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Invited Review

TRPV4 calcium entry channel: a paradigm for gating diversity

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Nilius, Bernd, Joris Vriens, Jean Prenen, Guy Droogmans, and Thomas Voets. TRPV4 calcium entry channel: a paradigm for gating diversity. *Am J Physiol Cell Physiol* 286: C195–C205, 2004;10.1152/ajpcell.00365.2003.—The vanilloid receptor-1 (VR1, now TRPV1) was the founding member of a subgroup of cation channels within the TRP family. The TRPV subgroup contains six mammalian members, which all function as Ca^{2+} entry channels gated by a variety of physical and chemical stimuli. TRPV4, which displays 45% sequence identity with TRPV1, is characterized by a surprising gating promiscuity: it is activated by hypotonic cell swelling, heat, synthetic 4α-phorbols, and several endogenous substances including arachidonic acid (AA), the endocannabinoids anandamide and 2-AG, and cytochrome *P*-450 metabolites of AA, such as epoxyeicosatrienoic acids. This review summarizes data on TRPV4 as a paradigm of gating diversity in this subfamily of Ca^{2+} entry channels.

transient receptor potential; calcium channels; vanilloid receptor

THE FREE INTRACELLULAR CA^{2+} CONCENTRATION ($[Ca^{2+}]_i$) is an important regulator of various cell functions. The most important mechanisms for increasing $[Ca^{2+}]_i$ are release of Ca^{2+} from intracellular stores and entry of extracellular Ca^{2+} via diverse Ca^{2+} entry channels. In the last 10 years, several novel Ca^{2+} entry channels belonging to the still expanding family of TRP cation channels have been discovered. More than 20 mammalian TRP genes have been identified, encoding membrane proteins with six transmembrane segments (TM1–TM6) and a putative pore region formed by a short hydrophobic stretch between TM5 and TM6 (for detailed reviews, see Refs. 11, 48, 49). On the basis of their homology, mammalian TRP proteins are classified into three subfamilies (50): TRPC (canonical), TRPV (vanilloid), and TRPM (melastatin). The core transmembrane channel structure of TRP channels resembles that of the pore-forming subunits of voltage-gated and cyclic nucleotide-gated channels and consists of a coassembly of four subunits (32).

THE TRPV SUBFAMILY

TRPV1 (VR-1), the founding member of the TRPV family, was identified by expression cloning as a capsaicin- and heat-gated channel (9). A similar expression cloning strategy for proteins responsible for reabsorption of Ca^{2+} in the kidney (31) and the gut (63) led to the discovery of TRPV5 (ECaC1) and TRPV6 (CaT1). The remaining three members (TRPV2–4) were identified by using electronic search strategies designed to recognize proteins related to TRPV1 or the related OSM-9 protein from *Caenorhabditis elegans* (for a detailed review, see Refs. 4, 27). Functionally, the six mammalian members of the TRPV subfamily can be subdivided in two groups: TRPV1 to TRPV4 are Ca^{2+}-permeable, nonselective cation channels with steep temperature dependence; TRPV5 and TRPV6 are highly Ca^{2+}-selective channels with low temperature sensitivity. TRPV channels are also present in invertebrates: *C. elegans* genome encodes five TRPVs, OCR-1 to OCR-4 and the above-mentioned OSM-9; *Drosophila melanogaster* expresses two TRPVs.

TRPV1 is an outwardly rectifying cation-selective ion channel with a preference for calcium ($P_{Ca}/P_{Na} \sim 10$) and magnesium ($P_{Mg}/P_{Na} \sim 5$) (9), which depends on a single aspartic acid residue in the pore region of the protein (23). TRPV1 is also activated by moderate heat (≥43°C) and low pH (≤5.9) and may act as an integrator of chemical and physical pain-eliciting stimuli. Gating by heat is direct, whereas mild acidosis (pH < 5.9) reduces the temperature threshold for activation and potentiates the responses to capsaicin (9, 30, 81). Capsaicin and the plant toxin resiniferatoxin are potent exogenous agonists of the vanilloid receptor (77). Endogenous agonists include the cannabinoid receptor agonist anandamide (arachidonoylethanolamide, AEA) and several eicosanoid products of lipoxygenases including 12-(*S*)- and 15-(*S*)-hydroperoxyeicosatetraenoic acids, 5-(*S*)-hydroxyeicosatetraenoic acid, and leukotriene B_4 (34, 66, 72, 105). TRPV1 mediates nociception and contributes to the detection and integration of diverse chemical and thermal stimuli (7).

TRPV2 (VRL-1), which is 50% identical to TRPV1, is insensitive to capsaicin and low pH and has a higher threshold for activation by heat (≥52°C) (8).

TRPV3, the last member of the TRPV family to be cloned, is thermosensitive in the physiological temperature range of 22 to 40°C (60, 73, 101).

TRPV4 (OTRPC4, VRL-2, VR-OAC, and TRP12) was first described as a channel activated by hypotonicity-induced cell swelling (42, 55, 74, 99), but it might, as discussed below in more detail, integrate a large variety of stimuli.

TRPV5 (ECaC1, CaT2) and the highly homologous TRPV6 (ECaC2, CaT1) were identified via an expression cloning strategy screening for Ca^{2+} influx-promoting genes in *Xenopus* oocytes, using cDNA libraries from rabbit distal tubule kidney cells and rat duodenum, respectively. Both proteins share ~80% homology at the amino acid level (61–65), are functionally very similar, and are able to form functional heterotetramers (32). TRPV5 and TRPV6 are highly Ca^{2+} selective

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($P_{Ca}/P_{Na} > 100$) and display anomalous mole fraction behavior, Mg^{2+} block, and Ca^{2+}-dependent feedback inhibition (54, 86–88). All these properties are linked to a single negatively charged aspartic acid residue in the pore region (D542 in TRPV5, D541 in TRPV6) (56).

TRPV4: STRUCTURE AND EXPRESSION

Within the TRPV subfamily, TRPV4 displays significantly stronger homology with TRPV1–TRPV3 than with TRPV5 and TRPV6 (Fig. 1). Species differences for TRPV4 are minimal (human/mouse 95.2/96.9%; human/rat 94.8/97.0%, mouse/rat 98.9/99.2% identity/similarity). TRPV4 consists of 871 amino acids with at least three ankyrin repeats in the NH_2 terminus (Fig. 2).

TRPV4 is expressed in a broad range of tissues, including lung, spleen, kidney, testis, fat, brain, cochlea, skin, smooth muscle, liver, and vascular endothelium (10, 18, 37, 42, 74, 99). In situ hybridization in the brain indicates expression, in the lamina terminalis of the mouse brain, in neurons of the arched vascular organ of the lamina terminalis, in the median preoptic area, the optic chiasm, neurons of the subfornical organ, the ventral hippocampal commissure, anterior hypothalamic structures, ependymal cells of the choroid plexus in the lateral ventricles, and dorsal root ganglia (DRG) neurons (14, 42, 74). Interestingly, TRPV4 mRNA but not the protein could be detected in the soma of DRG neurons, suggesting that there might exist a mechanism for the transport of the TRPV4 protein from the neuronal bodies to the sensory terminals (26). Direct functional measurement of endogenous TRPV4-mediated Ca^{2+} entry and/or whole cell currents have been described so far only for endothelial cells (94, 96, 97), keratinocytes (10), and DRG neurons (2).

