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Ion occupancy of the channel pore is critical for triggering excitation-transcription (ET) coupling

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ABSTRACT

During membrane depolarization the voltage-gated calcium channel (VGCC) activates gene expression in excitable cells by means of a signal-transduction pathway termed excitation transcription (ET) coupling. The L-type calcium channel Cav1.2 can drive nuclear activity by either the ERK-CREB pathway, the Ca^{2+}/calmodulin-dependent protein kinase II (CaMKII) cascade, or *via* the Ca^{2+}-dependent protein phosphatase calcineurin. The ERK-CREB pathway mediates nuclear activity *via* a direct interaction of the intracellular β subunit of VGCC with the Ras/GRF1 complex.

Here we show that ET coupling in HEK293 cells transfected with wt Cav1.2 or the Timothy mutant $Cav1.2^{G406R}$ is mediated by substituting Ca^{2+} with the impermeable lanthanum (La^{3+}). In the absence of extracellular Ca^{2+} or La^{3+}, ET coupling was not triggered. This implies that cation occupancy of the selectivity filter, as opposed to calcium influx, plays an essential role in depolarization triggered signaling to the nucleus.

ET coupling triggered by membrane depolarization in Cav1.2 transfected HEK293 cells and neuroendocrine PC12 cells was also supported by substituting Ba^{2+} for Ca^{2+} as the charge carrier. Since Ba^{2+} ions do not bind to calmodulin this implies activation of ET coupling *via* a Ca^{2+}/calmodulin-independent pathway. Together, these results suggest a model whereby nuclear signaling through the ERK-CREB pathway is driven by voltage-dependent conformational change that requires channel pore occupancy and is Ca^{2+} influx-independent. This model is also consistent with the previous observation that ET coupling can be driven by the Ca^{2+}-impermeable $Cav1.2^{L745P}$ mutant. Thus, the conversion of synaptic stimuli to transcriptional activation is mediated by the metabotropic function (Ca^{2+}-inflow independent) of Cav1.2, similar to the ion-influx independent depolarization-triggered transmitter release and transcription activation mediated by the NMDA receptors.

1. Introduction

Excitation-transcription (ET) coupling in neuronal cells is mediated by voltage-gated calcium channels (VGCC), and the N-methyl-D-aspartate-type (NMDA) receptors, the α-amino-3-hydroxy-5-methyl-4-isoxazolepropionate-type (AMPA) glutamate receptors [1]. Several possible signaling routes link VGCC to gene transcription, the Ras-ERK1/2 (extracellular signal-regulated kinases)-CREB (cAMP response element-binding protein) pathway, the Ca^{2+}/calmodulin dependent protein kinases (CaMKs), and the calcineurin (CaN) cascade [2–7]. The current general consensus holds that Ca^{2+} entry through the NMDA receptors or VGCC followed by Ca^{2+} binding to calmodulin (CaM), is responsible for ET coupling.

In these studies CREB-dependent transcription activation by VGCC was been shown to progress *via* Ca^{2+}/CaM-dependent protein kinase II (CaMKII) whereby Ca^{2+}/CaM is shuttled to the nucleus *via* a calmodulin-dependent kinase cascade [6], or *via* ERK-CREB pathways [8]. CaMKII regulates diverse enzymatic pathways: for example, in heart muscle CaMKII controls Ca^{2+} homeostasis, whereas in neurons, CaMKII regulates activity-dependent dendritic remodeling and long-term potentiation (LTP), which is considered a neurobiological correlate of learning and memory. Activation through these pathways has been shown to require Ca^{2+} influx.

A more recent study have shown that ET coupling *via* the ERK-CREB pathway can also be driven in an ion-inflow independent way, as demonstrated by $Cav1.2^{L745P}$, a Ca^{2+}-impermeable single point channel mutant [9]. Other downstream signaling events triggered by metabotropic activity of Cav1.2 have been shown using a Ca^{2+}-impermeable channel; these include excitation-secretion (ES) coupling [10–14], and excitation contraction (EC) coupling [15]. NMDA receptors can similarly act as metabotropic receptors (as opposed to ionotropic NMDA receptors), triggering excitation transcription (ET) coupling in an ion-

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inflow independent way, through conformational changes during ligand binding [16,17].

The contribution of Ca^{2+} influx through Cav1.2 to intracellular signaling pathways has also been examined, by replacing Ca^{2+} with pore-impermeable cations. It was demonstrated that La^{3+}, which was used as a charge carrier in the absence of Ca^{2+}, supports depolarization induced catecholamine release in chromaffin cells, and insulin release in pancreatic cells [18–20], consistent with catecholamine triggered by the Cav1.2 impermeable mutant Cav1.2^{L775P} [21]. These studies led to suggest that Cav1.2 communicates directly with cytosolic signaling proteins prior to Ca^{2+} inflow. Accordingly, this signaling requires a membrane depolarization voltage step concomitantly with cation binding at the selectivity filter during channel opening, converting the selectivity filter from a single to multiple ion occupancy-pore [11].

In the present study we used La^{3+} as a charge carrier to further characterize the mechanism of depolarization-triggered transcription *via* the Ras-ERK-RSK-CREB pathway in PC12, and HEK293 cells.

It is known that the impermeable La^{3+} displays a similar ionic radius (1.06 Å) to Ca^{2+} (0.99 Å) and binds to the selectivity filter of the channel pore without permeation into the cell [22]. Therefore, La^{3+} can be used as a tool to separate the events of Ca^{2+} binding to the channel pore from Ca^{2+} influx in ET coupling.

We also examined nuclear activation mediated *via* Cav1.2 in PC12 cells using barium ions, which bind to the channel pore and are conducted into the cell. Ba^{2+} with the large ionic radius (1.42 Å) compared to Ca^{2+}, does not bind to CaM [23]. Therefore, a support of nuclear activation by Ba^{2+} would be mediated independently of the Ca^{2+}/CaM-dependent cascade. The use of Ba^{2+}could also clarify the role of the IQ motif of the Ras-exchange-proteins or GRF1 and GRF2 that require Ca^{2+} binding, which were previously suggested to participate in ET coupling *via* the Ras-ERK-CREB pathway [24,25].

Our results show that either the impermeable La^{3+} ions or Ba^{2+} support ET coupling in HEK293 and PC12 cells. Thus, the signaling from the voltage-gated calcium channel to gene transcription appears to be dependent on channel pore occupancy and independent of intracellular Ca^{2+} rise or Ca^{2+} influx.

2. Materials and methods

2.1. Materials lipofectamine® 3000 (Invitrogen; life technologies, Inc. Waltham, MA)

Nifedipine in 0.001% DMSO was used at a final concentration of 8 µm (Sigma); FPL-64176, methyl-2,5-dimethyl-4-(2-phenylmethyl) benzoyl-1*H*-pyrrole-3-carboxylate, was from Sigma; Verapamil HCl (Sigma Aldrich, St. Louis, MO); FPL in 0.0001% DMSO was used at a final concentration of 0.5 µM.

To avoid precipitation of insoluble $La(OH)_3$ and $Ln(CO)_3$ or loss of La^{3+} to the container surface, millimolar stock solution of $LaCl_3$ was freshly prepared before each experiment.

