Opening of ligand-gated cation channel families by calpain inhibitors[1]

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Abstract The class of $Ca2^+$-permeable cation channels is composed of large families with six transmembrane segments including transient receptor potential, vanilloid receptor (VR), polycystin, epithelial calcium channels and melastatin (MLS). However, most of them are functionally silent and unexpressed in mammalian cells. An investigation of associated proteins made us believe that the blockade of calpain opens the silent channels. Using 1 μM of blockers in whole cellular patch pipette fill we measured currents of Chinese hamster ovary cells transfected by VR-like 1 and 2, polycystin-2, or a MLS-like new member (MLS3S). Significant conductance of every clone with a characteristic rectification by blockers was demonstrated. The permeability of $Ca2^+$ to them is similar to that reported. Western blot suggested that blockers did not affect the assembly of the protein but enabled its cleavage. Therefore, investigation of these families with the blockers may boost our knowledge of electrophysiologic function. © 2002 Published by Elsevier Science B.V. on behalf of the Federation of European Biochemical Societies.

Key words: Calcium channel; Protease; Calpastatin

1. Introduction

Influx of Ca^{2+} into cells is a fundamental process and is widely observed in physiologic or pathophysiologic phenomena. The Ca^{2+} permeability is achieved as a cation flow, which does not precisely discriminate between monovalent and divalent cations. Ca^{2+} permeability is driven through the cation channel and activated by voltage, ligand, or mechanical stress. The voltage-independent, ligand-gated, non-selective cation currents are widely observed and encoded by large molecular families including transient receptor potential (TRP) [1–5], vanilloid receptor (VR) [6], epithelial calcium channel (ECaC) [9–11] and polycystins [12–16]. All of these possess six transmembrane segments (TMSs) and a putative pore region between the fifth and sixth TMS with a unique N-terminal. Most of these channels are weakly expressed in common organisms, e.g. as in mammalian cells or *Xenopus* oocytes, and that limits the systems in which we can explore knowledge of their physiologic functions.

Drosophila photoreceptors provide a first clue to investigating the TRP cation channel. TRP reveals six TMSs with a pore, the structure of which is similar to the voltage-dependent K channel family. TRP is coupled with Gq protein and requires metabolites of inositol phosphates for activation [1–4]. TRP currents appeared after removal of intracellular Ca^{2+} or Ca^{2+}-store depletion with thapsigargin [5]. Using expression cloning, the six TMSs are generated in higher quantity. VR (VR1), cloned by employing a capsaicin-induced rise in intracellular calcium, is another example of cation channel with six TMSs [6]. Physical or chemical irritants (or insulin) are required to open the VR family [7,8]. On the other hand, six TMSs were also discovered in analysis of disease. Genes corresponding to polycystic kidney disease show PKD1 and 2 [12,13]. Especially, PKD2 shows a similar structure to six TMSs. PKDL, a family of PKD but unrelated to disease, has a cation channel expressed [16]. Melastatin (MLS) has been cloned by a differential display unique in melanocytes metastasis [17,18]. MLS family distributes widely and reveals a similar structure but its function remains obscure. An exception is the ECaC family, which opens as a highly Ca^{2+}-permeable channel and is only expressed in mammalian Chinese Hamster ovary (CHO) cells [11]. The reason for the functional silence for most of the clones above is unknown. One explanation is found in the requirement for an associated protein that introduces channels toward the membrane surface. Another reason might possibly be an intracellular mammalian specific regulatory protein which may inhibit ion permeation of these channels. Because of the high degree of expression of channel molecule in these cells, the associated proteins should be abundant in situ.

During an investigation of associated proteins, we found frequent binding of an enzyme, protease, to the C-terminal adjacent to sixth TMS of VR-like SIC channel [19]. Genomic analysis revealed that VR-like 2 (AB021875) is more appropriate for doing further analysis than SIC, since VRl2 as well as VRl1 have homology to VR1. Using the following cDNA clones: VRl1 (AB022332) and VRl2, MLS-like clones (MLS3), and human PKD2, we explored their functional expressions by modifying this enzyme in mammalian CHO cells.