TRPV4: FUNCTIONAL HALLMARKS

The exogenous agonist 4α-phorbol 12,13-didecanoate (4αPDD) activates a large current in TRPV4-expressing cells (Fig. 3, *A–C*), which is transient in the presence of Ca^{2+} (Fig. 3*A*) and shows a complex time course comprising potentiation, subsequent inhibition by higher $[Ca^{2+}]_i$, and desensitization of the agonist response (see below). In the absence of Ca^{2+}, the current decays more slowly (Fig. 3, *D–F*). Clearly resolvable inward currents can be measured with Ca^{2+} or Mg^{2+} as the only permeating extracellular cation, demonstrating that both divalent cations can permeate TRPV4 channels. Permeability values relative to Na^+ are 6–10 for Ca^{2+} and 2–3 for Mg^{2+} (42, 55, 74, 75, 91, 94). Current-voltage relationships display

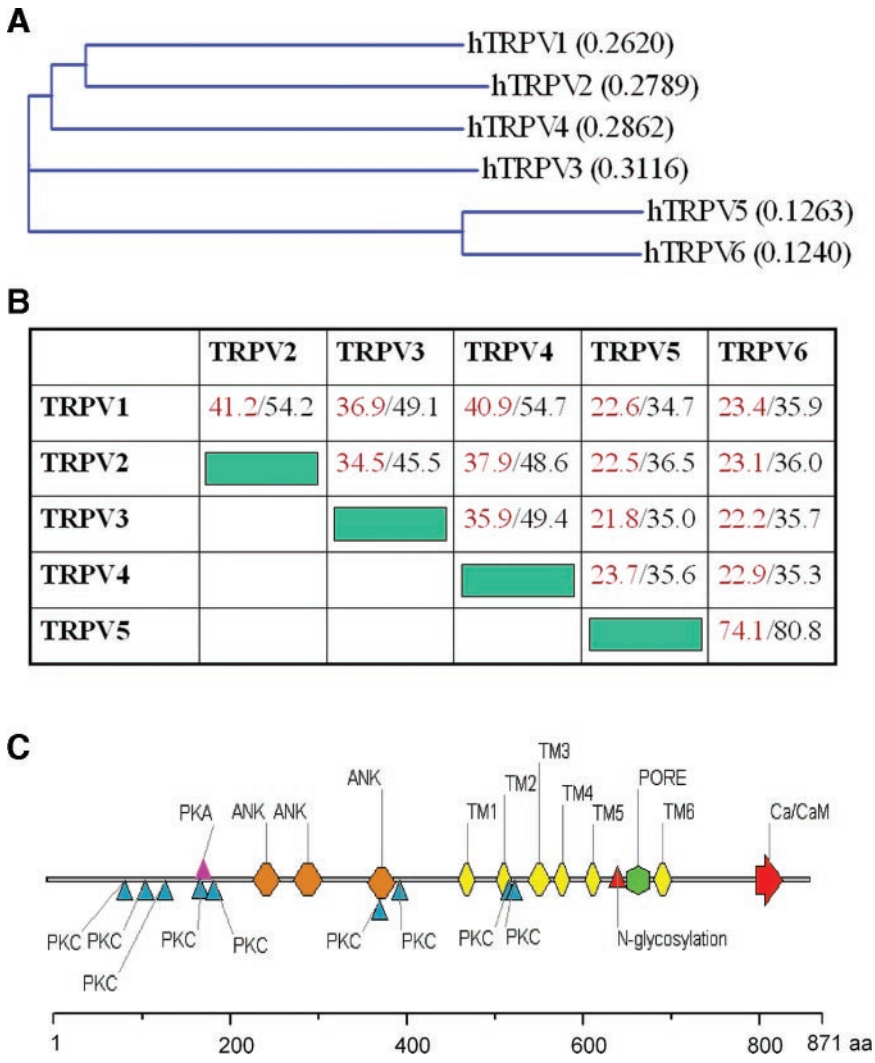

Fig. 1. The TRPV family. *A*: dendrogram of the TRPV family, based on homology (data obtained from Vector NT, pairwise comparison). Accession nos. are NP_542437 (hTRPV1), NP_057197 (hTRPV2), AF 514998.1 (hTRPV3), NP_067638 (hTRPV4), NM_019841 (hTRPV5), and NP_06116 (hTRPV6). Phylogenetic distance was calculated using the Neighbor Joining method (Vector NTI 8). *B*: identity (red) and similarity (black) of the human TRPV (hTRPV) members. *C*: putative domain structure of m(h)TRPV4. Indicated are putative PKC (blue triangles) and PKA phosphorylation sites (pink triangle), ankyrin binding repeats (ANK), the 6 transmembrane regions (TM1–TM6), a glycosylation site close to the pore (red triangle), and the pore region (see also Fig. 5). Red arrow indicates a Ca^{2+}/calmodulin binding site; aa, amino acid. A tyrosine kinase site is present in the first ankyrin repeat (Y253, not shown).

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  1  MADPGDGPRA APGEVAEPPG DESGTSGGEA FPLSSLANLF EGEEGSSSLS
 51  PVDASRPAGP GDGRPNLRMK FQGAFRKGVP NPIDLLESTR YESSVVPGPK
101  KAPMDSLFDY GTYRHHPSDN KRWRRKVVEK QPQSPKAPAP QPPPILKVFN
151  RPILFDIVSR GSTADLDGLL SFLLTHKKRL TDEEFREPST GKTCLPKALL
201  NLSNGRNDTI PVLLDIAERT GNMREFINSP FRDIYYRGQT SLHIAIERRC
251  KHYVELLVAQ GADVHAQARG RFFQPKDEGG YFYFGELPLS LAACTNQPHI
301  VNYLTENPHK KADMRRQDSR GNTVLHALVA IADNTRENTK FVTKMYDLLL
351  LKCSRLFPDS NLETVLNNDG LSPLMMAAKT GKIGVFQHII RREVTDEDTR
401  HLSRKFKDWA YGPVYSSLYD LSSLDTCGEE VSVLEILVYN SKIENRHEML
451  AVEPINELLR DKWRKFGAVS FYINVVSYLC AMVIFTLTAY YQPLEGTPPY
501  PYRTTVDYLR LAGEVITLFT GVLFFFTSIK DLFTKKCPGV NSLFVDGSFQ
551  LLYFIYSVLV VVSAALYLAG IEAYLAVMVF ALVLGWMNAL YFTRGLKLTG
601  TYSIMIQKIL FKDLFRFLLV YLLFMIGYAS ALVTLLNPCT NMKVCDEDQS
651  NCTVPTYPAC RDSETFSAFL LDLFKLTIGM GDLEMLSSAK YPVVFILLLV
701  TYIILTFVLL LNMLIALMGE TVGQVSKESK HIWKLQWATT ILDIERSFPV
751  FLRKAFRSGE MVTVGKSSDG TPDRRWCFRV DEVNWSHWNQ NLGIINEDPG
801  KSEIYQYYGF SHTVGRLRRD RWSSVVPRVV ELNKNSSADE VVVPLDNLGN
851  PNCDGHQQGY APKWRTDDAP L
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Fig. 2. Sequence of mTRPV4 (accession no. NP_071300) with the ankyrin binding repeats underlined in black and TM1–TM6 marked with red bars above the amino acid code. The pore region is indicated in blue type and the calmodulin binding site in red type.

slight outward rectification in the presence of extracellular Ca^{2+} and reverse at a positive potential. Outward rectification is also evident at the single-channel level (Fig. 4). Single-channel conductance is 90–100 pS for outward currents and 50–60 pS for inward currents (74, 75, 96, 97). Ruthenium red (RR) reversibly inhibits inward but not outward currents (Fig. 3, *G–I*).