Anti-α2 antibodies were prepared by D. Tobi [26].

Antibodies used:

Protein	Company	Catalogue #
pERK1/2 phospho p44/42, Thr 202/Tyr 204 mouse mAb	Cell Signaling Tech, Danvers, MA, USA	9101
ERK2 polyclonal rabbit Ab	Santa Cruz, Dallas, TX, USA	Sc154
pCREB, Ser133, D1G6, monoclonal rabbit Ab (	Cell Signaling Tech, Danvers, MA, USA	9198
CREB (86B10) Mouse mAb	Cell Signaling Tech., Danvers, MA, USA	9104
Phospho-p90RSK (Thr 573)	Cell Signaling Tech., Danvers, MA, USA	9346
RSK1,2,3, rabbit mAb	Cell Signaling Tech., Danvers, MA, USA	9341S
β-catenin, mouse IGg1	BD, transduction laboratories USA	610154
MeCP2, N-17, polyclonal goat Ab	Santa Cruz, Dallas, TX, USA	Sc5755
BDNF, polyclonal rabbit IgG Ab	Abcam, Cambridge, UK	Ab220679
cFos (Ab-5)	Calbiochem, Sigma-Aldrich USA	PC38
Phospho cJun (Ser73)	New England Biolabs, USA	3270
Anti- rabbit	Cell Signaling Tech., Danvers, MA, USA	7074
Anti-mouse	Cell Signaling Tech, Danvers, MA, USA	7076
Anti-goat	Santa Cruz, Dallas, TX, USA	Sc2378

2.2. Methods

2.2.1. Cells

HEK293 and PC12 cells were cultured in Dulbecco's modified Eagle's medium (DMEM) plus 10% fetal calf serum, 100 units/mL penicillin, 3 mM glutamine-alanine, and 1 µg/mL streptomycin at 37 °C in a humidified atmosphere with 5% CO_2. Cells were maintained in serum-free DMEM plus 10% fetal calf serum, 100 units/mL penicillin, 3 mM glutamine-alanine, and 1 µg/mL streptomycin for 16 h at 37 °C in 5% CO_2.

2.2.2. Transfection of HEK293 cells

HEK293 cells were plated at a density of $8.3 \times 10^4/cm^2$ on collagen (rat tail), Roche Diagnostics, Mannheim, Germany), coated plates and incubated for 16 h in 24-well plates (BD Biosciences, San Jose, CA, USA) and allowed to reach 70–80% confluency in 1 ml of culture medium.

Transfection mixtures contained N-acetylcysteine-amide to improve cell viability. The cells were transiently transfected on the next day with 1.0 µg of each plasmid cDNA encoding human L-type channel subunit $\alpha_1$1.2 (Z34815.1), β2b (M80545), and $\alpha_2\delta$ (M86621), or various $\alpha_1$1.2, mutants in the indicated combinations using the Lipofectamine®3000 transfection reagent, according to the manufacturer's instructions. The cells were incubated at 37 °C, 5% CO_2 for 6 h in Opti MEM® (Thermo Fisher Scientific, Waltham, MA) containing the transfection mixtures and subsequently replaced by regular DMEM containing 10% FBS solution. The cells were incubated for 72 h before depolarization.

2.2.3. Membrane depolarization and ERK-RSK and CREB activation

Prior to depolarization, the cells were starved for 2 h in serum-free DMEM supplemented with 2 mM L-alanyl glutamine. To begin the experiment an equal volume of either a control solution or a stimulatory solution were added to the wells, resulting in a basal or a depolarizing level of KCl. The control solution consisted of 125 mM NaCl, 30 mM Glucose, 1 mM $MgCl_2$, 2 mM $CaCl_2$, 40 mM $NaHCO_3$, and 1 mM NaH_2PO_4. The final concentration of KCl after adding at equal volume of the control solution to the DME (originally 5 mM KCl) was 2.5 mM.

The stimulatory solution consisted of 135 mM KCl, 30 mM Glucose, 1 mM $MgCl_2$, 2 mM $CaCl_2$, 40 mM $NaHCO_3$, and 1 mM NaH_2PO_4. When added to the cells at equal volume this resulted in a final concentration of 70 mM KCl. After the addition of control or depolarizing solutions, the cells were incubated for 3 min, and washed with 1 ml of PBS before lysis.

In experiment using La^{3+} the cells were washed twice with 1 ml of a Ca^{2+}-free solution containing 125 mM NaCl, 30 mM Glucose, 1 mM $MgCl_2$, 40 mM $NaHCO_3$, and 1 mM NaH_2PO_4, prior to the depolarization step. The basal solution contained 125 mM NaCl, 3 mM KCl, 30 mM Glucose, 1 mM $MgCl_2$, 40 mM $NaHCO_3$, 1 mM NaH_2PO_4, and 0.1 mM $LaCl_3$. The assay was carried out as described above for 3 min by adding equal volume of the control solution for which in addition to the above contains 0.1 mM $LaCl_3$ (as indicated), to the control wells. To effect depolarization an equal volume of a solution containing 135 mM KCl, 30 mM Glucose, 1 mM $MgCl_2$, 0.1 mM $LaCl_3$, 40 mM $NaHCO_3$, and 1 mM NaH_2PO_4 was added to the stimulated wells. Thus, the final KCl

concentration consisted of either 2.5 mM (bas) solution, or 70 mM (70 K; dep). The osmolarity was similar in all solutions. In all experiments after depolarization the cells were washed once with PBS and then lysed. Cell proteins were separated on SDS-PAGE [9]. Phosphorylated ERK1/2, RSK, and CREB were identified by immunobloting using the corresponding antibodies to the phospho-proteins. The net stimulation under depolarization conditions (K70) was quantified after subtracting a basal phosphorylation level at non-depolarizing conditions (K2.5), and normalizing with the non-phosphorylated proteins [9].

2.2.4. Western blotting

Protein extracts. For the preparation of protein extracts, HEK293, or PC12 cells were lysed in 0.1 ml of buffer containing 150 mM Tris, pH 6.8, 10% Glycerol, 0.6% SDS, Bromophenol Blue and 100 mM β-Mercaptoethanol. Lysates were collected and heated at 100 °C for 5 min. The lysates were then centriguged for 3 min at 20,000xg, saving the supernatants. Protein concentration of the lysates was determined by using Coomassie Brilliant Blue staining. For western blot analysis 20–60 µg protein were loaded on 10% SDS-PAGE gel and electrophoresed. After blotting to a nitrocellulose membrane (Whatman, Maidstone) the blots were blocked for 1 h at RT in TBS-T (25 mM Tris – HCl pH 7.4, 0.9% NaCl and 0.02% Tween-20) with 4% Difco skim milk (BD, Franklin Lakes, NJ).

