



Depolarizing Effectors of Bradykinin Signaling in Nociceptor Excitation in Pain Perception

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Abstract

Inflammation is one of the main causes of pathologic pain. Knowledge of the molecular links between inflammatory signals and pain-mediating neuronal signals is essential for understanding the mechanisms behind pain exacerbation. Some inflammatory mediators directly modulate the excitability of pain-mediating neurons by contacting the receptor molecules expressed in those neurons. For decades, many discoveries have accumulated regarding intraneuronal signals from receptor activation through electrical depolarization for bradykinin, a major inflammatory mediator that is able to both excite and sensitize pain-mediating nociceptor neurons. Here, we focus on the final effectors of depolarization, the neuronal ion channels, whose functionalities are specifically affected by bradykinin stimulation. Particular G-protein coupled signaling cascades specialized for each specific depolarizer ion channels are summarized. Some of these ion channels not only serve as downstream effectors but also play critical roles in relaying specific pain modalities such as thermal or mechanical pain. Accordingly, specific pain phenotypes altered by bradykinin stimulation are also discussed. Some members of the effector ion channels are both activated and sensitized by bradykinin-induced neuronal signaling, while others only sensitized or inhibited, which are also introduced. The present overview of the effect of bradykinin on nociceptor neuronal excitability at the molecular level may contribute to better understanding of an important aspect of inflammatory pain and help future design of further research on the components involved and pain modulating strategies.

Key Words: Bradykinin, Pain, Nociceptor neuron, Ion channel, Depolarization

INTRODUCTION

Inflammation is frequently accompanied by pain, where several inflammatory pain mediators generated from inflamed tissues have been known to contribute to this pain induction, e.g., bradykinin, nerve growth factors, prostaglandins, and a group of cytokines (Patapoutian *et al.*, 2009). These mediators stimulate the primary nociceptor neurons innervating inflamed locations. The resultant firing of electrical signals is then transmitted to the brain, leading to the perception of pain. Acquiring information on the nature of the stimulatory mechanisms may help to improve therapeutic pain control strategies, and the relevant approaches at cellular and molecular levels have been conducted for many years. Rarely found regarding the properties for other mediators, bradykinin is able to induce action potential firing of the nociceptors as well as to sensitize those to other stimulations. The mechanisms seem to involve various ion channels that function as the final effec-

tors of excitatory outcomes. Although important frames for the molecular signaling that support the mechanisms were built in late 20th century, the molecular identities and detailed properties of most of the ionotropic players were reported during the 21st century.

As early as the 1950s, the hypothesis that bradykinin mediates pain via nociceptor excitation started to be confirmed in various experimental settings with *in vitro* and *in vivo* animal models, as well as human subjects. Administration of bradykinin to human skin and muscle clearly elicited pain perception (Armstrong *et al.*, 1957; Whalley *et al.*, 1987; Manning *et al.*, 1991; Kindgen-Milles *et al.*, 1994; Babenko *et al.*, 1999). Injections to the skin, vascular areas, and the peritoneal cavity caused nocifensive reflexes in model animals including mice, rats, cats, rabbits, dogs, and monkeys (Kumazawa and Mizumura, 1976; Steranka *et al.*, 1988; Walter *et al.*, 1989; Khan *et al.*, 1992; Hong and Abbott, 1994; Griesbacher *et al.*, 1998; Katanosaka *et al.*, 2008). Fiber recordings revealed that

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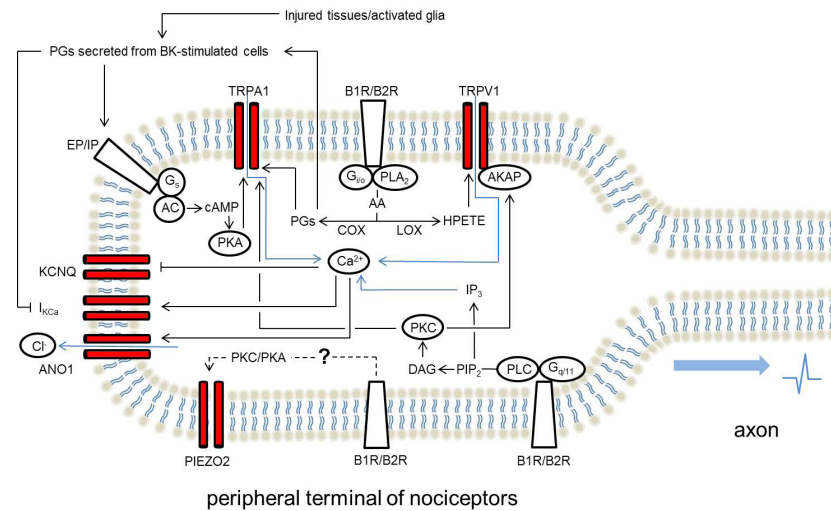


Fig. 1. Summary of the roles of important effector ion channels which account for bradykinin-induced excitation of pain-mediating nociceptors. AA, arachidonic acid; AC, adenylate cyclase; AKAP, A kinase anchoring protein; ANO1, anoctamin 1; B1R, bradykinin receptor B1; B2R, bradykinin receptor B2; BK, bradykinin; cAMP, 3',5'-cyclic adenosine monophosphate; COX, cyclooxygenase; DAG, diacylglycerol; EP/IP, prostaglandin E2 receptor and prostaglandin I2 receptor; HPETE, hydroperoxyeicosatetraenoic acid; IKCa, Ca²⁺-activated K⁺ channels; IP3, inositol 1,4,5-trisphosphate; KCNQ, voltage-gated K⁺ channel subfamily KCNQ; LOX, lipoxygenase; PG, prostaglandin; PIP2, phosphatidylinositol 4,5-bisphosphate; PKA, protein kinase A; PKC, protein kinase C; PLA2, phospholipase A2; TRPA1, transient receptor potential ankyrin subtype 1; TRPV1, transient receptor potential vanilloid subtype 1.

the nociceptor depolarization initiated those painful outcomes (Juan and Lembeck, 1974; Chahl and Iggo, 1977; Dray *et al.*, 1992; Soukhova-O'Hare *et al.*, 2006), in which models utilizing testis-spermatoc nerve and skin-saphenous nerve preparations have greatly contributed to the provision of fundamental information on bradykinin-controlling sensory modalities and phases, nociceptor categorizing, and signaling participants (Beck and Handwerker, 1974; Kumazawa and Mizumura, 1976). As a result, it is now firmly known that the polymodal nociceptors comprising the unmyelinated C and thinly myelinated A δ neurons are responsible for bradykinin-induced pain, that the B2 receptor is more constitutively responsible for bradykinin detection than the B1 receptor, and that both discharging of action potentials and lowering of its threshold can be caused by bradykinin action (Mizumura *et al.*, 2009).

