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Sphingosine-1-phosphate induces Ca^{2+} mobilization via TRPC6 channels in SH-SY5Y cells and hippocampal neurons

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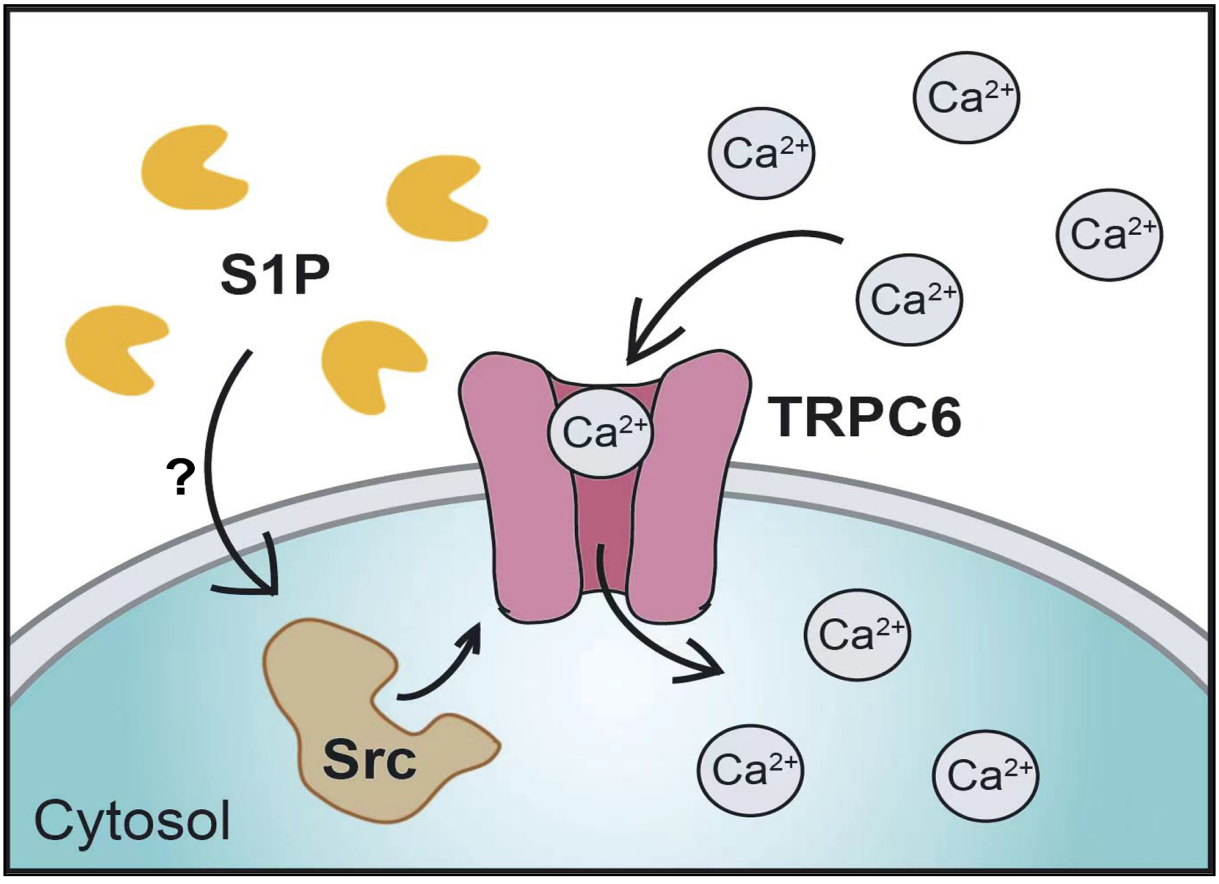
Graphical abstract



S1P activates TRPC6 in a Src-dependent way to induce Ca^{2+} mobilization in SH-SY5Y cells and hippocampal neurons.

Public summary

■ Sphingosine-1-phosphate (S1P) evokes global Ca^{2+} signals in neurons.

■ TRPC6 channels mediate S1P-induced Ca^{2+} influx in SH-SY5Y cells and hippocampal neurons.

■ S1P activates TRPC6 channels in a Src-dependent manner.

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Abstract: Sphingosine-1-phosphate (S1P) is a widely expressed biologically active sphingolipid that plays an important role in cell differentiation, migration, proliferation, metabolism and apoptosis. S1P activates various signaling pathways, some of which evoke Ca^{2+} signals in the cytosol. Few studies have focused on the mechanism by which S1P evokes Ca^{2+} signals in neurons. Here, we show that S1P evokes global Ca^{2+} signals in SH-SY5Y cells and hippocampal neurons. Removal of extracellular calcium largely abolished the S1P-induced increase in intracellular Ca^{2+}, suggesting that the influx of extracellular Ca^{2+} is the major contributor to this process. Moreover, we found that S1P-induced Ca^{2+} mobilization is independent of G protein-coupled S1P receptors. The TRPC6 inhibitor SAR7334 suppressed S1P-induced calcium signals, indicating that the TRPC6 channel acts as the downstream effector of S1P. Using patch-clamp recording, we showed that S1P activates TRPC6 currents. Two Src tyrosine kinase inhibitors, Src-I1 and PP2, dramatically inhibited the activation of TRPC6 by S1P. Taken together, our data suggest that S1P activates TRPC6 channels in a Src-dependent way to induce Ca^{2+} mobilization in SH-SY5Y cells and hippocampal neurons.

Keywords: sphingosine-1-phosphate; calcium; TRPC6 channel; Src kinase; SH-SY5Y; neuron

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1 Introduction

Sphingosine-1-phosphate (S1P) is a bioactive sphingolipid that is present at high levels in the blood and various tissues[1, 2]. It is derived from another bioactive sphingolipid, ceramide. The enzyme ceramidase converts ceramide into sphingosine, which is subsequently phosphorylated by sphingosine kinases (SphK) to produce S1P[3]. After being synthesized in the cytosol, S1P can be transported out of the cell through non-specific ABC-binding cassette transporters or a specific spinster-2 transporter[4] and activates G-protein coupled S1P receptors expressed on the plasma membrane (PM). There are five subtypes of S1P receptors, named $S1P_{1\text{-}5}$[5]. The binding of S1P to these receptors activates different G-proteins, which in turn activate various downstream signaling pathways and regulate several fundamental processes, including cell differentiation, migration, proliferation, stem cell programming, metabolism and apoptosis[6–8].