2.2.5. Statistics

Phosphorylated MEK, ERK1/2, RSK, and CREB were identified by immunoblotting, using the corresponding antibodies to the phospho-protein. The values shown are averages (± SEM) of duplicates or triplicates, carried out in two or three independent experiments, as indicated, and normalized with the corresponding non-phosphorylated proteins, α2δ subunit, or β-catenin. Different conditions were compared with the depolarized cells (70 K; dep) or wt channel. Statistical significance between two groups was evaluated with Student's test. One-way analysis of variance (ANOVA) followed by Tukey's HSD test was used for analysis between three or more groups. Statistics were performed in GraphPad Prism 5. In the figures the criterion for statistical significance was set at $*P < 0.05$, $**P < 0.01$, and $***P < 0.001$.

3. Results

3.1. The impermeable cation La^{3+} supports depolarization-triggered ERK1/2 CREB phosphorylation through binding at the channel pore

Previously we have shown that the human L-type channel Cav1.2 and the cation impermeable mutant Cav1.2^{L745P} mediate depolarization-triggered transcriptional activation [9]. To gain further insight into the role of Ca^{2+} influx in Cav1.2 mediated-ET coupling, lanthanum (La^{3+}) was substituted for Ca^{2+} in nominally Ca^{2+}-free solution. Similar to Ca^{2+} La^{3+} occupies the channel pore and exhibits tight binding at the selectivity filter, unable to permeate into the cell [22]. HEK293 cells were transfected with the three channel subunits $\alpha_1$1.2/α2 δ/β2b (see [9]) and 72 h later were washed to remove Ca^{2+} and treated for 3 min with 70 mM KCl (70 K; dep) or 2.5 mM KCl (bas) solutions containing La^{3+}, at the indicated concentrations. The concentrations of La^{3+} used were based on previous known levels of La^{3+} that optimally mediate stimulus secretion coupling in PC12 cells and pancreatic islets [18,19].

As shown, membrane depolarization activated the ERK-RSK-CREB pathway appeared to be maximal at 0.1 mM La^{3+} in a nominally Ca^{2+}-free solution (Fig. 1A and B). Activation of the ERK-CREB pathway was evaluated by monitoring ERK1/2, RSK (ribosomal protein S6 kinase), and CREB phosphorylation during stimulation (dep) compared to control (bas) and normalized to total ERK1/2, RSK, or CREB as well as to α2δ (Fig. 1B). Maximal activation at 0.1 mM La^{3+} is parallel to the maximal catecholamine release seen at 0.1 mM La^{3} in PC12 cells [18].

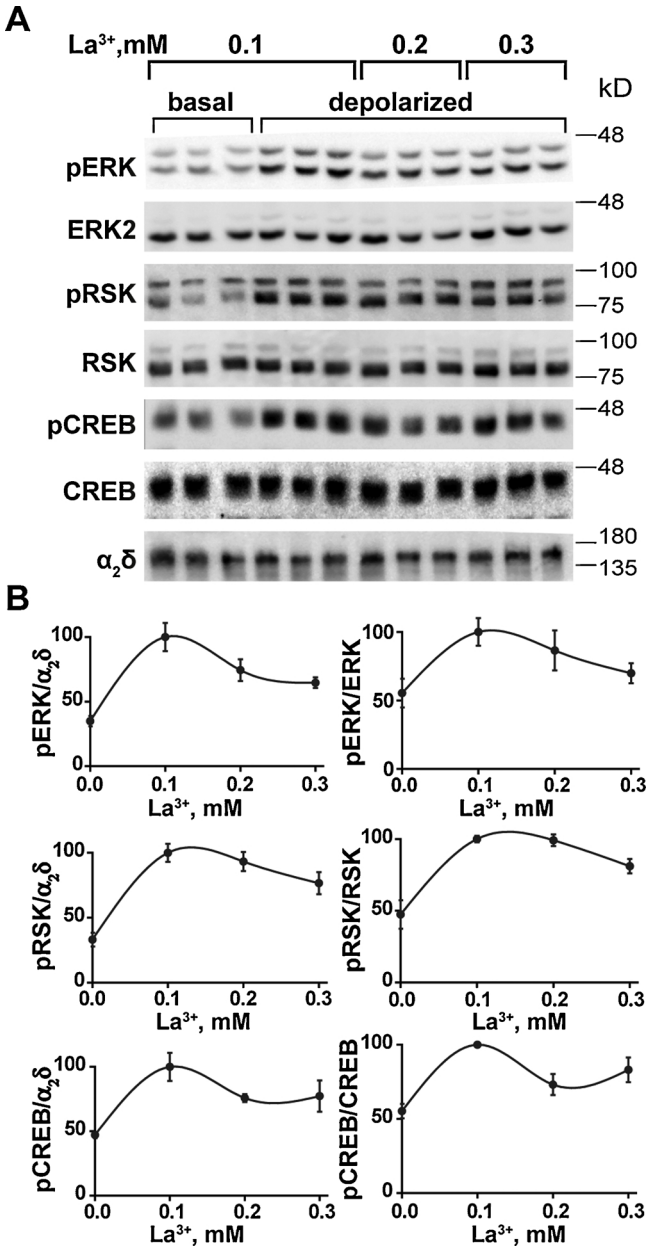

Fig. 1. Membrane depolarization drives ERK1/2, RSK and CREB activation using La^{3+} ions as the charge carrier.
(**A**) HEK293 cells were transiently transfected with Cav1.2 channel subunits $\alpha_1$1.2/α2δ/β2b. After 72 h the cells were treated with non-depolarizing solution (2.5 K; bas) containing 0.1 mM La^{3+} or depolarizing (70 K; dep) solutions containing 0.1 mM, 0.2 mM or 0.3 mM La^{3+} in the absence of Ca^{2+}, for 3 min. The cells were harvested, lysed and proteins in cell extracts were resolved by SDS-PAGE, and analyzed by western blot analysis (**Materials and methods**). Transfection efficiency was normalized using the corresponding anti-α2δ antibodies (**B**) Quantification of phosphorylated proteins, pERK1/2 (lower band), pRSK (lower band) and pCREB as well as α2δ was performed by densitometry and plotted with a linear regression program. The values shown are averages (± SEM) of triplicates.

Next, we compared gene activation mediated by wt Cav1.2 and the Timothy channel Cav1.2^{G406R}. The Timothy channel is a mutated Cav1.2, harboring a single missense mutation G406R located within the IS6 region of the $\alpha_1$1.2 pore forming subunit. The Timothy syndrome manifests itself with cardiac arrhythmias, adrenal gland dysfunction, and autism spectrum disorder (ASD) [27].

