Activation of H₂O₂-Induced VSOR Cl⁻ Currents in HTC Cells Require Phospholipase C γ 1 Phosphorylation and Ca²⁺ Mobilisation

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Key Words

Volume-sensitive chloride channels • Hydrogen peroxide • Phospholipase C • IP₃ • Ca²⁺

Abstract

Volume-sensitive outwardly rectifying (VSOR) Cl⁻ channels participate in several physiological processes such as regulatory volume decrease, cell cycle regulation, proliferation and apoptosis. Recent evidence points to a significant role of hydrogen peroxide (H₂O₂) in VSOR Cl⁻ channel activation. The aim of this study was to determine the signalling pathways responsible for H₂O₂-induced VSOR Cl⁻ channel activation. In rat hepatoma (HTC) cells, H₂O₂ elicited a transient increase in tyrosine phosphorylation of phospholipase C γ 1 (PLC γ 1) that was blocked by PP2,

a Src-family protein kinases inhibitor. Also, H₂O₂ triggered an increase in cytosolic [Ca²⁺]_i that paralleled the time course of PLC γ 1 phosphorylation. The H₂O₂-induced [Ca²⁺]_i rise was prevented by the generic phospholipase C (PLC) inhibitor U73122 and the inositol 1,4,5-trisphosphate-receptor (IP₃R) blocker 2-APB. In line with these results, manoeuvres that prevented PLC γ 1 activation and/or [Ca²⁺]_i rise, abolished H₂O₂-induced VSOR Cl⁻ currents. Furthermore, in cells that overexpress a phosphorylation-defective dominant mutant of PLC γ 1, H₂O₂ did not induce activation of VSOR Cl⁻ currents. All these H₂O₂-induced effects were independent of extracellular Ca²⁺. Our findings suggest that activation of PLC γ 1 and subsequent Ca²⁺_i mobilisation mediate H₂O₂-induced VSOR Cl⁻ currents, indicating that H₂O₂ operates via redox-sensitive signalling pathways akin to those activated by osmotic challenges.

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Introduction

VSOR Cl⁻ channels are ubiquitously expressed in most mammalian cells types playing a pivotal role in maintaining normal cell volume. Increasing evidence support the notion that these channels participate in several other physiological processes, such as salt transport and metabolic stress, apoptosis, and cell cycle regulation [1-5]. VSOR Cl⁻ currents have been well studied and characterised, however, intracellular signal transduction pathways responsible for VSOR Cl⁻ channel activation under these diverse physiological circumstances remain less understood and appear to be rather contradictory [6]. The mechanisms of activation and regulation of VSOR Cl⁻ channels comprise, depending on the cell type studied, a large variety of factors including ionic strength, macromolecular crowding, intracellular Ca²⁺, G proteins, arachidonic acid and metabolites, PKC, cytoskeleton proteins and tyrosine kinase-mediated phosphorylation of several proteins [7-13].

A growing body of evidence suggests that H₂O₂ serves as an intracellular signalling molecule participating in several physiological processes [14]. For instance, intracellular H₂O₂ influences the function of various proteins, including protein tyrosine phosphatases (PTPs), protein tyrosine kinases transcription factors [15, 16] and ion channels [17, 18]. In this context, it has been recently demonstrated in various cell types that H₂O₂, generated upon NAD(P)H oxidase activation, exerts a central role in VSOR Cl⁻ channel activation [19, 20].

In this study, we explored the mechanisms by which H₂O₂, in the absence of cell swelling, induces VSOR Cl⁻ channel activation. Our results show that at micromolar concentrations, H₂O₂ triggers VSOR Cl⁻ current activation via PLC γ 1 phosphorylation and IP₃-mediated intracellular Ca²⁺ mobilisation. Furthermore, these suggest that H₂O₂ exerts its primary role in VSOR Cl⁻ channel activation by acting through redox-sensitive signalling pathways and not directly as a redox modulator of VSOR Cl⁻ channels.

Materials and Methods

Cell culture

HTC cells passages 2-8 were grown at 37°C in a 5% CO₂-95% air atmosphere in DMEM (GIBCO, USA) supplemented with 5% foetal calf serum (GIBCO, USA), 2 mM L-glutamine and 50 U/mL penicillin-streptomycin (Sigma, USA). All experiments were performed at room temperature.

