

## Full-length article

# Synergistic actions of diacylglycerol and inositol 1,4,5 trisphosphate for Ca<sup>2+</sup>-dependent inactivation of TRPC7 channel<sup>1</sup>

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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<sup>2+</sup>-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<sup>2+</sup>-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<sup>2+</sup> into the bath, and recovery from this inhibition was only partial after the Ca<sup>2+</sup> removal, unless vigorous intracellular Ca<sup>2+</sup> buffering with 10 mmol/L 1,2 bis(2-aminophenoxy)ethane-*N,N,N',N'*-tetraacetic acid (BAPTA) (plus 4 mmol/L Ca<sup>2+</sup>) 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<sup>2+</sup> removal. Interestingly, the inclusion of inositol 1,4,5 trisphosphate (IP<sub>3</sub>; 100 μmol/L) in the patch pipette rendered the OAG-induced current susceptible to the persistent Ca<sup>2+</sup>-mediated inhibition independent of the IP<sub>3</sub> receptor in the majority of the tested cells, as evidenced by the inability of heparin and thapsigargin in reversing the effect of IP<sub>3</sub>. **Conclusion:** The present results suggest that Ca<sup>2+</sup> entry via the activated TRPC7 channel plays a critical role in inactivating the channel where the cooperative actions of DAG and IP<sub>3</sub> are essentially involved.

## Introduction

Transient receptor potential (TRP) is a vast gene superfamily which contains more than 30 isoforms ranging from yeast to mammals<sup>[1–3]</sup>. 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<sup>[4]</sup>. 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<sup>[4]</sup>.

The mammalian TRP superfamily is composed of 6

subfamilies: the canonical TRP (TRPC), the vanilloid receptor TRP (TRPV), the melastatin or long TRP, the mucopolins, the polycystins, and ankyrin transmembrane protein 1<sup>[3]</sup>. 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<sup>2+</sup> entry observed in many non-excitable tissues<sup>[1–3]</sup>. 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<sup>[1–3]</sup>. TRPC7 is the latest cloned TRPC isoform and least elucidated for its channel regulation.

Ca<sup>2+</sup> entry through the channel pore plays essential roles

in the regulation of many  $\text{Ca}^{2+}$  permeable channels. It is well known that the L-type voltage-gated  $\text{Ca}^{2+}$  current decays much faster in the presence of extracellular  $\text{Ca}^{2+}$  than its surrogate  $\text{Ba}^{2+}$ <sup>[5]</sup>. The ubiquitous  $\text{Ca}^{2+}$  sensor calmodulin (CaM) tethered to the proximal segment of the cytoplasmic C-terminus of the  $\alpha_1\text{C}$  subunit, upon entry of  $\text{Ca}^{2+}$ , is thought to reorientate to cause conformational rearrangements<sup>[6–8]</sup>. 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<sup>[9,10]</sup>. This non-selective cationic channel undergoes rapid inactivation or desensitization during the agonist application in  $\text{Ca}^{2+}$ -containing bathing solution<sup>[11]</sup>, but the underlying mechanism is controversial. While Numazaki *et al*<sup>[12]</sup> pointed out that  $\text{Ca}^{2+}$ /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<sup>[13]</sup>. In addition, Bhave *et al*<sup>[14]</sup> showed that cAMP dependent phosphorylation antagonizes the desensitization leading to the resensitization of the channel.

