The Transient Receptor Potential Protein Homologue TRP6 is the Essential Component of Vascular α₁-Adrenoceptor–Activated Ca²⁺-Permeable Cation Channel

Ryuji Inoue, Takaharu Okada, Hitoshi Onoue, Yuji Hara, Shunichi Shimizu, Shinji Naitoh, Yushi Ito, Yasuo Mori

Abstract—The Drosophila transient receptor potential protein (TRP) and its mammalian homologues are thought to be Ca²⁺-permeable cation channels activated by G protein (Gq/11)–coupled receptors and are regarded as an interesting molecular model for the Ca²⁺ entry mechanisms associated with stimulated phosphoinositide turnover and store depletion. However, there is little unequivocal evidence linking mammalian TRPs with particular native functions. In this study, we have found that heterologous expression of murine TRP6 in HEK293 cells reproduces almost exactly the essential biophysical and pharmacological properties of α₁-adrenoceptor–activated nonselective cation channels (α₁-AR–NSCC) previously identified in rabbit portal vein smooth muscle. Such properties include activation by diacylglycerol; S-shaped current-voltage relationship; high divalent cation permeability; unitary conductance of 25 to 30 pS and augmentation by flufenamate and Ca²⁺; and blockade by Cd²⁺, La³⁺, Gd³⁺, SK&F96365, and amiloride. Reverse transcriptase–polymerase chain reaction and confocal laser scanning microscopy using TRP6-specific primers and antisera revealed that the level of TRP6 mRNA expression was remarkably high in both murine and rabbit portal vein smooth muscles as compared with other TRP subtypes, and the immunoreactivity to TRP6 protein was localized near the sarcoclemmal region of single rabbit portal vein myocytes. Furthermore, treatment of primary cultured portal vein myocytes with TRP6 antisense oligonucleotides resulted in marked inhibition of TRP6 protein immunoreactivity as well as selective suppression of α₁-adrenoceptor–activated, store depletion–independent cation current and Ba²⁺ influx. These results strongly indicate that TRP6 is the essential component of the α₁-AR–NSCC, which may serve as a store depletion–independent Ca²⁺ entry pathway during increased sympathetic activity. (Circ Res. 2001;88:325-332.)

Key Words: receptor-operated Ca²⁺ channel ■ transient receptor potential protein ■ α₁-adrenoceptor

The α₁-adrenoceptor (α₁-AR) is distributed widely in the vascular system and plays a central role in control of systemic blood pressure via sympathetic nerves. Stimulation of the α₁-AR leads to activation of G protein (Gq/11)–coupled phospholipase Cβ (PLCβ), which catalyzes formation from phosphoinositide of 2 major metabolites, inositol 1,4,5-triphosphate (IP₃) and diacylglycerol (DAG), thereby causing a release of stored Ca²⁺ and an accompanying sustained Ca²⁺ entry.¹ The α₁-AR–activated nonselective cation channel (α₁-AR–NSCC) is thought to contribute to this Ca²⁺ entry in both direct and indirect ways, since it is activated by DAG and allows preferential movement of divergent cations and secondarily evokes Ca²⁺ entry through the voltage-dependent pathway by depolarizing the membrane.²–⁴ Despite this potential physiological importance, no clues elucidating the molecular entity of α₁-AR–NSCC have been obtained so far.

The transient receptor potential (trp) gene and its closest relative trp1 (trp-like) were originally identified in investigation of abnormal visual transduction of Drosophila melanogaster and were later shown to encode Ca²⁺-entry channels that open during activation of the rhodopsin/G protein/PLC/IP₃, signaling cascade.⁵ Subsequently, their 7 mammalian homologous genes (trp1 to trp7) have been cloned, in the hope of elucidating the molecular counterparts of native receptor-operated Ca²⁺ entry channels (ROCCs) in mammals (including those activated by store depletion; G protein; or second messengers such as IP₃, DAG, arachidonic acid, and Ca²⁺) on stimulation of G protein–coupled receptors (GPCRs) or tyrosine kinase–coupled receptors (RTKs).⁶–¹¹ Although functional expression of these mammalian trp-encoding proteins (TRP homologues) in the heterologous system demonstrated the appearance of phosphoinositide

© 2001 American Heart Association, Inc.

Circulation Research is available at http://www.circresaha.org
turnover–linked Ca\(^{2+}\)–permeable cation conductance (channel activity or Ca\(^{2+}\) fluorescence increase), it remains unclear how they correspond to particular ROCCs in the native system, except for some studies implicating TRP1, TRP3, TRP4, and TRP5 in store-operated or capacitative Ca\(^{2+}\) entry.\(^{6,10,12,13}\)

In this study, we have obtained the first clear evidence that a mammalian TRP homologue, TRP6,\(^{14}\) the human isoform of which was previously shown to act as a DAG-activatable cation channel rather than the store depletion–operated Ca\(^{2+}\) channel (SOC),\(^{10,15}\) is likely to be the molecular identification of the \(\alpha_1\) AR–NSCC that has also been reported to be activated by DAG in rabbit portal vein smooth muscle.\(^{16}\) To examine this, we made a detailed comparison between recombinantly expressed TRP6 protein and the \(\alpha_1\) AR–NSCC using molecular and electrophysiological techniques, and examined the expression of TRP6 mRNA and the functional significance of TRP6 proteins in portal vein smooth muscle, employing the reverse transcriptase–polymerase chain reaction (RT-PCR), immunocytochemistry and antisense strategy.

**Materials and Methods**

**Recombinant Expression, Electrophysiology, and Fluorescence Measurements**

HEK293 cells were transfected with one of the recombinant plasmids pCI-neo-mTRP6, -mTRP3, or -mTRP7\(^{7,18}\) and were used for electrophysiological experiments within 48 to 72 hours. For antisense experiments, myocytes enzymatically dissociated from the rabbit portal vein smooth muscle\(^{19}\) were maintained in a short-term culture (3 to 4 days) in a laminin (20 \(\mu\)g/mL)-coated dish containing DMEM supplemented with 2% FBS plus antibiotics and TRP antisense or sense oligonucleotides (5 \(\mu\)mol/L) (Table 1). Cells were then reseeded on coverslips and used within 12 to 24 hours for electrophysiological and fluorescent measurements.

Whole-cell and single-channel current recordings and data analyses were performed as described elsewhere.\(^{18,19}\) Test solutions were topically applied using the so-called “Y-tube” fast solution exchange device. Bath solution contained (in mmol/L) Na\(^+\) 135, K\(^+\) 5, Mg\(^{2+}\) 1.2, Ca\(^{2+}\) 2, Cl\(^-\) 151.4, glucose 5, and HEPES 5. \(\alpha_1\) internal solution for whole-cell recording contained (in mmol/L) Cs\(^+\) 140, Mg\(^{2+}\) 2, Cl\(^-\) 24, aspartate 120, Na\(_{ATP}\) 2, phosphocreatine 5, BAPTA 10, Ca\(^{2+}\) 4, glucose 10, and HEPES 10. In the experiments shown in Figures 3A and 3B, 1 mmol/L EGTA instead of 10 mmol/L BAPTA/4 mmol/L Ca\(^{2+}\) was added in this solution. Pipette solution for cell-attached recording contained (in mmol/L) Na\(^+\) 140, \(\alpha_1\) \(\mathrm{Ca}^{2+}\) 1, Mg\(^{2+}\) 1.2, tetraethylammonium 10, Cl\(^-\) 154.4, glucose 5, HEPES 10, and 100 \(\mu\)mol/L ATP or carboc Cheryl (CCh).