Electrophysiological measurements

For electrophysiological experiments, cells were grown on 12-mm cover slips and directly mounted on the stage of an inverted microscope (Nikon Diaphot, Nikon America, USA). Bath solution contained (mM): 5 NaCl, 95 NMDGCl, 2 CaCl₂, 1 MgCl₂, 100 sorbitol and 10 Hepes, pH 7.4, adjusted with Tris. Pipette solution contained (mM): 5 NaCl, 133 CsCl, 1 MgCl₂, and 10 Hepes, pH 7.2 adjusted with Tris. Patch-clamp pipettes were made from thin borosilicate (hard) glass capillary tubing with an outer diameter of 1.5 mm (Clark Electromedical, UK), using a BB-CH puller (Mecanex, Switzerland). Whole-cell currents were recorded with an EPC-7 (List Medical, Germany) amplifier. Command voltage protocols and whole cells currents acquisition were controlled by pClamp 8 (Molecular Devices Corporation, USA) via a laboratory interface (Digidata 1200, Molecular Devices Corporation, USA). The acquisition rate was 1 kHz. Membrane cell capacitance ranged from 20 to 30 pF. In all experiments the nystatin perforated-patch whole-cell configuration was used as described [21].

Western blot experiments and analysis

Cells were stimulated, as indicated, in the medium described above and lysed in a cold buffer (50 mM Tris, pH 7.4, 150 mM NaCl, 1 mM EGTA, 1% glycerol, 1% Triton X-100, 1 mM NaF, 1 mM sodium orthovanadate and Protease Inhibitor Cocktail Set 1 (Calbiochem, USA) and centrifuged (10000 x g 15 min at 4°C). Precleared proteins were immunoprecipitated with an anti-PLC γ 1 (TransLab, USA) antibody. Proteins were separated in a 10% SDS-PAGE gel and subsequently transferred to PVDV membranes. Phosphorylated PLC γ 1 (pY-PLC γ 1) was identified using a polyclonal antibody that recognises the phosphorylated form of the phospholipase C γ 1 containing a phosphate on tyrosine 783 (Abcam Ltd., UK). Total PLC γ 1 was detected with the same antibody used for immunoprecipitation. Peroxidase-conjugated goat-anti rabbit (Sigma, USA) was used to reveal the anti-pY-PLC γ 1 antibody. After membrane stripping, total PLC γ 1 antibody was detected with a peroxidase-conjugated goat-anti mouse (Sigma, USA) antibody. Bands were scanned and intensity values were obtained with ImageJ software (NIH, USA, rsb.info.nih.gov/ij/). The phosphorylated fraction of PLC γ 1 was defined as the ratio of the densitometry change of pY-PLC/ PLC γ 1 for each experiment.

Calcium measurements

[Ca²⁺]_i was measured by dual-wavelength emission ratiometric laser scanning confocal microscopy, using the Ca²⁺-sensitive fluorescent dyes fluo-3 and fura-red [12]. HTC cells were loaded with fluo-3 and fura-red for 20-45 min at room temperature in the presence of 5 μ M fluo-3 acetoxymethyl ester (fluo-3-AM) and 15 μ M fura-red-AM, dissolved in pluronic acid/DMSO (Invitrogen, USA) in the bath solution described above. Cells were thoroughly washed and experiments were started after 30 minutes. Each cover slip was placed in a perfusion chamber mounted on an Axiovert 200 inverted fluorescence microscope equipped with a LSM 5 Pascal laser scanning confocal system (Carl Zeiss, USA). Dyes were excited with the 488 nm line of an argon-krypton laser and emission

Fig. 1. H_2O_2 -induced PLC γ 1 phosphorylation. (A) Representative immunoblot for pY783-PLC γ 1 of HTC cells exposed to 200 μM H_2O_2 for 0.5, 2, 5 and 10 minutes. Time 0 corresponds to control conditions (no H_2O_2 added). (B) Time course of tyrosine phosphorylation of pY783-PLC γ 1 normalised against total PLC γ 1 expressed as fold increase in cells exposed to 200 μM H_2O_2 in the presence of 2 mM external Ca^{2+} (○, control), in the absence of external Ca^{2+} (●) and preincubated with PP2 (20 μM , ▽). *, $P < 0.05$ with respect to control, $N = 4$ independent experiments. Error bars are shown if bigger than symbols.

was detected simultaneously at 515–530 nm (fluo-3) and long pass ≥ 670 nm (fura-red) with a 40x/1.4 NA oil immersion objective. Changes in $[\text{Ca}^{2+}]_i$ were measured in a field-of-view consisting of 10–30 cells. Fluo-3 and fura-red fluorescence emission intensity were acquired every 30 s. Changes in $[\text{Ca}^{2+}]_i$ were inferred from changes in the relative fluorescence ratio, calculated by dividing R at each time point by R_0 , the fluorescence ratio measured as the average fluorescence ratio 1–2 minutes before stimulation.

