

Research Report

T-type calcium channel trigger p21^{ras} signaling pathway to ERK in Ca_v3.1-expressed HEK293 cells

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Accepted 3 May 2005

Available online 27 July 2005

Abstract

We constructed a new cell line which stably expressed Ca_v3.1 and Kir2.1 subunits in HEK293 cells (HEK293/Ca_v3.1/Kir2.1) in order to investigate the unknown cellular signaling pathways of T-type voltage-dependent calcium channels. The new cell line has a stable resting membrane potential and can activate T-type Ca²⁺ channels by KCl-mediated depolarization. We showed that Ca_v3.1 activation resulted in the level of p21^{ras}-GTP in the cells being rapidly decreased during the first 2 min, and then recovering between 2 min and 15 min. The kinetics of ERK activation following Ca_v3.1 stimulation was also investigated. ERK activation was decreased from 2 min to 5 min after KCl stimulation, which means that Ca_v3.1 activation reduced ERK activity in the very early stages of activation. In addition, similar results for Ca_v3.1 activation were also shown in the case of Sos1, Grb2, and Shc, which means that Ca_v3.1 activation triggers p21^{ras} and that this signal is transferred to ERK by Sos1, Grb2, and Shc.

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Theme: Cellular and molecular biology

Topic: Calcium channel structure, function, and expression

Keywords: Ca_v3.1 T-type calcium channel; HEK293/Ca_v3.1/Kir2.1 cells; p21^{ras}; ERK

1. Introduction

Voltage-dependent calcium channels (VDCC) in neurons and other cells have been divided into high and low voltage-activated (HVA and LVA or T-type) classes [7,8,26]. T-type VDCCs were found to mediate the low-threshold spike and to be involved in rebound burst firing, oscillations, and resonance [20]. T-type VDCCs characteristically begin to activate at about –70 mV under

physiological conditions, producing a maximum current at between –40 and –20 mV [7,20]. The current waveform is transient with a division into rapidly and more slowly inactivating subtypes [9,21]. Although a class of LVA T-type calcium channel has been cloned and its native role has been identified, the molecular function of T-type VDCCs has so far eluded investigation [12,14,23]. T-type VDCCs have properties which differ from those of HVA VDCCs, such as a more negative voltage range for their activation and inactivation and more rapid gating kinetics [28]. It has been reported that T-type VDCCs participate in cardiac pacemaking [18], as well as the regulation of vascular tone and hormone secretion [24,37].

In sensory neurones, endogenous Ras is involved in the tonic upregulation of VDCC [15], and the activation of the

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Ras-MAPK pathway was also shown to suppress endogenous T-type VDCC in Swiss 3T3 cells [33]. It remains to be elucidated how activation of the MAPK pathway inhibits T-type Ca^{2+} channels. To investigate the unknown cellular signaling pathways of T-type Ca^{2+} channels, we have reached the need of making a useful cell line which has a stable resting membrane potential and can activate T-type Ca^{2+} channels by biochemical tools such as KCl or channel activators. Among the inwardly rectifying family of potassium channel subunits (Kir), Kir2 family members show strong rectification, preferentially passing potassium ions into the cell. It has been demonstrated that Kir2.1 is one of major determinants of resting membrane potential in many cell types [22]. We therefore devised human embryonic kidney (HEK293) cells stably transfected with Kir2.1 subunit (HEK293/ $\text{Ca}_v3.1$ /Kir2.1) from the cells stably express $\text{Ca}_v3.1$ subunit, a subset of T-type α_1 subunits (HEK293/ $\text{Ca}_v3.1$) [34].

We also performed yeast two hybrid assay using intracellular domains of $\text{Ca}_v3.1$ as baits. In this assay we found several binding candidates in human brain library (manuscript in preparation). These included RanBPM, a novel Met-interacting protein which stimulates Ras activation by recruiting Sos [38]. Therefore, we investigated a link between T-type VDCCs and Ras activity.

In the present study, we showed that $\text{Ca}_v3.1$ activation reduces the activation of p21^{ras} in HEK293/ $\text{Ca}_v3.1$ /Kir2.1 cells. After the activation of $\text{Ca}_v3.1$ by KCl, the level of p21^{ras} -GTP rapidly decreased for the first 2 min, and then recovered between 2 min and 15 min. We also found the $\text{Ca}_v3.1$ activation was correlated with ERK activity, as well as with other signaling molecules, such as Sos1, Grb2, and Shc.

2. Materials and methods

2.1. Cloning of human Kir2.1 cDNA

The full-length gene for human Kir2.1 cDNA was amplified from a human brain cDNA library in plasmid (Takara, Kyoto) with cKir2.1 forward (5'-CCGCTCGAGGCCGCCATGGGCAAGTGTGAG-3') and cKir2.1 reverse (5'-CCGGAATTGCATATCTCCGATTCTCGCC-3') as primers. The PCR product was cloned into the *Xho*I/*Eco*RI site of pCMVpuro (Clontech).

2.2. Generation of stably transfected HEK293/ $\text{Ca}_v3.1$ /Kir2.1 cells

HEK293 cells stably expressing human $\text{Ca}_v3.1$ subunit of T-type Ca^{2+} channels were kindly provided from Dr. Perez-Reyes (University of Virginia) and grown in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal bovine serum, penicillin (100 units/ml), streptomycin (100 $\mu\text{g}/\text{ml}$), and geneticin (500 $\mu\text{g}/\text{ml}$). For the generation

of HEK293/ $\text{Ca}_v3.1$ /Kir2.1 cell line, Kir2.1 (cloned into pCMVpuro) was transfected with HEK293/ $\text{Ca}_v3.1$ cells using pEQPAM3 and pVSV-G (Clontech) for the production of a retrovirus including the Kir2.1 gene. A standard calcium phosphate transfection procedure was performed. These retrovirus-infected HEK293/ $\text{Ca}_v3.1$ cells positively selected with puromycin (1 $\mu\text{g}/\text{ml}$) in DMEM medium to generate HEK293/ $\text{Ca}_v3.1$ /Kir2.1 cell line which stably expressed both $\text{Ca}_v3.1$ and Kir2.1 subunits. Cells were incubated in a humid atmosphere of 5% CO_2 and 95% air at 37 °C.

