ZnT-1 enhances the activity and surface expression of T-type calcium channels through activation of Ras-ERK signaling

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ZnT-1 is a putative zinc transporter that confers cellular resistance from zinc toxicity. Here we studied the effects of ZnT-1 on the expression and function of T-type calcium channels. In Xenopus oocytes expressing voltage-gated calcium channel CaV3.1 or CaV3.2, ZnT-1 enhanced the low-threshold calcium currents (I_{cT}) to 182 ± 15 and 167.95 ± 9.27% of control, respectively (P < 0.005 for both channels). As expected, ZnT-1 also enhanced ERK phosphorylation. Coexpression of ZnT-1 and nonactive Raf-1 blocked the ZnT-1-mediated ERK phosphorylation and abolished the ZnT-1-induced augmentation of I_{cT}. In mammalian cells (Chinese hamster ovary), coexpression of CaV3.1 and ZnT-1 increased the I_{cT} to 166.37 ± 6.37% compared with cells expressing CaV3.1 alone (P < 0.01). Interestingly, surface expression measurements using biotinylation or total internal reflection fluorescence microscopy indicated marked ZnT-1-induced enhancement of CaV3.1 surface expression. The MEK inhibitor PD-98059 abolished the ZnT-1-induced augmentation of surface expression of CaV3.1. In cultured murine cardiomyocytes (HL-1 cells), transient exposure to zinc, leading to enhanced ZnT-1 expression, also markedly retained in intracellular compartments, mutations in ZnT-1 also enhanced ERK phosphorylation. Coexpression of ZnT-1 and CaV3.1 increased the I_{cT} by 10.220.32.246 on June 26, 2017 http://ajpcell.physiology.org/ Downloaded from
the surface expression of the LTCC α-subunit (30). ZnT-1 has a well-documented role in conferring resistance against zinc toxicity (14, 39, 40, 53). In addition, ZnT-1 binds to the amino-terminal regulatory portion of Raf-1 kinase and promotes its enzymatic activity, thereby activating the Ras-ERK signaling cascade (7, 23). Thus, as already outlined before, ZnT-1 may have important and broad regulatory roles, only parts of which are directly related to zinc homeostasis (3, 13).

LTCC and TTCC are coexpressed in many tissues and appear to have different functions in a variety of cells, such as cardiomyocytes, pancreatic β-cells, and neurons (17, 38, 45). The goal of the present study was to determine the effects of ZnT-1 on CaV3.1 and CaV3.2 compared with its recently described effect on LTCC function. Our results indicate that, in contrast to the inhibitory effect of ZnT-1 on LTCC, ZnT-1 markedly augments the activity of CaV3.1 and CaV3.2 channels. We further demonstrate that this functional augmentation relates to ZnT-1-induced activation of Ras-ERK signaling and is associated with enhanced surface expression of the channels. These findings document a novel regulatory function of ZnT-1 and a new mode of regulation of TTCC channels by the Ras-ERK signaling cascade.

EXPERIMENTAL PROCEDURES

Expression of CaV3.1, CaV3.2, and ZnT-1 in Xenopus oocytes. Oocytes of mature female Xenopus laevis frogs were excised and prepared as previously described (4, 30). Complementary cRNAs were synthesized by in vitro transcription with T7 or an SP6 Amplicap High-Yield Message Maker Kit (Epicentre Technologies, Madison, WI). The recombinant plasmids used in these reactions were pGEM-HE-1H and pGEM-HE-1G (42, 43), pSPCA1 [rabbit skeletal muscle (35)], and pXen1-ZnT1 [rat SLC30A (23), a kind gift from Dr.}

