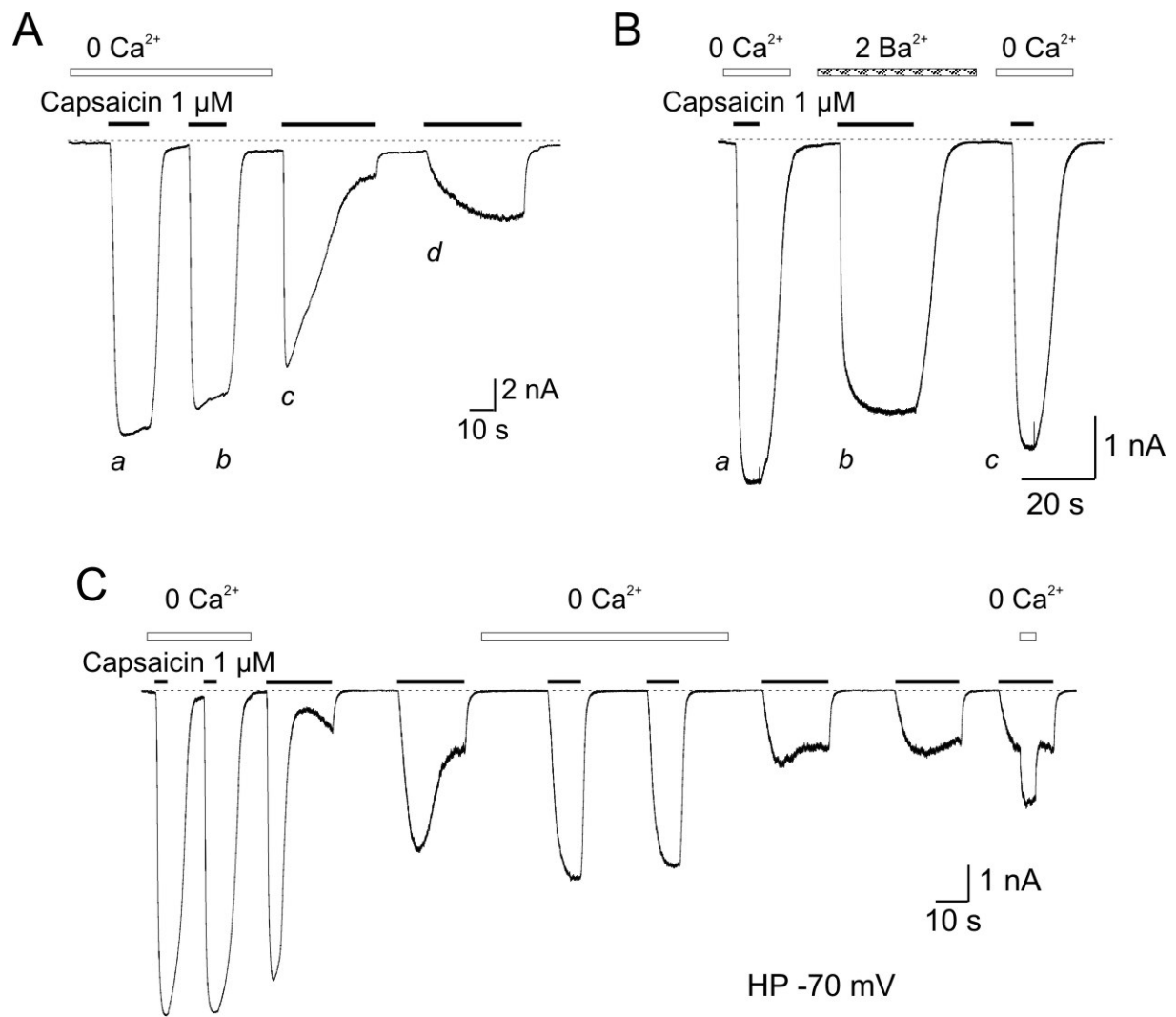


Fig. 2

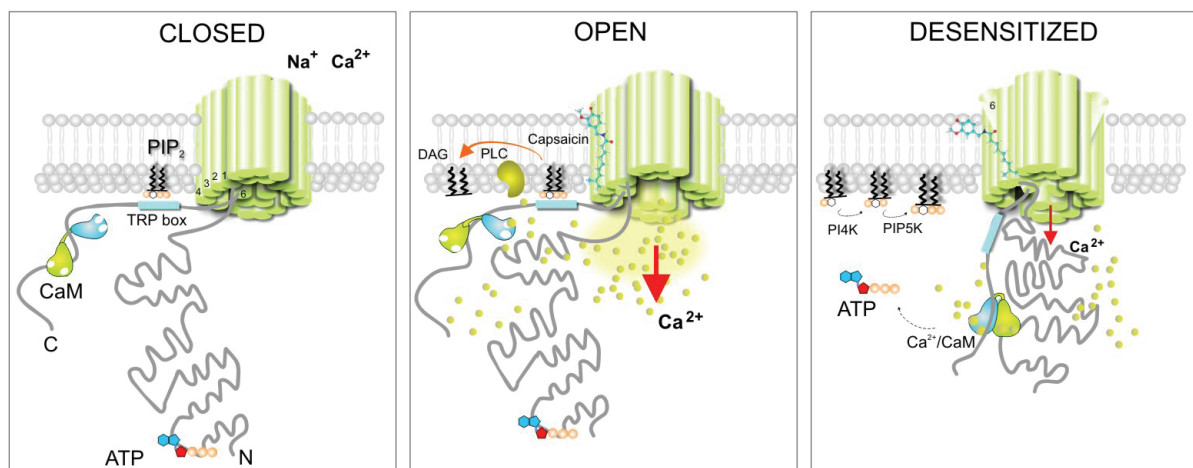


Fig. 3