HEK293 cells transfected with wt Cav1.2 ($\alpha_1$1.2/β2b/α2δ) or with

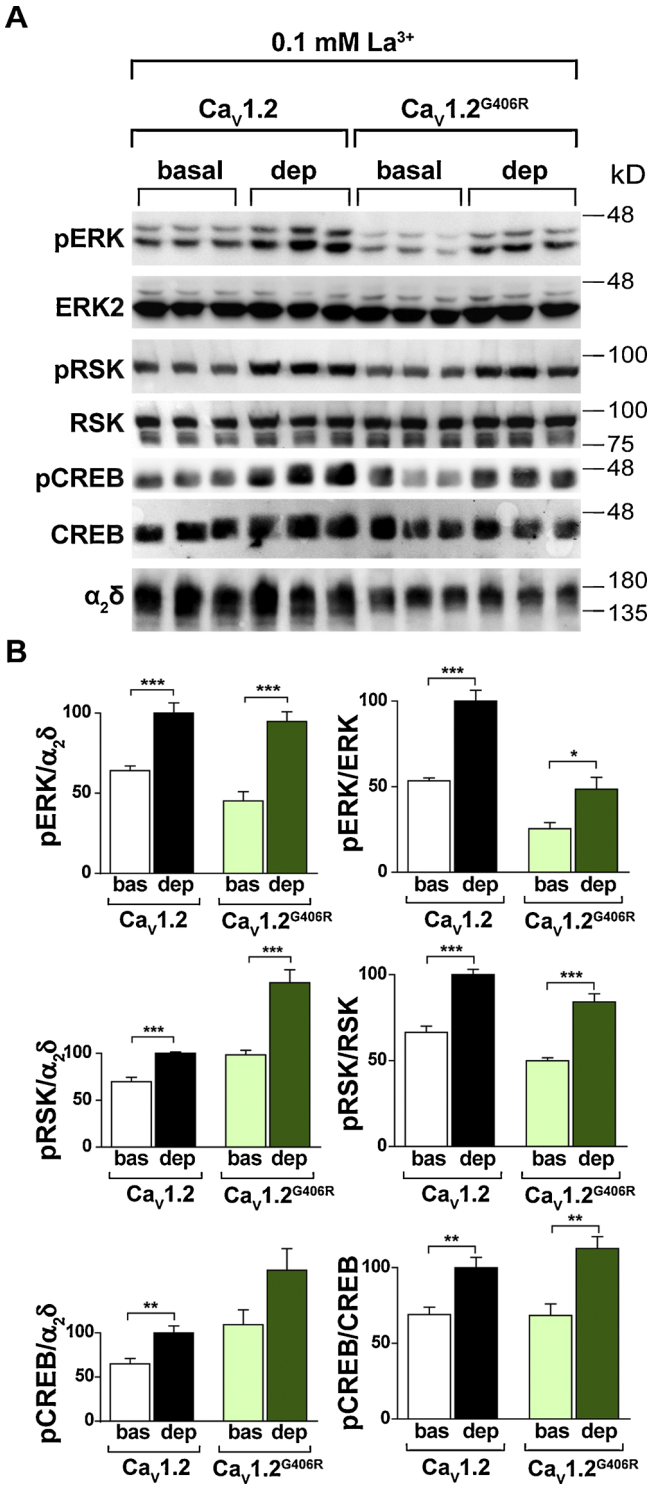

Fig. 2. Impermeable La^{3+} ions activate depolarization-triggered ERK1/2, RSK and CREB phosphorylation.
(**A**) HEK293 cells were transiently transfected with Cav1.2 channel subunits $\alpha_1$1.2/α2δ/β2b or the Timothy channel $\alpha_1 1.2^{G406R}$/α2δ/β2b. At 72 h after transfection the cells were treated with non-depolarizing solution (2.5 K; bas) containing 0.1 mM La^{3+} or depolarizing (70 K; dep) solution containing 0.1 mM La^{3+} for 3 min, as indicated. The cells were harvested, lysed, and proteins in cell extracts were resolved by SDS-PAGE and analyzed by western blot analysis (**Materials and methods**). Transfection efficiency was monitored using the anti-α2δ antibodies (**B**) Quantification of ERK1/2 (lower band), RSK and CREB phosphorylation normalized using the corresponding non-phosphorylated proteins, ERK2, RSK and CREB, as well as α2δ, was performed by densitometry and plotted with a linear regression program. The values shown are averages (± SEM) of triplicates of two independent experiments (see second experiment in **Fig. S1**). Student's *t*-test (two populations) was performed; *p value < 0.05; **p value < 0.01; ***p < 0.001.

the Cav1.2^{G406R} ($\alpha_1 1.2^{G406R}$/β2b/α2δ) were depolarized 72 h after transfection by 70 K (dep) for 3 min, or treated with a non-depolarizing control solution containing 2.5 mM KCl (bas) for 3 min (Fig. 2). The charge carriers used were 0.1 mM Ca^{2+} or 0.1 mM La^{3+} with no added Ca^{2+} in the external solution, and not buffered by Ca^{2+} chelators.

La^{3+} appeared to support gene activation mediated by both wt Cav1.2 and the Timothy channel Cav1.2^{G406R} (Figs. 2; **S1**). Channel opening and cation binding at the selectivity filter, without permeation, seemed to be sufficient for induction of downstream intracellular signaling. The ion-flow independent activation is reminiscent of downstream signaling triggered by the NMDARs in an ion-flow independent mode [17,28–30], consistent with ET coupling driven by the impermeable mutants Cav1.2^{L745P} [9].

3.2. The effect of Cd^{2+}, verapamil, and FPL on ERK activation with Ca^{2+} substituted for La^{3+}

Next we explored the inhibitory effects of: 1) verapamil (Ver;), a selective Cav1.2 blocker that binds intracellularly to the open pore 2) Cd^{2+}, a general VGCC pore blocker that prevents Ca_V1.2 from conducting Ca^{2+} without affecting depolarization, and 3) FPL-64176, a Cav1 channel agonist, known to increase Cav1 open probability. At 72 h after transfection the cells were treated with either depolarizing solution (final 70 mM KCl) or a non-depolarizing solution (final 2.5 mM KCl) in the absence of Ca^{2+}, or La^{3+}, or in the presence of 0.1 mM La^{3+} with or without Cd^{2+} (200 μM), verapamil (20 μM), or FPL-64176 (2 μM) (Fig. 3). There was no significant increase in depolarization-triggered ERK1/2 phosphorylation in the absence of Ca^{2+} or La^{3+} (Fig. 3A and B). This is consistent with the importance of pore occupancy in Cav1.2 activating intracellular pathways [9,18,20,11–13]. As shown, ERK1/2 phosphorylation was virtually abolished by Cd^{2+}, or verapamil (back to control dep levels without a charge carriers). As expected the Cav1.2 channel agonist FPL-64176, potentiated depolarization-driven ERK phosphorylation (Fig. 3A and B). Hence, the use of La^{3+} did not alter the inhibitory effects of Cd, Ver, or the potentiation by FPL-64176 on nuclear activity, compared to those observed when Ca^{2+} was used as the charge carrier [9].

3.3. Ba^{2+} ions support ET coupling in HEK293 cells

The role of Ca^{2+}/CaM complex in ET coupling was examined by Ca^{2+} substituted with Ba^{2+}, which does not bind to CaM nor does it displace Ca^{2+} from Ca^{2+}/CaM complex [23].

HEK293 cells transfected with wt Cav1.2 ($\alpha_1$1.2/β2b/α2δ) channel and depolarized 72 h after transfection with 70 K for 3 min (dep), or treated with a control solution containing 2.5 mM KCl for 3 min (bas). The charge carriers were 2 mM Ba^{2+} or 2 mM Ca^{2+}. As shown the extent of membrane-depolarization triggered ERK1/2 and CREB phosphorylation was not affected by exchanging Ca^{2+} for Ba^{2+} (Fig. 4; see also **Fig. S2**). Hence, nuclear activity driven by elevating intracellular Ba^{2+} excludes activation *via* the Ca^{2+}/CaM-cascade or *via* calcineurin, the Ca^{2+}-dependent protein phosphatase. These results are consistent with a minor effect of the CaM inhibitor trifluoperazine on ERK-CREB activation [9,31]. It also agrees with a lack of an effect on ET coupling of Cav1.2^{I1264A}, a mutated IQ motif that does not bind Ca^{2+}/CaM [9].

