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Protein Kinase C Can Inhibit TRPC3 Channels Indirectly Via Stimulating Protein Kinase G

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There are two known phosphorylation-mediated inactivation mechanisms for TRPC3 channels. Protein kinase G (PKG) inactivates TRPC3 by direct phosphorylation on Thr-11 and Ser-263 of the TRPC3 proteins, and protein kinase C (PKC) inactivates TRPC3 by phosphorylation on Ser-712. In the present study, we explored the relationship between these two inactivation mechanisms of TRPC3. HEK cells were first stably transfected with a PKG-expressing construct and then transiently transfected with a TRPC3-expressing construct. Addition of 1-oleoyl-2-acetyl-*sn*-glycerol (OAG), a membrane-permeant analog of diacylglycerol (DAG), elicited a TRPC3-mediated $[Ca^{2+}]_i$ rise in these cells. This OAG-induced rise in $[Ca^{2+}]_i$ could be inhibited by phorbol 12-myristate 13-acetate (PMA), an agonist for PKC, in a dose-dependent manner. Importantly, point mutations at two PKG phosphorylation sites (T11A-S263Q) of TRPC3 markedly reduced the PMA inhibition. Furthermore, inhibition of PKG activity by KT5823 (1 μM) or H8 (10 μM) greatly reduced the PMA inhibition of TRPC3. These data strongly suggest that the inhibitory action of PKC on TRPC3 is partly mediated through PKG in these PKG-overexpressing cells. The importance of this scheme was also tested in vascular endothelial cells, in which PKG plays a pivotal functional role. In these cells, OAG-induced $[Ca^{2+}]_i$ rise was inhibited by PMA, which activates PKC, and by 8-BrcGMP and S-nitroso-*N*-acetylpenicillamine (SNAP), both of which activate PKG. Importantly, the PMA inhibition on OAG-induced $[Ca^{2+}]_i$ rise was significantly reduced by PKG inhibitor KT5823 (1 μM) or DT-3 (500 nM), suggesting an important role of PKG in the PMA-induced inhibition of TRPC channels in native endothelial cells. J. Cell. Physiol. 207: 315–321, 2006. © 2005 Wiley-Liss, Inc.

The TRPC channel family consists of seven nonselective cation channels that, based on their sequence homology, can be divided into four subgroups TRPC1, TRPC4,5, TRPC3,6,7, and TRPC2 (Montell, 2005). Within the TRPC family, TRPC3 is one of the most extensively characterized channels (Putney et al., 2004). Expression of the channels can be found in a wide variety of cell types including vascular endothelial cells, neurons, airway smooth muscle cells, etc. (Minke and Cook, 2002; Li et al., 2003; Nilius et al., 2003). TRPC3 channels are activated via G-protein-coupled receptors, which stimulate phospholipase C (PLC) (Minke and Cook, 2002; Li et al., 2003). PLC enhances Ca^{2+} influx either via inositol 1,4,5-trisphosphate ($InsP_3$)-mediated Ca^{2+} store-dependent mechanism (Kiselyov et al., 1998) or via diacylglycerol (DAG)-mediated Ca^{2+} store-independent mechanism (Hofmann et al., 1999). These two mechanisms are not mutually exclusive. A recent report showed that, dependent upon the expression levels of TRPC3 proteins, TRPC3 could switch from the store-operated phenotype to the store-independent phenotype and vice versa (Vazquez et al., 2003). Ca^{2+} may also modulate the activity of TRPC channels (Zhang et al., 2001). A calmodulin binding site is located in the C-terminus of TRPC3 that overlaps with its $InsP_3R$-binding domain. Ca^{2+}-calmodulin may modulate the activity of TRPC3 under resting condition (Zhang et al., 2001).

Protein phosphorylation is another important means of TRPC3 regulation. For example, a nonreceptor tyrosine kinase Src is required for receptor- and DAG-mediated activation of TRPC3 (Vazquez et al., 2004). Another nonreceptor tyrosine kinases Fyn can directly phosphorylate TRPC6 and as a consequence increases the channel activity (Hisatsune et al., 2004).

Protein phosphorylation is not only important for activation mechanism, but is also important for inactivation mechanism of TRPC3 channels. Several studies showed that treatment of TRPC3-overexpressing cells with PMA, an activator for protein kinase C (PKC), completely abolished 1-oleoyl-2-acetyl-*sn*-glycerol (OAG)-induced activation of TRPC3 (Trebak et al., 2003; Venkatachalam et al., 2003); and this inhibition was reversed by GF 109203X, a highly selective and potent inhibitor of multiple PKC subtypes (Venkatachalam et al., 2003). These data provided evidence that the TRPC3 activity is negatively regulated by PKC. A recent study from Trebak et al. (2005) showed that the PKC inhibition is due to direct PKC phosphorylation on Ser-712 of TRPC3 proteins. Protein kinase G (PKG) is another kinase that is capable of downregulating the activity of TRPC3 (Kwan et al., 2004). Our recent study showed that PKG can phosphorylate TRPC3 at Thr-11 and Ser-263, and as a result, reduces the activity of TRPC3 (Kwan et al., 2004). Interestingly, Hou et al. (2003) recently showed that PKG itself is activated by PKC through direct phosphorylation at Thr-58 of PKG1α proteins, raising the possibility that PKC may inhibit TRPC3 indirectly by activating PKG.

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In the present study, we explored the role of PKG in PKC-induced inhibition of TRPC3. Our results showed that PKC can indeed inhibit TRPC3 indirectly by activating PKG, and that this pathway has an important contribution to the overall PKC inhibition on TRPC3, especially in those cells that have high PKG activity. In addition, we confirmed that PKC could also inhibit TRPC3 by direct phosphorylation on Ser-712. These studies provide a full mechanistic scheme for the PKC inhibition of TRPC3.