2.3. Intracellular Ca^{2+} imaging

The acetoxymethyl-ester form of fura-2 (fura-2/AM; Molecular probes, Eugene, OR) was used as the fluorescent Ca^{2+} indicator. Cells were incubated for 40–60 min at room temperature with 5 μM fura-2/AM and 0.001% Pluronic F-127 in a HEPES-buffered solution composed of the following (in mM): 150 NaCl, 5 KCl, 1 MgCl_2 , 2 CaCl_2 , 10 HEPES, 10 glucose, whose pH was adjusted to 7.4 with NaOH. The cells were then washed with HEPES-buffered solution and placed on an inverted microscope (Olympus, Japan). The cells were illuminated using a xenon arc lamp, and the required excitation wavelengths (340 and 380 nm) were selected by means of a computer-controlled filter wheel (Sutter Instruments, CA). Data were acquired every 2 s and a shutter installed in the light path was closed between exposures to protect the cells from phototoxicity. Emitter fluorescence light was reflected through a 515-nm long-pass filter to a frame transfer cooled CCD camera, and the ratios of emitted fluorescence were calculated using a digital fluorescence analyzer and converted to intracellular-free Ca^{2+} concentration ($[\text{Ca}^{2+}]_i$). All imaging data were collected and analyzed using Universal Imaging software (West Chester, PA).

2.4. Immunoprecipitation and Western blot analysis

Stimulation of cells was terminated by washing twice with ice-cold phosphate-buffered saline (PBS). The cells were then lysed with 50 mM HEPES buffer, at pH 7.5, containing 150 mM NaCl, 10% glycerol, 1% Triton X-100, 1.5 mM MgCl_2 , 1 mM EGTA, 10 $\mu\text{g}/\text{ml}$ leupeptin, 10 $\mu\text{g}/\text{ml}$ aprotinin, 1 mM phenylmethylsulfonyl fluoride, and 0.2 mM sodium orthovanadate for 20 min at 4 °C. The cells were scraped from the dishes and centrifuged at 15,000 $\times g$ for 20 min at 4 °C. The resulting supernatant was mixed with the antibodies which had been bound to protein A/G plus agarose (Santa Cruz Biotechnology, Santa Cruz, CA), rotated for 1 h at 4 °C, and then the beads were spun down and washed with lysis buffer. The resulting immunoprecipitates were boiled in sample buffer, electrophoresed in SDS polyacrylamide gel, transferred to a PVDF membrane (Millipore, Bedford, MA), and then blotted with the indicated antibodies.

2.5. In-vivo Ras activation assay

HEK293/Ca_v3.1/Kir2.1 cells were treated with or without 150 mM KCl and lysed for a subsequent in vivo Ras activation assay. Cell lysates (1 mg) prepared from HEK293/Ca_v3.1/Kir2.1 cells were incubated with 40 μg of Raf-1 RBD agarose (Upstate Biotech) at 4 °C for 2 h. The agarose beads were collected, washed, resuspended in the sample buffer (Upstate Biotech), and resolved on an SDS–PAGE gel, followed by Western blotting with anti-Ras monoclonal antibody (Upstate Biotech). The cell lysates, incubated with GDP and GTPγS (Upstate Biotech), were served as negative and positive controls, respectively. The cell lysates of each sample were used for Western blotting with anti-Ras monoclonal antibody as a loading control.

2.6. ERK activity assay

This assay was performed according to a previously described method [16]. Cells were stimulated with 150 mM KCl and harvested as described above. Lysates, containing 500 μg protein, were immunoprecipitated with 2 μg of anti-p42^{ERK} antibody (Santa Cruz Biotechnology). Immunoprecipitation was carried out at 4 °C for 2 h before adding protein A/G plus agarose (Santa Cruz Biotechnology) and incubating for an additional 1 h. The immunoprecipitates were washed twice in lysis buffer and twice in 20 mM HEPES buffer, at pH 7.4, containing 10 mM MgCl₂ and 0.2 mM sodium orthovanadate and incubated in 0.25 mg/ml myelin basic protein (MBP), 50 μM ATP, and 5 μCi [γ -³²P]ATP for 15 min at 30 °C. The reaction was terminated by the addition of 2× sample buffer and the protein was subjected to electrophoresis in a 12% SDS–PAGE gel and visualized by autoradiography.

3. Results

3.1. High KCl-mediated [Ca²⁺]_i increase in HEK293/Ca_v3.1/Kir2.1 cells

In order to investigate the unknown cellular signaling pathways of T-type Ca²⁺ channels, we constructed a new cell line which stably expressed Ca_v3.1 and Kir2.1 subunits in HEK293 cells (HEK293/Ca_v3.1/Kir2.1) [34]. To confirm the activation of Ca_v3.1 T-type Ca²⁺ channels directly in HEK293/Ca_v3.1/Kir2.1 cells, we measured KCl-mediated changes in intracellular Ca²⁺ concentration ([Ca²⁺]_i) using fura-2-based digital imaging techniques. In HEK293/Ca_v3.1 cells, there was no detectable change of [Ca²⁺]_i by 150 mM treatment of KCl (data not shown). However, application of high concentration of KCl (150 mM, 30 s) produced a transient increase of [Ca²⁺]_i in HEK293/Ca_v3.1/Kir2.1 cells. Treatment of 150 mM KCl induced the increase of [Ca²⁺]_i from 75 to 120 nM in a regular HEPES-buffered solution (Fig. 1). Furthermore, this increase was totally blocked by co-treatment of 10 μM mibefradil, the potent T-type Ca²⁺ channel blocker, with partial recovery. These results indicate that the increase of [Ca²⁺]_i by KCl-induced depolarization is fully responsible for Ca_v3.1 channel activated in HEK293/Ca_v3.1/Kir2.1 cells. Altogether, the detailed functional analysis performed in this study by comparing HEK293/Ca_v3.1 and HEK293/Ca_v3.1/Kir2.1 cells provides a compelling evidence that the stable expression of Kir2.1 channels only made it possible to construct a cell line which could activate T-type Ca²⁺ channels by KCl-mediated depolarization [34].