3.4. Ba^{2+} ions support ET coupling in PC12 cells

Next, we exmained depolarization-triggered nuclear activity in the

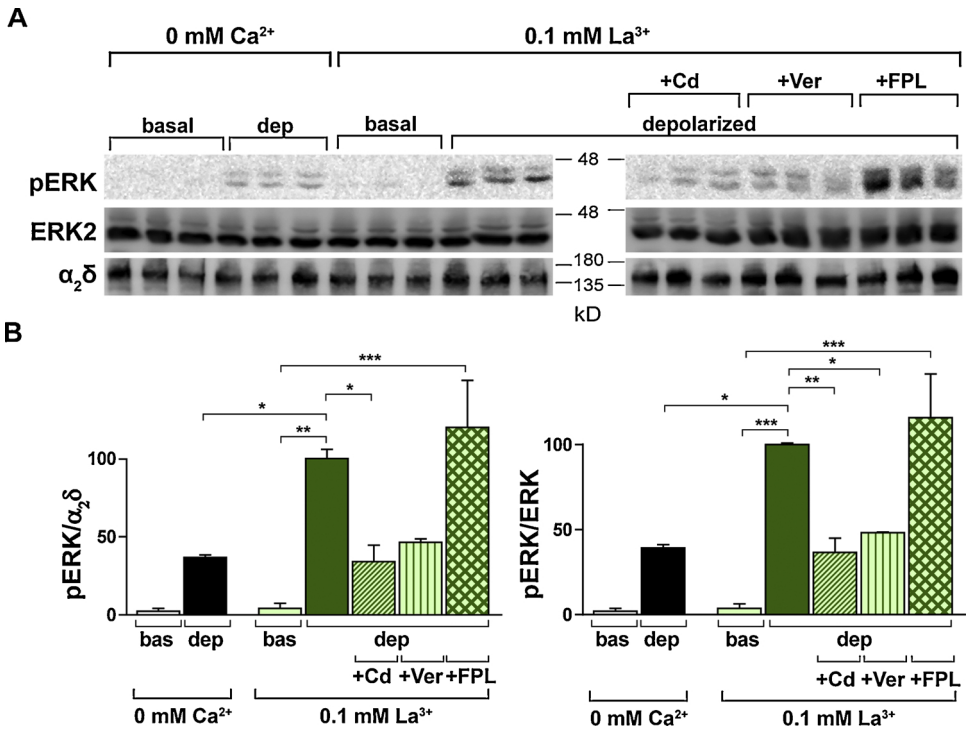

Fig. 3. The effect of Cd^{2+}, verapamil and FPL-64176 on ERK phosphorylation driven by membrane depolarization with La^{3+}. (**A**) HEK293 cells transiently transfected with Cav1.2 channel subunits $\alpha_1$1.2/α2δ/β2b were treated 72 h after transfection with non-depolarizing solution (2.5 K; bas) or depolarizing (70 K; dep) solution for 3 min, in the absence of Ca^{2+} or La^{3+}, or in the presence of 0.1 mM La^{3+}, without or with, Cd^{2+} (200 µM), verapamil (Ver; 20 µM) or FPL-64176 (2 µM), as indicated. The cells were harvested, and cell extract proteins were lysed, resolved on SDS-PAGE, and analyzed by a western blot analysis (**Materials and methods**). Transfection efficiency was monitored using anti-α2δ antibodies (**B**) ERK phosphorylation (lower band) was quantified by densitometry using $\alpha_2\delta$ subunit antibodies (*left*) or the non-phosphorylated ERK2 (*right*), and plotted with a linear regression program. The values shown are averages (± SEM) of triplicates. One way analysis ANOVA was performed; *p value < 0.05; **p value < 0.01; ***p < 0.001.

neuroendocrine pheochromocytoma PC12 cells.

Initially, we determined the contribution of the L-type channels to gene activation in these cells using nifedipine, the selective L-type channel blocker. Membrane depolarization was triggered by 70 mM KCl (70 K; 3 min) compared to a control silution of 2.5 mM KCl (bas; 3 min), in the presence or in the absence of nifedipine (8 µM). As shown phosphorylation of ERK1/2 was completely inhibited in the presence of nifedipine, showing the predominance of L-type channel in mediating depolarization-triggered nuclear activity in PC12 cells (Fig. 5**)**. It is well documented that the contribution of Ca_V1 channels to the overall Ca^{2+} entry in the cell through a plethora of VGCCs is the smallest, yet Cav1 are responsible for depolarization-triggered catecholamine release in chromaffin and in PC12 cells [18], and for depolarization triggered nuclear activity in PC12 [32] and in neuronal cells [8].

We substituted Ca^{2+} with Ba^{2+} and examined depolarization-triggered ERK1/2 and CREB phosphorylation with or without nifedipine (8 µM) (Fig. 6; see also **Fig. S3**). A significant increase in phosphorylated ERK1/2 and CREB during membrane depolarization (70 K; 3 min) was observed with Ca^{2+} as the charge carrier, and activation was inhibited by nifedipine. In the presence of Ba^{2+} however, the basal phosphorylation (without depolarization) of ERK1/2 and CREB was significantly higher. This elevated basal activity, which could be attributed to Ba^{2+} blockade of voltage-gated K^+ channel, or to a Cav1.2-independent nuclear activation, or both, masked the additional activation during membrane depolarization. Nifedipine blocked phosphorylation of ERK1/2 and CREB, either in Ca^{2+} or Ba^{2+}. These results confirm L-type channel mediated ERK1/2 and CREB singling using Ba^{2+} as the charge carrier (Fig. 6; see also **Fig. S3**).

3.5. Barium ions support gene transcription in PC12 cells

The effects of calcium and barium on transcriptional activation triggered by Cav1.2 was further investigated. PC12 cells were depolarized (70 K; 3 min) and allowed to incubate for an additional period of 60 min at 37 °C. Elevation of phospho-cFos, BDNF, MeCP2, or c-Jun expression was demonstrated with either Ca^{2+} or Ba^{2+} (Fig. 7). Depolarization-triggered gene expression of cFos, BDNF, MeCP2, or cJun was inhibited when the cells were incubated with nifedipine (8 µM), indicating L-type channel involvement in triggering nuclear activity. No significant differences were observed by substituting Ca^{2+} for Ba^{2+}. These results demonstrate the lack of contribution of the Ca^{2+}/CaM kinase dependent cascade, or the Ca^{2+}-dependent protein phosphatase calcineurin, to ET in PC12 cells.