THE TRPV4 PORE

The ultimate proof that a membrane protein forms a functional channel is the identification of its pore and experimental evidence about mutations in the putative pore region that alter permeation properties. Significant progress in the identification of the molecular determinants of TRP channel pores has been achieved for TRPV1, TRPV4, TRPV5, and TRPV6 channels (23, 32, 56, 89–91). For these channels, point mutations have been described in the linker between TM5 and TM6 that affect Ca^{2+} selectivity, relative monovalent permeability, and blocker sensitivity, providing convincing evidence that, as in the other six TM channels, this linker forms the pore loop containing the selectivity filter.

Figure 5 shows an amino acid sequence alignment of the putative pore regions of the six mammalian TRPV channels, illustrating the high sequence conservation for TRPV1–4. Interestingly, there is also significant homology with the residues in and surrounding the selectivity filter of the KcsA potassium channel, the so-called K^+ channel "signature sequence" (TXXTXGYGD) (17, 103). The sequence similarities may indicate conserved pore structures for these cation channels. The GYG motif in the pore of the K^+-selective channel is changed into a GMG motif for TRPV1, -2, and -4 and a GLG motif for TRPV3. This difference between TRPV1, -2, and -4 on one hand and TRPV3 on the other hand might explain the remarkably higher single-channel conductance of TRPV3 (172 pS at +60 mV vs. ~100 pS for TRPV1, -2 and -4) (101).

The aspartate residue D682 is an important determinant of the Ca^{2+} sensitivity of the TRPV4 pore (Fig. 6). Neutralizing this aspartate to alanine causes a moderate reduction of the relative permeability for divalent cations and of the degree of outward rectification, without significantly altering monovalent permeability. Neutralizing D672 has only minor effects, whereas neutralization of both aspartates causes a much stronger reduction of Ca^{2+} permeability and channel rectification than D682 alone and shifts the permeability sequence for monovalent cations from Eisenman IV to I. Moreover, neutralizing D682 but not D672 strongly reduces the channel's affinity for RR (Fig. 7). In contrast, neutralization of the only positively charged residue in the putative pore region, K675, has no obvious effects on the properties of the TRPV4 channel pore. Interestingly, a mutation to M680 in the region of the K^+ channel signature sequence, which is likely an equivalent of the GYG motif in K^+ channels, strongly reduces whole cell current amplitude and impairs Ca^{2+} permeation. Therefore, it is reasonable to speculate that these mutated residues form part of the TRPV4 selectivity filter and that the architecture of the TRPV4 pore is comparable to that of K^+ channels.

ACTIVATION MECHANISMS

Synthetic TRPV4 agonists. Although TRPV4 was originally considered to be a channel activated upon hypotonic cell swelling, functional characterization of the channel was greatly advanced by the discovery that the synthetic 4αPDD acts as a robust and direct channel activator. This phorbol ester, which has only weak PKC-activating potency ($ED_{50} > 25$ μM) and does not activate TRPV1 or other TRPV channels, is the most potent known activator of TRPV4 with an ED_{50} of 200–400 nM (94). The phorbol 12,13-didecanoate 20-homovanillate phorbol-vanillate (PDDHV), a potent activator of TRPV1 (78), fails to activate TRPV4 channels in inside-out patches. However, PDDHV activates TRPV4 currents in whole cell record-

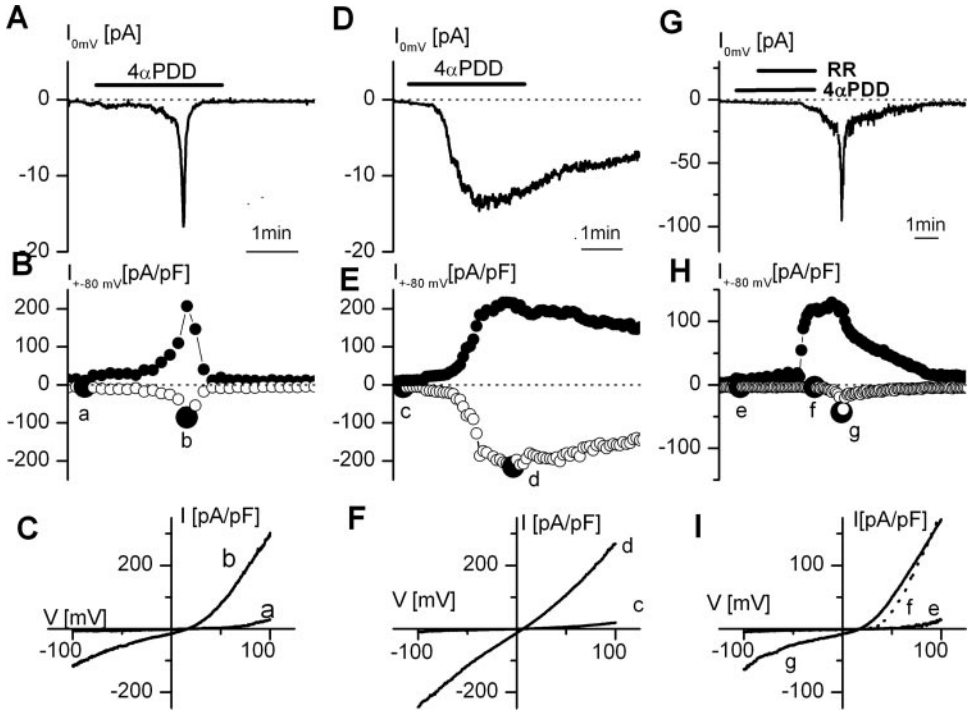


Fig. 3. Activation of TRPV4 by 4α-phorbol 12,13-didecanoate (4αPDD). *A*: at a holding current of 0 mV, application of 1 μM 4αPDD induced an inward current (*I*) that typically appeared with some delay and rapidly inactivated in the presence of extracellular Ca^{2+}. *B*: time course of currents at +80 (●) and −80 mV (○) measured from repetitively applied voltage ramps from −100 to +100 mV (holding potential 0 mV). *C*: current-voltage (*I-V*) relationships measured at times indicated by *a* and *b* in *B*. Note the outward rectification in the presence of extracellular Ca^{2+}. *D*: same protocol as in *A*, at holding current of 0 mV, but in the absence of extracellular Ca^{2+}. Note the delayed inactivation. *E*: time course of currents activated by 4αPDD at +80 and −80 mV. *F*: I-V curves at times labeled *c* and *d* in *E*. Note the near absence of rectification in Ca^{2+}-free solution. *G*: inhibition of currents through TRPV4 by ruthenium red (RR; 1 μM). Inward currents were completely blocked (holding current 0 mV). After RR was washed out, a large inward current appeared. *H*: current traces at +80 and −80 mV. Note that in the presence of RR, 4αPDD activated an outward current but no inward current, indicating a voltage-dependent block of TRPV4 by RR. The inward current appeared after RR was washed out. *I*: *I-V* curves at times labeled *e–g* in *H*. Block by RR is shown by *trace f*. Note the absence of the inward current (compare with *C*). However, after RR was washed out, the typical outwardly rectifying *I-V* curve reappeared [1.5 mM extracellular $[Ca^{2+}]$ ($[Ca^{2+}]_e$) present].