Ba\(^{2+}\) fluorescence was measured using a dual-excitation wavelength spectrophotometer (CAM 230, Nihon Bunko). Fura-2–loaded cells (incubated with 2 \(\mu\)mol/L, fura-2–acetoxymethyl ester for 30 to 45 minutes) were alternately illuminated by UV lights (340 and 380 nm, 100 Hz), and the emitted fluorescence was collected after filtering at 510 nm (±30 nm). The extent of Ba\(^{2+}\) influx was assessed as the ratio of fluorescence intensity at 340 and 380 nm excitation. All experiments were performed at 24°C to 26°C.

All data are expressed as mean±SEM. Student t test and 1-way ANOVA were used for single- and multiple-comparison statistical analyses, respectively.

**RT-PCR**

Total RNA was extracted from the whole rabbit and murine portal veins or isolated smooth muscles, and first-strand cDNA generated from 1 \(\mu\)g of total RNA was subjected to PCR amplification using TRP homologue-specific primers. The PCR protocol was as follows: 10 cycles of 30 seconds at 94°C, 30 seconds at 95°C, 30 seconds at 64°C, and 1 minute at 68°C. Followed by 30 cycles of 30 seconds at 94°C, 30 seconds at 60°C, and 1 minute at 68°C. PCR products were identified by hybridization with \(\beta\)-P-5'-end-labeled synthetic oligonucleotide probes. For the PCR primers and oligonucleotide probes used, see the supplementary information (available at http://www.circresaha.org).

**Immunocytochemistry**

Anti-mouse TRP6 rabbit antiserum was raised against the C-terminal sequence, LIRKLGERLSLEPKLEESRR, and used for immuno-staining of portal vein myocytes adherent on coverslips. The protocol used was the following: fixation in 4% paraformaldehyde, 20 minutes; permeabilization in 0.2% Triton/PBS, 15 minutes; preincubation in 10% normal goat serum/PBS, 1 hour; incubation in FITC-labeled anti-rabbit goat antiserum, 1 hour. Immunostained cells were observed under a confocal laser scanning microscope (LSM 510, Zeiss; krypton/argon; excitation, 488 nm; emission, 505 nm) with an optical section of 0.8 to 1.1 \(\mu\)m.

An expanded Materials and Methods section can be found in an online data supplement available at http://www.circresaha.org.

**Results and Discussion**

**Murine TRP6 Is a DAG-Activated Channel**

Human embryonic kidney 293 (HEK293) cells transiently expressing murine TRP6 protein (mTRP6) exhibited virtually no spontaneous currents under normal conditions (current density at \(-60\) mV, 0.15±0.1 pA/pF; \(n=31\)). Exogenously applied ATP and CCh dose-dependently produced inward currents in these cells (ED\(_{50}\), 4.5 and 9.1 \(\mu\)mol/L, respectively; \(n=5\) to 6), whereas no discernible currents were activated by these agonists in control cells transfected with the empty vector (15 of 15 cells). The ATP and CCh-induced inward currents in mTRP6-expressing cells (hereafter designated as mTRP6 currents) were strongly inhibited by pretreatment with suramin (100 \(\mu\)mol/L; by 93±3%, \(n=5\)) and atropine (1 \(\mu\)mol/L; by 91±5%, \(n=5\)), respectively, thus suggesting that the endogenous P\(_{2Y}\) and muscarinic receptors in HEK293 cells mediate their activation.

It has recently been reported that the human TRP6 channels expressed in CHO-K1 cells with the G\(_{q/11}\)-coupled H1 histamine receptor are activated by DAG through a mechanism independent of protein kinase C (PKC).\(^{15}\) We therefore tested whether this applies to mTRP6 recombinantly expressed in HEK293 cells at the whole-cell current level. As summarized in Figure 1A, (1) significant suppression of mTRP6 current activation occurred in the presence of the PLC inhibitor U73122 (10 \(\mu\)mol/L) and with intracellular perfusion of GDP\(_{BS}\) (100 \(\mu\)mol/L), but not by the PKC inhibitor calphostin C (1 \(\mu\)mol/L) or intracellular perfusion of...
Figure 1. Activation profile, voltage-dependent gating, and ionic selectivity of mTRP6. A, mTRP6 current density at −60 mV. Results are shown for (in μmol/L) ATP 100, CCh 100, OAG (left inset) 100, RH80267 (RHC) 100, U73122 10, calphostin C (calcC) 1, thapsigargin (TG) 2, with CCh (GTP, heparin, or GDPβS), GTP 100 μmol/L, heparin 1 mg/mL, or GDPβS 300 μmol/L included in the pipette. Right inset, GTPγS (100 μmol/L) was included in the pipette. Numbers of experiments are given in parentheses. *P<0.05, ANOVA and pooled-variance f test. B and C, I-V relationships of mTRP6 under 2 biionic conditions and that of α1-AR–NSCC from the rabbit portal vein (RPV) under conventional whole-cell clamp. D, mTRP6 channel activities at 4 different potentials from the same cell-attached patch. Resting membrane potential of HEK293 cell was almost nulled by excess K+ (128 mM) in the bath. E and F, open probability vs voltage relationships of single mTRP6 channel (n=11 to 12). Solid line indicates best linear fit of data points; mp, membrane potential; and Vp, pipette potential.

The activation profile of mTRP6 described above is strongly reminiscent of a native second messenger–activated cation channel, ie, the α1-AR–NSCC, in the rabbit portal vein smooth muscle.15,16 Thus, to determine a possible molecular correspondence between TRP6 and the native α1-AR–NSCC, we made a detailed comparison of their biophysical and pharmacological properties in terms of patch-clamp technique (see below).

mTRP6 Shows a Voltage Dependence Similar to That of α1-AR–NSCC

The current-voltage (I-V) relationship of mTRP6 showed a marked voltage-dependent inhibition at strongly negative potentials (−40 mV; Figure 1B). This inhibition, which is also observed for mTRP520 but not for other TRP subtypes, is unlikely due to ion permeation blockade by divergent cations such as Ca2+ and Mg2+, as the degree of inhibition was not appreciably affected by adding 2 mmol/L Ca2+ and 1.2 mmol/L Mg2+ in divalent cation-free bath solution (in mmol/L, Ca2+: 0, Mg2+: 0, and mmol/L Na+: 140). At potentials slightly positive to the reversal potential (Erev) of mTRP6 (0 to 30 mV), there is a range in which little current flows in the outward direction, whereas at more positive potentials (>30 mV), a prominent outward rectification is seen (Figure 1B). A very similar I-V relationship (5 shape and outward rectification) was also obtained for the α1-AR–NSCC current recorded under the same experimental conditions (Figure 1C; see also References 19 and 21).