4. Discussion

Long-term learning and plasticity require transcriptional activation in a process called excitation- transcription (ET) coupling. Recent studies support the role of Ca^{2+}/CaM in triggering ET coupling, even though the contributions of Ca^{2+} influx to this process was not fully investigated, see recent review [33]. In the present study, we examined ET coupling triggered in HEK293 and PC12 cells *via* the ERK1/2-RSK-CREB-pathway. We showed that ET coupling can be triggered using Ca^{2+}, Ba^{2+}, or La^{3+}, schematically shown in Fig. 8. No ET coupling was observed in the absence of divalent or trivalent cations in the extracellular media, highlighting the importance of cation occupancy of the selectivity filter as opposed to cation influx. Ba^{2+} binds to the selectivity filter of Cav1.2 and permeates into the cell, and supports depolarization-triggered gene transcription *via* the ERK-RSK-CREB pathway, in spite of its inability to bind to calmodulin and activate the Ca^{2+}/CaM enzymatic cascade.

4.1. Metabotropic Cav1.2 activity can induce gene activation

La^{3+} has a similar ionic radius (1.06 Å) to Ca^{2+} (O.99 Å), by virtue of which it binds to the EEEE locus, the sole high-affinity Ca^{2+} binding motif in the pore of the VGCC [22,34–36].

La^{3+} ion with higher affinity than Ca^{2+} ion, competes for binding at the selectivity filter. When both Ca^{2+} and La^{3+} ions are present, calcium permeation is blocked by La^{3+}. Therefore, La^{3+} in the presence of Ca^{2+} is widely used as a channel blocker. In contrast, in the absence of Ca^{2+}, La^{3+} functions as a charge carrier and performs in a manner similar to Ca^{2+} without permeation.

Cav1.2 function in Excitation Secretion (ES) coupling is also supported by La^{3+}. For example depolarization-evoked catecholamine release in chromaffin cells, and insulin release triggered by high-glucose

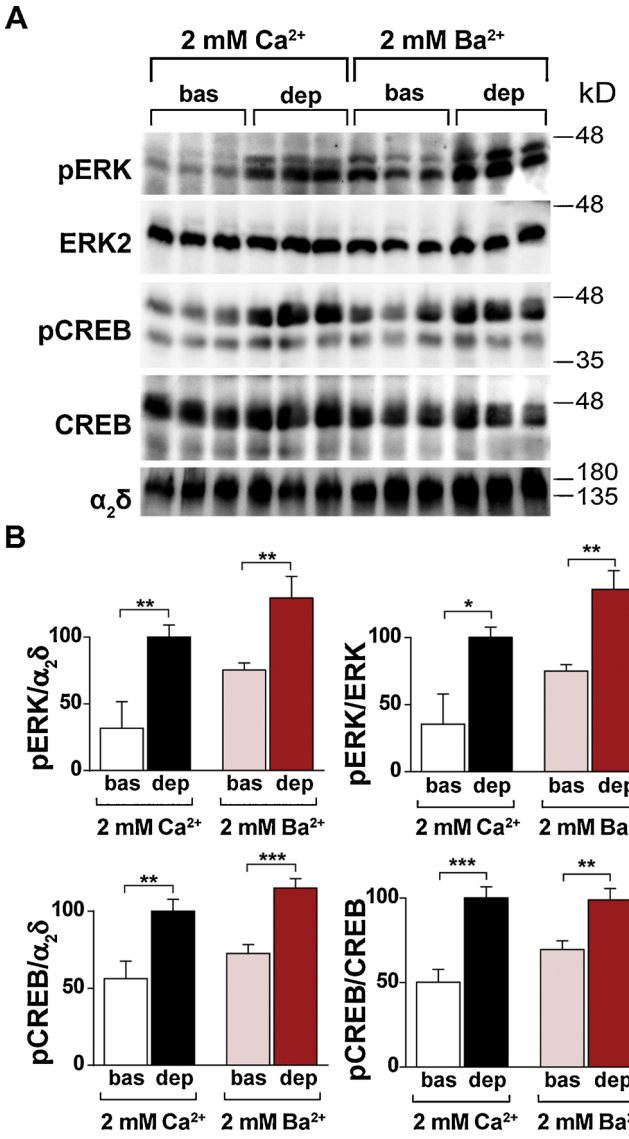

Fig. 4. Ba^{2+} ions support ET coupling by activating ERK1/2 and CREB phosphorylation in HEK293 cells transfected with Cav1.2 channel subunits.

(A) HEK293 cells were transiently transfected with Cav1.2 channel subunits $\alpha_1$1.2/α2δ/β2b. After 72 h the cells were treated with control solution (2.5 K; bas) or depolarizing (70 K; dep) solution for 3 min. The charge carriers were 2 mM $CaCl_2$ or 2 mM $BaCl_2$, as indicated. The cells were harvested after depolarization (70 K; 3 min), and proteins in cell extracts were lysed, resolved by SDS-PAGE, and analyzed by western blot analysis. Transfection efficiency was normalized using anti-α2δ antibodies **(B)** Quantification of phosphorylated ERK1/2 (lower band) and phosphorylated CREB (upper band) was performed by densitometry and plotted with a linear regression program. The values shown are averages (± SEM) of triplicates of three independent experiments (see two additional experiments in **Fig. S2**), normalized to $\alpha_2\delta$-subunit (*left*) or to the non-phosphorylated ERK and CREB, respectively (*right*); One way analysis of variance (ANOVA) test was performed; **p value < 0.01; ***p < 0.001.

in pancreatic islets are also supported by substituting La^{3+} for Ca^{2+} [18–20].

Here we showed that La^{3+} supports depolarization-triggered ERK1/2, RSK, and CREB phosphorylation in HEK293 cells expressing wt Cav1.2 or the mutated G406R Timothy channel. The Timothy channel is a Cav1.2 single point mutant characterized by a loss of voltage-dependent inactivation (VDI), and impaired calcium-dependent inactivation (CDI), both of which lead to an increase in intracellular calcium

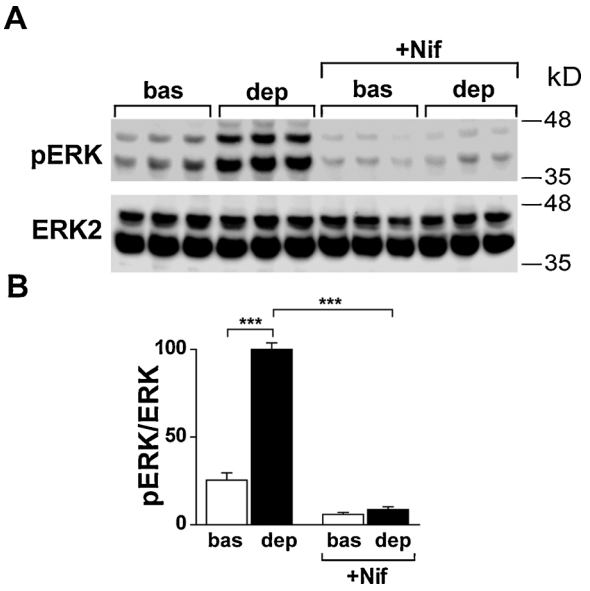

Fig. 5. Nifedipine blocks depolarization triggered ERK1/2 phosphorylation in PC12 cells.

