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# Negatively-charged Amino Acids near and in Transient Receptor Potential (TRP) Domain of TRPM4 Channel Are One Determinant of Its $Ca^{2+}$ Sensitivity

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Running title: Amino acids in TRP domain of TRPM4 affect $Ca^{2+}$ sensitivity

**Keywords:** Ion channel; Transient receptor potential channels (TRP channels); Calcium; Manganese; Phosphoinositide; Electrophysiology; Site-directed mutagenesis; Molecular biology; Physiology

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**Background:** Transient receptor potential melastatin 4 (TRPM4) channel is activated by intracellular $Ca^{2+}$.

**Results:** Cobalt potentiated activation of TRPM4 by $Ca^{2+}$. Mutations of negatively charged amino acids in TRP domain reduced $Ca^{2+}$ sensitivity.

**Conclusion:** The acidic amino acids are required for the proper activation of TRPM4 by $Ca^{2+}$.

**Significance:** A novel role of TRP domain in TRPM4 was suggested.

## ABSTRACT

**Transient Receptor Potential (TRP) channel Melastatin subfamily member 4 (TRPM4) is a broadly expressed nonselective monovalent cation channel. TRPM4 is activated by membrane depolarization and intracellular $Ca^{2+}$, which is essential for the activation. The $Ca^{2+}$ sensitivity is known to be regulated by calmodulin and membrane phosphoinositides, such as PI(4,5)$P_2$. Although these regulators must play important roles in controlling TRPM4 activity, mutation analyses of the calmodulin binding sites have suggested that $Ca^{2+}$ binds to TRPM4 directly. However, the intrinsic binding sites in TRPM4 remain to be elucidated. Here, by using patch-clamp and molecular biological techniques, we show that**

**there are at least two functionally different divalent cation binding sites and the negatively charged amino acids near and in the TRP domain in C-terminal tail of TRPM4 (D1049 and E1062 of rat TRPM4) are required for maintaining the normal $Ca^{2+}$ sensitivity of one of the binding sites. Applications of $Co^{2+}$, $Mn^{2+}$, or $Ni^{2+}$ to the cytosolic side potentiated TRPM4 currents, increased the $Ca^{2+}$ sensitivity, but were unable to evoke TRPM4 currents without $Ca^{2+}$. Mutations of the acidic amino acids near and in the TRP domain, which are conserved in TRPM2, TRPM5, and TRPM8, deteriorated the $Ca^{2+}$ sensitivity in the presence of $Co^{2+}$ or $PI(4,5)P_2$ but hardly affected the sensitivity to $Co^{2+}$ and $PI(4,5)P_2$. These results suggest a novel role of the TRP domain in TRPM4 as a site responsible for maintaining the normal $Ca^{2+}$ sensitivity. These findings provide more insights into the molecular mechanisms of the regulation of TRPM4 by $Ca^{2+}$.**

Transient Receptor Potential Channel Melastatin (TRPM), a subfamily of the TRP ion channel, consists of eight channels, TRPM1 to TRPM8. Among the TRPM channels, TRPM4, as well as TRPM5, forms a $Ca^{2+}$-activated nonselective monovalent cation channel, which does not conduct divalent cations such as $Ca^{2+}$ (1) although other TRPM channels permeate them (2). TRPM4 does not differentiate $Na^+$ and $K^+$ (3,4), and its opening affects cell functions through membrane depolarization. Unlike TRPM5, TRPM4 has a relatively broad tissue expression pattern (4). In those tissues, TRPM4 has been implicated in several physiological functions, for example, immune response (5-7) and constriction of cerebral arteries (8,9). Additionally, its mutations in the TRPM4 gene have been associated with cardiac conduction dysfunction in human patients (4,10-12). Furthermore, it has been shown that TRPM4 mediates axonal and neuronal degeneration in experimental autoimmune encephalomyelitis and multiple sclerosis (13).

TRPM4 channel activity is increased by membrane depolarization, but it absolutely requires intracellular $Ca^{2+}$ (14). Thus, the most important regulator of TRPM4 activity is the intracellular $Ca^{2+}$. However, the activation mechanisms of TRPM4 by $Ca^{2+}$ have not been completely clarified. Calmodulin is thought to play an important role in the activation of TRPM4 by $Ca^{2+}$ through its binding to the C-terminal tail of TRPM4 because it has been reported that deletion mutants of calmodulin binding sites showed strongly impaired current activation by reducing $Ca^{2+}$ sensitivity (14). For example, although wild-type TRPM4 has shown large currents in the presence of 100 μM $Ca^{2+}$, the mutants have shown negligible currents under the same conditions (14). However, the mutants were still able to be activated by very high concentrations (e.g. 1 mM) of $Ca^{2+}$ and positive voltages (14). Furthermore, TRPM5, which shows the highest homology to TRPM4 (4), has been suggested to be activated by $Ca^{2+}$ directly rather than through calmodulin because calmodulin modulators did not affect TRPM5 (15). These

findings imply that there are unidentified intrinsic $Ca^{2+}$ binding sites in TRPM4 as mentioned elsewhere (4,14). Moreover, a membrane phospholipid, Phosphatidylinositol-4,5-bisphosphate ($PI(4,5)P_2$, $PIP_2$), has been shown to restore the $Ca^{2+}$ sensitivity of TRPM4 after desensitization (16,17). For example, the $EC_{50}$ for $Ca^{2+}$ after desensitization was reported to be 520 μM, and that after application of $PIP_2$ was 120 μM (16). Positively charged amino acids in a C-terminal pleckstrin homology (PH) domain were identified as important determinants of $PIP_2$ action (17). However, the mechanism of how $PIP_2$ increases the $Ca^{2+}$ sensitivity of TRPM4 has not been revealed.

The C-terminal cytosolic tail of TRPM4, which is important for the regulation of its activity, contains the TRP domain and TRP box. The TRP domain refers to a homologous block of about 25 residues immediately C-terminal to S6 that is loosely conserved in almost all TRP mammalian subfamilies (18). The TRP domain encompasses a highly conserved 6-amino acid TRP box (18). The TRP domain of TRPM8, TRPM5, and TRPV5 has been suggested to serve as a $PIP_2$-interacting site (19). However, it has been shown that the TRP box and TRP domain of TRPM4 are not the main determinants of $PIP_2$ action (17). Therefore, the functional role of the TRP domain and TRP box in TRPM4 remains elusive.

$Ca^{2+}$ binding sites of $Ca^{2+}$-regulated proteins exhibit diverse divalent cation selectivities. Thus, the divalent cation selectivities of binding sites have been used as a powerful tool for distinguishing properties of different $Ca^{2+}$ binding sites in conjunction with the molecular biological approaches. For example, it has been shown that the large-conductance $Ca^{2+}$-activated $K^+$ channel, BK channel, has three divalent cation binding sites, the so-called $Ca^{2+}$-bowl, RCK1 domain, and E399-related low-affinity sites, of which divalent cation selectivities are different (20,21). On the basis of such an idea, it has been shown that $Sr^{2+}$ and $Ba^{2+}$ do not substitute for $Ca^{2+}$ in TRPM4 activation (22). However, not much has been done to reveal the overall mechanisms of the activation of TRPM4 by $Ca^{2+}$, such as the number of binding sites in TRPM4 and their roles in the activation by $Ca^{2+}$.

The objective of this paper is to obtain further understanding of the mechanisms underlying the activation of TRPM4 by intracellular $Ca^{2+}$. In order to reveal the properties of divalent cation binding sites of TRPM4, we firstly examined the effects of larger variety of divalent cations applied to the cytosolic side of the channel. Secondly, we explored the amino acid residues responsible for the activation by $Ca^{2+}$ using single amino acid mutagenesis approaches. Among several mutants of the amino acid residues in the cytosolic C-terminal tail of TRPM4, we found two negatively-charged amino acids near and in the TRP domain of TRPM4 to be important determinants of $Ca^{2+}$ sensitivity.

## EXPERIMENTAL PROCEDURES

*Animal Ethics Approval* − All animal experiments

were performed in accordance with guidelines from and protocols approved by the Institutional Animal Care and Use Committee (IACUC), Graduate School of Veterinary Medicine, Hokkaido University and the Committee on Animal Experimentation, Niigata University School of Medicine.

*Molecular cloning and Site-directed Mutagenesis* − TRPM4b, the long form of TRPM4, forms a functional channel and considered to be the significant variant (3). Therefore, we refer to TRPM4b as TRPM4 in this paper. Rat TRPM4 was cloned from mRNA of the stria vascularis in the cochlea because $Ca^{2+}$-activated nonselective currents were recorded from the apical membrane of marginal cells in freshly isolated stria vascularis using inside-out mode of patch clamp technique (data not shown) as similarly reported from those of guinea pig (23) and gerbil (24). Additionally, the expression of TRPM4 in the marginal cells has been confirmed by immunohistochemistry more recently (25). RNA was extracted from stria vascularis in the cochleae of BN/SsNSlc male rats (5-6 weeks old) using NucleoSpin RNA XS (Takara Bio, Otsu, Japan). cDNA was synthesized using PrimeScript II Reverse Transcriptase (Takara Bio). The full length of the open reading frame of TRPM4 cDNA was amplified as two overlapped N-terminal and C-terminal fragments by PCR using a high fidelity polymerase (PrimeSTAR GXL, Takara Bio) and the following primers: 5' GGC GGC TGA GAG AAA TAC ACG GAG C 3' (N-terminal Forward) and 5' GTC ACT CCA GGG GGC TTG TTC AAA G 3' (N-terminal Reverse), 5' AAC TTT TCC GTG GGG ACA TCC AGT G 3' (C-terminal Forward) and 5' CAT GGG GTC TAC GGT GAG GAC AAG G 3' (C-terminal Reverse), respectively. The primers were designed based on the reported sequence of rat TRPM4 cDNA (Genbank accession #NM_001136229.1). The two fragments of TRPM4 were cloned in TA-vector (Takara Bio) and sequenced. The nucleic acid and amino acid sequences of the cloned rat TRPM4 were identical to those recorded in database (#NM_001136229.1 and #NP_001129701.1, respectively (26)). The N-terminal and C-terminal fragments, which contained no PCR errors, were subcloned and combined in a bicistronic expression vector pIRES2-EGFP (Takara Bio). Site-directed mutagenesis of TRPM4 cDNA in pIRES2-EGFP was accomplished using the PrimeSTAR Mutagenesis Basal kit (Takara Bio). Mutations were verified by DNA sequencing.

*Cell culture and transfection* − HEK 293T cells were cultured in DMEM (Dulbecco's modified Eagle's medium; Sigma-aldrich, St. Louis, MO) supplemented with 10% FBS (Moregate Biotech, Bulimba, Australia, or Thermo Fisher Scientific, Waltham, MA. Their local distributors were Hana-nesco Bio, Tokyo, Japan and Thermo Fisher Scientific K.K., Yokohama, Japan, respectively.) and penicillin/streptomycin (1000 U/ml and 1000 μg/ml, respectively, Thermo Fisher Scientific) at 37 °C in a 5% $CO_2$ incubator. Cells were

transiently transfected with plasmids using TransIT-293 Transfection Reagent (Takara Bio). The cells were plated on coverslips the following day. Patch-clamp recordings were made two days after transfection from EGFP positive cells, which were identified with an inverted microscope (Diaphot 300, Nikon, Tokyo, Japan) equipped with a super high-pressure mercury lamp light source (C-SHG, Nikon) for excitation of green fluorescence from EGFP.

*Electrophysiology* − HEK 293T cells on coverslips were transferred to a bath mounted on the stage of the inverted microscope and superfused with a standard NaCl rich solution containing 145 mM NaCl, 5 mM KCl, 1 mM $MgCl_2$, 1 mM $CaCl_2$, 10 mM D-glucose, and 10 mM HEPES (pH = 7.4 with NaOH). The pipette solution contained 145 mM NaCl, 1 mM $MgCl_2$, 1 mM $CaCl_2$, and 10 mM HEPES (pH = 7.4). Before patch excision, the bath solution was changed to mainly a solution containing 145 mM NaCl, 10 mM HEPES (pH = 7.4), and 1 mM $CaCl_2$. In many cases, a divalent cation-free solution contained 5 mM EGTA to chelate divalent cations but in some experiments, a nominally divalent cation-free solution was also used, which was made simply without adding divalent cations. Dichloride salts of divalent cation were used. The concentration of free $Ca^{2+}$ in solutions less than 10 μM was adjusted by adding an appropriate amount of $CaCl_2$, calculated using a software webmaxc (http://www.stanford.edu/~cpatton/webmaxcS.htm ), to solutions containing 5 mM EGTA. In some experiments, o-phenylenedioxydiacetic acid (o-PDDA, Sigma-aldrich) was used as a divalent cation chelator and free divalent cation concentrations were calculated with the stability constants reported elsewhere (27). Na-fluoride (NaF, 145 mM) was also used as a $Ca^{2+}$-chelator (21,28). Osmolality of solutions were measured using Vapro Vapor Pressure 5600 (Wescor Inc, Logan, UT, USA). The osmolality of most solutions were near physiological range e.g. that of the pipette solution was 283 ±2 mOsm/kg, that of nominal $Ca^{2+}$-free solution was 276 ±1 mOsm/kg, and that of the solution containing 10 mM $CaCl_2$ was 301 ±0 mOsm/kg. However, the osmolality of the solution containing 30 mM $CaCl_2$ was 352 ±1 mOsm/kg. The results of the present study might be partially affected by the change in osmolality as a background effect. The speed of perfusion was about 1.5 ml/min. The bath solution around a patch membrane was cleared within 10 sec in most cases or within 15 sec at the latest.

