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Synergistic actions of diacylglycerol and inositol 1,4,5 trisphosphate for Ca^{2+}-dependent inactivation of TRPC7 channel[1]

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Key words

canonical transient receptor potential 7; calcium; inactivation, diacylglycerol; inositol 1,4,5 trisphosphate

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Abstract

Aim: The aim of the present study was to explore the mechanism for the Ca^{2+}-dependent inactivation of the canonical transient receptor potential (TRPC) 7 channel expressed in human embryonic kidney 293 cells. **Method:** The whole-cell patch-clamp technique was used in the study. **Results:** With Ca^{2+}-free external solution, the perfusion of 100 μmol/L carbachol to, or dialysis of the cell with 100 μmol/L guanosine 5'-3-*O*-(thio)triphosphate (GTPγS), induced large inward currents, respectively. These currents were rapidly inhibited by the addition of 1 mmol/L Ca^{2+} into the bath, and recovery from this inhibition was only partial after the Ca^{2+} removal, unless vigorous intracellular Ca^{2+} buffering with 10 mmol/L 1,2 *bis*(2-aminophenoxy)ethane-*N,N,N',N'*-tetraacetic acid (BAPTA) (plus 4 mmol/L Ca^{2+}) was employed. In contrast, the current induced by a membrane-permeable analog of diacylglycerol (DAG), 1-oleoyl-2-acetyl-sn-glycerol (OAG; 100 μmol/L) did not undergo the inhibition persisting after Ca^{2+} removal. Interestingly, the inclusion of inositol 1,4,5 trisphosphate (IP_3; 100 μmol/L) in the patch pipette rendered the OAG-induced current susceptible to the persistent Ca^{2+}-mediated inhibition independent of the IP_3 receptor in the majority of the tested cells, as evidenced by the inability of heparin and thapsigargin in reversing the effect of IP_3. **Conclusion:** The present results suggest that Ca^{2+} entry via the activated TRPC7 channel plays a critical role in inactivating the channel where the cooperative actions of DAG and IP_3 are essentially involved.

Introduction

Transient receptor potential (TRP) is a vast gene superfamily which contains more than 30 isoforms ranging from yeast to mammals[1–3]. The proteins of this family are predicted to have 6 transmembrane (TM) domains and intracellularly located amino and carboxyl termini. Like many voltage-gated channels, a lipophilic hairpin structure between the fifth and the sixth TM domain is predicted to form the channel pore[4]. Functional studies *in vitro* show that most channels of this family have poor selectivity over cations and can be activated through G protein-coupled receptors or tyrosine kinase receptors linked to phospholipase C[4].

The mammalian TRP superfamily is composed of 6 subfamilies: the canonical TRP (TRPC), the vanilloid receptor TRP (TRPV), the melastatin or long TRP, the mucolipins, the polycystins, and ankyrin transmembrane protein 1[3]. Among these, TRPCs share the highest similarities to its prototype, *Drosophila* TRPs, and are likely to be the molecular candidate that mediate the native receptor-operated and store-operated Ca^{2+} entry observed in many non-excitable tissues[1–3]. The 7 members in TRPC can be subclassified into 3 subgroups according to the structural and functional similarities: TRPC3/6/7, TRPC1/4/5, and TRPC2[1–3]. TRPC7 is the latest cloned TRPC isoform and least elucidated for its channel regulation.

Ca^{2+} entry through the channel pore plays essential roles in the regulation of many Ca^{2+} permeable channels. It is well known that the L-type voltage-gated Ca^{2+} current decays much faster in the presence of extracellular Ca^{2+} than its surrogate Ba^{2+}[5]. The ubiquitous Ca^{2+} sensor calmodulin (CaM) tethered to the proximal segment of the cytoplasmic C-terminus of the a1c subunit, upon entry of Ca^{2+}, is thought to reorientate to cause conformational rearrangements[6–8]. TRPV1 is probably the best-defined channel in the TRP superfamily that serves as a poly-modal detector of many pain-producing chemicals and physical stimuli in the dorsal root ganglia neurons[9,10]. This non-selective cationic channel undergoes rapid inactivation or desensitization during the agonist application in Ca^{2+}-containing bathing solution[11], but the underlying mechanism is controversial. While Numazaki *et al*[12] pointed out that Ca^{2+}/CaM binded to a 35 aa segment of the C-terminus of the channel, resulting in desensitization, the involvement of calcineurin-dependent dephosphorylation was long regarded by others to be involved in the process[13]. In addition, Bhave *et al*[14] showed that cAMP dependent phosphorylation antagonizes the desensitization leading to the resensitization of the channel.

TRPC7 is highly sensitive to extracellular Ca^{2+} (Ca^{2+}_o) in the physiological range, being almost completely inhibited by 1 mmol/L Ca^{2+}_o [IC_{50}(half inhibition-concentration value) =0.11 mmol/L][15]. A detailed examination with single channel recordings revealed that rapid permeation blockade by external Ca^{2+} and the tonic inhibitory actions of internal Ca^{2+} via Ca^{2+}/CaM both underlie this inhibition. According to our preliminary results, the inhibition of TRPC7 induced by the transient application of 1 mmol/L Ca^{2+}_o was hardly washable, suggesting that some long-term effects of Ca^{2+}_o still function in the channel inactivation regulation. To explore the mechanisms underlying this long-term effect and to broaden our understanding towards this highly Ca^{2+}-sensitive TRPC7 channel, we performed further patch-clamp experiments in the present study.