S1P is an important regulator of intracellular calcium signals. In yeast, intracellular S1P promotes the influx of calcium ions by activating calcium ion channels on the cell membrane, causing an increase in the intracellular calcium ion concentration ($[Ca^{2+}]_i$)[9]. In mammalian cells, S1P regulates calcium signals through a more complex mechanism, which includes both extracellular and intracellular pathways. Extracellular S1P can directly activate calcium channels on the cell membrane or act through S1P receptors[10]. Some S1P receptors are coupled to the activation of phospholipase C (PLC) and subsequent formation of inositol trisphosphate (IP_3) and diacylglycerol (DAG). IP_3 binds to its receptor on the endoplasmic reticulum (ER) and causes calcium mobilization from the ER calcium store. DAG may interact with PM Ca^{2+}-permeable channels to modulate Ca^{2+} entry from the extracellular environment[11]. On the other hand, intracellular S1P has been found to directly induce the release of calcium from intracellular stores, but the mechanism is still unclear[12, 13]. Due to the different distributions of related signaling molecules, S1P regulates calcium signals differently in different cells.

Intracellular calcium serves as an important second messenger that regulates many fundamental activities in eukaryotic cells. In the nervous system, calcium plays more extensive roles in regulating a great variety of neuronal processes, such as synaptic transmission, neuroplasticity, neurodevelopment, and neuronal excitability[14]. Interestingly, S1P is also involved in the regulation of these processes[15], indicating an important physiological significance of S1P-induced calcium signaling in the nervous system. A previous study showed that in astrocytes, S1P can induce an increase in intracellular calcium concentration by activating channels on the cell membrane[16]. However, the regulatory mechanism of S1P on calcium signaling in neurons is still unclear.

Transient potential receptor canonical 6 (TRPC6) is a

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calcium-permeable ion channel belonging to the TRPC family. Human TRPC6 is expressed ubiquitously and is involved in the regulation of cellular function and calcium signaling in a variety of cell types[17, 18]. The function of TRPC6 in the nervous system has been extensively studied[19], but its role in S1P-induced calcium signaling in neurons remains unclear.

Here, by means of calcium imaging and patch-clamp techniques, we explored the effect of S1P on calcium signals in SH-SY5Y cells and cultured hippocampal neurons and revealed an underlying mechanism involving TRPC6 and Src kinase.

2 Materials and methods

2.1 Cell culture

SH-SY5Y cells were cultured in Dulbecco's modified Eagle medium (DMEM)/F12 1∶1 (1∶1, HyClone) supplemented with 20% FBS (Biological Industries), 10000 units/mL penicillin and 10 mg/mL streptomycin (Biosharp), and 1× glutagro supplement (200 mmol/L L-alanyl-L-glutamine, Corning) at 37 °C in a humidified incubator with 5% CO_2. HEK293T cells were cultured in Dulbecco's modified Eagle medium (DMEM) (Biological Industries) supplemented with 10% FBS (Biological Industries), 10000 units/mL penicillin and 10 mg/mL streptomycin (Biosharp), and 1× glutagro supplement (Corning) at 37 °C in a humidified incubator with 5% CO_2. Cells were passaged using 0.25% trypsin-EDTA solution with phenol red (Beyotime) and plated onto 35 mm culture dishes with thin glass bottoms for calcium imaging or 12 mm poly-L-lysine-coated coverslips for patch clamp 12 h before experiments.

Hippocampal neurons were prepared from the hippocampi of 0- to 2-day-old C57BL/6N mice. The hippocampi were cut into tiny pieces and digested with 20 units/mL papain (Worthington) for 30 min at 37 °C. Cells were dissociated by pipetting up and down, plated onto 12 mm coverslips, and maintained in DMEM (Biological Industries) supplemented with 10% F-12 (Gibco), 10% bovine calf serum (BCS) (Sigma-Aldrich), 10000 units/mL penicillin, 10 mg/mL streptomycin (Biosharp), and 1× glutagro supplement (Corning) at 37 °C in a humidified incubator with 5% CO_2. Twenty-four hours later, the culture medium was changed to Neurobasal-A (Gibco) supplemented with 1× B-27 (Gibco), 10000 units/mL penicillin, 10 mg/mL streptomycin (Biosharp), and 1× glutagro supplement (Corning). The neurons were used for experiments at DIV 8–15.

2.2 TRPC6 plasmid transfection

The pcDNA3.1-TRPC6 plasmid was a gift from Dr. Dejian Ren (University of Pennsylvania). pcDNA3.1-TRPC6 and pEGFP-N1 plasmids at a proportion of 20∶3 were cotransfected into HEK293T cells using jetPRIME (Polyplus-transfection) reagent according to the manufacturer's instructions.

2.3 Measurement of cytosolic Ca^{2+} concentrations

SH-SY5Y cells cultured on 35 mm culture dishes with thin glass bottoms and hippocampal neurons grown on 12 mm poly-L-lysine-coated coverslips were used for calcium imaging. A high-affinity ratiometric Ca^{2+} fluorescent dye Fura-2 was used to monitor the intracellular concentration of Ca^{2+}. An increase in intracellular calcium ions causes the fluorescence signal of Fura-2 to increase under 340 nm excitation (F340) and to decrease under 380 nm excitation (F380), thus increasing the F340/F380 ratio. Cells were incubated with 2 μmol/L membrane-permeant Fura-2 acetoxymethyl ester (Fura-2-AM, Beyotime) and 0.04% Pluronic F-127 (Beyotime) at 37 °C for 30 min in a solution containing 135 mmol/L NaCl, 5.4 mmol/L KCl, 1.8 mmol/L $CaCl_2$, 0.9 mmol/L $MgCl_2$, 10 mmol/L HEPES, and 10 mmol/L glucose (pH 7.4 by NaOH) and then washed three times with PBS. For calcium-free experiments, $CaCl_2$ was substituted with 1 mmol/L EGTA. Cytosol Ca^{2+} concentration was monitored and captured by live-cell imaging using a calcium imaging system consisting of a DG-5 wavelength switcher (Sutter Instrument), an ORCA-Flash4.0 LT+ CMOS camera (Hamamatsu), and a Ti2 inverted microscope (Nikon).