Axopatch 200B patch-clamp amplifier, digidata 1322A, and the pCLAMP8 software (Axon Instruments, Union City, CA) were used to perform voltage clamp, data storage, and analysis. A reference Ag–AgCl electrode was connected into the bathing solution via an agar bridge filled with the aforementioned standard NaCl-rich bath solution. Patch electrodes had a resistance between 3 and 5 megaohms. The currents were filtered at 1 kHz with an internal four-pole Bessel

filter, and sampled at 5 kHz. Current–voltage (*I-V*) relations for the currents were studied using voltage ramps. The membrane potential held at −60 mV, and the command voltage was varied from −100 to +100 mV over a duration of 400 ms following a prepulse of −100 mV for 50 ms every 5 s. Because TRPM4 is activated by membrane depolarization, we analyzed the current amplitudes at −100 mV and +100 mV as the least and the most activated current among the currents evoked by the applied pulse, respectively. Furthermore, because the current amplitudes at −100 mV in some mutants were negligible, the current amplitudes at +100 mV were used for the analysis of dose-response. All experiments were performed at room temperature.

*Data analysis* − Dose-response curves were fits of the averages with the Hill equation:

$$I = I_{max} \times \frac{C^n}{{EC_{50}}^n \times C^n}$$

where $I_{max}$ is the maximum currents, $C$ is the concentration of substance being tested, $EC_{50}$ is the concentration for half-maximal effect, $n$ is the Hill coefficient. The relationship between $EC_{50}$ for $Ca^{2+}$ and the concentration of $Co^{2+}$ was analyzed with the pseudo Hill equation:

$$y = y_{max} + (y_{min} - y_{max}) \times \frac{C^n}{{EC_{50}}^n \times C^n}$$

where $y_{max}$ is the $EC_{50}$ for $Ca^{2+}$ in the absence of $Co^{2+}$ and $y_{min}$ is the minimal $EC_{50}$ for $Ca^{2+}$ among those estimated in the presence of $Co^{2+}$, $C$ is the concentration of $Co^{2+}$, $EC_{50}$ is the concentration of $Co^{2+}$ for half-maximal effect, $n$ is the pseudo Hill coefficient. Chord conductance-voltage curves were fitted with the Bolzmann equation:

$$G = G_{max} \times (1 - \frac{1 - f}{1 + e^{(V_m - V_{1/2})/dx}})$$

where $G_{max}$ is fixed to the normalized conductance at +100 mV, $V_m$ is the membrane potential, $V_{1/2}$ is the membrane potential at which the conductance is half of $G_{max}$, $dx$ is the slope factor, and $f$ is the voltage-independent conductance fraction. All curves were obtained by fitting the data averages.

The results are reported as means ±S.E. of independent experiments (n), where n refers to the number of cells patched. Statistical significance was evaluated using Student's two-tailed paired or unpaired t test or Dunnett's test as appropriate. A value of $P < 0.05$ was considered significant.

## RESULTS

### *Effects of divalent cations on TRPM4 currents*

Rat TRPM4 showed outward rectifying currents after the inside-out patch excision in the presence of 1 mM $Ca^{2+}$ in the perfusate, and the currents were decreased probably mainly by a decline in the sensitivity of the channels to $Ca^{2+}$ (14), which is most likely due to the loss of $PIP_2$ as in well-studied human TRPM4 (16,17,29) (Fig. 1A). The TRPM4 currents were abolished when the $Ca^{2+}$ in the bath solution, which faces the cytoplasmic side of the ion channel, was removed (Fig. 1A). Although it has been reported that HEK 293 cells express endogenous TRPM4 (1,30), no $Ca^{2+}$-activated currents were recorded from our mock transfected HEK 293T cells, and on the

contrary the background outward currents were increased when $Ca^{2+}$ in the solution was removed (in the presence of 1 mM $Ca^{2+}$: 9.2 ±2.3 pA; in the absence of $Ca^{2+}$: 11.9 ±2.2 pA at +100 mV, $n$ = 8, typical *I-V* curves are shown in the inset to Fig. 1B). The discrepancy might be due to differences in cell clones or in culture conditions such as serum. The $Ca^{2+}$-inhibitable currents may be endogenous TRPM7 currents (31), which are inhibited by millimolar concentrations of intracellular $Ca^{2+}$ as well as $Mg^{2+}$ (32,33). At least as macroscopic currents the background endogenous TRPM4 currents had little effect on the analysis of heterologously expressed TRPM4 currents in our system.

First of all, we examined effects of divalent cations on the desensitized TRPM4 currents. After the current amplitudes became almost stable in the presence of 1 mM $Ca^{2+}$, 1 mM of several divalent cations ($Ca^{2+}$, $Co^{2+}$, $Mn^{2+}$, $Ni^{2+}$, $Mg^{2+}$, $Ba^{2+}$, $Sr^{2+}$, $Cd^{2+}$, and $Zn^{2+}$) were co-applied with 1 mM $Ca^{2+}$ to the intracellular perfusate. When the current amplitude was judged to have reached a steady state, the relation of the current amplitude to the previous data value at +100 mV was 98.8 ±0.6% (n = 80) and that at −100 mV was 100.8 ±2.0% (n = 80). However, the current amplitudes were gradually slightly decreased throughout the measurements in many cases. Therefore, in order to eliminate the influence of variations in current amplitudes among the time of measurement and among the patch membranes, the current amplitudes were normalized to those recorded in the presence of 1 mM $Ca^{2+}$ right before exposure to the other divalent cations. $Co^{2+}$, $Mn^{2+}$, and $Ni^{2+}$ potentiated TRPM4 currents (Fig. 1A-C), made the *I-V* curve of TRPM4 currents linear (Fig. 1B), and increased the voltage-independent conductance fraction (Fig. 1D). The currents potentiated by $Co^{2+}$ were inhibited by a TRPM4 inhibitor, fulfenamic acid (34) (Sigma-aldrich, 100 μM, Fig. 2). However, 100 μM FA did not completely inhibit the $Co^{2+}$-potentiated TRPM4 currents. The slight reduction of the inhibition by FA might be due to an allosteric change of TRPM4 structure by the binding of $Co^{2+}$, which may also cause the increase in the current amplitudes. In excised patches from mock transfected cells, 1 mM $Co^{2+}$ or even 10 mM $Mn^{2+}$, co-applied with 1 mM $Ca^{2+}$, did not evoke any currents in comparison with the $Ca^{2+}$-free condition (inset to Fig. 1B, $n$ = 6). $Mg^{2+}$ had no effect on TRPM4 currents (Fig. 3). $Cd^{2+}$ and $Zn^{2+}$ abolished TRPM4 currents and chelation with EDTA was required to reactivate TRPM4 currents fully with $Ca^{2+}$ (Fig. 3). Effects of $Ba^{2+}$ and $Sr^{2+}$ were not consistent; they blocked TRPM4 currents 4 out of 7 ($Ba^{2+}$) or 4 out of 8 ($Sr^{2+}$) membrane-patch recordings, respectively, but showed no effect on the currents in other cases (Fig. 3). The results, which indicate that $Ba^{2+}$ and $Sr^{2+}$ at least do not activate TRPM4, are partially consistent with the report that $Ba^{2+}$ and $Sr^{2+}$ cannot substitute for $Ca^{2+}$ in TRPM4 channel activation (22).

***Existence of two functionally different divalent cation binding sites***

Preliminary experiments using nominally $Ca^{2+}$-free solution showed that effects of $Co^{2+}$, $Mn^{2+}$, and $Ni^{2+}$ without $Ca^{2+}$ were none or weaker than those in the presence of 1 mM $Ca^{2+}$. Therefore, we assumed the effects of $Co^{2+}$, $Mn^{2+}$, and $Ni^{2+}$ were dependent on $Ca^{2+}$. To test the effects of $Co^{2+}$, $Mn^{2+}$, and $Ni^{2+}$ in the absence of $Ca^{2+}$, we used fluoride as a $Ca^{2+}$ chelator because common divalent cation chelators, such as EGTA and EDTA, bind to $Co^{2+}$, $Mn^{2+}$, and $Ni^{2+}$ with a higher affinity than $Ca^{2+}$. In solutions containing 145 mM NaF, because of the lack of solubility of $CaF_2$, the fluoride ion removes free $Ca^{2+}$ from such solutions so that the effective free $Ca^{2+}$ concentrations can be considered less than 20 nM (21,28). In contrast, 1 mM $Mn^{2+}$, $Co^{2+}$, and $Ni^{2+}$ remain soluble (21,28). Perfusion with the $Ca^{2+}$-free solution containing fluoride eliminated the TRPM4 currents at the same level as that containing EGTA (Fig. 4A). One mM $Mn^{2+}$, $Co^{2+}$, and $Ni^{2+}$ did not evoke any currents in the $Ca^{2+}$-free solution containing fluoride (Fig. 4A).

We cannot completely exclude the possibility that the fluoride itself inhibited TRPM4 and so that TRPM4 currents were not evoked by $Mn^{2+}$, $Co^{2+}$, and $Ni^{2+}$ in the presence of fluoride. Therefore, we also used another divalent cation chelator, *o*-phenylenedioxydiacetic acid (o-PDDA, the same with 1,2-Phenylenedioxydiacetic acid, Sigma-aldrich). The stability constants of o-PDDA for $Ca^{2+}$ and $Co^{2+}$ are 3.1 and 1.1, respectively (27), which means that o-PDDA binds to $Ca^{2+}$ with a higher affinity than $Co^{2+}$. On the other hand, the stability constants of $Mn^{2+}$ and $Ni^{2+}$ are 2.8 and 1.6, respectively (27). That means $Co^{2+}$ has the least inhibitory effect on the $Ca^{2+}$-chelating action of o-PDDA among these three divalent cations. Therefore, we used $Co^{2+}$ in this experiment. Even when the free $Ca^{2+}$ concentration was controlled by the chelation with 6 mM o-PDDA, 1 mM $Ca^{2+}$ evoked TRPM4 currents, and 1 mM $Co^{2+}$ potentiated the currents in the presence of $Ca^{2+}$ but did not evoke any currents in the absence of $Ca^{2+}$ (Fig. 4B).

These results indicate not only that the effects of $Co^{2+}$, $Mn^{2+}$, and $Ni^{2+}$ were dependent on $Ca^{2+}$ but also there are at least two functionally different divalent cation binding sites in TRPM4 and/or its associated proteins. One is a relatively $Ca^{2+}$-specific binding site, which has a negligible affinity for $Co^{2+}$, $Mn^{2+}$, and $Ni^{2+}$, and the other is a biding site for $Co^{2+}$, $Mn^{2+}$, and $Ni^{2+}$ (Fig. 4C). The $Ca^{2+}$-dependence of the effects of $Co^{2+}$, $Mn^{2+}$, and $Ni^{2+}$ implies that only the binding of divalent cations to the 2nd binding site do not open the TRPM4 channel and the binding of $Ca^{2+}$ to the 1st binding site is probably necessary to open the TRPM4 channel.

***Mutations of negatively-charged amino acids near and in TRP domain reduce $Ca^{2+}$sensitivity and voltage dependence***

To explore the divalent cation binding sites of TRPM4, we performed analysis of point mutations causing a single amino acid substitution in the cytosolic C-terminal region. Among the mutations of several negatively-charged acidic amino acids, we found mutations of two amino

acids near and in the TRP domain that altered the function of the TRPM4 channel. One is the aspartate just before the TRP domain (1049th aspartate: D1049 of rat TRPM4) and the other is the glutamate in the TRP domain (1062nd glutamate: E1062 of rat TRPM4) (Fig. 5A). These amino acids are conserved across species (*Mus muscles*: Genbank accession # NP_780339, *Homo sapiens*: #NP_001182156, *Pan troglodytes*: # JAA33534, and *Danio rerio*: # NP_001275744). Intriguingly, the amino acids are also conserved in the other (directly or indirectly) $Ca^{2+}$-sensitive TRPM channels, TRPM5 (3), TRPM2 (35), and TRPM8 (36) (Fig. 5A). The other acidic amino acids which we tested were E1112, D1133, D1136, D1138, E1140, D1150, E1161, D1163, E1170, and E1172. The mutants of these amino acids did not show clear differences from wild-type (WT) TRPM4.

After desensitization, WT TRPM4 currents were evoked by 0.3 mM $Ca^{2+}$ and saturated by 3 mM $Ca^{2+}$ at +100 mV (Fig. 5B and C). Analyses of the currents at +100 mV showed that the mutations of D1049 to asparagine (D1049N) and to alanine (D1049A) only slightly, and those of E1062 to glutamine (E1062Q) and to alanine (E1062A) comparatively largely reduced $Ca^{2+}$ sensitivity, respectively (Fig. 5B and C). The values of normalized current amplitudes of the mutants except D1049A were significantly different from those of WT at 1 and/or 3 mM $Ca^{2+}$. The $EC_{50}$ values for $Ca^{2+}$ and Hill coefficients were summarized in Table 1. The maximal current amplitudes of WT and mutants evoked by $Ca^{2+}$ were summarized in Table 2. The surface expression level, which is assumed from the current amplitudes, may be also affected by the mutations but it is not correlated to the $Ca^{2+}$ sensitivity. All the mutants maintained the reactivity to $Co^{2+}$, $Mn^{2+}$, and $Ni^{2+}$ since their currents were potentiated by application of 1 mM $Co^{2+}$ (Fig. 5B), $Mn^{2+}$, or $Ni^{2+}$ (Fig. 6). Double mutants of D1049N and E1062Q were active but their steady state currents were too small to analyze (data not shown).