Material and methods

Cell culture and transfection Human embryonic kidney 293 (HEK293) cells were cultured in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum. For transfection, the cells were reseeded in a 35 mm culture dish and were allowed to grow to a 40%–50% confluency. The cells were then transfected with a mixture of 2 μg plasmid vector (pCI-neo) incorporating murine TRPC7 DNA and 0.4 μg pCI-neo-πH3-CD8 (cDNA of the T cell antigen CD8) with the aid of 20 μL of the transfection reagent SuperFect (Qiagen, Germany). In some cases (Figure 1F), 2 μg plasmid DNA for a Ca^{2+}-insensitive mutant calmodulin (replacement with alanine of the aspartate residues 21, 57, 94, and 130 in 4 E–F hands of calmudulin) was cotransfected. Approximately 24 h later, the cells were trypsinized and reseeded onto coverslips precoated with 100 μmol/L poly-L-lysine. Electrophysiological experiments were performed within 24–48 h.

Electrophysiological recording Two modes of patch-clamp recording technique were employed in the present study, that is, nystatin-perforated and conventional whole-cell recording. For both recordings, microglass pipettes with 2.5–4.0 MΩ resistance (when filled with Cs-pipette solution) were used. Voltage generation and current signal acquisition were implemented by a high-impedance, low-noise patch-clamp amplifier (EPC9; HEKA Electronics, Lambrecht/Pflaz Germany). For the construction of the current–voltage curve (Figure 1B), sampled data were low-pass filtered at 1 kHz and stored on a computer hard disc after digitization at 5 kHz. Long-term recordings (current traces, such as Figure 1A) were performed in conjunction with an A/D, D/A converter, Powerlab/400 (AD instruments, Bella Vista, New South Wales, Australia) at a sampling rate of 100 Hz to diminish the size of the data; data evaluation was made with accessory software, Chart v3.6. All the drugs were administered by a "Y-tube" device that enabled us to quickly change the solution around the cell.

Solutions Solutions with the following composition were used. The pipette solution for the nystatin-perforated recording (in mmol/L) was as follows: 140 CsCl, 2 $MgCl_2$, 10 HEPES (4-(2-hydroxyethyl)piperazine-1-ethanesulfonic acid), and 10 glucose (adjusted to pH 7.2 with Tris base); the poorly-buffered pipette solution for conventional whole-cell recording (in mmol/L) was: 120 CsOH, 120 aspartate, 20 CsCl, 2 $MgCl_2$, 0.1 BAPTA (1,2 *bis*(2-aminophenoxy)ethane-*N*,*N*,*N'*, *N'*-tetraacetic acid) or EGTA (ethylene glycol-bisc2-aminoethylether)-*N*,*N*,*N'*,*N'*-tetraacetic acid), 0.04 $CaCl_2$, 10 HEPES, 2 ATP, 0.1 GTP (guanosine 5'-triphosphate), and 10 glucose (adjusted to pH 7.2 with Tris base); the highly-buffered pipette solution for conventional whole-cell recording (in mmol/L)was : 120 CsOH, 120 aspartate, 20 CsCl, 2 $MgCl_2$, 10 BAPTA (or EGTA), 4 $CaCl_2$, 10 HEPES, 2 ATP, 0.1 GTP, and 10 glucose (adjusted to pH 7.2 with Tris base). In total, 100 μmol/L GTPγS, 100 μmol/L inositol 1,4,5 trisphosphate (IP_3), inositol 1,3,4,5 tetraphosphate (IP_4), or 1,4 bisphosphate (IP_2) was added to the poorly-buffered or highly-buffered pipette solutions when necessary. The normal external solution contained (in mmol/L) 140 NaCl, 5 KCl, 1.2 $MgCl_2$, 1 EGTA, 10 HEPES, and 10 glucose (adjusted to pH 7.4 with Tris base). For the Ca^{2+}-containing external solution, 1 mmol/L $CaCl_2$ was added with the omission of EGTA.