2.4 Electrophysiology

An Axopatch 200B patch-clamp amplifier, a Digidata 1550B data acquisition system, and pClamp software (Molecular Devices) were used to record whole-cell currents of HEK293T cells. Recording pipettes were made from borosilicate glass tubes using a P-1000 puller (Sutter Instrument). To record the TRPC6 currents, a bath solution containing 140 mmol/L NaOH, 4 mmol/L KOH, 1 mmol/L $MgCl_2$, 2 mmol/L $CaCl_2$, 10 mmol/L HEPES, and 5 mmol/L glucose (pH 7.4 by approximately 138 mmol/L methanesulfonic acid) was used. Using the chloride channel-impermeable methanesulfonate ion as the predominant anion in the bath solution largely eliminated the endogenous chloride current generated by chloride influx in HEK293T cells. The pipette solution contained 145 mmol/L CsCl, 2 mmol/L $MgCl_2$, 0.3 mmol/L $CaCl_2$, 10 mmol/L EGTA, 10 mmol/L HEPES, and 2 mmol/L ATP-Mg (pH 7.2 by CsOH). For the perforated patch-clamp, 250 μg/mL amphotericin B was added to the pipette solution. After obtaining electrical access (whole-cell or perforated patch), a ramp voltage protocol (−100 mV to +100 mV; V_h = 0 mV) or a step voltage protocol (10 mV step, from −100 mV to +100 mV; V_h = 0 mV) were used to elicit the TRPC6 current. We used a holding potential of 0 mV because it is close to the resting membrane potential of HEK293T cells (approximately −20 mV to −30 mV) and does not induce a TRPC6 current. The current amplitudes measured at +100 mV were used for data analysis.

2.5 Statistical analysis

All data are presented as the means ± SEMs and analyzed by *t*-test. *P* values less than 0.05 were considered statistically significant and are denoted as follows in the figures: $*P < 0.05$, $**P < 0.01$, and $***P < 0.001$.

3 Results

3.1 S1P evokes global Ca^{2+} signals in SH-SY5Y cells

In SH-SY5Y cells loaded with the intracellular calcium indicator Fura-2-AM, we found that extracellular application of 10

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μmol/L S1P caused a robust increase in F340/F380, suggesting an elevation of the intracellular Ca^{2+} concentration (Fig. 1a–c). The vast majority of cells responded to S1P but not to vehicle (Fig. 1d–f), further indicating that the increase in intracellular Ca^{2+} is caused by S1P.

3.2 Extracellular Ca^{2+} mainly contributes to S1P-evoked Ca^{2+} signals

The increased cytosolic calcium concentration caused by S1P may be due to the entry of extracellular Ca^{2+} or the release of Ca^{2+} from intracellular calcium stores. When we completely removed Ca^{2+} from the bath solution, S1P did not evoke a significant alteration in fluorescence. Only transient Ca^{2+} elevation of low amplitude without subsequent sustained increases in the F340/F380 ratio was observed (Fig. 2a, b). The statistical results showed that the increase in the F340/F380 ratio and the percentage of cells responsive to S1P were significantly reduced under Ca^{2+}-free conditions, indicating that extracellular Ca^{2+} is a main contributor to intracellular Ca^{2+} oscillations (Fig. 2c).

We then preincubated SH-SY5Y cells with 100 μmol/L 2-APB (2-aminoethyl diphenylborinate), an inositol 1,4,5-trisphosphate (IP_3) receptor antagonist that can inhibit the IP_3-induced release of Ca^{2+} from intracellular stores[20]. We observed that 2-APB did not elicit changes in intracellular Ca^{2+} signals regardless of whether the extracellular fluid contained calcium. However, under Ca^{2+}-containing conditions, S1P could still induce an increase in $[Ca^{2+}]_i$ in 2-APB-pretreated cells. When extracellular Ca^{2+} was omitted, S1P-evoked responses were completely inhibited by 2-APB (Fig. 2d, e, f). This explained the transient fluctuations of the calcium signals caused by S1P in the absence of extracellular Ca^{2+}, as shown in Fig. 2a, b. Collectively, these data suggest that extracellular Ca^{2+} entry rather than internal Ca^{2+} release plays a major role in S1P-evoked Ca^{2+} signals.

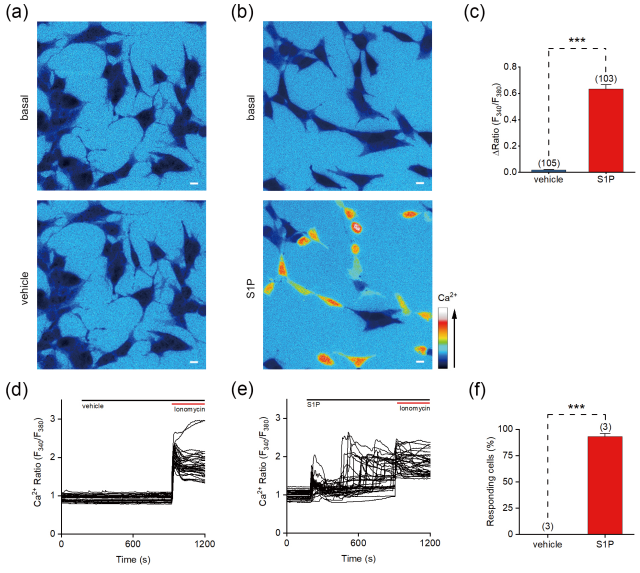

Fig. 1. S1P evokes Ca^{2+} signals in SH-SY5Y cells. (a, b) Representative pseudocolor images of the Fura-2 AM fluorescence ratio (F340/F380) responding to vehicle (a) and 10 μmol/L S1P (b) in SH-SY5Y cells. (c) Changes in the F340/F380 ratio (ΔRatio) of cells induced by vehicle or S1P. (d, e) Representative F340/F380 ratios of SH-SY5Y cells in response to vehicle (d) or 10 μmol/L S1P (e). (f) Percentage of cells responding to the application of vehicle or S1P (ΔRatio > 0.2). Scale bar = 10 μm. The data are presented as the means ± SEMs. ***$P < 0.001$.