The mTRP6 current is cationic, as it was completely abolished when all external cations were substituted by large impermeant cations such as N-methyl-D-glucamine but was not affected on total anion substitution with benzenesulfonate. Erev of the mTRP6 current was also close to 0 mV under near-physiological conditions (Figure 1B). The value of Erev was significantly shifted toward more positive potentials, when divergent cations such as Ca2+, Ba2+, and Sr2+ were the sole charge-carrying cations in the bath (by 29.7±3.2, 25.8±3.2, and 16.5±9.9 mV, respectively [n=5], at 100 mmol/L; for actual I-V see the dotted curve in Figure 1B). The relative permeabilities of mTRP6 determined from such Erev measurements under biionic conditions22 (see also supplementary information available at http://www.circresaha.org) were

\[
P_C: P_{Ca}: P_{Ba}: P_{Sr} = 1.0: 4.0: 5.4: 3.2: 3.5: 1.3: 1: 1.2: 2.0: 1.0: 0.7: 0.7: 0.58\]  

These results strongly suggest that mTRP6 is several times more permeable to Ca2+ and Ba2+ than to monovalent cations such as Na+ (Eisenman sequence III).

Single-channel activities accounting for the reversal potential and voltage dependence of mTRP6 currents (hereafter designated as mTRP6 channels) were recorded from cell-attached patches of mTRP6-expressing HEK293 cells (19 of 45 patches) but not from those of the empty vector-expressing cells (0 of 38 patches). As displayed in Figure 1D, the polarity of mTRP6 channels reversed at ≈0 mV, and their openings became less frequent on hyperpolarization (Figures 1E and 1F). The slope conductance of mTRP6 channels calculated from the inward portion of I-V relationship gave a unitary

current.
conductance of ~28 pS on average, under normal ionic conditions (Figure 1E).

These biophysical properties of mTRP6 currents and channels are very similar to those of the α₁-AR–NSCC, i.e., in unique voltage dependence (S shape and outward rectifying I-V), unitary conductance of 25 to 30 pS, and preferential permeation of Ca²⁺ and Ba²⁺ relative to Na⁺ (E₉ᵣ of α₁-AR–NSCCs in Ba²⁺-rich external solution [26.4 mV with 89 mmol/L Ba²⁺] is comparable with that of mTRP6 current [25.8 mV with 100 mmol/L Ba²⁺; this study]).

Similar Pharmacology of mTRP6 and α₁-AR–NSCC

To further confirm the similarity between mTRP6 and the α₁-AR–NSCCs, we next investigated the effects of a nonspecific but frequently used cation channel blocker flufenamate on mTRP6 currents. This compound has previously been shown to uniquely “enhance” the mTRP6 currents. This compound has nonspecific but frequently used cation channel blocker dependence on Ca²⁺, and Ba²⁺-rich external solution [26.4 mV with 89 mmol/L Ba²⁺] is comparable with that of mTRP6 current (25.8 mV with 100 mmol/L Ba²⁺; this study).

![Figure 2. Effects of cation channel blockers on mTRP6 and α₁-AR–NSCC currents under conventional whole-cell clamp with holding potential of −60 mV. A through D, Representative records of the effects of flufenamate and Cd²⁺ on mTRP6 (A), mTRP7 (B), and α₁-AR–NSCC activated by 100 μmol/L phenylephrine (Phe) recorded from rabbit portal vein smooth muscle (RPV) (C and D). E, Potentiating effects of flufenamate (100 μmol/L) under various conditions. The following were used (in μmol/L): ACh 100 or ATP 100 with and without indomethacin (indo) 10, RHC80267 (RHC) 100, or GTP·S 100 included in the pipette. Data for α₁-AR–NSCC, mTRP3, and mTRP7 currents are also shown. F, Concentration-inhibition curves for mTRP6 (●) and α₁-AR–NSCC (○) currents by Cd²⁺ (n = 5 to 15) in divergent cation-free bath solution. Curves are best nonlinear fits to the Hill equation, 1/(1 + [(Cd²⁺)/Kᵢ]ⁿ), where (Cd²⁺), Kᵢ, and n denote the concentration of Cd²⁺ applied, dissociation constant, and Hill coefficient, respectively.

| Currents assessed at −60 mV in Ca²⁺, Mg²⁺-free bath solution with Cs⁺ internal solution, from 5 to 15 cells. |
|---|---|---|---|---|
| α₁-AR–NSCC | mTRP6 | α₁-AR–NSCC | mTRP6 |
| IC₅₀, μmol/L | 253 | 4.0 | 1.9 | 4.2 | 129 |
| Cd²⁺ | 213 | 3.4 | 2.3 | 5.1 | 154 |
| La⁺³ | 235 | 2.5 | 2.5 | 3.5 | 2.5 |
| SK&F96365 | Amloride | 213 | 3.4 | 2.3 | 5.1 | 154 |
but the latter may be more susceptible to Ca2\textsuperscript{+}-induced immediate increase (Figure 3D). The immediate Ca2\textsuperscript{+}-induced increase was accompanied by a markedly increased current noise, which reflects the increased mTRP6 channel or \(\alpha\text{-AR–NSCC}\) conductance. On average, the unitary conductance of mTRP6 estimated by noise analysis increased from 7.4 \(\pm\) 0.7 to 20.0 \(\pm\) 1.9 pS (\(n=13\)) for a [Ca2\textsuperscript{+}] change from 0 to 1 mmol/L (Figure 3F).

Very similar values were also obtained for the \(\alpha\text{-AR–NSCC}\) in the present study (8.9 \(\pm\) 1.1 versus 19.5 \(\pm\) 2.1 pS; \(n=7\)) and by others.\textsuperscript{32} These results strongly suggest that the potentiating action of Ca2\textsuperscript{+} on mTRP6 is essentially the same as on the \(\alpha\text{-AR–NSCC}\).

Figure 4. Dominant expression over other TRP homologues and cellular distribution of TRP6 in the portal vein smooth muscle. A, Autoradiogram of blot hybridization analyses of TRP6 and cyclophillin cDNA fragments amplified by RT-PCR from total RNA of whole mouse brain, mouse portal vein, rabbit portal vein, and smooth muscle isolated from rabbit portal vein (left panel) and of TRP1, TRP3, TRP4, TRP5, and TRP7 cDNA fragments amplified from total RNA of mouse brain and mouse portal vein (right 2 panels). B, Confocal images of TRP6 immunoreactivity in single rabbit portal vein myocytes without or with preabsorption of anti-TRP6 antibody by the immunizing peptide (b and e) and their DIC images (a and d). Inset c is a 2-fold magnification with enhanced contrast from a part boxed in panel b. Anti-TRP6 indicates anti-TRP6 antibody; IP, immunizing peptide; and DIC, differential interference contrast.