3.2. Ca_v3.1 activation rapidly reduces p21^{ras}-GTP in HEK293/Ca_v3.1/Kir2.1 cells

The activity of p21^{ras} is regulated through its binding of guanine nucleotides, the GTP-bound active form of the

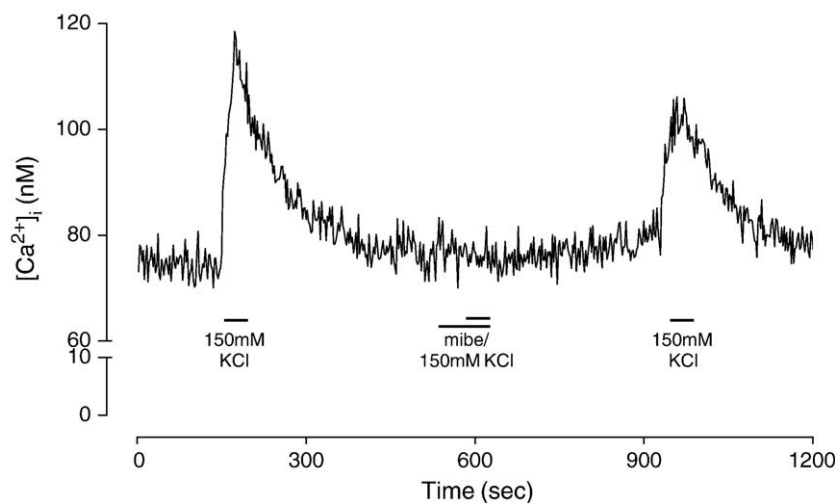


Fig. 1. High KCl-mediated [Ca²⁺]_i increase in HEK293/Ca_v3.1/Kir2.1 cells. [Ca²⁺]_i recording in HEK293/Ca_v3.1/Kir2.1 cells by membrane depolarization using 150 mM KCl. Application of T-type calcium channel blocker, mibefradil (1 μM, 1 min), reduced the 150 mM KCl (30 s)-induced [Ca²⁺]_i increase by 93.6% in this cell line.

protein being able to interact with effector proteins such as Raf-1 and phosphatidylinositol 3-kinase (PI-3-K) [4]. The kinetics of $\text{Ca}_v3.1$ stimulated p21^{ras} activation has, as yet, not been studied.

In the present study, p21^{ras} activation was assessed by measuring the amount of p21^{ras} -GTP, using Raf1-RBD and the p21^{ras} monoclonal antibody described in the Materials and methods section. Firstly, we investigated whether the activation of $\text{Ca}_v3.1$ reduces the activation of p21^{ras} . The initial decrease in p21^{ras} -bound GTP, which peaks at 2 min, is followed by a subsequent increase in p21^{ras} -bound GTP which returns to its initial value at 15 min and thereafter decreases again to attain the zero level at 120 min post-stimulation (Fig. 2A). The application of 150 mM KCl over a period of 2 h triggers a transient increase in $[\text{Ca}^{2+}]_i$ (about 130 nM, peak level = 133 nM) (Fig. 2C). This increase of $[\text{Ca}^{2+}]_i$ was completely inactivated ($[\text{Ca}^{2+}]_i$ level = 56.7 ± 0.6 nM) after 15 min. These data provide evidence that the early period of stimulation of $\text{Ca}_v3.1$ results in a decrease in the amount of p21^{ras} -GTP in HEK293/ $\text{Ca}_v3.1$ /Kir2.1 cells. This sudden decrease of p21^{ras} -GTP is recovered at 15 min after stimulation, and then gradually decreases again up until 2 h. Compared to the HEK293/ $\text{Ca}_v3.1$ /Kir2.1 cells, in which KCl stimulation altered the level of p21^{ras} -GTP, no such change was observed in the HEK293/ $\text{Ca}_v3.1$ cells (Fig. 2B). The application of 150 mM KCl over a period of 1 h did not alter the response to $\text{Ca}_v3.1$ in any of the HEK293/

$\text{Ca}_v3.1$ cells ($[\text{Ca}^{2+}]_i$ level = 58.8 ± 1.2 nM, $n = 11$) (Fig. 2D). These data showed that the very early inactivation of p21^{ras} is solely dependent on the stimulation of $\text{Ca}_v3.1$ by KCl.

3.3. The effect of $\text{Ca}_v3.1$ activation on ERK

The p21^{ras} effectors, which are known to predominantly mediate growth stimulatory signals, are the serine/threonine Raf kinases [25]. To date, p21^{ras} is known to interact, through the effector region, with the N-terminal domain of Raf, regulating its kinase activity [25,36]. Raf activates MEK by phosphorylating its two regulatory serine residues [2]. MEK1 and MEK2 are dual specific kinases capable of phosphorylating regulatory serine/threonine and tyrosine residues on ERK (also referred to as p44 and p42 MAP kinase) [27]. Thus, when activated, ERK is itself able to phosphorylate the serines and threonines of cytoplasmic and nuclear proteins, such as transcription factors, thereby transducing proliferative and differentiation signals to the nucleus [32]. ERK can also phosphorylate membrane proteins including ion channels [1].