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

## Material and methods

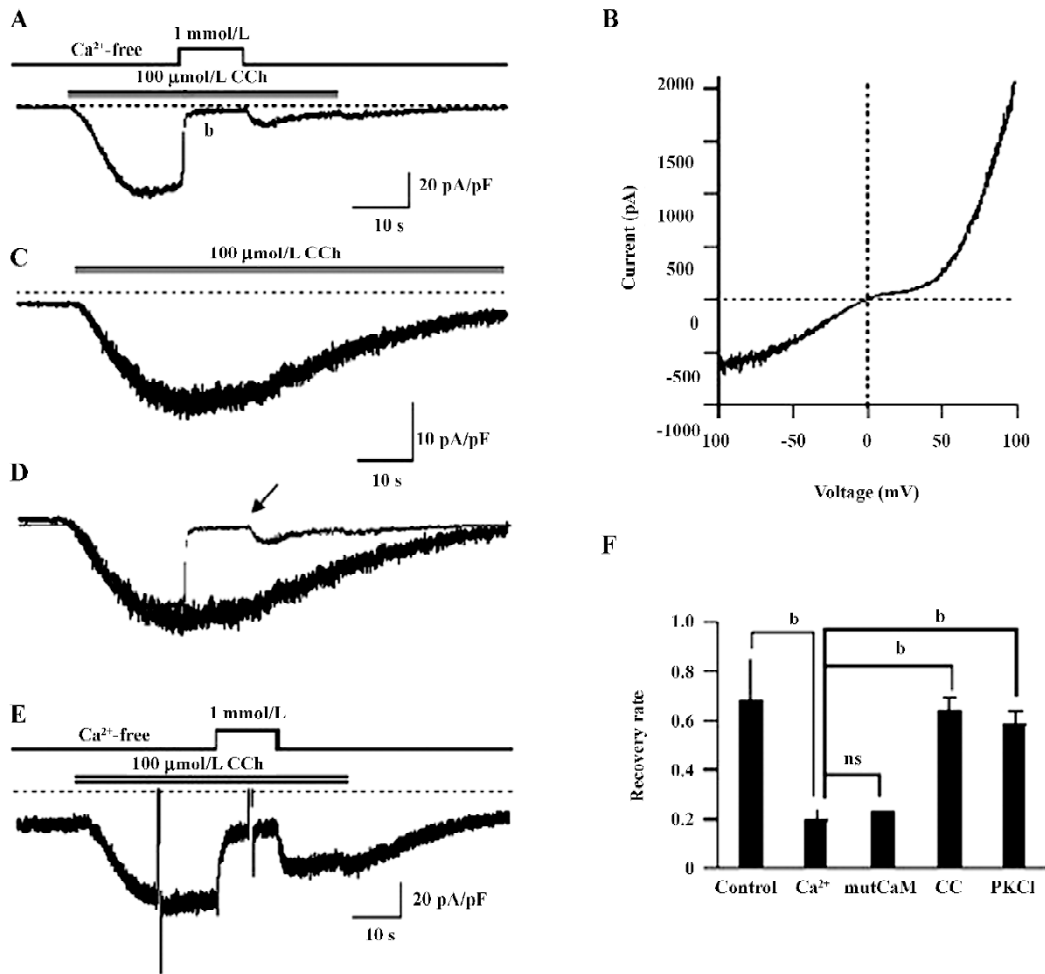
**Cell culture and transfection** Human embryonic kidney 293 (HEK293) cells were cultured in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum. For transfection, the cells were reseeded in a 35 mm culture dish and were allowed to grow to a 40%–50% confluency. The cells were then transfected with a mixture of 2  $\mu\text{g}$  plasmid vector (pCI-neo) incorporating murine TRPC7 DNA and 0.4  $\mu\text{g}$  pCI-neo- $\pi\text{H3-CD8}$  (cDNA of the T cell antigen CD8) with the aid of 20  $\mu\text{L}$  of the transfection reagent SuperFect (Qiagen, Germany). In some cases (Figure 1F), 2  $\mu\text{g}$  plasmid DNA for a  $\text{Ca}^{2+}$ -insensitive mutant calmodulin (replacement

with alanine of the aspartate residues 21, 57, 94, and 130 in 4 E–F hands of calmodulin) was cotransfected. Approximately 24 h later, the cells were trypsinized and reseeded onto coverslips precoated with 100  $\mu\text{mol/L}$  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 M $\Omega$  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<sub>2</sub>, 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<sub>2</sub>, 0.1 BAPTA (1,2 bis(2-aminophenoxy)ethane-*N,N,N',N'*-tetraacetic acid) or EGTA (ethylene glycol-bis(2-aminoethylether)-*N,N,N',N'*-tetraacetic acid), 0.04 CaCl<sub>2</sub>, 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<sub>2</sub>, 10 BAPTA (or EGTA), 4 CaCl<sub>2</sub>, 10 HEPES, 2 ATP, 0.1 GTP, and 10 glucose (adjusted to pH 7.2 with Tris base). In total, 100  $\mu\text{mol/L}$  GTP $\gamma\text{S}$ , 100  $\mu\text{mol/L}$  inositol 1,4,5 trisphosphate (IP<sub>3</sub>), inositol 1,3,4,5 tetrakisphosphate (IP<sub>4</sub>), or 1,4 bisphosphate (IP<sub>2</sub>) 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<sub>2</sub>, 1 EGTA, 10 HEPES, and 10 glucose (adjusted to pH 7.4 with Tris base). For the  $\text{Ca}^{2+}$ -containing external solution, 1 mmol/L CaCl<sub>2</sub> was added with the omission of EGTA.