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[1] Sequence data have been submitted to the DDBJ/EMBL/GenBank databases under accession numbers AB021875 (VRL2) and AB022332 (VRL1), respectively.

Abbreviations: CHO, Chinese hamster ovary

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2. Materials and methods

2.1. Two-hybrid screening

To investigate associated proteins of a VR family member (VRl2), the C-terminal amino acid (A.A. #736-end) was employed as bait for binding proteins from a mouse kidney library by yeast two-hybrid screening (Clontech, Matchmaker ver. 2, CA, USA).

2.2. Expression of cDNA and detection of the VRl2 protein

The cloned cDNAs (VRl1 and VRl2) were ligated to the mammalian expression vector, pCMV-SPORT (Gibco-BRL, MD, USA). Human PKD2 cDNA was inserted into pCEP4 (Invitrogen, MD, USA). Six-histidine-tag was added to the C-terminal using a pHM6 vector (Roche Molecular Biochemicals, Germany). Northern blot, Western blot, and the expression in *Xenopus* oocytes were performed as previously described [20]. The membrane fraction of the oocytes (0.1 mg) was incubated with Ca-dependent protease (5 U/ml, Sigma, St. Louis, MO, USA) in buffer containing 10 mM dithiothreitol, 10% glycerol, 100 mM HEPES, 1 mM ATP and 5 mM $CaCl_2$ for 3 h at 37°C. The reaction was modified by incubation either with calpastatin (0.1 mM), leupeptin (1 mM), E64 (1 mM) or EGTA (5 mM). A part of the samples was loaded to SDS-denatured acrylamide gel, in which VRl2 was detected by an antibody against the C-terminal (PKWRTDDAPL). The specificity of this antibody was determined by using an excess amount of antigen.

To obtain VRl2-expressed membrane of mammalian cells, CD4 and VRl2 were co-transfected to CHO cells. They were incubated with 10 μM dE64 (membrane-permeable E64) in bath solution containing 140 mM NaCl, 1 mM $MgCl_2$, 3 mM HEPES (pH 7.4) with 5 mM $CaCl_2$ for 1 h. The cells were homogenized by 10 strokes and centrifuged at $5000 \times g$ for 10 min in 0.3 M sucrose, 25 mM imidazole, 1 mM EDTA, and 1 mM PMSF. The supernatant was incubated with anti-CD4 antibody-coated glass-beads (Dynabeads M-450, Dynal, Oslo, Norway) for 20 min at room temperature. The CD4-positive membrane fraction was then collected by brief centrifugation ($5000 \times g$ for 1 min) and dissolved in RIPA (150 mM NaCl, 50 mM Tris pH 7.4, 0.05% NP40, and 0.1% SDS). The sample of 0.01 mg was loaded to an SDS-denatured gel, in which VRl2 and CD4 were detected by anti-His antibody (anti-His6 HRP, Invitrogen) and anti-CD4 antibody (CD4 Ab2, NeoMarkers, CA, USA), respectively. The immune complex was detected by ECL™ (Amersham, OH, USA).