Fig. 1. Zinc transporter-1 (ZnT-1) enhances the activity of voltage-gated calcium channel (CaV) 3 expressed in Xenopus oocytes. A: representative current traces from single oocytes coinjected with cRNA for CaV3.1, with or without ZnT-1. B: representative steady-state current-voltage (I-V) relationship of CaV3.1 Ba2+ currents from a batch of oocytes coinjected with cRNA for CaV3.1, with or without ZnT-1. Data represent means ± SE of the currents recorded from control (squares) and ZnT-1-expressing oocytes (circles). C: normalized peak currents from 4 independent experiments. Coexpression of ZnT-1 enhanced the peak CaV3.1 current by 82 ± 15% (n = 57 and 65 oocytes, with and without ZnT-1, P < 0.005). D: voltage dependence of the fractional conductance (G/Gmax), derived from the data shown in A. Fitting the data with the Boltzmann equation yielded half-Gmax voltage of −33.7 ± 1.1 and −39.5 ± 2 mV (P < 0.05) for controls and ZnT-1-expressing oocytes, respectively. **P < 0.01. E–H: similar data as in A–D for oocytes expressing CaV3.2 channels. E: representative current traces from single oocytes. F: representative steady-state I-V relationship. G: normalized peak currents from 4 independent experiments. Coexpression of ZnT-1 enhanced the normalized peak CaV3.2 current by 67.95 ± 9.27% [n = 31 and 29 oocytes (nos. in parentheses), with and without ZnT-1, P < 0.005]. H: voltage dependence of the fractional conductance (G/Gmax), derived from the data shown in E. Fitting the data with the Boltzmann equation yielded half-Gmax voltage of −27.97 ± 0.44 and −33.48 ± 0.54 mV (P < 0.05) and e-effective charge of gating of 4.894 ± 0.254 and 6.440 ± 0.663 (P < 0.01) for controls and ZnT-1 expressing oocytes, respectively. **P < 0.01.
Anthony Muslin. Oocytes were injected with equal quantities (2.5 ng) of cRNA of either CaV3.1 or CaV3.2, with or without cRNA of ZnT-1 (2.5 ng). In addition, αβγ was injected into all oocytes. Injected oocytes were stored at 18°C for 4 days in NDE96 solution before their use for experiments.

Barium current recordings in Xenopus oocytes. Current was monitored by a two-electrode voltage clamp (TEVC) using Gene Clamps 500 amplifier (Molecular Devices, Sunnyvale, CA), as previously described (4, 30). The bath solution contained the following (in mM): 40 Ba(OH)2, 50 NaOH, 2 KOH, and 5 HEPES (titrated to pH 7.5 with methanesulfonic acid). Currents were measured in response to 200-ms-long voltage-clamp pulses generated every 2 s from a holding potential of −80 mV to test potentials between −80 and +50 mV. Barium currents in water-injected oocytes or cells injected with the cRNA of ZnT-1 alone were negligible.

Cell culture and transfection. Human embryonic kidney cells (HEKT-293) and Chinese hamster ovary (CHO) cells were maintained in high-glucose Dulbecco’s modified Eagle’s medium supplemented with (vol/vol) 1% penicillin-streptomycin, 1% L-glutamine, and 10% fetal bovine serum at 37°C in a humidified 5% CO2 incubator. HEKT-293 cells were subcultured into 60-mm dishes 1 day before transfection and seeded to reach 50–70% confluence. Transient transfection of HEKT-293 cells was carried out utilizing calcium phosphate transfection protocol. CHO cells were transfected utilizing
Lipofectamine 2000 (Invitrogen, Carlsbad, CA), according to the manufacturer’s instructions. The recombinant plasmids used for expressing CaV3.1 and ZnT-1 were as follows: pEGFP-N1 [murine CaV3.1-EGFP (enhanced green fluorescent protein), a kind gift from Dr. Norbert Klugbauer], SLC30A-pJJ19 [rat ZnT-1 tagged with myc (rZnT-Myc), a kind gift from Dr. Anthony Muslin], SLC30A-pJJ19 [rat ZnT-1 tagged with myc (rZnT-Myc), a kind gift from Dr. Anthony Muslin], and CaV3.1-EGFP (enhanced green fluorescent protein), a kind gift from Dr. Norbert Klugbauer. Cells transfected with pEGFP-N1 were cotransfected with either SLC30A-pJJ19 or an empty pcDNA3 vector. The total amounts of plasmids and transfection reagent were similar in each treatment group. Measurements were performed 36–48 h after transfection. In some experiments, ERK signaling was inhibited, by incubating the cells with the MEK-1 inhibitor PD-98059 (25 

