TRP Channels and Pain

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Abstract

Nociception is the process whereby primary afferent nerve fibers of the somatosensory system detect noxious stimuli. Pungent irritants from pepper, mint, and mustard plants have served as powerful pharmacological tools for identifying molecules and mechanisms underlying this initial step of pain sensation. These natural products have revealed three members of the transient receptor potential (TRP) ion channel family—TRPV1, TRPM8, and TRPA1—as molecular detectors of thermal and chemical stimuli that activate sensory neurons to produce acute or persistent pain. Analysis of TRP channel function and expression has validated the existence of nociceptors as a specialized group of somatosensory neurons devoted to the detection of noxious stimuli. These studies are also providing insight into the coding logic of nociception and how specification of nociceptor subtypes underlies behavioral discrimination of noxious thermal, chemical, and mechanical stimuli. Biophysical and pharmacological characterization of these channels has provided the intellectual and technical foundation for developing new classes of analgesic drugs.

Contents

SENSING NOXIOUS STIMULI: NOCICEPTION AS A DISTINCT SOMATOSENSORY MODALITY

Somatosensation, colloquially referred to as touch, actually encompasses several submodalities that include touch (detection of light mechanical stimuli), proprioception (detection of mechanical displacement of muscle and joints), thermosensation (detection of cool and warmth), and nociception (detection of noxious mechanical, thermal, or chemical stimuli that give rise to pain sensations) (Gardner et al. 2000). Except for chemical sensitivity, nociception could simply be considered an extreme version of touch and temperature sensation. Indeed, one of the great debates in this field has revolved around the question of whether (and to what extent) nociception represents not just a psychophysically distinct aspect of somatosensation but a mechanistically distinct one, as well.

Noxious stimuli are initially detected by primary afferent sensory nerve fibers that innervate a peripheral target and transmit this information to neurons within the dorsal horn of the spinal cord, and thence to the brain, via ascending neural circuits (**Figure 1**) (Basbaum & Jessell 2000). Two opposing models have been put forth to explain how primary afferent neurons encode noxious percepts. According to the pattern theory, pain is produced when a stimulus of sufficient intensity elicits a pattern of activity across functionally indistinct sensory nerve fibers; the resulting pattern is then deconvoluted within the central nervous system (spinal cord and brain) to generate a specific percept representing the noxious mechanical, thermal, or chemical stimulus applied to the peripheral receptive field (Melzack & Wall 1965). In contrast, the specificity theory posits that pain is produced by activation of sensory neuron subtypes that are tuned to detect a stimulus of a specific quality and/or intensity (e.g., noxious heat versus cold) (Bessou & Perl 1969, Perl 2007, Sherrington 1903). As such, information about the modality and intensity of a stimulus is encoded, at least in part, by the primary afferent nerve fiber itself, even before signals reach the

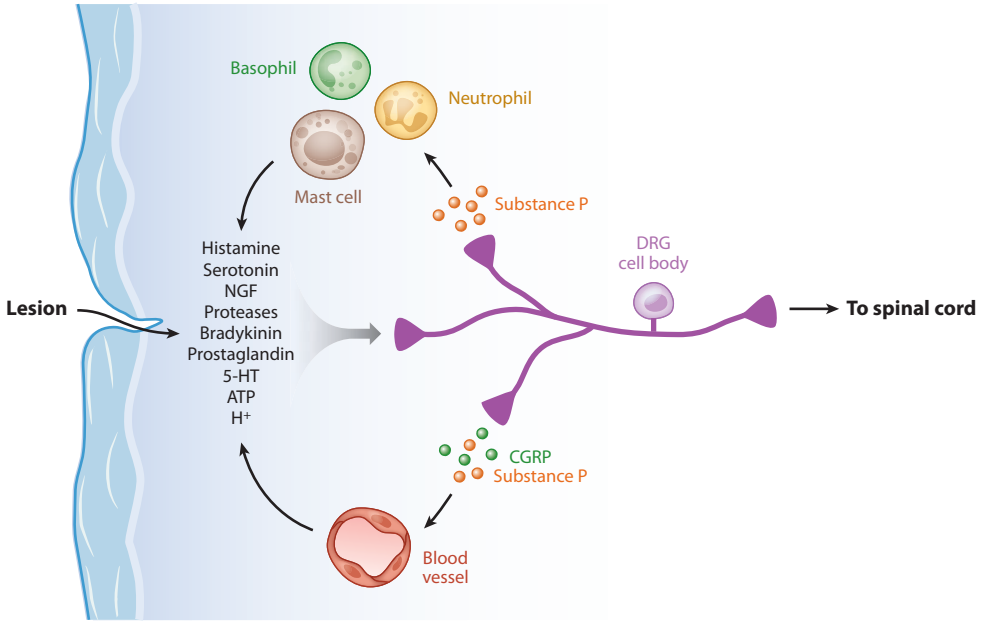


Figure 1

Sensitization of nociceptors by tissue injury and inflammation. Primary afferent nociceptors (*purple neurons*) are activated by noxious thermal, mechanical, or chemical stimuli. Once activated, these nerve fibers transmit noxious signals to secondary neurons in the spinal cord dorsal horn, and thence to the brain, eliciting a percept of acute discomfort or pain. In addition to these centrally transmitted signals, nociceptors are unique in their ability to also signal antidromically, such that they release transmitters not only from central terminals in the spinal cord but also from stimulated peripheral terminals. In particular, some nociceptors release the peptides substance P and CGRP, which elicit vasodilation, vascular leakage, and other responses from nearby peripheral cell types. These actions produce or release a panoply of local signaling molecules, a process referred to as neurogenic inflammation. The inflammatory soup so produced includes neurotrophins, prostanoids and other bioactive lipids, extracellular protons and nucleotides, and monamines, each of which interacts with receptors on the nociceptor terminal to enhance its sensitivity to physical or chemical stimuli. This phenomenon represents a key peripheral mechanism whereby tissue injury promotes pain hypersensitivity. Abbreviation: DRG, dorsal root ganglion.

central nervous system. That is to say, the specificity theory predicts the existence of the nociceptor as a functionally distinct subtype of somatosensory neuron that is devoted to the detection and transduction of noxious, high-intensity stimuli capable of causing tissue damage and eliciting pain (Sherrington 1906). This controversy is not merely academic because each side makes predictions as to how noxious stimuli are encoded; whether receptors and other functionally important molecules are expressed by specific subsets of somatosensory nerve fibers; and, thus, whether and to what extent primary afferent nerve fibers constitute effective targets for analgesic therapy.

As is so clearly demonstrated in other sensory systems (e.g., vision, olfaction, and taste), the cloning and functional characterization of sensory receptors provides the definitive molecular tools with which to address such questions and elucidate the logic of stimulus detection and perception (Julius & Nathans 2012). Indeed, the study of transient receptor potential (TRP) channels has helped resolve long-standing controversies in the pain field by validating the existence of the nociceptor (and thus the specificity theory), while providing a molecular framework for understanding peripheral mechanisms underlying stimulus detection, injury-evoked sensitization,

and psychophysical coding (Basbaum et al. 2009, Caterina & Julius 1999, Patapoutian et al. 2009, Woolf & Ma 2007).

NATURAL PRODUCTS AS PROBES OF THE PAIN PATHWAY

Natural products from medicinal plants have proven to be among the most powerful and important tools for identifying and manipulating key elements of the pain pathway. Two notable examples include morphine from the opium poppy and salicylate from the willow bark, pharmacological agents that were key to discovering opioid receptors and cyclooxygenases, respectively (Snyder 1977, Vane & Botting 1998) (**Figure 2*a***). The profound influence of these natural products on pain research and therapeutics is highlighted by the fact that they represent the two main classes of pain medicines still in use today, namely, opiate analgesics and nonsteroidal anti-inflammatory drugs (Jancsó et al. 1977).

Morphine and aspirin suppress pain, but some plants generate compounds that elicit pain, presumably as a chemical defense mechanism to ward off foraging mammals and other predators. The most familiar of these proalgesic compounds is capsaicin, the pungent agent that gives hot chili peppers their zing (**Figure 2*a***). Capsaicin and related vanilloid compounds robustly and selectively excite primary afferent sensory neurons (Jancsó et al. 1977), eliciting a sensation of acute burning pain accompanied by local vasodilation and inflammation, followed by hypersensitivity to heat (thermal hyperalgesia) and, to some degree, touch (mechanical allodynia) (Jancsó et al. 1967). Thus, sensitivity to capsaicin has long been regarded as a defining functional hallmark of presumptive nociceptors, igniting a decades-long search for its site and mechanism of action.

But capsaicin is not the only plant-derived agent that produces irritation or pain; others include menthol, the cooling agent from mint, as well as isothiocyanate and thiosulfinate compounds that account for the pungency of wasabi, garlic, onions, and other mustard and allium plants (**Figure 2*a***). Like capsaicin, these compounds have been used extensively in cellular and behavioral studies of acute and persistent pain and, most recently, to identify proteins and sensory neurons that contribute to these processes (Basbaum et al. 2009). Remarkably, each of these structurally distinct classes of irritants has evolved to elicit discomfort and pain through a common molecular mechanism in which they target specific members of the TRP channel family on primary afferent nerve fibers; these include TRPV1 (the capsaicin receptor), TRPM8 (the menthol receptor), and TRPA1 (the wasabi receptor) (**Figure 2*b***). Functional and histological analyses have shown that TRPV1, TRPM8, and TRPA1 exhibit distinct patterns of expression within primary sensory ganglia, providing pharmacologic and genetic strategies for identifying and manipulating sensory neuron subtypes that contribute to particular aspects of nociception and pain.