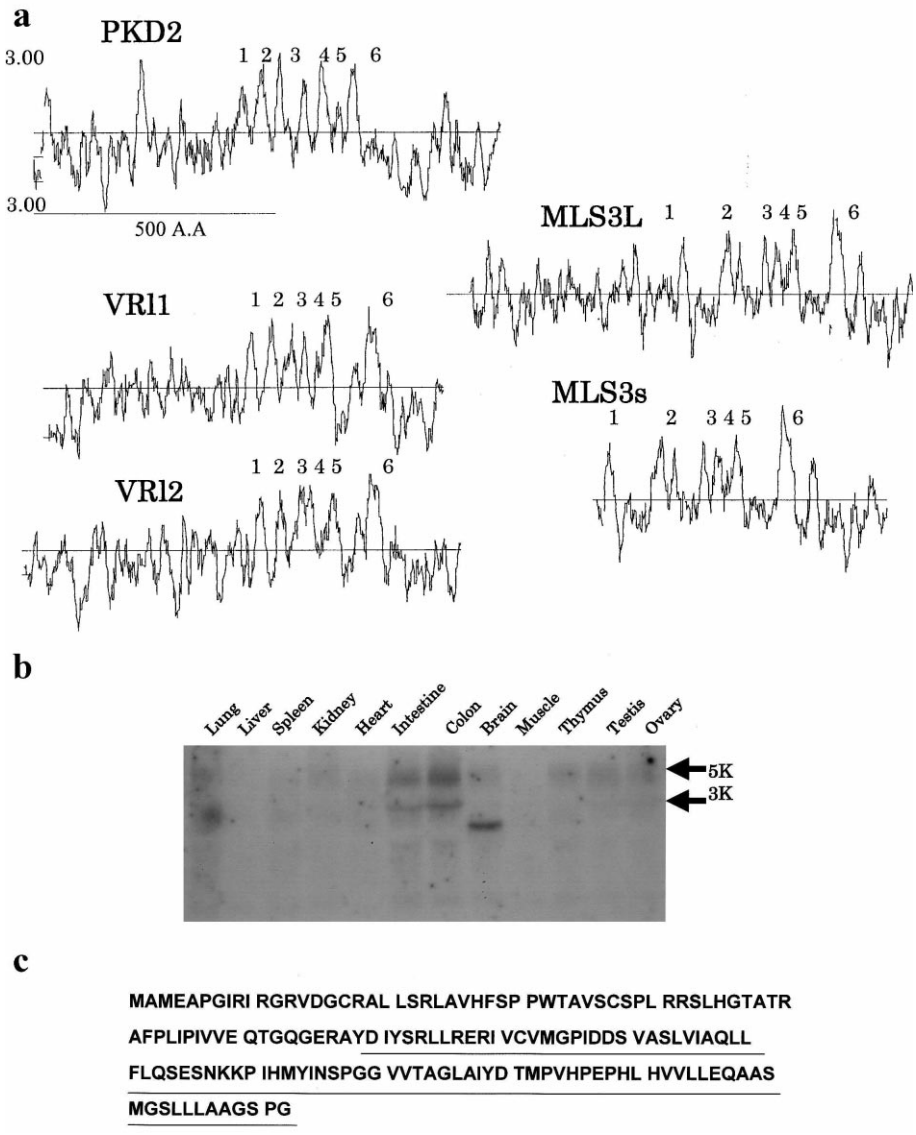

Fig. 1. Molecules examined in this study. a: Hydropathy plot of VRl1, VRl2, PKD2, MLS3L, and MLS3S reveals putative six TMSs (1–6) by Kyte–Doolittle. A.A.: amino acid number. b: Northern blot analysis of the expression of MLS3 mRNA was performed. Full-length cDNA was used as a probe. 2 μg of total RNA from mouse tissues was charged per lane. The migration positions of rRNA are indicated on the left. Arrows indicate long MLS3L around 5 kb and short MLS3S around 3 kb. c: Cloned amino acids alignment of the binding protein to VRl2 C-terminal in mouse kidney library. Line indicates a common alignment of an active center of Ca-dependent protease.

