Molecular Cloning and Functional Characterization of a Novel Receptor-activated TRP Ca\(^{2+}\) Channel from Mouse Brain*  

(Received for publication, November 4, 1997, and in revised from, January 28, 1998)  

Takaharu Okada‡, Shunichi Shimizu‡, Minoru Wakamori‡, Akito Maeda‡, Tomohiro Kurosaki‡, Naoyuki Takada‡§, Keiji Imoto‡, and Yasuo Mori‡§  

From the ‡Department of Information Physiology, National Institute for Physiological Sciences, Okazaki 444, Japan, and the §Department of Molecular Pharmacology and Biophysics, University of Cincinnati College of Medicine, Cincinnati, Ohio 45267-0228, and the ¶Department of Molecular Genetics, Institute for Liver Research, Kansai Medical University, Moriguchi 570, Japan  

Characterization of mammalian homologues of Drosophila TRP proteins, which induce light-activated Ca\(^{2+}\) conductance in photoreceptors, has been an important clue to understand molecular mechanisms underlying receptor-activated Ca\(^{2+}\) influx in vertebrate cells. We have here isolated cDNA that encodes a novel TRP homologue, TRP5, predominantly expressed in the brain. Recombinant expression of the TRP5 cDNA in human embryonic kidney cells dramatically potentiated extracellular Ca\(^{2+}\)-dependent rises of intracellular Ca\(^{2+}\) concentration ([Ca\(^{2+}\)]\(_i\)) evoked by ATP. These [Ca\(^{2+}\)]\(_i\) transients were inhibited by SK&F96365, a blocker of receptor-activated Ca\(^{2+}\) entry, and by La\(^{3+}\). Expression of the TRP5 cDNA, however, did not significantly affect [Ca\(^{2+}\)]\(_i\) transients induced by thapsigargin, an inhibitor of endoplasmic reticulum Ca\(^{2+}\)-ATPases. ATP stimulation of TRP5-transfected cells pretreated with thapsigargin to deplete internal Ca\(^{2+}\) stores caused intact extracellular Ca\(^{2+}\)-dependent [Ca\(^{2+}\)]\(_i\) transients, whereas ATP suppressed [Ca\(^{2+}\)]\(_i\) in thapsigargin-pretreated control cells. Furthermore, in ATP-stimulated, TRP5-expressing cells, there was no significant correlation between Ca\(^{2+}\) release from the internal Ca\(^{2+}\) store and influx of extracellular Ca\(^{2+}\). Whole-cell mode of patch-clamp recording from TRP5-expressing cells demonstrated that ATP application induced a large inward current in the presence of extracellular Ca\(^{2+}\). Omission of Ca\(^{2+}\) from intrapipette solution abolished the current in TRP5-expressing cells, whereas 10 mM intrapipette Ca\(^{2+}\) was sufficient to support TRP5 activity triggered by ATP receptor stimulation. Permeability ratios estimated from the zero-current potentials of this current were \(P_{\text{Ca}}/P_{\text{Na}}/P_{\text{Cs}} = 14.3:1.5:1\). Our findings suggest that TRP5 directs the formation of a Ca\(^{2+}\)-selective ion channel activated by receptor stimulation through a pathway that involves Ca\(^{2+}\) but not depletion of Ca\(^{2+}\) store in mammalian cells.  

Calcium (Ca\(^{2+}\)) influx across the plasma membrane plays a vital role in the regulation of diverse cellular processes, ranging from ubiquitous activities like gene expression to tissuespecific functions such as neurotransmitter release and muscle contraction, by controlling the cytosolic free Ca\(^{2+}\) concentration ([Ca\(^{2+}\)]\(_c\)). Recently, in addition to the well characterized modes of Ca\(^{2+}\) entry through voltage-dependent Ca\(^{2+}\) channels and ligand-gated cation channels, receptor-activated Ca\(^{2+}\) influx that occurs as a second phase of phosphatidylinositol (PI)1-dependent response, has been recognized for its physiological significance (3). Diverse ion channels activated by various triggers have been recognized to be responsible for the receptor-activated Ca\(^{2+}\) influx (3). Among members of the group, recent attention was particularly directed to capacitative Ca\(^{2+}\) entry (CCE; in other words, Ca\(^{2+}\) release-activated current (ICRAC), or store-operated channel), that is activated through Ca\(^{2+}\) release from the intracellular Ca\(^{2+}\) store, endoplasmic reticulum (ER), induced by inositol 1,4,5-trisphosphate (IP3) and consequent depletion of Ca\(^{2+}\) from the store (2–9). Diffusible small molecules (10, 11), IP3 metabolites (12), and direct coupling of IP3 receptors or small GTP-binding (G) proteins with the channel proteins (5, 6, 9) have been proposed to be involved in the activation of this Ca\(^{2+}\) entry pathway. Other plasma membrane ion channels directly activated by second messengers such as Ca\(^{2+}\), IP3, and inositol 1,3,4,5-tetraphosphate (IP4) (13–16) are also categorized as receptor-activated Ca\(^{2+}\) channels (3).  