Electrical recordings of T currents in CHO cells. Patch-clamp recordings were performed using whole cell configuration. Signals were amplified using an Axopatch 200B patch-clamp amplifier (Molecular Devices, Sunnyvale, CA) sampled at 2 kHz and filtered at 800 Hz via an four-pole Bessel low-pass filter. Data were acquired using pClamp 9.2 software (Molecular Devices) and an IBM-compatible Pentium IV computer in conjunction with a DigiData 1322A interface (Molecular Devices). The patch pipettes were pulled from borosilicate glass (Warner Instrument) with a resistance of 3–6 MΩ. The compound was dissolved in DMSO alone was diluted 1:500 and added to all other wells of these experiments, as DMSO alone was diluted 1:500 and added to the culture 2 h before the experiment.

Western blot analysis. To harvest cellular proteins, cultured cells were washed three times in phosphate-buffered saline (PBS), scraped with a rubber-policeman, and homogenized by sonication in 150 μl

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Fig. 4. NAF blocks the ZnT-1 induced augmentation of I_{caT} in Xenopus oocytes expressing CaV3.2 channels. A: representative current traces from single oocytes coinjected with cRNA for CaV3.2, CaV3.2 + ZnT-1, and CaV3.2 + ZnT-1 + NAF. Selected recordings are from a similar batch of oocytes (excised, injected, and recorded together under similar conditions). B: steady-state I-V relationship of Ba^{2+} currents that were measured in oocytes expressing CaV3.2, CaV3.2 + ZnT-1, and CaV3.2 + ZnT-1 + NAF. Values are means ± SE of the currents recorded in 3–4 independent experiments from 28, 25, and 24 oocytes for each of the above conditions, respectively. C: normalized peak currents as depicted in B. Coexpression of ZnT-1 enhanced the peak I_{caT} in a similar manner as in Fig. 1. The expression of NAF led to complete inhibition of the effect of ZnT-1 (P = 0.37, relative to the control oocytes expressing CaV3.2 only). D: voltage dependence of the fractional conductance (G/G_{max}), derived from the data shown in A. Fitting the data with the Boltzmann equation yielded half-G_{max} voltage of −27.48 ± 0.61, −30.85 ± 0.51, and −24.09 ± 0.28 mV, and z-effective charge of gating of 6.197 ± 0.341, 6.563 ± 0.401, and 5.829 ± 0.277 for controls, oocytes expressing ZnT-1, and oocytes expressing CaV3.2, ZnT-1, and NAF, respectively. **P < 0.01.
Choi homogenizing buffer containing the following (in mM): 20 Tris·HCl, 320 sucrose, 0.2 EDTA, 0.5 EGTA, and a protease inhibitor mixture (Boehringer Complete Protease Inhibitor Mixture, Roche Molecular Biochemicals). The homogenate was cleared at 14,000 g for 15 min at 4°C, and the supernatant frozen and stored at −80°C for future use. *Xenopus* oocytes were lysed with Nonidet P-40 lysis buffer (0.5% Nonidet P-40; Igepal), containing the following (in mM): 137 NaCl, 50 NaF, 5 EDTA, 10 Tris (pH 7.5), 1 NaVO₃, and a protease inhibitor mixture as above. Lysates were cleared by low-speed centrifugation (820 g for 15 min at 4°C), and the supernatant frozen and stored at −80°C for future use.