The mutations of D1049 and E1062, except E1062A, changed also the voltage dependence. D1049N, D1049A, and E1062Q mutants lost the inward currents evoked by $Ca^{2+}$ at −100 mV (Fig. 5B). As shown in Figure 7A, the *I-V* relationships of D1049N, D1049A, and E1062Q mutants had a strongly outward rectification in the presence of 3 mM $Ca^{2+}$. Curiously, the *I-V* curve of E1062A resembled with that of WT TRPM4 (Fig. 7A). The ratios of currents at −100 mV to those at +100 mV of D1049N, D1049A, and E1062Q mutants were significantly smaller than that of WT TRPM4 (Fig. 7B). For each construct, *G-V* curves at different [$Ca^{2+}$] were obtained from currents evoked by ramp pulses and fit with Boltzmann functions (Fig. 7C). A raise in [$Ca^{2+}$] increased the voltage-insensitive conductance fraction of WT TRPM4 and the E1062A mutant but scarcely affected that of D1049N, D1049A, and E1062Q mutants (Fig. 7C). These results indicate that D1049 and E1062 are necessary for the normal $Ca^{2+}$ sensitivity and voltage dependence of TRPM4. Moreover, the mechanisms controlling

these two properties must be related but not completely coupled with each other since the E1062A mutation affected only one of them.

***Mutations of D1049 and E1062 decrease the $Ca^{2+}$ sensitivity of the apparently $Ca^{2+}$-specific binding site***

Which divalent cation binding sites, suggested by their different affinities for divalent cations, are affected by the mutations of D1049 and E1062? To answer the question, we evaluated the affinities of the 1st and the 2nd binding sites. First, we found that $Co^{2+}$ increased $Ca^{2+}$ sensitivity of WT TRPM4. As shown in Fig. 8A, in the presence of 1 mM $Co^{2+}$, $Mn^{2+}$, or $Ni^{2+}$, TRPM4 currents were substantially evoked by 0.1 mM $Ca^{2+}$. The $Ca^{2+}$ dose-response curves for TRPM4 currents were shifted to the left by the co-application of $Co^{2+}$ (Fig. 8B). $EC_{50}$ for $Ca^{2+}$ was decreased by the co-application of $Co^{2+}$ (e.g. 0.96 mM without $Co^{2+}$, 107 μM with 1 mM $Co^{2+}$). These results suggest that the binding of $Co^{2+}$ to the 2nd binding site increased the affinity for $Ca^{2+}$ of the 1st binding site. The $EC_{50}$ for $Co^{2+}$ was 57.5 μM, which was calculated from the shift of $EC_{50}$ for $Ca^{2+}$ and is deemed to reflect the affinity of the 2nd binding site.

$Ca^{2+}$ sensitivities of D1049N and E1062Q mutants were likewise increased by co-application of $Co^{2+}$ (Fig. 8C and 8D). The $EC_{50}$ for $Ca^{2+}$ of D1049N and E1062Q mutant currents were decreased by the co-application of $Co^{2+}$ as in WT TRPM4 although the absolute values of $EC_{50}$ for $Ca^{2+}$ were different from that of WT TRPM4 (Fig. 8E). The $EC_{50}$ for $Ca^{2+}$ normalized in the absence of $Co^{2+}$ are shown in the inset to Fig. 8E. The $EC_{50}$ for $Co^{2+}$ of D1049N and E1062Q were 72.4 μM and 126 μM, respectively, which were similar to the aforementioned $EC_{50}$ for $Co^{2+}$ of WT TRPM4 (57.5 μM, Table 1).

In all the constructs, 1 mM $Co^{2+}$ was high enough to reach maximum decrease in the $EC_{50}$ for $Ca^{2+}$ (Fig. 8E). Therefore, we evaluated the $EC_{50}$ for $Ca^{2+}$ of all the constructs in the presence of 1 mM $Co^{2+}$, which is deemed to reflect the affinity of the 1st binding site for $Ca^{2+}$ in the situation that the 2nd binding site is filled with $Co^{2+}$. The $Ca^{2+}$ dose-response curves of D1049 and E1062 mutants were shifted to the right in comparison with that of WT TRPM4 and the mutations of E1062 showed a more pronounced effect than those of D1049 (Fig. 8F). The values of normalized current amplitudes of the all mutants were significantly different from those of WT at 0.1, 0.3, and/or 1 mM $Ca^{2+}$.

As summarized in Table 1, the mutations of D1049 and E1062 increased the values of $EC_{50}$ for $Ca^{2+}$ in the presence of 1 mM $Co^{2+}$ rather than the values of $EC_{50}$ for $Co^{2+}$ as compared with WT TRPM4. Assuming that the $EC_{50}$ for $Ca^{2+}$ in the presence of 1 mM $Co^{2+}$ reflects the affinity of the 1st binding site and the $EC_{50}$ for $Co^{2+}$ reflects affinity of the 2nd binding site, mutations of D1049 and E1062 most likely mainly affected the 1st binding site and decreased its $Ca^{2+}$ sensitivity.

***Mutations of D1049 and E1062 reduce the $Ca^{2+}$ sensitivity in the presence of $PIP_2$ but do not***

***affect the sensitivity to $PIP_2$***

$PIP_2$ is a membrane phosphoinositide that strongly enhances TRPM4 activity by increasing the $Ca^{2+}$ sensitivity and shifting its voltage dependence towards negative potentials (16,17). It is important to reveal whether D1049 and E1062 play a role in the activation of TRPM4 by $Ca^{2+}$ under a more physiological condition, i.e. in the presence of $PIP_2$. A water-soluble form of $PIP_2$, diC8-PI(4,5)$P_2$ (30 μM, CellSignals, Columbus, OH), was applied to the cytosolic perfusate. In the presence of $PIP_2$, the currents of WT TRPM4 and also D1049N and E1062Q mutants were evoked by lower concentrations of $Ca^{2+}$ compared with the desensitized currents in the absence of $PIP_2$ (Fig.9A and compare with Fig. 5B). The $Ca^{2+}$ dose-response curves of WT TRPM4, D1049N, and E1062Q in the presence of $PIP_2$ show that mutations of D1049 and E1062 decrease $Ca^{2+}$ sensitivity which is elevated by $PIP_2$ (Fig. 9B). The $EC_{50}$ for $Ca^{2+}$ in the presence of $PIP_2$ are summarized in Table 1.

To exclude the possibility that the rightward shifts of $Ca^{2+}$ dose-response curves of D1049N and E1062Q mutants compared with WT TRPM4 were due to reduction of sensitivities for $PIP_2$, the affinities for $PIP_2$ of the constructs were evaluated. Typical time-courses of the currents of WT TRPM4, D1049N, and E1062Q in the presence of several concentrations of $PIP_2$ are shown in Fig. 9C. The $Ca^{2+}$ concentrations in perfusate were 0.1 mM, 0.3 mM, or 1 mM for WT TRPM4, D1049N, or E1062Q, respectively. They were close to the $EC_{50}$ for $Ca^{2+}$ of each construct in the presence of 30 μM $PIP_2$. The currents of all the constructs at +100 mV and also −100 mV were increased by $PIP_2$ (Fig. 9C). The dose-response curves for the effect of $PIP_2$ of WT TRPM4, D1049N, and E1062Q were similar (Fig. 9D). In particular, $EC_{50}$ for $PIP_2$ of WT TRPM4, D1049N, and E1062Q were almost the same (Table 1).

These results suggest that D1049 and E1062 are necessary for the normal $Ca^{2+}$ sensitivity maintained by binding with $PIP_2$ and moreover, $PIP_2$ probably increases the affinity for $Ca^{2+}$ of the 1st binding site, which is related to D1049 and E1062 (Fig. 10). Negligible effects of the mutations on the sensitivity to $PIP_2$ indicate that the structure of $PIP_2$ binding site in the cytosolic C-terminal tail of TRPM4 (17) was not disturbed by the mutations.

## DISCUSSION

The present study provides several new insights into the regulation of TRPM4 activity by intracellular $Ca^{2+}$ as summarized in Fig. 10. First, there are at least two functionally different divalent cation binding sites in TRPM4 and/or associated proteins. One is the comparatively $Ca^{2+}$-specific binding site and the other is the binding site for $Co^{2+}$, $Mn^{2+}$, and $Ni^{2+}$. A binding of $Ca^{2+}$ to the former (1st binding site) is required for the opening of TRPM4 channel. A binding of a divalent cation to the latter (2nd binding site) increases the $Ca^{2+}$ sensitivity of the 1st binding site and make the channel less voltage-dependent as in the effect of $PIP_2$. The relief of inactivation at hyperpolarized membrane potentials has a great

influence on the channel activity at physiological membrane potentials. Next, the mutations of D1049 and E1062 decreased their $Ca^{2+}$ sensitivity mainly by decreasing the $Ca^{2+}$ sensitivity of the 1st binding site, and they had little effect on the affinities of the 2nd binding site and the $PIP_2$ binding site. Additionally, the modulations of voltage dependence by $Co^2$ and $PIP_2$ must have not been affected by the mutations because the inward currents of the mutants were increased by them as in WT TRPM4. Finally, the binding of $Ca^{2+}$ to the 1st binding site (or at least a binding site other than the 2nd binding site) probably also makes the channel less voltage-dependent because the modulation of voltage dependence by high concentrations of $Ca^{2+}$ was lost by the D1049N, D1049A, or E1062Q mutations, which had little effect on the modulation of voltage dependence through the 2nd binding site.

The reason why we conclude the mutations of E1062 and D1049 affected primarily the 1st binding site (comparatively $Ca^{2+}$-specific binding site) rather than the 2nd binding site (binding site for $Co^{2+}$) is because the mutations of these amino acid residues increased the $EC_{50}$ for $Ca^{2+}$ in the presence of $Co^{2+}$ rather than the $EC_{50}$ for $Co^{2+}$. For example, the D1049N mutant showed about 4 times larger $EC_{50}$ for $Ca^{2+}$ in the presence of 1 mM $Co^{2+}$ than WT (shown in Fig. 8F and Table 1) but hardly affected the $EC_{50}$ for $Co^{2+}$ (WT: D1049N = 1: 1.3) (shown in Fig. 8E and Table 1). The former, $EC_{50}$ for $Ca^{2+}$ in the presence of 1 mM $Co^{2+}$, can be presumed to reflect the $Ca^{2+}$ affinity of the 1st binding site for the following reason. One mM $Co^{2+}$ is high enough to reduce $EC_{50}$ for $Ca^{2+}$ maximally, as shown in Fig. 8E, indicating that the effect of the binding of $Co^{2+}$ to the 2nd binding site was saturated. Thus, the increases in the currents by $Ca^{2+}$ in the presence of 1 mM $Co^{2+}$ were supposed to be due to the $Ca^{2+}$ binding to the 1st binding site. The latter, $EC_{50}$ for $Co^{2+}$, which was calculated from the effect of the decreases in $EC_{50}$ for $Ca^{2+}$ caused by $Co^{2+}$ shown in Fig. 8E, can be supposed to reflect the affinity for $Co^{2+}$ of the 2nd binding site because the $Co^{2+}$ affinity of the 1st binding site is inferred to be negligible.

Manganese and cobalt are essential mineral micronutrients for humans (37, 38). An important question is whether these divalent cations can regulate TRPM4 channel activity under physiological conditions. It was reported that the intracellular free $Mn^{2+}$ concentration in ovine brain tissue was below 0.5 μM (39). We were unable to find literature in which an absolute intracellular free $Co^{2+}$ concentration was measured under normal conditions. However, the serum concentration of cobalt in humans has been reported to be 1.8 nM, which is below that of manganese (7.3 nM) (40). Therefore, the intracellular concentration of free $Co^{2+}$ may be similar to that of free $Mn^{2+}$ or less. In the present study, more than 10 μM of $Co^{2+}$ (Fig. 8E) or $Mn^{2+}$ (data not shown) were necessary for the occurrences of their effects on TRPM4, which were over their inferred physiological intracellular concentrations. Additionally, because the effects of $Co^{2+}$ and $Mn^{2+}$ were reversible (e.g. Fig. 1A),

$Co^{2+}$ and $Mn^{2+}$ probably do not constitutively bind to TRPM4. Thus, although the increase of inward currents at negative potentials, which is elicited by binding of $Co^{2+}$ or $Mn^{2+}$ to the 2nd binding site, is advantageous for the control of physiological membrane potentials by TRPM4, there is no data clearly indicating that $Co^{2+}$ and $Mn^{2+}$ regulate TRPM4 activity under physiological conditions thus far.