An important clue for understanding the molecular basis of receptor-activated Ca\(^{2+}\) influx was first attained through the finding of a Drosophila visual transduction mutation transient receptor potential (trp), whose photoreceptors fail to generate the Ca\(^{2+}\)-dependent sustained phase of receptor potential and to induce subsequent Ca\(^{2+}\)-dependent adaptation to light (17, 18). Inasmuch as the gene products of the trp and trp-like (trpl) gene (TRP and TRPL) comprise the light-activated, PI-dependent Ca\(^{2+}\) conductance in Drosophila photoreceptors (19), the original hypothesis that the counterparts of TRP and TRPL are responsible for CCE in vertebrate cells was based upon analogy between the phototransduction mechanism in Drosophila and the PI-dependent signal transduction processes in vertebrates (18). In fact, recent molecular characterization has unveiled the existence of multiple genes encoding TRP homologues in vertebrate cells (20–25), and cDNA expression experiments of

* This work was supported by research grants from the Ministry of Education, Science, Sports, and Culture, the Japan Society for the Promotion of Science, and the Mochida Memorial Foundation for Promotion of Medicine and Pharmacy. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.  

The nucleotide sequence(s) reported in this paper has been submitted to the GenBank™/EBI Data Bank with accession number(s) AF029983.  

† To whom correspondence should be addressed: Dept. of Information Physiology, National Institute for Physiological Sciences, Okazaki 444, Japan Tel.: 81-564-55-7855; Fax: 81-564-55-7853; E-mail: moriy@nips.ac.jp.

1 The abbreviations used are: PI, phosphatidylinositol; IP3, inositol 1,4,5-trisphosphate; IP4, inositol 1,3,4,5-tetraphosphate; CCE, capacitative Ca\(^{2+}\) entry; \(I_{\text{CRAC}}\), Ca\(^{2+}\) release-activated current; ER, endoplasmic reticulum; PCR, polymerase chain reaction; kb, kilobase pairs; RT, reverse transcriptase; HEK, human embryonic kidney; HBS, HEPES-buffered saline.
TRP proteins present some lines of supportive evidence for the hypothesis that TRP and its homologues except TRPL are CCE channels (20, 23–31). However, the hypothesis is still controversial (19, 32, 33). Achariya et al. (33) demonstrated that photoreceptors from Drosophila with homozygous loss-of-function mutation of IP3 receptors were indistinguishable from wild-type controls in sensitivity, kinetics, and adaptation of response to light. Furthermore, most significantly, low IP3 concentrations can induce substantial Ca2+-release from the stores without activating Ca2+-entry at all in rat leukemia cells, suggesting that even the activation of CCE is not that tightly coupled to Ca2+-release from the IP3-sensitive stores (12).

Thus, criteria of activation trigger, other than depletion of Ca2+-store, should be considered in functionally establishing cloned TRP channels to be correlated with native Ca2+-channels responsible for receptor-activated Ca2+-influx, including CCE.

We have here isolated cDNA that encodes a novel TRP homologue, TRP5, predominantly expressed in the brain. The recombinant expression of the TRP5 cDNA in human embryonic kidney (HEK) cells potentiated an extracellular Ca2+-dependent increase of [Ca2+]i, evoked by ATP, but not by an inhibitor of ER Ca2+-ATPases, thapsigargin. Whole-cell mode of patch-clamp recordings from TRP5-expressing cells demonstrated that ATP application induced a large inward current in the presence of extracellular Ca2+, which reversed at a positive potential. Our findings suggest that TRP5 directs the formation of a highly Ca2+-permeable ion channel that can be activated through receptor-operational pathways other than depletion of Ca2+ from Ca2+-stores in brain neurons.

**EXPERIMENTAL PROCEDURES**

cDNA Cloning and Sequence Determination—A mixture of oligo(dT)-primed cDNAs synthesized from the brain (BALB/c or 129/SvJ) brain poly(A+) RNA was subjected to PCR amplification using a Marathon cDNA amplification kit (CLONTECH). Degenerate oligonucleotide primers used were 5′-TGGGCGCTTCA/TATTGCTGAGAATCT/TA-CTGGAGA-3′ and 5′-G/TGATATCGA/GCCAA/CTTCCA/CTTC/CTC-3′. Obtained PCR products were subsequently subcloned into the T/A cloning plasmid, pCRII (Invitrogen, Carlsbad, CA), to yield pTRP15. Sequence comparison with a PCR product amplified using a pair of specific oligonucleotide primers T5–1 and T5–2 (see above), and the sequence in the clone 657-base pair into the 657-base pair of the 5′-GCATAGAATCGTCTTCG-3′ and the other 13 657-base pair of the 657-base pair of the 5′-ATGAACTTACAACTCAGGA-3′ and the other 13