Drugs 1-oleoyl-2-acetyl-sn-glycerol (OAG), GTPγS,

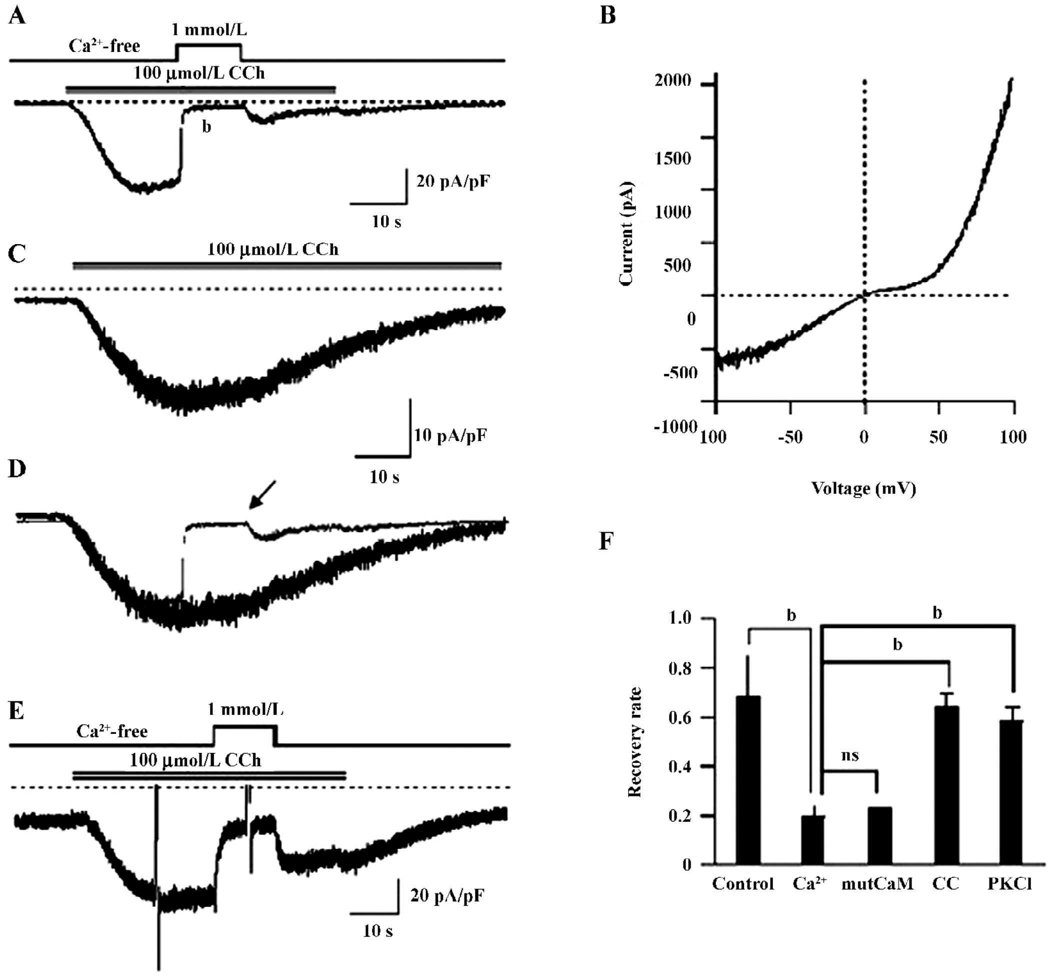

Figure 1. Ca^{2+}-dependent inactivation of I_{TRPC7} induced by CCh, nystatin-perforated recording, holding potential (HP) =60 mV. (A) typical trace to show that the addition of 1 mmol/L Ca^{2+} following the full activation of TRPC7 caused strong and a hardly washable inhibition of the current (indicated by the asterisk). (B) typical current–voltage curve of the TRPC7 current. (C) typical TRPC7 current recorded in Ca^{2+}-free bathing solution. Note the natural time course of the current decay at continuous CCh perfusion. (D) superimposing the traces of A (gray) and C (black). Arrows indicate the smaller remaining current after perfusion of Ca^{2+} for 10 s. (E) typical TRPC7 trace recorded with 5 μM PKC–IP_{19-36} in the pipette solution (0.04 mmol/L Ca^{2+} plus 0.1 mmol/L BATPA), conventional whole-cell recording. (F) comparison of the recovery rate of I_{TRPC7} (the ratio of currents amplitude after to before Ca^{2+} application; in the control, it is the relative amplitude of I_{TRPC7} 10 s after the peak to the maximum) with or without (control) calphostin C (CC), PKC–IP_{19-36} (PKCI) treatments, and mutCaM co-expression, respectively (n=6–8). $^{b}P<0.05$. ns, no statistical significance

heparin, thapsigargin (TG), and protein kinase C (PKC) inhibitory peptide (PKC–IP_{19-36}) were purchased from Calbiochem (La Jolla, CA, USA); IP_2, IP_4, carbachol (CCh), and HEPES were from Sigma (St Louis, MO, USA); ATP, GTP, EGTA, and BAPTA were from Dojindo (Kumamoto, Japan).

Statistics All data are expressed as means±SEM. Paired and unpaired Student's *t*-test was used to evaluate statistical significance.

Results

Ca^{2+}-dependent inactivation of TRPC7 current HEK293 cells overexpressing mouse TRPC7 exhibited large inward currents following the addition of 100 μmol/L CCh (35.7±5.2 pA/pF, n=21; Figure 1A). The current–voltage (I-V) curve showed a double-rectifying property with a reversal potential near 0 (Figure 1B), which represents the typical I–V relationship of the TRPC3/6/7 subfamily[1]. Little current was recorded with empty vector-expressing cells (0.89±0.61 pA/pF, *n*=10). Thus, the currents induced by CCh in the present study resulted from the heterologously-expressed TRPC7 channel (designated as I_{TRPC7} hereafter).

The application of 1 mmol/L $\text{Ca}^{2+}_{\text{o}}$ after full activation by CCh caused a rapid decline of I_{TRPC7} (Figure 1A). According to Shi *et al*[15], this inhibition can be assigned to the blockade of cation permeation by external Ca^{2+}, which accounts for approximately 80% of the observed inhibition and the intracellular Ca^{2+}/CaM-dependent tonic inhibition, which accounts for the remaining 20%. The inhibition of I_{TRPC7} by $\text{Ca}^{2+}_{\text{o}}$ was long lasting since only a small fraction of the current was recovered after $\text{Ca}^{2+}_{\text{o}}$ was washed out (Figure 1A). Superimposition of traces with (Figure 1A; indicated by an arrow) and without (Figure 1C) transient $\text{Ca}^{2+}_{\text{o}}$ application clearly demonstrates that $\text{Ca}^{2+}_{\text{o}}$ greatly accelerates the inactivation process of I_{TRPC7} (Figure 1D). As summarized in Figure 1F, the recovery rate of I_{TRPC7} (the ratio of current amplitudes after to before Ca^{2+} application; in the control, this ratio was calculated as the relative amplitude of I_{TRPC7} 10 s after the peak to the maximum) at the 2 conditions showed a statistically significant difference. The persistent inhibition of I_{TRPC7} by $\text{Ca}^{2+}_{\text{o}}$ is unlikely due to the remaining function of intracellular CaM, since a similar recovery rate was observed when a Ca^{2+}-insensitive mutant calmodulin (mutCaM) was co-expressed with TRPC7 (Figure 1F). Consistent with the previous report[15], however, PKC is rather involved in the process since both pretreatment of the cells with calphostin C (500 nmol/L), a potent inhibitor of PKC, and inclusion of $\text{PKC–IP}_{19–36}$ (5 μmol/L) in the pipette solution antagonized the persistent Ca^{2+} inhibition and accordingly raised the recovery rate, respectively (Figure 1E,1F).