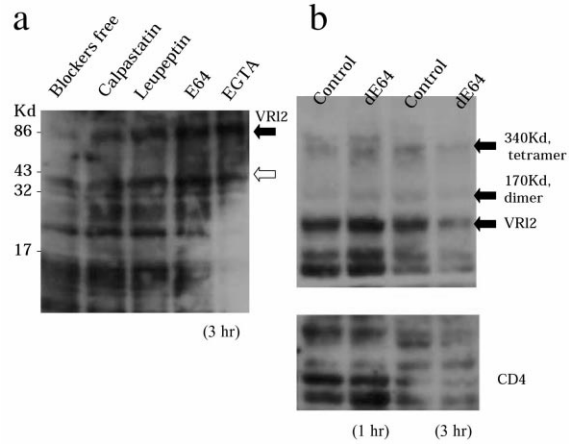

Fig. 2. Calpain-dependent cleavage of VRl1 and VRl2 and influence of E64d. a: VRl2-expressed *Xenopus* oocyte membrane was incubated with 5 mM $CaCl_2$, 10 μM calpain inhibitors (lanes 2–4) or 5 mM EGTA for 3 h. VRl2 protein was detected by C-terminal antibody. Closed arrow indicates VRl2 protein and open arrow indicates a non-specific protein, which remained after incubation with an excess amount of antigen. b: Influence of cell-permeable calpain blocker, dE64, on the expression of VRl2 was examined. VRl2-expressed CHO cells were incubated with 5 mM $CaCl_2$, or 10 μM dE64 for 1 h (lanes 1 and 2) and 3 h (lanes 3 and 4). VRl2 protein was detected by anti-His6 antibody. Arrow indicates VRl2 corresponding bands, monomer, dimer and tetramer (upper panel). The membrane protein, CD4, was concomitantly detected by anti-CD4 antibody. Arrow indicates the corresponding band (lower panel).

2.3. Electrophysiology

Patch clamp recordings were carried out according to methods described previously [11,19]. The GFP-positive cells were visualized by fluorescence measurement (CAM 2000 System, Jasco, Tokyo, Japan) with an emission of 490 nm. Currents were recorded at room temperature with an EPC-9 patch clamp amplifier (HEKA, Pfalz, Germany). Applied voltage and sampling were controlled by a computer system (HEKA, Pfalz, Germany). The bath solution contained 140 mM NaCl, 1.0 mM $MgCl_2$, 1.0 mM $CaCl_2$, and 3 mM HEPES (pH 7.4). The whole-cell patch pipette contained a filtered solution of 150 mM CsCl, 1 mM EGTA (Ca^{2+} = 200 nM adjusted by $CaCl_2$ to mimic intracellular milieu), and 3 mM HEPES (pH 7.2). The bath solution was substituted with 100 mM NMDGCl/40 mM NaCl, or with 100 mM Na aspartate/40 mM NaCl to calculate the permeability ratio of $NMDG^+$ or Cl^-. To measure the Ca^{2+} over Cs^+ permeability ratio, the bath solution underwent substitution with 50 mM $CaCl_2$, and 3 mM HEPES. The permeability ratio was calculated as described [21].

$$P_{Ca}/P_{Cs} = s\alpha Cs[Cs]I\{\exp(E_{rev}F/RT)(1+\exp(E_{rev}F/RT))\}$$

$$/4\alpha Ca[Ca]o\ (\alpha Cs = 0.75,\ \alpha Ca = 0.25)\ .$$

3. Results

3.1. Cloning of channels

VRl2 was cloned from specific SIC C-terminal alignment as a probe from a mouse kidney cDNA library. VRl2 was detected in lung and kidney by Northern blot. The BLAST program searched homology of Trp-like transmembrane alignment, resulting in an MLS-like structure with TRP7-like TMSs (accession numbers AK000235, AK000048) [22].