**Reverse Transcriptase (RT)-PCR Amplification and Southern Blot Analysis—**Reverse transcription and PCR amplification from 1 μg of total RNA were performed using 37Th DNA polymerase (RT-PCR high Fidelity; Perkin Elmer, Norwalk, CT) in the presence of primer sets for TRP5 and cyclophycin were T5–1 and T5–2 (see above), and 5′-GCAGCCATGGTGAACCCCGAGG-3′ and 5′-GAAAATAGAGGTCGTTACCAGTG-3′ (GenBank™ accession no. X52803), respectively. The thermocycler was programmed to give an initial cycle consisting of 60 °C reverse transcription for 5 min and 94 °C denaturation for 2 min, followed by 40 cycles of 94 °C denaturation for 1 min and 55 °C annealing/extension for 1.5 min. The final cycle was followed by an additional extension at 52 °C for 7 min. To verify the identity of the PCR products, Southern blots were hybridized with 32P-end-labeled synthetic oligonucleotide probes 5′-ATGAACCTAAACATCCAGGA-3′ and 5′-CAGCATCACCGCGATGAGCCC-3′ for detection of TRP5 and cyclophycin mRNA, respectively. Hybridization was performed at 50 °C in 6× SSC, 50 mM sodium phosphate buffer (pH 7.0), 0.2% SDS, 0.1% polyvinylpyrrolidone, 0.1% Ficoll 400 (Amersham Pharmacia Biotech), 0.1% bovine serum albumin, and 0.2 mg/ml sonicated herring sperm DNA, as described previously (34).
Patch pipettes were made from borosilicate glass capillaries (1.5 mm, outer diameter; Hilgenberg, Malsfeld, Germany) using a model P-87 Flaming-Brown micropipette puller (Sutter Instrument, San Rafael, CA). Pipette resistance ranged from 2 to 4 megohms when filled with the pipette solution described below. Currents were sampled at 200 Hz after low-pass filtered at 1 kHz \((\text{2}^{3/2}\text{dB})\) using an 8-pole Bessel filter (900, Frequency Devices, Haverhill, MA) for Fig. 7 \((\text{A and B})\), sampled at 1 kHz for Fig. 7 \((\text{C})\), and analyzed with pCLAMP 6.02 software (Axon Instruments, Foster City, CA). The pipette solution for Fig. 7 contained (in mM): CsOH 105, aspartic acid 105, CsCl 40, MgCl\(_2\) 2, CaCl\(_2\) 3.2, EGTA 5, Na\(_2\)ATP 2, HEPES 5, adjusted to pH 7.2 with CsOH. Calculated free Ca\(^{2+}\) concentration was 200 nM. CaCl\(_2\) was 1.33 and 0.34 mM in the pipette solution containing calculated free Ca\(^{2+}\) concentration of 50 and 10 nM, respectively. The “0Ca\(^{2+}\)” external solution contained (in mM): NaCl 140, MgCl\(_2\) 1.2, CaCl\(_2\) 1.2, EGTA 10, glucose 10, HEPES 11.5, adjusted to pH 7.4 with NaOH (8 nM calculated free Ca\(^{2+}\)). The osmolarity of the external solutions was adjusted to about 325 mosM.

Rapid exchange of the external solutions was made by a modified “Y-tube” method (41).

**RESULTS**

**Primary Structure of TRP5**—Fig. 1A shows the amino acid sequence of the mouse brain TRP5 deduced from the open reading frame of the corresponding cDNA sequence. The translation initiation codon is assigned to the first in-frame methionine codon downstream of a stop codon. TRP5 is composed of 975 amino acid residues with a hydropathy profile revealing eight hydrophobic segments and hydrophilic N and C termini (Fig. 1B), similar to those of other TRP subtypes (21, 22, 24, 25, 42, 43). Sufficient length of hydrophobic regions to span the membrane, together with the lack of a hydrophobic N-terminal sequence indicative of the signal sequence, suggests that TRP5 is a membrane protein with a core of transmembrane segments and the flanking N- and C-terminal regions disposed on the
cytoplasmic side, like other TRP subtypes (21–25, 42, 43). Domains that form coiled-coil structure were predicted on each side of the hydrophobic core (Fig. 1A) (44). Potential cAMP- and cGMP-dependent protein kinase phosphorylation sites Ser212 and Thr167 are assigned to the putative cytoplasmic regions. Fig. 1C depicts the phylogenetic tree of the TRP family constructed by the neighbor-joining method (45), based on the sequence alignment carried out by the Clustalw program (46). Sequence identity/similarity between TRP5 and bCCE (25), a bovine counterpart of TRP4 (24), was relatively high (67/79%), compared with identities/similarities between TRP5 and other TRP homologues (36–46/57–66%). Homology of TRP5 with TRP homologues (36–46/57–66%). Homology of TRP5 with other TRP subtypes (21–25, 42, 43). Do-}

Southern blot hybridization using a TRP5-specific oligonucleotide probe, disclosed TRP5 expression not only in the brain regions, but also in liver, kidney, testis, and uterus (48). In addition to the main hybridizable PCR product of ~330 base pairs, which corresponds to the expected size, a second hybridizable product of ~250 base pairs was detected in the hindbrain region, liver, kidney, testis, and uterus, but not in the forebrain.