ings and also increases $[Ca^{2+}]_i$, suggesting that its vanillyl moiety has to be cleaved by intracellular esterases (Watanabe H, Vriens J, and Nilius B, unpublished observations). The TRPV4 current activated by 4αPDD is transient, and repetitive applications result in decreased responses, indicative of desensitization. The classic PKC activator phorbol 12-myristate 13-acetate (PMA), which is structurally similar to 4αPDD, displays a 10- to 50-fold lower potency than 4αPDD in activating TRPV4 channels (94). These data strongly suggest that 4αPDD acts via a mechanism distinct from the classic interaction of a phorbol 12,13-diester with a phorbol ester/diacylglycerol-type receptor target. The 4α configuration is apparently not essential for channel activation, because 4βPDD also activates TRPV4 in a similar concentration range (Watanabe H and Nilius B, unpublished observation; see also Fig. 8). TRPV4 does not contain a typical cysteine-rich phorbol-binding site, homologous to the C1 domains described for PKC and "nonkinase" phorbol ester receptors (40), and it is therefore unlikely that activation results from binding of 4αPDD to such a site. In addition, the region of best alignment with several PKCs, chimerins, and MUNC13 has very low homology and is located in the pore region (650H-699C), which makes it unlikely that phorbols are bound via a known motif to TRPV4.

Endogenous TRPV4 agonists. The potent activation of TRPV4 by 4αPDD fueled the search for possible endogenous TRPV4 agonists. Endocannabinoids are a class of endogenous lipids, including amides and esters of long-chain polyunsaturated fatty acids (15, 16, 45) that activate metabotropic cannabinoid receptors. The endocannabinoid anandamide (AEA) and the metabolite 12-hydroxyeicosatetraenoic acid are potent activators of TRPV1 (27, 72, 82, 104, 105). Recently, AEA and its metabolite arachidonic acid (AA) were found to cause a robust increase in intracellular Ca^{2+} and activate typical whole cell currents in TRPV4-expressing cells (96). AEA and the related endocannabinoid 2-arachydonyl glycerol (2-AG) (45) are transported into the cell through the action of a membrane transporter and degraded via a lipoxygenase. AEA is hydrolyzed to AA exclusively by fatty acid amidohydrolase (FAAH) (13, 15), whereas 2-AG can also be hydrolyzed through monoacylglycerol lipase and other esterases (84). Methanandamide, a nonmetabolizable analog of AEA, is not able to activate TRPV4, and phenylmethylsulfonyl fluoride, a selective FAAH inhibitor, inhibits the effects of AEA but not of AA, indicating that FAAH-dependent hydrolysis of AEA to AA is required for TRPV4 activation (96). Surprisingly, AA is not able to activate TRPV4 in cell free patches, indicating that cellular metabolism of AA is required for channel activation. ETYA, a nonspecific

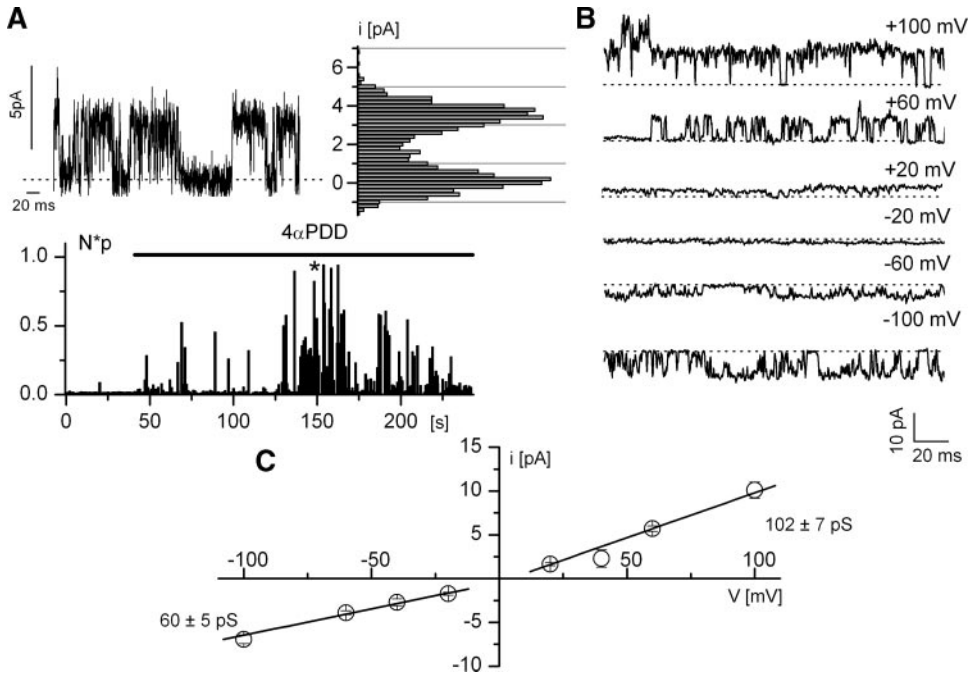
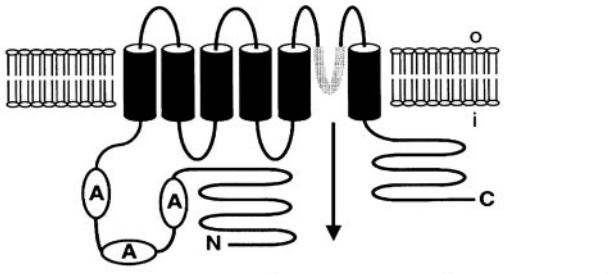


Fig. 4. Single-channel currents through TRPV4 activated by 4αPDD. *A*: cell-attached patch (+60 mV, 1.5 mM $[Ca^{2+}]_e$, 1 μM 4αPDD). Single-channel activity and amplitude histogram (*top*) are shown from the sweep labeled with a star (*bottom*), showing the time course of open probability (averaged current per sweep divided by single-channel current). Single-channel current was 3.7 pA. *B*: single TRPV4 channels at different potentials activated by 1 μM 4αPDD. *C*: single-channel current-voltage (*i-V*) relationship from more than 5 patches per voltage. From linear regressions, an inward conductance of 60 pS and outward conductance of 102 pS were calculated (currents from amplitude histograms).