Transient expression of dominant negative PLC γ 1

Sub-confluent cultures of HTC cells (~80%) were trypsinised, washed and resuspended in 0.1 mL Optimem (Gibco, USA) aliquots with 1 μg of rat tyrosine phosphorylation-defective mutant PLC γ 1^{Y783F} plasmid [22] and 0.2 μg of pEGFP-C1 vector (Clontech, USA). The aliquots were transferred to electroporation cuvettes (0.2-cm gap) and cells were subjected to an electroporation protocol consisting in an exponential decay pulse at 160 V and 500 μF capacitance using a GenePulser XCell electroporator (BioRad, USA). Electrophysiological experiments were performed 48 h post-transfection and transfected cells were identified by following GFP fluorescence.

Reagents

All reagents were of analytical grade and were purchased from Sigma (USA) and Merck (Germany). PP2, 4-amino-5-(4-chlorophenyl)-7-(*t*-butyl) pyrazolo [3,4-*d*] pyrimidine; PP3, 4-amino-7-phenylpyrazolo [3,4-*d*] pyrimidine; U73122, 1-[6-((17 β)-3-methoxyestra-1,3,5(10)-trien-17-yl)amino]hexyl]-1H-pyrrole-2,5-dione; U73343, 1-[6-((17 β)-3-methoxyestra-1,3,5(10)-trien-17-yl)amino]hexyl]-2,5-pyrrolidinedione; 2-APB, 2-aminoethoxydiphenyl borate; EGTA, ethyleneglycol-*bis*(*b*-aminoethyl)-*N,N,N',N'*-tetraacetic acid; EGF, epidermal growth factor and BAPTA-AM, 1,2-bis (2-aminophenoxy) ethane-*N,N,N',N'*-tetraacetic acid tetra(acetoxymethyl) ester were purchased from Calbiochem (San Diego, CA, USA).

In all experiments with PP3, PP2, U73343, U73122, cells were preincubated at the corresponding concentrations for 1 hour prior to the exposure to H_2O_2 or EGF. In experiments with 2-APB, cells were preincubated for 15 minutes and 2-APB was maintained throughout the experiments.

Statistics

All results are presented as means $\pm$ SEM from four to ten independent experiments. Statistical analysis of the data was performed using Statgraphics Plus 5.0 (Statistical Graphics Corp., USA). Unpaired Student's *t*-test was performed and considered significant at $P < 0.05$. Data from PLC γ 1 phosphorylation and intracellular Ca^{2+} time courses were analysed by repeated-measures ANOVA followed by the Bonferroni *post hoc* test and considered significant at $P < 0.05$.

Results and Discussion

H_2O_2 enhances PLC γ 1 phosphorylation

In contrast to other reactive oxygen species (ROS), such as hydroxyl radicals or superoxide anions, H_2O_2 is a rather mild oxidant that targets primarily deprotonated cysteine residues in diverse proteins, particularly protein tyrosine phosphatases [23–25], and protein tyrosine kinases [26, 27]. Although it has been reported that H_2O_2 induces the activation of VSOR Cl^- currents [4, 19], the mechanisms involved in this process remain unknown.

Fig. 2. Effect of U73122 and 2-APB on H_2O_2 -mediated $[\text{Ca}^{2+}]_i$. Time course of intracellular Ca^{2+} signals (averaged fluorescence ratio $\pm$ SEM). Cells were exposed to 200 μM H_2O_2 in isotonicity during the time depicted by the bar ($\circ$, control), in the absence of external Ca^{2+} ($\bullet$), in the presence of 2-APB (50 μM , $\triangle$) and preincubated with U73122 (10 μM , $\square$). *, $P < 0.05$ compared to 2-APB and U73122 data points. $N = 5$ independent experiments.

