during stimulation by PS or nifedipine. However, HEK293 cells transfected by TRPM3α1 showed a robust increase in current amplitude during stimulation by CIM0216. Here, we have further investigated the existence of an alternative ion permeation pathway in the TRPM3α1 isoform. This study aims to understand the mechanism of activation of the alternative ion permeation pathway in TRPM3 and to determine the necessary structural features to open the alternative pathway.

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Regulation of the TRPM3 Channel in Planar Lipid Bilayers

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Transient Receptor Potential Melastatin 3 (TRPM3) is a non-selective cation channel that can be activated with nifedipine and pregnenolone sulfate (PS). The role of TRPM3 in nociceptive neurons has been linked to the heat sensation and development of heat hyperalgesia during inflammation. However, the functional characterization of TRPM3 and its specific agonists remain poorly understood. To investigate biophysical and pharmacological properties of TRPM3 we aimed to incorporate the purified channel in planar lipid bilayers. TRPM3 was purified from HEK-293 cells using immunoprecipitation technique. The single channel activity was then examined under various conditions. We investigated TRPM3 activity with different agonists. Application of nifedipine (1-10 μM) resulted in dose-dependent increase of open probability of the channel. These results suggest that nifedipine can alone activate TRPM3. Unlike nifedipine, addition to the bilayers of PS, did not induce the channel openings alone and required co-application of phosphatidylinositol-4,5-bisphosphate ($PI(4,5)P_2$) or clotrimazole. TRPM3 demonstrated outward rectification upon activation with PS/$PI(4,5)P_2$, but linear current-voltage relationship with PS/clotrimazole. In both conditions, TRPM3 tended to inactivate at higher voltages (100-120 mV) and with increased concentrations of PS. Interestingly, in the absence of any agonists TRPM3 exhibited basal channel activity that sustained for several minutes. Previously TRPM3 was proposed to serve as a temperature sensor. We tested temperature sensitivity of TRPM3 and found that in the framework of lipid bilayers the channel did not exhibit any temperature-induced activation in the absence of agonists. In the presence of nifedipine TRPM3 demonstrated weak temperature dependence with $Q_{10}$of 1.5 at +100 mV and 1.7 at −100 mV. These results indicate that TRPM3 is unlikely to serve as a temperature sensor alone and may involve some alternative molecular mechanisms for temperature sensation.

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Biophysical Properties of the Alternative Ion Permeation Pore in TRPM3

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TRPM3 was identified as a Ca^{2+}-permeable, non-selective cation channel (P_{Ca}/P_{Na} of 1.57) that showed a small but constitutively single channel conductance of ~65 pS for Ca^{2+} and around ~130 pS for Cs^{+}. Like the structure of all TRP channels, TRPM3 channels contain six transmembrane spanning regions with a pore-forming reentrant loop between the fifth (S5) and the sixth (S6) transmembrane domain. It is generally accepted that, physical and chemical activating stimuli, lead to gating of a single, central cation-conducting pore formed by S5, S6 and the interconnecting pore loop. For TRPM3, stimulation by heat or chemical compounds like pregnenolone sulphate (PS) and nifedipine, will open the central pore and induces outwardly rectifying currents in TRPM3-expressing cells. In contrast to this view, evidence is shown for the existence of an alternative ion permeation pathway in TRPM3, distinct form the central pore, that can be specifically activated by the combined application of PS and clotrimazole (Clt). Recently, we have identified CIM0216, a single compound that is able to open both ion permeation pathways of TRPM3 by itself. The combined application of CIM0216 and La^{3+}, a blocker of the central pore, has allowed us to further investigate the biophysical properties of the alternative ion permeation pathway in TRPM3.