Following this, the molecular evidence has kept being corroborated regarding bradykinin receptor-mediated signals, using extended technologies such as culture platforms, molecular biology, genetics, and the patch clamp. Bradykinin acts on the B1 and B2 receptors that are among the metabotropic G protein-coupled receptors (GPCRs) expressed at the surface membrane (Burgess *et al.*, 1989; McGuirk *et al.*, 1989; McGehee and Oxford, 1991; Dray *et al.*, 1992; McGuirk and Dolphin, 1992). The majority of the downstream information was obtained from B2 studies, but as for many molecular processes, both receptors have been shown to share similar mechanisms of action (Petho and Reeh, 2012). Commonly, Gq/11-mediated phospholipase C (PLC) and Gi/o-mediated phospholipase A2 (PLA2) activation lead to diverse cellular effects. In nociceptor neurons, several depolarizing effectors are activated or positively regulated (sensitized) through such signaling, which are critical steps necessary for action potential firing or threshold lowering. Here we summarize the identities of the depolarizing molecules and bradykinin-related mechanisms for activation and sensitization.

TRANSIENT RECEPTOR POTENTIAL VANILLOID SUBTYPE 1 ION CHANNEL

Transient Receptor Potential Vanilloid subtype 1 ion channel (TRPV1) functions as a receptor and a cation channel in nociceptor sensory neurons. Sensitive to noxious temperature ranges (>43°C), protons (pH <5.5), and pungent chemicals (e.g., capsaicin), TRPV1 responds by opening its pore. Cation influx through TRPV1 depolarizes the nociceptor membrane, discharging action potentials when the membrane voltage reaches its firing threshold. Other mechanisms for activation and activity modulation have been revealed, and bradykinin has been shown to be tightly linked.

Bradykinin-induced activation of TRPV1 via arachidonic acid metabolism

TRPV1-mediated action potential spike generation upon bradykinin exposure has successfully been repeated in the primary sensory afferents from various sources, including cutaneous nociceptors, cardiac afferents, jejunal afferents, and tracheobronchial afferents (Fig. 1) (Carr *et al.*, 2003; Pan and Chen, 2004; Rong *et al.*, 2004; Lee *et al.*, 2005a). Research efforts have been put into seeking the link between bradykinin-initiated G protein signaling and depolarizing effector functions. Increased production of arachidonic acid by bradykinin and its further metabolism has been considered an important candidate for the signaling (Thayer *et al.*, 1988; Burgess *et al.*, 1989; Gammon *et al.*, 1989). Not only in neurons but also in other tissues, Gi/o mediated arachidonic acid liberation through bilayer digestion of PLA2 activated by bradykinin has been proposed to be involved (Burch and Axelrod, 1987; Gammon *et al.*, 1989; Yanaga *et al.*, 1991). The resultant excitation and sensitization of the nociceptor has also been demonstrated (Taiwo *et al.*, 1990; Ferreira *et al.*, 2004). The role of members of the lipoxygenase (LOX) in furthering arachidonic acid

metabolism has been raised for the immediate depolarization caused by bradykinin (McGuirk and Dolphin, 1992; Shin *et al.*, 2002; Carr *et al.*, 2003; Wu and Pan, 2007). Formation of 12-LOX products like 12(S)-hydroxyeicosatetraenoic acid (12(S)-HETE) was also confirmed in nociceptor neurons and skin (Wang *et al.*, 1999; Shin *et al.*, 2002; Tang *et al.*, 2005).

Production of arachidonic acid can result from PLC metabolism, as well as from PLA2-mediated one. Diacylglycerol (DAG) produced via PLC action may further be converted into arachidonic acid by DAG lipase and monoacylglycerol lipase (Gammon *et al.*, 1989; Allen *et al.*, 1992; Dray *et al.*, 1992). Attenuation of locally injected bradykinin-elicited acute nocifensive behaviors that reflect immediate sensory action potential discharge is achieved via PLC inhibitors, as well as PLA2 and 5-LOX inhibitors (Ferreira *et al.*, 2004).

LOX metabolites have been shown to activate TRPV1 (Hwang *et al.*, 2000). A number of HETEs, hydroperoxyeicosatetraenoic acids (HPETEs), and leukotrienes displayed this activity, where 12(S)-HPETE has the greatest potency. A later study from the same group reported the B2 receptor-PLA2-LOX axis for the signaling cascade for TRPV1 activation (Shin *et al.*, 2002). The same signaling paradigm was successfully repeated by a pharmacological approach for visceral afferents by using multiple TRPV1 blockers and LOX inhibitors (Carr *et al.*, 2003). LOX product pools that have TRPV1 activating potential and the neuronal outcomes other than nociception for example memory, are both being currently extended (Gibson *et al.*, 2008; Gregus *et al.*, 2012). Although the binding pocket for LOX-produced ligands or TRPV1 is less conclusively determined, their structural similarity with capsaicin as predicted by 3-dimensional simulation suggests that the capsaicin binding site, the S4-S5 intracellular linker region of TRPV1 protein is a strong candidate (Ikeda *et al.*, 2001b; Gao *et al.*, 2016).

Bradykinin-induced activation of TRPV1 via protein kinase C

Gq/11-activated PLC metabolizes the membrane bilayer phosphatidylinositol 4,5-bisphosphate (PIP2) into DAG and inositol 1,4,5-trisphosphate (IP3). The increased level of these two products upon bradykinin stimulation has been reported in nociceptor dorsal root ganglionic (DRG) neurons, indicating the activation of Gq/11-PLC axis by bradykinin (Thayer *et al.*, 1988; Burgess *et al.*, 1989; Gammon *et al.*, 1989; Kano *et al.*, 1994; Tang *et al.*, 2004). Both of the two messengers contribute to protein kinase C (PKC) activation.