TRP CHANNELS: A BRIEF PRIMER

TRP channels were first discovered in the fly eye, where light-activated rhodopsin stimulates phospholipase C (PLC) to hydrolyze the minor plasma membrane lipid, phosphatidyl-inositol bisphosphate (PIP_2). This, in turn, promotes gating of TRP channels to depolarize the photoreceptor cell (**Figure 3**) (Hardie 2007, Minke & Parnas 2006, Montell 2012). Although the basic outlines of this receptor-operated TRP channel signaling pathway were laid out several decades ago, the mechanism whereby PIP_2 hydrolysis leads to TRP channel gating remains enigmatic. Genetic and electrophysiological studies suggest that channel activation involves a combinatorial process requiring both depletion of PIP_2 and consequent generation of a phosphoinositide-derived second messenger(s), such as polyunsaturated fatty acid and/or intracellular protons (Estacion et al. 2001, Huang et al. 2010). According to this model, TRP channels in the fly eye are negatively

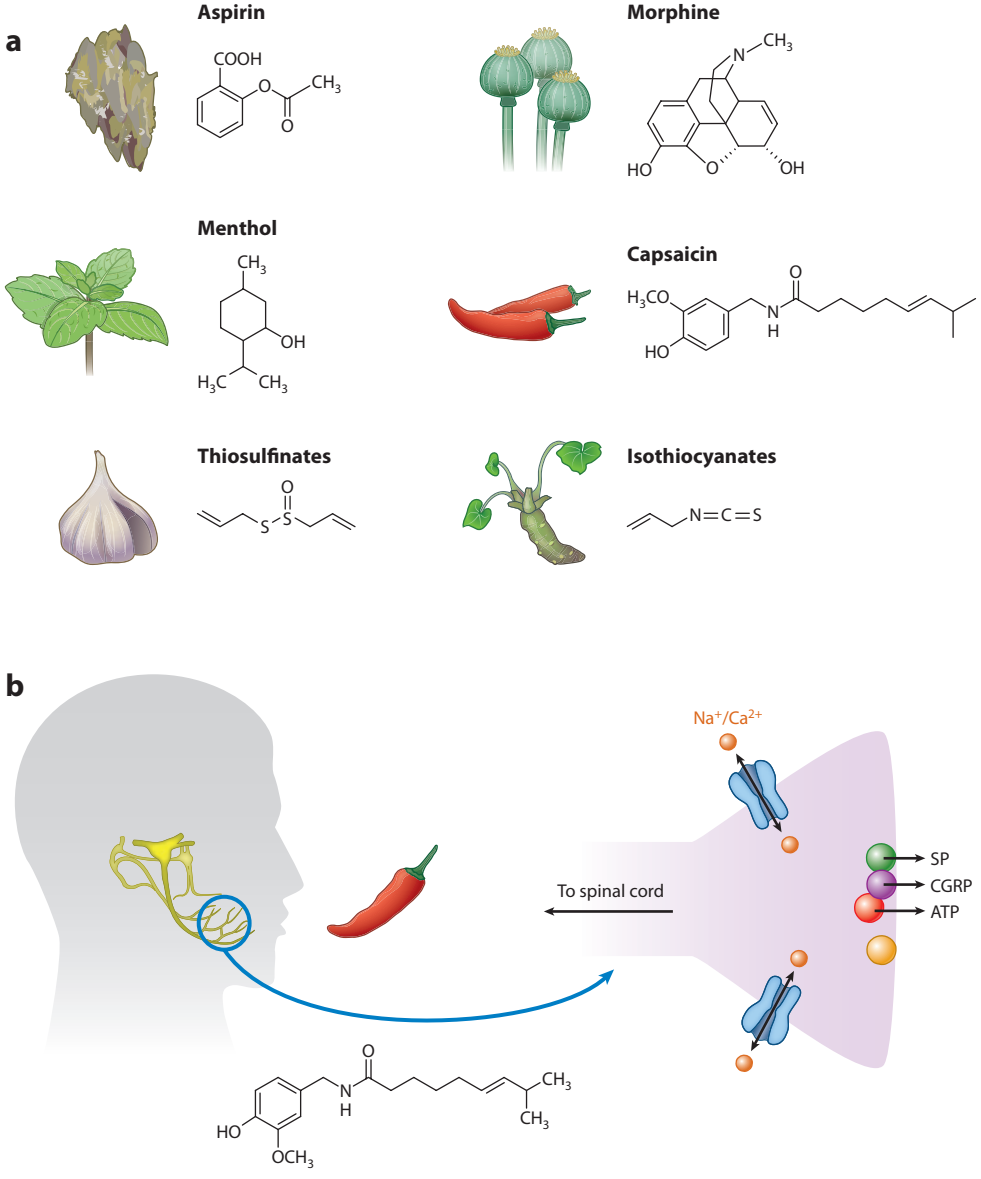


Figure 2

Natural products as probes of the pain pathway. (*a*) Natural plant products have provided the most powerful pharmacological probes for identifying and studying molecules and signaling mechanisms underlying nociception and pain. These include salicylic acid (related to aspirin) from willow bark; morphine from opium poppies; menthol from mint leaves; capsaicin from chili peppers; thiosulfinates from garlic, onions, and other allium plants; and isothiocyanates from wasabi and other mustard plants. (*b*) Capsaicin elicits acute pain and neurogenic inflammation by selectively activating excitatory transient receptor potential (TRP) ion channels on nociceptor nerve endings, promoting the release of neurotransmitters and peptides from both central and peripheral nerve terminals. Similar mechanisms underlie the actions of menthol, isothiocyanates, thiosulfinates, and related pungent irritants.

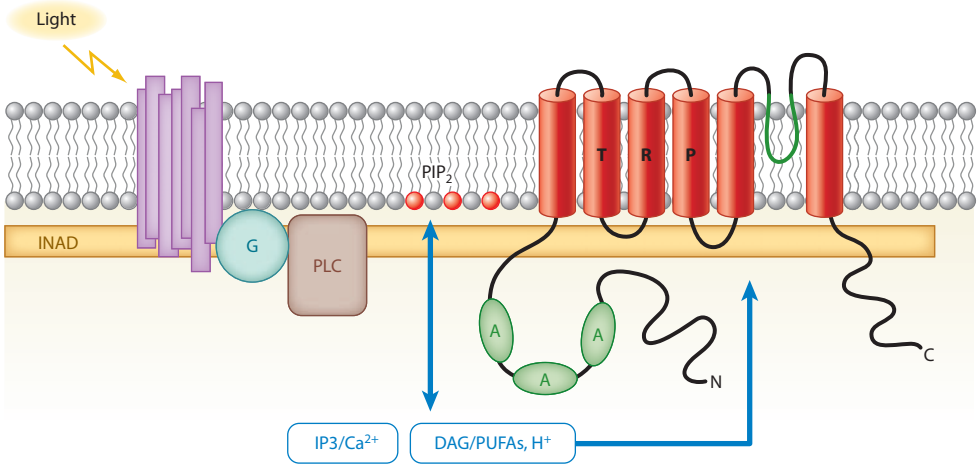


Figure 3

Drosophila transient receptor potential (TRP)—a model for the canonical receptor-operated TRP channel. TRP channels were first characterized in the fly eye, in which they depolarize the photoreceptor cells in response to light. Photon-evoked activation of G protein–coupled rhodopsin (*purple*) stimulates phospholipase C (PLC) to catalyze the hydrolysis of plasma membrane phosphatidyl-inositol-4,5-bisphosphate (PIP_2), yielding second messengers inositol triphosphate (IP3), diacylglycerol (DAG) and downstream polyunsaturated fatty acids (PUFAs), and intracellular protons. How these actions promote TRP channel activation remains a matter of discussion and debate, although most evidence points to the importance of lipid-channel interactions. Abbreviations: A, ankyrin repeats; C, carboxy terminus; G, heterotrimeric G protein; INAD, scaffolding protein for fly TRP and associated signaling molecules; N, amino terminus.

regulated by PIP_2 (and possibly other phosphoinositides), such that PLC-mediated hydrolysis removes this inhibitory constraint and allows the channel to then be fully activated by simultaneously produced metabolites or other physiologic stimuli.

Vertebrate TRP channels represent an extended protein family consisting of more than 30 distinct subtypes (Ramsey et al. 2006). Genetic studies have highlighted the importance of TRP channels in numerous biological processes (e.g., calcium adsorption, neuronal growth cone guidance, keratinocyte development, and sensory transduction), and TRP channelopathies are associated with a range of human disorders (e.g., polycystic kidney disease, skeletal dysplasia, and familial episodic pain syndrome) (Nilius 2007). Thus, understanding how these channels respond to physiological stimuli and drugs is of direct clinical and therapeutic relevance to diseases that affect virtually every major organ system in the body. However, when compared with most other ion channel superfamilies, such as those of the voltage- or ligand-gated ilk, the study of TRP channels is still in its infancy, and endogenous stimuli or physiological roles for many family members remain unknown. Moreover, whereas functional TRP channels likely resemble voltage-gated potassium channels in regard to their overall transmembrane topology and tetrameric subunit composition, relatively little is known about their structure or structure-activity relationships. These gaps in knowledge reflect several limitations, including (*a*) a dearth of pharmacological agents for manipulating channel function in vitro or in vivo; (*b*) a lack of detailed (i.e., crystallographic) structural information (except for some soluble intracellular domains); and (*c*) relatively low sequence similarity among TRP family members, which makes identification of conserved motifs and analysis of subtype chimeras challenging. As such, recent efforts have focused on

discovering novel, subtype-selective pharmacophores (including toxins and other natural products); developing unbiased, high-throughput screens for pinpointing functionally important channel domains; and applying protein purification and reconstitution methods to probe channel function in defined systems using biophysical and structural approaches. These limitations notwithstanding, somewhat rudimentary structure-function maps are beginning to emerge for certain TRP channel subtypes, which are discussed here primarily in the context of TRPV1 and TRPA1.

Although TRP channels are widely divergent in their primary structures and (known or presumed) physiologic functions, many resemble ancestral fly channels in their ability to be activated or modulated by PLC-coupled receptors (Venkatachalam & Montell 2007). As in the fly eye, mechanisms underlying these receptor-operated pathways remain enigmatic, but existing results suggest that vertebrate TRPs have evolved to recognize distinct consequences of PLC activation, with some subtypes responding to lipid metabolism per se and others responding to inositol-triphosphate (IP3)-mediated Ca^{2+} release. In this way, neurotransmitters or hormones that activate PLC-coupled receptors can activate TRP channels or enhance their sensitivity to other physiological stimuli. Indeed, some TRP channels respond to multiple physiological inputs, enabling them to function as polymodal signal integrators capable of assessing complex changes in the cell or tissue environment. Despite the physiological relevance of such regulation—which, in the context of nociception, bears significantly on pathways leading to nociceptor sensitization and consequent pain hypersensitivity—the mechanistic and structural details underlying PLC-evoked channel modulation remain largely unresolved.