Ca^{2+} entry is involved in the Ca^{2+}-dependent inactivation of TRPC7 To determine whether the Ca^{2+}-dependent inactivation (CDI) of I_{TRPC7} is due to Ca^{2+} influx, we next employed 2 different pipette solutions with weak and high-buffering capacities for Ca^{2+}. As shown in Figure 2A and 2B, when recorded with high-buffering pipette solution (10 mmol/L BAPTA plus 4 mmol/L Ca^{2+}), a brief Ca^{2+} addition no longer facilitated the inactivation of I_{TRPC7}. As summarized in Figure 2C, the recovery rate of I_{TRPC7} with weak Ca^{2+} buffering was similar to that with the nystatin-perforated recording, but was significantly smaller than that obtained under strong Ca^{2+}-buffering conditions. These data preliminarily suggest that the source of Ca^{2+} that causes this persistent inhibition is due to Ca^{2+} entry because the Ca^{2+} buffer power in chelating the transient Ca^{2+} entry near the vicinity of the channel might be weak. To reinforce this idea, TG, a potent inhibitor of Ca^{2+}-ATPase in the endoplasmic reticulum (ER), was used to deplete the internal Ca^{2+} store. As summarized in Figure

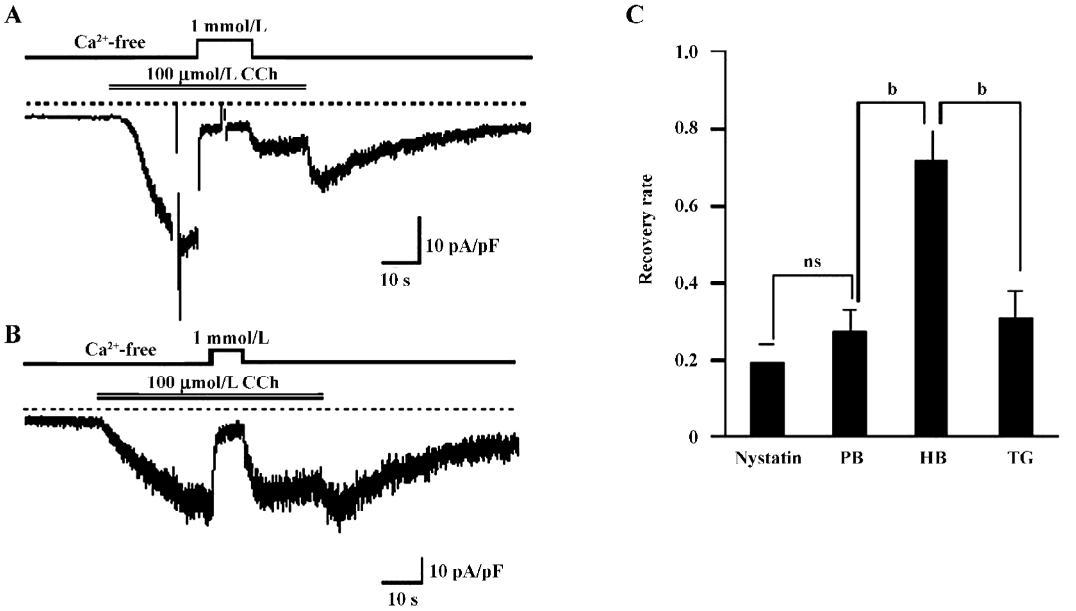

Figure 2. Essential role of Ca^{2+} entry in mediating the CDI of TRPC7, HP =–60 mV. (A) typical trace showing the CDI of I_{TRPC7} with poorly-buffered (PB) pipette solution (0.04 mmol/L Ca^{2+} plus 0.1 mmol/L BATPA), conventional whole-cell (cwc) recording. (B) typical trace showing that highly-buffered (HB) pipette solution (4 mmol/L Ca^{2+} plus 10 mmol/L BATPA) eliminated the weak reversibility of the $\text{Ca}^{2+}_{\text{o}}$-mediated inhibition, cwc recording. (C) comparison of the recovery rate of I_{TRPC7} between the nystatin-perforated recording with or without TG pretreatment, cwc recording with PB or HB pipette solutions (*n*=8–12). $^{\text{b}}P<0.05$. ns, no statistical significance.

2C, the recovery rate of the Ca^{2+} inhibition of I_{TRPC7} did not change significantly when the bathing solution contained 2 μmol/L TG. These results collectively suggest that Ca^{2+} entry through the activated TRPC7 channel accelerates the inactivation of the channel.

GTPγS-induced TRPC7 current shows similar CDI It is well established that Ca^{2+} affects the inactivation and/or desensitization of channels through acting on both the G protein-coupled receptor and its downstream effectors-mediated pathways[16]. To delineate the site of actions of Ca^{2+} on TRPC7, GTPγS (100 μmol/L) was included in the pipette solution. Dialysis of the cell with GTPγS resulted in the slow development of an inward current comparable to that evoked by CCh (Figures 3A,3B). The addition of Ca$^{2+}_o$ caused a dose-dependent inhibition of the current. Perfusion of 0.1 mmol/L Ca$^{2+}_o$ mildly inhibited the current, which was quickly reversed by the removal of Ca^{2+} (Figures 3A,3B). In contrast, with 1 mmol/L Ca$^{2+}_o$, which severely inhibited the current, the recovery from the inhibition became much less complete (Figure 3A,3C), and was rescued by the inclusion of high concentrations of BAPTA in the pipette (Figure 3B,3C). Because GTPγS directly activates the G protein and bypasses the receptor stimulation, these results suggest that the CDI of I_{TRPC7} does not involve a process upstream of G protein activation.