Evidence has been accumulating that PKC activation upon bradykinin exposure leads to an immediate excitation in studies with various platforms using ectopically receptor expressing cells (Crandall *et al.*, 2002), cultured sensory neurons (Rang and Ritchie, 1988; Burgess *et al.*, 1989; McGehee and Oxford, 1991; McGuirk and Dolphin, 1992), afferent nerve fibers (Mizumura *et al.*, 1997; Guo *et al.*, 1998, 1999), spinal cord-tail preparations (Dray *et al.*, 1988, 1992), or animals with nocifensive behaviors (Ferreira *et al.*, 2004). Suppression of excitatory responses by pharmacological inhibition of PKC and mimicking of depolarization when exposed to PKC-activating phorbol esters support the finding. The excitatory effect appears to be caused by the increased permeability of the neuronal membrane to both Na⁺ and K⁺ ions, indicating that nonselective cation channels are probably a final effector for this bradykinin-induced PKC action (Rang and Ritchie, 1988; Burgess *et al.*, 1989; McGehee and Oxford, 1991).

Further research revealed that TRPV1 participates as the ion-permeating effector and suggested that bradykinin leads to TRPV1 activation by PKC phosphorylation and that PKC ϵ plays a crucial role (Premkumar and Ahern, 2000; Vellani *et al.*, 2004). When strongly depolarized, sensory afferents secrete peptidergic transmitters, specifically Substance P and calcitonin gene-related peptide, which is augmented by PKC activation and reduced by PKC downregulation (Barber and Vasko, 1996). Two serine residues located in the intracellular S2-S3 linker and N-terminal end of TRPV1 protein were identified as PKC ϵ phosphorylation targets (Numazaki *et al.*, 2002; Bhave *et al.*, 2003; Choi *et al.*, 2014). Although neuronal excitation by PKC has readily been repeated, ectopic expression systems have often shown less effective induction of the inward current but relatively prominent sensitization of the TRPV1 response to other stimulation, which remains to be quantitatively dissected.

Bradykinin-induced sensitization of TRPV1 activity

In comparison with an acute excitatory action, constantly sensitized nociception caused by a mediator may more broadly explain pathologic pain mechanisms. Since TRPV1 is the major heat sensing molecule, heat hyperalgesia induced by bradykinin, which has long been studied in pain research, may putatively involve changes in TRPV1 activity. Therefore, here we provide an overview of the role of bradykinin in pathology-induced heat hyperalgesia and then discuss the evidence supporting the possible participation of TRPV1 in this type of bradykinin-exacerbated thermal pain. Different from acute nociception where data were produced mostly in B2 receptor setting, the focus may include both B1 and B2-mediated mechanisms underlying pathology-induced chronic nociception, since roles for inducible B1 may emerge in certain disease states. A number of specific pathologies may even show pronounced dependence on B1 function. Nonetheless, both receptors likely share the intracellular signaling mechanisms for effector sensitization.

B1 receptor-dependent pathologic pain: Since the 1980s, B2 receptor involvement has been extensively demonstrated in relatively short-term inflammation models primed with an adjuvant carrageenan or other mediator treatments (Costello and Hargreaves, 1989; Ferreira *et al.*, 1993b; Ikeda *et al.*, 2001a). On the other hand, B1 receptor seems to be more tightly involved in heat hyperalgesia in relatively chronic inflammatory pain models such as the complete Freund's adjuvant (CFA)-induced inflammation model. While B2 knockout mice failed to show any difference in comparison with wild types, either B1 knockouts or B1 antagonism leads to reduced heat hyperalgesia (Rupniak *et al.*, 1997; Ferreira *et al.*, 2001; Porreca *et al.*, 2006). Because of the ignorable difference in CFA-induced edema between wild types and B1 knockouts, B1 is thought to be involved in heightened neuronal excitability rather than inflammation itself (Ferreira *et al.*, 2001). In diabetic neuropathy models, B1 knockouts are resistant to development of the heat hyperalgesia, and treatment with a B1 antagonist was effective in preventing heat hyperalgesia in naïve animals (Gabra and Sirois, 2002, 2003a, 2003b; Gabra *et al.*, 2005a, 2005b). In a brachial plexus avulsion model, B1 knockouts but not B2 knockouts have shown prolonged resistance to heat hyperalgesia (Quintão *et al.*, 2008). Pharmacological studies on ultraviolet (UV) irradiation models have also shown B1 dominance (Perkins and Kelly, 1993; Perkins

et al., 1993; Gougat *et al.*, 2004). Both the peptidergic antagonist des-Arg⁹,Leu⁸-bradykinin and a synthetic B1 antagonist SSR240612 commonly prevented UV-induced heat hyperalgesia, whereas the effect of HOE 140, a B2 antagonist, was largely limited. The hyperalgesia was further aggravated by a relatively selective B1 agonist des-Arg⁹-bradykinin and reversed only by the B1 antagonist.

B1 & B2 receptor-dependent pathologic pain: In neuropathic pain models, both B1 and B2 receptor-mediated mechanisms are generally important (Levy and Zochodne, 2000; Yamaguchi-Sase *et al.*, 2003; Ferreira *et al.*, 2005; Petcu *et al.*, 2008; Luiz *et al.*, 2010). In the models of chronic constriction injury, infraorbital nerve constriction injury, and partial sciatic nerve ligation, selective pharmacological antagonism of either of the receptor types was effective against the putatively TRPV1-mediated heat hyperalgesia, as well as cold hyperalgesia and mechanical allodynia. Heat hyperalgesia occurring in a rat plantar incision model was once shown to be unrelated to bradykinin-mediated mechanisms (Leonard *et al.*, 2004). Later, a contradictory result that the heat hyperalgesia was partially reversed by treatment with either B1 or B2 receptor antagonist was obtained in a different laboratory (Füredi *et al.*, 2010). In the same model, treatment with an LOX inhibitor or a TRPV1 antagonist was also effective. Interestingly, in the same study, heat injury-evoked heat hyperalgesia was attenuated only by B2 antagonist treatment.