Dominant TRP6 mRNA and Protein Expression in Portal Vein

The almost identical electrophysiological and pharmacological properties of mTRP6 and \(\alpha\text{-AR–NSCC}\) described above strongly suggest that the TRP6 protein may be an essential molecular component of \(\alpha\text{-AR–NSCC}\). To test this possibility more directly, we examined the expression of TRP6 mRNA and protein in portal vein smooth muscles. As shown in Figure 4A, total RNA was isolated and subjected to reverse transcription combined with PCR amplification and Southern blot hybridization for determination of expressed TRP subtypes in portal vein smooth muscle cells. In the mouse portal vein, TRP6 RNA was abundantly expressed, whereas TRP1, TRP3, and TRP4 RNAs were present at much lesser levels, and TRP5 and TRP7 RNAs were undetectable. Abundance of TRP6 RNA was similarly found in the whole rabbit portal vein and the smooth muscle isolated from it, suggesting that smooth muscle cells are the major expression site for TRP6 RNA in the portal vein. Immunocytochemistry using anti-TRP6 antisera (see supplementary information, available at http://www.circresaha.org) revealed that TRP6 protein is localized near the sarcolemmal region of an acutely dissociated cell.
ated rabbit portal vein myocyte (Figures 4Bb and 4Bc), whereas no immunoreactivity was detected from the myocytes treated with FITC-labeled secondary antibody alone (data not shown) or with preabsorption of anti-TRP6 antibody by the immunizing peptide (Figure 4Be). These results strongly suggest that TRP6 is the dominant TRP subtype expressed in the portal vein smooth muscle.

**TRP6 Functions as the α₁-AR–Activated Ca^{2+} Entry Channel**

Finally, to determine whether the endogenously expressed TRP6 protein really functions as the α₁-AR–activated channels, we cultured myocytes enzymatically dissociated from the rabbit portal vein with the TRP6 antisense oligonucleotide that was expected to selectively inhibit TRP6 expression (Table 1). Three to 5 days of antisense oligonucleotide treatment almost completely abolished the expression of TRP6 protein in portal vein myocytes (Figure 5E), whereas in those treated with the sense oligonucleotide, substantial TRP6 immunoreactivity remained (Figure 5C). Correspondingly, the density of cation current activated by the α₁-AR agonist phenylephrine (100 μmol/L) was markedly decreased with the TRP6 antisense oligonucleotide (Figures 6B and 6D) compared with cells treated with the TRP6 sense oligonucleotide (Figures 6A and 6D) or antisense oligonucleotides for other TRP homologues detected in the portal vein by RT-PCR (Figure 6D). We also tested the contribution of TRP6 to Ca^{2+} entry through the α₁-AR–NSCC by measuring Ba^{2+} fluorescence (see supplementary information, available at http://www.circresaha.org), because Ba^{2+} is almost equally as permeable as Ca^{2+} through the mTPP6 channel (Figure 1B), whereas it permeates the native SOCs to a lesser extent than Ca^{2+}.33 The use of Ba^{2+} may also be advantageous to measure genuine influx, as it is not extruded or taken up into internal stores by Ca^{2+}-ATPases.34 As shown in Figure 6C and...
summarized in Figures 6E and 6F, treatment with TRP6 antise sense oligonucleotide significantly reduced the rate and peak of Ba2+ fluorescence ratio increase in response to the α1-AR activation but did not affect those evoked by store depletion per se (thapsigargin 2 μmol/L) (Figures 6E and 6F). These results strongly point to the functional importance of TRP6 protein as a Ca2+ entry pathway independent of SOCs during the α1-AR stimulation via sympathetic nerves in this muscle.

Striking similarity between recombinantly expressed mTRP6 and α1-AR–NSCC currents, ie, in activation profile (Figure 1A), unique voltage dependence (S-shaped and outward rectifying I-V), divergent cation permeability (Ca2+, Ba2+>Na+), unitary conductance (25±30 pS), efficacy of organic and inorganic blockers, and augmentation by flufenamate and external Ca2+, strongly suggests that the TRP6 protein is the essential molecular component of α1-AR–NSCC channels in the portal vein smooth muscle. This is further corroborated by the high expression level of TRP6 mRNA, localization of TRP6-specific immunoreactivity near the cell membrane, and marked inhibition of TRP6 protein expression and α1-AR–activated cation current and Ba2+ entry by the antisense strategy. Although possible roles of other endogenous TRPs (Figure 4A) or yet- unidentified accessory regulatory proteins, which may form a heteromultimer with TRP6, cannot be excluded, there is little doubt that TRP6 has central importance in fulfilling the function of α1-AR–NSCC in some vascular tissues as a store depletion– independent, receptor-activated Ca2+ entry pathway.

Looking at other native systems, there are groups of nonselective cation channels activated by GPCRs independently of store depletion that show considerable resemblance to the α1-AR–NSCC from an electrophysiological point of view. For example, muscarinic cation channels ubiquitously identified in gastrointestinal smooth muscle are of ≈25 pS in unitary conductance; severalfold more permeable to Ca2+ and Ba2+ than Na+; suppressed by hyperpolarization; sensitive to [Ca2+]i; and immediately potentiated by Ca2+, although their primary activator is likely to be the activated G(q)/protein.2-4 Some of the 30-pS Ca2+–activated nonselective cation channels in cardiac and epithelial tissues are also known to be voltage dependent and/or activated by GPCRs.35,36 Considering that many biologically important signals produced through GPCR or RTK stimulation (Ca2+, IP3, DAG, arachidonic acid, activated G protein, and store depletion signal, etc) are also recognized as key activators/modulators of TRPs,6-10,29-31,37-40 it is quite possible that a much broader range of ROCCs than currently envisaged may be associated with TRPs in some way. Consistent with this idea, the evidence is gradually accumulating that the TRPs are a requisite component of native Ca2+-permeable cation channels activated by GPCRs, RTKs, and other stimuli.12,13,41,42

Acknowledgments

This work is supported by Grant-in-Aid 12670088 to R.I. from the Japan Society for the Promotion of Sciences. We thank Prof A.F. Bradin, University Department of Pharmacology, Oxford University, for English correction of the manuscript; Drs Brian Seed and Gary Yellen for providing the pH3-CD8 plasmid; and Hiroshi Fuji, Miyo Ikeda, Emiko Mori, and Kumiko Saito for their expert technical assistance.