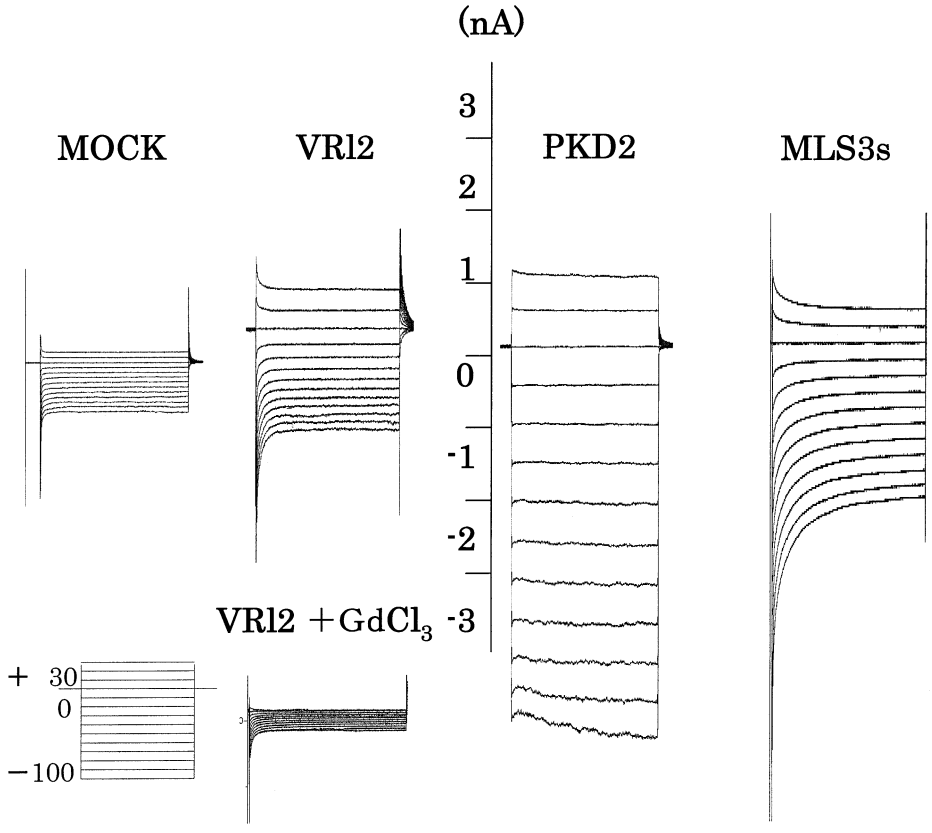

Fig. 3. Representative traces of cation channels. Whole cellular currents during voltage steps ranging from −100 mV to 30 mV (increment of 10 mV; holding potential 0 mV; duration of stimulation 100 ms) following expression of GFP marker (MOCK), and genes of interest (VRl2, PKD2, MRL3s) in CHO cells were recorded by patch clamp. Pipette contained 1 μM calpastatin for VRl2 and 1 μM leupeptin for MOCK, PKD2 and MRL3s. VRl2+GdCl3: $GdCl_3$ at 0.1 mM was added to VRl2 channel. Magnitude of the current is set to the scale provided in the center.

These two clones, named MLS3S and MLS3L in the present study, were purchased from Takara Co. (Osaka, Japan). The hydropathy of the channels examined here is aligned for comparison (Fig. 1a). All the channels have putative six TMS with a possible pore region between the fifth and sixth TMS. MLS3 is highly expressed in the gastrointestinal tract and possesses two isoforms, 5 kb (MLS3L) and 3 kb (MLS3S), by Northern blot analysis (Fig. 1b).

3.2. Interaction of the protease and VRl1 and VRl2

Duplicated screenings for binding proteins for C-terminal were examined, resulting in 80 positive clones. The majority of their sequences were equivalent to proteins in the nucleus or mitochondria, three to unknown enzymes and two to the following enzymes: aldehyde reductase and calpain. The sequence of the clone (Fig. 1c) revealed it to be homologous to an active center of the Ca^{2+}-dependent cysteine protease, calpain. Potency of GAL4 promoter was estimated by β-galactosidase activity under the manufacturer's protocol (ONPG method). Co-expression of the bait and the clone resulted in 56 units as compared to 10 in the negative control.

Various forms of the enzyme (CAPN1–13) have been extensively studied. Since the clone was only a fragment of the catalytic domain, we could not identify which molecule it was of this family [23,24]. Calpain is activated at a Ca^{2+} concentration which exceeds that necessary to digest a target protein. Several specific blockers are useful: calpastatin, leupeptin, and E64 were employed in the present study. Since many associated proteins may modify the cleavage in mammalian cells, the membrane fraction of VRl2-expressed *Xenopus* oocytes was used. It was incubated with calpain for 0.5, 1 and 3 h at 37°C. The cleavage of this protein was detected at 3 h by C-terminal antibody and protection of this cleavage was achieved with either calpastatin, leupeptin, E64 or EGTA (Ca free) (Fig. 2a). Thus VRl2 (and VRl1, data not shown) becomes a substrate for degradation by the enzyme. Further, to elucidate the effect of the blocker in CHO cells on VRl2 expression, tag-protein was added to enhance sensitivity of detection. The VRl2-transfected cultures were incubated with or without cell-permeable E64d (10 μM, 1 h and 3 h) in bath solution. CD4 is an antigen expressed in the membrane. Using CD4 as a marker to purify the plasma mem-