In this study, we investigated the kinetics of ERK activation following $\text{Ca}_v3.1$ stimulation. ERK activation was assessed both by the delayed mobility of the phosphorylated form on SDS-PAGE and the ability of immunoprecipitated p42^{ERK} to phosphorylate myelin basic

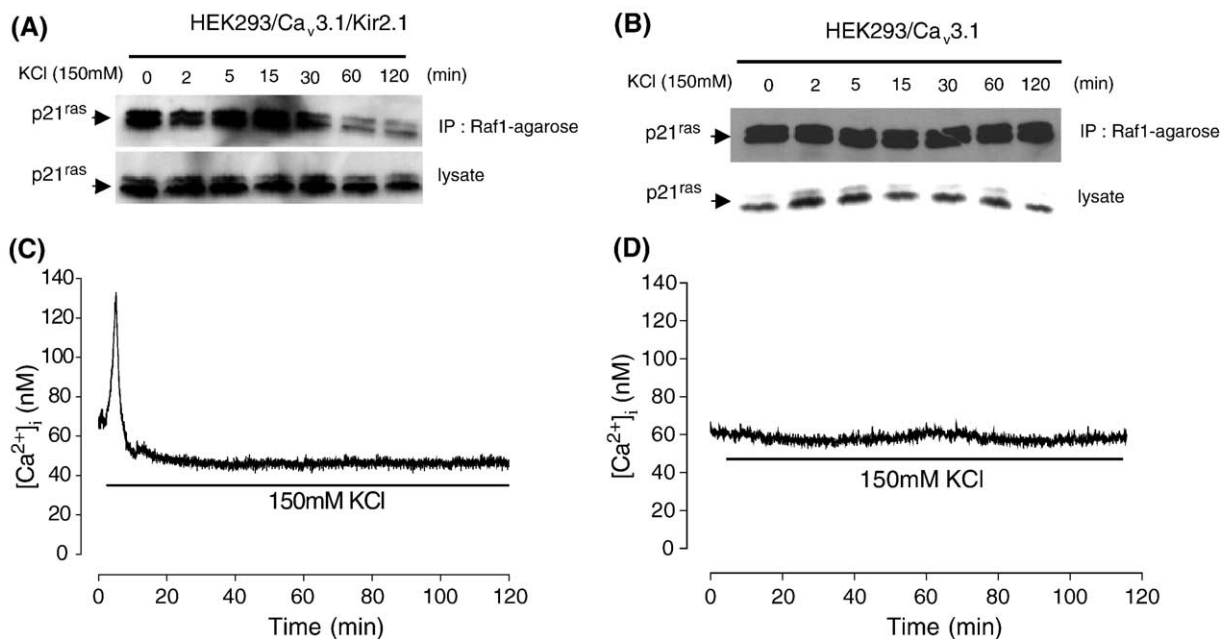


Fig. 2. Activation of $\text{Ca}_v3.1$ briefly reduces p21^{ras} in HEK293/ $\text{Ca}_v3.1$ /Kir2.1 cells. (A) HEK293/ $\text{Ca}_v3.1$ /Kir2.1 cells or (B) HEK293/ $\text{Ca}_v3.1$ cells which do not express Kir2.1 were exposed to 150 mM KCl and incubated for different periods of time. Cleared cell lysates were incubated with Raf-1 RBD. The beads were then washed, and the proteins were resolved by SDS-PAGE. The amount of activated Ras bound to Raf-1 RBD beads was determined by anti- p21^{ras} immunoblotting. Activation of $\text{Ca}_v3.1$ did not affect p21^{ras} in HEK293/ $\text{Ca}_v3.1$ cells (B). Cell lysates were also directly subjected to anti-Ras immunoblotting to determine the levels of Ras in each sample. (C) $[\text{Ca}^{2+}]_i$ recording of 150 mM KCl responses in HEK293/ $\text{Ca}_v3.1$ /Kir2.1 cells. Application of 150 mM KCl (over 2 h) triggers a transient increase in $[\text{Ca}^{2+}]_i$ (about 130 nM, peak level = 133 nM). This increase of $[\text{Ca}^{2+}]_i$ was completely inactivated ($[\text{Ca}^{2+}]_i$ level = 56.7 ± 0.6 nM) after 15 min. (D) $[\text{Ca}^{2+}]_i$ recording of 150 mM KCl responses in HEK293/ $\text{Ca}_v3.1$ cells. The application of 150 mM KCl (over 1 h) did not alter the responses to $\text{Ca}_v3.1$ in any of the HEK293/ $\text{Ca}_v3.1$ cells ($[\text{Ca}^{2+}]_i$ level = 58.8 ± 1.2 nM, $n = 11$).

protein (MBP) (Figs. 3A and B). As shown in Figs. 3A and B, the data obtained by these methods are consistent with the KCl-induced peak of ERK activation which occurred at 15 min. However, ERK activation decreased from 2 min to 5 min after KCl stimulation, which means that $Ca_v3.1$ activation reduced ERK activity in the very early stages of activation. According to our $p21^{ras}$ activation study, these variations in ERK activity in stimulated HEK293/ $Ca_v3.1$ /Kir2.1 cells are correlated with the amount of $p21^{ras}$ -GTP. The time course for ERK activation appears to be consistent with that of Ras activity and $Ca_v3.1$ activation suggesting that $Ca_v3.1$ activation regulates Ras/ERK activity.