**Drugs** 1-oleoyl-2-acetyl-sn-glycerol (OAG), GTP $\gamma\text{S}$ ,



**Figure 1.** Ca<sup>2+</sup>-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<sup>2+</sup> 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<sup>2+</sup>-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<sup>2+</sup> for 10 s. (E) typical TRPC7 trace recorded with 5 μM PKC–IP<sub>19–36</sub> in the pipette solution (0.04 mmol/L Ca<sup>2+</sup> 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<sup>2+</sup> 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<sub>19–36</sub> (PKCI) treatments, and mutCaM co-expression, respectively ( $n=6–8$ ). <sup>b</sup> $P<0.05$ . ns, no statistical significance

heparin, thapsigargin (TG), and protein kinase C (PKC) inhibitory peptide (PKC–IP<sub>19–36</sub>) were purchased from Calbiochem (La Jolla, CA, USA); IP<sub>2</sub>, IP<sub>4</sub>, 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

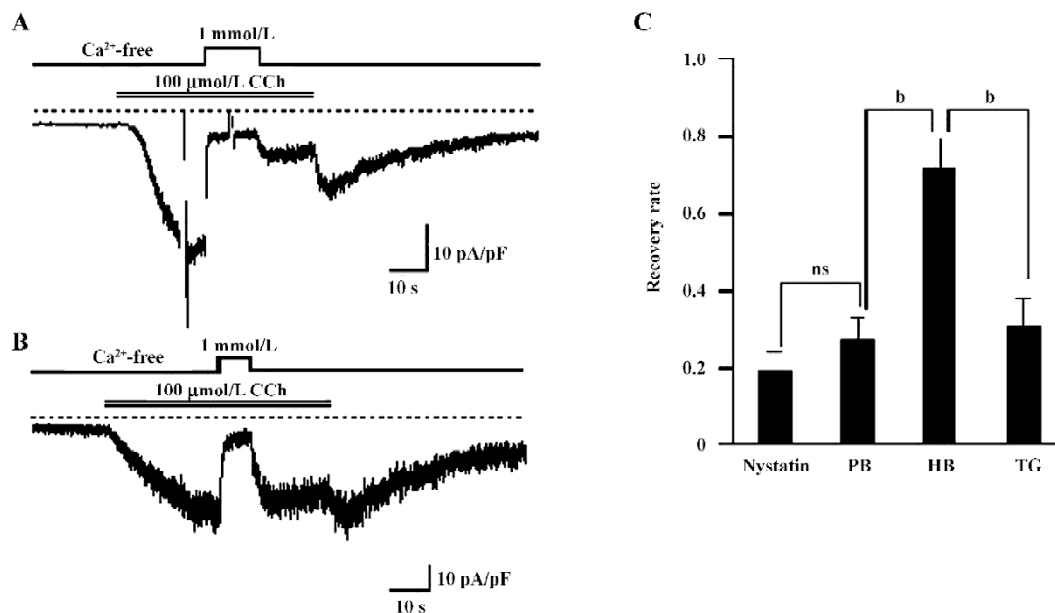
**Ca<sup>2+</sup>-dependent inactivation of TRPC7 current** HEK293 cells overexpressing mouse TRPC7 exhibited large inward currents following the addition of 100 μmol/L CCh (35.7±5.2 pA/pF,  $n=21$ ; Figure 1A). The current–voltage (*I*-*V*) curve showed a double-rectifying property with a reversal potential near 0 (Figure 1B), which represents the typical *I*-*V* relationship of the TRPC3/6/7 subfamily<sup>[1]</sup>. Little current was