MATERIALS AND METHODS

Materials

Human embryonic kidney cell line HEK293 and rat aortic smooth muscle cell line A7r5 were from ATCC (Manassas, VA). Primary antibody against PKG (KAP-PK005) was from StressGen (Victoria, BC, Canada), and that against S239 phosphorylated VASP (16C2) was from Nanotools (Antikörpertechnik, Germany). Antibodies against TRPC3 and TRPC6 were from Alomone Laboratories (Jerusalem, Israel). Fluo3/acetoxymethyl ester (Fluo3/AM) and pluronic F127 were from Molecular Probes, Inc. (Eugene, OR). Lipofectamine 2000, blasticidin, soybean trypsin inhibitor, phenylmethylsulfonyl fluoride (PMSF), fetal bovine serum (FBS), and culture medium DMEM were from Invitrogen (Carlsbad, CA). 8-BrcGMP, KT5823, H8 (N-[2-(ethylamino)ethyl]-isoquinoline sulfonamide), DT-3 (RQIKIWFQNRRMKWKK-LRK$_5$H-amide), SNAP (S-nitroso-*N*-acetylpenicillamine), OAG, PMA, aprotinin, and leupeptin were from Calbiochem (San Diego, CA). Nonidet P-40, GF 109203X, and trypsin were from Sigma (St. Louis, MO).

Cell culture

HEK293 cells and A7r5 cells were cultured in DMEM supplemented with 10% FBS. Rat aortic endothelial cells were isolated as described elsewhere (Kwan et al., 2000) and they were cultured in RPMI1640 supplemented with 10% FBS. Cells were grown at 37°C in a 5% CO_2 humidified incubator.

Clones and point mutations

PKG1α gene (2,213 bp) and human TRPC3 (Genbank accession no: NM_003305) were cloned as described elsewhere (Kwan et al., 2004). Point mutations were introduced by cloning *TRPC3* gene into pALTER-MAX vector (Promega, Madison, WI), followed by annealation with specially-designed mutagenic oligonucleotides and DNA elongation (Altered Sites II Mammalian Mutagenesis System, Promega). Mutagenic oligonucleotides were GCATCACTGCCATGCGTCT for construct T11A, TTGCATTGCATTTGGAGCTTCCGATA for S263Q, and CTCCACCTTTCAGTCTAGTTCCTGCCCCAA-AATCATTTGTTTATTTCATCA for S712A (the mutated nucleotides are underlined). Wild-type TRPC3, mutant TRPC3, and *PKG1α* genes were each cloned into a mammalian expression vector pcDNA6 (Invitrogen). All clones were autosequenced by ABI310 autosequencer (Perkin-Elmer-Cetus, Wellesley, MA) to verify the authenticity of the constructs.

Transient and stable transfection

Unless stated otherwise, HEK293 cells used for all studies were stably transfected with PKG1α, thus named PKG-HEK. These cells were then transiently transfected with or without TRPC3 constructs. The procedures were as described elsewhere (Kwan et al., 2004). Briefly, *PKG1α* gene cloned in pcDNA6 was stably transfected into HEK293 cells with Lipofectamine 2000. Transfection was done with 4 μg of DNA plasmid and 6 μl of Lipofectamine 2000 in 200 μl Opti-MEM reduced serum medium in 6-well plates, which contained $\sim 6 \times 10^4$ cells per well. Stable PKG-containing cell line was established under the selection pressure of blasticidin. pcDNA6 contains blasticidin resistance gene for selection of transfected eukaryotic cells. Blasticidin is a powerful nucleoside antibiotic that inhibits protein synthesis in eukaryotic cells. All nontransfected cells are killed by blasticidin within 7–10 days. This powerful technique allowed us to produce a stable PKG-HEK cell line. In most experiments, PKG-HEK cells were then transiently transfected with either wild-type or mutant TRPC3 construct. Functional studies were performed 3 days post-transfection. About 80% of HEK293 cells were successfully transfected with TRPC3 as determined by control transfection experiments using either GFP-tagged TRPC3 or β-galactosidase construct.

$[Ca^{2+}]_i$ measurements

Cell preparation and Ca^{2+} measurement were performed as described previously (Kwan et al., 2000). Briefly, the cells were loaded with Fluo-3/AM in a normal physiological solution (NPSS) that contained 140 mM NaCl, 1 mM KCl, 1 mM $CaCl_2$, 1 mM $MgCl_2$, 10 mM glucose, and 5 mM HEPES, pH 7.4. Ca^{2+} influx was initiated by OAG (100 or 300 μM) application. The cells were pre-treated with or without 1 or 2 mM 8-BrcGMP, 1 μM KT5823, 10 μM H8, 1 nM–10 μM PMA, 100 nM–10 μM GF 109203X, 500 nM DT-3 for 5 min before the experiments. The fluorescence signals were monitored and recorded by an MRC-1000 laser scanning confocal imaging system and the data were analyzed using Metaflour software. Each experiment had 10–20 cells. Changes in $[Ca^{2+}]_i$ were displayed as a ratio of fluorescence relative to the intensity before the application of OAG (F1/F0).

SDS–PAGE and immunoblots

The procedures were as described elsewhere (Kwan et al., 2004). Briefly, whole-cell lysates were extracted with detergent extraction buffer, which contained 1% (v/v) Nonidet P-40, 150 mM NaCl, 20 mM Tris-HCl, pH 8.0, with addition of 1 μg/ml soybean trypsin inhibitor, 5 μg/ml leupeptin, 5 μg/ml aprotinin, and 100 μM PMSF. Eighty micrograms of extracted proteins were applied to each lane and separated on 8% SDS–PAGE gel. The proteins were then blotted onto a PVDF membrane (Millipore, Billerica, MA). The membrane was incubated at 4°C overnight with appropriate antibodies (dilution 1:200) in TBS buffer containing 0.1% Tween 20 and 5% nonfat dry milk. Immunodetection was accomplished with horseradish-conjugated secondary antibodies, followed by ECL detection system (Amersham, Piscataway, NJ).

