1773-Pos
Complex Regulation of TRPV1 by Phosphoinositides
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TRPV1 is a nonselective highly calcium permeable cation channel present in the peripheral nervous system exclusively in polymodal nociceptors. A sensory integrator of several noxious stimuli, TRPV1 plays a crucial role in the development of inflammatory pain and hypersensitivity. Plasmamembrane phosphoinositides are recognized as important regulators of TRPV1 function; the precise nature of their effect is, however, controversial.
Phosphatidylinositol-4,5-bisphosphate (PIP_2) has initially been proposed to tonally inhibit TRPV1 via a C-terminal inhibitory domain. Furthermore, receptor-mediated depletion of PIP_2 was proposed to be involved in sensitization of TRPV1 in response to pro-inflammatory agents. However, in subsequent studies including our own, direct intracellular application of PIP_2 reproducibly potentiated TRPV1 currents rather than inhibiting them. In addition, PIP_2 depletion concurrent with robust TRPV1 activation is an important contributing factor to channel desensitization consistent with the activating effect of PIP_2. We attempt to address this controversy utilizing multiple independent approaches to selectively regulate plasmamembrane PIP_2 levels in heterologous expression systems. Our results show that TRPV1 currents in intact cells elicited by low to moderate, but not high agonist concentrations are potentiated in response to PIP_2 depletion. Conversely, increasing PIP_2 levels inhibits low but not high agonist-induced TRPV1 currents. These effects are reduced or absent in the mutant channel lacking the putative C-terminal inhibitory domain. The inhibitory effect of PIP_2 however was never observed in excised patches even at low agonist concentrations. Our results are consistent with an agonist concentration-dependent dual regulatory effect of PIP_2. The inhibitory effect furthermore appears to be indirect. Such dual effects of PIP_2 have previously been described for voltage-gated calcium channels as well as other TRP channels and raise important questions as to the identity of interacting molecules conferring the inhibitory effect and the physiological relevance of such complex regulation.

1774-Pos
TRPV1 Activation by Allyl Isothiocyanate
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Allyl isothiocyanate (mustard oil, MO) is a highly reactive electrophilic compound known to cause irritation, pain and inflammation. These effects are thus far thought to be mediated by activation of TRPA1, a Transient Receptor Potential (TRP) cation channel expressed in nociceptive neurons. Recent research has shown that TRPV1, the heat and capsaicin receptor, can be also activated by reactive compounds such as allicin and leek and onion extracts. Here, we show that both human and mouse TRPV1 are activated by MO, at concentrations at which TRPA1 undergoes fast desensitization and block. In Ca2+ imaging experiments of intact HEK293 cells, MO induces an increase of the intracellular Ca2+, which was not present when Ca2+ was omitted in the bath solution. Activation of TRPV1 by MO is dose-dependent and is caused by a shift of the voltage dependence of channel activation to more negative potentials, similar to the activation of TRPV1 by capsaicin. Stimulation of TRPV1 by MO can be observed in inside-out patches, indicating a membrane-delimited mechanism of activation. Furthermore, the heat-induced activation of TRPV1 could be sensitized with sub-activating MO concentrations.
Notably, MO was able to stimulate a large population of sensory neurons isolated from Trpa1 KO mice. This population was significantly reduced in Trpa1/Trpv1 double KO mice, indicating the physiological importance of TRPV1 activation by MO. WT, Trpa1 and Trpv1 KO mice displayed significantly stronger aversion to MO than double KO mice in forced drinking and open field exploration assays. The identification of TRPV1 as a novel target of MO is essential for the full understanding of the mechanisms of action of this compound in vivo and prompts to re-evaluate the results of previous research, in which MO was used as specific activator of TRPA1.

1775-Pos
Molecular Determinants of the Activation Gate of the TRPV1 Channel
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The ability of ion channels to transit among different conformations allows them to regulate different types of cellular functions. Transient Receptor Potential Vanilloid 1 (TRPV1)channels participate in several types of physiological responses such as pain detection and inflammation, little is known about how their structural components convert different types of stimuli into channel activity. To localize the activation gate of these channels, we used the substituted cysteine accessibility method (SCAM) and inserted cysteines along the S6 segment of the TRPV1 channel and assessed their accessibility to thiol-modifying agents and silver. Our results show that access to the pore of the TRPV1 is gated by the S6 both in response to capsaicin binding and to increases in temperature, that the pore-forming S6 segments are helical structures and that there are two constrictions in the pore. One located at residue L681 which hampers the access to large molecules and one located at residue Y671 which impedes the entrance of smaller ions a and constitutes the activation gate of these channels. These data have also allowed us to produce a model of this region in the structure of TRPV1 based on functional findings.