In this context, we have previously shown that hypotonicity-induced cell swelling increases H_2O_2 generation in HTC cells [19]. It is also known that in this same cell type, swelling evokes a transient tyrosine phosphorylation of PLC γ 1 subsequent IP_3 generation and increase in $[\text{Ca}^{2+}]_i$ [12]. Because H_2O_2 has been shown to activate PLC γ 1 in several cell types [28–30], we asked whether this isoform of PLC γ could participate in H_2O_2 -induced VSOR Cl^- channel activation. Phosphorylation of PLC γ 1 on Tyr-783 is essential for activation of this enzyme as well as for IP_3 generation [31]. Thus, a phosphospecific Y783 antibody was used to examine the time course of H_2O_2 -induced PLC γ 1 phosphorylation in HTC cells. As shown in Figures 1A–B, exposure of HTC cells to 200 μM H_2O_2 , a concentration twice the EC_{50} for VSOR Cl^- current activation [19], resulted in a transient increase in the Tyr-783 phosphorylated fraction of PLC γ 1. The increase (~ 1.7 -fold) in H_2O_2 -induced phosphorylation reached a maximum at approximately 2 minutes and returned to basal levels in 10 minutes. In various cell types, members of the Src family of non receptor tyrosine kinases (NRTKs) have been implicated in integrin-dependent PLC γ 1 tyrosine phosphorylation [32] and VSOR Cl^- channel activation [7, 33]. Based on the observation that PLC γ 1 is recruited by NRTKs [34], we investigated the effect of PP2, an inhibitor of the Src family of NRTKs [35], on H_2O_2 -induced Y783-PLC γ 1 phosphorylation. Incubation of HTC cells with PP2 significantly attenuated H_2O_2 -induced PLC γ 1 phosphorylation (Figure 1B). Worth mentioning, it has been shown that in HTC cells exposed to hypotonicity, cell swelling-induced PLC γ phos-

phorylation was significantly abrogated by PP2 [36].

Next, we explored the role of extracellular Ca^{2+} on PLC γ 1 phosphorylation. At variance with that observed in rat cardiomyocytes, where purinergic-induced PLC γ phosphorylation is lost in cells incubated in nominally Ca^{2+} -free buffer [37], in HTC cells the magnitude and the time course of the H_2O_2 -induced PLC γ 1 phosphorylation were unaffected by extracellular Ca^{2+} removal (no added Ca^{2+} plus 5 mM EGTA, Figure 1B).

H_2O_2 triggers $[\text{Ca}^{2+}]_i$ mobilisation

An important signalling pathway mediated by PLC γ 1 activation is the production of IP_3 and the activation of IP_3 receptors, which in turn leads to an increase in $[\text{Ca}^{2+}]_i$. In cultured rat astrocytes, micromolar concentrations of H_2O_2 have been demonstrated to trigger Tyr-783 PLC γ 1 phosphorylation followed by a rise in $[\text{Ca}^{2+}]_i$ [30]. Osmotic cell swelling-induced increase in $[\text{Ca}^{2+}]_i$ has been found in many cell types [38–41]. In HTC cells, osmotically-dependent $[\text{Ca}^{2+}]_i$ increase is related to IP_3 - and not to ryanodine receptor activation [12]. To explore further the involvement of PLC γ 1 and subsequent $[\text{Ca}^{2+}]_i$ increase in H_2O_2 -induced VSOR Cl^- current activation, relative changes in $[\text{Ca}^{2+}]_i$ were monitored in response to H_2O_2 . Figure 2 depicts the time course of $[\text{Ca}^{2+}]_i$ upon continuous exposure to 200 μM H_2O_2 . Under control conditions, H_2O_2 induces a transient rise in $[\text{Ca}^{2+}]_i$ which paralleled the time course observed for PLC γ 1 phosphorylation. The involvement of PLC γ 1 and its downstream effectors in H_2O_2 -induced $[\text{Ca}^{2+}]_i$ increase was demonstrated by inhibitory effects of both, the generic PLC