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Functional Analysis of the Thermosensor TRPM3 in Intact Sensory Fibers Using the Skin-Nerve Assay

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Transient receptor potential melastatin 3 (TRPM3) is a widely expressed calcium-permeable non-selective cation channel that is selectively activated by the neurosteroid pregnenolone sulphate (PS). Previous *in vitro* and *in vivo* experiments demonstrated that TRPM3 functions as a heat sensor in the somatosensory system. Although behavioral assays in combination with single-cell recordings on isolated sensory neurons can yield important insight into the role of TRPM3 in thermosensation, the procedure results in the isolation of neuronal cell bodies, devoid of the sensory nerve endings. Moreover, DRG neurons in primary culture lack the context of adjacent cells (e.g. keratinocytes), which are known to influence thermal sensation by the somatosensory system. The skin-nerve preparation is an *ex vivo* technique that bridges the gap between *in vitro* cellular recordings and behavioral assays. With this technique, we evaluated the functional role of TRPM3 in C-fibers. Our results indicate functional expression of TRPM3 in sensory terminals of a subset of mechano-heat sensitive C-fibers. Other types of C-fibers (mechano-cold, high- and low-threshold mechano) were insensitive to TRPM3 agonists. These experiments indicate a role for TRPM3 in heat sensation by C-fibers in the somatosensory system.

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Phosphoinositides as Co-Factors for the Ion Channel TRPM3

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The regulation of numerous ion channels by membrane lipids is widely known and well accepted. Of all the ion channel families, the modulation of members of Transient Receptor Potential (TRP) channels is complex, especially by phosphatidylinositol 4,5-bisphosphate (PtdIns(4,5)P2). PtdIns(4,5)P2, predominantly present at the cytoplasmic face of the plasma membrane, has been shown to be a positive regulator for all the members of the TRPM family, except TRPM3 and TRPM1. Here we show that PtdIns(4,5)P2 is required for the activity of TRPM3. TRPM3 is a Ca^{2+} permeable outwardly rectifying nonselective cation channel expressed in sensory neurons, brain, pancreas, kidney and vascular smooth muscle. In sensory dorsal root ganglion (DRG) neurons it was shown to function as a sensor for noxious heat. In DRG neurons as well as pancreatic beta cells, the neurosteroid pregnenolone sulfate has been shown to activate TRPM3.

In the current study we modulated the levels of PtdIns(4,5)P2 in Xenopus laevis oocytes and HEK cells heterologously expressing TRPM3. In inside-out patches, TRPM3 currents ran down rapidly after excision. This current could be restored by the exogenous application of naturally occurring arachidonyl-stearyl (AASt) PtdIns(4,5)P2 ,water soluble diC8 PtdIns(4,5)P2, diC8 PtdIns(3,4)P2, diC8 PtdIns(3,5)P2, diC8 PtdIns(3,4,5)P3 and MgATP to excised inside-out patches. The effect of MgATP was inhibited by PI4 kinase inhibitors (LY294002 and A1) and phosphatidylinositol specific PLC (PI-PLC) but not by a PKC inhibitor (Gö 6976). Thus the effect of MgATP can be attributed to restoration of membrane PtdIns(4,5)P2 and PtdIns(4)P. In addition, inducing the activity of PtdIns(4,5)P2-5-phosphatases in whole-cell patch clamp experiments also decreased TRPM3 currents. In conclusion, our data collected through excised inside-out and whole-cell patch clamp measurements suggest that TRPM3 requires PtdIns(4,5)P2 as a cofactor.

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Chemical Activation of Endogenous and Recombinant TRPM4 Channels

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TRPM4 (transient receptor potential melastatin 4) channels constitute Ca^{2+}-activated non-selective cationic currents in a variety of tissues (e.g. heart and brain) and in cells of the immune system. The channels are gated by elevated cytosolic Ca^{2+}, and channel activity is modulated by signalling molecules such as ATP and phospholipids. Activation of TRPM4 depolarises the membrane potential, which modulates the driving force for ions and the activity of ion channels. TRPM4 channel agonists and Ca^{2+}-independent gating have not been demonstrated yet.

In patch clamp experiments, the maleimide U73122, which is widely used as phospholipase C inhibitor, activated recombinant human TRPM4 and endogenous TRPM4 channels in cell lines. In contrast, U73122 inhibited TRPM3 channels and did not affect TRPM5 channel activity. TRPM4 current amplitudes were independent on phospholipid levels and, strikingly, U73122 activated TRPM4 even in absence of intracellular Ca^{2+}. As the structural analogue U73343 was ineffective, these findings suggested covalent modification as the underlying mechanism. In fact, pre-application of N-ethylmaleimide (NEM) to prevent further covalent modification abolished the activation of TRPM4 by U73122.