**Surface biotinylation.** The surface expression of CaV3 channels was assessed as previously described for CaV1.2 channels (30). Briefly, HEKT-293 cells were subcultured in 60-mm culture dishes achieving 50% confluence. The following day, HEKT-293 cells were cotransfected with a plasmid containing GFP-tagged CaV3.1 (pEGFP-N1) and either ZnT-1 (SLC30A-pJJ19) or an empty vector (pcDNA3). The experiments were carried out 48 h following the transfection. All procedures were done on ice at 0°C. Cell monolayers were washed three times with ice-cold PBS, incubated for 30 min at 4°C, and shaken gently, in the presence of 0.5 mg/ml of the membrane-impermeable reagent Sulfo-NHS-SS-biotin in PBS. The cells were then washed three times with PBS supplemented with 10% BSA (Sigma) and incubated in the same solution, but without the biotin, for an additional 5 min to remove any unbound NHS-SS-biotin. Following three additional cycles of PBS washing, the cells were scraped into Eppendorf tubes prechilled on ice, sonicated for 10 s, and centrifuged at 5,000 g for 10 min at 4°C. The supernatant was collected, and protein content was determined using the Bio-Rad assay kit with bovine serum albumin as a standard. All samples were adjusted to similar protein concentration. From each sample, 15 µl were retained and marked as “total protein.” Thirty microliters of Immobilized NeutrAvidin resin (Thermo Scientific) were added to the remaining samples, and the reaction mixture was gently rotated end over end at 4°C for 10 h. Thereafter, resin was washed with PBS, and the binding buffer was added to elute the biotinylated protein.

**Fig. 5.** ZnT-1 enhances the activity of CaV3.1 channels expressed in Chinese hamster ovary (CHO) cells. A: superimposed traces of $I_{CaT}$ in CHO cells expressing Ca3.1 without (middle) or with (right) ZnT-1. Cells were depolarized from a holding potential of −90 mV. Left: the voltage-clamp protocol. B: steady-state $I-V$ relationship of Ca$^{2+}$ currents. Values are means ± SE of the currents recorded from 6 cells in each group. C: normalized peak currents as depicted in A. The expression of ZnT-1 enhanced the relative normalized peak current by 66.37 ± 6.37% ($n$ = 6 for both, $P < 0.005$). D: calculated mean $G_{max}$ values for controls and ZnT-1-expressing cells (12.06 ± 0.37 and 16.71 ± 1.4 µS, respectively, $P < 0.01$). E: voltage dependence of the fractional conductance ($G/G_{max}$), derived from the data shown in A. Fitting the data with the Boltzmann equation yielded half-$G_{max}$ voltage of −37.9 ± 0.46 and −42.3 ± 0.45 mV ($P < 0.01$) and z-effective charge of gating of 4.63 ± 0.31 and 4.38 ± 0.13 ($P = 0.21$) for controls and ZnT-1-expressing cells, respectively. Of note, error bars are included, but are too small to be seen. **$P < 0.01$.**
spun down (6,000 g, 5 min) and washed three times with ice-cold harvest buffer. The released proteins were retained, marked as “membrane fraction”, and stored at −80°C for further analysis. Western blot analysis of the total protein and membrane fraction samples was done using anti-GFP antibody (Santa Cruz Biotechnology) for detecting GFP-tagged CaV3.1. Endogenous CaV3.1 channels in HL-1 cells were detected using anti-CaV3.1 and antibodies (Alomone Laboratories). Ponceau staining was used to confirm equal protein loading.

Total internal reflection fluorescence microscopy. Analysis of total internal reflection fluorescence microscopy (TIRFM) was done as previously described (30). CHO cells grown in a glass-bottom dish were cotransfected with CaV3.1/GFP, with or without ZnT-1. Twenty-four hours following transfection, the cells were fixed for 20 min with 4% paraformaldehyde/PBS and then imaged in standard PBS solution at room temperature. Imaging was performed on an in-house built prism-based TIRFM system, using the 488-nm line of an argon ion laser (Melles Griot) and imaged utilizing a SPOT charge-coupled device camera (Diagnostic Instruments, Sterling Heights, MI), mounted on a Zeiss Axioplan2 upright microscope with a ×40 1.3 numerical aperture objective (Zeiss, Jena, Germany). To quantify changes in TIRFM of EGFP-labeled proteins, fluorescent intensities were normalized according to the following equation: relative fluorescence intensity = F(t)/F(e), where F(t) is the intensity of TIRFM measurements, and F(e) is the intensity of epifluorescence measurements. The relative fluorescence intensity was determined using the Image J software package.