blocker of all AA-metabolizing enzymes (19, 71), prevents activation of TRPV4 currents by AA, which indicates that lipoxygenase (LOX), cyclooxygenase (COX), and cytochrome *P*-450 (CYP) metabolites of AA might act as potential activators of TRPV4 (96). Activation of TRPV4 by AA was insensitive to indomethacin, nordihydroguaiaretic acid, and a combination of these inhibitors, which ruled out an involvement of the COX and LOX pathways. Miconazole, an inhibitor of *P*-450 epoxygenase, and 17-octadecynoic acid (17-ODYA), an inhibitor of the *P*-450 epoxygenase and ω/ω-1-hydroxylases (71), both fully abolished the AA activation of TRPV4 (96). Importantly, the CYP inhibitors ETYA, miconazole, and 17-ODYA do not directly inhibit TRPV4 channels, because they can still be activated by 4αPDD in the presence of these blockers. Given that 5′,6′-epoxyeicosatrienoic acid (EET) and, to a lesser extent, 8′,9′-EET activate TRPV4 in a membrane-delimited fashion, it is most likely that the epoxygenase pathway is involved in TRPV4 activation. Thus AEA and AA apparently act as endogenous chemical agonists of TRPV4, activating the channels through CYP-dependent formation of 5′,6′-EET (96). It is unclear whether these endogenous ligands can directly bind to the channel. Activation of TRPM2 by AA depends on an **IS**XX**TKE** arachidonate recognition sequence (ARS) (28) that was first shown to be important for AA signaling in the two-pore-domain potassium channel TREK-1 (58). Such an ARS-like sequence, **LS**RK**FKD**, is present at the TRPV4 COOH-terminal end of the NH_2 terminus (amino acids 402–408 in mTRPV4). Its role in the activation of TRPV4 is unclear because the corresponding deletion mutant could not be functionally expressed (Vriens J, Prenen J, and Nilius B, unpublished observations).



TRPV1: YNSLYSTCLEL FKFTIGMG**D**L EFTE
TRPV2: YRGILEASLEL FKFTIGMG**E**L AFQE
TRPV3: YGSFSDAVLEL FKLTIGLG**D**L NIQQ
TRPV4: SETFSTFLL**D**L FKLTIG**M**G**D**L EMLS
TRPV5: YPTALFSTFEL F-LTII**D**GPA NYSV
TRPV6: YPMALFSTFEL F-LTII**D**GPA NYNV
KcsA: YPRALWWSVET A-T**T**V**GYGDL** -YPV

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Fig. 5. Alignment of the putative TRPV4 pore region with that of other TRPV channels and of the potassium channel KcsA. Transmembrane topology of TRPV channels (*top*) and alignment of their putative pore region with the bacterial potassium channel KcsA (*bottom*) are shown. Box marks the region with the highest homology among TRPV1, TRPV2, and TRPV4, supposedly the selectivity filter. Negatively charged residues and the crucial determinants for TRPV4 permeation within this region are in bold type, and those for TRPV4 are underlined. D672 and D682 in TRPV4 are indicated. GenBank accession nos. are CAC 20703 (TRPV4), CAB 89866 (TRPV1), NP_057197 (TRPV2), NP_062815 (TRPV5), AAG 41951 (TRPV6), and PIR S60172 (KcsA). A, ankyrin binding repeats.

TRPV4 activation by osmosensation and mechanical stimuli. Senses based on mechanosensation include hearing and balance mediated by mechanosensors of the inner ear hair cells and cutaneous touch sensation via the terminals of sensory cells that innervate the skin (22). Changes in cell volume affect other mechanosensors, e.g., osmosensitive neurosensory cells in the circumventricular organs measure the osmolality of the blood and communicate with neurosecretory cells, leading to the secretion of antidiuretic hormone (6). TRPV4 can be activated by exposing cells to hypotonicity, implying that this channel might be a cellular osmosensor (42, 55, 74, 99). The expression of TRPV4 in epithelial cells of kidney, in the stria

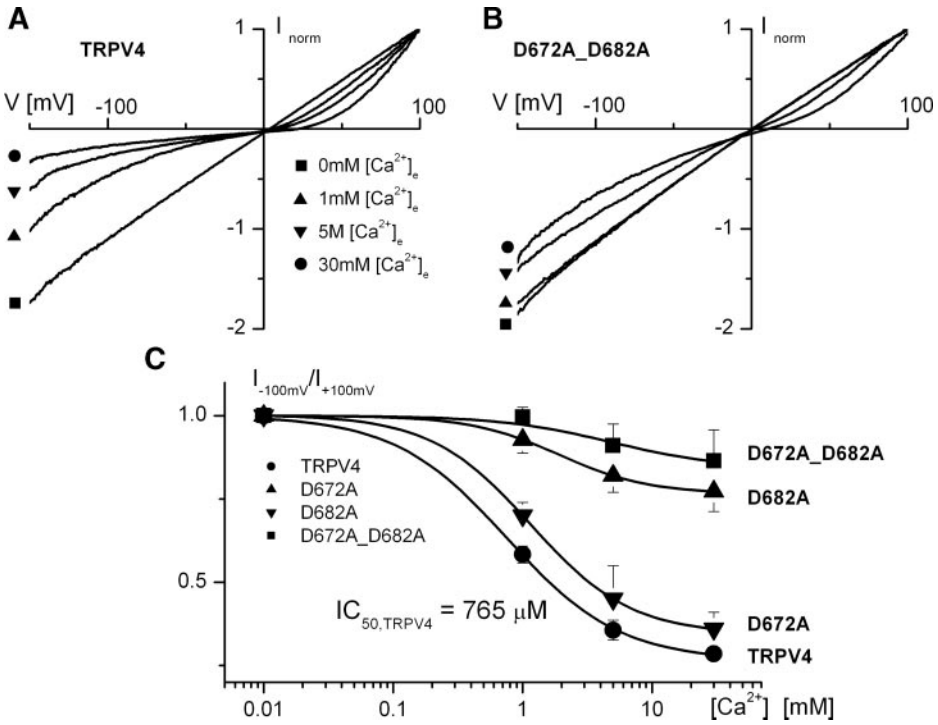


Fig. 6. Functional properties of the TRPV4 pore. *A*: *I-V* curve of whole cell currents normalized to +100 mV in the presence of different $[Ca^{2+}]_e$. Note the Ca^{2+}-dependent outward rectification representing the low-affinity block of inward currents through TRPV4 by $[Ca^{2+}]_e$. *B*: the double aspartate mutation D672A-D682A in the pore region strongly reduced the Ca^{2+}-dependent block of inward current. *C*: Ca^{2+} block represented as ratio of the current at −100 and +100 mV. Concentration for half-maximal block of the wild-type channel was 765 μM $[Ca^{2+}]_e$. Note that the pore mutations D682A or the double aspartate mutations, but not the mutation of D672 alone, decreased the low-affinity block by Ca^{2+}. These results indicate a low-affinity binding of Ca^{2+} in the TRPV4 pore, which is mainly determined by D682.

vascularis of the cochlea, in sweat glands, and in the osmosensory cells of the brain's circumventricular organs (14, 26, 42, 51, 74), is in agreement with such an osmosensor function.