RESULTS

PKC inhibits OAG-induced $[Ca^{2+}]_i$ rise in TRPC3-transfected HEK cells

It is well documented that TRPC3 channels are activated by DAG and its analog OAG (Hofmann et al., 1999; Ma et al., 2000). In the present study, application of OAG (300 μM) elicited a $[Ca^{2+}]_i$ rise in the HEK293 cells that are transiently transfected with *TRPC3* gene, but not in the control cells without TRPC3 transfection (Fig. 1A). This OAG activation of TRPC3 was not mediated by PKC, because it persisted in the presence of a PKC antagonist GF109203X at 100 nM (Fig. 1B) or 10 μM (data not shown). In addition, pre-treatment of the cells for 5 min with phorbol ester PMA (1 μM), which activates PKC, markedly attenuated OAG-induced $[Ca^{2+}]_i$ rise (Fig. 1C), suggesting that PKC actually inhibited, instead of mediating, OAG-induced activation of TRPC3. In the presence of GF109203X either at 100 nM (Fig. 1D) or 10 μM (data not shown), PMA lost its inhibitory effect on OAG-induced $[Ca^{2+}]_i$ rise (Fig. 1D), further confirming that PKC inhibited TRPC3. These data agree with the results from several other laboratories (Trebak et al., 2003, 2005; Venkatachalam et al., 2003), supporting the notion that PKC inhibit DAG- or OAG-induced activation of TRPC3.

In the present study, we investigated the OAG-induced $[Ca^{2+}]_i$ rise due to TRPC3, thus any endogenous OAG-induced Ca^{2+} influx could complicate our data interpretation. One previous report showed that HEK cells expressed endogenous TRPC1, TRPC3, and TRPC4 (Wu et al., 2002). The results from the same report also

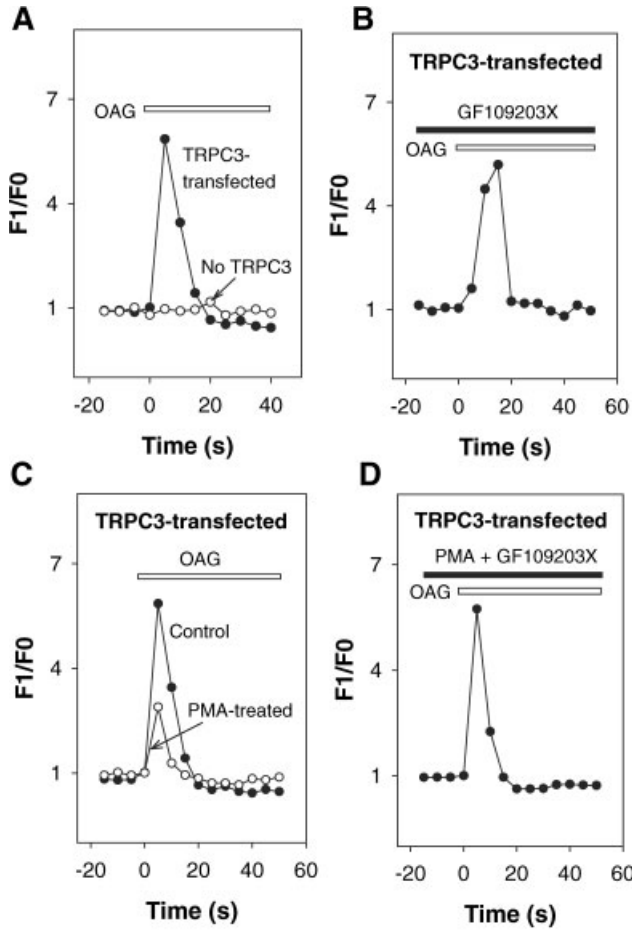

Fig. 1. OAG-induced $[Ca^{2+}]_i$ rise and the effect of PKC modulators in PKG-HEK cells. PKG-HEK cells were incubated in NPSS containing 1 mM Ca^{2+}. The bars on top of the traces indicate the time duration of chemical treatments. **A**: OAG (300 μM)-induced $[Ca^{2+}]_i$ rise in PKG-HEK cells that were either transiently transfected with TRPC3 or with a control vector pcDNA6. **B**: Effect of GF109203X (100 nM). **C**: Effect of PMA (1 μM). **D**: Effect of GF109203X (100 nM) + PMA (1 μM). PMA and GF109203X were introduced 5 min prior to OAG. Each trace is a representative taken from 4–8 experiments containing a total of 50–150 cells.

suggested that endogenous TRPC4 in HEK293 cells could contribute to OAG-induced Ca^{2+} influx. However, these results were not supported by our studies. Our previous immunoblot experiments failed to detect TRPC4 expression in HEK293 cells we used (Kwan et al., 2004, supporting Fig. 6). More importantly, consistent with the published results from several other laboratories (Hofmann et al., 1999; Ma et al., 2000; Venkatachalam et al., 2003), OAG (100–300 μM) failed to elicit a $[Ca^{2+}]_i$ rise in nontransfected HEK cells. Furthermore, Hofmann et al. (1999) found that TRPC4 and TRPC5 channels overexpressed in HEK cells were not sensitive to OAG challenge. One possible explanation for these discrepancies might be the differences in cell culture conditions or passage number of the cultured cells. Because OAG failed to elicit a $[Ca^{2+}]_i$ rise in nontransfected HEK293 cells in our conditions, the endogenous OAG-induced Ca^{2+} influx is not considered further in the present study.