Statistical analysis. Values were expressed as means ± SE. Student’s t-test or one-way ANOVA were used as required to determine statistical significance of differences between means. For results that were distributed in a non-Gaussian manner, the Mann-Whitney nonparametric test was used, as indicated. Statistical significance was set at P < 0.05. In Figs. 1–10, * signifies P < 0.05, and ** signifies P < 0.01.

RESULTS

ZnT-1 enhances TTCC function. Regulation of TTCC by ZnT-1 was first assessed by measuring Ba2+ currents in Xenopus oocytes expressing either CaV3.1 or CaV3.2 channels (Fig. 1). Overexpression of ZnT-1 augmented the peak IcaT of CaV3.1 and CaV3.2 expressing oocytes to 182 ± 15 and 167.95 ± 9.27% of control, respectively (Fig. 1, C and G), and enhanced the calculated maximum conductance (Gmax) of CaV3.1 and CaV3.2 expressing oocytes to 165.9 ± 8.4 and 129.2 ± 3.8% of control, respectively (not shown). In addition, ZnT-1 induced a small but significant shift of the half-Gmax voltage of CaV3.1 from −33.7 ± 1.1 to −39.5 ± 2 mV (Fig. 1D) and a similar shift of the half-Gmax voltage of CaV3.2 from −27.97 ± 0.44 to −33.48 ± 0.54 mV (Fig. 1H).

ZnT-1 enhances TTCC function through activation of Ras-ERK signaling. ZnT-1 has previously been shown to bind and activate Raf-1 kinase, leading to activation of Ras-ERK signaling (7, 23). Therefore, we investigated whether the regulatory effect of ZnT-1 on CaV3 channels is mediated by the activation of this signaling pathway. For this purpose, ZnT-1 and nonactive Raf-1 (NAF) were coexpressed in Xenopus oocytes. As expected, coexpression of NAF with ZnT-1 inhibited the ability of ZnT-1 to enhance ERK phosphorylation (Fig. 2). In addition, NAF completely inhibited the ZnT-1-induced enhancement of IcaT in oocytes expressing either CaV3.1 (Fig. 3) or CaV3.2 (Fig. 4). Expression of NAF in the absence of ZnT-1 seemed to have a mild inhibitory effect on IcaT by itself (not shown). Taken together, the results support the notion that ZnT-1 augments the activity of CaV3 channels through activation of Ras-ERK signaling. It should be noted that, in a different set of experiments, we also tested the effect of the MEK inhibitor PD-98059 on the ZnT-1-induced enhancement of IcaT in Xenopus oocytes. Although in some experiments the drug indeed blocked the effect of ZnT-1, unexplained variability did not allow definite conclusion using this drug in Xenopus oocytes.

ZnT-1 enhances TTCC function in mammalian cells. The effect of ZnT-1 on TTCC function was further assessed in mammalian cells. Similar to the findings in oocytes, IcaT increased to...
166.37 ± 6.37% in CHO cells coexpressing CaV3.1 and ZnT-1, compared with cells expressing CaV3.1 alone (Fig. 5, A–C). ZnT-1 also enhanced the calculated $G_{\text{max}}$ to 138.55 ± 1.4% of control (Fig. 5D) and induced a small, but significant, shift in the half-$G_{\text{max}}$ voltage from −37.90 ± 0.46 mV in control cells to −42.30 ± 0.45 mV in cells coexpressing CaV3.1 channels and ZnT-1 (Fig. 5E).