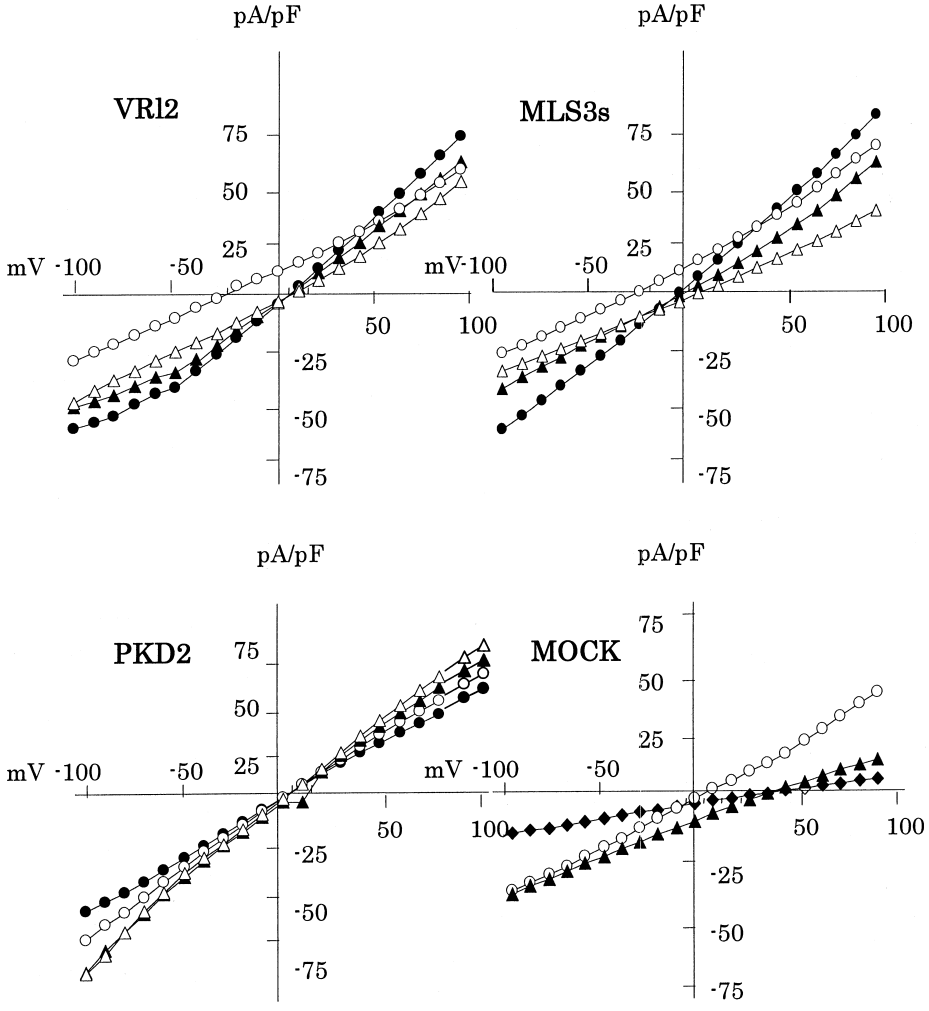

Fig. 4. Current–voltage relations of cation channels during substitution of bath solution. Mean normalized currents are plotted to voltage in NaCl (open circle), to $CaCl_2$ (closed triangle) bath solution through NMDGCl (closed circle) and Na aspartate (open triangle). Pipette contained 1 μM calpastatin for VRl2 ($n=8$) and 1 μM leupeptin for MOCK ($n=4$), PKD2 ($n=8$) and MRL3s ($n=8$). MOCK was the current in CHO cells transfected by GFP marker fluorescence in pipette containing 1 μM E64. Open diamond indicates current in the pipette free from any blockers ($n=4$).

brane, the VRl2 expressed on the membrane was isolated from the whole cellular extracts. We tried to compare the density and ratio of VRl2/CD4 before and after treatment with dE64. The treatment, however, did not influence the amount of expression or the assembly of their subunits on the membrane in six repeated detections (Fig. 2b).