**Functional Characterization of TRP5: Cytosolic Ca2+ Measurements—**HEK293 cells are capable of serving as an excellent expression system for studying functional properties of TRP5 as a receptor-activated Ca2+ channel, inasmuch as they have been known to endogenously express the P2 purinoceptor coupled to activation of Gq protein and phospholipase C (49). HEK cells were also reported for the absence of endogenous TRP5 expression (48). TRP5, together with a marker protein CD8, was transiently expressed in HEK cells, and intracellular Ca2+ concentration was monitored in transfectants and nontransfected control HEK cells using fura-2 as an indicator. In the presence of 2 mM extracellular Ca2+, application of 100 μM ATP to control cells induced a rapid rise in [Ca2+]i, that peaked within 30 s and gradually decreased to the resting level within 300 s (Fig. 3A). This transient rise in [Ca2+]i was presumed to be mainly due to release from the intracellular Ca2+ store, because omission of extracellular Ca2+ little affected on the peak level (Fig. 4A), whereas the decay phase was accelerated in the absence of extracellular Ca2+. When 100 μM ATP was applied to TRP5-transfected, CD8-positive cells in the presence of extracellular Ca2+, the peak [Ca2+]i level greatly increased (Fig. 3B). In TRP5-expressing cells, Ca2+ influx across the plasma membrane was likely to be a major cause of the [Ca2+]i,

![Image 323x496 to 539x729](https://example.com/image-url)

**Fig. 3.** ATP-induced [Ca2+]i transients in control and TRP5-transfected HEK cells in the presence of extracellular Ca2+. Cytosolic Ca2+ was measured in fura-2-loaded control HEK293 cells (A) or HEK293 cells transfected with TRP5 plus CD8 (B). The cells were treated with 100 μM ATP in the presence of 2 mM extracellular Ca2+. The duration of exposure to Ca2+-containing HBS and 100 μM ATP is indicated by the filled and hatched bars, respectively, above the graphs. C, dose-response relationships for maximum ATP-induced [Ca2+]i rises (∆[Ca2+]i) in individual control HEK293 cells (filled box) or HEK293 cells transfected with TRP5 plus CD8 cDNAs (filled circle) in the presence of 2 mM extracellular Ca2+, and in TRP5-transfected cells in the absence of extracellular Ca2+ (open circle). Data points are the means ± S.E. [Ca2+]i, (A and B) or the means ± S.E. ∆[Ca2+]i. (C) in 30–41 control HEK cells or 14–20 TRP5-transfected cells.
rise, because the amplitude of [Ca$^{2+}$]$_{i}$ rise was much smaller in the absence of extracellular Ca$^{2+}$ than that in the presence of extracellular Ca$^{2+}$ at ATP concentrations above 1 µM (Fig. 3C), and remained almost the same as that of [Ca$^{2+}$]$_{i}$ transient evoked in control cells in the absence of extracellular Ca$^{2+}$ (Fig. 4, A and B). [Ca$^{2+}$]$_{i}$ rise evoked by ATP in TRP5-expressing cells in the presence and absence of extracellular Ca$^{2+}$, and in control cells in the presence of extracellular Ca$^{2+}$, increased in a similar dose-dependent manner (Fig. 3C).

To separate contribution to [Ca$^{2+}$]$_{i}$ rise of Ca$^{2+}$ influx from that of Ca$^{2+}$ release, ATP was first applied in the absence of extracellular Ca$^{2+}$, and 2 mM Ca$^{2+}$ was then added to the extracellular solution when [Ca$^{2+}$]$_{i}$ returned to the resting level (3 min after addition of ATP). Addition of Ca$^{2+}$ to the extracellular solution only slightly raised [Ca$^{2+}$]$_{i}$ above the resting level in control cells (Fig. 4, A and C), whereas in TRP5-transfected cells, it elicited dramatic [Ca$^{2+}$]$_{i}$ transients (Fig. 4, B and C), which reached maximum in the presence of ATP ≥ 10 µM (Fig. 4C). The second [Ca$^{2+}$]$_{i}$ rise evoked by extracellular Ca$^{2+}$ did not seem to correlate with the preceding first [Ca$^{2+}$]$_{i}$ rise caused by ATP-dependent Ca$^{2+}$ release from the intracellular Ca$^{2+}$ store. The second [Ca$^{2+}$]$_{i}$ rise was 613 ± 77 (mean ± S.E.) nM for the TRP5-expressing cells that showed the first rise staying below 10 nM (n = 16), and was not significantly different from that (452 ± 40 nM) observed in the cells where the first rise was above 10 nM (n = 48). The time lag between start of ATP stimulation and addition of extracellular Ca$^{2+}$ did not significantly affect amplitude of [Ca$^{2+}$]$_{i}$, rise up to 5 min (Fig. 4D). Interestingly, after 3 min of stimulation by ATP in Ca$^{2+}$-free solution, thapsigargin induced intact [Ca$^{2+}$]$_{i}$ rise in untransfected cells (102 ± 4 nM, n = 53), as compared with control cells without ATP stimulation (113 ± 5 nM, n = 51) (see below). Furthermore, after initial application of ATP for 3 min and subsequent omission of ATP up to 5 min in Ca$^{2+}$-free solution, untransfected cells did not show significant [Ca$^{2+}$]$_{i}$ rise induced by the second application of ATP (n = 38). These results suggest that ATP receptors are rapidly desensitized by incubating with 100 µM ATP, and thereby internal stores are replenished with Ca$^{2+}$ within 3 min. Without ATP, [Ca$^{2+}$]$_{i}$ rise was not observed in TRP5-transfected cells that were immersed in the Ca$^{2+}$-containing solution after preincubation in the Ca$^{2+}$-free solution for up to 7 min (data not shown).