**ZnT-1 increases the surface expression of CaV3.1 channels.** Recently our laboratory showed that ZnT-1 modulates the activity of LTCC by reducing membrane surface expression of its α-subunit (30). In the present study, using biotinylation experiments, we evaluated the effect of ZnT-1 on the surface expression of TTCC. Coexpression of ZnT-1 and CaV3.1 in HEKT-293 cells led to an increase in the surface expression of the CaV3.1. A concomitant tendency of reduction was noted in the total expression of the channel. However, this tendency did not reach statistical significance (Fig. 6). Interestingly, inhibition of Ras-ERK signaling by the MEK-1 inhibitor PD-98059 abolished the ZnT-1-induced translocation of CaV3.1 channels to the plasma membrane (Fig. 7). TIRFM utilizing GFP-labeled CaV3.1 (CaV3.1:GFP) was applied to substantiate the surface expression findings and to directly quantify fluorescent-tagged CaV3.1 in the plasma membrane vicinity (28). A relative measure of the fluorescence at the near membrane was defined as the ratio between the TIRFM signal and the conventional epifluorescence signal, representing total protein expression. Coexpression of CaV3.1 and ZnT-1 increased the relative signal of the CaV3.1:GFP at the cell surface to 158.86 ± 12.5% of cells expressing CaV3.1:GFP alone. Importantly, consistent with the biotinylation results, application of the MEK-1 inhibitor PD-98059 abolished the ZnT-1-induced augmentation in the TIRFM signal of the CaV3.1:GFP (Fig. 8).

![Figure 7](http://apjcell.physiology.org/)

**Fig. 7.** The effect of ZnT-1 on CaV3.1 surface expression is Ras-ERK dependent. A: representative blot from 3 independent biotinylation experiments in which the surface expression of CaV3.1 α2δ-subunit was evaluated in HEKT-293 cells expressing CaV3.1:GFP without ZnT-1, CaV3.1:GFP with ZnT-1, and CaV3.1:GFP with ZnT-1 following 2 h of exposure to the MEK-1 inhibitor PD-98059. **Left:** total homogenate. **Right:** surface expression. Ponceau staining serves as loading control (bottom bands). B: summary of the densitometric analysis for CaV3.1 total expression. No significant difference was noted in the total expression of the channels in these groups. C: analysis as in B for the surface expression of CaV3.1. ZnT-1 increased the plasma membrane expression of CaV3.1 as noted in Fig. 4. PD-98059 totally blocked this effect of ZnT-1. D: immunoblot analysis for ERK phosphorylation in HEKT-293 cells expressing CaV3.1, CaV3.1 + ZnT-1, and CaV3.1 + ZnT-1 + PD-98059. **Top:** representative blots from 5 independent experiments. **Bottom:** normalized densitometry values. Note increased ERK phosphorylation in cells transfected with ZnT-1, which was inhibited by exposure of the cells to PD-98059. Values are means ± SE. **P < 0.01.**
Ras-ERK signaling promotes surface expression of CaV3.1 channels in HL-1 cells. Utilizing HL-1 cells, a cardiomyocyte cell line (12), which abundantly expresses TTCC (56), we validated the effect of endogenous ZnT-1 on the endogenous surface expression of CaV3.1. The expression of ZnT-1 in a variety of cells is regulated by extracellular zinc that activates the transcription factor MTF-1 (15, 37). Taking advantage of this phenomenon, our laboratory recently demonstrated that transient exposure of HL-1 cells to 100 μM ZnSO4 for 6 h markedly enhances ZnT-1 expression and phospho-ERK levels 36 h later (3). In the present study, we monitored changes in the surface expression of endogenous CaV3.1 channels under similar conditions. As expected, increased ZnT-1 expression following transient exposure to zinc (Fig. 9A) was associated with enhanced surface expression of endogenous CaV3.1 channels in the HL-1 cells (Fig. 9, B–D).

To determine whether activation of Ras-ERK signaling by means other than ZnT-1 expression can augment the surface expression of CaV3.1 channels, HL-1 cells were exposed to ET-1, a potent activator of ERK signaling (8). Indeed, exposure of HL-1 cells to 30 nM ET-1 led to enhanced ERK-1/2 phosphorylation (Fig. 10A) and to a concomitant increase in the surface expression of endogenous CaV3.1 channels (Fig. 10, B–D). The effect of ET-1 on CaV3.1 surface expression was abolished in the presence of the MEK inhibitor PD-98059, supporting the involvement of Ras-ERK in the trafficking of CaV3.1 channels.