3.4. $Ca_v3.1$ activation stimulates tyrosine phosphorylation of Shc and its association with Grb2 and Sos1

It has been shown that the Shc proteins are involved in activation of Ras via receptor tyrosine kinases [10,30]. In this study we investigated whether $Ca_v3.1$ activation could bring about Shc phosphorylation in HEK293/ $Ca_v3.1$ /Kir2.1 cells. As shown in Fig. 3C, a KCl-induced peak of phosphorylated Shc occurred at 15 min. However, phosphorylated Shc activation was decreased from 2 min to 5 min, i.e., in the very early period following KCl stimulation, which means that $Ca_v3.1$ activation reduced Shc phosphorylation in the very early stages. This process of phosphorylation persisted for at least 120 min, with a peak occurring at 15 min. The anti-Shc antibody was able to immunoprecipitate all three isoforms of Shc, as determined by immunoprecipitation and immunoblotting with the same antibody (Fig. 3C). Although the variation of phospho- $p52^{Shc}$ within each sample were not distinguishable, this phenomenon can be explained in light of a previous study [16]. Several agonists have been shown to induce Shc phosphorylation, not only at Tyr317 but also at Tyr239 and Tyr240 [16,17,35]. It has been reported that phosphorylation at positions Tyr239 and Tyr240 does not contribute to $p21^{ras}$ /ERK activation and has little involvement with Grb2

association [17]. Shc phosphorylation creates a binding site for the SH2 domain of the SH2/SH3 domain-containing adaptor protein, Grb2 [31]. The SH3 domains of Grb2 have been shown to interact with Sos1 [6,13]. This event leads to the recruitment of Sos1 to the membrane and enables it to activate $p21^{ras}$. To verify whether, and with what kinetics, Grb2 associates with Shc in HEK293/ $Ca_v3.1$ /Kir2.1 cells stimulated with KCl, we performed immunoprecipitations with anti-Shc antibody at different time points post $Ca_v3.1$ activation, followed by immunoblotting with an anti-Grb2 antibody. As shown in Fig. 4A, Grb2/Shc association suddenly increased between 5 and 15 min following the activation of $Ca_v3.1$. Moreover, to confirm the Grb2 to associate with the phosphorylated form of $p52^{Shc}$, we precipitated phospho- $p52^{Shc}$ with Grb2. As shown in Fig. 4B, Grb2 can precipitate phospho- $p52^{Shc}$ between 5 and 30 min and peak was shown at 15 min after the activation of $Ca_v3.1$. Immunoblotting analysis showed that the $p52^{Shc}$ isoform, which is the most prevalent isoform in HEK293/ $Ca_v3.1$ /Kir2.1, plays a major role in the association of Shc and Grb2.

3.5. Peak activation of $p21^{ras}$ and ERK in 15 min after $Ca_v3.1$ activation is correlated with Grb2/Shc interaction

To verify the association of Grb2 with Sos1, Sos1 immunoprecipitates were immunoblotted with anti-Grb2 antibody. As shown in Fig. 4C, Sos1 was able to associate with Grb2 in HEK293/ $Ca_v3.1$ /Kir2.1 cells and this association rapidly decreased at 2 min after KCl treatment and then recovered its initial level at 15 min. After about 30 min, the association began to decrease. All Sos1 immunoblotting experiments were generated by over-exposure. These results indicate that KCl stimulation increases the Grb2/Sos1 complex at 15 min, which correlates temporally with $p21^{ras}$ activation. Finally, we verified whether Sos1 is associated with Shc, as a result of the association of Grb2 with both Sos1 and Shc, after KCl stimulation, and whether

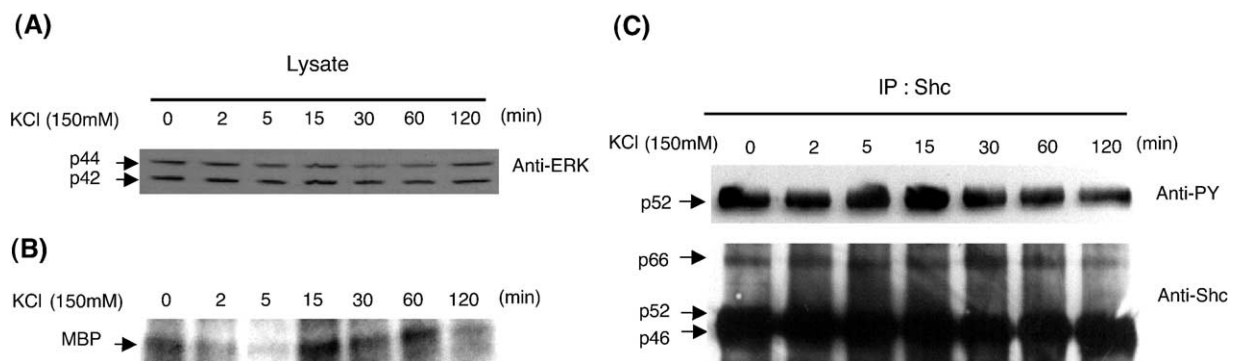


Fig. 3. Monophasic activation of ERK by KCl and Shc phosphorylation during the time period of KCl stimulation of HEK293/ $Ca_v3.1$ /Kir2.1 cells. HEK293/ $Ca_v3.1$ /Kir2.1 cells were stimulated with KCl (150 mM) for the times indicated. (A) Immunoblot detection of the two isoforms of ERK. Activation is verified by the appearance of bands with delayed mobility related to the phosphorylated form of the protein. (B) ERK activity assayed by the ability of the immunoprecipitated $p42^{ERK}$ to phosphorylate MBP as substrate. (C) Cell lysates prepared from HEK293/ $Ca_v3.1$ /Kir2.1 cells were immunoprecipitated with anti-Shc polyclonal antibody. Immunoprecipitates were analyzed by SDS-PAGE and immunoblotted with anti-phosphotyrosine (anti-PY) antibody and anti-Shc antibody as indicated. Shown here is a representative result; the experiment was repeated three times.