3.3 The S1P-induced Ca^{2+} response is independent of G-coupled S1P receptors

S1P can bind to five G protein-coupled S1P receptors, namely, $S1PR_1$, $S1PR_2$, $S1PR_3$, $S1PR_4$ and $S1PR_5$. Therefore, we next aimed to determine which S1P receptor is specifically involved in the effect of S1P on intracellular calcium levels. We first blocked $S1PR_2$ with its antagonist JTE-013 (2 μmol/L) and found that it failed to inhibit the S1P-induced Ca^{2+} response in SH-SY5Y cells (Fig. 3a, b). Similarly, both the selective antagonist for $S1PR_1$ and $S1PR_3$, VPC23019 (10 μmol/L), and the potent $S1PR_4$ antagonist CYM50358 (2 μmol/L), failed to inhibit S1P-induced Ca^{2+} responses (Fig. 3a, b). In addition, since there is no commercial $S1PR_5$ antagonist, we examined the involvement of the phospholipase C (PLC) pathway downstream of the S1P receptors. However, the PLC inhibitor U73122 (1 μmol/L) had no effect on S1P-induced Ca^{2+} responses (Fig. 3a, b). These agents could neither reduce S1P-evoked calcium signals nor decrease the proportion of responding cells, indicating that G-coupled S1P receptors are probably not the main factor that

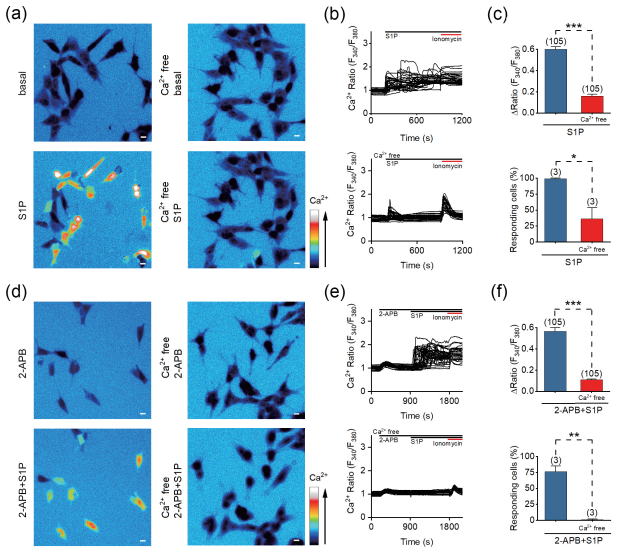

Fig. 2. S1P induces the influx of extracellular Ca^{2+}. (a) Representative pseudocolor images of F340/F380 in SH-SY5Y cells before and after the application of 10 μmol/L S1P in Ca^{2+}-containing (left) and Ca^{2+}-free (right) bath solution. (b) Representative traces of calcium signals responding to 10 μmol/L S1P treatment under Ca^{2+}-containing (upper) and Ca^{2+}-free (lower) conditions. (c) Statistics of the ΔRatio (F340/F380; upper) and percentage of responding cells (ΔRatio > 0.2) shown in (a) and (b). (d) Representative pseudocolor images of the F340/F380 ratio in SH-SY5Y cells treated with 100 μmol/L 2-APB (upper) or 100 μmol/L 2-APB + 10 μmol/L S1P (lower) in Ca^{2+}-containing (left) and Ca^{2+}-free (right) solution. (e) Representative F340/F380 ratios of SH-SY5Y cells in response to 100 μmol/L 2-APB + 10 μmol/L S1P in Ca^{2+} (upper) and Ca^{2+}-free (lower) solution. (f) Statistics of the ΔRatio (F340/F380; upper) and percentage of responding cells (ΔRatio > 0.2) shown in (d) and (e). Scale bar = 10 μm. The data are presented as the means ± SEMs. *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$.

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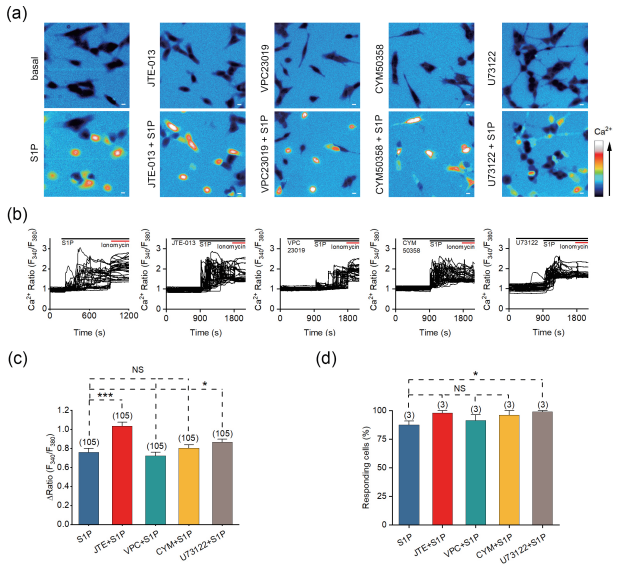

Fig. 3. The S1P-induced Ca^{2+} response is independent of G-protein coupled S1P receptors. (a) Representative pseudocolor images of F340/F380 in SH-SY5Y cells preincubated with the indicated antagonists and treated with 10 μmol/L S1P. (b) Representative traces of calcium signals responding to 10 μmol/L S1P treatment in SH-SY5Y cells incubated with the indicated antagonists. (c) Statistics of the ΔRatio (F340/F380). (d) Statistics of the percentage of responding cells. Scale bar = 10 μm. The data are presented as the means ± SEMs. $*P < 0.05$, $***P < 0.001$. NS indicates no significant difference.

mediates Ca^{2+} entry in this process (Fig. 3c, d).