Lanthanides La$^{3+}$ and Gd$^{3+}$ were reported to block currents induced by recombinant expression of Drosophila TRP, TRPL, human TRP1, TRP3 (23, 24, 50), and native Ca$^{2+}$ channels (51–53) and I$_{CRAC}$ (54). The imidazol derivative, SK&F96365, inhibits various types of ion channels including receptor-activated channels (55, 56). In Fig. 5, 100 µM ATP was added alone (Fig. 5A) or together with one of the agents (25 µM SK&F96365 (Fig. 5B) or 100 µM LaCl$_{3}$ (Fig. 5C)) to the Ca$^{2+}$-free extracellular solution, and 2 mM Ca$^{2+}$ was added 3 min later. As shown in Fig. 5 (B–D), 25 µM SK&F96365 and 100 µM LaCl$_{3}$ signifi-
Brain TRP Ca\(^{2+}\) Channel

**Fig. 6.** Thapsigargin-induced [Ca\(^{2+}\)] transient and ATP-induced [Ca\(^{2+}\)] changes after store depletion in control and TRP5-transfected HEK cells. Cytosolic Ca\(^{2+}\) was measured in fura-2-loaded control HEK293 cells (A and C) or HEK293 cells transfected with TRP5 plus CD8 (B and D). In A and B, the perfusion solution was changed to Ca\(^{2+}\)-free HBS containing 0.5 mM EGTA, and 2 \(\mu\)M thapsigargin (TG) was applied to the cells in the absence of extracellular Ca\(^{2+}\), which was followed by the addition of 2 mM extracellular Ca\(^{2+}\). In C and D, the cells were treated with 2 \(\mu\)M thapsigargin in the presence of extracellular Ca\(^{2+}\), then thapsigargin was replaced with 100 \(\mu\)M ATP. The duration of exposure to Ca\(^{2+}\)-containing HBS, Ca\(^{2+}\)-free HBS, 100 \(\mu\)M ATP, and 2 \(\mu\)M thapsigargin is indicated by the filled, open, hatched, and shaded bars, respectively, above the graphs. Data points are the means ± S.E. of [Ca\(^{2+}\)], in the indicated number of cells.

![Graph A](image1)

![Graph B](image2)

![Graph C](image3)

![Graph D](image4)

![Graph E](image5)

**Fig. 7.** Electrophysiological characterization of the TRP5 channel. A, shown is a time course of ionic current recorded from a control HEK293 cell at a holding potential of −50 mV. During application of ATP (indicated by the hatched bar above the current) to the control HEK293 cell, the external solutions were changed from the 0Ca\(^{2+}\) solution (open bar) to the 10Ca\(^{2+}\) solution (filled bar). Finally, ATP was washed out with the 0Ca\(^{2+}\) solution. In B, a time course of ionic current recorded from a HEK293 cell transfected with TRP5 plus CD8 is shown. In C, current-voltage relationships of the TRP5 channel are shown. Currents were evoked by 1.5-s negative voltage ramps from 40 to −70 mV. Five consecutive ramps were applied every 5 s in the 0Ca\(^{2+}\) solution or the 10Ca\(^{2+}\) solution with 100 \(\mu\)M ATP. The averaged currents were drawn. The currents shown in B and C were recorded from different TRP5-transfected cells.
the inward current was abolished quickly upon the removal of Ca\textsuperscript{2+}, although the fluctuation of currents remained until ATP was washed out. In the control cells (n = 7), 100 \mu M ATP did not induce significant ionic currents regardless of the presence of 10 mM Ca\textsuperscript{2+} in extracellular solution (Fig. 7A). When Ca\textsuperscript{2+} concentration in pipette solution was reduced to 50 nM, similar proportion of the CD8-positive, TRP5-transfected cells (11 out of 12 cells) showed responsiveness to ATP. However, usage of 10 mM free Ca\textsuperscript{2+} in intrapipette solution resulted in a slightly reduced number of the CD8-positive, TRP5-transfected HEK cells responsive to ATP, inducing inward currents in six out of nine cells. The CD8-positive, TRP5-transfected HEK cells measured using the EGTA-containing, Ca\textsuperscript{2+}-free pipette solution were not responsive to ATP (n = 5).