Presently, the mechanism whereby swelling activates TRPV4 is not yet fully solved. The NH_2-terminal intracellular domain of TRPV4 contains three or more ankyrin repeat domains that seem to be involved in responses to physical challenges, because TRPV4 activation is delayed if these ankyrin repeats are lacking (42) (Vriens J and Nilius B, unpublished observations). These repeats may anchor the channel to the cytoskeleton and form a mechanical link for gating. A different mechanism of hypotonicity-induced activation of TRPV4 proceeding via the phosphorylation of TRPV4 has been proposed recently (100). These authors observed in a heterologous expression model and in native murine distal convoluted tubule cells in culture a rapid cell swelling-induced tyrosine phosphorylation of TRPV4 mediated via a Lyn kinase-dependent phosphorylation of residue Y253 in the first ankyrin binding repeat. Mutation of this site abolished the hypotonicity-dependent activation of TRPV4. This mechanism is, however, controversial. We did not observe any effect on the swelling-induced response in the Y253F mutant (91a). An alternative possibility could be that hypotonic activation of TRPV4 acts through the above-described AA-EET-dependent pathway, downstream of swelling-induced, PLA_2-dependent AA release (3, 59).

Activation by heat. An emerging characteristic of TRPV channels is their distinct response to changes in temperature. TRPV1 is activated at temperatures above 42°C and shows a slight sensitization during repeated stimulations (8, 38). The temperature threshold for TRPV3 activation is about 39°C, but this channel shows strong sensitization during repetitive heat challenges (60, 73, 101). TRPV4 is activated at temperatures above ~27°C. In contrast to TRPV1 and TRPV3, it desensitizes upon repeated heat applications (26, 97). When constantly exposed to 37°C, TRPV4 can still respond to increased temperatures, i.e., its shows incomplete desensitization (26). Likely, TRPV4 is constitutively active at body temperature. Ca^{2+}-dependent inactivation is a possible adaptive mechanism to reduce channel open probability by the resulting increase in $[Ca^{2+}]_i$ (94, 95) (see also *Modulation by* Ca^{2+}). The mechanism of heat activation of TRPV4 is unclear. However, the observation that heat in contrast to, for example, 4αPDD or 5′,6′-EET does not activate TRPV4 channels in cell-free inside-out patches (10, 95) argues against direct activation and points to an indirect or messenger-mediated mechanism.

Modulation by Ca^{2+}. Intracellular Ca^{2+} is an important regulator of TRPV4 channels and, depending on the concentration, either potentiates or inhibits channel activity (75, 94, 95). Stimulation with 4αPDD activates TRPV4 current with a certain latency, followed by inactivation. This decay is accelerated by increasing the extracellular Ca^{2+} concentration and is delayed in the absence of extracellular Ca^{2+}. The ED_{50} for intracellular Ca^{2+}-dependent inactivation of TRPV4 is ~400–600 nM (94, 95), but the nature of this Ca^{2+}-dependent negative feedback mechanism has not yet been identified. Inactivation in the presence of extracellular Ca^{2+} was much slower in a mutant channel with a point mutation in the sixth transmembrane domain (F707A) (95).

An increase in intracellular Ca^{2+} was shown to first stimulate TRPV4 (75), and TRPV4 currents stimulated by hypotonic solutions or phorbol esters were strongly reduced at all potentials in the absence of extracellular Ca^{2+}. The permeant divalent cations Ba^{2+} and Sr^{2+} were less effective than Ca^{2+} in potentiating TRPV4. This effect depended on an intracellular site in the COOH terminus, to which calmodulin binds in a Ca^{2+}-dependent manner. This site, however, does not affect inactivation. A positively charged α-helical stretch VGRLRRDRWSSVVPRVV, similar to the COOH-terminal Ca^{2+}/calmodulin-binding motif in TRPV6 and with some similarity