DISCUSSION

TTCC are widely expressed in different cells and organs and possess several structural and electrophysiological properties that make them unique among other VGCC (42, 49). In addition, accumulating data indicate that abnormal function of these channels has a role in the pathophysiology of various disease states, such as absence epilepsy (52), peripheral neuropathy (22, 27, 34), cardiac hypertrophy (11), and heart failure-related arrhythmias (25). The results of the present study indicate that ZnT-1 augments the activity of CaV3 channels and increases their plasma membrane expression. In addition, the results indicate that the modulatory effect of ZnT-1 on the activity of TTCC depends on the known function of ZnT-1 as an activator of Ras-ERK signaling (23).

Electrophysiologically, the most prominent effect of ZnT-1, was a marked augmentation of TTCC currents. This finding was seen in both oocytes expressing CaV3.1 and CaV3.2 and was also confirmed in CHO cells expressing CaV3.1. A small but significant shift to the left in the half-\(G_{\text{max}}\) voltage was also consistently observed.

Fig. 8. Total internal reflection fluorescence microscopy (TIRFM) evaluation confirms the Ras-ERK-dependent effect of ZnT-1 on the surface expression of CaV3.1. A: CHO cells expressing CaV3.1:GFP alone (top row), CaV3.1:GFP with ZnT-1 (middle row), and CaV3.1:GFP with ZnT-1 following exposure to the MEK-1 inhibitor PD-98059 (bottom row). Images demonstrate TIRFM illumination (left, red) and epifluorescence (Epi; middle, green), both exclusively of GFP. A merged image of TIRFM and Epi is shown on the right. B: the ratio between the TIRFM and Epi was evaluated in each condition. ZnT-1 increased the ratio to 158.86 ± 12.5% of the control value. PD-98059 totally inhibited this effect of ZnT-1 with ratio values of 85.86 ± 8.3% of control (P < 0.05, CaV3.1 vs. CaV3.1 + ZnT-1, P = 0.22, CaV3.1 vs. CaV3.1 + ZnT-1 + PD; n = 43, 40, and 17 for cells in the three groups, respectively). Data were obtained from 3–4 independent experiments. Values are means ± SE. *P < 0.05.
observed. However, since we did not record the actual voltage traces of the TEVC amplifier, we cannot exclude the possibility that the left shift of the half-$G_{\text{max}}$ voltage was related to inadequate voltage control due to partial saturation of the TEVC amplifier. Nevertheless, the fact that we have seen this phenomenon in practically all oocyte batches (even with observed currents that were rather small as in Fig. 4) as well as in the patch clamped CHO cells (Fig. 5), suggests that this shift to the left is at least partially a true phenomenon.

The effect of ZnT-1 on the Ras-ERK signaling pathway was unveiled by a genetic screen in *Caenorhabditis elegans*, which identified the ZnT-1 invertebrate homolog CDF-1 as an activator of Ras-mediated signaling (7). In subsequent work, it was demonstrated that the COOH-terminus of either CDF-1 or ZnT-1 binds and promotes the biological activity of Raf-1 kinase in *Xenopus* oocytes (23). Recently, in two hybrid analyses, an interaction between ZnT-1 and Raf-1 was also noted (29). Thus the ability of ZnT-1 to augment ERK phosphorylation in the present study was expected. Interestingly, a prominent stimulatory effect of ZnT-3 on Ras-ERK signaling was also reported recently in hippocampal mossy fiber terminals (48). However, the involved molecular mechanism(s) was not evaluated in detail in this report. It will be interesting to further investigate whether the stimulatory effect of ZnT-3 on MAPK signaling shares common features with the effect of ZnT-1 on this pathway. Also, it will be intriguing to determine the relationship between the effect of ZnT-1 on Ras-ERK signaling and its other known modulatory functions in the context of zinc toxicity and the modulation of LTCC function (see below).