Current-voltage relationship of ionic current triggered by ATP in TRP5-expressing cells was examined using negative voltage ramps from 40 to -70 mV for 1.5 s. Five consecutive voltage ramps were applied. The averages of current traces generated by five consecutive ramps every 5 s in the 0Ca\textsuperscript{2+} external solution and the 10Ca\textsuperscript{2+} external solution with 100 \mu M ATP were drawn in Fig. 7C. The current-voltage relationship recorded in 10Ca\textsuperscript{2+} was nonlinear, showing a significant inward current at physiological potentials. Permeability ratios among Na\textsuperscript{+}, Ca\textsuperscript{2+}, and Ca\textsuperscript{2+} were estimated on the basis of the Goldman-Hodgkin-Katz equation using the reversal potentials in Fig. 7C. On the assumption that activity coefficients are 0.3 for Ca\textsuperscript{2+} and 0.75 for both Na\textsuperscript{+} and Ca\textsuperscript{2+}, the reversal potentials of 8 mV in the 0Ca\textsuperscript{2+} external solution and 17 mV in the 10Ca\textsuperscript{2+} solution lead to permeability ratios $P_{\text{Ca}}/P_{\text{Na}}P_{\text{Ca}} = 14.3:1:5.1$.

**DISCUSSION**

**Activation Mechanism of TRP5 Essential for Receptor-activated Ca\textsuperscript{2+} Influx**—In the present investigation, we have cloned and functionally characterized the mouse TRP homologue, designated as TRP5, predominantly expressed in the brain. Recombinant expression of the TRP5 cDNA in HEK cells potentiated transient increases in [Ca\textsuperscript{2+}], evoked by ATP in the presence of extracellular Ca\textsuperscript{2+} (Fig. 3, A and B). When Ca\textsuperscript{2+} was added to the extracellular solution after preincubating the cells in the Ca\textsuperscript{2+}-free solution under constant ATP stimulation, potentiation of transient [Ca\textsuperscript{2+}], rise induced by TRP5 expression became more prominent (Fig. 4, A and B). In this experiment, the second [Ca\textsuperscript{2+}], rise due to Ca\textsuperscript{2+} influx showed no significant correlation with the first [Ca\textsuperscript{2+}], rise due to Ca\textsuperscript{2+} release from IP\textsubscript{3}-sensitive internal stores in the absence of extracellular Ca\textsuperscript{2+}. In an extreme case, [Ca\textsuperscript{2+}], rise through Ca\textsuperscript{2+} influx was induced in a TRP5-expressing cell where Ca\textsuperscript{2+} release was hardly detectable. When thapsigargin was substituted for ATP to deplete the internal Ca\textsuperscript{2+} store by inhibiting ER Ca\textsuperscript{2+}-ATPases, the second [Ca\textsuperscript{2+}], rise due to CCE was not potentiated by TRP5 expression (Fig. 6, A and B). These results indicate that TRP5 is responsible for Ca\textsuperscript{2+} influx activated by ATP via mechanisms other than Ca\textsuperscript{2+} depletion from the internal Ca\textsuperscript{2+} store.

The independence of TRP5-activating cascades from depletion of the internal Ca\textsuperscript{2+} stores is confirmed by additional lines of experimental evidence. In the presence of extracellular Ca\textsuperscript{2+}, after the thapsigargin-induced [Ca\textsuperscript{2+}], transient decayed to a plateau level, another [Ca\textsuperscript{2+}], rise was induced in TRP5-expressing cells by ATP (Fig. 6D), which did not induce [Ca\textsuperscript{2+}], transients and even elicited slight decreases of [Ca\textsuperscript{2+}], in control cells (Fig. 6C). The lack of ATP-induced Ca\textsuperscript{2+} release from ER in control cells after thapsigargin treatment (Fig. 6C) indicates that the ATP-sensitive stores are included in the thapsigargin-sensitive stores, excluding the possibility that TRP5 is activated via depletion of the ATP-sensitive stores independent of the thapsigargin-sensitive stores. It is also unlikely from this finding that TRP5 activation is directly coupled with the Ca\textsuperscript{2+}-releasing process involving the IP\textsubscript{3} receptors. Furthermore, our results indicate that after 3 min of stimulation by ATP in Ca\textsuperscript{2+}-free solution, TRP5 channels are still activable (Fig. 4B), but endogenous receptor-activated channels including CCE channels, whose activation by thapsigargin is clearly seen in Fig. 6A, are dormant in HEK cells (Fig. 4A). This is presumably due to differences between the TRP5 channel and endogenous channels in susceptibility to effects of ATP receptor desensitization. Desensitization of ATP receptors within 3 min is suggested from our experimental observation that in Ca\textsuperscript{2+}-free solution, [Ca\textsuperscript{2+}], transient was not any more induced by ATP after initial application of ATP for 3 min and subsequent washing out of ATP for 5 min. Rapid desensitization of ATP receptors, compared with other types of receptors, was also reported by other groups (49). After a 3-min application of ATP in Ca\textsuperscript{2+}-free solution, [Ca\textsuperscript{2+}], rise induced by subsequent application of thapsigargin was intact, suggesting that internal stores were rapidly replenished with Ca\textsuperscript{2+}. Thus, after desensitization of ATP receptors and replenishment of Ca\textsuperscript{2+} stores, activation signals for TRP5 channel still persist, whereas the activation trigger for CCE is already abolished.