As mentioned above, physiologic stimuli for most members of the TRP family remain unknown, hampering our ability to uncover in vivo roles, mechanisms of activation, or structural underpinnings of stimulus detection and channel gating. TRP channels of the somatosensory pathway are notable exceptions because they are targeted by defensive natural products (plant-derived irritants and spider toxins) that elicit discomfort or pain, providing unique and powerful pharmacological tools with which to manipulate and probe channel function in a variety of biological systems. Another advantage derives more generally from the fact that sensory systems must evolve to detect and transduce signals that are most salient for the survival and reproduction of an organism (Julius & Nathans 2012). As such, comparative analysis of sensory receptors from different species represents a tremendously powerful way to identify residues or domains that specify stimulus detection or other functional attributes. Indeed, this approach has been quite fruitful in the study of sensory TRP channels and stands in contrast with the relatively meager success in comparing TRP channel subtypes within a given organism, where low sequence similarity among members of the superfamily has stymied identification of conserved functional domains or the analysis of chimeras generated from TRP channel homologs within a given species. For these reason, somatosensory TRPs have become favored subjects for elucidating basic principles underlying TRP channel pharmacology, structure, and regulation. What follows is a current view of physiological roles, activation mechanisms, and structure-function relationships for the nociceptive TRP channels, TRPV1, TRPM8, and TRPA1. This is not meant to be comprehensive but, rather, to highlight a few areas that are particularly interesting and relevant to the study of nociception and pain.

TRPV1: A HEAT-ACTIVATED ION CHANNEL AND INTEGRATOR OF INFLAMMATORY SIGNALS

TRPV1 (also known as VR1) is the founding member of a subfamily of thermosensitive TRP channels that enable primary afferent nociceptors, or other cells, to detect changes in ambient temperature over a wide physiologic range (Julius 2005, Patapoutian et al. 2003). TRPV1 is a

noxious heat–activated channel with a steep temperature coefficient ($Q_{10} > 20$) and a thermal activation threshold of ~43°C (Caterina et al. 1997). Genetically engineered (knockout) mice lacking TRPV1 show partial deficits in acute thermal nociception, which attests to a role for this channel in heat-evoked pain. Importantly, products of tissue damage and inflammation can dramatically decrease this threshold, and genetic and pharmacologic studies have shown that TRPV1 is an essential component of the cellular signaling mechanisms through which injury produces thermal hyperalgesia and pain hypersensitivity (Basbaum et al. 2009, Caterina et al. 2000, Davis et al. 2000).

TRPV1 marks a population of unmyelinated, slowly conducting neurons (C-fibers) that express the neuropeptides substance P, neurokinin A, and CGRP and constitute approximately 30–50% of all somatosensory neurons within rodent sensory ganglia (Kobayashi et al. 2005, Tominaga et al. 1998). Activation of these fibers promotes peptide-mediated vascular leakage, vasodilation, and other paracrine actions on both excitable and nonexcitable cells in the vicinity of the trauma, culminating in the production and release of a complex mixture of proinflammatory and proalgesic factors (including neurotrophins, bioactive lipids, extracellular protons and nucleotides, proteases, and monoamines), collectively referred to as the inflammatory soup. Once released, these factors act back on local sensory nerve endings to enhance their sensitivity to temperature or touch (**Figure 1**). This process of nerve fiber–initiated (neurogenic) inflammation, together with immunologically initiated inflammation, accounts for an important component of tissue injury–evoked pain hypersensitivity (McMahon et al. 2006, Meyer et al. 2006). Indeed, TRPV1 has come to represent the preeminent molecular marker for defining the nociceptor subpopulation that accounts for acute sensitivity to noxious heat, neurogenic inflammation, and thermal hyperalgesia. Activation of these peptidergic C-fibers likely contributes to acute and chronic pain associated with a wide range of pathophysiological conditions involving inflammation, such as arthritis, irritable bowel syndrome, pancreatitis, migraine headache, and cancer pain (Szolcsányi & Sandor 2012). Importantly, neurogenic inflammation helps to initiate a cycle in which injury-evoked nociceptor activation initiates or exacerbates the inflammatory response, leading to, for instance, further activation and sensitization of the nociceptor and ongoing inflammation. Thus, drugs targeting the nociceptor may not only diminish pain but also facilitate healing by interrupting this neuroinflammatory cycle.

Remarkably, somatosensory nerve fibers express receptors for most, if not all, components of the inflammatory soup, rendering them competent to respond to these factors through a variety of signaling mechanisms (Julius & McCleskey 2006). TRPV1 is an important target for many of these factors, which enhance sensitivity to heat by lowering the channel's thermal activation threshold. TRPV1 sensitization can occur in two ways: Some proalgesic agents (e.g., extracellular protons, anandamide and other bioactive lipids, capsaicin, and peptide toxins) interact directly with the channel to serve as positive allosteric modulators, whereas others (e.g., bradykinin, ATP, and nerve growth factor) stimulate their own receptors (of the G protein–coupled or tyrosine kinase variety) to activate PLC or other second messenger–signaling pathways. In this way, TRPV1 functions as a polymodal integrator of thermal and chemical signals, modulating nociceptor excitability in response to changes in the local tissue environment (Caterina & Julius 2001, Huang et al. 2006, Tominaga et al. 1998). Elucidating structural and biophysical underpinnings of these interactions remains a fascinating and important goal from both basic and translational standpoints.

An Emerging Functional Map of TRPV1

One relatively straightforward but important goal has been to identify regions of TRPV1 that specify sensitivity to direct-acting chemical ligands, including exogenous irritants, endogenous

proalgesic agents, and synthetic pharmacophores. This has been accomplished through standard mutational analyses, guided where possible by species-specific differences in ligand sensitivity.

The vanilloid site. One of the first such goals was to identify elements governing sensitivity to capsaicin and other vanilloid compounds. This was facilitated by a bit of neuroethological insight that relates to a phenomenon called directed deterrence, wherein mammals are sensitive to the noxious effects of capsaicin and thus repelled from the chili plant, whereas birds are indifferent to capsaicin and therefore are evolutionarily favored as vectors for seed dispersal (Tewksbury & Nabhan 2001). Analysis of chimeric rat/chicken TRPV1 channels, together with other site-directed mutational studies, suggests that capsaicin sensitivity is specified by residues within the second and third transmembrane (TM2 and TM3) regions and the intervening cytoplasmic loop (Jordt & Julius 2002), consistent with patch-clamp-recording studies suggesting that vanilloid agonists penetrate or cross the membrane to activate TRPV1 from the intracellular face of the bilayer (Jung et al. 1999). In a similar vein, species differences in efficacy and/or potency of other vanilloid compounds, such as resiniferatoxin, an ultrapotent TRPV1 agonist from *Euphorbia* cactus, or capsazepine, a synthetic antagonist, have also been exploited to map residues that specify ligand sensitivity (Gavva et al. 2004, Johnson et al. 2006). Taken together, these studies consistently highlight TM2 through TM4 as the region containing a putative vanilloid binding pocket, in which the vanillyl moiety or the aliphatic side chain is proposed to interact with aromatic and/or other hydrophobic residues in the cytoplasmic loop between TM2 and TM3 (**Figure 4*a***). At this point, however, a more accurate and detailed picture of the vanilloid binding site will likely require a high-resolution structure of a ligand-channel complex.

From a physiological standpoint, such information will be highly significant for a couple of reasons. First, the vanilloid binding site likely specifies interaction with endogenous factors such as anandamide and other bioactive lipids that bear structural similarity to capsaicin (so-called endovanilloids) (Jordt & Julius 2002). Thus, the vanilloid site represents an important locus at which certain components of the inflammatory soup enhance the (mammalian) channel's sensitivity to heat. Second, we know that for some species, the vanilloid antagonist capsazepine blocks activation not only by capsaicin but also by heat (Tominaga et al. 1998). Thus, delineating the atomic structure and relative location of this key allosteric site will likely provide great insight into the mechanism(s) by which chemical and physical stimuli gate the channel.

The proton site. Local tissue acidosis is a hallmark of injury and inflammation, and extracellular protons constitute a prominent component of the inflammatory soup. Protons excite or sensitize nociceptors by interacting with numerous molecular targets, including members of the acid-sensing ion channel (ASIC) and TRP channel families (Holzer 2009, McMahon et al. 2006, Meyer et al. 2006). Genetic and physiological studies suggest that ASICs contribute significantly to acute acid-evoked ischemic pain in skeletal or cardiac muscle, whereas TRPV1 plays a prominent role in acid-evoked sensitization of cutaneous and visceral nociceptors (Leffler et al. 2006, Sugiura et al. 2007, Yagi et al. 2006). ASIC channels can be activated by relatively mild decreases in extracellular pH and account for the large, transient component of proton-evoked currents seen in a variety of sensory neuron subtypes. TRPV1 is activated at lower pH values (threshold ~pH 6 at room temperature) and accounts for the smaller, persistent phase of proton-evoked currents seen in many small, unmyelinated neurons (Leffler et al. 2006). Whereas protons serve as bona fide TRPV1 agonists under conditions of extreme acidosis (pH < 6), they function as positive allosteric modulators under more moderate acidic conditions (pH 6.5), greatly enhancing sensitivity to heat, capsaicin, or other inflammatory agents. These dual actions likely contribute to nociceptor sensitization and pain hypersensitivity under a variety of pathological conditions (e.g., infection,