Bradykinin-induced heat hypersensitivity: Injection of bradykinin itself has also been shown to augment heat pain sensitivity in humans, monkeys, and rats (Manning *et al.*, 1991; Khan *et al.*, 1992; Schuligoi *et al.*, 1994; Griesbacher *et al.*, 1998). It is commonly likely that the heat sensitivity was left-shifted with lowered heat threshold by bradykinin injection. There are several different points when speculating possible mechanisms that could explain direct excitation and sensitization. Direct nociception in response to bradykinin generally undergoes strong tachyphylaxis, but such sensitization appears to be relatively persistent in time scale. In-depth analyses at the cellular or molecular levels that are mentioned below have shown that the sensitizing effect sometimes occurs in the absence of direct excitation (Beck and Handwerker, 1974; Kumazawa *et al.*, 1991; Khan *et al.*, 1992). Nonetheless, nociceptors that more readily fire upon bradykinin exposure appeared to tend to be more sensitized in heat responsiveness (Kumazawa *et al.*, 1991; Liang *et al.*, 2001). Common PKC-centered machinery is hypothesized to be responsible for both excitation and sensitization, which still requires further careful dissection to understand how those differentiated outcomes are realized.

The sensitizing action of bradykinin on nociceptors: After feline nociceptors were once demonstrated to be sensitized by acute bradykinin exposure of their termini in terms of heat-evoked spike discharges in an *in vivo* model, many similar *in vitro* or *ex vivo* results were produced, again for example, in rodent skin-saphenous nerve and canine testis-spermatic nerve models (Beck and Handwerker, 1974; Lang *et al.*, 1990; Kumazawa *et al.*, 1991). As shown in the *in vivo* experiments mentioned above, the potency and efficacy of heat-induced electrical responses were increased by bradykinin stimulation of the relevant receptive fields, which was mainly observed in unmyelinated C- or thinly myelinated A δ nociceptors with polymodality (Kumazawa *et al.*, 1991; Koltzenburg *et al.*, 1992; Haake *et al.*, 1996; Liang *et al.*, 2001). Such facilitation

occurred at lower doses than needed for bradykinin-evoked excitation, and moreover, subpopulations of nociceptors that were without bradykinin- or heat-evoked excitation in a naive stage became sensitive to heat by bradykinin exposure (Kumazawa *et al.*, 1991; Liang *et al.*, 2001). The observed population enlargement is unlikely to be due to an elevated expression of TRPV1 at the surface membrane as this failed to be demonstrated in a more recent study (Camprubi-Robles *et al.*, 2009). Although the experiment did not manipulate heat, research revealed that the capsaicin responses in trachea-innervating vagal C-fibers was sensitized by bradykinin, underlying cough exacerbation upon bradykinin accumulation as an adverse effect of treatment with angiotensin converting enzyme inhibitors for hypertension (Fox *et al.*, 1996). B2 receptor participation was confirmed in the models above.

TRPV1 as a principal actuator for bradykinin-induced heat sensitization: As mentioned above, PKC activation is involved in TRPV1 activation and sensitization. Electrophysiological recordings of canine testis-spermatic nerve preparations raised a role for PKC in the bradykinin-induced sensitization of the heat responses (Mizumura *et al.*, 1997). PKC ϵ phosphorylation initiated by bradykinin was proposed to sensitize the native heat-activated cation channels of cultured nociceptor neurons (Cesare and McNaughton, 1996; Cesare *et al.*, 1999). This was successfully repeated in TRPV1 experiments after its genetic identification and the temperature threshold for TRPV1 activation was lowered by PKC phosphorylation (Vellani *et al.*, 2001; Sugiura *et al.*, 2002). Not only to heat but also to other activators such as protons and capsaicin, TRPV1 responses were sensitized by PKC phosphorylation in several different experimental models (Stucky *et al.*, 1998; Crandall *et al.*, 2002; Lee *et al.*, 2005b; Camprubi-Robles *et al.*, 2009). However, it remains to be elucidated if inducible B1 receptor may utilize the same pathway.

Molecular mechanisms for TRPV1 sensitization by PKC phosphorylation: TRPV1 protein contains a number of target amino acid residues for phosphorylation by various protein kinases. The phosphorylation of these residues largely contributes to the facilitation of TRPV1 activity but it is likely that bradykinin mainly utilizes PKC for its TRPV1 sensitization according to an *in vitro* analysis of phosphorylated proteins (Lee *et al.*, 2005b). PKC has been shown to directly phosphorylate two TRPV1 serine residues that are located in the first intracellular linker region between the S2 and S3 transmembrane domains, and in the C-terminal (Numazaki *et al.*, 2002; Bhave *et al.*, 2003; Wang *et al.*, 2015). Mutant TRPV1 that was missing these target sequences were tolerant in terms of sensitization upon bradykinin treatment. Interestingly, an adaptor protein seems to be critical to access to the target residues by PKC. Members of A kinase anchoring proteins (AKAPs) are able to modulate intracellular signaling by recruiting diverse kinase and phosphatase enzymes (Fischer and McNaughton, 2014). The activity of some of ion channels is known to be controlled by this modulation when these proteins form a complex, the best known example being the interaction of TRPV1 with AKAP79/150 (AKAP79 indicates the human homolog of the rodent AKAP150) (Zhang *et al.*, 2008). Specifically, AKAP79/150 bound to the C-terminal of TRPV1 protein has been shown to greatly facilitate PKC action by physically recruiting it upon bradykinin stimulation.

Other proposed mechanisms for bradykinin-induced TRPV1 sensitization: As mentioned above, pharmacological inhibition

or genetic downregulation studies have demonstrated that PKC may be a predominant factor required for bradykinin-induced TRPV1 sensitization. In addition, different molecular mechanisms also appear to contribute to this sensitization under bradykinin-mediated pathway. PLC is an upstream enzyme of PKC, which consumes bilayer PIP2 to produce IP3 and DAG. PIP2 seems to bind to TRPV1 protein in its resting state, tonically inhibiting TRPV1 activity. In response to bradykinin stimulation however, PIP2 is consumed by Gq-mediated PLC, thereby disinhibiting TRPV1. Responses to capsaicin and protons have been shown to be augmented by this mechanism, although sensitization to heat by bradykinin remains to be explored (Chuang *et al.*, 2001; Prescott and Julius, 2003). As arachidonic acid metabolizing enzymes, LOXs generate a number of endogenous TRPV1 activators. Interestingly, 12-LOX inhibition was also effective at erasing bradykinin-induced heat sensitization of TRPV1 (Shin *et al.*, 2002). 12(S)-HPETE or 12(S)-HETE, its metabolites may therefore not only activate TRPV1, but also facilitate the heat response, which remains to be experimentally confirmed.