The sensitivity to $Mn^{2+}$ of TRPM4 might be important for the toxicity mechanisms of manganese. Excessive exposure or intake of manganese may lead to a condition known as manganism, which is caused by the preferential accumulation of manganese in brain areas rich in dopaminergic neurons (37). As manganese poisoning progresses, catecholamine levels decrease, most likely due to the loss of nigrostriatal dopaminergic neurons, and consequently, parkinsonian-like symptoms ensue (37). On the other hand, the pathological activation of TRPM4 in neurons has shown to mediate axonal and neuronal degeneration (13). Therefore, the contribution of TRPM4 activation by $Mn^{2+}$ to the pathogenesis of manganism needs to be addressed.

If the 2nd binding site plays a physiological role in the regulation of TRPM4 activity, it should have affinity for $Ca^{2+}$. Although, as discussed above, the effect of high concentrations of $Ca^{2+}$ on the voltage dependence is probably not due to the $Ca^{2+}$ binding to the 2nd binding site, the following results might suggest that $Ca^{2+}$ binds to the 2nd binding site and regulates the affinity of the 1st binding site. The differences in $EC_{50}$ for $Ca^{2+}$ between WT TRPM4 and the mutants of D1049 and E1062 in the absence of $Co^{2+}$ and $PIP_2$ (shown in Fig. 5C and Table 1) were smaller than those in the presence of $Co^{2+}$ or $PIP_2$ (shown in Fig. 8F and 9B and Table 1). If $Ca^{2+}$ can bind to the 2nd binding site, the small differences in $EC_{50}$ for $Ca^{2+}$ between WT and the mutants in the absence of $Co^{2+}$ and $PIP_2$ might be explained as follows. The $Ca^{2+}$ affinity of the 1st binding site in the absence of $Co^{2+}$ and $PIP_2$ might be near to (or perhaps in the case of the mutants of E1062, lower than) that of the 2nd binding site because the $Ca^{2+}$ affinity of the 1st binding site is not increased by $Co^{2+}$ nor $PIP_2$. Thus, the $EC_{50}$ for $Ca^{2+}$ in the absence of $Co^{2+}$ and $PIP_2$ might be a result of the binding of $Ca^{2+}$ to both binding sites and be more influenced by the $Ca^{2+}$ affinity of the 2nd binding site than in the presence of $Co^{2+}$ or $PIP_2$. The $Ca^{2+}$ affinity of the 2nd binding site is less affected by the mutations than the 1st one. Therefore, the differences in $EC_{50}$ for $Ca^{2+}$ between WT TRPM4 and the mutants in the absence of $Co^{2+}$ and $PIP_2$ were smaller than in the presence. Additionally, the Hill coefficient calculated from a fit of the $Ca^{2+}$ dose-response curve for WT TRPM4 currents at +100 mV in the absence of $Co^{2+}$ was 2.99 and that in the presence of 1 mM $Co^{2+}$ was 1.45 (Table 1). This decrease of the Hill coefficient supports the idea that $Ca^{2+}$ binds to more sites in the absence of $Co^{2+}$ than its presence. However, even if $Ca^{2+}$ can bind to the 2nd binding site at millimolar concentrations, it is unlikely that $Ca^{2+}$ affects the TRPM4 function through the binding

to the 2nd binding site at physiological concentrations.

Our results do not deny the involvement of calmodulin in the regulation of TRPM4. The 2nd binding site might be calmodulin because $Mn^{2+}$ has been shown to bind to and activate calmodulin (41-44). However, there are conflicting data. $Co^{2+}$ and $Ni^{2+}$ had the effects similar to $Mn^{2+}$ on TRPM4 currents in the present study, but $Co^{2+}$ and $Ni^{2+}$ have been reported to have little or no effect on calmodulin (42,43). That implies the effects of the divalent cations on TRPM4 cannot be explained only by calmodulin. Furthermore, we cannot completely exclude the possibility that the single amino acid mutations near and in the TRP domain prevent the binding of calmodulin, the binding sites of which have been suggested to be located in the C-terminal region following the TRP domain (14). However, it should be noted that the mutations of D1049 and E1062 did not interfere with the binding of $PIP_2$. The $PIP_2$ binding site of TRPM4 has been shown to be located among the calmodulin binding sites in the C-terminal tail ((17), Fig. 5A). If the structure of the calmodulin binding sites were disturbed by the mutations, it is highly likely that the sensitivity for $PIP_2$ was likewise affected by the mutations.

Intriguingly, $Mn^{2+}$, $Co^{2+}$, and $Ni^{2+}$, the ionic radii of which are similar to each other and smaller than that of $Ca^{2+}$ ($Mn^{2+}$: 0.80 Å, $Co^{2+}$: 0.74 Å, $Ni^{2+}$: 0.72 Å, and $Ca^{2+}$: 0.99 Å (20)), have been shown to be able to increase the apparent affinity of the BK channel for $Ca^{2+}$ through binding to the E399-related low-affinity binding site (21,28). The mechanisms of how $Mn^{2+}$, $Co^{2+}$, and $Ni^{2+}$ allosterically affect the affinity of the $Ca^{2+}$ binding site in TRPM4 might be similar to those in the BK channel. Additionally, $Mn^{2+}$, $Co^{2+}$, and $Ni^{2+}$ are the best activators of calcineurin, a serine/threonine protein phosphatase (45,46). Although calcineurin is also a calmodulin binding protein, $Mn^{2+}$, $Co^{2+}$, and $Ni^{2+}$ were more potent activators of calcineurin than $Ca^{2+}$ in the absence of calmodulin (47) through their binding to the divalent cation binding site in calcineurin (46). It would be interesting to examine whether calcineurin regulates TRPM4 or not. Moreover, information regarding the structures of divalent cation binding sites of the BK channel and calcineurin might be useful in searching for the binding site for $Mn^{2+}$, $Co^{2+}$, and $Ni^{2+}$ in TPRM4.

D1049 and E1062, which are required for the normal $Ca^{2+}$ sensitivity of TRPM4, are conserved in TRPM5, TRPM2, and TRPM8. TRPM5 is also a $Ca^{2+}$-sensitive channel and has been reported to be independent of calmodulin (15). Involvements of the comparable aspartate and glutamate of TRPM5 in its activation by $Ca^{2+}$ should be addressed. TRPM2 is activated in a synergistic fashion by intracellular ADP-ribose and $Ca^{2+}$ (48). It has been reported that the mutation of the calmodulin-binding domain, located in the N-terminal of TRPM2, made the channel nonfunctional (49). However, intriguingly, it has been also suggested that the $Ca^{2+}$ binding sites of TRPM2 were located in intracellular deep crevices near the pore entrance (50). It is noteworthy that the TRP domain in TPRM2 is just

beneath the 6th transmembrane pore forming domain. TRPM8 has been reported to be desensitized in a $Ca^{2+}$-dependent manner (51). Although the desensitization by $Ca^{2+}$ is considered to be due to the $Ca^{2+}$-mediated activation of PLC and subsequent $PIP_2$ hydrolysis near TRPM8 (51), the direct effects of $Ca^{2+}$ through the comparable aspartate and glutamate of TRPM8 might be partially involved in the desensitization.

Our study demonstrated that the TRP domain of TRPM4 plays a pivotal role in determining $Ca^{2+}$ sensitivity, which is a novel function of the TRP domain. As another example of functions of TRP domain in TRPM channels, it has been reported that the TRP domains of TRPM5 and TRPM8 channels are involved in the interaction with $PIP_2$ (19), which increases $Ca^{2+}$ sensitivity of TRPM4 (16,17). However, the TRP box and TRP domain of TRPM4 were shown not to be the main determinants of $PIP_2$ action (17). Our data also consistently indicate that the mutation of D1049 and E1062 did not affect the sensitivity to $PIP_2$. Thus, the TRP domain of TRPM4 determines its $Ca^{2+}$ sensitivity not by the modulating $PIP_2$ binding.

The simplest scenario how the mutations of D1049 and E1062 of TRPM4 reduced the $Ca^{2+}$ sensitivity is that these two negative residues participate in the formation of a $Ca^{2+}$ binding site. In this case, the contribution of D1049 to the formation of the 1st binding site may be smaller than that of E1062 because the effect on $EC_{50}$ for $Ca^{2+}$ of the mutation of D1049 was weaker than that of E1062. A second scenario is that the mutations disrupt the structure of a $Ca^{2+}$ binding site, which is located elsewhere. At present, there is no conclusive data showing which scenario is the case. Clarification of the crystal structure of TRPM4 will help to understand the structure-function relationship. A third scenario is that the $Ca^{2+}$ binding site is located in a different region and the mutations of D1049 and E1062 affected the allosteric mechanism that couples the signals of the $Ca^{2+}$ binding with the channel opening. There are examples of such allosteric functions of the TRP domain. Amino acids I696 and W697 in the TRP box of TRPV1 are critical for the efficient coupling of stimulus sensing and gate opening (52). The TRP domain of TRPM8 was also indicated to be involved in translating the initial ligand-binding event to the allosteric conformational changes that open the channel independently from the effect of $PIP_2$ (53). However, in the present study, the $EC_{50}$ for $Ca^{2+}$ were increased by the D1049 or E1062 mutations of TRPM4. Therefore, it is more conceivable that the initial $Ca^{2+}$-binding event is inhibited by the mutations of D1049 or E1062 rather than the allosteric mechanisms in the case of TRPM4. Lastly, the mutations of D1049 and E1062 might have changed the amount of plasma membrane surface expression of the channels because the maximal current amplitudes of the mutants except E1062A, which were evoked by $Ca^{2+}$ alone, were smaller than that of WT (Table 2). The maximal current amplitudes may reflect the expression level of the channel protein on the patch membrane although we cannot rule out the

possibility that the maximal current amplitudes are determined not only by the surface expression level but also by other unknown mechanisms. However, we measured the maximum reaction and analyzed the $EC_{50}$ for divalent cations. Even if the maximal current amplitudes are changed according to the different surface expression levels of the channel proteins, the $EC_{50}$ for divalent cations will not be affected. Thus, the change in surface expression level of the channel proteins does not affect our conclusion that the mutations of D1049 or E1062 reduce the $Ca^{2+}$ sensitivity of TRPM4. Additionally, as shown in Table 2, the maximal current amplitudes of WT and mutants are not correlated to the $Ca^{2+}$ sensitivities when E1062Q and E1062A are compared. Moreover, although the maximal current amplitudes of D1049N and E1062Q in the presence of $PIP_2$ were not significantly different from that of WT, the $EC_{50}$ for $Ca^{2+}$ of the mutants in the presence of $PIP_2$ were obviously larger than that of WT (Fig. 9B). Therefore, it is very unlikely that the surface expression level is the main determinant of the sensitivities to $Ca^{2+}$.

The mutations of D1049 and E1062 of TRPM4 also affected the voltage dependence. The voltage-independent conductance fraction and the currents amplitudes at −100 mV of WT TRPM4 were increased by the applications of $Ca^{2+}$, $Co^{2+}$, $Mn^{2+}$, $Ni^{2+}$, or $PIP_2$, which suggests that the inactivation of the currents at negative potentials was relieved by them. Though contrarily it has been reported that $Ca^{2+}$ did not change the voltage dependence of TRPM4 (1,54), the $Ca^{2+}$ concentrations at which the voltage dependence was affected in the present study (over 1 mM) were higher than those used in the articles (1,54). In contrast with WT TRPM4, the voltage-independent conductance fractions of D1049N, D1049A, and E1062Q mutants were not increased by $Ca^{2+}$. These results suggest not only that the voltage-sensing machinery can be regulated by $Ca^{2+}$ but also that the mutations except E1062A disrupt the coupling between the signal of $Ca^{2+}$ binding and the voltage-sensing machinery. It should be noted that the effects on the voltage dependence of $Co^{2+}$ and $PIP_2$ were not eliminated by the any mutations of D1049 or E1062. These results indicate that the mutants do not lose the capability to regulate the voltage-sensing machinery. Furthermore, the WT-like behavior of E1062A mutant suggests that the expression level on the plasma membrane of the channels might account for the different responses of the voltage dependence by $Ca^{2+}$. As summarized in Table 2, the maximal current amplitudes of the mutants, which were evoked by $Ca^{2+}$ alone, were significantly smaller than WT, except for E1062A. The surface expression level of E1062A, which may be the nearest to that of WT among the mutants, might cause the WT-like behavior of E1062A regarding the modification of voltage dependence by $Ca^{2+}$.

Finally, the time course of decrease in the current amplitudes after the first exposure to the calcium solution appears to vary between experiments even for the WT channel. Currently, we cannot clearly indicate the reason of the

variations. However, we assume that the variations were probably due to the variations in the initial ratio of the number of expressed channel proteins to the concentration of $PIP_2$ in a patch membrane. The concentrations of $PIP_2$ are probably similar among patch membranes. Thus, when the number of channel proteins is small and the ratio is low, the initial currents are large in comparison with the desensitized currents because the large percentage of channel proteins was initially potentiated by $PIP_2$. The loss of $PIP_2$ affected many channels so that the time course of the desensitization was sharp. When the number of channel proteins is large and the ratio is high, the opposite happens. Additionally, in some patch recordings, the currents increased slowly after patch excision (e.g. D1049N in Fig. 5B, WT in Fig. 9A). That's perhaps because the inside of the patch membrane was gradually opened after the vesicle formed. In those cases, the initial peak currents were not measured properly and thus the decrease in the current amplitudes appeared to be slow. On the other hand, the differences in the time course of the decrease in the current amplitudes between WT and mutants are probably mainly due to the difference in the $Ca^{2+}$-sensitivity among them.