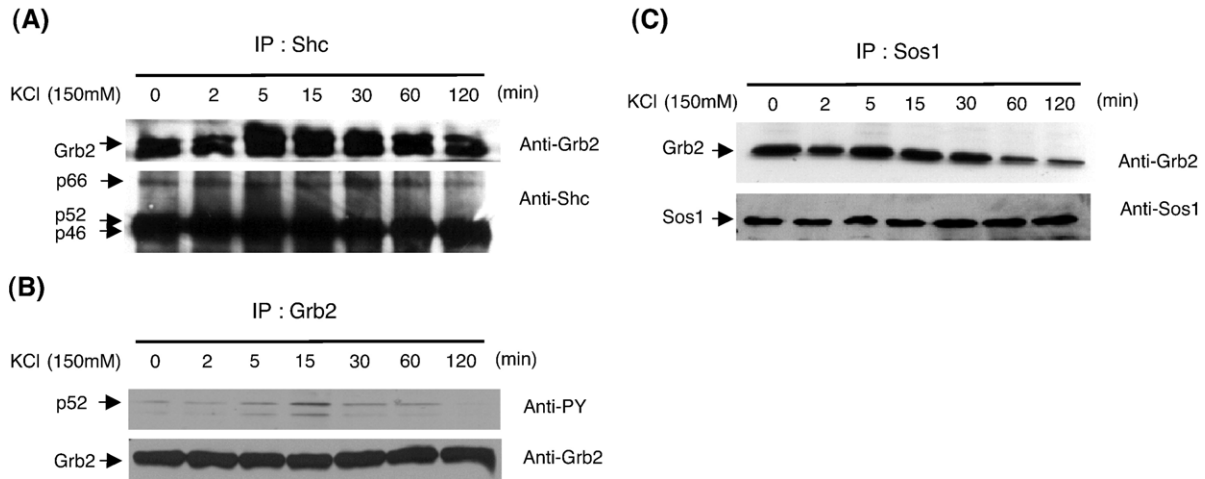


Fig. 4. Activation of $Ca_v3.1$ induces association of Shc/Grb2 and Sos1/Grb2. Lysates from HEK293/ $Ca_v3.1$ /Kir2.1 cells stimulated with KCl (150 mM) for the times indicated were immunoprecipitated with anti-Shc (A) or anti-Grb2 antibodies (B). The resultant immunoprecipitates were resolved by SDS–PAGE and then immunoblotted with anti-Grb2 and anti-PY antibodies, respectively. (C) HEK293/ $Ca_v3.1$ /Kir2.1 cells stimulated with KCl (150 mM) for the indicated times. Cell lysates were immunoprecipitated with anti-Sos1 antibody. Shown here is a representative result; the experiment was repeated three times.

the association of Sos1/Shc follows the kinetics of the association of Shc with Grb2. Sos1 was immunoprecipitated from KCl treated or untreated HEK293/ $Ca_v3.1$ /Kir2.1 cells and immunoblotted with anti-Shc antibody. In can be seen in Fig. 5A that Shc can precipitate Sos1 between 5 and 30 min, and that this precipitation peaks at 15 min after the activation of $Ca_v3.1$ by 150 mM KCl treatment. This result was also correlated with the $p21^{ras}$ activation event. However, we could not clearly verify the immunoprecipitation and immunoblotting of Sos1 in detail (Fig. 5B). This phenomenon can be explained by the relative excess of

cytosolic Grb2 and Shc compared to the small amount of Sos1 under the experimental conditions used in this study.

4. Discussion

For three decades Ca^{2+} has been known to affect cell proliferation and differentiation [3,5]. This provided the initial evidence of the potential link between Ca^{2+} and Ras signaling. Recently, increased intracellular Ca^{2+} resulting from Ca^{2+} influx through voltage-dependent ion channels was found to activate Ras in rat pheochromocytoma (PC12) cells and primary cultures of rat cortical neurons [11,29]. In this study, we showed that the activation of $Ca_v3.1$, a T-type voltage-dependent calcium channel, results in a rapid reduction of the $p21^{ras}$ signaling pathway, which peaks at 15 min, and that this signal was transferred to ERK. The construction of HEK293/ $Ca_v3.1$ /Kir2.1 cells specifically activating $Ca_v3.1$ was very useful in this assay. $Ca_v3.1$ activation resulted in the level of $p21^{ras}$ -GTP in the cells being rapidly decreased during the first 2 min and the level of $p21^{ras}$ -GTP gradually increased up to 15 min, then rapidly decreased again (Fig. 2A).

The initial decrease at 2 min was different with general signaling processes. Most signaling induced by stimulation is gradually increased or decreased by the time. It is possible that an initial decrease of $p21^{ras}$ signaling might be the resultant of using stably transformed HEK293/ $Ca_v3.1$ /Kir2.1 cells. The experiments for the identification of this behavior are now investigating.

ERK activation was observed at 15 min, as shown in Fig. 3B, and then gradually decreased. This phenomenon was also mentioned in another recent report [11]. From this data, it was concluded that the role of $Ca_v3.1$ in HEK293/ $Ca_v3.1$ /Kir2.1 activation is to trigger Ras signaling, and that this signal is transferred to ERK with a unique pattern. Recent

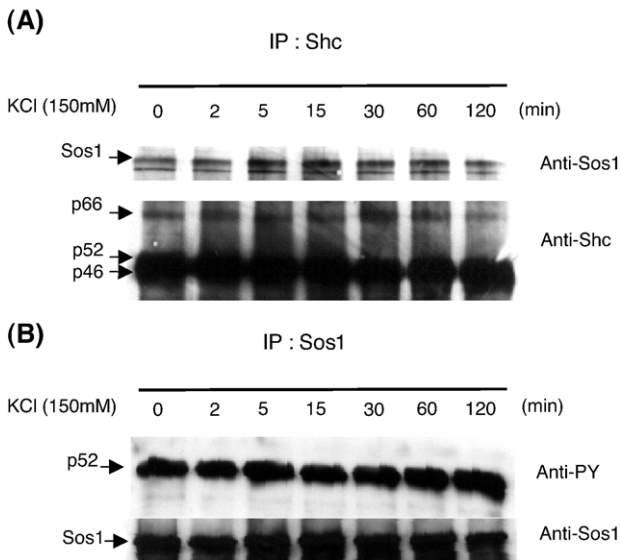


Fig. 5. Grb2 mediates the association of Sos1 and Shc. Lysates from HEK293/ $Ca_v3.1$ /Kir2.1 cells stimulated with KCl (150 mM) for the times indicated were immunoprecipitated with anti-Shc (A) or anti-Sos1 antibodies (B). The resultant immunoprecipitates were resolved by SDS–PAGE and then immunoblotted with anti-Shc and anti-Sos1 antibodies. Shown here is a representative result; the experiment was repeated three times.