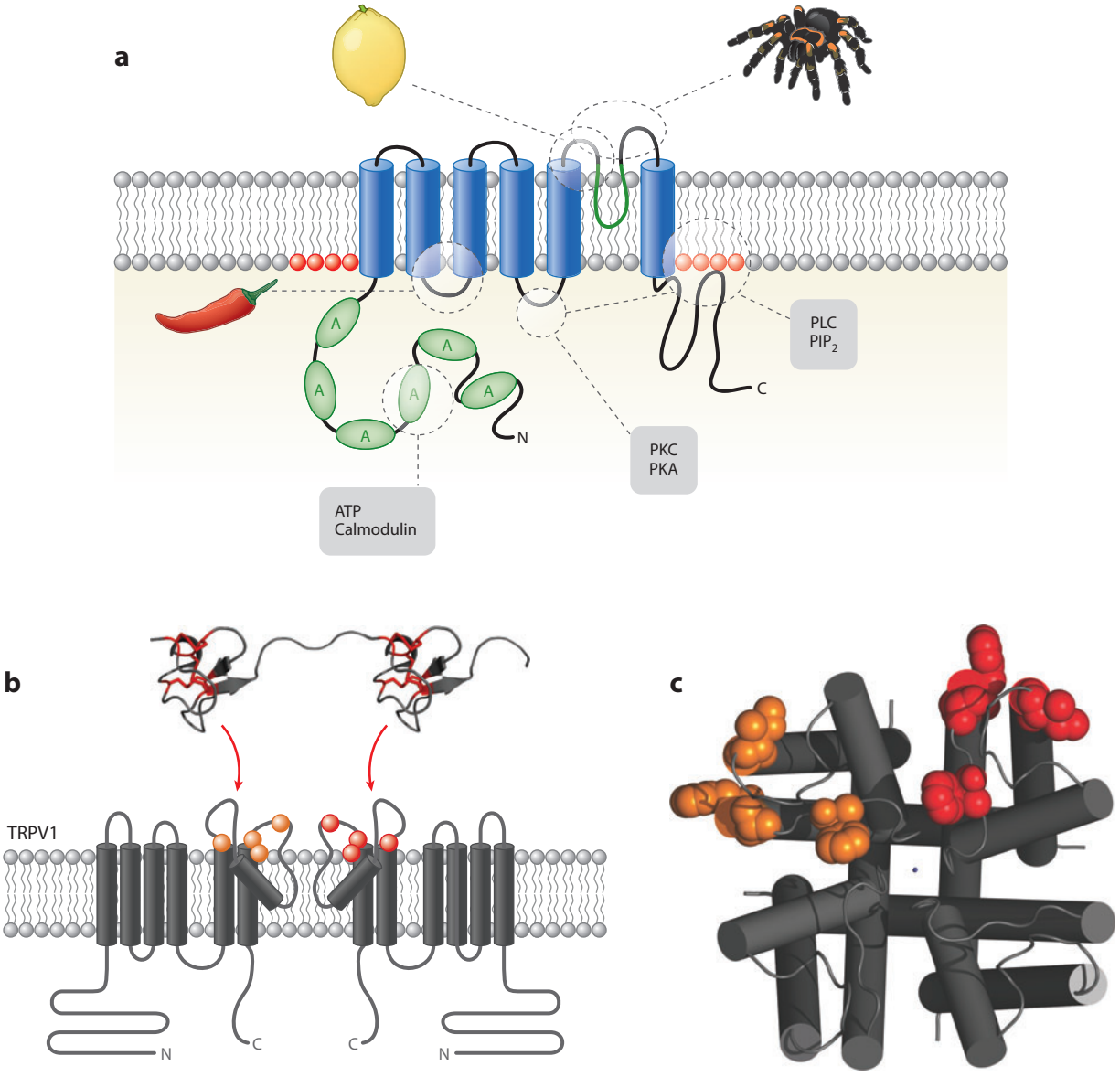


Figure 4

An emerging structure-function map for the capsaicin receptor, TRPV1. (*a*) Pharmacological and mutagenesis studies have identified TRPV1 domains that confer sensitivity to various stimuli or contribute to physiological modulation of the channel downstream of metabotropic receptors. These include sites of action for capsaicin (*chili pepper*) and related vanilloid ligands, extracellular protons (*lemon*), or peptide toxins from tarantula (*spider*). Regions implicated in channel modulation by cellular proteins and cytoplasmic second messengers are also indicated. (*b*) The bivalent double knot toxin (DkTx) from the Earth Tiger tarantula binds to the outer pore region of TRPV1, locking it in its open state. Four residues within this region define a putative footprint for toxin binding, illustrated schematically in a cross section of the channel showing just two diagonally opposed subunits for clarity. (*c*) Top-down view of proposed DkTx binding site on a homotetrameric TRPV1 channel. Orange and red spheres represent locations of amino acids (on neighboring channel subunits) that have been implicated in toxin binding. The TRPV1 pore domain was modeled after the structure of the bacterial KcsA potassium channel. Panels *b* and *c* were adapted with permission from Bohlen et al. (2010). Abbreviations: A, ankyrin repeats; C, carboxy terminus; N, amino terminus; PLC, phospholipase C.

arthritis, ischemia, tumor growth) in which tissue acidosis ranges from moderate to severe (Holzer 2009).

Electrophysiological recordings from excised plasma membrane patches clearly show that protons activate or sensitize TRPV1 only when supplied to the extracellular face of the bilayer (Tominaga et al. 1998), suggesting that protons interact with a region(s) of the channel facing outside of the cell. Indeed, structure-function studies have identified residues within extracellular domains that, when mutated, specifically alter proton sensitivity (Jordt et al. 2000, Ryu et al. 2007). Two important conclusions have emerged from these experiments. First, mutations at some amino acids (e.g., E600) perturb proton-mediated sensitization without abolishing proton-evoked activation, whereas mutations at other sites (e.g., E648 and T633) exhibit the converse phenotype. Thus, the ability of extracellular protons to sensitize TRPV1 under moderate acidic conditions can be uncoupled from their ability to activate the channel de novo at more extreme pH, which demonstrates that these different modalities of proton action occur through separable and distinct mechanisms. Second, most residues specifying proton sensitivity are located between TM5 and TM6, within the outer pore and pore helix domains adjacent to the presumptive ion permeation path (**Figure 4*a***). These observations, together with additional structure-function studies (noted below), implicate the outer pore and pore helix domains as key regulators of TRP channel gating. This notion has been subsequently validated by the discovery of novel pain-producing toxins that activate TRPV1.

The spider toxin site. Venoms from spiders, snakes, fish, cone snails, and scorpions contain a pharmacopoeia of toxins that modulate (usually block) receptor or channel function as a means of producing shock, paralysis, or death (Escoubas & Rash 2004, Lewis et al. 2012). Classic examples are provided by tetrodotoxin from puffer fish and Joro toxin from spiders, which inhibit voltage-gated sodium channels and ionotropic glutamate receptors, respectively. In addition to such small-molecule antagonists, there exist a tremendous diversity of genetically encoded peptide toxins that target a wide range of physiological processes. Well-known examples include α-bungarotoxin from elapid snakes and charybdotoxin from scorpions, which potently and specifically block nicotinic acetylcholine receptors and calcium- and voltage-gated potassium channels, respectively. These and other such toxins have proven essential for identifying key structural elements underlying ion channel function, including ligand-binding sites, voltage-sensing domains, and ion-permeation pathways (Bosmans & Swartz 2010, Miller 1995).

Historically, natural plant irritants have predominated in the pharmacological analysis of somatosensory function. However, metazoans must also defend themselves; thus, it stands to reason that they, too, possess specialized chemical mechanisms for repelling predators or competitors. Indeed, bites and stings from venomous creatures often produce acute pain and neurogenic inflammation (Chahl & Kirk 1975), leading one to wonder whether such responses are mediated through excitation of primary afferent sensory nerve fibers by specific venom components. With this as a guiding neuroethological framework, venoms from a variety of animals were screened for their ability to activate heterologously expressed TRP channels, which culminated in the identification of four novel peptide toxins from tarantulas that robustly activate TRPV1 (Bohlen et al. 2010, Siemens et al. 2006). All four of these so-called vanillotoxins belong to the extended family of inhibitor cystine knot (ICK) peptides commonly found in venoms from spiders, scorpions, and cone snails. The typical ICK motif is a short amphipathic peptide 25–50 amino acids in length that contains multiple disulfide bonds. This structure constrains the toxin into a rigid and compact shape, enhancing stability as well as avidity for membrane targets (Zhu et al. 2003).

Three of the vanillotoxins (VaTx1, 2, and 3) were purified from the venom of the Trinidad chevron tarantula (*Psalmopoeus cambridgei*), an arboreal spider from the West Indies. Each is a

34- or 35-residue peptide scaffolded by three intramolecular disulfide bonds. VaTx1 and VaTx2 share ~80-sequence similarity with heteroscodratoxin and related spider toxins that inhibit Kv2-type voltage-gated potassium channels by interacting with residues in the C-terminal half of TM3 and retarding movement of the adjacent TM4 voltage sensor domain during membrane depolarization (Phillips et al. 2005). Interestingly, VaTx1 is as potent an agonist of TRPV1 as it is an antagonist for Kv2.1 (Siemens et al. 2006), and in light of the presumed structural similarity between TRP and Kv channels (Ramsey et al. 2006), one might therefore assume that vanillotoxins also target the TM3–TM4 region of TRPV1 to promote channel gating. This, however, does not seem to be the case, as revealed by the analysis of a fourth tarantula toxin, called DkTx, that exhibits exceedingly high avidity and specificity for TRPV1.

DkTx was isolated from the Chinese bird spider (*Ornithoctonus huwena*, also known as the Earth Tiger tarantula), a rather aggressive burrowing species found in the tropical rain forests of southern China and Vietnam. Consistent with the spider's fearsome reputation, DkTx binds to TRPV1 with extremely high avidity and serves as an essentially irreversible agonist (Bohlen et al. 2010). Thus, compared with vanillotoxin-evoked responses, which decay over 1–2 min following washout, DkTx-evoked currents persist for a very long time, decreasing minimally (<20%) even after an extended (>15 min) washout period. DkTx is also an ICK peptide, but one very unique feature accounts for its near-irreversible binding; it is approximately twice the length of the typical ICK toxin and contains two ICK motifs in tandem, separated by a linker region. Biophysical and biochemical experiments show that antibody-like bivalency of this double-knot toxin (ergo DkTx) underlies its extremely high avidity, likely reflecting an exceedingly slow dissociation rate from its multimeric (i.e., homotetramer TRPV1) target.

Where on TRPV1 does DkTx bind to effect channel gating? Here, again, species-specific differences in ligand sensitivity could be exploited to address this question. *Xenopus* TRPV1 channels are resistant to DkTx; thus, analysis of frog-rat chimeras highlighted key amino acids that specify vanillotoxin sensitivity. These residues are clustered in extracellular loops between TM5 and TM6, delineating a potential footprint for DkTx on the outer pore domain of the channel (Bohlen et al. 2010) (**Figure 4*b***). Presumably, each of the toxin's two ICK domains interacts with individual subunits in the homotetrameric channel complex to stabilize the open state conformation. Whether these subunits are adjacent or orthogonal to each other is not known (**Figure 4*c***). In any case, the important conclusion is that vanillotoxins have presumably evolved to target the outer pore domain because this region likely undergoes substantial conformational rearrangement during channel gating. This hypothesis is supported by a host of mutagenesis studies indicating that the outer pore and pore helix domains of TRPV channels are critical for gating (Grandl et al. 2008, 2010; Myers et al. 2008; Ryu et al. 2007; Yeh et al. 2005) and is consistent with the fact that extracellular protons also target this region to effect allosteric modulation (see above).