In conclusion, the present study provides new insights to better understand the mechanisms underlying the activation of TRPM4. In particular, it demonstrated that the acidic amino acids near and in the TRP domain of TRPM4 play a pivotal role in the determination of $Ca^{2+}$ sensitivity. If the crystal structure of the C-terminal tail of TRPM4 is revealed, it will be clarified whether the TRP domain of TRPM4 is a direct binding site for $Ca^{2+}$ or not. At least, the functional observations by the present study will help to understand the correlation between the structure and function of TRPM4.

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## FOOTNOTES

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## FIGURE LEGENDS

**TABLE 1. $EC_{50}$ and Hill coefficients for $Ca^{2+}$, $Co^{2+}$, and $PIP_2$ of wild-type TRPM4 and mutants (D1049A, D1049N, E1062A, and E1062Q).** $EC_{50}$ and Hill coefficients for $Ca^{2+}$ were determined without additional conditions or also in the presence of 1 mM $Co^{2+}$ or 30 μM diC8-PI(4,5)$P_2$ ($PIP_2$). $EC_{50}$ and Hill coefficients for $Co^{2+}$ were calculated from the decrease of $EC_{50}$ for $Ca^{2+}$ by the simultaneous

application of $Co^{2+}$, shown in Fig. 8E. The $EC_{50}$ and Hill coefficients for $PIP_2$ were evaluated in the presence of $Ca^{2+}$, shown in Fig. 9D. The $Ca^{2+}$ concentrations used for wild-type (WT) TRPM4, D1049N, and E1062Q were 100 μM, 300 μM, and 1 mM $Ca^{2+}$, respectively. The ratios of the $EC_{50}$ of mutants to that of WT TRPM4 are shown in the parentheses. The current amplitudes at +100 mV were used for the calculation.

**TABLE 2. Maximal current amplitudes of wild-type TRPM4 and mutants (D1049A, D1049N, E1062A, and E1062Q).** The maximal current amplitudes at +100 mV in the presence of only $Ca^{2+}$, $Ca^{2+}$ and 1 mM $Co^{2+}$, or $Ca^{2+}$ and 30 μM $PIP_2$ are shown. The $Ca^{2+}$ concentrations, at which the current amplitudes reached maximum, are listed next to the current amplitudes. Shown are the mean ±S.E. from 3-7 patch recordings. * $P < 0.05$, ** $P < 0.01$ vs. WT (Dunnett's test).

**FIGURE 1. Intracellular $Mn^{2+}$, $Co^{2+}$, and $Ni^{2+}$ potentiate TRPM4 channel activity.** *A*, a representative time course of TRPM4 currents at +100 mV (filled circles) and −100 mV (open squares) in an inside-out patch using the voltage-ramp protocol. The arrow indicates the onset of inside-out mode. *B*, traces of inside-out currents evoked by ramp-pulse. The letters correspond to those described in A. (inset) *I-V* curves recorded from a mock transfected cell when the bath solutions were a $Ca^{2+}$-free solution (EGTA), a solution containing 1 mM $Ca^{2+}$, one containing 1 m $Ca^{2+}$ and 1 mM $Co^{2+}$, or one containing 1 mM $Ca^{2+}$ and 10 mM $Mn^{2+}$. *C*, averaged values of normalized current amplitudes showing the activation by the application of 1 mM divalent cations ($Ca^{2+}$, $Ni^{2+}$, $Co^{2+}$, and $Mn^{2+}$) in the presence of 1 mM $Ca^{2+}$. The current amplitudes at +100 mV (shaded bars) and −100 mV (unshaded bars) were normalized to the current amplitude recorded in the presence of 1 mM $Ca^{2+}$ right before exposure to the other divalent cations.. Shown are mean ±S.E. **: $P < 0.01$ vs. currents evoked by 1 mM $Ca^{2+}$. $n$ = 8-10. *D*, applications of 1 mM $Ni^{2+}$, $Mn^{2+}$, and $Co^{2+}$ make TRPM4 voltage-independent. The horizontal axis is the membrane potential and the vertical axis is the chord conductance normalized to that at +100 mV. Shown are the mean ±S.E. from 8-10 patch recordings. The lines are fits to the Boltzmann equation.

**FIGURE 2. TRPM4 currents potentiated by $Co^{2+}$ are inhibited by a TRPM4 inhibitor, flufenamic acid.** *A*, a typical time-course of TRPM4 channel currents at +100 mV (filled circles) and −100 mV (open squares) when 100 μM flufenamic acid (FA) was applied to the cytosolic side of TRPM4. FA was added to the perfusate which contained 1 mM $Ca^{2+}$ with and without 1 mM $Co^{2+}$. *B*, averaged values of normalized current amplitudes at +100 mV (shaded bars) and −100 mV (unshaded bars). The currents were normalized to the current amplitude recorded in the presence of 1 mM $Ca^{2+}$ right before exposure to the FA or 1 mM $Co^{2+}$. FA inhibited TRPM4 currents significantly regardless of whether TRPM4 currents

were potentiated by $Co^{2+}$. *: $P < 0.05$, **: $P < 0.01$. $n = 4$ or 6.

**FIGURE 3. Effects of other divalent cation on TRPM4 activity.** *A*, a typical time course of TRPM4 channel currents at +100 mV (filled circles) and −100 mV (open squares). One mM of $Mg^{2+}$ (a), $Ba^{2+}$, $Sr^{2+}$ (b), $Cd^{2+}$, or $Zn^{2+}$ (c) was applied to the intracellular side of the patch membrane with 1 mM of $Ca^{2+}$. *B*, summary of the change in current amplitudes by the application of 1 mM divalent cations ($Mg^{2+}$, $Ba^{2+}$, $Sr^{2+}$, $Cd^{2+}$, or $Zn^{2+}$) in the presence of 1 mM $Ca^{2+}$. The current amplitudes at +100 mV (shaded bars) and −100 mV (unshaded bars) were normalized to the current amplitude recorded in the presence of 1 mM $Ca^{2+}$ right before exposure to the other divalent cations. *: $P < 0.05$, **: $P < 0.01$ vs. currents evoked by 1 mM $Ca^{2+}$. $n = 4$-11.

**FIGURE 4. $Ca^{2+}$ dependence of the effects of $Mn^{2+}$, $Co^{2+}$, and $Ni^{2+}$.** *A* (a), a representative time course of TRPM4 currents at +100 mV (filled circles) and −100 mV (open squares) showing that neither 1 mM $Co^{2+}$, $Ni^{2+}$, nor $Mn^{2+}$ evoked any currents when the $Ca^{2+}$ was chelated by 145 mM fluoride. (b), summary of the normalized currents at +100 mV (shaded bars) and −100 mV (unshaded bars). Shown are mean ±S.E. The normalized current amplitudes in the presence of $Co^{2+}$, $Ni^{2+}$, or $Mn^{2+}$ were not significantly different from those under the $Ca^{2+}$-free condition using EGTA. $n = 3$ or 4. *B* (a), a typical time course of TRPM4 currents when the free $Ca^{2+}$ and $Co^{2+}$ concentrations were adjusted to 0 or 1 mM using a chelating agent, o-phenylenedioxydiacetic acid (o-PDDA, 6 mM). (b), summary of the normalized currents at +100 mV (shaded bars) and −100 mV (unshaded bars). Shown are mean ±S.E. The normalized current amplitudes in the presence of $Co^{2+}$ (1 mM) were not significantly different from those under the $Ca^{2+}$-free condition using o-PDDA. Application of $Co^{2+}$ (1 mM) in the presence of $Ca^{2+}$ (1 mM) increased TRPM4 current amplitudes significantly. **: $P < 0.01$. $n = 5$. *C*, a diagram of two divalent cation binding sites of TRPM4 and their agonists. As the effects of $Co^{2+}$, $Mn^{2+}$, and $Ni^{2+}$ are dependent on $Ca^{2+}$, it is suggested that there are at least two divalent cation binding sites in TRPM4. The 1st binding site is sensitive to $Ca^{2+}$ but insensitive to $Co^{2+}$, $Mn^{2+}$, and $Ni^{2+}$. Accordingly, it is relatively $Ca^{2+}$-specific. The 2nd binding site is sensitive to $Co^{2+}$, $Mn^{2+}$, and $Ni^{2+}$.

**FIGURE 5. Mutations of the acidic amino acids (D1049 and E1062) near and in the TRP domain reduce $Ca^{2+}$ sensitivity.** *A*, positions of the acidic amino acids which were mutated. (Upper) predicted membrane topology of TRPM4 and the binding sites for $PIP_2$ and calmodulin (CaM) in the C-terminal tail are illustrated. (Lower) an alignment of cDNA sequences around the TRP domain of rat TRPM channels is shown. The aspartate (D1049) and the glutamate (E1062) near and in the TRP domain of rat TRPM4 are conserved in rat TRPM5 (Genbank accession #NP_001178825.1), TRPM2 (#NP_001011559.1), and

TRPM8 (#NP_599198.2) but not in TRPM1 (#NP_001032823.1), TRPM3 (NP_001178491.1), TRPM6 (#XP_219747.6), nor TRPM7 (NP_446157.2). *B*, typical time courses of currents of WT TRPM4 and mutants (D1049N, D1049A, E1062Q, and E1062A). Intracellular $Ca^{2+}$ concentration was increased from 0 to 30 mM. Arrows indicate the onset of inside-out mode. Application of 1 mM $Co^{2+}$ potentiated all mutants. *C*, dose-response curves for the effect of intracellular $Ca^{2+}$ on WT TRPM4 and mutants. Shown are the mean ±S.E. from 3-7 patch recordings. Current amplitudes are normalized to the maximum currents. The lines are fits to the Hill equation. **P* < 0.05, ** *P* < 0.01 vs. WT (Dunnett's test).

**FIGURE 6. $Mn^{2+}$ and $Ni^{2+}$ potentiate the currents of the mutants.** Representative time courses of currents of D1049N (A), D1049A (B), E1062Q (C), and E1062A (D) at +100 mV (filled circles) and −100 mV (open squares). One mM of $Mn^{2+}$, $Co^{2+}$, $Ni^{2+}$, or $Ca^{2+}$ was applied to the intracellular side of the patch membrane with 1 mM of $Ca^{2+}$.

**FIGURE 7. Mutations of the acidic amino acids (D1049N, D1049A, and E1062Q) alter voltage dependence.** *A*, typical *I-V* curves of $Ca^{2+}$-dependent currents recorded from the inside-out patch membrane expressing WT TRPM4 and mutants (D1049N, D1049A, E1062Q, and E1062A). The $Ca^{2+}$-dependent currents were obtained by subtracting the currents under the $Ca^{2+}$-free condition from the currents evoked by 3 mM $Ca^{2+}$. *B*, summary of the ratio of currents at −100 mV to those at +100 mV. The ratios of D1049N, D1049A, and E1062Q mutants were significantly different with that of WT TRPM4. Shown are the mean ±S.E. from 4-6 patch recordings. *: *P* < 0.05, **: *P* < 0.01 (Dunnett's test). *C*, changes of chord conductance of WT TRPM4 and mutants by intracellular $Ca^{2+}$ concentrations. The values are normalized to the conductance at +100 mV. Shown are the mean ±S.E. from 3-6 patch recordings. The lines are fits to the Boltzmann equation.

**FIGURE 8. $Co^{2+}$ increases the $Ca^{2+}$ sensitivity of the apparently $Ca^{2+}$-specific (1st) binding site and the mutations of D1049 and E1062 decrease the sensitivity to divalent cations of the 1st binding site rather than that of the $Co^{2+}$ binding site.** *A*, typical time courses of WT TRPM4 currents, showing that the $Ca^{2+}$ sensitivity is increased by the simultaneous application of 1 mM $Co^{2+}$ (a), $Mn^{2+}$ (b), or $Ni^{2+}$ (c). *B-D*, dose-response curves for the effect of intracellular $Ca^{2+}$ on WT TRPM4 (B), D1049N (C), and E1062Q (D) mutants in the absence or presence of $Co^{2+}$ (0.01 mM to 1 mM). Shown are the mean ±S.E. from 3-9 patch recordings. The lines are fits to the Hill equation. *E*, application of $Co^{2+}$ reduces $EC_{50}$ for the activation by $Ca^{2+}$ of WT TRPM4 (black circles), D1049N (red triangles), and E1062Q (blue squares) mutants. The horizontal axis shows $Co^{2+}$ concentration. The vertical axis shows the $EC_{50}$ for $Ca^{2+}$. The lines are fit to the pseudo Hill equation. (inset) $EC_{50}$ for $Ca^{2+}$ are normalized to that in the absence of $Co^{2+}$

for the sake of comparison of $EC_{50}$ for $Co^{2+}$ between WT TRPM4 and the mutants. *F*, dose-response curves for the effect of intracellular $Ca^{2+}$ on WT TRPM4 (black circles), D1049N (blue triangles), D1049A (red inverted triangles), E1062Q (green diamonds), and E1062A (pink squares) mutants in the presence of 1 mM $Co^{2+}$. Shown are the mean ±S.E. from 3-10 patch recordings. The lines are fits to the Hill equation. **P* < 0.05, ** *P* < 0.01 vs. WT (Dunnett's test).