PKG inhibits OAG-induced $[Ca^{2+}]_i$ rise

Previous studies showed that, in TRPC3-overexpressing HEK cells, thapsigargin treatment enhanced Ca^{2+} influx (Kwan et al., 2004), and that this type of store-operated Ca^{2+} influx mediated by TRPC3 was inhibited by PKG (Kwan et al., 2004). In the present study, we activated TRPC3 by OAG (300 μM) (in a store-independent mode), and examined whether PKG could also inhibit OAG-induced activation of TRPC3. Our results show that 8-BrcGMP at 1 mM attenuated, and at 2 mM almost completely abolished the OAG-induced $[Ca^{2+}]_i$ rise (Fig. 2A). KT5823 (1 μM), a potent and highly specific PKG inhibitor, and H8 (10 μM), another PKG inhibitor, reversed the inhibitory effect of 8-BrcGMP (Fig. 2A,B). Taken together, our data clearly indicate that the stimulatory effect of OAG on TRPC3 is inhibited by PKG. Note that the HEK cells used in these experiments (Figs. 1 and 2) were stably transfected with *PKG 1α* genes. These cells expressed a much higher level of PKG than that of wild-type HEK cells (Fig. 2C). Note also that H8 is a relatively nonspecific PKG inhibitor. Its IC50 for PKG is ~0.2 μM, IC50 for PKA is ~1 μM, and IC50 for PKC is ~15 μM. At the concentration we used (10 μM), H8 can potentially inhibit PKA, and in a less degree PKC. Therefore, the data of H8 experiments can only be interpreted together with the data of KT5823 experiments.

PKC acts on PKG to inhibit TRPC3

A recent study showed that PKG is one of downstream targets of PKC (Hou et al., 2003). This was verified here. In these experiments, PKG activity was estimated by the standard VASP Ser-239 phosphorylation method. VASP is a well-characterized substrate for PKG. The VASP phosphorylation at Ser-239 by PKG can be determined by a monoclonal antibody (16C2) (Smolenski et al., 1998). Figure 3A shows that treatment of TRPC3-transfected PKG-HEK cells with PMA (1 μM) for 5 min markedly enhanced the amount of phosphorylated VASP, indicating that PMA indeed stimulated PKG activity.

Functional studies were then carried out to investigate whether the inhibition of TRPC3 by PMA

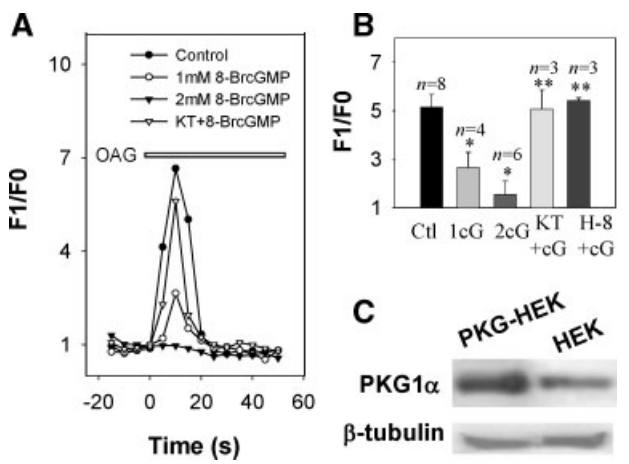

Fig. 2. Effect of PKG modulators on OAG-induced $[Ca^{2+}]_i$ rise in PKG-HEK cells. **A**: Representative traces of OAG (300 μM)-induced $[Ca^{2+}]_i$ rise in TRPC3-transfectd PKG-HEK cells. Shown were the effect of 1 mM 8-BrcGMP, 2 mM 8-BrcGMP, and KT5823 (1 μM) + 8-BrcGMP (1 mM). Each trace is a representative taken from 3–8 experiments containing a total of 40–120 cells. **B**: Summary of the peak OAG-induced $[Ca^{2+}]_i$ rise as in A. Also shown is the effect of H8 (10 μM) + 8-BrcGMP (1 mM). 8-BrcGMP, KT5823, and H8 were introduced 5 min prior to OAG. Mean ± SE (n = 3–8). $^*P < 0.05$ versus control; $^{**}P < 0.05$ versus 1cG. **C**: (Upper part) a representative immunoblot (from n = 3) with an anti-PKG antibody; lower part: an immunoblot with antibody against β-tubulin, indicating that an equal amount of protein was loaded onto each lane. Ctl, control; KT, KT5823; 1cG, 1 mM 8-BrcGMP; 2cG, 2 mM 8-BrcGMP.

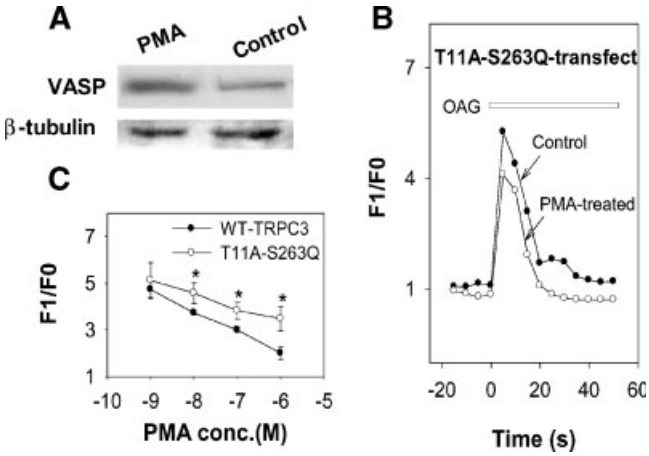

Fig. 3. Effect of point mutations at functional PKG phosphorylation sites on the PMA inhibition in PKG-HEK cells. **A**: (Upper part) representative immunoblot (from n = 3) of PKG-HEK cells with antibody against phosphorylated VASP (16C2). Cells were pre-incubated with 1 μM PMA for 5 min before the total proteins were extracted. Control had no pre-treatment. Only one main VASP band of 50 kDa was consistently observed. Another VASP band of 46 kDa (not shown) was very weak and was only observed occasionally. Lower part: An immunoblot with antibody against β-tubulin, indicating that an equal amount of protein was loaded onto each lane. **B**: Representative traces, showing the effect of PMA (1 μM) on OAG (100 μM)-induced $[Ca^{2+}]_i$ rise in T11A-S263Q-transfected PKG-HEK cells. Control had no PMA. Each trace is a representative taken from 6–9 experiments containing a total of 70–170 cells. **C**: Comparison of dose-dependent PMA inhibition on the OAG-induced peak $[Ca^{2+}]_i$ rise in PKG-HEK cells that were transfected either with wild-type TRPC3 or T11A-S263Q. Mean ± SE (n = 4–10). $^*P < 0.05$ versus WT-TRPC3.