Whereas TRP and voltage-gated channels are thought to share similar overall structures (Ramsey et al. 2006), toxins that modify gating have apparently evolved to target different domains, likely reflecting the relative importance of these domains to the gating process. Remarkably, this distinction can even be made by a single toxin, as suggested by the fact that mutations affecting VaTx1-mediated inhibition of Kv2.1 reside within the S3-S4 region, whereas those affecting VaTx1-evoked activation of TRPV1 map to the outer pore domain (Bohlen et al. 2010). How a small ICK peptide can engage in interactions with two distinct, nonoverlapping sites is a fascinating structural puzzle that may help to reveal conformational secrets underlying differential gating mechanisms for these distant ion channel cousins.

Cytoplasmic termini and intracellular loops as key regulatory sites. Sensitization of TRPV1 by inflammatory factors involves not only direct actions by allosteric agents but also modulation by

second messenger–signaling pathways, akin to the role of ancestral fly TRP as a receptor-operated channel. In this regard, various intracellular domains have been recognized as important loci for modulation by phosphorylation or by interaction with other proteins, such as calmodulin, or with plasma membrane lipids, most notably PIP_2 and other phosphoinositide derivatives. The inflammatory soup contains several proalgesic agents that sensitize TRPV1 and elicit thermal hyperalgesia by activating Gq/PLC-signaling pathways. This includes activation of G protein–coupled receptors by bradykinin, extracellular nucleotides, or monamines, as well as activation of receptor tyrosine kinases by neurotrophins, most notably nerve growth factor (McMahon et al. 2006). In each case, the cellular consequences include the hydrolysis of PIP_2, increased cytoplasmic free calcium, and activation of protein kinase C, each of which exerts a modulatory action on TRPV1.

The most straightforward, and perhaps least controversial, mechanism of sensitization involves protein kinase C–mediated phosphorylation of TRPV1 at two main intracellular sites (S502 and S800) located within the loop connecting TM3 and TM4 and the proximal region of the C-terminal tail, respectively (Bhave et al. 2003, Cesare & McNaughton 1996, Numazaki et al. 2002, Premkumar & Ahern 2000, Vellani et al. 2001) (**Figure 4*a***). Pharmacological inhibition of PKC, or mutation of these sites to nonphosphorylatable residues, diminishes TRPV1 sensitization by agents that activate Gq-coupled receptors. Still unclear is the biochemical or structural basis whereby phosphorylation enhances channel sensitivity, although, as noted below, interaction with anionic membrane lipids may factor into this mechanism.

As in the case of TRP channels in the fly eye, depletion of PIP_2 per se may play an important role in PLC-mediated sensitization of TRPV1. Pharmacological and mutagenesis studies support a model in which a positively charged, lysine/arginine-rich region(s) of the channel's C-terminal tail interacts with the negatively charged phospholipid head groups on the inner leaflet of the plasma membrane (Chuang et al. 2001, Prescott & Julius 2003, Ufret-Vincenty et al. 2011), reminiscent of well-studied interactions between PIP_2 and cytoplasm-facing residues of inwardly rectifying potassium channels (Gamper & Rohacs 2012, Hansen et al. 2011, Hilgemann et al. 2001). PLC-mediated PIP_2 hydrolysis would release the TRPV1 C terminus, thereby relieving the channel from an inhibitory constraint imposed by this membrane-tethering interaction (Prescott & Julius 2003) (**Figure 4*a***). Again, as in the case of fly TRP, PIP_2 depletion alone is insufficient to activate TRPV1 under most physiological conditions and requires another cooperating stimulus, such as heat or extracellular protons. However, disrupting interaction between PIP_2 and the C terminus sensitizes TRPV1, thereby lowering the stimulus intensity (i.e., thermal activation threshold) required for gating. Interestingly, one of the functionally important PKC sites (S800) falls within this putative PIP_2 interaction zone, which suggests one potential mechanism whereby PKC-mediated phosphorylation could contribute to channel sensitization by enhancing repulsion between the C terminus and negatively charged membrane phospholipids.

This model is entirely consistent with the well-validated and widely accepted observation that activation of PLC-coupled receptors robustly potentiates TRPV1-mediated responses to heat, capsaicin, or other direct-acting stimuli. It is also supported by the fact that natural evolutionary variation within the C-terminal region is associated with species-specific tuning of TRPV1 thermal activation profiles. This is most dramatically illustrated by analysis of TRPV1 in vampire bats, in which a cohort of somatosensory nerve fibers innervate specialized facial structures (pit organs) that detect infrared heat from warm-blooded prey. The vampire bat TRPV1 gene gives rise to two splice variants that differ only in the length of the C terminus. The long isoform is essentially identical to TRPV1 channels expressed by mice and humans and has a characteristic thermal activation threshold of ~40°C. The short isoform, which is expressed exclusively by somatosensory neurons that innervate the head and face, lacks 62 amino acids from the C terminus, being truncated at precisely the start of a putative PIP_2-interacting domain (Gracheva et al. 2011). Interestingly, this

truncated channel has a much lower thermal activation threshold (~30°C), in keeping with its postulated role as a highly sensitive detector of radiant body heat from prey.

Nonetheless, this model is not without controversy, and several objections have been raised. One concerns the broader issue of how phosphoinositide lipids interact with TRP channels. Whereas there is general agreement that such interactions occur (Rohacs & Nilius 2007), there is dissent as to whether PIP_2 and other phosphoinositide lipids exert positive or negative regulatory effects (or both) on TRPV1 (Gamper & Rohacs 2012, Klein et al. 2008, Lukacs et al. 2007). Other issues, such as the precise location of putative PIP_2 binding sites within the TRPV1 C terminus (Prescott & Julius 2003, Ufret-Vincenty et al. 2011) and the involvement of cellular phosphoinositide-binding proteins (Kim et al. 2008, Ufret-Vincenty et al. 2011), have also been disputed. Existing controversies and inconsistencies may reflect the complexity of cell-based experimental systems, as well as uncertainties as to the identity of lipids, lipid-modifying enzymes, scaffolding proteins, and other factors in whole cells versus excised membrane patches.

One approach to resolving these controversies, at least in regard to defining a channel's intrinsic sensitivity to a given stimulus or modulator, is to reconstitute heterologously expressed and purified channel protein into a fully defined synthetic lipid environment. This has been accomplished for TRPM8 and TRPV1, demonstrating in each case that these channels are intrinsically sensitive to thermal stimuli, as well as to modulation by PIP_2 (Zakharian et al. 2010, Cao et al. 2013). Interestingly, PIP_2 exerts opposite effects on these channels, serving as a positively acting, obligatory cofactor for TRPM8 while exerting negative, inhibitory actions on TRPV1. These studies have also shown that other phosphoinositide species, such as PI and PI4P, now known to be present at the plasma membrane at levels rivaling that of PIP_2, can also modulate TRP channel activity. Thus, TRP channels are intrinsically sensitive to phosphoinositide lipids, but the functional outcome varies with channel subtype, likely reflecting the relationship between a given channel, PLC-coupled receptors, and other signaling mechanisms that control phosphoinositide lipid metabolism. At this point, resolution of existing controversies will likely require high-resolution crystallographic structures of TRP channels in different phospholipid environments, as were recently achieved for inwardly rectifying potassium channels (Hansen et al. 2011).

To date, the only high-resolution structural information for TRP channels is that determined for N-terminal regions of TRPV1 and related members of the TRPV subfamily (Gaudet 2009). N-terminal domains for these channels, and some other TRP subtypes, contain strings of ankyrin repeat domains (ARDs). ARDs are 33–amino acid–long motifs which, when tandemly arrayed, form scaffolds for a wide range of protein-protein interactions. Although it is assumed that ARDs play a similar role in TRP channels, no specific intra- or intermolecular interactions have so far been identified. Nonetheless, removal or disruption of these motifs is usually deleterious to channel function, which attests to their importance for assembly, trafficking, and/or gating. Crystallographic analysis of the TRPV1 N terminus shows that it contains six consecutive ankyrin repeats, with an interesting surprise; namely, the second repeat binds a molecule of adenosine triphosphate through interaction of two lysine side chains (K155 and K160) with the phosphodiester moiety (Kwak et al. 2000, Lishko et al. 2007) (**Figure 4*a***). High (millimolar) concentrations of ATP (or nonhydrolyzable analogs) enhance TRPV1 currents when applied to the inner surface of the membrane (Kwak et al. 2000), possibly through this interaction with the ankyrin-rich region. TRPV1 is also subject to calcium-dependent desensitization (Tominaga et al. 1998, Wood 1993), which may involve interaction between Ca^{2+}-calmodulin and one or more cytoplasmic regions at the N and C termini of the channel (Lau et al. 2012, Numazaki et al. 2003, Rosenbaum et al. 2004). ATP and calmodulin have been proposed to compete for a common binding site within the ARD cluster, which suggests a mechanism for nucleotide-dependent regulation (Lishko et al. 2007). Although such models remain speculative, mutation of K155 or K160 clearly renders TRPV1

constitutively active and/or refractory to desensitization (Lishko et al. 2007, Myers et al. 2008), attesting to the importance of this N-terminal region in controlling channel activity.

Finally, in addition to the PIP_2 hypothesis described above, a different regulatory mechanism has been proposed that also centers on the cytoplasmic C terminus. In this scenario, a 14–amino acid binding site (residues 736–749) within this domain serves as a docking site for a multiprotein complex consisting of protein kinases and phosphatases (PKC-e, PKA, and PP2B) bound to the scaffolding protein, AKAP79/150. Activation of PLC-coupled receptors promotes association of this complex with the TRPV1 C terminus, which in turn controls the phosphorylation status of S502, thereby regulating trafficking of the channel to the plasma membrane. Evidence supporting this model comes from measurements of capsaicin-evoked currents, but whether and how this mechanism accounts for shifts in the thermal activation threshold remain to be determined (Zhang et al. 2008).

TRPM8: A COLD-ACTIVATED CHANNEL

TRPM8 (also known as CMR1) is activated by a variety of natural and synthetic cooling agents, such as menthol, as well as by thermal (cold) stimuli. Native or cloned TRPM8 channels show a remarkably steep temperature dependence of gating ($Q_{10} \sim 40$) with an average activation threshold <26°C. Like TRPV1, TRPM8 forms a homotetrameric nonselective cation channel with substantial permeability to calcium ions (McCoy et al. 2011, McKemy et al. 2002, Peier et al. 2002, Yudin & Rohacs 2012).