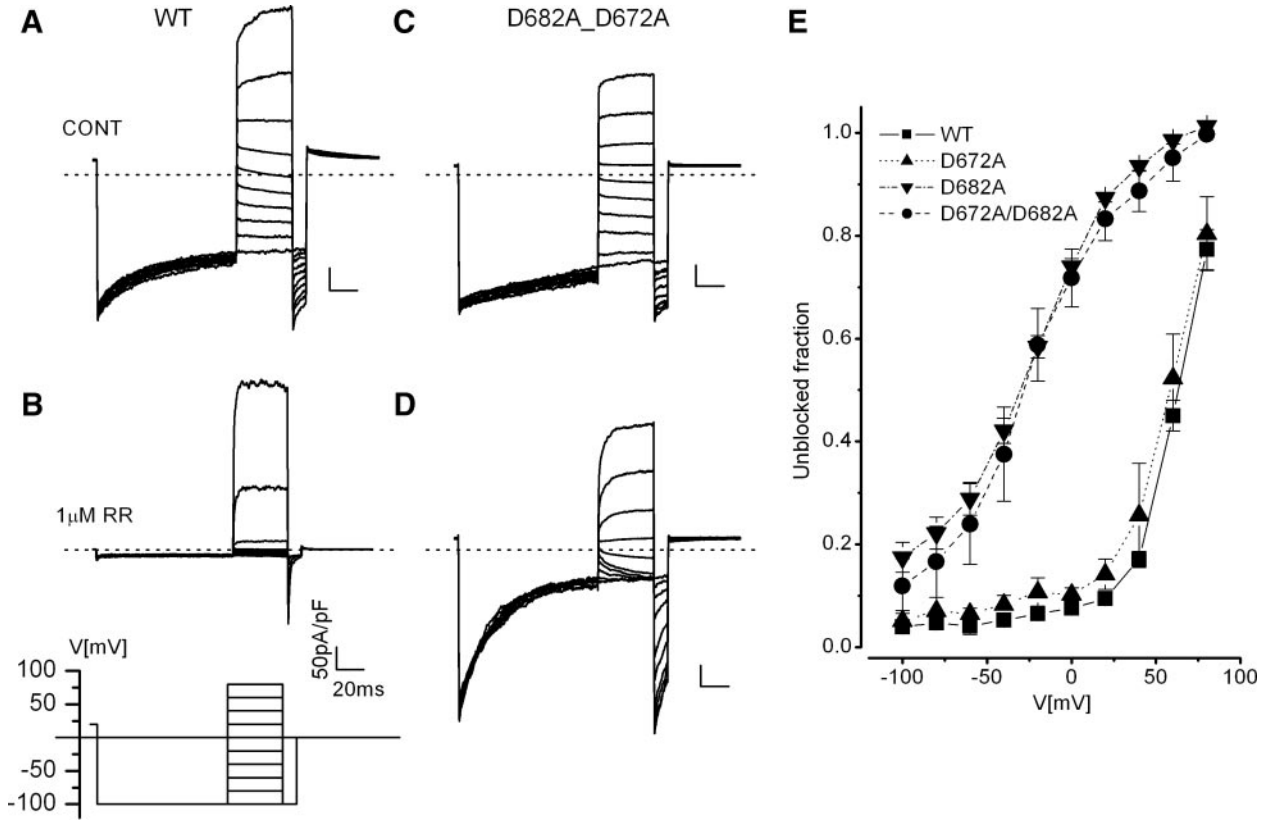


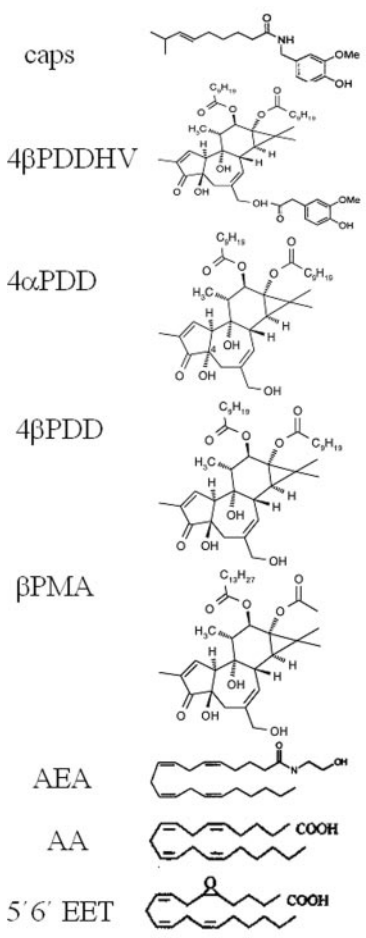
Fig. 7. Block of TRPV4 by RR. *A*: currents are shown through wild-type (WT) TRPV4. Channels were activated by 1 μM 4αPDD. Holding potential was +20 mV. The voltage protocol consisted of a hyperpolarizing prestep to −100 mV, followed by test steps from −100 to +80 mV spaced by 20 mV and a further step back to −100 mV (see *B*, *inset*; $[Na^+]_e$ = 150 mM, $[Ca^{2+}]_e$ = 5 mM). The slow decay of the inward current is likely due to inhibition by Ca^{2+}. *B*: 1 μM RR completely abolished the inward current but did not affect the outward currents in WT TRPV4 channels. This again indicates that the block of TRPV4 by RR is voltage dependent. *C*: the double mutant D672A-D682A currents are similar to those for the WT; however, the Ca^{2+}-dependent decay was delayed. *D*: RR had much less effect on the mutant channel than on the WT. Inward currents were still large and decayed slowly, probably due to the slower entrance of RR into the pore vestibule. *E*: voltage dependence of the block by 1 μM RR for WT TRPV4 and the 3 mutants. The voltage at the abscissa is the test potential after the first step to −100 mV. The unblocked fraction in the presence of RR was obtained by measuring peak tail currents during the second step to −100 mV and normalizing them to the current in the absence of the blocker (see also Ref. 91).

to the PKC pseudosubstrate site (52), has been identified in the COOH terminal of TRPV4 starting at position 814 (75). By mutagenesis, it has been shown that this motif is the structural determinant of Ca^{2+}-dependent potentiation (75). The same site seems essential for the spontaneous opening of TRPV4 channels in the absence of any agonist (75). This spontaneous activation might be responsible for the observed elevated Ca^{2+} levels in nonstimulated TRPV4-expressing cells (42, 74, 96, 97, 99). Interestingly, mutant channels with a single mutation in the COOH terminus of TRPV4 (E797) were constitutively open, i.e., spontaneous activation seemed to be increased (95), suggesting that this site may interfere with Ca^{2+} binding at the neighboring calmodulin-binding motif.

Modulation by phosphorylation. The mechanism of TRPV1 activation and potentiation by PKC-dependent phosphorylation has been investigated in detail (39, 57, 67, 85). It has recently been shown that PMA, a known activator of PKC, also activates TRPV4 (21). Concentrations of PMA that are subthreshold at room temperature (94) activate TRPV4 at 37°C through a PKC-dependent pathway. The PMA activation of TRPV4 is dramatically reduced in the presence of the PKC inhibitors calphostin C and staurosporine (21), indicating that phorbols activate TRPV4 via PKC-independent and -dependent mechanisms. The potentiating effect of PKC stimulation on TRPV4 activation by other stimuli, such as endogenous agonists, cell swelling, and heat, has not yet been studied in detail. Putative PKC phosphorylation sites are indicated in Fig. 1. Probably, S88, S134, and S528 are the most likely candidates for mediating functional effects.

Remarkably, modulation by lipids, such as phosphatidylinositol 4,5-bisphosphate (PIP_2), is still completely unknown for TRPV4. The COOH terminus of TRPV1 contains a modular PIP_2 binding site (a cluster of basic residues interspersed by hydrophobic amino acids, e.g., L**R**SS**R**VSG**R**HW**K**NFALV-PLL**R**EASA**R**D**R**QSAQPEEVYL**R**QFSS for hTRPV1). Binding of PIP_2 to this site causes tonic inhibition of the channels, and PLC-mediated hydrolysis sensitizes the channel for activation by capsaicin, protons, and heat (68). This site, however, is not conserved in TRPV4, but all TRPV4s contain a low-homology site with six basic amino acids between residues 400 and 446 whose possible functional impact is still unknown.

Interference of various stimuli. TRPV4 is coexpressed with TRPV3 in mouse keratinocytes (10). Heat responses were significantly enhanced under hypotonic conditions and inhib-



	TRPV1	TRPV4
caps	340nM	none
4βPDDHV	60nM	400nM, cleavage to PDD
4αPDD	none	185 - 400nM
4βPDD	none	340-500nM
βPMA	sensitize PKC	3 μM
AEA	1 – 10 μM	2 μM
AA	none	1.8 μM
5′6′ EET	none	150nM

Fig. 8. Comparison of the pharmacology of activation of TRPV1 and TRPV4 by phorbols and fatty acids. Shown are the structures of agonists for TRPV1 and TRPV4. TRPV1 agonists seem to require the vanillyl moiety. For the phorbols, the 4α vs. 4β structure is indicated by a dashed and solid triangle, respectively. K_d values for TRPV1 are from Ref. 77, and values for TRPV4 are from Refs. 94 and 96 and from Watanabe H and Nilius B [unpublished data for 4βPDD and 4β-12,13-didecanoate 20-homovanillate phorbol-vanillate (PDDHV); 4αPMA has not yet been tested].

ited under hypertonic conditions in these cells. 4αPDD also augmented the responsiveness to heat, i.e., a concentration of 4αPDD that is subthreshold at room temperature activates TRPV4 at higher temperatures (10, 21). Similar synergistic effects have also been observed for the responses of TRPV1 channels to capsaicin, heat, and protons.

TRPV4 expressed in human embryonic kidney (HEK) cells also clearly shows this stimulus interdependence of activation. At room temperature, activation by hypotonic cell swelling, shear stress, and PKC is modest or absent, but 4αPDD induces a clear effect. At elevated temperatures (37°C), TRPV4 is rapidly activated by all stimuli. Temperature appears to be a critical modulator of TRPV4 channel gating, leading to activation of the channel by a diverse range of microenvironmental chemical and physical signals (21). It is obvious from these data that the precise threshold for TRPV4 activation depends on the cellular context and environmental history of the channel. Activity-dependent changes in channel state, channel phosphorylation, or dephosphorylation (100); changes in osmolarity; activation of downstream signaling pathways; and protein-protein interactions such as heteromultimeric channel formation may all cause diversity in activation parameters. Heat does not affect the 5′,6′-EET-induced increase in $[Ca^{2+}]_i$, but this increase is reduced under hyposmotic conditions (Vriens J and Nilius B, unpublished data). Likely, the heat-sensitive pathway is different from the swelling-induced pathway.