References

1. Minneman KP. α1-Adrenergic receptor subtypes, inositol phosphates, and sources of cell Ca2+. Pharmacol Rev. 1988;40:87–119.
2. Carl A, Lee HK, Sanders KM. Regulation of ion channels in smooth muscle. Am J Physiol Cell Physiol. 1996;271:C9–C34.
3. Kuriyama H, Kitamura K, Itoh T, Inoue R. Physiological features of visceral smooth muscle cells, with special reference to receptors and ion channels. Physiol Rev. 1998;78:811–920.
4. Kotlikoff ML, Herrera G, Nelson MT. Calcium permeant ion channels in smooth muscle. Rev Physiol Biochem Pharmacol. 1999;134:147–199.
5. Hardie RC, Minke B. Novel Ca2+ channels underlying transduction in Drosophila photoreceptors: implications for phosphoinositide-mobilized Ca2+ mobilization. Trends Neurosci. 1993;16:371–376.
6. Birnbaumer L, Zhu X, Jiang M, Boulay G, Peyton M, Vannier B, Brown D, Platano D, Sadeghi H, Stefani E, Birnbaumer M. On the molecular basis and regulation of cellular capacitative calcium entry: roles for Trp proteins. Proc Natl Acad Sci USA. 1996;93:15195–15202.
7. Montell C. New light on TRP and TRPL. J Pharmacol Exp Ther. 1997; 55:755–763.
8. Barrett G. Receptor-activated Ca2+ inflow in animal cells: a variety of pathways tailored to meet different intracellular Ca2+ signalling requirements. Biochem J. 1999;337:153–169.
9. Putney JW Jr, McKay RR. Capacitative calcium entry channels. BioEssays. 1999;21:38–46.
10. Hofmann T, Schaefer M, Schultz G, Gundermann T. Transient receptor potential channels as molecular substrates of receptor-mediated cation entry. J Mol Med. 2000;78:14–25.
11. Fasolato C, Innocenti B, Pozzan T. Receptor-activated Ca2+ influx: how many mechanisms for how many channels? Trends Pharmacol Sci. 1994; 15:77–83.
12. Liu J, Wang W, Singh BB, Lockitch T, Jadlowiec J, O’Conell B, Weller R, Zhu MX, Ambulkar IS. Trp1, a candidate protein for the store-operated Ca2+ influx mechanism in salivary gland cells. J Biol Chem. 2000;275:3403–3411.
13. Wu X, Babnigg G, Villedare ML. Functional significance of human trp1 and trp3 in store-operated Ca2+ entry in HEK-293 cells. Am J Cell Physiol. 2000;278:C526–C36.
14. Boulay G, Zhu X, Peyton M, Jiанг M, Hurs R, Stefani E, Birnbaumer L. Cloning and expression of a novel mammalian homolog of Drosophila Transient Receptor Potential (Trp) involved in calcium entry secondary to activation of receptors coupled by the Gq class of G-protein. J Biol Chem. 1997;272:29672–29680.
15. Hofmann T, Obukhov AG, Schaefer M, Harteneck C, Gudermann T, Schultz G. Direct activation of human TRPC6 and TRP3 channels by diaacylglycerol. Nature. 1999;397:259–263.
16. Helliwell RM, Large WA. α1-Adrenoceptor activation of a non-selective cation current in rabbit portal vein by 1,2-diacyl-sn-glycerol. J Physiol (Lond). 1997;499:417–428.
17. Mori Y, Takada N, Okada T, Wakamori M, Imoto K, Waniuchi H, Oka H, Oba A, Ikenaka K, Kurosaki T. Differential distribution of TRPC Ca2+ channel isoforms in mouse brain. Neuroreport. 1998;9:507–515.
18. Okada T, Inoue R, Yamazaki K, Maeda A, Kurosaki T, Yamakuni T, Tanaka I, Shimizu S, Ikenaka K, Imoto K, Mori Y. Molecular and functional characterization of a novel mouse transient receptor potential protein homologue TRP7: Ca2+-permeable cation channel that is constitutively activated and enhanced by stimulation of G protein-coupled receptor. J Biol Chem. 1999;274:27359–27370.
19. Inoue R, Kuriyama H. Dual regulation of cation-selective channels by muscarinic and α1-adrenergic receptors in the rabbit portal vein. J Physiol (Lond). 1993;465:427–448.
20. Okada T, Shimizu S, Wakamori M, Maeda A, Kurosaki T, Takada N, Imoto K, Mori Y. Molecular cloning and functional characterization of a novel receptor-activated TRP Ca2+ channel from mouse brain. J Biol Chem. 1998;273:10279–10287.
21. Helliwell RM, Large WA. Dual effect of external Ca2+ on noradrenaline-activated cation current in rabbit portal vein smooth muscle cells. J Physiol (Lond). 1996;492:75–88.
22. Lewis CA. Ion-concentration dependence of the reversal potential and the single channel conductance of ion channels at the frog neuromuscular junction. J Physiol (Lond). 1979;286:447–445.
23. Byrne NG, Large WA. Membrane ionic mechanisms activated by noradrenaline in cells isolated from the rabbit portal vein. J Physiol (Lond). 1988;404:557–573.

24. Popp R, Englert HC, Lang HJ, Gögelein H. Inhibitors of nonselective cation channels in cells of the blood-brain barrier. In: Siemen D, Hescheler J, eds. Nonselective Cation Channels. Basel, Switzerland: Birhäuser Verlag; 1993:213–218.

25. Chen S, Inoue R, Ito Y. Pharmacological characterization of muscarinic receptor-activated cation channels in guinea-pig ileum. Br J Pharmacol. 1993;109:793–801.

26. Chen XZ, Vassilev PM, Basora N, Peng JB, Nomura H, Segal Y, Brown EM, Reeders ST, Hediger MA, Zhou J. Polycystin-L is a calcium-regulated cation channel permeable to calcium ions. Nature. 1999;401:383–386.

27. Yamada K, Waniishi Y, Inoue R, Ito Y. Fenamates potentiate the α1-adrenoceptor-activated nonselective cation channels in rabbit portal vein smooth muscle. Jpn J Pharmacol. 1996;70:81–84.

28. Obkhov AG, Schultz G, Lückhoff A. Regulation of heterologously expressed transient receptor potential-like channels by calcium ions. Neuroscience. 1998;85:497–495.

29. Hardie RC, Raghu P. Activation of heterologously expressed Drosophila TRPL channels: Ca2+ is not required and IP3 is not sufficient. Cell Calcium. 1998;24:153–163.

30. Zimmer S, Trost C, Cavalié A, Philipp S, Flockerzi V. The Trp protein is a Ca2+-calmodulin activated non-selective cation (CAN) channel. Naunyn Schmiedebergs Arch Pharmacol. 1997;355:R67. Abstract.

31. Zit C, Obukhov AG, Strubing C, Zobel A, Kalkbrenner F, Lückhoff A, Schultz G. Expression of TRP3 in Chinese hamster ovary cells results in calcium-activated cation currents not related to store depletion. J Cell Biol. 1997;138:1333–1341.
The Transient Receptor Potential Protein Homologue TRP6 Is the Essential Component of Vascular α1-Adrenoceptor–Activated Ca\textsuperscript{2+}-Permeable Cation Channel

Ryuji Inoue, Takaharu Okada, Hitoshi Onoue, Yuji Hara, Shunichi Shimizu, Shinji Naitoh, Yushi Ito and Yasuo Mori

Circ Res. 2001;88:325-332
doi: 10.1161/01.RES.88.3.325

Circulation Research is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
Copyright © 2001 American Heart Association, Inc. All rights reserved.
Print ISSN: 0009-7330. Online ISSN: 1524-4571

The online version of this article, along with updated information and services, is located on the World Wide Web at:
http://circres.ahajournals.org/content/88/3/325

Data Supplement (unedited) at:
http://circres.ahajournals.org//subscriptions/

Permissions: Requests for permissions to reproduce figures, tables, or portions of articles originally published in Circulation Research can be obtained via RightsLink, a service of the Copyright Clearance Center, not the Editorial Office. Once the online version of the published article for which permission is being requested is located, click Request Permissions in the middle column of the Web page under Services. Further information about this process is available in the Permissions and Rights Question and Answer document.