TRPM8 is robustly expressed by ~15% of all somatosensory neurons encompassing mostly small-diameter, unmyelinated C-fibers, as well as a minor cohort of lightly myelinated Aδ fibers. A subset of these TRPM8-positive cells may coexpress TRPV1 (with estimates ranging from 0–30% depending on detection strategy) (Bautista et al. 2007, Dhaka et al. 2008, Kobayashi et al. 2005, Mishra et al. 2011, Takashima et al. 2010) and/or CGRP, but a substantial fraction of these neurons are not labeled by other known somatosensory or nociceptive markers (e.g., substance P, P2×3 purinergic receptors, IB4 lectin, or neurotrophin receptors), placing them in their own anatomically and functionally unique subpopulation of primary afferent neurons (Kobayashi et al. 2005). This profile of TRPM8 function and expression is generally consistent with the known characteristics of most cold-sensitive neurons and nerve fibers, including temperature threshold, prevalence, fiber types, and pharmacology (Reid & Flonta 2001). TRPM8 expression has also been detected outside of the somatosensory system, including bladder, prostate, and several types of tumors and transformed cells. However, the function of the channel or its mechanism of activation in these nonneural settings remains unclear (McCoy et al. 2011).

TRPM8 Accounts for Cold Sensation In Vivo

TRPM8 knockout mice exhibit profound deficits in both normal (acute) cold sensation and injury-evoked cold hypersensitivity (Bautista et al. 2007, Colburn et al. 2007, Dhaka et al. 2007), solidifying the role of TRP channels as molecular thermometers in vivo. Indeed, these animals show near-complete loss of cold sensitivity, whether measured in cultured neurons, isolated nerve fibers, or awake-behaving animals. Thus, TRPM8-deficient animals cannot discriminate between a warm and a cold surface (and show little or no preference for the former) over a wide range of temperatures that humans consider to be innocuously cool (15–30°C) or noxiously cold (<15°C). At the extreme end of the scale (<5°C), mutants show apparent avoidance of the cold surface in some behavioral paradigms (but not in others), but it is not clear whether this represents a bona fide TRPM8-independent component of cold sensitivity, a preference for the warm surface

owing to thermoregulatory drive, or a response to tissue injury (McCoy et al. 2011). In contrast to TRPV1, which under normal conditions confers sensitivity to thermal stimuli only within the noxious range, TRPM8 plays a role in both innocuous and noxious cold sensation in vivo (Bautista et al. 2007, Dhaka et al. 2007, Knowlton et al. 2010, Yudin & Rohacs 2012). Thus, although the restricted pattern of TRPM8 expression upholds the specificity theory in its broadest sense, its role in transducing stimuli of both innocuous and noxious intensities blurs the functional definition of the nociceptor, at least in regard to this particular modality of somatosensation. Importantly, TRPM8-deficient mice retain normal sensitivity to noxious heat and mechanical stimuli, demonstrating specificity of the observed phenotypes for cold sensation, as well as functional segregation of these somatosensory/nociceptive modalities. What remains to be determined is whether TRPM8-expressing fibers are functionally heterogeneous, perhaps with those coexpressing TRPV1 predominating in behavioral responses to noxious cold. Also at issue is whether central integration of inputs from hot (TRPV1-expressing) and cold (TRPM8-expressing) fibers underlies the perception of warmth.

Tissue inflammation or nerve injury associated with trauma, diabetes, viral infection, or chemotherapy can result in extreme hypersensitivity to innocuous cooling or noxious cold. Genetic ablation or pharmacological inhibition of TRPM8 in rodents substantially attenuates cold hypersensitivity produced by nerve injury (Calvo et al. 2012, Colburn et al. 2007). Thus, while cold hypersensitivity likely involves changes to numerous ion channels that regulate neural excitability, TRPM8 clearly plays an important role in this class of chronic pain syndromes (Malkia et al. 2011). Cold stimuli can also be analgesic in the context of inflammation or other injuries, and this phenomenon also appears to be TRPM8 dependent (Dhaka et al. 2007, Proudfoot et al. 2006).

The Cooling Agent Site(s)

Just as capsaicin functions as a positive allosteric modulator of TRPV1, menthol and other cooling agents enhance sensitivity of TRPM8 to cold, which enables the channel to open at warmer temperatures, thereby mimicking a psychophysical sensation of cold. Which region(s) of the channel specifies sensitivity to these ligands, and how does this compare to what we currently know about the interaction of TRPV1 with capsaicin or other modulatory compounds? Once again, a comparison of species orthologs has helped to address this question: Whereas rat TRPM8 is robustly activated by the supercooling agent icillin, the chicken channel is insensitive to this synthetic agonist (but still sensitive to menthol and cold). Three residues within the TM2-TM3 intervening loop and proximal region of TM3 account for this differential pharmacological profile, revealing remarkable similarity to the location of residues critical for activation of TRPV1 by capsaicin (Chuang et al. 2004). Furthermore, a high-throughput mutagenesis screen identified a residue within TM2 of mouse TRPM8 that abrogates menthol sensitivity (Bandell et al. 2006), which further implicates this region of the channel as an important determinant of cooling agent sensitivity. Two residues in the cytoplasmic C terminus were also identified in this screen, within a conserved region called the Trp domain, just proximal to TM6. Functional analysis suggests that TM2 mutants affect menthol potency and binding, whereas those in the Trp domain alter efficacy and may affect activation steps downstream of ligand binding. Interestingly, the Trp domain has also been identified as a putative PIP_2-interacting region involved in channel modulation.

TRPM8 Modulation

Compared with TRPV1, somewhat less is known about whether or how TRPM8 is modulated by inflammatory agents or cell signaling pathways, but possible mechanisms have been described

(Malkia et al. 2011, Yudin & Rohacs 2012). One particularly interesting example pertains to the phenomenon wherein activation of TRPM8 is accompanied by a calcium-dependent desensitization, or tachyphylaxis (McKemy et al. 2002), which may underlie sensory adaptation to cold. This process is initiated when calcium influx through activated TRPM8 channels stimulates calcium-sensitive PLC, leading to PIP_2 turnover (Daniels et al. 2009, Yudin et al. 2011). As noted above, PIP_2 is a positive regulator of TRPM8; thus, its depletion promotes channel rundown. Furthermore, mutagenesis experiments suggest that, as in the case of TRPV1, a positively charged putative PIP_2-interacting domain resides in the C-terminal cytoplasmic tail of TRPM8, specifically within the Trp domain (Rohács et al. 2005). Because PIP_2 hydrolysis has opposite effects on TRPV1 and TRPM8, an inflammatory agent that activates a PLC-coupled receptor could potentially produce hyperalgesia by sensitizing heat-response fibers while simultaneously suppressing any counteracting analgesic action from cold-sensitive fibers.

How Do TRPV1 and TRPM8 Detect Thermal Stimuli?

Understanding how TRPV1 and TRPM8 detect thermal stimuli is arguably the most intriguing structure-function question for these channels. Liposome reconstitution studies have shown that TRPV1 and TRPM8 are intrinsically temperature sensitive and that their activation by thermal stimuli does not require other cellular proteins or second messengers. In each case, substantial conformational rearrangements are believed to accompany gating because large changes in enthalpy and entropy are observed during temperature-evoked activation (Brauchi et al. 2004, Cao et al. 2013, Yao et al. 2010, Zakharian et al. 2010). Models based on principles of thermodynamics and protein folding suggest that such conformational transitions will be similar, if not identical, for these channels, irrespective of their physiologic thresholds (Clapham & Miller 2011), but what is lacking is a structural or biochemical picture of how these transitions occur. For instance, are there specific residues or domains that detect changes in ambient temperature, and which regions of the channel move during temperature-evoked gating?

Some TRP channels exhibit a degree of voltage sensitivity, and thermal stimuli may elicit gating by shifting their voltage-dependent activation curves toward physiologic membrane potential (Voets et al. 2004). However, it is not clear that voltage sensitivity underlies temperature sensitivity, per se, as opposed to being an independent partial activator that simply modulates temperature and chemical sensitivity through allosteric coupling (Matta & Ahern 2007). Furthermore, structural elements that might account for voltage sensitivity of TRPV1 or TRPM8 have not been clearly identified, which limits mechanistic exploration of this hypothesis. Random and directed mutagenesis studies have implicated several TRP channel domains as key determinants of temperature sensitivity, including N and C termini and the outer pore (Brauchi et al. 2007; Cordero-Morales et al. 2011; Grandl et al. 2008, 2010; Yang et al. 2010; Yao et al. 2011). Still at issue, however, is whether these (or any) single, discrete domains function as bona fide temperature sensors or truly selective determinants of temperature-evoked gating (Clapham & Miller 2011, Myers et al. 2008, Papakosta et al. 2011).

TRPA1: A CHEMORECEPTOR FOR ENVIRONMENTAL IRRITANTS AND INFLAMMATORY AGENTS

In the context of nociception, mammalian TRPA1 (also known as ANKTM1) was initially suggested to function as a detector of noxious cold and to account for a component of cold sensitivity not mediated by TRPM8 (Story et al. 2003). Although this hypothesis remains controversial (Caspani & Heppenstall 2009), current evidence suggests that TRPA1 plays little, if any, role in acute cold sensation but more likely contributes to injury-evoked cold hypersensitivity

(del Camino et al. 2010, Knowlton et al. 2010, Moran et al. 2011). Irrespective of this cold controversy, there is widespread agreement that TRPA1 plays an important role in chemonociception by serving as a detector of chemical irritants that elicit acute and inflammatory pain (Andrade et al. 2012, Basbaum et al. 2009, Bautista et al. 2012).