Complicated results were obtained from research exploring the role of a different group of arachidonic acid metabolizing enzymes, cyclooxygenases (COXs), as mentioned in the *Unsolved problems* section. COX activation undoubtedly contributes as a downstream from bradykinin signaling to nociceptor sensitization and excitation but it remains under debate where among nociceptors and surrounding cells COXs plays the predominant role. Whatever the major source of prostaglandins is, the activation of neuronal G-protein-coupled receptors, including EP1, EP4, and IP, by secreted prostaglandins causes activation of not only PKC, but also protein kinase A (PKA) that also phosphorylates and sensitizes TRPV1 (Moriyama *et al.*, 2005).

Unsolved problems

While the major heat sensor TRPV1 has been massively studied regarding bradykinin-induced nociception, the contribution of thermosensitive cousins of TRPV1 in the same superfamily, including TRPV2 and TRPV3, have largely been unexplored. Interestingly, another polymodal TRPV member, TRPV4, which is sensitive to warm temperatures and mechanical stretches, has very recently been shown to mediate at least mechanical hyperalgesia of a mouse under paclitaxel-induced peripheral neuropathy via bradykinin-B1/B2-PKC ϵ axis (Costa *et al.*, 2018). In addition, for bradykinin-induced sensitization, effector components other than such direct temperature sensors are possibly involved. For example, knockouts of a tetrodotoxin-insensitive voltage-gated Na⁺ channel, Nav1.9, have displayed a blunted phenotype in bradykinin-induced heat hyperalgesia (Amaya *et al.*, 2006).

Still, it remains as theoretical predictions how exactly PKC underlies both activation and sensitization of TRPV1. It is reasonable to hypothesize that these differential outcomes might result from phosphorylation of different serine residues, which possibly contributes to channel gating in different manners. Alternatively, at the point of AKAP79/150 action, the differential roles of PKC could be diverged. Although it seems to be limited to a particular tissue like cutaneous areas, the transcellular mechanism involving prostaglandins may exclusively be engaged in sensitization.

The central molecular mechanisms for TRPV1 activation and sensitization have firmly been shown to engage voltage-

dependence (Voets *et al.*, 2004). The relevant stimuli, including heat, capsaicin, protons, endogenous ligands, phosphorylations, etc., appear to converge into the leftward shift of TRPV1 voltage-dependence. In this regard, given multiple stimuli may be additive or synergistic for enhancing TRPV1 voltage sensitivity, which can be seen as one stimulus facilitates the response to others (Vyklícký *et al.*, 1999). Accordingly, bradykinin-induced phosphorylation may left-shift the effect of heat on TRPV1 voltage-dependence, leading to augmented firing of the nociceptors upon heat stimulation. An extreme shift may enable TRPV1 activation by ambient temperatures, which can be seen as bradykinin directly excites the neurons. Since TRPV1 is known to essentially undergo Ca²⁺-induced desensitization to itself, Reeh and colleagues have suggested that, prior to desensitization, bradykinin may induce short-term direct firing, and that the relatively blunted shift of TRPV1 sensitivity may look as if its lowered heat threshold during desensitized state (Reeh and Pethö, 2000; Liang *et al.*, 2001). A newly found mechanism unrelated to voltage dependence or even to other signal transductions mentioned above has recently been proposed. Exocytic trafficking of TRPV1-containing vesicle may selectively contribute to the sensitization of peptidifergic nociceptors, which awaits replication (Mathivanan *et al.*, 2016).

The major tissue type where bradykinin induces COX-dependent prostaglandin secretion remains elusive. While nociceptor neurons has been raised as a crucial source of prostaglandins in the pharmacological inhibition of COXs and labeling of COX expression (Mizumura *et al.*, 1987; Kumazawa *et al.*, 1991; Dray *et al.*, 1992; Rueff and Dray, 1993; Vasko *et al.*, 1994; Weinreich *et al.*, 1995; Maubach and Grundy, 1999; Jenkins *et al.*, 2003; Oshita *et al.*, 2005; Inoue *et al.*, 2006; Tang *et al.*, 2006; Jackson *et al.*, 2007), other studies have failed to corroborate this finding and have instead suggested surrounding tissues innervated by neuronal termini (Lembeck and Juan, 1974; Lembeck *et al.*, 1976; Juan, 1977; Franco-Cereceda, 1989; McGuirk and Dolphin, 1992; Fox *et al.*, 1993; Sauer *et al.*, 1998; Kajekar *et al.*, 1999; Sauer *et al.*, 2000; Pethö *et al.*, 2001; Shin *et al.*, 2002; Ferreira *et al.*, 2004). Possibly, COXs in non-neuronal cells may be of more importance during the initial stages of bradykinin action and a relatively long term exposure (~hours or longer) is needed for the induction of neuronal expression of COXs (Oshita *et al.*, 2005). However, the relative importance of COX-1 and COX-2 needs to be fully assessed (Jackson *et al.*, 2007; Mayer *et al.*, 2007). In addition, many lines of pharmacological evidence for COX participation include the reduction in bradykinin-evoked immediate excitation of nociceptors by COX inhibition. On the other hand, the protein kinase-mediated molecular mechanisms of bradykinin action mentioned above only explain sensitized heat responses.

TRANSIENT RECEPTOR POTENTIAL ANKYRIN SUBTYPE 1 ION CHANNEL

Transient Receptor Potential ankyrin subtype 1 (TRPA1) is a comparably important TRP channel in nociception with regards to polymodality. The opening of TRPA1 depolarizes polymodal nociceptors in response to temperatures <17°C, mechanical stretches, and reactive irritants (e.g., mustard oil, cinnamaldehyde, air pollutants, prostaglandins with α,β -

carbonyl carbon, etc.) (Bang and Hwang, 2009). Inflammatory pain mediators such as bradykinin also appear to positively modulate TRPA1 activity, leading to pain exacerbation.