A previous study showed that S1P can transactivate the epidermal growth factor receptor (EGFR) by activating $S1PR_3$[21]. Our data above suggest that $S1PR_3$ is not involved in S1P-induced calcium signaling in neurons, thus also ruling out the involvement of EGFR transactivation. To further verify this speculation, we treated SH-SY5Y cells with the specific EGFR inhibitor AG1478 (1 μmol/L) and found that it failed to inhibit the S1P-induced Ca^{2+} response (Fig. 4a–c).

3.4 S1P induces calcium signals in high-K^+ solution and Na^+-free solution

In neurons, the activation of voltage-gated calcium channels by membrane potential depolarization is an important pathway for extracellular calcium influx. To test whether S1P induces calcium signals by activating this pathway, we treated SH-SY5Y cells with a bath solution containing 100 mmol/L K^+ solutions to induce depolarization of membrane potential. A high K^+ concentration induced transient calcium signals in these cells (Fig. 5a, b). However, S1P could still induce calcium signals of similar strength in high-K^+ solution as in low-K^+ solution (Fig. 5a–c), indicating that depolarization and subsequent activation of voltage-gated calcium channels may not be the main mechanism of S1P-induced calcium signals. In addition, we replaced all the sodium ions in the bath solution with NMDG ions and found that the S1P-induced calcium signals were not reduced, which ruled out the participation of sodium ion channels (Fig. 5a–c).

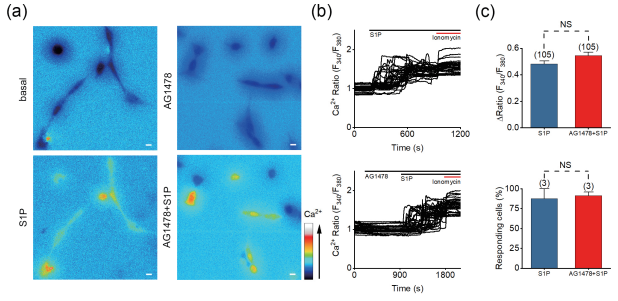

Fig. 4. S1P-induced Ca^{2+} response is independent of the transactivation of EGFR. (a) Representative pseudocolor images of F340/F380 in SH-SY5Y cells preincubated with 1 μmol/L AG1478 and treated with 10 μmol/L S1P. (b) Representative traces of calcium signals responding to 10 μmol/L S1P treatment in SH-SY5Y cells incubated with 1 μmol/L AG1478. (c) Statistics of the ΔRatio (F340/F380) and statistics of the percentage of responding cells. Scale bar = 10 μm. The data are presented as the means ± SEMs. NS indicates no significant difference.

3.5 S1P-induced Ca^{2+} mobilization is mainly mediated by TRPC6 channels in both SH-SY5Y cells and hippocampal neurons

S1P may interact with other ion channels on the plasma membrane (PM) to modulate Ca^{2+} entry from the extracellular fluid. Transient receptor potential canonical (TRPC) channels are PM-localized nonselective cationic channels that permeate Ca^{2+}. It was reported that in astrocytes, S1P could cause elevation in intracellular Ca^{2+} via the TRPC6 channels[16]. Therefore, we first investigated the effect of the potent and selective TRPC6 channel inhibitor SAR7334 on S1P-induced Ca^{2+} signals in SH-SY5Y cells. The results showed that the application of 1 μmol/L SAR7334 in bath solution significantly suppressed the increase in the F340/F380 ratio and the proportion of responding SH-SY5Y cells elicited by 10 μmol/L S1P (Fig. 6a–c). Then, we tested the effect of 0.1 μmol/L SAR7334 in cultured hippocampal neurons. Similarly, 10 μmol/L S1P facilitated calcium influx, and SAR73334 exhibited a strong blocking effect on S1P-induced Ca^{2+} responses (Fig. 6d–f). The inhibitory function of SAR73334 on Ca^{2+} signals indicates that TRPC6 plays a vital role in S1P-induced intracellular Ca^{2+} elevation in neurons.

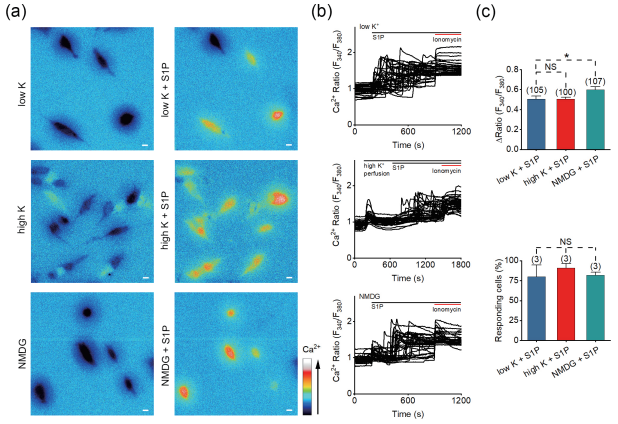

Fig. 5. S1P induces calcium signals in high-K^+ solution and Na^+-free solution. (a) Representative pseudocolor images of F340/F380 showing the S1P-induced calcium signals in SH-SY5Y cells with low-K^+ (5.4 mmol/L; upper), high-K^+ (100 mmol/L; middle) or Na^+-free (lower) solution treatment. (b) Representative traces of calcium signals responding to low-K^+ (upper), high-K^+ (middle) or Na^+-free (lower) treatment in SH-SY5Y cells. (c) Statistics of the ΔRatio (F340/F380; upper) and percentage of responding cells (ΔRatio > 0.2; lower) shown in (a) and (b). Scale bar = 10 μm. The data are presented as the means ± SEMs. $*P < 0.05$. NS indicates no significant difference.