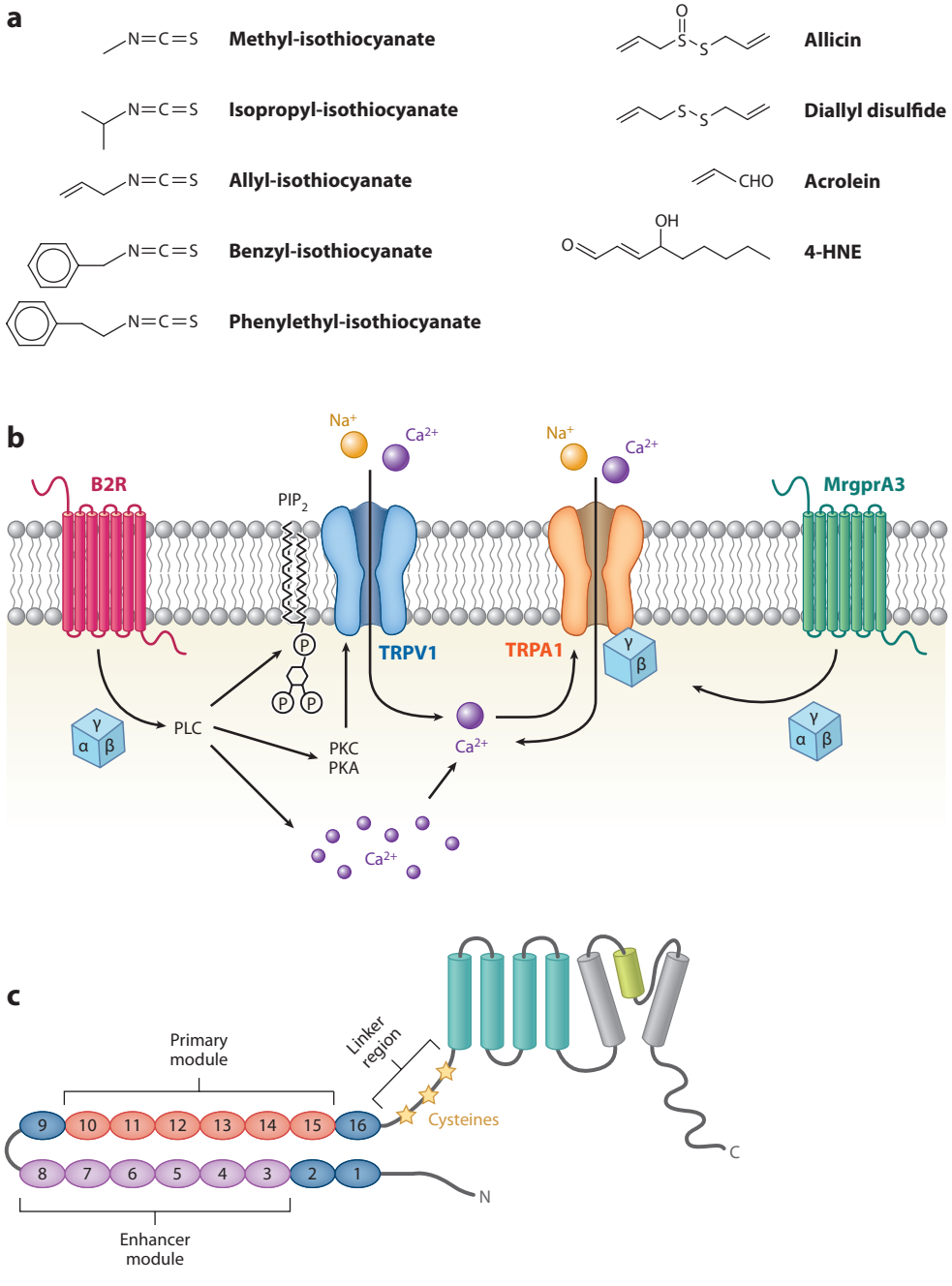
TRPA1 is activated by isothiocyanates and thiosulfinates that constitute pungent agents from mustard (e.g., wasabi) and allium (e.g., garlic and shallot) plants, respectively (Bandell et al. 2004, Bautista et al. 2005, Jordt et al. 2004, Macpherson et al. 2005) (**Figure 5*a***). Pharmacological and genetic studies have shown that TRPA1 plays an essential role in the nociceptive response to these and other environmental irritants (Bautista et al. 2006, Bessac et al. 2009, Kwan et al. 2006, Macpherson et al. 2007b, McNamara et al. 2007). For example, TRPA1 is targeted by acrolein and formalin, both highly toxic and volatile air pollutants, and by 2-pentenal, a metabolic by-product of chemotherapeutic agents that produces life-threatening and extremely painful inflammatory syndromes, such as hemorrhagic cystitis (Ahluwalia et al. 1994). Activation of TRPA1-expressing nerve fibers in the lung similarly promotes neurogenic inflammation, likely exacerbating airway constriction and cough in those suffering from asthma and other respiratory disorders (Andrè et al. 2008, Bessac et al. 2008, Caceres et al. 2009, Muroi & Undem 2011). TRPA1 is also activated by endogenous products of oxidative or nitrative stress, such as 4-hydroxynonenal, which is produced when reactive oxygen species peroxidate membrane lipids (Andersson et al. 2008, Trevisani et al. 2007). These and other reactive carbonyl species are commonly associated with chronic inflammatory syndromes, such as rheumatoid arthritis, diabetes, pancreatitis, colitis, asthma, and other obstructive pulmonary disorders. In each of these cases, there is likely a substantial neurogenic contribution to chronic inflammation. Indeed, within mammalian sensory ganglia, TRPA1 is expressed exclusively by peptidergic C-fibers (Bautista et al. 2005, Kobayashi et al. 2005), which, as described above, initiate neurogenic inflammation through the release of substance P, neurokinin A, and CGRP. Moreover, all TRPA1-positive neurons coexpress TRPV1 and thus belong to the cohort of C-fiber nociceptors that mediate acute sensation of noxious heat, as well as thermal hyperalgesia. As such, TRPA1 is well suited—both pharmacologically and anatomically—to contribute to a wide variety of inflammatory pain syndromes owing to the action of both environmental irritants and endogenous proalgesic agents (Bautista et al. 2006, 2012).

Figure 5

The wasabi receptor, TRPA1, is a detector of chemical irritants. (*a*) A variety of compounds activate TRPA1, including exogenous irritants and endogenous products of tissue injury and inflammation. The agents shown here include isothiocyanates and α,β-unsaturated aldehydes, both of which exhibit strong electrophilic reactivity as the functional attribute underlying their ability to activate TRPA1 channels. (*b*) In addition to direct activation by electrophilic irritants, TRPA1 functions as a receptor-operated channel that can be activated or sensitized by G protein–coupled signaling pathways. Two such mechanisms have been proposed: (i) A GPCR, such as the B2R bradykinin receptor, activates phospholipase C (PLC) to mobilize release of intracellular calcium. Increased cytoplasmic calcium then activates TRPA1. (ii) Activation of a GPCR, such as the MrgprA3 puritogen receptor, promotes the release of free $G\beta\gamma$, which serves as the downstream cytoplasmic activator of TRPA1. In addition to these proposed mechanisms, TRPA1 can be activated or sensitized by other events that enhance cytoplasmic calcium levels, such as activation of TRPV1 or other calcium-permeable channels. (*c*) Structure-function studies point to the TRPA1 cytoplasmic amino terminus as an important site for channel modulation. Electrophilic agonists covalently modify three key cysteine residues within the linker region connecting the ankyrin repeat domain to the transmembrane core of the channel (ankyrin repeats shown as colored ovals). Modulation of the channel by other factors, such as cytoplasmic calcium or heat (for snake or insect TRPA1 orthologs), is specified by two modules within the ankyrin repeat domain, as indicated. Panel *c* adapted with permission from Cordero-Morales et al. (2011). Abbreviations: C, carboxy terminus; N, amino terminus; PLC, phospholipase C.

Activation by Covalent Modification

TRPA1 agonists are exceedingly diverse in their chemical structures, begging the question of how ligand specificity is achieved. The unifying trait is not chemical structure, per se, but rather chemical reactivity; most TRPA1 agonists are strong electrophiles capable of forming reversible covalent adducts to thiol moieties of available cysteine residues (Hinman et al. 2006, Macpherson



et al. 2007a, Sadofsky et al. 2011). Remarkably, TRPA1-deficient mice lack specific somatosensory or behavioral responses to acrolein, formalin, or 4-hydroxynonenal, which demonstrates that a specific physiological response can be generated through this unusual scenario of ligand-receptor interaction, despite its rather indiscriminate chemical mechanism.

Cysteine residues throughout TRPA1 are susceptible to electrophilic modification, but sensitivity to these agents is determined by just a few cysteines within the channel's cytoplasmic N terminus (Hinman et al. 2006, Macpherson et al. 2007a). Among mammalian TRP channels, TRPA1 sports the longest N-terminal tail, possessing a string of ~16 ankyrin repeats. In human TRPA1, three critical cysteine residues are clustered within the so-called linker region that connects the ankyrin-rich domain to the transmembrane core; mutation of all three residues renders the channel insensitive to activation by isothiocyanates, except at very high (>100 μM) concentrations, where modification of a nearby lysine elicits small and persistent responses (Hinman et al. 2006). For some electrophiles, other regions may contribute to channel activation. For example, a single cysteine residue within the TM6 domain of hTRPA1 has been implicated in determining sensitivity to the thioaminal-containing compounds (Chen et al. 2008).

TRPA1 Is Also a Receptor-Operated Channel

In addition to direct gating by chemical agents, TRPA1 is activated by inflammatory factors (such as bradykinin or ATP) that stimulate Gq-PLC-coupled receptors (Bandell et al. 2004, Jordt et al. 2004). Downstream second messengers most critical to TRPA1 modulation have not been fully identified, but current evidence points to cytoplasmic Ca^{2+} as an important player (**Figure 5*b***). Calcium exerts dual effects on TRPA1, including initial activation or potentiation, followed by long-lasting inactivation (Jordt et al. 2004, Wang et al. 2008). Both aspects of regulation can be observed when calcium is supplied to the intracellular face of cell-free excised membrane patches, which suggests that calcium exerts its effects on TRPA1 by interacting directly with an intracellular domain(s) of the channel (Nagata et al. 2005, Wang et al. 2008). However, underlying mechanisms remain controversial; some groups favor direct binding of calcium to an EF-hand-like motif in the N-terminal domain (Doerner et al. 2007, Zurborg et al. 2007), and others suggest an indirect, calmodulin-independent process of a still-unknown nature (Nilius et al. 2011, Wang et al. 2008).

In addition to being activated by PLC-evoked release of calcium from intracellular stores, TRPA1 may also amplify responses initiated by other calcium-permeable channels, such as TRPV1, further promoting sensitization to thermal or chemical stimuli (Bautista et al. 2006, 2012). Recent pharmacological studies further suggest that some GPCRs, such as the chloroquine-activated itch receptor MrgprA3, activate TRPA1 through a mechanism involving direct coupling to Gβγ subunits (Bautista et al. 2012, Wilson et al. 2011) (**Figure 5*b***). Channel domains specifying proposed interactions with calcium or Gβγ subunits have not yet been delineated, nor have proposed interactions with Gβγ been directly tested using electrophysiological methods.