recorded with empty vector-expressing cells ( $0.89 \pm 0.61$  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  $Ca^{2+}_o$  after full activation by CCh caused a rapid decline of  $I_{TRPC7}$  (Figure 1A). According to Shi *et al*<sup>[15]</sup>, 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  $Ca^{2+}_o$  was long lasting since only a small fraction of the current was recovered after  $Ca^{2+}_o$  was washed out (Figure 1A). Superimposition of traces with (Figure 1A; indicated by an arrow) and without (Figure 1C) transient  $Ca^{2+}_o$  application clearly demonstrates that  $Ca^{2+}_o$  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  $Ca^{2+}_o$  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<sup>[15]</sup>, 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 PKC-IP<sub>19-36</sub> (5  $\mu$ mol/L) in the pipette solution antagonized the persistent  $Ca^{2+}$  inhibition and accordingly raised the recovery rate, respectively (Figure 1E, 1F).

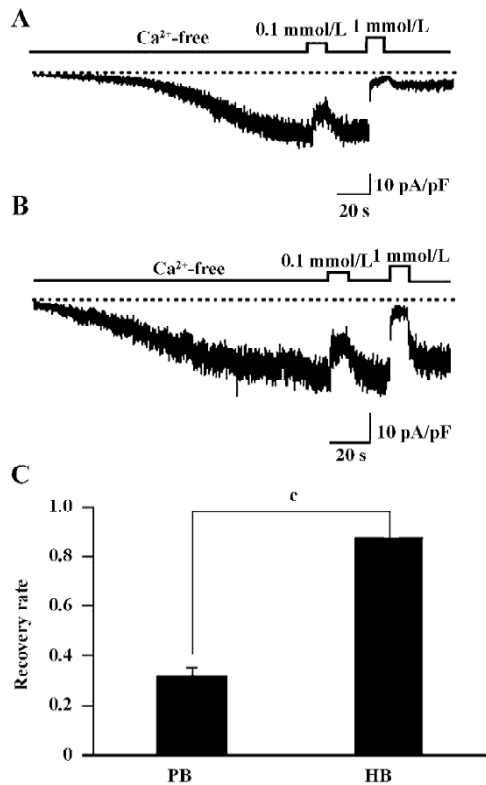
**Ca<sup>2+</sup> entry is involved in the Ca<sup>2+</sup>-dependent inactivation of TRPC7** To determine whether the  $Ca^{2+}$ -dependent inactivation (CDI) of  $I_{TRPC7}$  is due to  $Ca^{2+}$  influx, we next employed 2 different pipette solutions with weak and high-buffering capacities for  $Ca^{2+}$ . 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  $Ca^{2+}_o$ -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 ( $n=8-12$ ). <sup>a</sup> $P < 0.05$ . ns, no statistical significance. <sup>b</sup> $P < 0.05$ .

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

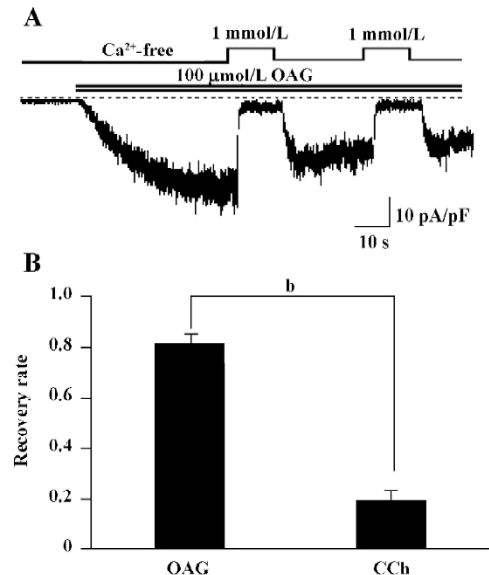
**GTP $\gamma$ S-induced TRPC7 current shows similar CDI** It is well established that  $\text{Ca}^{2+}$  affects the inactivation and/or desensitization of channels through acting on both the G protein-coupled receptor and its downstream effectors-mediated pathways<sup>[16]</sup>. To delineate the site of actions of  $\text{Ca}^{2+}$  on TRPC7, GTP $\gamma$ S (100  $\mu\text{mol/L}$ ) was included in the pipette solution. Dialysis of the cell with GTP $\gamma$ S resulted in the slow development of an inward current comparable to that evoked by CCh (Figures 3A,3B). The addition of  $\text{Ca}^{2+}_o$  caused a dose-dependent inhibition of the current. Perfusion of 0.1  $\text{mmol/L}$   $\text{Ca}^{2+}_o$  mildly inhibited the current, which was quickly