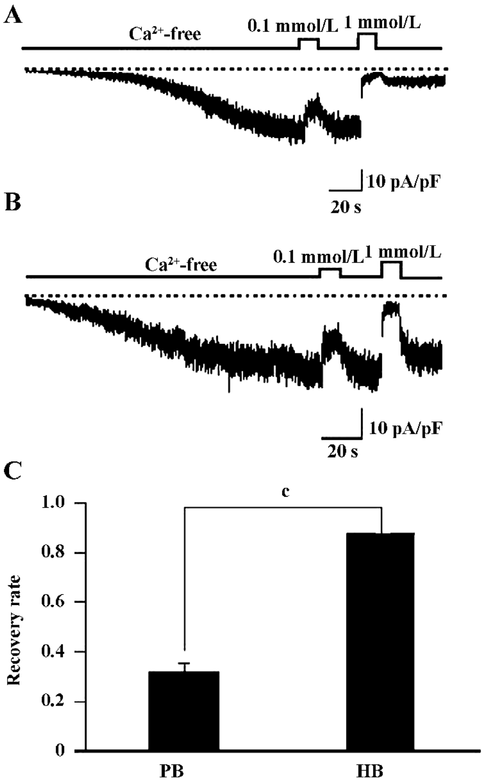

Figure 3. TRPC7 current induced by GTPγS underwent similar Ca^{2+} entry-dependent CDI, cwc recording with 100 μmol/L GTPγS in the pipette solution, HP =–60 mV. (A) 1 mmol/L Ca$^{2+}_o$ caused a strongly irreversible inhibition of the GTPγS-induced current at the PB condition (0.04 mmol/L Ca^{2+} plus 0.1 mmol/L BATPA). (B) 1 mmol/L Ca$^{2+}_o$ caused a potent but easily washable inhibition of the current when intracellular Ca^{2+} was severely buffered with a high concentration of BAPTA (4 mmol/L Ca^{2+} plus 10 mmol/L BATPA). (C) summary of the recovery rate of the current amplitude after to before perfusion of 1 mmol/L Ca^{2+} (n=7). $^{c}P<0.01$.

OAG-induced TRPC7 current exhibits no obvious CDI The members of the TRPC3/6/7 subfamily are known to be activated by 1 of the major phosphatidylinositide metabolites, diacylglycerol (DAG). This action is thought to be direct on the channel protein, independent of PKC or IP_3[20]. Thus, the use of DAG instead of CCh may enable us to explore the above-mentioned actions of Ca^{2+} in simpler conditions in which the role of IP_3 can be excluded. For this purpose, we used a membrane-permeable analog of DAG, OAG. Perfusion of 100 μmol/L OAG induced an inward current with the hallmarks of I_{TRPC7} (ie the reversal potential near 0; the ohmic current–voltage relationship with a slight double-rectifying property; blockade by millimolar extracellular Ca^{2+}; data not shown). The sustained inhibition after application of 1 mmol/L Ca$^{2+}_o$ seen for CCh- and GTPγS-induced I_{TRPC7} was almost completely abolished in OAG-induced I_{TRPC7} (Figure 4A). The

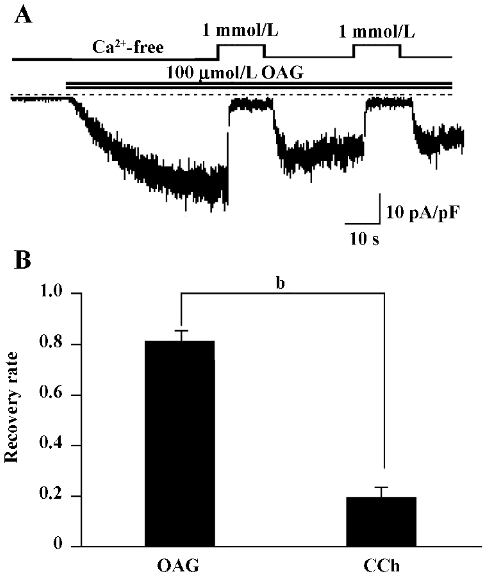

Figure 4. OAG-induced current showed good repetitivity between continual applications of 1 mmol/L Ca$^{2+}_o$, nystatin-perforated recording, HP =–60 mV. (A) typical trace showing that 100 μmol/L OAG-induced current was reproducible after the repeated addition of 1 mmol/L Ca^{2+}. (B) comparison of the recovery rates of CCh- and OAG-induced currents after the transient application of Ca^{2+} (n=9). $^{b}P<0.05$.

recovery from the Ca^{2+}-dependent inhibition was much more complete (80%) in OAG-induced I_{TRPC7} as compared with that observed with CCh (circa 20%; Figure 4B).

IP_3 regresses the CDI of OAG induced currents The most important difference in activating the TRP channels between DAG and GTPγS/CCh is no involvement of the IP_3 pathway in the former. Therefore, 100 μmol/L (the commonly-used full concentration) IP_3 was added into the pipette solution to see whether the Ca^{2+}-induced persisting inhibition of I_{TRPC7} could be restored. As illustrated in Figure 5A and summarized in Figure 5C, in more than half of the tested cells (6 of 11 cells), the intrapipette inclusion of IP_3 caused a prominent, irreversible Ca^{2+} inhibition of I_{TRPC7} evoked by OAG. The effect of IP_3 appeared to be direct since no such regressions of CDI were observed when other metabolites of inositol phosphate, such as IP_4 and IP_2, were included in the pipette solution (Figure 5B,5C).