Evolutionary Tuning of TRPA1: Chemosensation Versus Thermosensation

As noted above, a role for mammalian TRPA1 in cold sensation is controversial (Caspani & Heppenstall 2009, Knowlton et al. 2010). However, TRPA1-like channels from some nonmammalian species are thermosensitive and likely contribute to one or more aspects of heat sensation in vivo, as first proposed in insects (flies and mosquitoes) (Hamada et al. 2008, Matsuura et al. 2009, Viswanath et al. 2003). In the vertebrate world, TRPA1 is used by certain snake species (pit vipers, boas, and pythons) to detect infrared radiation (Gracheva et al. 2010), in much the same way that vampire bats use a variant of TRPV1 for this purpose. Interestingly, heat-sensitive TRPA1 channels from these vertebrate or invertebrate organisms can be activated by wasabi and

other electrophilic irritants but with greatly reduced potency compared with non-heat-sensitive mammalian orthologs, despite the fact that snake and mammalian TRPA1 possess the same critical cysteines required for electrophile sensitivity. The analysis of snake-human chimeras suggests that the N-terminal cytoplasmic region of TRPA1 is a portable unit that determines sensitivity to heat or chemical irritants (Cordero-Morales et al. 2011). According to this model, the N terminus is organized into a bimodal arrangement in which thermal and chemical sensitivities are conferred by two independent, ankyrin repeat–rich modules; together, these modules establish a combinatorial code such that the one closest to the transmembrane core (AR10–15) predominates in establishing stimulus sensitivity (thermal or chemical), and the one closest to the N terminus (AR3–8) serves as an enhancer (**Figure 5*c***).

The current structure-function map of TRPA1 is rudimentary, and the models proposed above are certainly in the working stage. Nonetheless, existing mutagenesis and physiological data point to the N terminus as an especially important locus for the detection and integration of stimuli, including electrophiles, calcium, and heat. Many questions remain, such as whether and how the ankyrin-rich regions interact with one another or with the linker region to effect channel gating, and how this is altered by covalent cysteine modification, changes in ambient temperature, or calcium-dependent modulation. Here, again, structural models of the large N-terminal domain (which accounts for half of the subunit mass) and the channel proper will likely be required to fully elucidate these regulatory mechanisms.

CONCLUDING REMARKS

TRP Channels and Neural Specificity

TRP channels now serve as key molecular and functional landmarks for subsets of somatosensory neurons, and their identification and characterization have helped to validate the basic tenets of the specificity theory of nociception (Woolf & Ma 2007). Beyond that, they have been instrumental in elucidating a rudimentary outline of the circuitry underlying the initial phase of stimulus detection and coding by primary afferent sensory nerve fibers. For example, although there is some overlap in expression of TRPV1 and TRPM8 channels, they mainly define distinct subpopulations of primary afferent neurons. Thus, to a first approximation, these channels reveal a system in which hot and cold information is transmitted to the CNS via discrete labeled lines (**Figure 6**). Indeed, this anatomical picture is consistent with physiological and behavioral phenotypes of TRPV1 and TRPM8 knockout mice, which show modality-specific deficits in hot and cold sensation, respectively. Additionally, peptidergic nerve fibers expressing TRPA1 and TRPV1 can be viewed as a labeled line specifying neurogenic inflammatory actions at the periphery and conveying information about heat hypersensitivity to the spinal cord (**Figure 6**). Furthermore, mice in which TRPV1-expressing fibers are selectively ablated show complete loss of noxious heat sensitivity but retain normal sensitivity to cold or noxious mechanical stimuli, which argues for yet another, independent neural pathway devoted to mechano-nociception, one that is distinct from the TRPV1-positive population of C-fibers (Cavanaugh et al. 2009, Mishra & Hoon 2010). These models are clearly rudimentary and not uniformly accepted (Abrahamsen et al. 2008), but TRP channels and their genes provide an important genetic and functional toehold for establishing an initial map of the nociceptive circuitry that connects peripheral receptive fields to postsynaptic neurons in the spinal cord dorsal horn.

Therapeutic Potential

Opiates are fantastically effective pain suppressors, but they are plagued by serious side effects (for instance, tolerance, addiction, constipation, and respiratory depression) owing to actions at

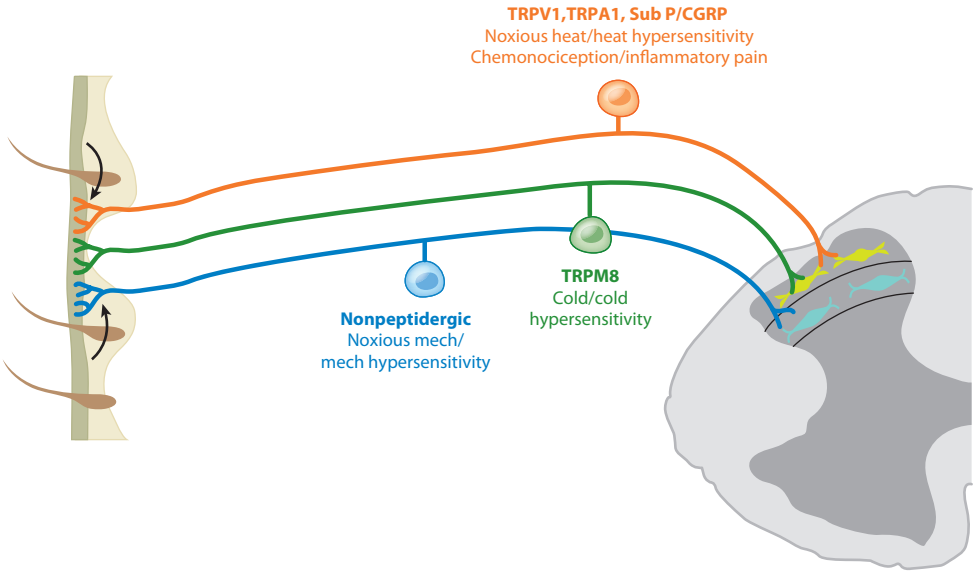


Figure 6

Transient receptor potential (TRP) channels define functionally distinct nociceptor populations. Functional studies have shown that TRPV1-expressing nociceptors (*orange*) mediate sensitivity to noxious heat, whereas TRPM8-expressing neurons (*green*) mediate sensitivity to cold. Thus, to a first approximation, these TRP channels define functionally distinct subpopulations of primary afferent nociceptors. A subset of TRPV1-positive neurons coexpress TRPA1 and the neuropeptides substance P (Sub P) and CGRP, making them important players in chemonociception, neurogenic inflammation, and inflammatory pain. Behavioral studies suggest that sensitivity to noxious mechanical stimuli is mediated by nociceptor subpopulations (*blue*) distinct from these aforementioned groups. While rudimentary, the scenario outlined here is meant to convey the idea that nociceptors display aspects of labeled line specificity, as determined, in part, by TRP channel expression and functionality.

receptors outside of the pain pathway. Similarly, nonsteroidal anti-inflammatory drugs are effective at treating inflammatory pain, but their use is limited by gastrointestinal, renal, and cardiovascular risk owing to inhibition of cyclooxygenase enzymes throughout the body. In principle, analgesics that act at nociceptor-specific targets have the advantage of biological specificity and—for some applications—selective delivery via topical application, local injection, or inhalation (Patapoutian et al. 2009, Premkumar & Abooj 2012). The TRP channels discussed here may satisfy these criteria because, although they have been described in tissues and cell types outside of the somatosensory system, their expression (whether measured histologically or functionally) is substantially higher in nociceptors (Cavanaugh et al. 2009, Mishra et al. 2011). Furthermore, with the exception of predicted deficits in nociception, knockout mice lacking TRPV1, TRPM8, or TRPA1 are fertile, viable, and phenotypically normal, which suggests that drugs acting at these channels will have effects that are largely determined by their actions on somatosensory neurons. The expectation is that TRPV1 and/or TRPA1 blockers will be effective for treating one or more chronic inflammatory pain syndromes; TRPM8 antagonists should be efficacious in treating less common, but nonetheless serious, conditions of cold hypersensitivity brought on by nerve injury or treatment with chemotherapeutic agents.

Among these three TRP channel targets, compounds acting at TRPV1 are furthest along the drug discovery pathway. Many selective, potent, and chemically diverse antagonists have been

developed by the pharmaceutical industry, several of which have now been through various stages of preclinical and clinical testing (Szallasi & Sheta 2012, Szolcsányi & Sandor 2012). Promisingly, numerous trials have validated predictions from in vitro studies of channel function, as well as cellular and behavioral phenotypes of TRPV1 knockout mice. That is to say, TRPV1 antagonists are analgesic in a variety of rodent pain models (García-Martínez et al. 2002; Ghilardi et al. 2005; Honore et al. 2005, 2009; Schwartz et al. 2011), and some have shown positive results in clinical pain trials (Chizh et al. 2007, Krarup et al. 2011). On the negative side, some TRPV1 antagonists are reported to produce on-target adverse effects that have raised concerns. One of these is diminished acute sensitivity to noxious heat (but not cold), which could compromise protective avoidance of hot objects (Rowbotham et al. 2011, Szallasi & Sheta 2012). The other is a transient hyperthermia characterized by an increase in core body temperature, typically in the range of 1–2°C (Gavva et al. 2008). It has been known for many years that capsaicin promotes a transient hypothermia associated with heat loss through cutaneous vasodilation, sweating, and perhaps other afferent-evoked thermoregulatory actions (part of the attraction of eating hot peppers in hot climates) (Caterina et al. 2000, Issekutz et al. 1950, Jancsó-Gábor et al. 1970). Moreover, pharmacological antagonism of TRPM8 produces dose-dependent hypothermia in wild-type but not TRPM8-deficient mice (Knowlton et al. 2011), which further substantiates the connection between thermosensitive TRP channels, peripheral thermosensation, and thermoregulatory mechanisms. Is it possible to develop a TRPV1 antagonist whose analgesic action can be split off from thermoregulatory effects? The ideal drug would block the sensitizing actions of inflammatory agents without altering basal heat sensitivity. Interestingly, some of the newer-generation TRPV1 blockers do not produce hyperthermia and thus hold promise for overcoming existing limitations (Lehto et al. 2008, Reilly et al. 2012). The mechanistic basis behind these differential antagonist effects is currently unclear but could reflect their actions at distinct modulatory sites on the channel. It is still a bit too early to know whether TRP channel antagonists will emerge as the next generation of smart and effective analgesics, but cautious optimism might be an appropriate watchword.

DISCLOSURE STATEMENT

The author is an inventor on patents (held by the University of California) related to the use of TRPV1 and TRPM8 for drug discovery. He also consults for biopharmaceutical firms that have an interest in TRP channels as therapeutic targets.

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