PC12 cells were treated with non-depolarizing control solution (2.5 K; basal) or with a depolarizing solution (70 K; dep) for 3 min. The cells were harvested, lysed, and phosphorylated ERK1/2 in the cell extracts were resolved by SDS-PAGE, and analyzed by western blot analysis. Quantification of phosphorylated ERK1/2 (lower band) was performed by densitometry and plotted with a linear regression program. The values shown are averages (± SEM) of two independent experiments normalized to the corresponding non-phosphorylated proteins; One-way analysis of variance (ANOVA) test was performed;***p < 0.001.

[37,38]. The calcium overload typical of the Timothy channel is considered responsible for cardiac arrhythmias, and long term dysregulations. By substituting La^{3+} for Ca^{2+} we were able to exclude calcium inflow, and thereby negate the impact of Ca^{2+}-overload in depolarization-triggered gene activation. The ion-inflow independent conformational change that triggers nuclear activation *via* the Timothy channel implies an alternative mechanism responsible for long-term dysregulation such as ASD, which are associated with this mutant.

Our observation that La^{3+} supports depolarization-triggered gene activation programs is consistent with nuclear activity mediated also by the Ca^{2+} impermeable channel mutant, $Cav1.2^{L745P}$ [9]. These results show that Cav1.2 may also trigger intracellular signaling independently of Ca^{2+} inflow, confirming the metabotropic role of Cav1 in ES coupling, and EC coupling [13,15,20,39].

Previous studies have shown that glutamate binding to the NMDAR could induce conformational changes in the intracellular domain of the NMDAR and trigger nuclear activity independent of ion-flux. This metabotropic function of the NMDAR was shown to be associated with induction of long-term depression [28,29,16,30].

4.2. *Ba^{2+} substitution for Ca^{2+} supports gene activation*

Gene activation in the superior cervical ganglion, which participates in synaptic potentiation, learning and memory is activated by members of the CaM-kinase family of CaM-dependent enzymes [40–43]. The multifunctional serine/threonine CaMKII is highly expressed in the brain, and acts as a major calcium signaling protein [44]. The neuronal CaMKII is activated by Ca^{2+}/CaM binding, which removes the autoinhibition of CaMKII [45]. It was shown that Ca^{2+}/CaM is shuttled to the nucleus when Ca^{2+}/CaM-γCaMKII is phosphorylated by βCaMKII, and activates CREB-dependent transcription [6,46].

It is known that in addition to Ca^{2+}, CaM binds various divalent and trivalent metal cations, which alter its conformation [47]. Several

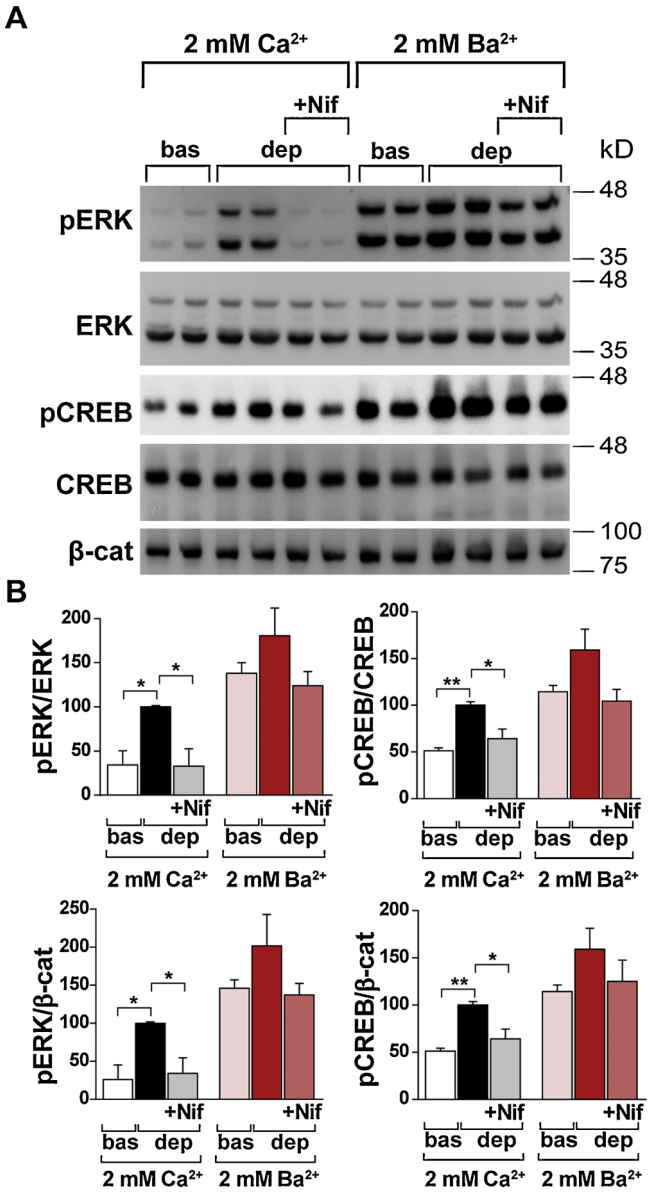

Fig. 6. Ba^{2+} ions activate depolarization-induced ERK1/2 and CREB phosphorylation *via* Cav1.2 in PC12 cells.

(**A**) PC12 cells treated with or without nifedipine (8 μM) were treated with non-depolarizing solution (2.5 K; bas) or depolarizing solution (70 K; dep) for 3 min. The charge carriers used were 2 mM $CaCl_2$ or 2 mM $BaCl_2$, as indicated. Phosphorylated ERK1/2 and CREB were resolved by SDS-PAGE and analyzed by western blot analysis (**B**) Quantification of phosphorylated ERK1/2 (lower band) and CREB was performed by densitometry and plotted with a linear regression program. The values shown are averages (± SEM) of duplicates of two independent experiments normalized to the non-phosphorylated ERK2, non-phosphorylated CREB, or to beta catenin (see the additional experiment in **Fig. S3**); One-way analysis of variance (ANOVA) followed by Tukey's HSD test was used for analysis between three or more groups; *p value < 0.05; **p value < 0.01.