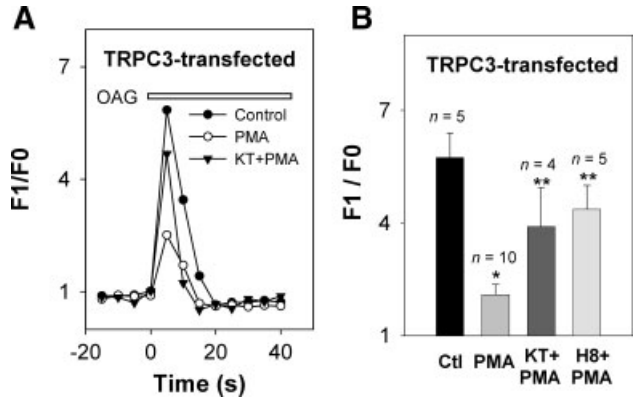

Fig. 4. Effect of PKG modulators on the PMA inhibition in PKG-HEK cells. **A**: Representative traces, showing the effect of PMA (1 μM), KT5823 (1 μM) + PMA (1 μM) on OAG (100 μM)-induced $[Ca^{2+}]_i$ rise in wild-type TRPC3-transfected PKG-HEK cells. Control had no PMA or KT5823. Each trace is a representative taken from 4–10 experiments containing a total of 50–200 cells. **B**: Summary of OAG-induced peak $[Ca^{2+}]_i$ rise as in A. Also shown is the effect of H8 (10 μM) + PMA (1 μM). PMA, KT5823, and H8 were introduced 5 min prior to OAG. Mean ± SE (n = 4–10). $^*P < 0.05$ versus control; $^{**}P < 0.05$ versus PMA. Ctl, control; KT, KT5823.

was mediated through PKG. Point mutations were generated (T11A and S263Q) to disrupt two PKG phosphorylation sites on TRPC3 proteins (Kwan et al., 2004). By comparing Figures 1C and 3B, it is clear that the mutation at two PKG phosphorylation sites (T11A-S263Q) did not affect OAG activation of TRPC3, but it greatly reduced the PMA inhibition on OAG-induced $[Ca^{2+}]_i$ rise. Figure 3C shows that PMA inhibited the OAG-induced $[Ca^{2+}]_i$ rise in a dose-dependent manner. Importantly, the PMA inhibition was much smaller in T11A-S263Q-expressing cells than in wild-type TRPC3-expressing cells (Fig. 3C). These results strongly suggest that the PMA inhibition is, in a large degree, due to PKG phosphorylation on TRPC3. In order to make our data comparable to those published by Trebak et al., who reported the role of Ser-712 in PMA inhibition of TRPC3, for the experiments in Figures 3 and 6 we used 100 μM OAG to stimulate TRPC3. OAG at 100 or 300 μM OAG caused a similar magnitude of $[Ca^{2+}]_i$ rise in TRPC3-transfected cells [F1/F0 = 5.17 ± 0.38 (n = 15) for 100 μM OAG; F1/F0 = 5.20 ± 0.24 (n = 16) for 300 μM OAG].

One concern is whether PKC may phosphorylate Thr-11 and Ser-263, but this is unlikely. Thr-11 and Ser-263 are PKG phosphorylation sites within the consensus sequences of $(R/K)_{2\text{-}3}$-X-S/T, whereas PKC phosphorylation sites have a different consensus sequence of S/T-X-R/K. Furthermore, the results from Trebak et al. suggest that TRPC3 has only one important PKC phosphorylation site at Ser-712 (Fig. 4 of Trebak et al., 2005). Therefore, the possibility of direct PKC phosphorylation on either Thr-11 or Ser-263 can be excluded.

To further substantiate the role of PKG in the PMA inhibition of TRPC3, we utilized PKG inhibitors. As shown in Figure 4, PMA (1 μM) inhibited the OAG-induced $[Ca^{2+}]_i$ rise in TRPC3-transfected HEK cells (Fig. 4A). However, in the presence of PKG inhibitor KT5823 (1 μM) or H8 (10 μM), the PMA inhibition was markedly reduced (Fig. 4A,B). These data further support the notion that PKC inhibits TRPC3 through PKG.

PKC inhibits TRPC3 by two independent mechanisms, direct phosphorylation on Ser-712 and indirectly through PKG

Taken together the above-mentioned results with those published by Trebak et al. (2005), it appears that PKC may inhibit TRPC3 by two different mechanisms, direct phosphorylation on Ser-712 and indirectly through PKG. An obvious next question is the relative contribution of these two pathways toward the overall PKC inhibition on TRPC3. To address this question, we utilized several mutant TRPC3 constructs. Those include S712A, T11A-S263Q, and the mutant T11A-S263Q-S712A, which have all three PKC and PKG sites mutated. Figure 5A compares the dose-dependent PMA inhibition on OAG-induced $[Ca^{2+}]_i$ rise between the cells transfected with different TRPC3 constructs (Fig. 5A). Only the peak amplitudes of OAG-induced $[Ca^{2+}]_i$ rise are compared. It can be seen that, the PMA inhibition was the highest in wild-type TRPC3, reduced in both S712A and T11A-S263Q, and completely abolished in T11A-S263Q-S712A (Fig. 5A). Figure 5B summarizes the percentage of PMA inhibition on the OAG-induced $[Ca^{2+}]_i$ rise. PMA (1 μM) inhibited the OAG-induced $[Ca^{2+}]_i$ rise by 78% (n = 4) in the cells transfected with wide-type TRPC3. The inhibition was reduced to 53% in S712A-transfected cells, and to 40% in T11A-S263Q-transfected cells. No significant PMA inhibition was observed in the cells transfected with T11A-S263Q-S712A (Fig. 5B). These results support that PKC inhibits TRPC3 by two different mechanisms, direct PKC phosphorylation on Ser-712 and indirectly through PKG. Compared to direct PKC phosphorylation pathway, the PKG-mediated indirect pathway appears to have a slightly bigger contribution towards the overall PKC inhibition on TRPC3 (Fig. 5B).