Bradykinin-induced activation of TRPA1 via arachidonic acid metabolism

In an early study where cinnamaldehyde was first found as a specific agonist for TRPA1, bradykinin also displayed an ability to activate TRPA1 via intracellular signaling. In a heterologous expression system co-transfected with DNAs encoding B2 receptor and TRPA1, immediate TRPA1-specific responses occurred upon bradykinin perfusion, as measured by TRPA1-mediated electrical currents and Ca^{2+} influx (Bandell *et al.*, 2004). Perfusions of a membrane-permeable DAG analog and an arachidonic acid analog also replicated this response, indicating that the bradykinin pathway may utilize PLC (perhaps together with DAG lipase) for TRPA1 activation and possibly PLA2. Although further downstream signaling has not been thoroughly explored, it is also possible that other substances more metabolized from arachidonic acid can activate TRPA1. For example, a number of prostaglandins (PGs) have also been shown to activate TRPA1 (Andersson *et al.*, 2008; Materazzi *et al.*, 2008). The PGs include 15-deoxy- Δ 12, 14-PGJ2, Δ 12-PGJ2, PGA1, PGA2, and 8-iso PGA2, all of which contain a reactive carbon that can covalently bind to reactive serine or cysteine residues in TRPA1 protein in the same manner that mustard oil and cinnamaldehyde interact (Hinman *et al.*, 2006; Macpherson *et al.*, 2007). Because the PGs are non-enzymatically generated from COX products such as PGH2 and PGE2, the bradykinin-mediated COX activation mentioned above may be linked to depolarization resulting from TRPA1 activation. Whatever the strongest contributor among the metabolites is, bradykinin appears to depolarize nociceptor neurons not only via TRPV1 but also via TRPA1, which was confirmed in TRPA1 knockout studies through action potential firing and nocifensive behaviors (Bautista *et al.*, 2006; Kwan *et al.*, 2006). TRPA1 knockouts have also exhibited reduced hypersensitivity in response to bradykinin (Bautista *et al.*, 2006; Kwan *et al.*, 2006).

Bradykinin-induced sensitization of TRPA1 activity

Molecular mechanisms for TRPA1 sensitization by bradykinin: Not only activation, but also sensitization of TRPA1 when exposed to bradykinin occurs in nociceptor neurons (Fig. 1). The same research group has suggested that there exist two parallel signaling pathways for bradykinin-induced TRPA1 sensitization, which were the PLC and PKC pathways (Dai *et al.*, 2007; Wang *et al.*, 2008). However, this awaits further confirmation because of some discrepancies. The Gq/11-mediated PLC pathway was raised first (Dai *et al.*, 2007). Without further requirement of downstream signaling such as PKC activation, bilayer PIP2 consumption has been demonstrated to disinhibit TRPA1, which seems to adequately explain enhanced TRPA1 activity observed when exposed to a known specific agonist for TRPA1. This study proposed that the membrane PIP2 intrinsically masks the channel's activity in the resting state, which was confirmed by an independent study showing that the addition of intracellular PIP2 inhibits TRPA1 opening (Kim *et al.*, 2008). Two other studies have shown the opposite effect, where TRPA1 is directly activated by PIP2 (Akopian *et al.*, 2007; Karashima *et al.*, 2008), while another group failed to show this activation (Kim and Cavanaugh,

2007). TRPV1 has once been demonstrated to be either positively or negatively modulated by the presence of PIP2, which may depend on the extent of channel activation, which is not shown yet to be the case for TRPA1 modulation (Lukacs *et al.*, 2007).

Another proposed mechanism for TRPA1 sensitization by bradykinin is via the PKA. As mentioned above, TRPV1 can be sensitized in a similar manner, but PKA action appears to take a relatively long time (~10 minutes) and requires PG synthesis as an upstream signal. However, fast sensitization of TRPA1 was shown to be dependent on Gs-mediated adenylyl cyclase activity and subsequent PKA activation but unlikely with PG production. Such Gs-mediated signaling by bradykinin stimulation has been reported to occur in different cell types (Stevens *et al.*, 1994; Liebmann *et al.*, 1996; Bae *et al.*, 2003). TRPA1, as well as TRPV1, needs further repetition in this regard.

Evidence from nociceptors and animals: Formalin and mustard oil are TRPA1-selective activators that were used as experimental stimulants for nociceptor excitation in the pain research field prior to their relationship with TRPA1 being discovered. Acute nocifensive behaviors are typically evoked by intraplantar administration of either of formalin or mustard oil, and were shown to be significantly facilitated by injections in the same location of bradykinin itself or bradykinin receptor specific agonists (De Campos *et al.*, 1998; Wang *et al.*, 2008). In addition to these chemical-specific modalities, TRPA1 seems to be involved in noxiously mechanical ones to an extent due to its intrinsic mechanosensitivity (Kwan *et al.*, 2006; Petrus *et al.*, 2007; Brierley *et al.*, 2009; Kwan *et al.*, 2009; Yu and Ouyang, 2009). Nociceptor firing in response to mechanical stimuli was significantly diminished in TRPA1-deficient mice or by pharmacological antagonism (Brierley *et al.*, 2005; Brierley *et al.*, 2009; Yu and Ouyang, 2009).

Therefore, it is worth speculating the relationship between TRPA1 and the molecular mechanisms underlying bradykinin-elicited mechanical hypersensitivities that have been proposed from behavioral studies. Protein kinase G (PKG) has been relatively unexplored with regards to TRPA1 modulation, and PKG inhibition has been shown to reduce bradykinin-induced mechanical hyperalgesia (Nakamura *et al.*, 1996). The same study actually suggested that the nitric oxide synthase (NOS)-guanylate cyclase (GC)-PKG cascade mediates the mechanical hypersensitivity. NOS is possibly activated by PLC-IP3-mobilized Ca^{2+} . However, NO itself is known to react with TRPA1 protein and seemed to be inadequate to lead to hyperalgesia despite the heightened level of NO, indicating that further signal amplification via subsequent GC and PKG activation may be required.