Effect of IP_3 is IP_3 receptor independent The main biological function of IP_3 is to cause Ca^{2+} release through binding to the IP_3 receptor (IP_3R) on the ER. To examine whether the recovery of CDI caused by IP_3 is due to the activation of IP_3R, heparin, a potent IP_3R antagonist (0.5 mg/mL), was included in the pipette solution. Interestingly, this compound failed to eliminate the effect of IP_3 to restore the Ca^{2+}-dependent inhibition in OAG-induced I_{TRPC7} (Figure 6A). The similar results were obtained when TG (2 μmol/L) was present in the bathing solution (Figure 6B). Taken together, these data strongly suggest that the role of IP_3R would be, if any, only minor in mediating the effect of IP_3.

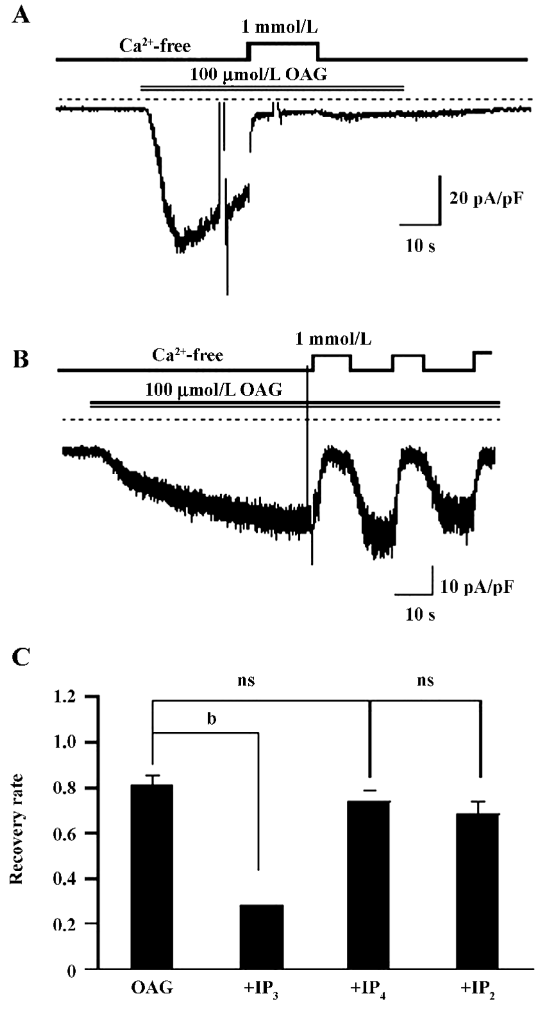

Figure 5. IP_3 regressed the irreversibility of the Ca^{2+}-mediated inhibition of the OAG-induced current, cwc recording, HP =–60 mV. (A) typical example showing that the inclusion of 100 μmol/L IP_3 in the pipette solution renders the current hardly washable after the removal of the external Ca^{2+}. (B) typical trace showing that the inclusion of 100 μmol/L IP_4 fails to restore the irreversibility of the current. (C) comparison of the recovery rate of the OAG-induced current with or without IP_3, IP_4, IP_2 (100 μmol/L) in the pipette solution (*n*=6–9). ${}^{b}P<0.05$. ns, no statistical significance.

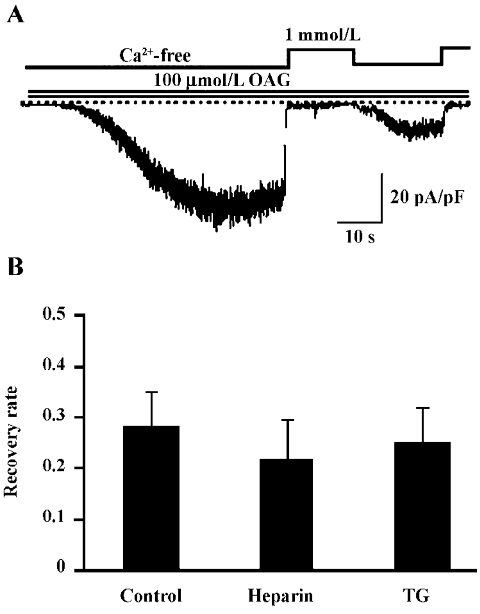

Figure 6. Independence of IP_3R in mediating the effect of IP_3, cwc recording, HP =–60 mV. (A) typical trace showing that the inclusion of heparin (0.5 mg/mL) in the pipette solution did not eliminate the poor reversibility of Ca^{2+} inhibition caused by IP_3. (B) summary of the recovery rate of Ca^{2+} inhibition with or without (control) heparin and TG (2 μmol/L) treatment, respectively (*n*=6–10).

Discussion

Inactivation/desensitization is the gating process in which channels become non-conductive to intensive and/or prolonged activation stimuli. The biological significance of this process is to restrict the excessive ion flux and maintain the pertinence of chemical and electrical signals. A multitude of factors are known to participate in the inactivation process, such as membrane potential, ions, calmodulin, lipids, and kinases. Amongst these, the Ca^{2+}-dependent inactivation is the common feature observed for many Ca^{2+}-permeable cation channels, including L-type Ca^{2+} channels, the NMDA receptor, and TRP channels, where PKC often plays a pivotal role[7,21–23]. The TRPC7 channel is subject to the negative regulation of PKC according to Shi *et al*[15], where their conclusion was made by comparison of the current density of I_{TRPC7} with either the activator or inhibitor of PKC in the pipette solution that contains different concentrations of Ca^{2+} (Figure 9C, right column), but the source of Ca^{2+} (Ca^{2+} entry or Ca^{2+} release) that activates PKC remains elusive. One notable finding of the present study is that Ca^{2+} entry through the activated TRPC7 channel can activate PKC even during a brief application of a physiological concentration of $Ca^{2+}{}_o$ after the full activation of the channel (Figure 1).