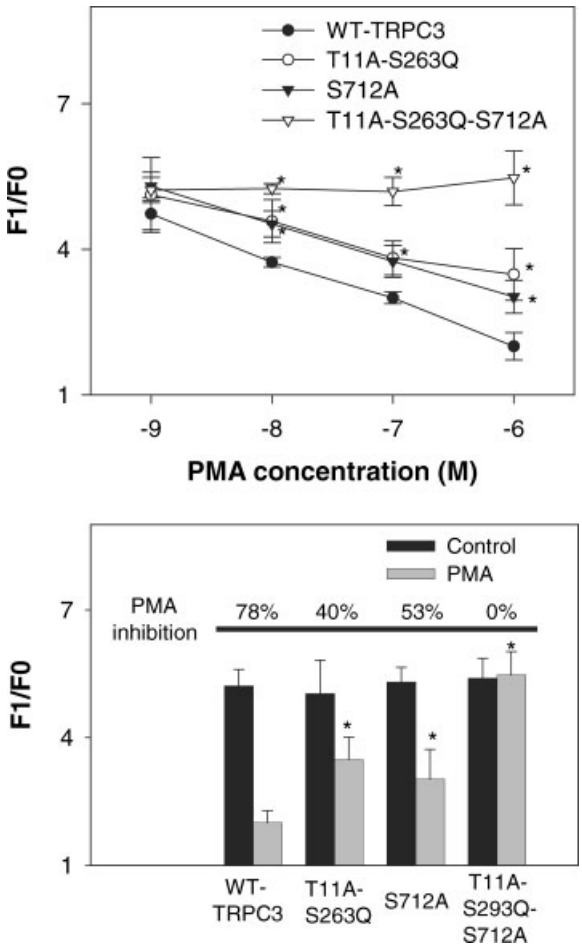

Fig. 5. Effect of point mutations at PKC site and/or PKG sites on the PMA inhibition in PKG-HEK cells. PKG-HEK cells transfected with different TRPC3 constructs were incubated in NPSS containing 1 mM Ca^{2+}. OAG (100 μM) was added to induce Ca^{2+} influx. PMA was introduced 5 min prior to OAG. Control had no PMA. **A**: Comparison of dose-dependent PMA inhibition on OAG-induced peak $[Ca^{2+}]_i$ rise. **B**: Comparison of the percentage inhibition caused by 1 μM PMA on OAG-induced peak $[Ca^{2+}]_i$ rise. The percentage of PMA inhibition is illustrated on top of the bar chart. Mean ± SE (n = 4–10). $^*P < 0.05$ versus PMA-treated WT-TRPC3.

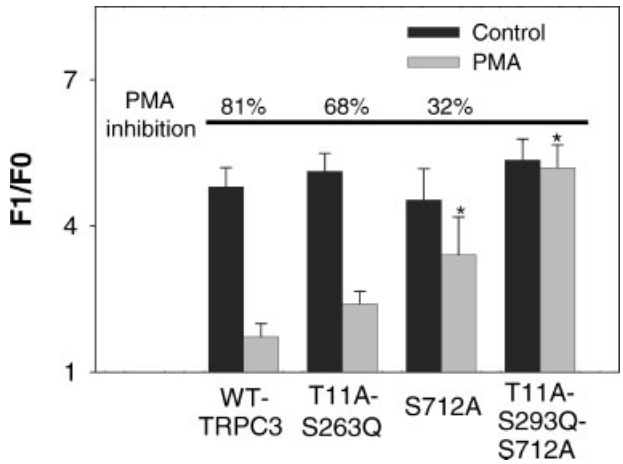

Fig. 6. Effect of point mutations at PKC site and/or PKG sites on the PMA inhibition in nonPKG-transfected HEK cells. NonPKG-transfected HEK cells in NPSS were challenged with OAG (100 μM) to induce Ca^{2+} influx. PMA (1 μM) was introduced 5 min prior to OAG. Only the peak values of OAG-induced $[Ca^{2+}]_i$ rise were compared. Control had no PMA. Mean ± SE (n = 4–8). $^*P < 0.05$ versus PMA-treated WT-TRPC3.

In nonPKG-transfected HEK cells, PKC inhibits TRPC3 mostly via direct PKC phosphorylation on Ser-712

Presumably, the importance of PKG regulation should correlate with the expression level of PKG. To verify this, we examined the PMA inhibition on TRPC3 in nonPKG-transfected HEK293 cells, which expresses PKG at a low level (Fig. 2C). In these cells, PMA (1 μM) was still able to inhibit the TRPC3-mediated $[Ca^{2+}]_i$ rise, and disruption of PKC phosphorylation site (S712A) could still reduce the PMA inhibition (Fig. 6). However, the disruption of PKG phosphorylation sites (T11A-S263Q) only had small effect on the PMA inhibition (Fig. 6). This is in sharp contrast to the results obtained in PKG-overexpressing cells, in which the mutant T11A-S263Q markedly reduced the PMA inhibition (Fig. 5A,B). These results suggest that in nonPKG-transfected HEK cells, PKC inhibited TRPC3 mostly via direct PKC phosphorylation on Ser-712.