Other studies have raised the role of the PLA2-COX pathway in the development of bradykinin-induced mechanical hyperalgesia (Taiwo and Levine, 1988; Taiwo *et al.*, 1990). COX induction by bradykinin may require a transcellular process in the sensitized heat responses mentioned above. In a multitude of studies on this mechanical hypersensitivity, details particularly including complicated upstreams for the COX mechanism have been described (Ferreira *et al.*, 1993a; Ferreira *et al.*, 1993b; Poole *et al.*, 1999; Cunha *et al.*, 2007). Collectively, the causality seems to involve a transcellular mechanism and to be that the sequential secretions of TNF- α , other cytokines including interleukin-1 β (IL-1 β), IL-6, and IL-8, and then finally prostaglandins and sympathetic amines. It appears that the

major sources of TNF- α and sympathetic amines may be vicinal macrophages and sympathetic nerve termini respectively, indicating that the bradykinin-induced mechanical hyperalgesia may also involve transcellular processes. Seeing as surgical sympathectomy alleviated mechanical, but not heat hyperalgesia, the downstream sympathetic amines from the postganglionic neurons may only control the peripheral mechanical function of nociceptors (Koltzenburg *et al.*, 1992; Meyer *et al.*, 1992; Schuligoi *et al.*, 1994).

Interestingly, the occurrence of mechanical hypersensitivity appears to depend on the location of bradykinin accumulation (Manning *et al.*, 1991; Khan *et al.*, 1992; Khasar *et al.*, 1993). This may again indicate that not only the changes in the functionality of nociceptors but also transcellular interactions where specific cellular components that additionally participate are important. In accordance with a study showing that mechanical hyperalgesia was induced by intradermal injections of bradykinin or PGE₂, but not readily by subcutaneous treatments, later studies using a diverse range of nociceptor markers demonstrated that nociceptor termini are differentially distributed in terms of the depth of the skin layer, and that a more superficial subpopulation may supposedly be responsible for mechanical hyperalgesia (Khasar *et al.*, 1993; Zylka *et al.*, 2005; Cavanaugh *et al.*, 2009). Interestingly, it has recently been demonstrated that TRPA1 in the central terminal of nociceptors also contribute to the development of mechanical hyperalgesia by participating in B1 receptor-dependent spinal neuroinflammation where intracellular and transcellular mechanisms may operate in a similar manner as mentioned above (Meotti *et al.*, 2017).

TRPA1 contributes to bradykinin-induced heat hyperalgesia: Although TRPA1 is not intrinsically sensitive to hot temperatures, TRPA1 knockouts have exhibited a blunted phenotype in bradykinin-induced heat hyperalgesia when compared to wild type littermates (Bautista *et al.*, 2006). In the same study, however, CFA-induced heat hyperalgesia was not affected by TRPA1 deletion. TRPA1 may only mildly influence TRPV1-based heat sensation. Intracellular Ca²⁺ elevated first by TRPV1 opening in response to heat was once proposed to link TRPV1 activation to the subsequent TRPA1 activation. However a current theory is that a part of TRPV1 and TRPA1 proteins may be physically coupled to form a sensory complex located on the surface of the nociceptor terminal (Staruschenko *et al.*, 2010; Fischer *et al.*, 2014; Weng *et al.*, 2015).

Difference between TRPA1 and PIEZO2: Piezo-type mechanosensitive ion channel component 2 (PIEZO2) is a recently discovered cation channel that has been shown to be a sensor responsible for innocuous touch and proprioception by displaying rapidly-inactivating feature with a low mechanical threshold and by being expressed in a medium to large diameter non-nociceptive population of sensory neurons, whereas TRPA1 represents a slowly-inactivating component with a high mechanical threshold. Interestingly, one study has shown that a fraction of the nociceptive subpopulation expresses PIEZO2 that can be functionally modified in response to bradykinin signaling (Dubin *et al.*, 2012). Unlike TRPA1, PIEZO2 was only sensitized but not activated by bradykinin exposure. Its mechanosensitivity and response efficacy were both heightened with a moderate threshold shift. PKA and PKC are involved in the enhancement in mechanosensitivity, but surprisingly, nothing upstream of PKC activation related to Gq/11- or Gi/o-coupled PLC activation was associated.

Ca²⁺-ACTIVATED Cl⁻ CHANNELS

The two aforementioned TRPs are not the only excitatory effector ion channels involved in bradykinin-induced nociceptor firing. Many studies have shown that incapacitating TRPs by using antagonist pharmacology or gene deletion did not completely ablate the action potential discharges of nociceptors upon bradykinin exposure (Dray *et al.*, 1990; Dickenson and Dray, 1991; Zahner *et al.*, 2003; Kollarik and Undem, 2004; Rong *et al.*, 2004; Qin *et al.*, 2006; Katanosaka *et al.*, 2008; Brierley *et al.*, 2009). An explanation as to the unknown excitatory component, i.e., the role for Cl⁻ channels has been emerging. Chemical inhibition of Ca²⁺-activated Cl⁻ channels (CaCCs) was effective in reducing bradykinin-induced firing in an *ex vivo* tissue-nerve model from the guinea pig airway sensory system and in *in vitro* cultured nodose neurons (Oh and Weinreich, 2004; Lee *et al.*, 2005a). In sensory neurons, it is well known that the intracellular Cl⁻ concentration is high and that opening of Cl⁻ channels leads to the efflux of Cl⁻, resulting in depolarization, which was confirmed by the same studies. In pathologic conditions, like chronic exposure to major inflammatory pain mediators in the periphery including prostaglandins, nerve growth factor (NGF), and ATP, the intracellular Cl⁻ concentration is known to even further be elevated owing to the facilitated activities of surface Na⁺-K⁺-2Cl⁻ cotransporters (NKCCs), indicating that Cl⁻ channel opening may contribute more to nociceptor firing than non-inflamed situations (Sung *et al.*, 2000; Labrakakis *et al.*, 2003; Funk *et al.*, 2008; Price *et al.*, 2009).