1776-Pos
Interactions between Quaternary Ammoniums and the Gate of the TRPV1 Channel
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TRP channels play a fundamental role in neuronal signalling and in the detection of painful stimuli and inflammatory processes. The TRPV1 (vanilloid 1) channel functions as an integrator of noxious chemical and physical signals known to cause pain. Structural and functional information of the pore domain shows that access to the pore is gated by the S6 in response to capsaicin and temperature. Our group recently found the presence of two intracellular constrictions: L681 which obstructs the ion conduction pathway for large molecules and Y671 which obstructs the ion conduction pathway for small permeating molecules and constitutes de activation gate of TRPV1 channels. Quaternary ammoniums (QA) are a family of pore blockers that have been successfully used in structure-function studies. Previous results using QA on TRPV1 show that these compounds block the channel in a state-dependent fashion. Since it has been shown that aromatic residues interact with quaternary ammoniums by direct hydrophobic interactions we decided to test if the actions of tetrabutylammonium (TBA) are mediated by the interaction with the aromatic residue Y671 . Our preliminary results indicate that this residue is not involved in the binding of TBA to TRPV1.

1777-Pos
Sensitization of Vanilloid Receptors TRPV3
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Vanilloid receptors of the transient receptor potential family have functions in thermal sensation and nociception. Among them, TRPV3 is expressed in skin keratinnocytes and has also been implicated in flavor sensation in oral and nasal cavities as well as being a molecular target of some allergens and skin sensitizers. The channel displays a unique property that repeated stimulation results in gradual increases of its activity, a process that is known as sensitization and is observed in both native cells and cell lines. Transient calcium release from internal stores has been thought to underlie the sensitization process through a mechanism involving relief of Ca2+-dependent inhibition of the channel due to calmodulin binding at the distal N-terminal. In support of the hypothesis is the differential effect of the calcium chelators BAPTA and EGTA, where BAPTA, which has a fast buffering kinetics, is able to modulate the sensitization, while EGTA is ineffective. Here we suggest an alternative mechanism for the sensitization process. We distinguish two types of sensitizations; one is reversible and the other irreversible. The irreversible sensitization is intrinsic to the gating of the channel, while the reversible one such as that mediated by BAPTA is attributable to a modulation effect. We show that analogs of BAPTA that apparently lack Ca2+ buffering capability similarly sensitize the channel. We conclude that the sensitization of the channel, including the effects of BAPTA, also involves a membrane-delimited mechanism.

1778-Pos
Ca2+ Inhibition of Cation Conductance through TRPV1 Receptors
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TRPV1 receptors are polymodal cation channels that show a marked permeability to Ca2+. In the present study, we used single channel electrophysiology and whole cell patch clamp photometry to further study the interaction of extracellular Ca2+ ([Ca2+]o) with the recombinant TRPV1 receptor expressed in HEK293 cells. In the presence of and 140 mM [NaCl]o, we observed that

[Ca2+]o attenuates the amplitude of capsaicin (0.5 microM)-evoked single channel currents through TRPV1 receptors within a physiologically relevant concentration range (Kd = 2 mM, Hill slope = 1.1). The inhibition was observed at a range of positive and negative membrane potentials, being more pronounced at negative potentials. Use of patch clamp photometry revealed that at −60 mV in the presence of 10 mM [Ca2+]o, which is almost maximally effective for inhibiting single channel current amplitudes (56%), the fractional of the current carried by Ca2+ current was only 40%. Thus, Na+ still carries most of the cation current through the TRPV1 receptor even when Ca2+ is likely occupying the site responsible for its inhibiting cation conductance. Finally, we observed that neutralizing the charge on single amino acids located in the mouth of the putative pore and known to contribute to Ca2+ selection by TRPV1 receptors, Asp646, Glu648 and Glu651, did not alter the inhibitory effect of 2 mM [Ca2+]o. To summarize, although Ca2+ has been reported to increase the open probability of TRPV1 receptors, this divalent cation also attenuates conductance through the channel pore via an unknown mechanism seemingly discreet from that contributing to the high Ca2+ permeability of these receptors.