evidence suggests that p21^{ras} reduces the L-type calcium channel current in cardiac Myocytes [19]. In this report, p21^{ras} was found to diminish L-channel promoter activity through the Raf-MEK-ERK pathway. It was reported that p21^{ras} reduces the T-type calcium channel density in Swiss 3T3 fibroblasts [19,33]. Similar results for Ca_v3.1 activation were also shown in the case of Sos1, Grb2, and Shc. Sos1 is normally localized to the cell cytosol, however, in response to growth factor stimulation, it is recruited to the plasma membrane. p21^{ras} is localized to the interior of the plasma membrane, so simply targeting Sos to this membrane efficiently regulates p21^{ras} activation. This is a key concept in the activation–deactivation cycle of p21^{ras}. Sos is recruited to the plasma membrane in complexes with the adaptor proteins, growth-factor receptor-bound protein 2 (Grb2) and Src-homology-2 domains containing Shc, which bind to phosphotyrosine-binding domains. In our results, Sos1, Grb2, and Shc were correlated with p21^{ras} kinetics after Ca_v3.1 activation, which means that Shc, Grb2, and Sos are involved in the activation pathways upstream of Ras and it is activation of Ras that triggers ERK activation via Raf and MEK. The data presented here will likely improve our understanding of T-type calcium channel signaling in the brain and may provide a basic understanding of how an ion channel activates transcriptional regulation and cell signaling pathways.

Acknowledgments

This work was supported by the intramural program of KIST, the National Research Laboratory Program (M10400000046-04J0000-04610), Real Time Molecular Imaging Project, the Brain Research Center of the 21st Century Frontier Research Program (M103KV010004-03K2201-00420), and the 21C Frontier Functional Proteomics Project from the Korean Ministry of Science and Technology. The authors extend their appreciation to Dr. Perez-Reyes for providing HEK293 cells stably expressing human Ca_v3.1 T-type Ca²⁺ channels.

References

- [1] J.P. Adams, A.E. Anderson, A.W. Barga, K.T. Dineley, R.G. Cook, P.J. Pfaffinger, J.D. Sweatt, The A-type potassium channel Kv4.2 is a substrate for the mitogen-activated protein kinase ERK, *J. Neurochem.* 75 (92) (2000) 2277–2287.
- [2] D.R. Alessi, Y. Saito, D.G. Campbell, P. Cohen, G. Sithanandam, U. Rapp, A. Ashworth, C.J. Marshall, S. Cowley, Identification of the sites in MAP kinase kinase-1 phosphorylated by p74raf-1, *EMBO J.* 13 (1994) 1610–1619.
- [3] S.D. Balk, J.F. Whitfield, T. Youdale, A.C. Braun, Roles of calcium, serum, plasma, and folic acid in the control of proliferation of normal and Rous sarcoma virus-infected chicken fibroblasts, *Proc. Natl. Acad. Sci. U. S. A.* 70 (1973) 675–679.
- [4] D. Barnard, B. Diaz, L. Hettich, E. Chuang, X.F. Zhang, J. Avruch, M. Marshall, Identification of the sites of interaction between c-Raf-1 and Ras-GTP, *Oncogene* 10 (1995) 1283–1290.
- [5] M.J. Berridge, Calcium signalling and cell proliferation, *BioEssays* 17 (1995) 491–500.
- [6] L. Buday, J. Downward, Epidermal growth factor regulates p21ras through the formation of a complex of receptor, Grb2 adapter protein, and Sos nucleotide exchange factor, *Cell* 73 (1993) 611–620.
- [7] E. Carbone, H.D. Lux, A low voltage-activated calcium conductance in embryonic chick sensory neurons, *Biophys. J.* 46 (1984) 413–418.
- [8] E. Carbone, H.D. Lux, Kinetics and selectivity of a low-voltage-activated calcium current in chick and rat sensory neurones, *J. Physiol.* 386 (1987) 547–570.
- [9] C.F. Chen, P. Hess, Mechanism of gating of T-type calcium channels, *J. Gen. Physiol.* 96 (1990) 603–630.
- [10] Y. Chen, D. Grall, A.E. Salcini, P.G. Pelicci, J. Pouyssegur, E. Obberghen Schilling, Shc adaptor proteins are key transducers of mitogenic signaling mediated by the G protein coupled thrombin receptor, *EMBO J.* 15 (1996) 1037–1044.
- [11] P.J. Cullen, P.J. Lockyer, Integration of calcium and Ras signalling, *Nat. Rev., Mol. Cell Biol.* 3 (2002) 339–348.
- [12] R.E. Dolmetsch, U. Pajvani, K. Fife, J.M. Spotts, M.E. Greenberg, Signaling to the nucleus by an L-type calcium channel-calmodulin complex through the MAP kinase pathway, *Science* 294 (2001) 333–339.
- [13] S.E. Egan, B.W. Giddings, M.W. Brooks, L. Buday, A.M. Sizeland, R.A. Weinberg, Association of Sos Ras exchange protein with Grb2 is implicated in tyrosine kinase signal transduction and transformation, *Nature* 363 (1993) 45–51.
- [14] S.I. Ertel, E.A. Ertel, Low-voltage-activated T-type Ca²⁺ channels, *Trends Pharmacol. Sci.* 18 (1997) 37–42.
- [15] E.M. Fitzgerald, A.C. Dolphin, Regulation of rat neuronal voltage-dependent calcium channels by endogenous p21-ras, *Eur. J. Neurosci.* 9 (1997) 1252–1261.
- [16] M. Foschi, S. Chari, M.J. Dunn, A. Sorokin, Biphasic activation of p21ras by endothelin-1 sequentially activates the ERK cascade and phosphatidylinositol 3-kinase, *EMBO J.* 16 (1997) 6439–6451.
- [17] N. Gotoh, M. Toyoda, M. Shibuya, Tyrosine phosphorylation sites at amino acids 239 and 240 of Shc are involved in epidermal growth factor-induced mitogenic signaling that is distinct from Ras/mitogen-activated protein kinase activation, *Mol. Cell. Biol.* 17 (1997) 1824–1831.
- [18] N. Hagiwara, H. Irisawa, M. Kameyama, Contribution of two types of calcium currents to the pacemaker potentials of rabbit sino-atrial node cells, *J. Physiol.* 395 (1988) 233–253.
- [19] P.D. Ho, J.S. Fan, N.L. Hayes, N. Saada, P.T. Palade, C.C. Glembofski, P.M. McDonough, Ras reduces L-type calcium channel current in cardiac myocytes. Corrective effects of L-channels and SERCA2 on [Ca(2+)](i) regulation and cell morphology, *Circ. Res.* 88 (2001) 63–69.
- [20] J.R. Huguenard, Low-threshold calcium currents in central nervous system neurons, *Annu. Rev. Physiol.* 58 (1996) 329–348.
- [21] J.R. Huguenard, D.A. Prince, A novel T-type current underlies prolonged Ca(2+)-dependent burst firing in GABAergic neurons of rat thalamic reticular nucleus, *J. Neurosci.* 12 (1992) 3804–3817.
- [22] H.J. Jongasma, R. Wilders, Channelopathies, Kir2.1 mutations jeopardize many cell functions, *Curr. Biol.* 11 (2001) 747–750.
- [23] J.H. Lee, A.N. Daud, L.L. Cribbs, A.E. Lacerda, A. Pereverzev, U. Klockner, T. Schneider, E. Perez-Reyes, Cloning and expression of a novel member of the low voltage activated T-type calcium channel family, *J. Neurosci.* 19 (1999) 1912–1921.
- [24] V. Leuranguer, A. Monteil, E. Bourinet, G. Dayanithi, J. Nargeot, T-type calcium currents in rat cardiomyocytes during postnatal development: contribution to hormone secretion, *Am. J. Physiol.: Heart Circ. Physiol.* 279 (2000) 2540–2548.
- [25] C.J. Marshall, MAP kinase kinase kinase, MAP kinase kinase and MAP kinase, *Curr. Opin. Genet. Dev.* 4 (1994) 82–89.
- [26] B. Nilius, P. Hess, J.B. Lansman, R.W. Tsien, A novel type of cardiac calcium channel in ventricular cells, *Nature* 316 (1985) 443–446.