3.3. Expression of the ligand-gated channel families with blockers

The opening of these channels with the blockers is shown in Fig. 3. The control basal current without the blockers was measured in VRl1-, VRl2- MLS3S- or PKD2-transfected CHO cells. All of the basal currents fluctuated within ±0.2 nA in the voltage range from −100 mV to 100 mV with 10–30 pF cells. Thus we judged that they did not open in this setting.

When pipette solution contained calpastatin (1 μM) or leupeptin (1 μM), a significant current appeared within 1 min. In GFP-transfected control cells, outward-rectified current was noticed but not amplified over ±1 nA. The current was driven by Cl^-, since substitution of Cl^- with aspartate shifted the reversal potential (V_{rev}) to positive (20 mV <) and addition of 0.1 mM Gd^{3+} did not influence the current ($n = 4$). VR1 and VRl2 revealed outward-rectified currents driven by Na^+/Cs^+, and this is characteristic of this family. The magnitude of the current was over ±2 nA, and it was completely blocked by 0.1 mM Gd^{3+} and not influenced by substitution of the anion. PKD2 showed a large current without a time-dependent component. On the other hand, MLS3S showed time-dependent fast decay, which is characteristic in the Trp family and in CCE (capacitive calcium entry) [3–5,22]. Both openings were blunted by 0.1 mM Gd^{3+}.

Next, current–voltage relations and ion selectivity of these channels were examined (Fig. 4, Table 1). The bath solution was changed from NaCl to $CaCl_2$ by using NMDGCl and Na aspartate. Reversal potentials of these currents were altered during the substitution of the solutions. These voltages of eight experiments in each channel provided P_X/P_{Cs} as summarized in Table 1. PKD2 showed less selectivity to both cations and anions, as compared to the other three. After the measurement, the currents driven by cations were abolished by addition of $GdCl_3$. If the current was not abolished (resistance over 100 MΩ), the data were discarded.

The opening of a channel may not be related to the kind of blocker. For VRl2, calpasatatin was the primary opener selected, giving 18 openings on 20 patches. The following two trials (with leupeptin or E64) provided successful openings (two openings on two patches). We thus considered calpasatatin to be the primary opener. In the same way, leupeptin for VRl1 and PKD2, and E64 for MLS3 were primary openers. We used the primary openers to obtain the results in Table 1.

Table 1
Permeability ratio over Cs of cation channels

	Na	NMDG	Cl	Ca
VRl2	1.1	0.01 <	0.01 <	9
VRl1	0.9	0.01 <	0.01 <	9
PKD2	1.1	1	0.1	4.8
MLS3S	2.2	0.01 <	0.01 <	4

Permeability ratio is calculated by mean reversal potentials during substitution of the solution ($n = 8$).

4. Discussion

Among the binding proteins to VRl2, we found a candidate, calpain, which modifies their function. The blockers are highly specific to the enzyme [23]. However, the effect on the cation channel appeared to be non-specific, since the blockers activated unrelated but structurally similar molecules, PKD2 and MLS3, in addition to a related molecule, VRl1. Furthermore, the blockers altered the current in untransfected–basal control. CHO cells generally revealed a little underlying Cl^- current in the present setting. The current was exaggerated by using the blocker. Therefore, discrimination between the expressed and underlying current is very important. The underlying current was driven by Cl^- as suggested by the ion substitution study. Most of the current was blocked by 0.1 mM flufenamate, but not altered by 0.1 mM $GdCl_3$. It did not exceed 1 nA (normalized current of 0.05 nA/pF). Current–voltage relation of the underlying current was slightly outwardly rectified. VRl2 and VRl1 showed similarly rectified currents. They were, however, completely blocked by 0.1 mM $GdCl_3$ and their reversal potential was not affected by substitution of the anion. We can therefore identify the opening by the magnitude of the current and the blockade of $GdCl_3$ added after the measurement.