1779-Pos

Distinct Modulations of Human Capsaicin Receptor by Proton and Magnesium Through Different Domains

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The capsaicin receptor (TRPV1) is a nonselective cation channel that integrates multiple painful stimuli, including capsaicin, protons and heat. Protons facilitate the capsaicin- and heat -induced currents by decreasing thermal threshold or increasing agonist potency for TRPV1 activation. In the presence of saturating capsaicin, rat TRPV1 (rTRPV1) reaches full activation, with no further stimulation by protons. Human TRPV1 (hTRPV1), a species ortholog with high homology to rTPRV1, is potentiated by extracellular protons and magnesium, even at saturating capsaicin. We investigated the structural basis for protons and magnesium modulation of fully capsaicin-bound human receptors. By analysis of chimeric channels between hTRPV1 and rTRPV1,We mapped the required domain and a single amino acid residue responsible for further potentiation of capsaicin efficacy by protons. We also showed that magnesium ions could also exert similar effects for capsaicin activation of human TRPV1, but through a different functional domain. Our results demonstrate that capsaicin efficacy of hTRPV1 correlates with the extracellular ion milieu, and unravel the relevant structural basis of modulation by protons and magnesium.

1780-Pos

Interactions between DAG, IP_3 and PIP_2 Govern Activation of Heterotetrameric TRPC6/C7 Channel Activity in Rabbit Portal Vein Myocytes

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Previously we have shown that synergism between inositol-1,4,5-trisphoshate (IP_3) and diacylglycerol (DAG) mediates activation of TRPC6-like channel activity by noradrenaline (NA, Albert & Large, 2003) in rabbit portal vein myocytes. Moreover, a recent study showed that endogenous phosphatidylinositol-4,5-bisphosphate (PIP_2) produced a marked inhibitory action on TRPC6 activity in mesenteric artery myocytes (Albert et al, 2008). In the present work we investigated interactions between DAG, IP_3 and PIP_2 in regulating TRPC6-like activity in portal vein myocytes using patch clamp and immunoprecipitation methods.

In inside-out and cell-attached patches, bath application of respectively 10 μM IP_3 and the cell-permeable IP_3 analogue, 10 μM 6-IP_3, both potentiated OAG-induced TRPC6-like channel activity by 3-fold but had no effect when applied on their own. In inside-out patches, pre-treatment with 20 μM wortmannin, to deplete endogenous PIP_2 levels, increased OAG-evoked channel activity by 75-fold compared to control patches. Moreover, anti-PIP_2 antibodies activated TRPC6-like activity in quiescent inside-out patches. In wortmannin-treated inside-out patches, 10 μM diC8-PIP_2 inhibited OAG evoked channel activity (IC_{50} = 0.74 μM) which was rescued by over 50 % by co-application of 10 μM IP_3. Anti-TRPC6 and anti-TRPC7 antibodies inhibited TRPC6-like activity induced by NA by over 80%, but channel activity was unaffected by other TRPC antibodies. Co-immunoprecipitation studies showed association between TRPC6 and TRPC7 proteins and that both these channel proteins interacted with PIP_2. Pretreated with 6-IP_3, reduced association between PIP_2 and TRPC7 but not TRPC6, whereas OAG reduced PIP_2 interactions with TRPC6 but not TRPC7.

These results indicate that endogenous PIP_2 has a pronounced inhibitory action on TRPC6/TRPC7 heteromeric channels in portal vein myocytes. Moreover channel activation by DAG requires both this triglyceride and IP_3 to remove associations between PIP_2 and these channel proteins.