There is a common CaM/IP_3 receptor (IP_3R) binding domain on the C terminus of all TRPC subfamily members[24]. It is proposed that IP_3R and CaM competitively bind to this domain, thereby regulating the channel activity[25]. According to Shi *et al*[15], Ca^{2+}/CaM is a prerequisite for the activation of TRPC6 channels, whereas it exerts tonic inhibition on TRPC7 channels, as evidenced by the increased macroscopic TRPC7 current (by approximately 20%), partial relief from $Ca^{2+}{}_o$-induced inhibition, and the rightward shift of Ca^{2+} sensitivity of single TRPC7 channels by treatment with calmidazolium (a specific CaM inhibitor) or co-expression of Ca^{2+}-insensitive mutant CaM. However, the sustained inhibition of I_{TRPC7} presented after the washout of $Ca^{2+}{}_o$, which was not examined in detail in this study, could not be relieved by inhibiting CaM. As clearly demonstrated in Figure 1F, the co-expression of mutCaM failed to recover the TRPC7 channels from $Ca^{2+}{}_o$-induced inhibition, which was instead reverted by an organic PKC inhibitor calphostin C, the PKC-specific inhibitory peptide, or vigorous intracellular Ca^{2+} buffering (Figures 1,2). These results strongly suggest that Ca^{2+} entering through the activated TRPC7 channel during receptor stimulation can give rise to 2 different modes of the channel inactivation, one being rapid and dependent on CaM, while the other persisting via the activation of PKC.

With respect to the mechanism responsible for the PKC-mediated inhibition of the TRPC7 channel, a noteworthy finding is the differential reversibility from the $Ca^{2+}{}_o$-induced inhibition of CCh and OAG-induced currents (Figures 1A–4A). Despite both agonists being capable of evoking I_{TRPC7}, recovery from the $Ca^{2+}{}_o$-induced inhibition was much faster and much more complete with OAG than CCh. However, after the intracellular perfusion of IP_3, the recovery of OAG-induced I_{TRPC7} from the inhibition became as comparably slow as CCh used to evoke the current (Figure 5). The action of IP_3 seemed to be specific and direct since its effect was not mimicked by its 2 main metabolites, IP_4 and IP_2. Furthermore, this effect of IP_3 was not prevented by heparin or TG (Figure 6). Considering that both OAG and CCh can activate PKC, the main difference must reside on the actions of IP_3, which is only produced in response to CCh stimulation. Therefore, one plausible picture that we envisage is that Ca^{2+} entry through the activated TRPC7 channel facilitates the activation of PKC with DAG, and consequent phosphorylation of the channels by the activated PKC leads to their inactivation. IP_3 may be necessary to stabilize this “inactivation” state, for example, by inhibiting the dephosphorylation of the channel protein via phosphatases, thereby causing a retarded recovery from the inactivation. Importantly, this stabilization seems to occur through an as yet unknown IP_3R-independent mechanism. A similar but oppositely-operating synergism between DAG and IP_3 has been observed for the activation process of the recombinant TRPC7 channel[15] and a native TRPC6-like channel, α_1-adrenoceptor cation channel[26], where IP_3 potentiates the extent of channel activation evoked by DAG in a heparin-insensitive manner[26]. However, IP_3 has also been reported to activate the TRPC7 channel in an IP_3R-dependent manner in chicken DT40 cells, when the channel is expressed at a low level[27]. Such complex aspects of the TRPC7 channel activation and inactivation make it difficult to examine the mechanisms underlying it separately. In addition, since the effect of IP_3 on targets other than IP_3R is just recently emerging[28], there is no evidence yet available for exploring the molecular basis for the extra-IP_3R actions of IP_3 on the TRPC7 channels. It would thus be better in future to determine whether these putative IP_3-dependent regulatory sites exist on TRPC channels per se, auxiliary proteins, or both, and then to elucidate their molecular identifications. It may also be worthwhile to explore the mechanism underlying Ca^{2+}-induced inhibition observed in this study, by means of mutagenesis of a PKC phosphorylation motif which has commonly been identified on the C-terminus of theTRPC3/6/7 subfamily (Ser^{712} in TRPC3)[29].

In conclusion, the present study has revealed a novel mechanism for the negative regulation of the TRPC7 channels by intracellular Ca^{2+} via PKC activation, which requires the cooperative action of DAG and IP_3. This mechanism would act as an effective brake against the excessive or prolonged activation of TRPC7 during receptor stimulation, and may be important to regulate a number of pathophysiological processes, such as apoptosis, where the TRPC7 channel likely plays a certain role[30,31]. Further studies, such as a phosphorylation analysis of the activated channel and molecular elucidation of the cooperation of DAG and IP_3 by extensive screening of responsible proteins, may help to promote our understanding about the inactivation mechanism of TRPC7.

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References

1 Minke B, Cook B. TRP channel proteins and signal transduction. Physiol Rev 2002; 82: 429–72.

2 Montell C. Physiology, phylogeny, and functions of the TRP superfamily of cation channels. Sci STKE. www.stke.org/cgi/content/full/OC_sigtrans;2001/90/re1, 1–17.

3 Clapham DE, Julius D, Montell C, Schultz G. International union of pharmacology. XLIX. Nomenclature and structure-function relationships of transient receptor potential channels Pharmacol Rev 2005; 57: 427–50.

4 Owsianik G, Talavera K, Voets T, Nilius B. Permeation and selectivity of TRP channels. Annu Rev Physiol 2006; 68: 685–717.

5 Lacinova L, Hofmann F. Ca^{2+}- and voltage-dependent inactivation of the expressed L-type Ca(v)1.2 calcium channel. Arch Biochem Biophys 2005; 437: 42–50.