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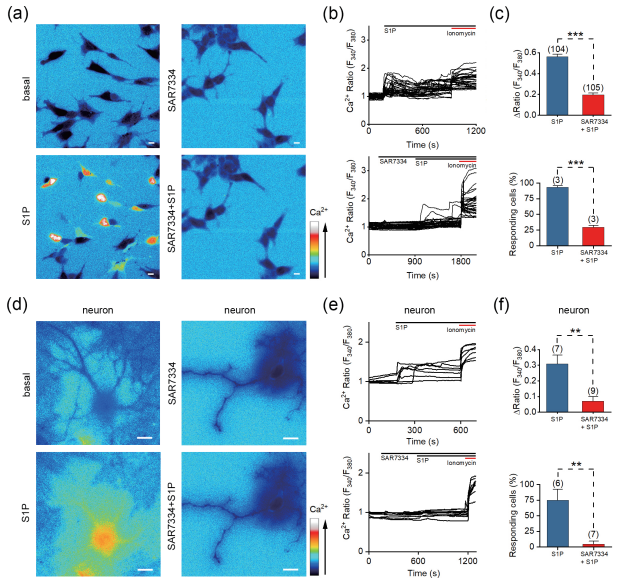

Fig. 6. S1P-induced Ca^{2+} mobilization is mediated by the TRPC6 channel in both SH-SY5Y cells and hippocampal neurons. (a) Representative pseudocolor images of F340/F380 showing the S1P-induced calcium signals in SH-SY5Y cells preincubated with vehicle (left) or 1 μmol/L SAR7334 (right). (b) Representative traces of calcium signals responding to 10 μmol/L S1P treatment in SH-SY5Y cells preincubated with vehicle (upper) or 1 μmol/L SAR7334 (lower). (c) Statistics of the ΔRatio (F340/F380; upper) and percentage of responding cells (ΔRatio > 0.2) shown in (a) and (b). (d) Representative pseudocolor images of F340/F380 showing the S1P-induced calcium signals in hippocampal neurons preincubated with vehicle (left) or 0.1 μmol/L SAR7334 (right). (e) Representative traces of calcium signals responding to 10 μmol/L S1P treatment in hippocampal neurons preincubated with vehicle (upper) or 0.1 μmol/L SAR7334 (lower). (f) Statistics of the ΔRatio (F340/F380; upper) and percentage of responding cells (ΔRatio > 0.2) shown in (c) and (d). Scale bar = 10 μm. The data are presented as the means ± SEMs. $^{**}P < 0.01$, $^{***}P < 0.001$.

3.6 S1P activates TRPC6 channels in a Src-dependent manner

Based on these results, we predicted that S1P could activate the TRPC6 channel. To test this hypothesis, we used the whole-cell patch-clamp technique to record TRPC6 currents and then examined the effect of S1P. Since neurons express many types of ion channels that may interfere with the recording of TRPC6 currents, we reconstituted TRPC6 currents in HEK293T cells. In the cells transiently transfected with TRPC6, but not in vector-transfected control cells, we recorded typical whole-cell TRPC6 currents (Fig. 7a–c). Unfortunately, these currents showed obvious "run-down" phenomena and were not suitable for detecting the effect of S1P. The amplitudes of the currents decreased to approximately a quarter of the original size within 5 min (Fig. 7d, e). The "run-down" of the currents was likely due to the dilution of cytoplasmic substances associated with the TRPC6 activity in the whole-cell recording configuration. To avoid this, we used a perforated patch clamp to record TRPC6 currents and found that the currents were stable (Fig. 7f).

Next, we investigated the effect of S1P on TRPC6 using the perforated patch-clamp method. In control HEK293T cells transfected with empty vector, extracellular application of 10 μmol/L S1P had no effect on endogenous currents (Fig. 8a–c). In TRPC6-transfected HEK293T cells, 10 μmol/L S1P significantly increased TRPC6 currents (Fig. 8d–f). From the representative currents elicited with ramp protocols (Fig. 8d) or step protocols (Fig. 8e), we could see that both the inward and the outward currents increased significantly after S1P application and thus displayed a complex "double rectification" in the *I-V* curve, which was consistent with previously published study[22].

Previous studies have shown that Src family kinases can phosphorylate and activate TRPC6[23]. In addition, S1P has been shown to activate Src kinase[24, 25]. Therefore, we speculate that S1P may activate TRPC6 through Src kinase. To test this hypothesis, we incubated TRPC6-transfected cells with 2 μmol/L Src kinase family inhibitor Src-I1 or 1 μmol/L PP2 for 10 min before recording. As expected, both Src-I1 and PP2 blocked the activation of TRPC6 by S1P (Fig. 8g–l).

4 Discussion

We have shown that S1P induces cytosolic Ca^{2+} mobilization in SH-SY5Y cells and primary mouse hippocampal neurons. The influx of extracellular Ca^{2+} is the main contributor to the increase in intracellular Ca^{2+}. S1P has long been known to play an important role in calcium signaling. One of the classical downstream mechanisms is that S1P binds to its G protein-coupled receptors on the plasma membrane and activates PLC, which in turn hydrolyzes PIP_2 into IP_3 and DAG. Subsequently, IP_3 releases calcium from the ER, while DAG may activate calcium channels on the plasma membrane and facilitate receptor-operated calcium entry (ROCE)[26, 27]. However, we found that the S1P receptors are not involved in the S1P-induced Ca^{2+} signal in SH-SY5Y cells and hippocampal neurons. Instead, S1P activates TRPC6 channels in a Src-dependent way to mediate the entry of extracellular Ca^{2+}.

Transient receptor potential canonical channels (TRPCs) are nonselective cation channels that can be activated by both GPCRs and receptor tyrosine kinases[28]. As Ca^{2+}-permeable channels, TRPCs regulate Ca^{2+} influx and maintain cytosolic and ER Ca^{2+} levels and are therefore important for calcium homeostasis and calcium signaling in the nervous system[29]. For example, Ca^{2+} influx through TRPC5 is critical for hippocampal neuronal axon formation and is responsible for the activation of Ca^{2+}/calmodulin kinase kinase (CaMKK) signaling cascades[30]. TRPC3 and TRPC6 channels are involved in brain-derived trophic factor (BDNF)-induced sustained elevation of intracellular Ca^{2+}[31]. Under diabetic conditions, TRPC6 knockout directly leads to neuronal loss, decreased neuronal activity, and cognitive function impairment[32].