1781-Pos

Isoform-Selective Physical Coupling of TRPC3 Channels to IP3 Receptors in Smooth Muscle Cells Regulates Arterial Contractility

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Many vasoconstrictors bind to phospholipase C (PLC)-coupled receptors on arterial smooth muscle cells, leading to an intracellular inositol 1,4,5-trisphophate (IP_3) elevation and vasoconstriction. IP_3-induced vasoconstriction can occur independently of intracellular Ca^{2+} release and via IP_3 receptor (IP_3R) and canonical transient receptor potential (TRPC) channel activation, but signaling mechanisms mediating this effect are unknown. Here, we studied the mechanisms by which IP_3Rs stimulate TRPC channels in smooth muscle cells of resistance-size cerebral arteries. Immunofluorescence resonance energy transfer (immuno-FRET) microscopy in smooth muscle cells indicated that endogenous type 1 IP_3Rs (IP_3R1) are in close spatial proximity to TRPC3, but distant from TRPC6 or TRPM4 channels. Endothelin-1 (ET-1), a PLC-coupled receptor agonist, elevated the immuno-FRET signal between IP_3R1 and TRPC3, but not between IP_3R1 and TRPC6 or TRPM4. IP_3R1 co-immunoprecipitated with TRPC3, but not with TRPC6. An antibody targeting TRPC3 channels and TRPC3 channel knockdown with short hairpin RNA inhibited IP_3-induced non-selective cation current (I_{Cat}) activation, whereas an antibody to TRPC6 and TRPC6 channel knockdown had no effect. Biotinylation indicated that ET-1 did not alter total or plasma membrane-localized TRPC3. RT-PCR demonstrated that a calmodulin and IP_3R binding (CIRB) domain is present on the C-terminus of both TRPC3 and TRPC6 channels. A CIRB domain peptide attenuated IP_3- and ET-1-induced I_{Cat} activation. A peptide corresponding to the IP_3R region that can interact with TRPC channels activated I_{Cat}. A HIV-1 TAT-conjugated CIRB domain peptide reduced IP_3- and ET-1-induced vasoconstriction in pressurized arteries. These data indicate that IP_3 stimulates direct coupling between IP_3R1 and membrane-resident TRPC3 channels in arterial smooth muscle cells, leading to I_{Cat} activation and vasoconstriction. Data also indicate that close spatial proximity between IP_3R1 and TRPC3 establishes this isoform-selective functional interaction.

1782-Pos

Molecular and Structural Basis of Dual Regulation of a Canonical TRP Channel by Calmodulin

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The canonical transient receptor potential (TRPC) channels are widely distributed and have diverse biological functions. They are activated by stimulation of phospholipase C-coupled receptors, resulting in membrane depolarization and Ca^{2+} influx, which in turn feedback to regulate the channel activity through the Ca^{2+}-binding protein calmodulin (CaM) and other signaling pathways. Previous biochemical studies indicate that TRPC subunits contain one to four putative CaM-binding sites. One of these sites is named the ''CaM-IP_3receptor binding'' or CIRB site (because it also interacts with an IP_3 receptor fragment *in vitro*). The CIRB site is conserved in all seven TRPC subunits. CaM exerts either stimulatory or inhibitory effects on different TRPC channels. However, the molecular mechanism of CaM modulation of TRPC channels is unclear. We have solved the crystal structure of the complex of CaM and the CIRB site of TRPC5 channels, which regulate growth cone morphology and neurite growth, and require CaM for agonist-induced activation. The structure shows that the two lobes of a single Ca^{2+}-bound CaM (Ca^{2+}/CaM) bind two CIRB peptides arranged in parallel. This peptide dimerizes only in the presence of Ca^{2+}/CaM, suggesting that Ca^{2+}/CaM binding to the CIRB site may induce major conformational changes in intact channels. Structure-based mutagenesis studies show that Ca^{2+}/CaM binding to the CIRB site is not required for agonist-induced channel activation, but it safeguards the channel against inhibition produced by CaM binding to another site on the channel. We have identified this inhibitory site and found it to be a novel CaM-binding motif that can interact with not only Ca^{2+}/CaM but also CaM_{1234}, a mutant CaM deficient in binding Ca^{2+}. Our results provide new insights into the intricate feedback regulation of a canonical TRP channel.

1783-Pos

TRPC3 is Essential for Maintenance of Skeletal Muscle Cells

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During membrane depolarization associated with skeletal excitation-contraction (EC) coupling, L-type Ca^{2+} channels (dihydropyridine receptor (DHPR) in the transverse (t)-tubule membrane) undergo conformational changes that are transmitted to Ca^{2+}-release channel (ryanodine receptor