- [27] D.M. Payne, A.J. Rossomando, P. Martino, A.K. Erickson, J.H. Her, J. Shabanowitz, D.F. Hunt, M.J. Weber, T.W. Sturgill, Identification of the regulatory phosphorylation sites in pp42/mitogen-activated protein kinase (MAP kinase), *EMBO J.* 10 (1991) 885–892.
- [28] E. Perez-Reyes, Molecular physiology of low-voltage-activated T-type calcium channels, *Physiol. Rev.* 83 (2003) 117–161.
- [29] L.B. Rosen, D.D. Ginty, M.J. Weber, M.E. Greenberg, Membrane depolarization and calcium influx stimulate MEK and MAP kinase via activation of Ras, *Neuron* 12 (1994) 1207–1221.
- [30] J. Sadoshima, S. Izumo, The heterotrimeric G protein-coupled angiotensin II receptor activates p21^{ras} via the tyrosine kinase-Shc-Grb2-Sos pathway in cardiac myocytes, *EMBO J.* 15 (1996) 775–787.
- [31] A.E. Salcini, J. McGlade, G. Pelicci, I. Nicoletti, T. Pawson, P.G. Pelicci, Formation of Shc-Grb2 complexes is necessary to induce neoplastic transformation by overexpression of Shc proteins, *Oncogene* 9 (1994) 2827–2836.
- [32] R. Seger, E.G. Krebs, The MAPK signaling cascade, *FASEB J.* 9 (1995) 726–735.
- [33] M.W. Strobeck, M. Okuda, H. Yamaguchi, A. Schwartz, K. Fukasawa, Morphological transformation induced by activation of the mitogen-activated protein kinase pathway requires suppression of the T-type Ca²⁺ channel, *J. Biol. Chem.* 274 (1999) 15694–15700.
- [34] T. Kim, J. Choi, S. Kim, O. Kwon, S.-Y. Nah, Y.S. Han, H. Rhim, The biochemical activation of T-type Ca²⁺ channels in HEK293 cells stably expressed Ca_v3.1 and Kir2.1 subunits, *BBRC* 324 (2004) 401–408.
- [35] G.P. Van der, S. Wiley, G.D. Gish, T. Pawson, The Shc adaptor protein is highly phosphorylated at conserved, twin tyrosine residues (Y239/240) that mediate protein–protein interactions, *Curr. Biol.* 6 (1996) 1435–1444.
- [36] A.B. Vojtek, S.M. Hollenberg, J.A. Cooper, Mammalian Ras interacts directly with the serine/threonine kinase Raf, *Cell* 74 (1993) 205–214.
- [37] C. Wagner, B.K. Kramer, M. Hinder, M. Kieninger, A. Kurtz, T-type and L-type calcium channel blockers exert opposite effects on renin secretion and renin gene expression in conscious rats, *Br. J. Pharmacol.* 124 (1998) 579–585.
- [38] D. Wang, Z. Li, E.M. Messing, G. Wu, Activation of Ras/Erk pathway by a novel MET-interacting protein RanBPM, *JBC* 277 (2002) 36216–36222.