From our experiments, some insights can be gained into the activation mechanism for TRP5. Present data imply an important role of Ca\textsuperscript{2+} in activation of TRP5. Whole-cell inward currents in ATP-stimulated, TRP5-transfected cells measured using the pipette solution containing free Ca\textsuperscript{2+} exhibited rapid and dramatic increases upon addition of Ca\textsuperscript{2+} to the extracellular solution (Fig. 7B), whereas those that were measured using the EGTA-containing, Ca\textsuperscript{2+}-free pipette solution were not responsive to ATP. [Ca\textsuperscript{2+}], could be lowered to 10 nM, which is considerably lower than physiological [Ca\textsuperscript{2+}], to elicit TRP5 current in HEK cells, whereas higher percentage of the TRP5 current-positive cells was obtained when [Ca\textsuperscript{2+}], was elevated to 50 nM and 200 nM. This observation suggests that TRP5 is activable in the physiological range of [Ca\textsuperscript{2+}], even at basal levels. Ca\textsuperscript{2+} may act through Ca\textsuperscript{2+}-binding proteins such as calmodulin and Ca\textsuperscript{2+}-dependent enzymes, although it is yet preliminary to make any conclusion with regard to the Ca\textsuperscript{2+} effect.

In thapsigargin-treated, TRP5-expressing cells, ATP primed the activity of TRP5 presumably not through [Ca\textsuperscript{2+}], elevation (Fig. 6D), given that the action of ATP on [Ca\textsuperscript{2+}], was toward decrease from slightly elevated levels in thapsigargin-treated control cells (Fig. 6C). This excludes the possibility that Ca\textsuperscript{2+} is a sole activation trigger for TRP5, strongly suggesting involvement of other factors in TRP5 activation. It is possible that slight decrease from the elevated level (in the presence of thapsigargin) optimizes [Ca\textsuperscript{2+}], in the range that activates but does not inactivate TRP5. Activation of G\textsubscript{q} protein, phospholipase C-\(\beta\), and protein kinase C, and production of phosphoinositide metabolites such as IP\textsubscript{3} and IP\textsubscript{4}, which are all triggered by stimulation of ATP receptors, should be considered as candidate activators of TRP5.

The results obtained are also indicative of a role of Ca\textsuperscript{2+} as a negative regulator for TRP5. In the presence of extracellular Ca\textsuperscript{2+}, [Ca\textsuperscript{2+}], transients induced by ATP stimulation decreased almost to the basal level (Fig. 3B) at the time when the second [Ca\textsuperscript{2+}], rise induced by Ca\textsuperscript{2+} addition with time lag of 3 min after ATP stimulation reached peak (Fig. 4B). Negative regulatory action of Ca\textsuperscript{2+} has been reported for Drosophila TRP, TRPL (30), and CCE in Xenopus oocytes (58).

Human TRP3 has been reported to form a nonselective cation channel that is not sensitive to Ca\textsuperscript{2+} store depletion (59, 60). Specifically, Zitt et al. (59) have shown that Ca\textsuperscript{2+} neither
act alone or act together with calmodulin directly on the TRP3 protein to activate the channel. Since the submission of the first version of this manuscript, we have learned that mouse TRP6 encodes a nonselective cation channel stimulated by the muscarinic M5 receptor, but not by intracellular store depletion (61). It is therefore possible that the Ca\(^{2+}\)-selective TRP5 channels are activated via common activation mechanisms that operate in opening the TRP3 channel and/or the TRP6 channel.

Functional Correlation of TRP Homologues and Native Receptor-activated Ca\(^{2+}\) Channels—Functional correspondence between cloned TRP homologues and Ca\(^{2+}\) channels responsible for receptor-activated Ca\(^{2+}\) influx, including CCE, in native preparations is still very controversial. Ca\(^{2+}\) selectivity in permeation has been one of the important criteria in correlating recombinant TRP homologues with native Ca\(^{2+}\) channels (32). Our whole-cell current measurements using patch pipettes filled with the solution containing 200 mM free Ca\(^{2+}\) demonstrated that rapid exchange of the external 0Ca\(^{2+}\) solution with the 10Ca\(^{2+}\) solution elicited instantaneous and dramatic increases of inward TRP5 currents (Fig. 7 B), which reversed at positive potentials (Fig. 7 C). This, together with the permeability ratios \(P_{\text{Ca}^{2+}} / P_{\text{Na}^{+}} / P_{\text{Ba}^{2+}} = 14.3:1.5:1\) calculated from the reversal potentials, indicates that TRP5 is selective for Ca\(^{2+}\) over monovalent cations. Of the other recombinantly expressed TRP homologues, Drosophila TRP and mammalian TRP4 were demonstrated for Ca\(^{2+}\) selectivity (25, 26, 30), whereas Drosophila TRPL and mammalian TRP1 and TRP3 were rather classified as nonselective cation channels (23, 27, 30, 59, 62). In the native systems, the TRP and TRPL components of light-activated current isolated through usage of trpl and trp mutant photoreceptors showed ion selectivity comparable with those of the recombinant TRP and TRPL (19, 63). Among the receptor-activated Ca\(^{2+}\) channels in vertebrate cells, known for diversity in ion permeation properties (3), some display ion selectivity that may correspond well to TRP homologues. However, establishing functional correlation of TRP with native receptor-activated Ca\(^{2+}\) channels becomes considerably unsuccessful by introduction of activation trigger as a second distinguishing criterion. Although \(I_{\text{Ca}^{2+}}\) in TRP5 is similar to TRP5 in selectivity for Ca\(^{2+}\) over Na\(^{+}\), Ba\(^{2+}\), and Mn\(^{2+}\) (64), depletion of the intracellular Ca\(^{2+}\) store activates \(I_{\text{Ca}^{2+}}\) and the nonselective cation channels TRP1 (23) and TRP3 (62), but not Ca\(^{2+}\)-selective TRP5. In contrast to TRP5, IP\(_3\) and Ca\(^{2+}\)-sensitive channels in endothelial cells are highly permeable not only to Ca\(^{2+}\), but also to other divalent cations such as Ba\(^{2+}\) and Mn\(^{2+}\) (16). It has been also reported that Ba\(^{2+}\) or monovalent cations are as permeant as Ca\(^{2+}\) in other receptor-activated Ca\(^{2+}\) channels triggered by second messengers such as IP\(_3\) or by activation of G-proteins (3). Thus, each vertebrate TRP homologue expressed in heterologous systems does not really correspond to the native receptor-activated Ca\(^{2+}\) channels in both the two functional criteria: activation trigger and Ca\(^{2+}\) selectivity.