**FIGURE 9. D1049N and E1062Q mutants showed lower sensitivity to $Ca^{2+}$ than WT TRPM4 in the presence of $PIP_2$.** *A*, typical time courses of currents of WT TRPM4 and mutants (D1049N and E1062Q) in the presence of 30 μM diC8-PI(4,5)$P_2$ ($PIP_2$). Intracellular $Ca^{2+}$ concentration was raised from 0 to 10 mM. *B*, dose-response curves for the effect of intracellular $Ca^{2+}$ on TRPM4 and mutants (D1049N and E1062Q) in the presence of 30 μM diC8-PI(4,5)$P_2$. Current amplitudes are normalized to the maximum currents. Shown are the mean ±S.E. from 3-5 patch recordings. The lines are fits to the Hill equation. **P* < 0.05, ** *P* < 0.01 vs. WT (Dunnett's test). Data at 0.3 mM $Ca^{2+}$ were not statistically analyzed because the value of WT at the concentration was not measured. *C*, typical time courses of currents of WT TRPM4 and mutants (D1049N and E1062Q) when the concentration of diC8-PI(4,5)$P_2$ was increased from 3 to 60 μM. Intracellular $Ca^{2+}$ concentrations were 100 μM, 300 μM, and 1 mM for WT TRPM4, D1049N mutant, and E1062Q mutant, respectively. *D*, dose-response curves for the effect of intracellular diC8-PI(4,5)$P_2$ on WT TRPM4 and mutants (D1049N and E1062Q). Current amplitudes are normalized to the maximum currents. Shown are the mean ±S.E. from 3-6 patch recordings. The lines are fits to the Hill equation.

**FIGURE 10. Suggested regulatory mechanisms of the TRPM4 activation.** The bindings of divalent cation to the 2nd binding site and the binding of $PIP_2$ to its binding site have similar effects. They increase $Ca^{2+}$ sensitivity of the 1st binding site and make TRPM4 less voltage-dependent. Hence, these sites function as regulatory sites. The binding of $Ca^{2+}$ to the 1st binding site opens the channel and also affects voltage dependence. The mutations in D1049 and E1062 attenuate the $Ca^{2+}$ sensitivity of the 1st binding site and inhibit the effects on the voltage dependence through the 1st $Ca^{2+}$ binding site although the mutations hardly affect the function of the regulatory sites.

$EC_{50}$ (mutants/WT), Hill coefficient

| | $Ca^{2+}$ | $Ca^{2+}$ w/ $Co^{2+}$ | $Co^{2+}$ | $Ca^{2+}$ w/ $PIP_2$ | $PIP_2$ |
|---|---|---|---|---|---|
| TRPM4 WT | 0.96 mM (1.0), 2.99 | 107 μM (1.0), 1.45 | 57.5 μM (1.0), 1.04 | 42.2 μM (1.0), 1.26 | 9.96 μM (1.0), 2.82 |
| D1049N | 1.17 mM (1.2), 6.74 | 417 μM (3.9), 2.82 | 72.4 μM (1.3), 1.78 | 288 μM (6.8), 2.68 | 9.80 μM (1.0), 1.83 |
| D1049A | 1.21 mM (1.3), 3.62 | 304 μM (2.8), 1.41 | - | - | - |
| E1062Q | 3.65 mM (3.8), 4.24 | 1067 μM (10.0), 2.10 | 126 μM (2.2), 2.42 | 809 μM (19.0), 4.47 | 9.95 μM (1.0), 2.58 |
| E1062A | 2.78 mM (2.9), 3.23 | 856 μM (8.0), 1.18 | - | - | - |

Table 1, Yamaguchi, *et al.*

Maximal current amplitudes

| | only $Ca^{2+}$ | | in the presence of $Co^{2+}$ | | in the presence of $PIP_2$ | |
|---|---|---|---|---|---|---|
| | Currents (pA) | $[Ca^{2+}]_i$ | Currents (pA) | $[Ca^{2+}]_i$ | Currents (pA) | $[Ca^{2+}]_i$ |
| TRPM4 WT | 4134 ±818 | 3 mM | 2870 ±544 | 3 mM | 5420 ±636 | 1 mM |
| D1049N | ** 617 ±416 | 3 mM | * 904 ±298 | 3 mM | 4032 ±486 | 1 mM |
| D1049A | ** 882 ±196 | 10 mM | * 1020 ±541 | 3 mM | - | - |
| E1062Q | ** 113 ±17 | 10 mM | * 468 ±123 | 10 mM | 3563 ±709 | 3 mM |
| E1062A | 2471 ±1305 | 10 mM | 3606 ±815 | 30 mM | - | - |