Our data suggested that S1P-induced Ca^{2+} signals can be suppressed by the TRPC6 inhibitor SAR7334 in both SH-SY5Y cells and hippocampal neurons. In addition, we found that the Src kinase inhibitors Src-I1 and PP2 suppressed basal TRPC6 currents and abolished the S1P-induced activation of TRPC6. Therefore, we discovered a new S1P-Src-TRPC6 pathway that regulates neuronal calcium signaling. Src can be directly activated by G proteins[33]. Previous studies have demonstrated that S1P can activate c-Src through its G protein-coupled receptors[34, 35]. However, in the present study, we found that S1P receptors are not involved, and how S1P activ-

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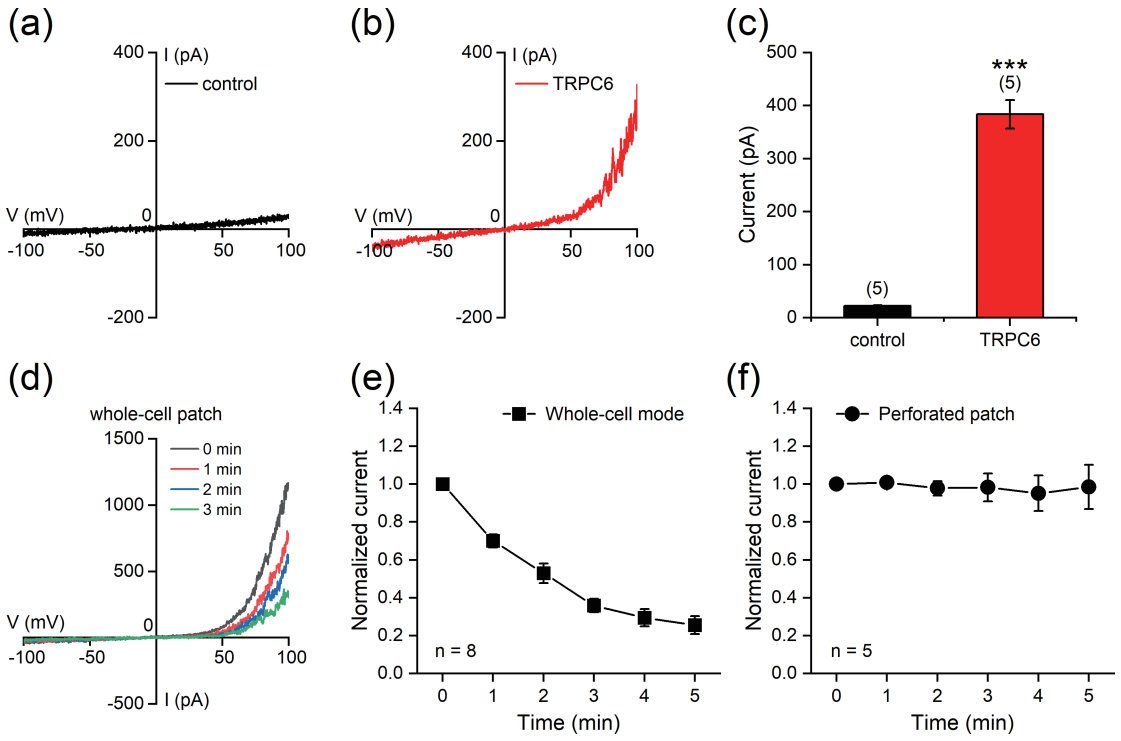

Fig. 7. Reconstitution of the TRPC6 current in HEK293T cells. (a, b) Representative whole-cell currents recorded in HEK293T cells transfected with empty vector (a) or TRPC6 (b). The currents were elicited with ramp protocols (−100 mV to +100 mV in 1 s). (c) Current amplitudes measured at +100 mV. (d) Representative currents recorded using whole-cell patch-clamp in TRPC6-transfected HEK293T cells at different time points after the start of recording. (e, f) Stability of the currents under whole-cell patch-clamp mode (e) or perforated patch-clamp mode (f). The current amplitudes (measured at +100 mV) at different time points were normalized to the values at the start of recordings. The data are presented as the means ± SEMs. ***$P < 0.001$.

ates Src kinase is still unclear. In addition, our data obtained from high-K^+ and Na^+-free experiments suggested that voltage-gated calcium channels or sodium channels may not be involved in S1P-induced calcium signaling, but whether other types of channels are involved remains to be studied. In other cell types, it is possible that other types of ion channels play a more important role in S1P-induced calcium signaling.

The synthases (SphK1/SphK2) and receptors of S1P ($S1PR_{1-5}$) are widely expressed in the brain, suggesting the importance of the S1P signaling pathway to the nervous system[36]. With the help of newly developed powerful tools, such as transgenic animals and specific inhibitors, the physiological function of S1P in the nervous system and its role in neurological diseases have been extensively studied. S1P deficiency caused by SphK knockout disturbs neurogenesis, including neural tube closure and angiogenesis, and causes embryonic lethality, suggesting that S1P is essential for neural development[37]. S1P is also involved in the regulation of synaptic transmission. By acting on presynaptic receptors, S1P stimulates neurotransmitter secretion and regulates synaptic plasticity[38, 39]. In addition, accumulating evidence links S1P to aging and neurodegenerative diseases such as Alzheimer's disease (AD) and Parkinson's disease (PD). In aging brains or in AD/PD patients and model animals, S1P levels were decreased, while another bioactive sphingolipid, ceramide, an upstream molecule in the S1P synthesis pathway, was increased[40, 41]. S1P has been found to be involved in the generation of neuronal Aβ. The inhibition of SphKs and the subsequent decrease in S1P levels inhibit Aβ production by inhibiting BACE1 activity in primary neuronal cultures and in the brains of mice[42]. Although S1P promotes the production of Aβ, it simultaneously inhibits the toxicity of Aβ to neurons, thus showing a neuroprotective effect[43]. In an MPTP-induced PD mouse model, remarkable downregulation of SphK2 expression in the substantia nigra region was observed. Loss of SphK2 decreases ATP and increases ROS levels in vitro, but the application of exogenous S1P reverses this process[44]. Since S1P has been shown to inhibit cell death in multiple studies, it is considered a potent neuroprotective factor.