Heteromultimer formation by multiple TRP isoforms (30) may be necessary to elicit native type receptor-activated Ca\(^{2+}\) entry. In our expression studies of TRP5, there was no clear functional indication for presence of heterogeneous populations of heteromultimer and homomultimer, although Garcia and Schilling (48) have shown expression of TRP1, TRP3, TRP4, and TRP6 mRNAs in HEK 293 cells. This may derive from the usage of ATP receptor stimulation in activating Ca\(^{2+}\) entry, or low mRNA expression levels of endogenous TRP isoforms compared with the level of TRP5 overexpression. It would be necessary to characterize functional properties of neuronal recep-
Brain TRP Ca\textsuperscript{2+} Channel

49. Bischof, G., Serwold, T. F., and Machen, T. E. (1997) Cell Calcium 21, 135–142
50. Sinkins, W. G., Vaca, L., Hu, Y., Kunze, D. L., and Schilling, W. P. (1996) J. Biol. Chem. 271, 2955–2960
51. Boland, L. M., Brown, T. A., and Dingledine, R. (1991) Brain Res. 563, 142–150
52. Lacampagne, A., Gannier, F., Argibay, J., Garnier, D., and Le Guennec, J. Y. (1994) Biochim. Biophys. Acta 1191, 205–208
53. Chang, W., Chen, T. H., Gardner, P., and Shoback, D. (1995) Am. J. Physiol. 269, E864–E877
54. Hash, M. and Penner, R. (1993) J. Physiol. 465, 359–386
55. Clementi, E., and Meldolesi, J. (1996) Cell Calcium 19, 269–279
56. Waldron, R. T., Short, A. D., and Gill, D. L. (1997) J. Biol. Chem. 272, 6440–6447
57. Inesi, G., and Sagara, Y. (1994) J. Membr. Biol. 141, 1–6
58. Petersen, C. C. H., and Berridge, M. J. (1994) J. Biol. Chem. 269, 32246–32253
59. Zitt, C., Obukhov, A. G., Strubing, C., Zobel, A., Kalbbrunner, F., Luckhoff, A., and Schultz, G. (1997) J. Cell Biol. 138, 1333–1341
60. Zhu, X., Jiang, M., Birnbaumer, L. (1998) J. Biol. Chem. 273, 133–142
61. Boulay, G., Zhu, X., Peyton, M., Jiang, M., Hurst, R., Stefani, E., and Birnbaumer, L. (1997) J. Biol. Chem. 272, 29672–29680
62. Birnbaumer, L., Zhu, X., Jiang, M., Boulay, G., Peyton, M., Vannier, B., Brown, D., Platano, D., Sadeghi, H., Stefani, E., and Birnbaumer, M. (1996) Proc. Natl. Acad. Sci. U. S. A. 93, 15195–15202
63. Hardie, R. C., and Minke, B. (1992) Neuron 8, 643–651
64. Hoth, M., and Penner, R. (1992) Nature 353, 353–356
65. Funayama, M., Goto, K., and Kondo, H. (1996) Mol. Brain Res. 43, 259–266
66. Kyte, J., and Doolittle, R. F. (1982) J. Mol. Biol. 157, 105–132
67. Sulston, J., Du, Z., Thomas, K., Wilson, R., Hillier, L., Staden, R., Halloran, N., Green, P., Thierry-Mieg, J., Qiu, L., Dear, S., Coulson, A., Craxton, M., Durbin, R., Berks, M., Metzstein, M., Hawkins, T., Ainscough, R., and Waterston, R. (1992) Nature 356, 47–48
68. Sakura, H., and Ashcroft, F. M. (1997) Diabetologia 40, 528–532