However, unlike the report by Trebak et al. (2005), in which the point mutation S712A completely abolished the PMA inhibition of TRPC3, our results show that PMA could still inhibit TRPC3 by 32% even after the point mutation at Ser-712 in nonPKG-transfected HEK cells (Fig. 6). Figure 5B shows that disruption of all three PKG and PKC phosphorylation sites completely abolished the PMA inhibition, suggesting that the PMA inhibition was exclusively mediated through PKC and PKG and that no other mechanisms (pathways) were involved. Therefore, if S712A fails to abolish the PMA inhibition, the PKG sites Thr-11 and Ser-263 must be involved. These data suggest that the discrepancy of the results between Trebak et al. and us might be due to the difference in PKG activity in different batches of HEK cells. PKG is an unstable enzyme and its activity tends to decrease during cell culture and cell passages (Draijer et al., 1995). We used the HEK cells of early passage (passage 2–8) (ATCC), in which certain amount of endogenous PKG can be detected (Kwan et al., 2004). It is possible that the PKG level may be higher in the HEK cells we used than the cells used by Trebak et al. (2005).

The PKG-mediated indirect pathway has an important contribution to the overall PKC inhibition on OAG-induced $[Ca^{2+}]_i$ rise in vascular endothelial cells

Our hypothesis implies that, in cell types with moderate to high PKG expression level, the PKG-mediated indirect pathway has an important contribution to the overall PKC inhibition on TRPC channels. We used the primary isolated rat aortic endothelial cells to test this hypothesis. Application of OAG caused a $[Ca^{2+}]_i$ rise in these cells, consistent with the notion that TRPC3 and TRPC6 are expressed in these cells (Fig. 7A). PMA inhibited the OAG-induced $[Ca^{2+}]_i$ rise in a dose-dependent manner (Fig. 7B) and the inhibition reached 84% with 1 μM PMA (Fig. 7B,C). Activation of PKG either by exogenous application of 8-BrcGMP (2 mM) or by a NO donor SNAP (100 μM), which increases endogenous cGMP level in vascular endothelial cell (Kwan et al., 2000), also inhibited OAG-induced $[Ca^{2+}]_i$ rise. In the presence of KT5823 (1 μM), 8-BrcGMP lost its inhibitory effect, confirming that cGMP acts through PKG to exert its effect. Importantly, pre-treatment of the cells with KT5823 (1 μM) or DT-3 (500 nM) (Dostmann et al., 2000), both of which selectively inhibit

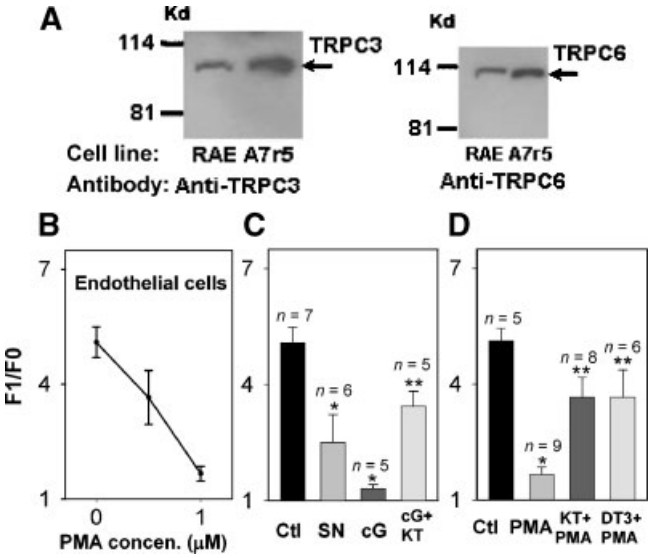

Fig. 7. Effect of PKC and PKG activity on OAG-induced $[Ca^{2+}]_i$ rise in primary rat aortic endothelial cells. **A**: representative immunoblots with antibodies against TRPC3 and TRPC6. Positive signals (TRPC3, ~90 kDa; TRPC6, ~110 kDa) were detected in the primary cultured rat aortic endothelial cells (RAE). Expression in rat smooth muscle cell line A7r5 was used as positive controls. **B**: Dose-dependent PMA inhibition on OAG-induced peak $[Ca^{2+}]_i$ rise. The primary rat aortic endothelial cells in NPSS were challenged with OAG (300 μM) to induce Ca^{2+} influx. PMA was introduced 5 min prior to OAG. Mean ± SE (n = 3–6). **C**: Summarized data showing the effect of SNAP (100 μM), 8-BrcGMP (2 mM), and 8-BrcGMP (2 mM) + KT5823 (1 μM) on OAG-induced peak $[Ca^{2+}]_i$ rise. **D**: effect of PMA (1 μM), KT5823 (1 μM) + PMA (1 μM), and DT-3 (500 nM) + PMA (1 μM) on OAG-induced peak $[Ca^{2+}]_i$ rise. Control had no treatment. Mean ± SE (n = 5–7). $^*P < 0.05$ versus control; $^{**}P < 0.05$ versus cGMP (in C) or PMA (in D). Ctl, control; cG, 8-BrcGMP; SN, SNAP; KT, KT5823.

PKG activity, markedly reduced the PMA inhibition (Fig. 7D), supporting the notion that PKG-mediated indirect pathway contributes to PMA inhibition on TRPC channels in native endothelial cells.