6 Stotz SC, Zamponi GW. Structural determinants of fast inactivation of high voltage activated Ca^{2+} channels. Trends Neurosci 2001; 24: 176–81.

7 Cens T, Rousset M, Leyris JP, Fesquet P, Charnet P. Voltage- and calcium-dependent inactivation in high voltage-gated Ca^{2+} channels. Prog Bioph Mol Biol 2006; 90: 104–17.

8 Findlay I. Physiological modulation of inactivation of L-type Ca^{2+} channels: one switch. J Physiol 2003; 554: 275–83.

9 Caterina MJ, Schumacher MA, Tominaga M, Rosen TA, Levine JD, Julius D. The capsaicin receptor: a heat activated ion channel in the pain pathway. Nature 1997; 389: 816–24.

10 Szallasi A, Cruz F, Geppetti P. TRPV1: a therapeutic target for novel analgesic drugs? Trends Mol Med 2006; 12: 545–54.

11 Tominaga M, Tominaga T. Structure and function of TRPV1. Pflugers Arch 2005; 451: 143–50.

12 Numazaki M, Tominaga T, Takeuchi K, Murayama N, Toyooka H, Tominaga M. Structural determinant of TRPV1 desensitization interacts with calmodulin. Proc Natl Acad Sci USA 2003; 100: 8002–6.

13 Docherty RJ, Yeats JC, Bevan S, Boddeke HW. Inhibition of calcineurin inhibits the desensitization of capsaicin-evoked currents in cultured dorsal root ganglion neurons from adult rats. Pflugers Arch 1996; 431: 828–37.

14 Bhave G, Zhu W, Wang H, Brasier DJ, Oxford GS, Gereau RW. cAMP-dependent protein kinase regulates desensitization of the capsaicin receptor (VR1) by direct phosphorylation. Neuron 2002; 35: 721–31.

15 Shi J, Mori E, Mori Y, Mori M, Li J, Ito Y, *et al.* Multiple regulation by calcium of murine homologues of transient receptor potential proteins TRPC6 and TRPC7 expressed in HEK293 cells. J Physiol 2004; 561: 415–32.

16 Gainetdinov RR, Premont RT, Bohn LM, Lefkowitz RJ, Caron MG. Desensitization of G protein-coupled receptors and neuronal functions. Ann Rev Neurosci 2004; 27: 107–44.

17 Swope SL, Moss SJ, Raymond LA, Huganir RL. Regulation of ligand-gated ion channels by protein phosphorylation. Adv Second Messenger Phosphoprotein Res 1999; 33: 49–78.

18 Kamp TJ, Hell JW. Regulation of cardiac L-type calcium channels by protein kinase A and protein kinase C. Circ Res 2000; 87: 1095–102.

19 Albert AP, Large WA. Signal transduction pathways and gating mechanisms of native TRP-like cation channels in vascular myocytes. J Physiol 2006; 570: 45–51.

20 Hardie RC. TRP channels and lipids: from Drosophila to mammalian physiology. J Physiol 2007; 578: 9–24.

21 Zhang S, Ehlers MD, Bernhardt JP, Su CT, Huganir RL. Calmodulin mediates calcium-dependent inactivation of N-methyl-d-aspartate receptors. Neuron 1998; 21: 443–53.

22 Krupp JJ, Vissel B, Thomas CG, Heinemann SF, Westbrook GL. Interactions of calmodulin and alpha-actinin with the NR1 subunit modulate calcium-dependent inactivation of NMDA receptors. J Neurosci 1999; 19: 1165–78.

23 Nilius B, Voets T. Diversity of TRP channel activation. Novartis Found Symp 2004; 258: 140–9.

24 Tang J, Lin Y, Zhang Z, Tikunova S, Birnbaumer L, Zhu MX. Identification of common binding sites for calmodulin and inositol 1,4,5-trisphosphate receptors on the carboxyl termini of trp channels. J Biol Chem 2001; 276: 21 303–10.

25 Zhang Z, Tang J, Tikunova S, Johnson JD, Chen Z, Qin N, *et al.* Activation of Trp3 by inositol 1,4,5-trisphosphate receptors through displacement of inhibitory calmodulin from a common binding domain. Proc Natl Acad Sci USA 2001; 98: 3168–73.

26 Albert AP, Large WA. Synergism between inositol phosphates and diacylglycerol on native TRPC6-like channels in rabbit portal vein myocytes. J Physiol 2003; 552: 789–95.

27 Vazquez G, Bird GS, Mori Y, Putney JW Jr. Native TRPC7 channel activation by an inosital trisphosphate receptor-dependent mechanism. J Biol Chem 2006; 282: 25 250–8.

28 Kanematsu T, Takeuchi H, Terunuma M, Hirata M. PRIR, a novel Ins(1,4,5)P3 binding protein, functional significance in Ca^{2+} signaling and extension to neuroscience and beyond. Mol Cells 2005; 20: 305–14.

29 Trebak M, Hempel N, Wedel BJ, Smyth JT, Bird GS, Putney JW Jr. Negative regulation of TRPC3 channels by protein kinase C-mediated phosphorylation of serine 712. Mol Pharmacol 2005; 67: 558–63.

30 Foller M, Kasinathan RS, Duranton C, Wieder T, Huber SM, Lang F. PGE2-induced apoptotic cell death in K562 human leukaemia cells. Cell Physiol Biochem 2006; 17: 201–10.

31 Satoh S, Tanaka H, Ueda Y, Oyama J, Sugano M, Sumimoto H, *et al.* Transient receptor potential (TRP) protein 7 acts as a G protein-activated Ca^{2+} channel mediating angiotensin II-induced myocardial apoptosis. Mol Cell Biochem 2006; 294: 205–15.