Fig. 3. Effect of PP2, U73122 and 2-APB on H_2O_2 -induced VSOR Cl^- currents. (A) Representative steady-state nystatin-perforated whole-cell, control (upper panel) and H_2O_2 -induced ($200\text{ }\mu\text{M}$) VSOR Cl^- current traces triggered by a voltage step protocol from -100 to 100 mV in 20 mV increments from a holding potential of -30 mV . The test pulse (2000 ms) was preceded by a prepulse to -100 mV lasting 200 ms and followed by a postpulse to -60 mV lasting 200 ms . The dashed line represents zero current. (B) Representative experiments showing the time course of development of Cl^- currents evoked by $200\text{ }\mu\text{M}$ H_2O_2 in isotonicity in the presence of 2 mM external Ca^{2+} ($\circ$), in a Ca^{2+} -free external solution plus 5 mM EGTA ($\bullet$), in the presence of 2-APB ($50\text{ }\mu\text{M}$, ∇), preincubated with PP2 ($20\text{ }\mu\text{M}$, $\triangle$) and preincubated with U73122 ($10\text{ }\mu\text{M}$, $\square$). Currents were measured at 80 mV every 15 s and normalised by cell capacitance. (C) Summary of the data for maximal current at 80 mV (% of control, 49.6 pA/pF) obtained from experiments described in (B), *, $P < 0.05$ respect to control, $N = 6\text{--}8$ independent experiments.

antagonist U73122 and the IP_3 receptor antagonist 2-APB. To identify the source of Ca^{2+} , experiments were carried out in the absence of external Ca^{2+} . As shown, the amplitude and time course of the $[\text{Ca}^{2+}]_i$ signal were unaffected by external Ca^{2+} removal, indicating that intracellular Ca^{2+} stores are the main source for H_2O_2 -induced $[\text{Ca}^{2+}]_i$ increase. Although it has been reported, that U73122 and 2-APB both have effects on $[\text{Ca}^{2+}]_i$ by blocking store-operated Ca^{2+} channels [42, 43], the experiments performed in the absence of extracellular Ca^{2+} confirmed that U73122 and 2-APB, at the concentrations used, inhibited Ca^{2+} mobilisation and not Ca^{2+} influx through store-operated Ca^{2+} channels.

H_2O_2 -induced VSOR Cl^- currents require the activation of Src-kinase and PLC-dependent pathways

If H_2O_2 -induced VSOR Cl^- channel activation relies upon PLC γ 1 activity and Ca^{2+} release from IP_3 -dependent stores, then it would be anticipated that inhibition of either NRTKs, PLC or the IP_3 receptor should prevent VSOR Cl^- channel activation. To test this hypothesis, HTC cells were exposed to either PP2, U73122 or 2-APB and whole-cell Cl^- currents under the nystatin-perforated configuration were recorded. As depicted in Figure 3A, exposure of cells to $200\text{ }\mu\text{M}$ H_2O_2 in isotonicity elicited a robust outwardly rectifying Cl^- current, indistinguishable from the Cl^- currents triggered by hypot-

onic challenges [19]. Figure 3B illustrates representative experiments of the time course of development of outwardly rectifying Cl^- currents elicited by $200\text{ }\mu\text{M}$ H_2O_2 in HTC cells exposed to these compounds. As shown, PP2, U73122 and 2-APB significantly inhibited H_2O_2 -induced VSOR Cl^- currents, whereas the inactive analogues PP3 and U73343 were ineffective, confirming that a PLC-dependent signalling pathway leading to increase in $[\text{Ca}^{2+}]_i$ is required for H_2O_2 -induced VSOR Cl^- currents. Prevention of this rise in $[\text{Ca}^{2+}]_i$ by preincubation of cells with BAPTA-AM (30 min , $75\text{ }\mu\text{M}$) strongly inhibited H_2O_2 -induced VSOR Cl^- currents ($6.4 \pm 2.4\%$ of control, $N = 6$). However, the importance of increased intracellular Ca^{2+} appears not to be universal, as activation of VSOR Cl^- channels in Ehrlich ascites tumour cells has also been observed under strong $[\text{Ca}^{2+}]_i$ buffering conditions with a $[\text{Ca}^{2+}]_i$ concentration of only 25 nM [44]. Despite the fact that both, PLC γ 1 phosphorylation and $[\text{Ca}^{2+}]_i$ signals proved to be independent from extracellular