We thought that the blocker might open an endogenous cation channel. That appeared unlikely however, since PKD2 opened by the blocker revealed its own characteristics such as linear current–voltage and poor selectivity for NMDG [14,15]. Generally, expression of PKD2 has not been achieved in mammalian cells. PKD1 is required to open the PKD2 channel [15]. Otherwise, PKD2 successfully expresses in insect cells [14]. Both reports [14,15] represent an electrophysiologic characteristic similar to the present results. Therefore, calpain opened the exogenous PKD2 cDNA rather than the modified endogenous ones.

MLS is a gene which has not been expressed as a channel in mammalian cells. The structure with six TMSs is similar to that of the Trp family. MLS, however, has a characteristic long N-terminal, though it is not known to have function as a channel. Likewise, VRl1, VRl2, and PKD2 have six TMSs with a hydrophobic long N-terminal. We found that the TMS of MLS3 had a significant homology to Trp7 and its N-terminal had a homology to MLS. MLS3S lacks the long N-terminal and co-localizes with its long form in intestine and colon. We tried to detect current in CHO cells with co-transfection of the two clones, but failed to detect a significant (over 0.5 nA at −100 mV) current ($n = 6$, data not shown). Thus we do not find a meaning in the presence of the two forms, MLS3L and S. One of the characteristics of Trp7 currents is a time-dependent rapid decay [22], which was observed in the expression of MLS3S in the present study. Thus MLS might become a channel with a certain stimulus or milieu.

The mechanism of the opening by the blocker is not known, but it at least involves a cleavage of C-terminal by the enzyme. VRl1 and VRl2 were cleaved by calpain but the time course of the functional activation and biochemical cleavage was quite different. We tried to detect another possibility that the blocker modified the expression toward the membrane or assembly of the subunit. We note that the histidine-tagged protein gains resolution to identify the labeled protein [25]. Thus we employed this strategy to clarify this possibility.

However, the results did not suggest this possibility. Even with the negative results, there are still other possibilities than cleavage underlying the mechanism of the activation. Our hybrid study that suggests the fact that the active center of calpain binds to C-terminal alignment might be used to argue that degrades would be less than 30 K_d by using the C-terminal antibody. The cleavage by calpain of VR1l and VR1l2 revealed several degrades, of sizes higher than expected. Since the C-terminal of VR1l2 is a site to bind calpain, we made N- or C-terminal deleted mutants. Surprisingly, both the mutants were able to induce the Gd^{3+}-sensitive outward-rectified current when the pipette contained E64, though not so frequently (4/10 in N-deleted and 2/10 in C-deleted mutants, data not shown). Therefore, the mechanism of the opening by the blockers still involves possibilities other than protection of the cleavage by calpain.

There are some data that calpain regulates calcium influx through voltage-dependent [26,27] NMDA receptor [28] and leaky Ca^{2+} entry [29]. The mechanism of run-down of voltage-dependent Ca^{2+} channel in heart involves calpain cleavage. The α-subunit is reported to be site-responsive to this mechanism. However, several studies agree that proteolysis is only partially responsible for the run-down, and therefore unidentified factor(s) in the cytoplasm contribute to the mechanism [30]. Therefore, there might be factor(s) related to calpain that up-regulate not only the voltage-dependent Ca^{2+} channel but also the Ca^{2+}-permeable non-selective cation channels and even possibly the underlying Cl^- channels. Activation of the ligand-gated cation channel families by calpain may be suggested by non-specific Ca^{2+} leakage as measured by fluorescence dye [29]. However, none of the direct evidence for this activation has been described at the molecular level. As far as we know, this is the first report that blockers against calpain are useful in the investigation of ligand-gated cation channels when they are functionally silent, although the molecular mechanism or relevance to physiologic regulation remains obscure.

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