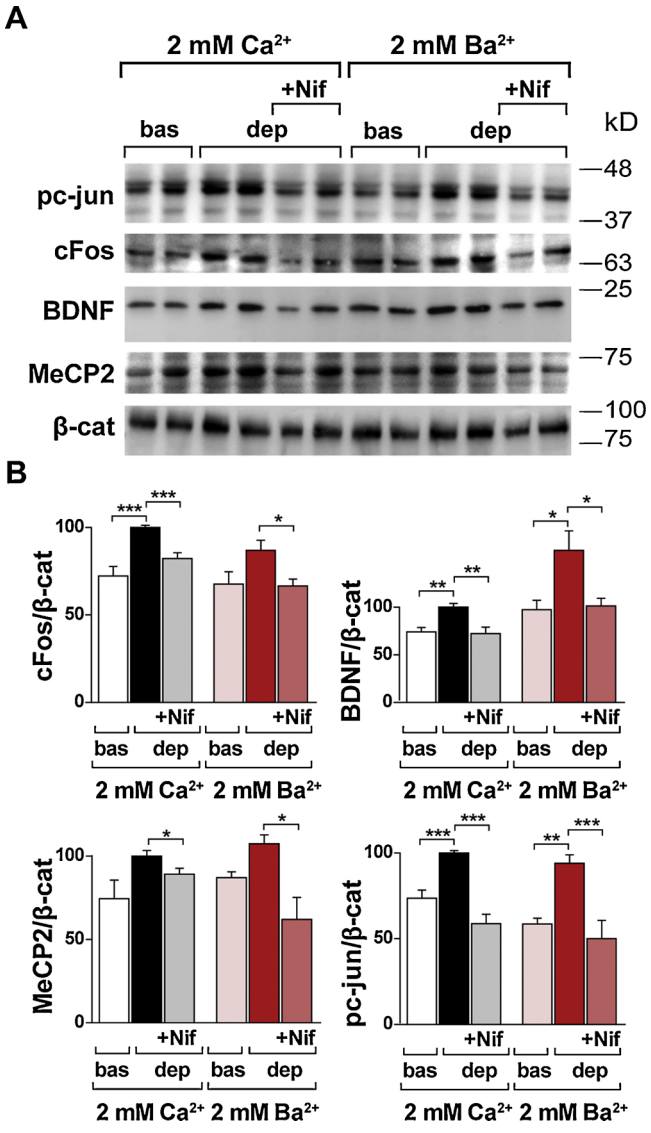

Fig. 7. Ba^{2+} ions support depolarization-triggered gene expression in PC12 cells.

(**A**) PC12 cells were treated with depolarizing solution (70 K; dep) or non-depolarizing control solution (2.5 K; bas) for 3 min, using 2 mM $CaCl_2$ or $BaCl_2$ as charge carriers, in the presence or in the absence of nifedipine (8μM). After depolarization the cells were incubated for 60 min at 37 °C, harvested, lysed, and resolved by SDS-PAGE. The expression of phospho-cJun, cFos, BDNF, and MeCP2 was determined by western blot analysis using the corresponding antibodies **(B)** Quantification was performed by densitometry and plotted with a linear regression program. The values shown are averages (± SEM) of four independent experiments normalized to β-catenin (see duplication experiments **Fig. S4**). One-way analysis of variance (ANOVA) followed by Tukey's HSD test was used for analysis between three or more groups; *p value < 0.05; **p value < 0.01; ***p < 0.001.

cations having ionic radii in the range of 1 ± 0.2 Å could substitute for Ca^{2+} in binding to CaM, while Ba^{2+} with a larger ionic radius (1.34 Å) is unable to bind to CaM or displace Ca^{2+} from Ca^{2+}/CaM complex. Indeed, ion current-dependent inactivation of Cav1.2 mediated through Ca^{2+}/CaM binding to the C-terminal of the $\alpha_1$1.2 subunit is ~100 times less effective when Ba^{2+} replaces external Ca^{2+} [48,49]. Also, Ba^{2+} is unable to support CaM-stimulated phosphodiesterase activity [23]. We found however, that Ba^{2+} supports Cav1.2 depolarization-triggered phosphorylation of ERK1/2, and CREB, and increases the expression of cJun, cFos, BDNF, and MeCP2 in PC12 cells. These results suggest that gene transcription using Ba^{2+} ions is not mediated *via* Ca^{2+}/CaM dependent pathways even though barium ions bind to the channel pore and mimic channel activities such as exocytosis. Hence, transcriptional activation in PC12 cells is triggered independently of the Ca^{2+}/CaM-dependent enzymatic cascade. This data is also consistent with an only small effect of the CaM inhibitor trifluperazine on ERK-CREB activation, and no effect of the IQ motif mutant Cav1.2^{I1643A} on ET coupling [9]. We cannot however, exclude a Ca^{2+}-independent activation of CaM/CaMKII or CaM/GRF1, *via* the IQ motif, because several proteins have been shown to interact with CaM in a Ca^{2+}-

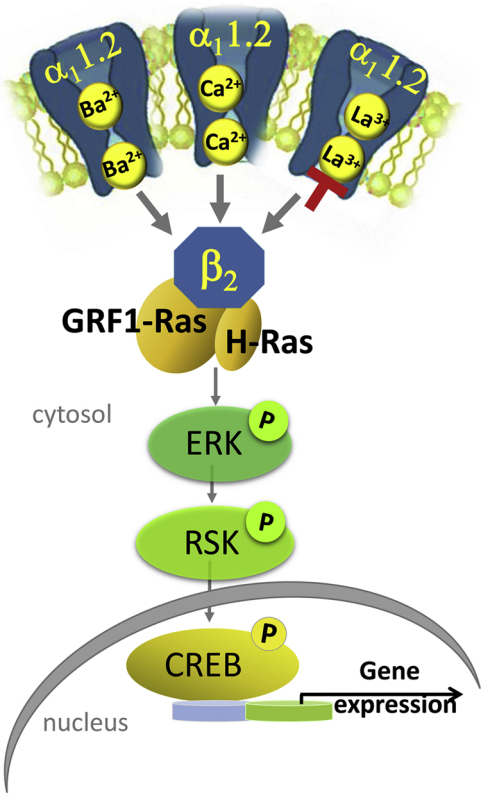

Fig. 8. Depolarization triggered transcriptional activation is supported by divalent and trivalent cations that bind to the Cav1.2 channel pore. Depolarization-triggered nuclear activity *via* the Ras-ERK-RSK-CREB pathway, is mediated by the auxiliary β subunit of the VGCC, which interacts directly with the Ras/GRF1 complex. This excitation-transcription coupling is triggered by substitution Ca^{2+} with the impermeable cation La^{3+} or by the permeable Ba^{2+} cation. ET coupling supported by La^{3+} indicates ion-flux independent activity. ET coupling supported by Ba^{2+}, which does not bind to calmodulin and is unable to activate Ca^{2+}/CaM dependent proteins, suggests a Ca^{2+} independent gene activation pathway. We propose a model in which a conformational change that is critically dependent on cations occupancy of the open channel pore ($\alpha_1$1.2), is conveyed through the β subunit and onto the H-Ras/GRF1 complex, activates nuclear signaling *via* the ERK-RSK-CREB pathway.

independent manner in a form of 'apocalmodulin', which is active when cellular Ca^{2+} is low [50–52].

In summary, our study shows that barium ions are as effective as calcium ions in triggering ET coupling, indicating a Ca^{2+}/CaM-independent pathway. Lanthanum ions also mediate ET coupling. It appears that cation occupancy of the channel pore, as opposed to Ca^{2+} inflow, is critical for ET coupling. These results highlight the metabotropic function of wt Cav1.2 and the Timothy channel Cav1.2^{G406R}. They indicate that cation binding at the open channel pore acts prior to and independent of ion inflow, as previously shown in depolarization-triggered catecholamines or insulin release, similar to specific ligand-binding at membrane receptors.

Authors contributions

DA supervised research; E. S. and MT performed research; and analyzed the data. All authors commented on the paper.

Ethics approval

Not applicable

Declaration of Competing Interest

None.

Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.ceca.2019.102102.

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