Fig. 4. EGF-induced VSOR Cl^- currents and effect of $\text{PLC}\gamma 1^{\text{Y783F}}$ dominant negative mutant on H_2O_2 -induced VSOR Cl^- currents. (A) Representative experiments showing the time course of current development evoked by 500 ng/mL EGF in isotonicity (control, $\circ$) and preincubated with U73122 (10 μM , $\square$). Currents were measured at 80 mV every 15 s and normalised by cell capacitance. (B) Summary of the data for maximal current at 80 mV (% of control, 44.5 pA/pF) obtained from experiments described in (A), *, $P < 0.01$ respect to control, $N = 4$ independent experiments. (C) Representative experiments showing the time course of current development evoked by 200 μM H_2O_2 in mock-transfected (control, $\circ$) and $\text{PLC}\gamma 1^{\text{Y783F}}$ transfected HTC cells ($\triangle$). Currents were measured at 80 mV every 15 s and normalised by cell capacitance. (D) Summary of the data for maximal current at 80 mV (% of control, 49.6 pA/pF) obtained from experiments described in (C), *, $P < 0.01$ respect to control, $N = 4$ independent experiments.

Ca^{2+} in our system, we questioned whether extracellular Ca^{2+} could affect H_2O_2 -induced VSOR Cl^- currents. To that end, experiments were performed in the absence of external Ca^{2+} . As indicated in Figure 3B, the current amplitude as well as the time course remained unchanged upon external Ca^{2+} removal, indicating that Ca^{2+} mobilisation from intracellular stores would suffice for H_2O_2 -induced VSOR Cl^- current activation. Of note, 100 μM ryanodine did not inhibit H_2O_2 -induced VSOR Cl^- currents ($93.8 \pm 4.5\%$ of control, $N = 5$), indicating that 2-APB-sensitive internal stores are adequate to sustain the observed $[\text{Ca}^{2+}]_i$ increase and thus, ruling out a role for caffeine-sensitive Ca^{2+} stores.

$\text{PLC}\gamma 1$ is known to be activated through tyrosine phosphorylation by growth factor receptors such as epidermal growth factor receptor [45]. We have previously reported that EGF in isotonicity elicited VSOR Cl^- current activation in HTC cells [19]. Therefore, we explored next whether EGF-induced VSOR Cl^- currents were sensitive to PLC blockade. As shown in Figure 4A-B, EGF-

induced VSOR Cl^- currents were significantly inhibited by the generic PLC inhibitor U73122 ($13.8 \pm 5.5\%$ of control, $N = 4$).

Because of the inherent lack of specificity of the PLC blocker U73122, inhibition of H_2O_2 -induced VSOR Cl^- currents by this compound should be interpreted with caution. To address this point more directly, we used HTC cells transiently transfected with a phosphorylation-defective dominant negative mutant ($\text{PLC}\gamma 1^{\text{Y783F}}$) of rat $\text{PLC}\gamma 1$ [22]. Given that phosphorylation of $\text{PLC}\gamma 1$ on Tyr-783 is essential for activation of this enzyme as well as for IP_3 generation [31], it can be anticipated that in transfected HTC cells H_2O_2 would not induce VSOR Cl^- current activation. As expected, Cl^- current was elicited by exogenous addition of H_2O_2 in mock-transfected cells but was significantly reduced in cells transfected with the dominant negative construct ($4.7 \pm 2.7\%$ of control, $N = 4$, Figure 4C-D), confirming that $\text{PLC}\gamma 1$ activity is essential for activating H_2O_2 -induced VSOR Cl^- currents.

In summary, our results demonstrate that exogenous

H₂O₂ induces VSOR Cl⁻ current activation by essentially sharing the key signalling pathways involved in hypotonicity-mediated current activation [12, 36, 46, 47]. Hence, the role of H₂O₂ as a second messenger molecule in VSOR Cl⁻ channel activation acting through a PLC-dependent signalling pathway is emphasized. Furthermore, the results demonstrate that H₂O₂ does not primarily exert its effects by directly oxidising VSOR Cl⁻ channels, although this possibility can not be completely ruled out at this point. A simple model accounting for both our present results and previous findings would envisage that in HTC cells, generated H₂O₂ regardless the stimuli,

promotes Src-mediated PLCγ1 phosphorylation leading to intracellular Ca²⁺ mobilisation, activation of Ca²⁺-sensitive pathways and subsequent VSOR Cl⁻ channel activation.

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