Reprints: Information about reprints can be found online at:
http://www.lww.com/reprints

Subscriptions: Information about subscribing to Circulation Research is online at:
http://circres.ahajournals.org//subscriptions/
**Supplementary information**

**Materials and Methods**

**cDNA cloning**

Oligo(dT)-primed, size-selected (>1 kilobase pairs (bp)) cDNA libraries constructed in the λ Uni-ZAP XR vector (Stratagene) using poly(A)+ RNA from the brain of adult BALB/c or postnatal 14 days-old (P14) C57BL/6J (B6) mice were screened by hybridization with cDNA inserts from p8283, that carry human TRP3 cDNA ¹ to yield mouse TRP6 clones λm2 (554-2892) and λm44 (1219-3064 followed by poly(dA) tract). To obtain the further upstream region of mouse TRP6 cDNA, rapid amplification of 5′-cDNA ends (5′-RACE) was performed using the Marathon cDNA Amplification Kit (Clontech, Palo Alto, CA). The template cDNAs were constructed from P14 C57BL/6J (B6) mouse brain poly(A)+ RNA. The specific antisense primers used for nested 5′-RACE PCR were 5′-ATGCTGGACTTGCCAGACCTTGTATGC-3′ and 3′-GTCCCATCTTCATCATAGGC-5′ for first and second PCR, respectively. The resulting ~0.7-kbp cDNA fragment was subcloned into pBluescript SK(+) (Stratagene) to yield a clone pRACE6 (-41 to 638).

**Construction of expression plasmid**

PCR product amplified from the clone pRACE6 using sense primers (5′-GGGTCGACGGGTTTTAATTTTCTTCAATGTTCCACATGAGCCA GAGCCCGAG-3′) designed to contain the untranslated leader (UTL) sequence from the alfalfa mosaic virus, a consensus sequence for translation initiation, and nucleotide residues 1-17 of TRP6 and antisense primers (5′-
AGCATCCCACAATCGAGACAAGTTTTC-3’) was digested with SalI and XhoI. Another PCR product amplified from λm44 using sense primers (5’-CCAGCAAATGAAAGCCTTTGGACCTCTGC-3’) and antisense primers (5’-ATGGCGGGCGCAATTTATAATCACC-3’) was digested with HindIII and NotI. The resulting fragments containing partial TRP6 cDNAs were ligated with the XhoI(567)/HindIII(1945) fragment from λm2 and the 5.5-kb SalI/NotI fragment from pCI-neo (Promega, Madison, WI) to yield pCI-neo-mTRP6.

Recombinant expression in HEK293 cells

Partially confluent HEK293 cells (40–60%) were co-transfected with two plasmid DNAs, πH8-CD8 containing the T-cell antigen CD8 cDNA, and either pCI-neo-mTRP6 pCI-neo-mTRP3, pCI-neo-mTRP7 or the vector pCI-neo, with a molar ratio of 5:1, using SuperFect Transfection Reagent (Qiagen, Hilden, Germany). Transfected cells were re-seeded on cover slips after trypsinization and placed in a 24-well tissue culture plate (Primaria, Falcon) filled with Dulbecco’s modified Eagle Medium (DMEM) complemented with 10% fetal bovine albumin (FBS), 20 units/ml penicillin and 30μg/ml streptomycin. mTRP6-expressing cells were selected as those positive to CD8 antibodies conjugated with polystrene microspheres (Dynabeads, M450 CD8; Dynal, Oslo, Norway). All experiments were performed within 48-72 h after transfection.

Cell dispersion and primary culture

Albino rabbits of either sex weighing 1.8-2.0kg (Nippon White) were anesthetized with intravenous injection of sodium pentobarbitone (40mg/kg) and killed quickly by exsanguination. After opening the abdominal cavity, an approximately 1cm long
segment of portal vein was excised between the hepatic and splenic bifurcations. After removing connective tissues with a fine scissors and forceps, thin strips of portal vein muscle measuring ca. 10mm x 2mm were made and incubated successively in nominally Ca\textsuperscript{2+}-free Krebs solution and one containing 2mg/ml collagenase (Sigma type I) at 35°C for 10 and 35min, respectively. Thereafter, the digested strips were thoroughly rinsed with Ca\textsuperscript{2+}-free Krebs solution and minced into small pieces, which were mechanically agitated using a blunt tipped Pasteur pipette to yield a sufficient number of single cells.

For acute electrophysiological measurements as shown in Fig.1, 2 and 3, dissociated portal vein myocytes were used within 3h after cell dispersion. For antisense experiments (Fig.6), the myocytes were seeded in a 35mm culture dish pre-coated with laminin (20µg/ml), and maintained in a short term culture in DMEM containing 2% FBS plus antibiotics and either antisense or sense oligonucleotides (5µM). Three to four days later, adherent myocytes in the dish were trypsinized and re-seeded on cover slips, and used for electrophysiological and fluorescent measurements within 12 - 24h.

Antisense and sense oligonucleotides for TRP1, 3 and 6 used are listed in Table 1. In different series of experiments, we used another sets of antisense and sense oligonucleotides for these TRP homologues; TRP1 (5'-GCAGCTAAAATGACAGGTGC-3'; 5'-GCACCTGTCATTTTAGCTGC-3'); TRP3 (GCTCTGCTGATTCCAGATCT: AGATCTGGAATCACGAGGC; TRP6 (5'-GTGAAGGAAAGCTGCGTGTCG-3'; 5'-GCACACGCAGCCTTCCTTCAC-3'). The obtained results were consistent with those summarized in Fig.6D, indicating that TRP6 antisense treatment markedly reduces the density of Phe (100µM)-induced cationic current.
The composition of Ca\(^{2+}\)-free Krebs solution was (mmol/L): 140 Na\(^+\), 6 K\(^+\), 1.2 Mg\(^{2+}\), 148.4 Cl\(^-\), 10 glucose, 10 HEPES (adjusted at pH 7.4 with Tris base).

**Reverse Transcriptase (RT)-PCR Amplification and Southern Blot Analysis**

Reverse transcriptase (RT)-PCR amplification and southern blot analysis for TRP expression were performed as described previously.\(^2\) Albino rabbits and B6 mice were killed by exsanguination under anesthesia (see above) and cervical dislocation, respectively, and portal veins were excised out. Total RNA was extracted from the whole rabbit and murine portal veins or smooth muscles mechanically dissected from the former (endothelium was denuded), using the total RNA extraction kit (RNeasy Minikit, Qiagen, Germany), according to the manufacturer's instructions. First strand DNA was generated from total RNA of 1\(\mu\)g using random primer (hexamer) and reverse transcriptase (Superscript II, Gibco-BRL) with a reaction volume of 10\(\mu\)L. PCR amplification was performed in a 50\(\mu\)L scale with Taq polymerase using a Takara PCR thermal cycler. The program of PCR amplification consisted of 30s denaturation at 94\(\textdegree\)C followed by 30s denaturation at 94\(\textdegree\)C, 30s annealing at 64\(\textdegree\)C, 1 min extension at 68\(\textdegree\)C for 10 cycles, and 30s denaturation at 94\(\textdegree\)C, 30s annealing at 60\(\textdegree\)C, 1min extension at 68\(\textdegree\)C for 30 cycles. PCR products were then hybridized with \(^{32}\)P-5'end-labeled synthetic oligonucleotide probes (specific radioactivity: 3.0 - 4.0 \(\mu\)Ci/pmol), and autoradiographed for 5 h. Pairs of primers (sense and antisense; 5' to 3') used were CTTCCAGCTGATAGCAAATCAT and TCGGAATTCTGACAGATCTTGGC for TRP1, GACTGCAAGGATCTGGAACTGG and GGAGTATTATTGCCACGTTGTGC for TRP3, AGACATTTCTAGCCTCGCGTTCG and CTAATCTTGGATTCAGTCACC for TRP4,
TATCTACTGCCTAGTACTACTGG and GCAATGAGCTGGTAGGATTTATTC for TRP5, \(\text{CCTGTTTCTCATGGATGGAGATG}\) and \(\text{CATTCCAAATCATAAAGGCTACAACACC}\) for TRP6, \(\text{AGATCATCTCGGAAGGGCTGTACG}\) and \(\text{CGTCTTCCTCGATTTTCCTGATAGGAG}\) for TRP7, and \(\text{GCAGCCATGGTCACCCCCACCG}\) and \(\text{GAAATTAGAGCTGTCACAGTCGG}\) for cyclophilin. \(^{32}\)P-5'-end-labeled synthetic oligonucleotide probes used in hybridization were \(\text{CTTCTCCAAAGACGATCTGCT}\) for TRP1, \(\text{ACAGCATTCTCAATCAGCCAA}\) for TRP3, \(\text{GCTCTCTACAATAACGTCAGC}\) for TRP4, \(\text{ATGAACCTAACAACCTGCAAGG}\) for TRP5, \(\text{AGGCATGATATGGGCTGAATG}\) for TRP6, \(\text{AAGGTCCGAAGCTCTCATTTGGCTG}\) for TRP7, and \(\text{TGGATGGCAAGCATGTGGTCTTTTGG}\) for cyclophilin. The RT-PCR primers and oligonucleotide probes for TRP6 and cyclophilin were designed based on the regions conserved between mouse and human TRP6 sequences and between mouse and rat cyclophilin sequences, respectively.