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

reversed by the removal of  $\text{Ca}^{2+}$  (Figures 3A,3B). In contrast, with 1  $\text{mmol/L}$   $\text{Ca}^{2+}_o$ , 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 GTP $\gamma$ S directly activates the G protein and bypasses the receptor stimulation, these results suggest that the CDI of  $I_{\text{TRPC7}}$  does not involve a process upstream of G protein activation.

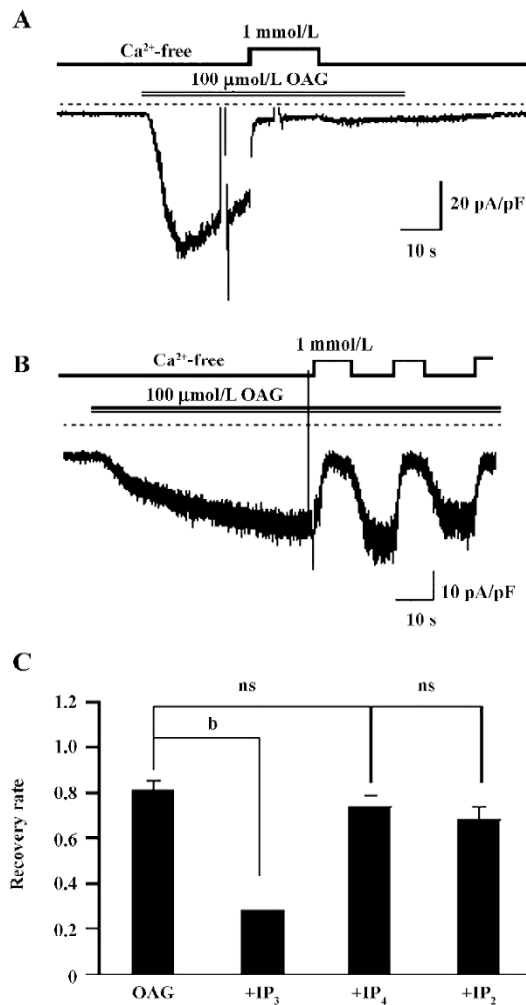
**OAG-induced TRPC7 current exhibits no obvious CDI** The members of the TRPC3/6/7 subfamily are known to be activated by 1 of the major phosphatidylinositide metabolites, diacylglycerol (DAG). This action is thought to be direct on the channel protein, independent of PKC or IP<sub>3</sub><sup>[20]</sup>. Thus, the use of DAG instead of CCh may enable us to explore the above-mentioned actions of  $\text{Ca}^{2+}$  in simpler conditions in which the role of IP<sub>3</sub> can be excluded. For this purpose, we used a membrane-permeable analog of DAG, OAG. Perfusion of 100  $\mu\text{mol/L}$  OAG induced an inward current with the hallmarks of  $I_{\text{TRPC7}}$  (ie the reversal potential near 0; the ohmic current-voltage relationship with a slight double-rectifying property; blockade by millimolar extracellular  $\text{Ca}^{2+}$ ; data not shown). The sustained inhibition after application of 1  $\text{mmol/L}$   $\text{Ca}^{2+}_o$  seen for CCh- and GTP $\gamma$ S-induced  $I_{\text{TRPC7}}$  was almost completely abolished in OAG-induced  $I_{\text{TRPC7}}$  (Figure 4A). The



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

recovery from the Ca<sup>2+</sup>-dependent inhibition was much more complete (80%) in OAG-induced I<sub>TRPC7</sub> as compared with that observed with CCh (circa 20%; Figure 4B).