POSSIBLE PHYSIOLOGICAL FUNCTIONS FOR TRPV4

One key question remains: What are TRPV4 channels good for? The ability of this unique channel to respond to a broad variety of signals has evoked hypotheses about its possible involvement in processes ranging from sensory detection and thermoregulation to regulation of vascular tone and signaling in the brain. At present, most of this is still speculative, but the recent creation of TRPV4-deficient mice will allow a direct testing of these hypotheses.

Keratinocytes are capable of detecting modest temperature elevations, which contribute to warmth perception and/or cutaneous thermoregulation. In a recent study, strong evidence was provided for an involvement of TRPV4 in these responses (10). In addition to peripheral temperature sensing, TRPV4 might also play a role in regulating thermogenesis. TRPV4 is expressed in the preoptic and anterior hypothalamus (26, 42), the control center of thermogenesis that contains specialized warm- and cool-sensitive neurons, which are also activated by hyposmolarity (1, 5, 33, 83). The high level of TRPV4 expression in endothelial cells (94, 97) may hint to another role in thermoregulation by influencing the vasomotor activity of peripheral vessels. The involvement of TRPV4 in thermosensation and thermoregulation might become clearer in mice lacking TRPV4.

The basal level of TRPV4 activity at normal body temperature will undoubtedly contribute to Ca^{2+} homeostasis and might influence the growth and differentiation state of cells expressing TRPV4. Primary keratinocytes maintain an undifferentiated proliferative phenotype at low extracellular Ca^{2+}, whereas exposure to higher Ca^{2+} inhibits proliferation, changes cell morphology and induces terminal differentiation (10, 102). In endothelial cells, temperature-sensitive Ca^{2+} entry through TRPV4 could have important consequences, e.g., for a steady-state production of nitric oxide, and might contribute to the known vasoconstriction and vasodilatation of peripheral blood vessels induced by cooling and warming, respectively (46). In addition, the temperature sensitivity of endothelial TRPV4 might suggest a role in mediating inflammatory pathophysiology in fever, e.g., by changing barrier properties that depend on Ca^{2+} influx (79).

Until now, TRPV4 is the only TRP channel that has been put forward as a potential constituent of a mammalian mechanotransducer (42), although its biophysical properties do not really match those of a mechanosensitive channel, because pressure applied to TRPV4-expressing cell-attached patches does not activate this channel (74). Nevertheless, it is an interesting possibility that TRPV4 is involved in mechanosensing, e.g., in endothelial cells via a mechanostimulation of PLA_2 and subsequent activation by AA and 5′,6′-EET (96). Interestingly, TRPV4 responds to shear stress, which might be especially important for endothelial cell function (21, 53). The proposed mechanosensitivity of TRPV4 has also made it a candidate gene for inherited dominant nonsyndromic hearing impairment (25, 27).

The TRPV4 activators AEA and 2-AG likely play an important role in the control of the vascular tone and potentially in shock conditions (44, 69, 70, 92, 93, 105). Interestingly, their effects could not be fully explained by an action on CB1 and CB2 receptors or on TRPV1 channels (24, 29, 35, 36, 92). Our data about the activation of TRPV4 by AEA and 2-AG might provide the missing link for the action of these compounds on endothelium.

Endocannabinoids are potent neuromodulators that may mainly act as retrograde messengers (20, 98). The finding that endocannabinoids are involved in TRPV4 activation identifies a new molecular target for cannabinoids and provides a link to modulation of synaptic function (16). In this respect, it might be of interest that the gene locus for the human TRPV4 channel is associated with bipolar affective disorder (14).

It has been shown that TRPV4 has a physiological role in rat primary afferent neurons and is involved in the detection of osmolarity in nociceptors (2). TRPV4 is thus a sensory transducer for osmotic stimulus-induced nociception. The TRPV4 protein is transported in sensory nerve distally toward the peripheral nerve endings. Single-fiber recordings on C-fibers showed an activation due to a hypotonic stimulus and, in addition, an enhanced production of the hyperalgesic inflammatory mediator prostaglandin E_2. It was also shown that this osmotransduction causes nociception and induced pain-related behavior in mice. This is the first report on the role of TRPV4 in pain signaling. Thus we conclude that TRPV4 might be a new target for the development of novel analgesics.

The recently described TRPV4-deficient mouse shows a markedly reduced sensitivity of the tail to pressure and acidic nociception, which is compatible with a role of TRPV4 in mechanosensation. The threshold to noxious stimuli and the conduction velocity of myelinated nerves responding to stimuli were also impaired, indicating that TRPV4 might be essential for the normal detection of pressure by a high-threshold mechanosensor (76). Another functional role of TRPV4 suggested by the Suzuki group is its putative role in osmoregulation (47). TRPV4 is expressed in the cerebral circumventricular organs (42), which is important for regulation of water input and/or osmolarity in the body. In TRPV4-deficient mice, water intake behavior, or serum osmolarity, and serum vasopressin (AVP), were not changed. During short-term salt ingestion, however, serum AVP and AVP secretion were significantly increased. In brain slices, hyperosmolarity exaggerated AVP secretion. It was concluded that TRPV4 might transmit a negative signal for AVP. The underlying mechanism is unclear, because in this case hyperosmolarity might be able to activate TRPV4.

Some clues for the functional role of TRPV4 may be obtained from TRPV subfamily members in *C. elegans* and *Drosophila*. OSM-9, one of the five *C. elegans* TRPV channels, is present in chemosensory and mechanosensory neurons, and OSM-9-deficient worms have defective olfactory and mechanosensory responses (12). Together with other TRPV channels (e.g., OCR-2), OSM-9 is essential for the diverse sensory functions and localized in sensory cilia (80). Importantly, the different *C. elegans* TRPV channels promote the targeting of each other to cilia. Likely, different combinations of TRPV proteins allow cell type-specific regulation of channel function and localization, and combinations of TRPV proteins may direct different functions to distinct subcellular locations. The *D. melanogaster* genome includes two predicted TRPV genes (43, 80). One gene encodes an 833-amino acid protein called Nanchung (Nan), which shares several topological hallmarks with TRPV4. Functional expression of Nan results in a Ca^{2+}-permeable channel activated by cell swelling. Nan is exclusively expressed in chordotonal neurons and is localized in the sensory cilia of the *Drosophila* antennas. Antennal sound-evoked potentials are completely absent in mutants lacking Nan. This TRPV channel therefore acts, at least in *Drosophila*, as a chordotonal mechanotransducer that is essential for hearing (41).

NOTE ADDED IN PROOF

After acceptance of this paper, the W. Liedtle laboratory published impressive data on the involvement of TRPV4 in osmoregulation. TRPV4-deficient mice drink less water, become more hyperosmolar, have a decreased blood level of antidiuretic hormone, and show an impaired response to hyper- and hyposmolar stimuli. Data indicate that TRPV4 is a necessary osmotic sensor in the circumventricular organs in the mammalian CNS (Liedtke W and Friedman JM. Abnormal osmotic regulation in *trpv4*$^{-/-}$ mice. *Proc Natl Acad Sci* October 27, 2003; 10.1073/pnas.173541610).

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