Delicate molecular approach was conducted by a group for determining the genetic identity of CaCC of rodent nociceptors contributing to bradykinin-induced excitation and the underlying activation mechanisms (Liu *et al.*, 2010; Jin *et al.*, 2013). The central player is anoctamin 1 (ANO1, also known as TMEM16A), a relatively newly discovered ion channel. The studies dissected how the intracellular Ca²⁺ was affected by bradykinin stimulation and revealed two phases, and the first of which may more depend on intracellular mobilization by IP₃. This indicates that bradykinin-evoked G protein signaling leads to PLC activation, resulting in productions of IP₃ and DAG as mentioned above (Thayer *et al.*, 1988; Burgess *et al.*, 1989; Kano *et al.*, 1994; Kozaki *et al.*, 2007), followed by the activation of IP₃ receptors on the endoplasmic reticulum (ER) membrane. This causes Ca²⁺ release from the ER, completing the first Ca²⁺ phase. This firstly elevated cytoplasmic Ca²⁺ activates ANO1. ANO1 was shown to physically couple to IP₃ receptor type 1 and bradykinin B2 receptor to compose a signaling complex, enabling the efficient utilization of the locally increased levels of Ca²⁺. The second phase of Ca²⁺ elevation may depend on the influx of extracellular Ca²⁺, resulting from activation of voltage-gated Ca²⁺ channels by ANO1-elicited depolarization, and of TRP channels that are highly Ca²⁺ permeable. Such ANO1-dependent bradykinin-mediated nociception was again confirmed in an *in vivo* study using tissue-specific ANO1-deficient mice (Advillin/Ano1^{fl/fl}) that lost ANO1 expression mainly in DRG neurons (Lee *et al.*, 2014).

K⁺ CHANNEL INHIBITION

The decreased activity of resting K⁺ channels may contribute to depolarization. Indeed, two studies that were mentioned

previously, exploring the outcomes of the first phase of Ca^{2+} elevation in response to bradykinin stimulation have proposed that together with CaCC activation, K^+ channel inhibition is also involved in nociceptor firing during this first phase (Oh and Weinreich, 2004; Liu *et al.*, 2010). Two different K^+ -permeating components were identified as contributors by the two studies respectively, as explained in the following section.

KCNQ voltage-gated K^+ channels

The outward K^+ current mediated by the opening of the KCNQ channel (also known as Kv7) refers to the M current as it was first found as a downstream effector of M2 muscarinic receptor signaling. A fraction of KCNQ channels open in the resting state and control the resting membrane potential and action potential rheobase (Delmas and Brown, 2005). The M current can be inhibited in the early phase of the intracellular Ca^{2+} wave caused by bradykinin exposure (Liu *et al.*, 2010). Further inhibition of the KCNQ-mediated current by a synthetic specific antagonist potentiated bradykinin-induced firing while its activation using the channel opener retigabine diminished it. Acutely pretreated retigabine also prevented nociceptive behaviors caused by intraplantar bradykinin injection in *in vivo* observations. Additionally, chelation of the early Ca^{2+} rise but not PKC or PLA2 inhibition reversed the closing of the K^+ channel in *in vitro* nociceptor assays, indicating that the Gq/11-coupled-PLC-IP3- Ca^{2+} cascade is required for the K^+ channel contribution and that no other signaling downstream of PLC or other branches of G protein signaling seems to be involved. The genetic identity of the KCNQ subtypes responsible for the underlying molecular mechanisms involved in bradykinin-induced signaling remain to be elucidated. Very recently, KCNQ3 and KCNQ5 have been raised as major Kv7 subtypes that depolarize murine and human visceral nociceptors upon B2 receptor stimulation (Peiris *et al.*, 2017).

Ca^{2+} -activated K^+ channels

Another K^+ component altered by bradykinin stimulation has been shown to be mediated by Ca^{2+} -activated K^+ channels (I_{KCa}). With regards to the action potential phase, these K^+ currents typically compose a slow component of the afterhyperpolarization (AHP). AHP is responsible for spike frequency accommodation in repeated firing. A shortened AHP resulting from Ca^{2+} -activated K^+ channel inhibition causes sustained or increased firing frequencies (Weinreich and Wonderlin, 1987; Cordoba-Rodriguez *et al.*, 1999). The contribution of the bradykinin-induced channel blockade to the alteration of nodose neuronal firing may reflect this paradigm (Oh and Weinreich, 2004).

CONCLUSIONS

Bradykinin is among the major pain mediators during inflammation. Peripherally produced bradykinin alters the electrical functions of nociceptor sensory neurons that are the forefront initiators of the ascending signals of the sensory neural pathway for pain perception. Bradykinin generally enhances their excitability, greatly contributing to the generation and exacerbation of pain. At the cellular level, bradykinin not only acutely excites the neurons but also electrically sensitizes them. Through intracellular signaling, mostly composed of G-protein coupled ones, it has been hypothesized that

bradykinin may finally augment the depolarizing activities of some specific effector ion channels expressed in the nociceptor neurons. Currently, an array of ion channels have been shown to be affected in this paradigm. Here we overviewed six important ion channels in their role as a depolarizing effector. The opening of TRPV1, TRPA1, and ANO1 all seem to enact this role in the direct induction of neuronal firing by bradykinin. TRPV1 and TRPA1 also appear to be involved in sensitized neuronal function in a longer duration. PIEZO2 is an emerging ion channel that functions as a mechanical pain-specific sensitizing effector. KCNQ and Ca^{2+} -activated K^+ channels may contribute to the initial excitation through their functional downregulation. Linker signals between bradykinin receptor activation and depolarizing effectors are currently being revealed in greater depth (summarized in Fig. 1).

The consistent expansion of information has broadened the knowledge of the molecular nature of bradykinin-induced inflammatory pain and has validated bradykinin signaling as an analgesic target. In particular, the B2 receptor inhibitor HOE 140 and capsaicin-mediated TRPV1 incapacitation seem to have promising outcomes from a multitude of clinical researches (Backonja *et al.*, 2008; Song *et al.*, 2008). Nonetheless, efforts to establish the excitatory mechanism mediated by relatively recent found effectors such as ANO1 and K^+ channels are still required. Further, unknown component may be present for the nociceptive neuronal actions of bradykinin. For example, pharmacological antagonism of purinergic P2X3 ion channel has once been shown to be effective specifically at bradykinin induced mechanical hyperalgesia, which needs to be confirmed by further molecular approaches (de Oliveira Fusaro *et al.*, 2010). Among nociceptor-specific voltage-gated Na^+ channels, Nav1.9 may particularly be affected under bradykinin-including pathologic condition but the mechanism remains elusive (Vaughn and Gold, 2010). Further accumulation of the knowledge will contribute to more precise understanding of the depolarization mechanisms and to development of more sophisticated painkilling strategies.

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