There are no known phosphorylation sites for MAPK, including ERK-1/2, on the CaV3 channels (20). However, a role of Ras-ERK signaling in the regulation of TTCC expression and function has been previously reported. Specifically, studies in cardiac myocytes have shown that the angiotensin II-induced enhancement of T currents is mediated by angiotensin 1...
(AT1) receptors and involves the activation of MEK-1/2 pathway (18). In their study, Ferron et al. (18) reported an increase in the mRNA level of CaV3.1 and an increase in the current density of both CaV3.1 and CaV3.2 following AT1 activation. Recently, these findings were substantiated when Morishima et al. showed an increase in the mRNA levels and current density for both CaV3.1 and CaV3.2 following 24-h stimulation of AT1 receptors in rat neonatal cardiomyocytes, in conjunction with increased phosphorylation of ERK-1/2 (36). In the present study, looking specifically on the surface expression of CaV3.1 in heterologously expressed systems, a substantial increase was noted in the presence of ZnT-1 and confirmed by two independent experimental methods: biotinylation experiments and TIRFM analysis. Using biotinylation experiments of HL-1 cells, we could further demonstrate that transient exposure to zinc, leading to the induction of ZnT-1 expression and Ras-ERK activation (3), augments the trafficking of endogenous CaV3.1 channels to the plasma membrane. Of note, this manipulation did not affect the total expression of CaV3.1 (Fig. 9). To further evaluate the involvement of Ras-ERK signaling in trafficking of CaV3.1 channels, we exposed HL-1 cells to ET-1, a potent activator of ERK signaling (8) known to potentiate \( I_{\text{CaT}} \) in cardiomyocytes (19, 21). Indeed, ET-1 induced marked activation of ERK signaling and concomitantly augmented the trafficking of endogenous CaV3.1 channels to the plasma membrane (Fig. 10). Moreover, the effect of ET-1 on TTCC trafficking was abolished in the presence of PD-98059, supporting the notion that this effect of ET-1 is indeed dependent on Ras-ERK activation. Unfortunately, single HL-1 cells demonstrate huge variability in their TTCC currents (55, 56). Therefore, experiments to assess the effects of various manipulations on TTCC currents are not straightforward in these cells.

Further experiments are needed to elucidate the molecular mechanisms underlying Ras-ERK activation-induced trafficking of CaV3 channels. In addition, it will be important to evaluate whether various proteins in the Ras-ERK cascade may associate with the CaV3 channels following activation of this signaling pathway, and whether ZnT-1 may be involved in such interaction. Of note, in the present study, enhanced surface expression was only shown for CaV3.1. Therefore, we cannot exclude the possibility that ZnT-1 affect CaV3.1 and CaV3.2.
CaV3.2 through different mechanisms, depending on the channel type. Interestingly, a recent study by Dey et al. (16) found that the stimulatory effect of leukemia inhibitory factor on TTCC function in HEK-T-293 cells was associated with increased membrane expression of GFP-tagged CaV3.2 channels and was blocked by the MEK-1/2 inhibitor U-0126.

LTCC and TTCC are coexpressed in a variety of cells and organs and appear to have differential roles in functional terms (17, 38, 45). Our laboratory’s recent reports indicate that ZnT-1 inhibits the LTCC by interaction with its β-subunit, which leads to a reduction in the surface expression of the LTCC α-subunit (30). Therefore, based on our present and previous findings, it appears that ZnT-1 has an important functional role in regulation of the delicate balance between the surface expression and function of LTCC and TTCC. While the actual domain of interaction between ZnT-1 and the β-subunit has not yet been identified, it will be intriguing to evaluate whether there is a relationship between the ability of ZnT-1 to bind Raf-1 and thereby augment I_{CaT}, and its ability to interact with the LTCC β-subunit, leading to LTCC inhibition.

ZnT-1 is a member of a 10-gene protein family and has a putative role in zinc homeostasis. Among the ZnT proteins, ZnT-1 is the most ubiquitously expressed and is the only putative role in zinc homeostasis. Among the ZnT proteins, ZnT-1 on the activity of CaV3.1 and CaV3.2 channels. The stimulatory effect of ZnT-1 on TTCC function is in contrast to the stimulatory effect of ZnT-1 on the activity of CaV3.1 and CaV3.2 channels. The above findings of ZnT-1 on the activity of CaV3.1 and CaV3.2 channels.

REFERENCES

ZnT-1 REGULATES T-TYPE CALCIUM CHANNELS