Table 2, Yamaguchi, *et al.*

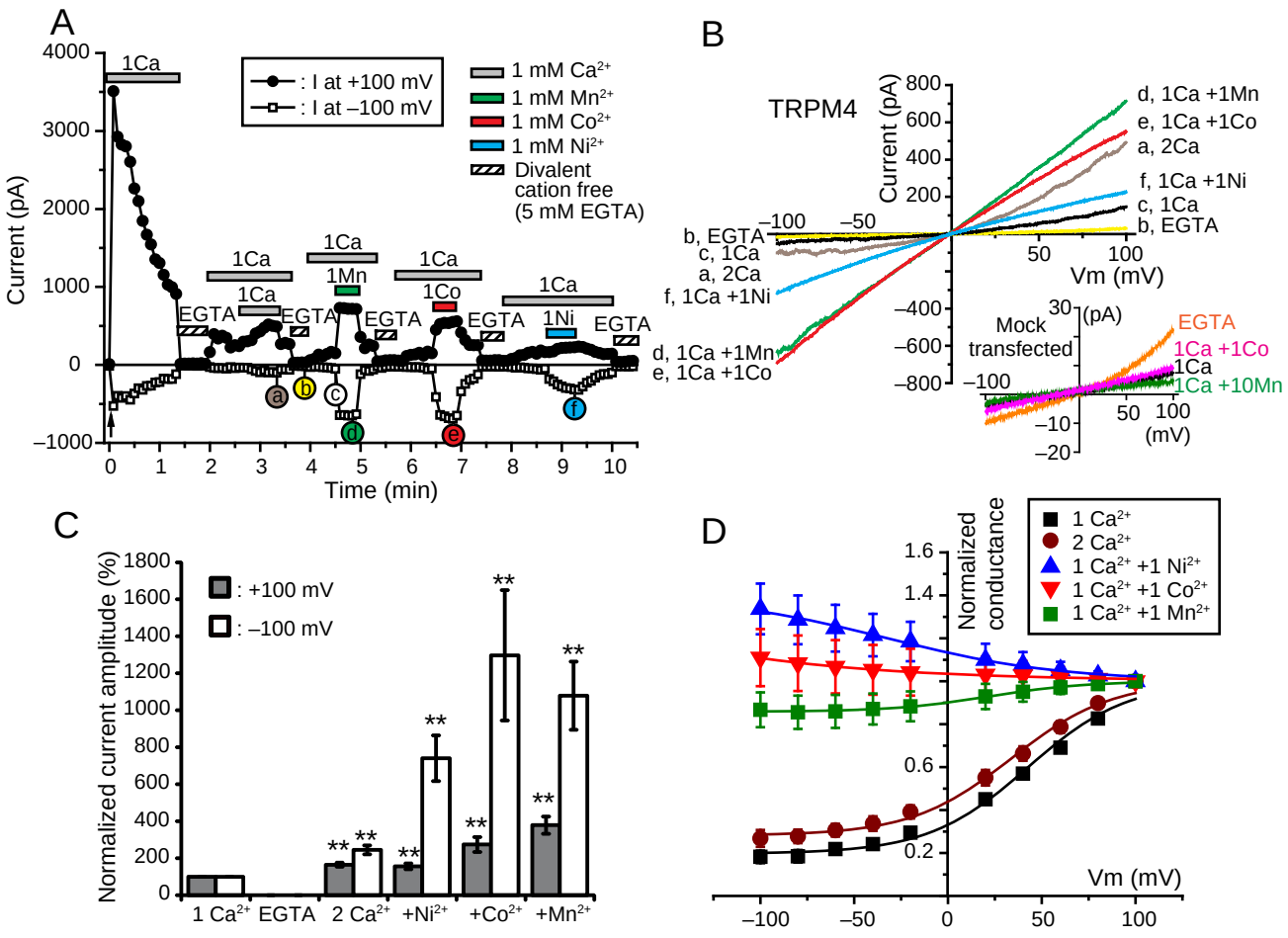


Figure 1, Yamaguchi, *et al.*

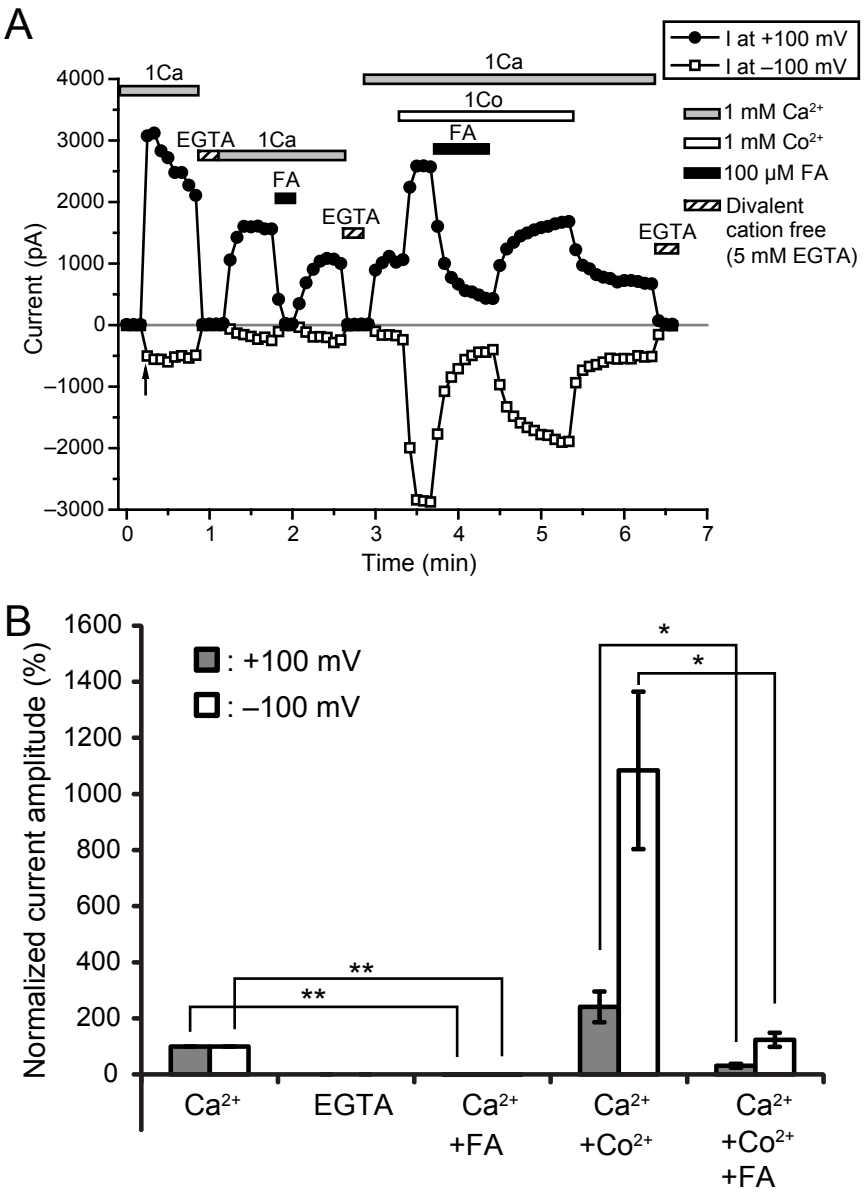


Figure 2, Yamaguchi, *et al.*

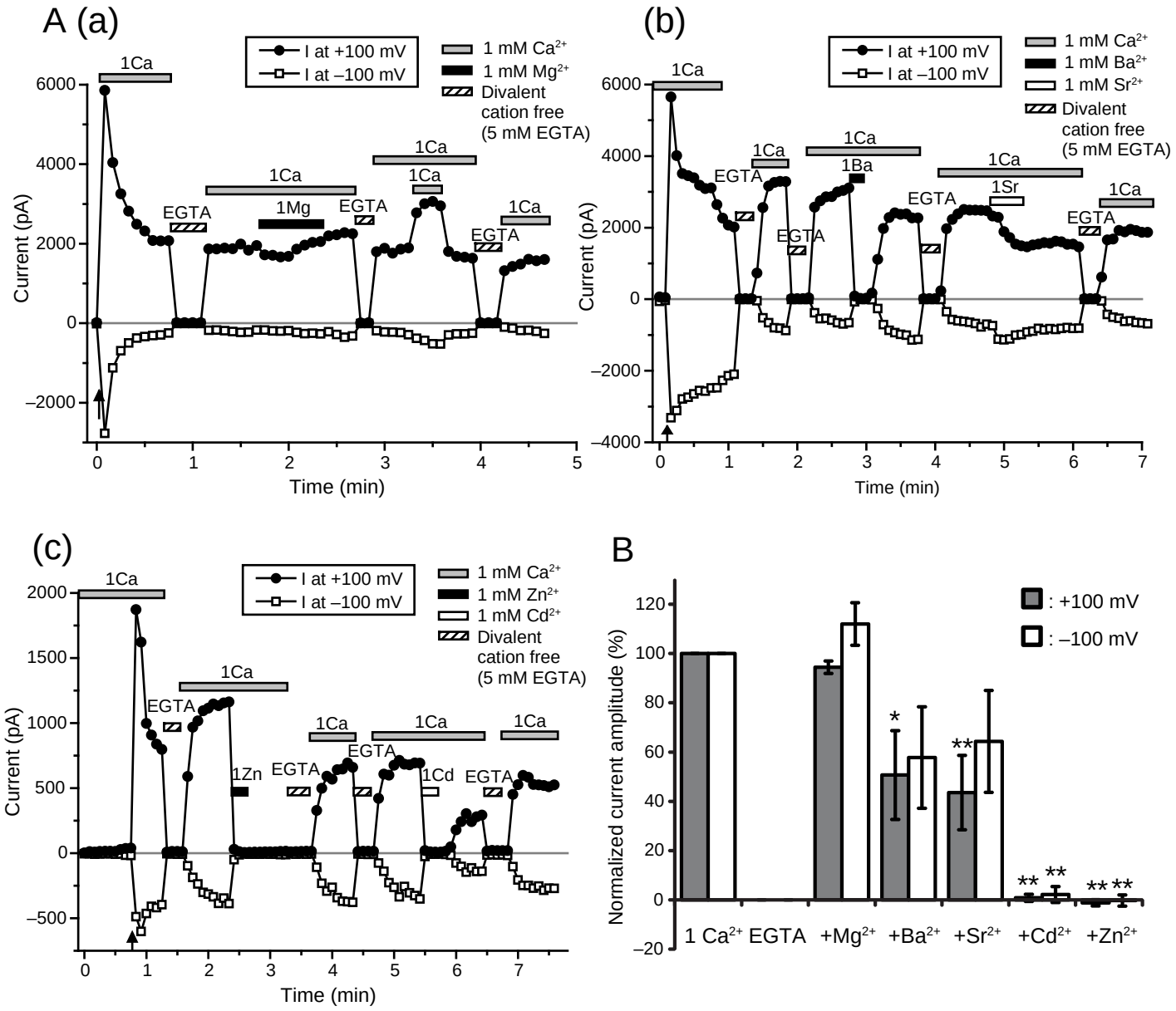


Figure 3, Yamaguchi, *et al.*

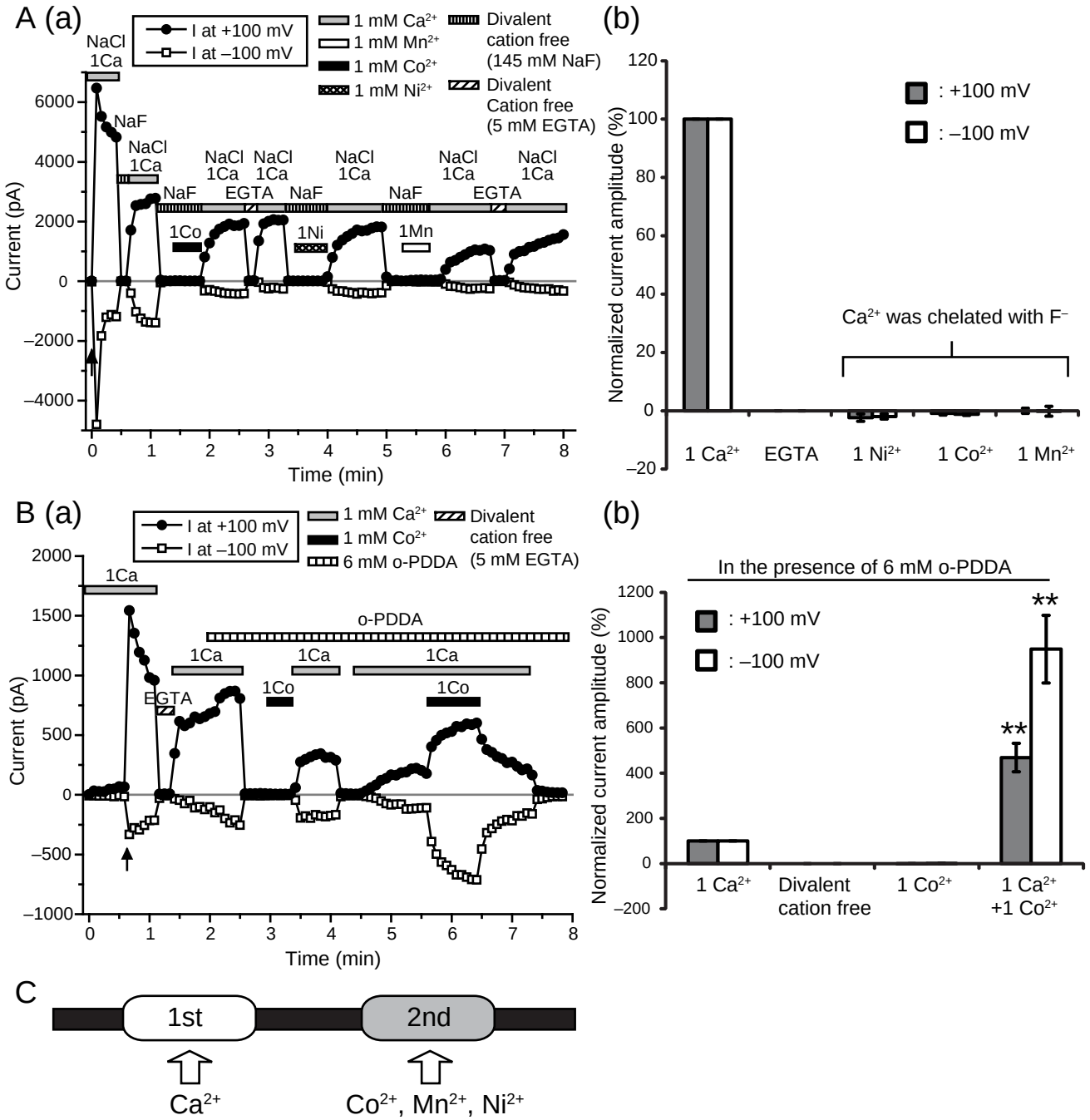


Figure 4, Yamaguchi, *et al.*

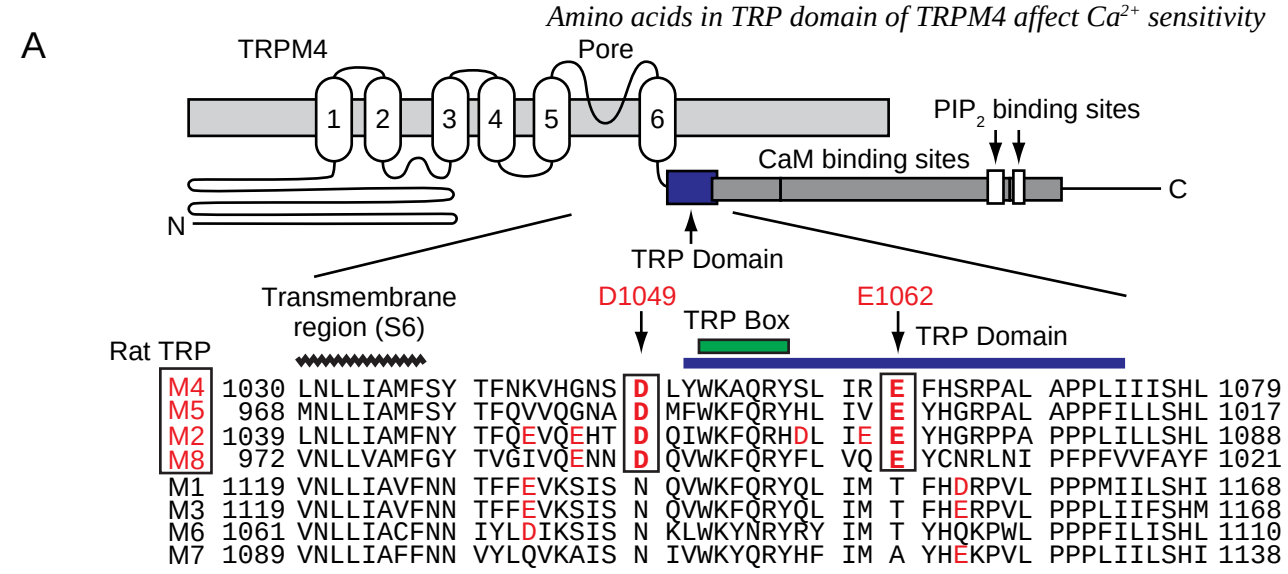




Figure 5, Yamaguchi, *et al.*