The amplification efficiency of the primer sets used in our PCR experiments was assessed by varying the concentration of cDNA templates using the same PCR protocol as described above. The concentrations of cDNA templates necessary for 50\% of saturable yield of amplification reaction were as follows (in mol/L): TRP1, \(5.4 \times 10^{-18}\); TRP3, \(1.4 \times 10^{-18}\); TRP4, \(6.5 \times 10^{-18}\); TRP5, \(5.3 \times 10^{-18}\); TRP6, \(3.0 \times 10^{-17}\); TRP7, \(5 \times 10^{-18}\). On the other hand, saturable yields of amplification per reaction were similar among the primer sets of TRP homologues (0.8 to 1.2 x \(10^{-12}\) mole). Thus, the apparent order of PCR amplification efficiency was TRP3 > TRP7 • TRP5 • TRP1 > TRP4 > TRP6.
Electrophysiology

Recording of whole-cell and single channel currents and data analysis were performed using the standard patch clamp system described elsewhere. The input resistance of electrodes used for whole-cell recording ranged from 2.5-3.5MΩ (Cs'-internal solution; see below), and 80-90% of access resistance (5-7MΩ) was electronically compensated. For long-term recordings (>1min), the current signal was obtained using MacLab/4 (digitized at 100Hz; AD Instruments, New South Wales, Australia) after low-pass filtering at 50Hz, from which the mean current amplitude and noise variance were calculated for every 1s segment. The liquid junction potential between pipette and bath solutions (~6mV) was corrected when constructing current-voltage relationships.

Nystatin-perforated recording was performed as described elsewhere. Briefly, nystain (Sigma) dissolved in dimethyl sulfoxide (50mM in stock) was diluted 200 times in Cs'-internal solution and ultrasonicated for 5-10min. A patch pipette was back-filled with this solution after shortly dipping its tip in a nystatin-free internal solution, and a ‘giga’ seal was formed on the cell membrane as quickly as possible. The time course of cell membrane perforation by nystatin was monitored by applying small voltage step pulses (2mV) and experiments were not started until the access resistance dropped to less than 20 MΩ.

For flash photolysis, HEK293 cells were loaded with 100 - 200 μM caged IP₃ (Dojin, Kumamoto, Japan) via the patch pipette until equilibrated (5 - 10min). A high pressure mercury lamp (intensity, 320mW/10mm² at 360nm) was used to generate UV light, and short flashes (20 -50ms) required to cause a maximum increase in [Ca²⁺] (determined by separate fluorescent experiments) were applied through an electronic shutter (HB-10103AF, Nikon, Tokyo) controlled by a voltage pulse generator (SEN-7103, Nihon
Kohden, Japan).

Test solutions were topically applied using the so-called ‘Y-tube’ fast solution exchange device. Bath solution consisted (mmol/L): 135 Na⁺, 5 K⁺, 1.2 Mg²⁺, 2 Ca²⁺, 151.4 Cl⁻, 5 glucose, 10 HEPES; Cs⁺ internal solution for whole-cell recording (mmol/L): 140 Cs⁺, 2 Mg²⁺, 24 Cl⁻, 120 aspartate, 2Na₂ATP, 5 phosphocreatine, 10BAPTA/4Ca²⁺, 10 glucose, 10 HEPES. In Fig.3A and B, 1mmol/L EGTA instead of 10BAPTA/4Ca²⁺ was added in this solution; pipette solution for cell-attached recording (mmol/L): 140 Na⁺, 1 Ca²⁺, 1.2 Mg²⁺, 10 tetraethylammonium, 154.4 Cl⁻, 5 glucose, 10 HEPES, 100μM ATP or carbachol.

All experiments were performed at 24 - 26°C, using a commercial warmer unit (TC-344B, Warner Instrument, USA).

Calculation of relative permeabilities

The relative permeability of mTRP6 to Na⁺ for a given cation was calculated by the modified Goldman-Hodgkin-Katz equation,⁶ using the activity coefficients of 0.77 and 0.524 for monovalent and divalent cations, respectively. These values are taken from a textbook of physical chemistry (Table 10.4; In Physical Chemistry, 4th Edition, Atkins, P.W., Oxford University Press, 1990).

Ba²⁺ fluorescence measurements

Ba²⁺ fluorescence measurements were performed using a dual excitation wavelength spectrofluorometer (CAM 230, Nihon Bunko). After incubating with 2μM fura2/AM containing saline for 45 – 60min at room temperature, fura2-loaded cells were transferred into a recording chamber and thoroughly washed. The cells were alternately
illuminated by two near-visible UV lights (340 and 380nm) delivered at a frequency of 100Hz, and the emitted fluorescence was passed through an objective lens (Fluor 40, Nikon, Japan) and collected by a photomultiplier tube after filtering at 510nm (±30nm). Background fluorescence at 340 and 380nm (including the autofluorescence from the cell) was determined as averages from control cells and corrected later. The extent of Ba$^{2+}$ influx was assessed as the ratio of fluorescence intensity at 340 and 380nm excitation. Rapid switching of solution was performed using the 'Y-tube' device.

Statistics

All data are expressed as mean ± s.e.m.. Statistical significance of differences between give sets of data was evaluated by Student $t$-test for single comparison and one way ANOVA for multiple comparison.