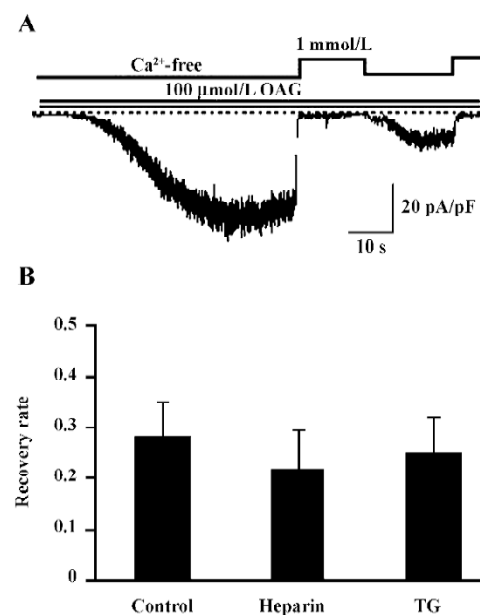
**IP<sub>3</sub> regresses the CDI of OAG induced currents** The most important difference in activating the TRP channels between DAG and GTPγS/CCh is no involvement of the IP<sub>3</sub> pathway in the former. Therefore, 100 μmol/L (the commonly-used full concentration) IP<sub>3</sub> was added into the pipette solution to see whether the Ca<sup>2+</sup>-induced persisting inhibition of I<sub>TRPC7</sub> could be restored. As illustrated in Figure 5A and



**Figure 5.** IP<sub>3</sub> regressed the irreversibility of the Ca<sup>2+</sup>-mediated inhibition of the OAG-induced current, cwc recording, HP = -60 mV. (A) typical example showing that the inclusion of 100 μmol/L IP<sub>3</sub> in the pipette solution renders the current hardly washable after the removal of the external Ca<sup>2+</sup>. (B) typical trace showing that the inclusion of 100 μmol/L IP<sub>4</sub> fails to restore the irreversibility of the current. (C) comparison of the recovery rate of the OAG-induced current with or without IP<sub>3</sub>, IP<sub>4</sub>, IP<sub>2</sub> (100 μmol/L) in the pipette solution (n=6-9). <sup>b</sup>P<0.05. ns, no statistical significance.

summarized in Figure 5C, in more than half of the tested cells (6 of 11 cells), the intrapipette inclusion of IP<sub>3</sub> caused a prominent, irreversible Ca<sup>2+</sup> inhibition of I<sub>TRPC7</sub> evoked by OAG. The effect of IP<sub>3</sub> appeared to be direct since no such regressions of CDI were observed when other metabolites of inositol phosphate, such as IP<sub>4</sub> and IP<sub>2</sub>, were included in the pipette solution (Figure 5B,5C).

**Effect of IP<sub>3</sub> is IP<sub>3</sub> receptor independent** The main biological function of IP<sub>3</sub> is to cause Ca<sup>2+</sup> release through binding to the IP<sub>3</sub> receptor (IP<sub>3</sub>R) on the ER. To examine whether the recovery of CDI caused by IP<sub>3</sub> is due to the activation of IP<sub>3</sub>R, heparin, a potent IP<sub>3</sub>R antagonist (0.5 mg/mL), was included in the pipette solution. Interestingly, this compound failed to eliminate the effect of IP<sub>3</sub> to restore the Ca<sup>2+</sup>-dependent inhibition in OAG-induced I<sub>TRPC7</sub> (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<sub>3</sub>R would be, if any, only minor in mediating the effect of IP<sub>3</sub>.



**Figure 6.** Independence of IP<sub>3</sub>R in mediating the effect of IP<sub>3</sub>, 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<sup>2+</sup> inhibition caused by IP<sub>3</sub>. (B) summary of the recovery rate of the recovery rate of the OAG-induced current 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  $\text{Ca}^{2+}$ -dependent inactivation is the common feature observed for many  $\text{Ca}^{2+}$ -permeable cation channels, including L-type  $\text{Ca}^{2+}$  channels, the NMDA receptor, and TRP channels, where PKC often plays a pivotal role<sup>[7,21–23]</sup>. The TRPC7 channel is subject to the negative regulation of PKC according to Shi *et al*<sup>[15]</sup>, where their conclusion was made by comparison of the current density of  $I_{\text{TRPC7}}$  with either the activator or inhibitor of PKC in the pipette solution that contains different concentrations of  $\text{Ca}^{2+}$  (Figure 9C, right column), but the source of  $\text{Ca}^{2+}$  ( $\text{Ca}^{2+}$  entry or  $\text{Ca}^{2+}$  release) that activates PKC remains elusive. One notable finding of the present study is that  $\text{Ca}^{2+}$  entry through the activated TRPC7 channel can activate PKC even during a brief application of a physiological concentration of  $\text{Ca}^{2+}_o$  after the full activation of the channel (Figure 1).