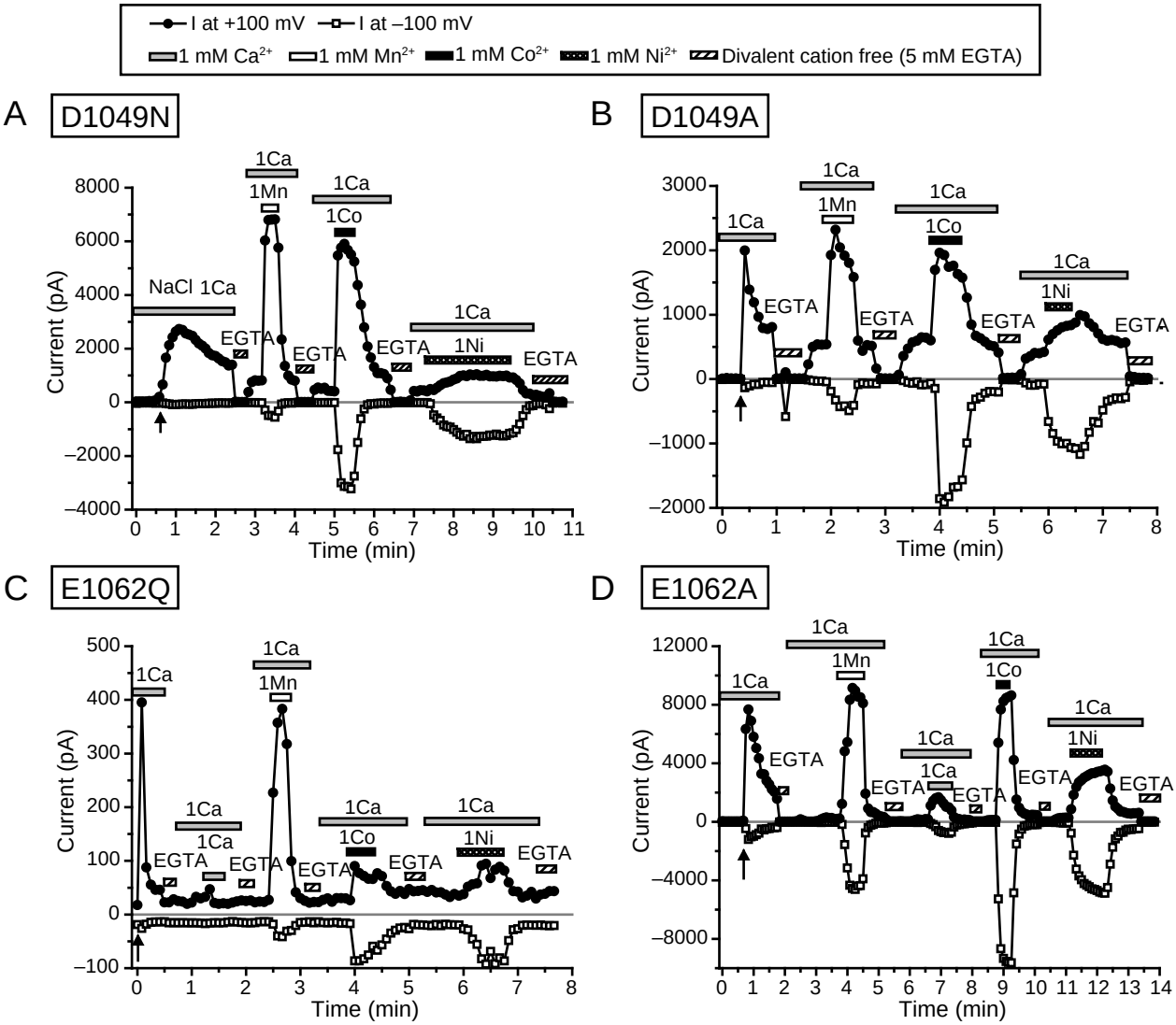


Figure 6, Yamaguchi, *et al.*

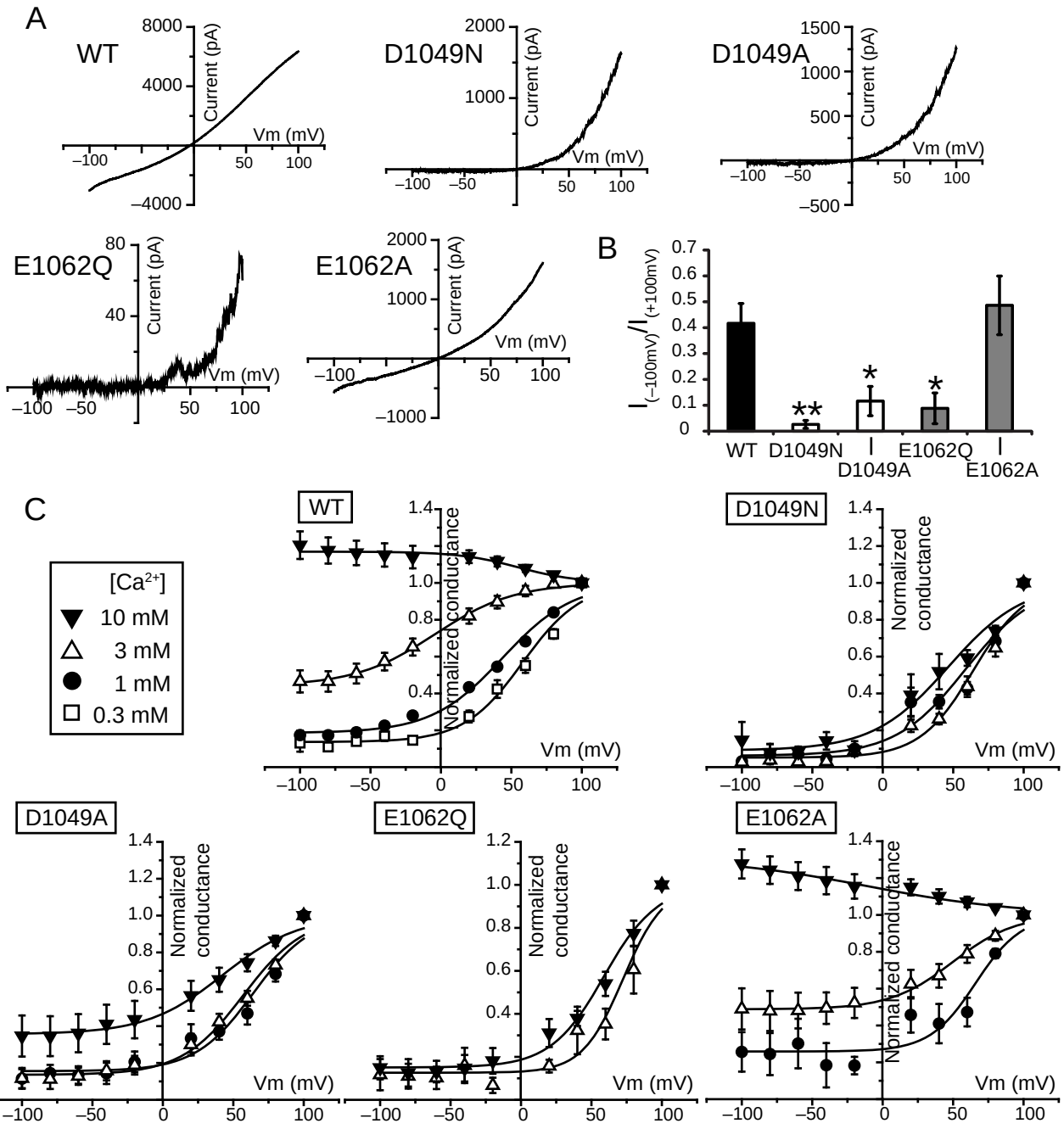


Figure 7, Yamaguchi, *et al.*

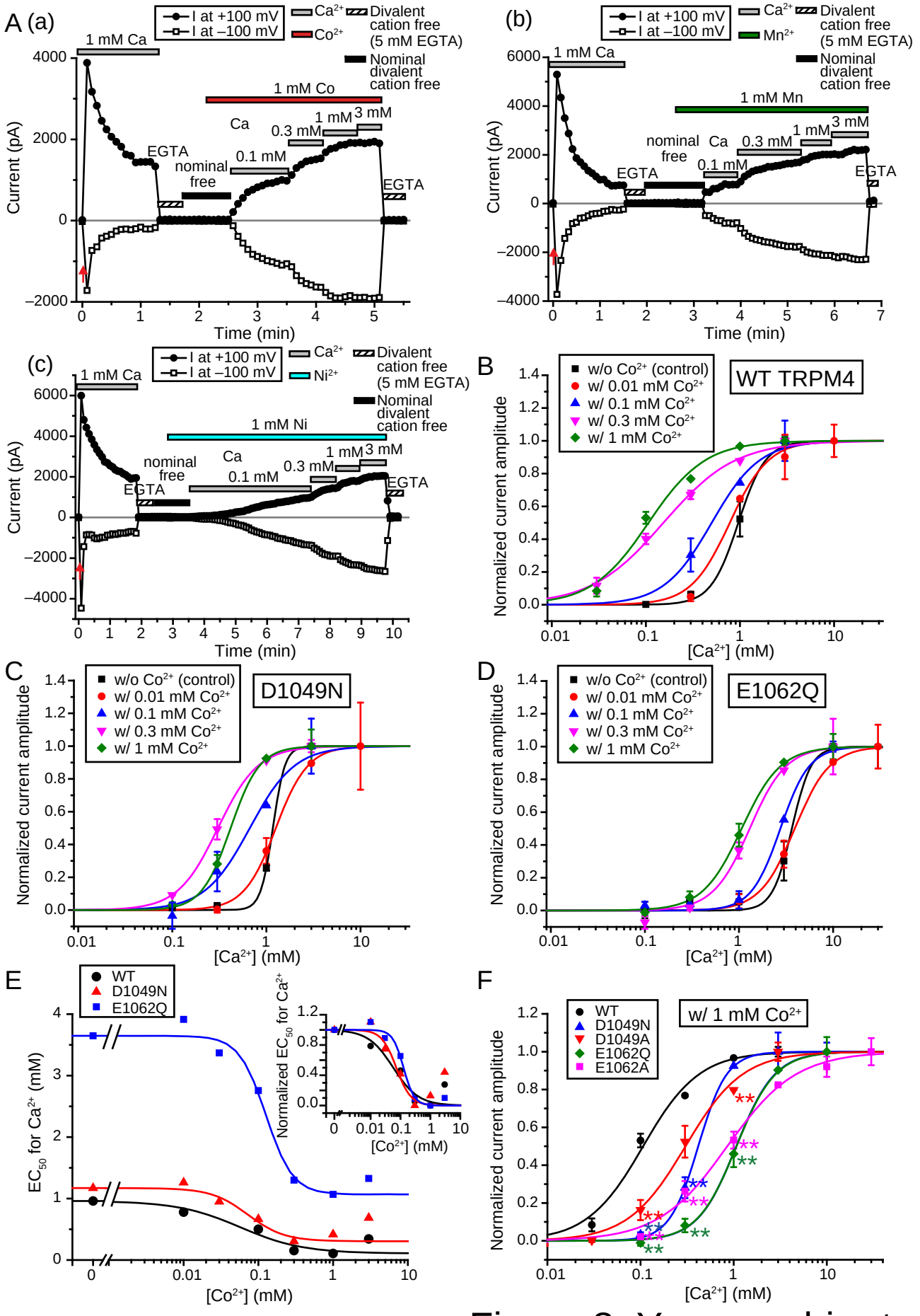




Figure 8, Yamaguchi, *et al.*

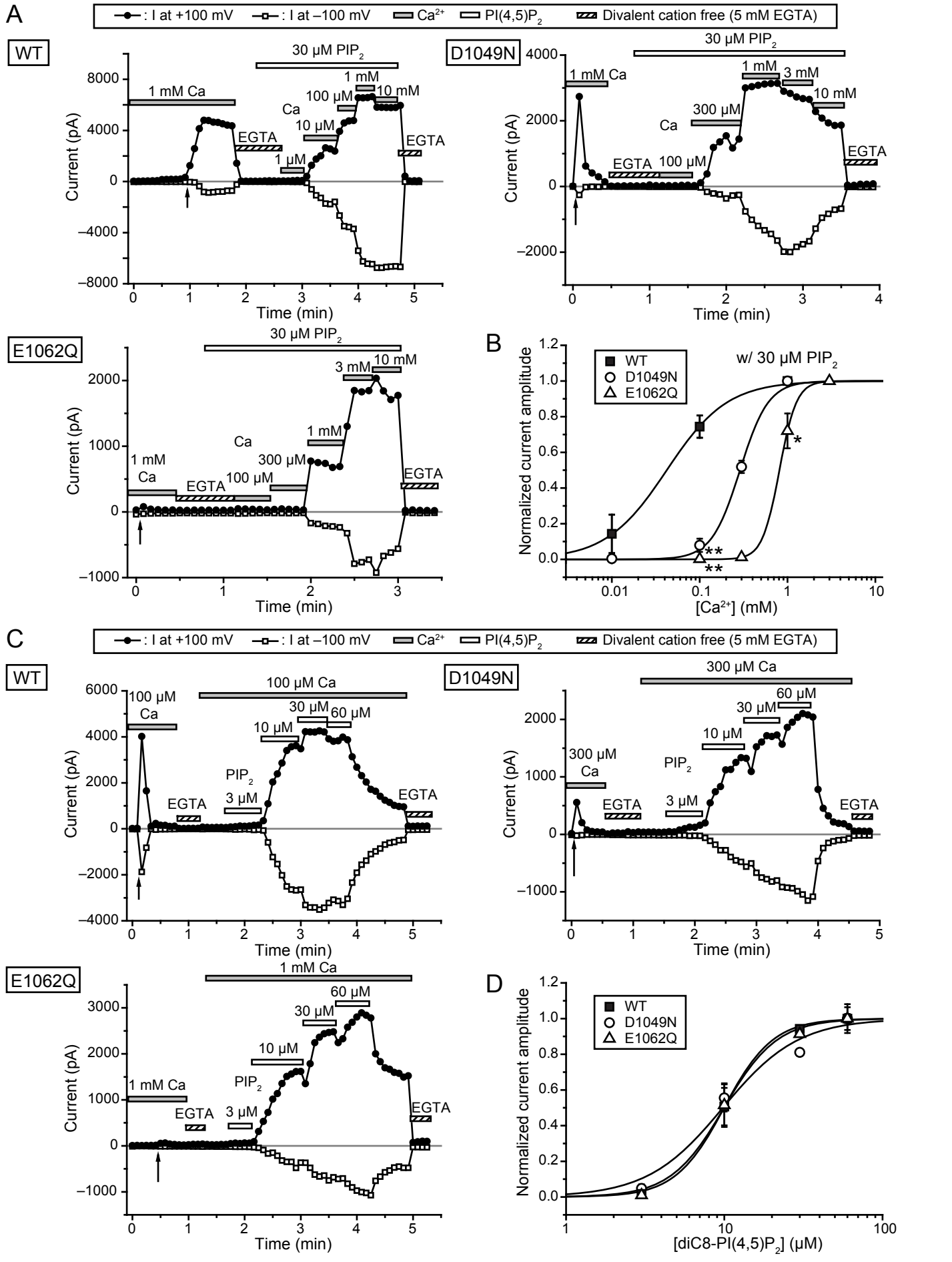




Figure 9, Yamaguchi, *et al.*

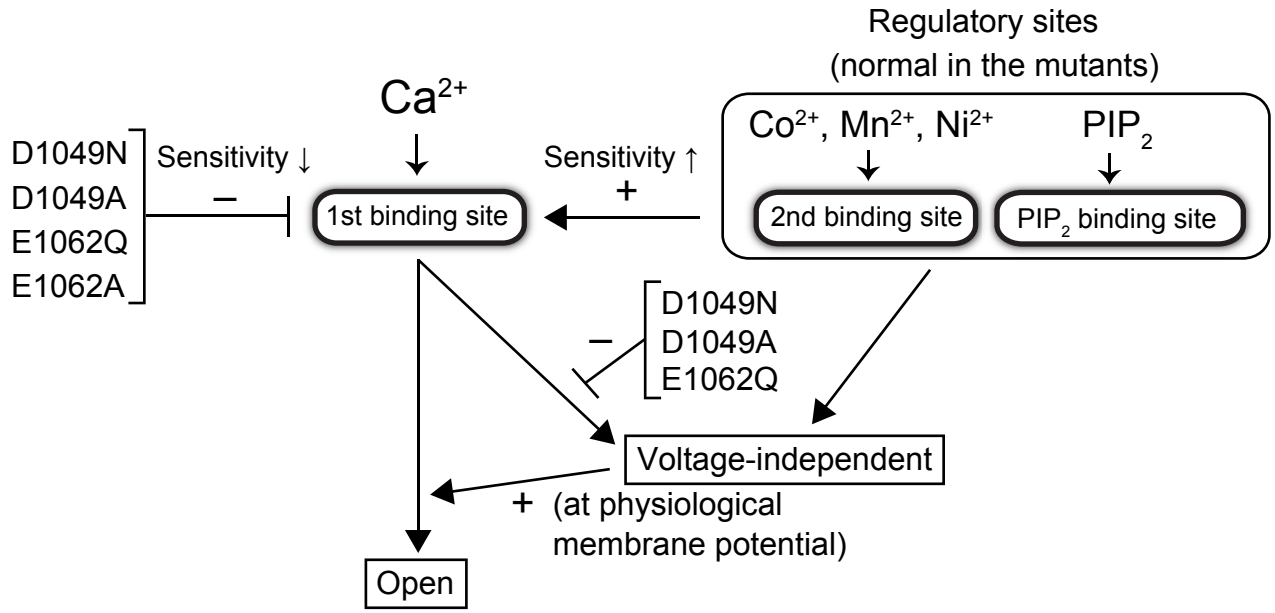


Figure 10, Yamaguchi, *et al.*