Immunocytochemistry

Anti-mouse TRP6 rabbit antiserum was raised against the C-terminal sequence, LIRKLGERLSELPLKLEESRR. Myocytes freshly dissociated from the rabbit portal vein muscle were seeded on coverslips pre-coated with either poly-L-lysine (50µg/ml) or laminin (20µg/ml), and incubated in 2% FBS containing DMEM for 2h or cultured with antisense and sense oligonucleotides for 3 -5 days. These myocytes on the coverslip were fixed with 4% paraformaldehyde for 20min and permeabilized with 0.2% Triton/phosphate-buffered saline (PBS) for 15min at room temperature. After rinsing in PBS several times, the myocytes were pre-incubated for 1hr with 10% normal goat serum (NGS)/PBS to prevent non-specific binding of antibodies, and incubated successively with 1:1000 diluted TRP6 antiserum for 1hr, and with fluorescein
isothiocyanate (FITC)-labeled anti-rabbit goat antiserum (Jackson) for 1hr after washing with 1% NGS/PBS. Pre-absorption of anti-TRP6 antibody by the immunizing peptide (IP; see above) was performed by adding 3μg/ml IP to 1:1000 diluted anti-TRP6 antibody. Immunostained cells were observed under a confocal laser scanning microscope (LSM 510, Zeiss) equipped with a krypton/argon laser source. A single-wavelength of 488nm was used for excitation, and the emitted fluorescence at 505nm was collected through an objective lens with a 63 times magnification (Plan-Apochromat), and an optical section of 0.8 - 1.1μm was projected on a single plane.

**Western blot analysis of TRP6.**

Cells were lysed and subjected to standard SDS/PAGE gels. In short, proteins were transferred to nitrocellulose (Hybond ECL, Amersham Pharmacia Biotech, Buckinghamshire, England) in buffer containing 150 mM glicine, 20 mM tris-base, and 10 % (vol/vol) methanol at 600mA, 4°C, 2 h. Blots were blocked with TBS plus 0.1 % Tween 20 and blocking reagent (Western blotting analysis system, Amersham Pharmacia Biotech) for 40 min at room temperature. For detection of TRP6 protein, the anti-TRP6 antibody was diluted 1:200 in TBS plus 0.1 % Tween 20, and incubated with blots for 50 min at room temperature. Blots were washed with TBS plus 0.1% Tween 20 for 30 min at room temperature. Antibodies retained on the blots were visualized with Western blotting analysis system from Amersham Pharmacia Biotech.

As shown in Fig.suppl-1, the polyclonal antibody raised to the C-terminal sequence of TRP6 specifically recognized bands at 100 and 110-120 kDa in HEK293 cells transfected with TRP6 cDNA, but not in those with other TRP homologue cDNAs. Using the anti TRP6 antibody, the effect of treatment with TRP6 antisense
oligonucleotide on TRP6 protein levels expressed in cultured rabbit portal vein myocytes was examined by Western blot analysis. Compared to the sense oligonucleotide treatment, antisense treatment significantly diminished the intensity of the 100 kDa band detected using the anti-TRP6 antisera. The 100 kDa band was also detected in the lung, in which the most abundant expression of TRP6 RNA has been reported among various tissues. These results suggest that antisense treatment efficiently reduced the TRP6 protein level in cultured portal vein myocytes.

Effects of L-type Ca\(^{2+}\) channel and \(\alpha_1\)-AD-NSCC blockers on Phe-evoked Ba\(^{2+}\) fluorescence increase.

To evaluate the extent of contribution of L-type voltage-dependent Ca\(^{2+}\) channels as well as \(\alpha_1\)-AD-NSCCs to Phe-induced Ba\(^{2+}\) fluorescence increase in primary cultured portal vein myocytes (Fig.6), we tested the effects of blockers for these channels. As shown in Fig.suppl.2, the specific blocker for L-type Ca\(^{2+}\) channel, nifedipine (10\(\mu\)M), did not significantly affect the Ba\(^{2+}\) fluorescence increase evoked by Phe (100\(\mu\)M). In contrast, pretreatment with 25\(\mu\)M SK&F96365, which inhibited the \(\alpha_1\)-AD-NSCC current by about 80\%, markedly reduced the fluorescence. In accordance with this, voltage-dependent Ca\(^{2+}\) currents were almost undetectable from the myocytes in a 5-day primary culture by the whole-cell clamp recording.

References

1. Mori Y, Takada N, Okada T, Wakamori M, Imoto K, Wanifuchi H, Oka H, Oba A, Ikenaka K, Kurosaki T. Differential distribution of TRP Ca\(^{2+}\) channel isoforms in mouse brain. *Neuroreport*. 1998;9:507-515.
2. Okada T, Shimizu S, Wakamori M, Maeda A, Kurosaki T, Takada N, Imoto K, Mori Y. Molecular cloning and functional characterization of a novel receptor-activated TRP Ca^{2+} channel from mouse brain. *J Biol Chem.* 1998;273:10279-10287.

3. Inoue R, Kuriyama H. Dual regulation of cation-selective channels by muscarinic and $\alpha_1$-adrenergic receptors in the rabbit portal vein. *J Physiol (Lond).* 1993;465:427-448.

4. Okada T, Inoue R, Yamazaki K, Maeda A, Kurosaki T, Yamakuni T, Tanaka I, Shimizu S, Ikenaka K, Imoto K, Mori Y. Molecular and functional characterization of a novel mouse transient receptor potential protein homologue TRP7. Ca^{2+}-permeable cation channel that is constitutively activated and enhanced by stimulation of G protein-coupled receptor. *J Biol Chem.* 1999;274:27359-27370.

5. Chen S, Inoue R, Ito Y. Pharmacological characterization of muscarinic receptor-activated cation channels in guinea-pig ileum. *Br J Pharmacol.* 1993;109:793-801.

6. Lewis CA. Ion-concentration dependence of the reversal potential and the single channel conductance of ion channels at the frog neuromuscular junction. *J Physiol (Lond).* 1979;286:417-445.

7. Boulay G, Zhu X, Peyton M, Jiang, M, Hurs R, Stefani E, Birnbaumer L. Cloning and expression of a novel mammalian homolog of *Drosophila Transient Receptor Potential* (Trp) involved in calcium entry secondary to activation of receptors coupled by the $G_q$ class of G-protein. *J Biol Chem.* 1997;272:29672-29680.

**Figure legends**

**Figure suppl-1.** Efficient suppression of TRP6 expression by antisense oligonucleotide in cultured portal vein myocytes.
Expression of TRP6 protein was characterized by Western blot analysis for HEK293 cells transiently transfected with TRP1, TRP4, or TRP6 cDNA, crude membrane fractions of rabbit lung, and cultured rabbit portal vein myocytes treated with antisense (6AS) or sense (6S) TRP6 oligonucleotide. Molecular weight standards are 208, 127, and 85 kDa. RPV: rabbit portal vein myocytes.

**Figure suppl-2.** Effects of L-type and cation channel blockers on Phe-induced Ba\(^{2+}\) fluorescence increase.

Portal vein myocytes in a 5-day primary culture with DMEM were stimulated by Phe (100µM) for 200 - 250s in Ca\(^{2+}\)-free bath solution and, 2mM Ba\(^{2+}\) was introduced into the bath using a slow gravity-fed superfusion system. Ba\(^{2+}\) fluorescence was measured using a video image analysis system (Argus-20/CA. Hamamatsu Photonics, Hamamatsu, Japan). No statistically significant difference was found between control cells (without blockers) and those pretreated with nifedipine (10µM). P values are the results of unpaired t-test.
Fig.suppl-1