There is a common CaM/ $\text{IP}_3$  receptor ( $\text{IP}_3\text{R}$ ) binding domain on the C terminus of all TRPC subfamily members<sup>[24]</sup>. It is proposed that  $\text{IP}_3\text{R}$  and CaM competitively bind to this domain, thereby regulating the channel activity<sup>[25]</sup>. According to Shi *et al*<sup>[15]</sup>,  $\text{Ca}^{2+}/\text{CaM}$  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  $\text{Ca}^{2+}_o$ -induced inhibition, and the rightward shift of  $\text{Ca}^{2+}$  sensitivity of single TRPC7 channels by treatment with calmidazolium (a specific CaM inhibitor) or co-expression of  $\text{Ca}^{2+}$ -insensitive mutant CaM. However, the sustained inhibition of  $I_{\text{TRPC7}}$  presented after the washout of  $\text{Ca}^{2+}_o$ , 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  $\text{Ca}^{2+}_o$ -induced inhibition, which was instead reverted by an organic PKC inhibitor calphostin C, the PKC-specific inhibitory peptide, or vigorous intracellular  $\text{Ca}^{2+}$  buffering (Figures 1,2). These results strongly suggest that  $\text{Ca}^{2+}$  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  $\text{Ca}^{2+}_o$ -induced inhibition of CCh and OAG-induced currents (Figures 1A–

4A). Despite both agonists being capable of evoking  $I_{\text{TRPC7}}$ , recovery from the  $\text{Ca}^{2+}_o$ -induced inhibition was much faster and much more complete with OAG than CCh. However, after the intracellular perfusion of  $\text{IP}_3$ , the recovery of OAG-induced  $I_{\text{TRPC7}}$  from the inhibition became as comparably slow as CCh used to evoke the current (Figure 5). The action of  $\text{IP}_3$  seemed to be specific and direct since its effect was not mimicked by its 2 main metabolites,  $\text{IP}_4$  and  $\text{IP}_2$ . Furthermore, this effect of  $\text{IP}_3$  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  $\text{IP}_3$ , which is only produced in response to CCh stimulation. Therefore, one plausible picture that we envisage is that  $\text{Ca}^{2+}$  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.  $\text{IP}_3$  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  $\text{IP}_3\text{R}$ -independent mechanism. A similar but oppositely-operating synergism between DAG and  $\text{IP}_3$  has been observed for the activation process of the recombinant TRPC7 channel<sup>[15]</sup> and a native TRPC6-like channel,  $\alpha_1$ -adrenoceptor cation channel<sup>[26]</sup>, where  $\text{IP}_3$  potentiates the extent of channel activation evoked by DAG in a heparin-insensitive manner<sup>[26]</sup>. However,  $\text{IP}_3$  has also been reported to activate the TRPC7 channel in an  $\text{IP}_3\text{R}$ -dependent manner in chicken DT40 cells, when the channel is expressed at a low level<sup>[27]</sup>. 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  $\text{IP}_3$  on targets other than  $\text{IP}_3\text{R}$  is just recently emerging<sup>[28]</sup>, there is no evidence yet available for exploring the molecular basis for the extra- $\text{IP}_3\text{R}$  actions of  $\text{IP}_3$  on the TRPC7 channels. It would thus be better in future to determine whether these putative  $\text{IP}_3$ -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  $\text{Ca}^{2+}$ -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 the TRPC3/6/7 subfamily (Ser<sup>712</sup> in TRPC3)<sup>[29]</sup>.

In conclusion, the present study has revealed a novel mechanism for the negative regulation of the TRPC7 channels by intracellular  $\text{Ca}^{2+}$  via PKC activation, which requires the cooperative action of DAG and  $\text{IP}_3$ . 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<sup>[30,31]</sup>. Further studies, such as a phosphorylation analysis of the activated channel and molecular elucidation of the cooperation of DAG and IP<sub>3</sub> by extensive screening of responsible proteins, may help to promote our understanding about the inactivation mechanism of TRPC7.

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