Interestingly, TRPC6 also commonly exhibits a protective role in neurological disorders. A previous study reported that inhibiting the degradation of TRPC6 suppressed neuronal death caused by cerebral ischemia[45]. In an animal model of epilepsy, researchers found decreased expression of TRPC6 in the hippocampus. Enhancing the function or expression level of TRPC6 protected neurons from seizure-induced injury[46]. TRPC6 has also been shown to be neuroprotective in neurodegeneration, especially in AD. The expression level of TPRC6 was decreased in various types of cells in AD patients and in neurons differentiated from induced pluripotent stem cells (iPSCs) derived from AD patients[47, 48]. Increasing the expression of TRPC6 can reduce the levels of Aβ and phosphorylated tau[47, 49]. Activation of TRPC6 or store-operated Ca^{2+} entry enhanced hippocampal function in AD model mice, suggesting that the neuroprotective effect of TRPC6 is related to its regulation of calcium signaling[50]. The S1P-induced activation of TRPC6 and downstream calcium signaling discovered in the present study may be one of the potential mechanisms of the neuroprotective effect of S1P. In the

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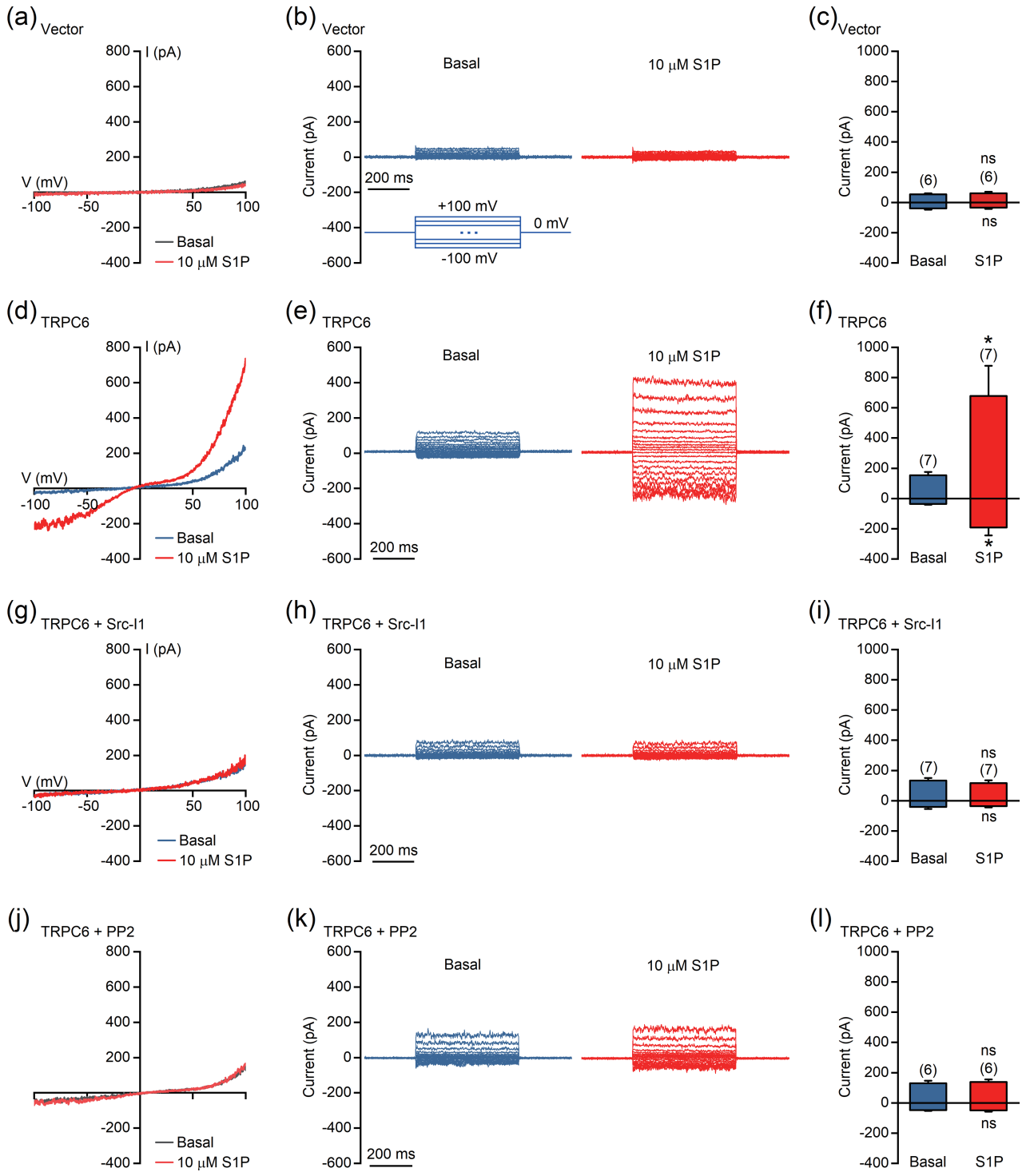

Fig. 8. S1P activates TRPC6 in a Src-dependent manner. (a–l) Perforated patch-clamp recordings were performed in HEK293T cells transfected with empty vector (a–c) or TRPC6 (d–i). Cells used in (g–l) were treated with 2 μmol/L Src-I1 (g–i) or 1 μmol/L PP2 (j–l) for 10 min before recording. (a, d, g, and j) Representative currents recorded with ramp protocols (−100 to +100 mV in 1 s). (b, e, h, and k) Representative currents recorded with step protocols (250 ms step pulses from −100 to +100 mV, 10 mV step; V_h = 0 mV). (c, f, i, and l) Amplitudes of the inward and outward currents shown in (a), (d), (g), and (j), respectively. The data are presented as the means ± SEMs. *P < 0.05. NS indicates no significant difference.

present study, we did not use related disease models to verify this hypothesis and hope to investigate it in future work.

Taken together, the S1P-Src-TRPC6 pathway of neuronal calcium signaling may play an important role in the physiological functions of the nervous system and in neurological diseases. Activating this signaling pathway is expected to become a new strategy for the treatment and drug development of neurodegenerative diseases.

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Conflict of interest

The authors declare that they have no conflict of interest.

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