DISCUSSION

It is well recognized that PKC can inhibit the activity of TRPC3 and TRPC6 (Zhang et al., 2001; Trebak et al., 2003; Venkatachalam et al., 2003). Evidence also shows that TRPC5 channels, which belong to another TRPC subfamily, are also negatively regulated by PKC (Venkatachalam et al., 2003). Based on these findings, Venkatachalam et al. (2003) proposed that the PKC-mediated inhibition of TRPC channels might be a general component in the feedback regulation of multiple TRPC isoforms including TRPC3,6,7 and TRPC4,5. An important next question is the detailed molecular mechanism of PKC-mediated inhibition on TRPC channels. Recently, Trebak et al. (2005) showed that PKC inhibits TRPC3 by directly phosphorylating on Ser-712 of the TRPC3 proteins. In the present study, we provided compelling evidence that PKC can also inhibit TRPC3 channels indirectly through PKG. PKC activates PKG, which subsequently inhibits TRPC3 by phosphorylation on Thr-11 and Ser-263. The relative importance of this PKG-mediated indirect pathway may rely on cellular PKG level. It has a large contribution to the overall PKC inhibition of TRPC3 in PKG-overexpressing HEK cells, whereas it only has a small contribution in nonPKG-transfected HEK cells, which only express PKG at low level. In nonPKG-transfected HEK cells, PKC inhibits TRPC3 mostly via direct PKC phosphorylation on Ser-712. A point to note is that some in vivo cell types are well known to have high/moderate PKG activity and they also express multiple TRPC isoforms. These include vascular smooth muscle cells, vascular endothelial cells, platelets, etc. (Hassock et al., 2002; Feil et al., 2003; Yip et al., 2004; Facemire et al., 2004). It is likely that this PKG-mediated indirect pathway may have an important contribution to the overall PKC inhibition on TRPC-mediated Ca^{2+} influx in these cell types. This was indeed confirmed in the primary isolated rat aortic endothelial cells, in which the inhibition of PKG by KT5823 or DT-3 caused a marked reduction in the PMA-induced inhibition of OAG-induced $[Ca^{2+}]_i$ rise (Fig. 7D).

An increasing body of evidence implicated an important role of protein kinases in the regulation of TRP channels. In most cases, phosphorylation leads to an increased channel activity. For example, PKC phosphorylation increases the activity of TRPM4 (Nilius et al., 2005), while Src phosphorylation enhances the activity of TRPM7 (Jiang et al., 2003). Multiple protein kinases, including PKC, CaMKII, and PKA, can activate or sensitize TRPV1 (Nagy et al., 2004). Lyn kinase may activate TRPV4 through phosphorylation on Tyr-253 (Xu et al., 2003), though this result has been disputed (Vriens et al., 2004). Within TRPC family, Src is required for DAG-mediated activation of TRPC3 (Vazquez et al., 2004), and Fyn directly phosphorylates TRPC6 proteins, resulting in an increased channel activity (Hisatsune et al., 2004). Limited amount of data indicate that phosphorylation also plays a role in the inactivation of TRP channels. PKC inhibits the activity of two TRPC subfamilies, TRPC3,6,7 and TRPC4,5 (Zhang and Saffen, 2001; Venkatachalam et al., 2003; Trebak et al., 2005), and PKG inhibits store-operated Ca^{2+} influx mediated by TRPC3 (Kwan et al., 2004). We previously hypothesized that the PKG-mediated inhibition on TRPC was an important component of a negative feedback loop to tightly control cytosolic Ca^{2+} levels. In this negative feedback loop, TRPC3 allows Ca^{2+} influx. The elevation of cytosolic $[Ca^{2+}]_i$ stimulates endothelial cell nitric oxide synthase activity, leading to increased production of cGMP. This secondary messenger will activate PKG; the ensuing phosphorylation inhibits TRPC3 channel activity and completes a PKG-dependent negative feedback loop. This model could at least be applied to certain cell types such as vascular endothelial cells, in which Ca^{2+} influx is known to be regulated by nitric oxide (NO)/cGMP/PKG in a negative feedback fashion (Kwan et al., 2000; Yao and Huang, 2003). In the present study, we show that two known feedback mechanisms, that is, PKC- and PKG-mediated feedback mechanisms, are interconnected. The action of PKC on TRPC3 is partly mediated through PKG. In other words, two pathways merge at PKG (Fig. 8). This adds another dimension to phosphorylation-mediated regulation of TRPC channels. Revelation of the novel link between PKC, PKG, and TRPC channels has widespread physiological implications, because PKC, PKG, and $[Ca^{2+}]_i$ are all important intracellular signal molecules, the activities/levels of which can be regulated under different physiological and pathological conditions.

There are two functionally important PKG phosphorylation sites in human TRPC3, that is, Thr-11 and Ser-263. PKG phosphorylation at each of these two sites downregulates TRPC3 activity (Kwan et al., 2004). Protein sequence alignment shows that these two sites are conserved in human TRPC6 and TRPC7. Therefore, it is likely that the PKG-mediated indirect pathway may also contribute to overall PKC inhibition of TRPC6 and

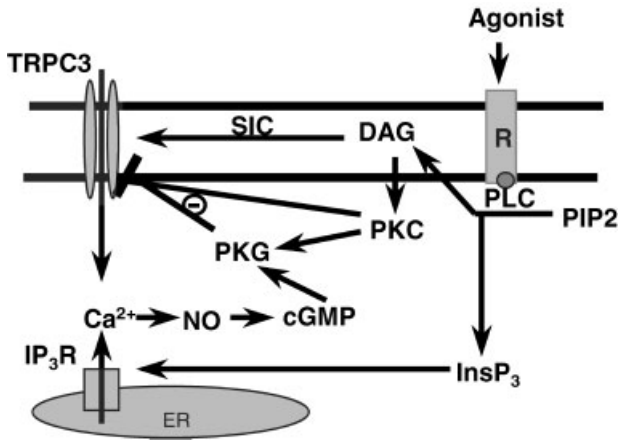

Fig. 8. A diagram summarizing the regulation of TRPC3. Stimulation of membrane receptor activates TRPC3 channels either via the store-independent Ca^{2+} influx mechanism mediated through DAG or via the store-operated Ca^{2+} influx mechanism due to $InsP_3$-mediated store Ca^{2+} release. An increased concentration of cytosolic Ca^{2+} activates NOS, which leads to the production of NO and cGMP. Elevated cGMP activates PKG, which inhibits TRPC3 channels in a negative feedback loop. Elevation of DAG stimulates PKC, which inhibits TRPC3 channels by two independent pathways, directly phosphorylating the channel and indirectly via activating PKG.

TRPC7 in a manner similar to that of TRPC3. Whether a similar scheme can also be applied to TRPC4 and/or TRPC5 is more of a question. Venkatachalam et al. (2003) showed that the activity of TRPC4 and TRPC5 is downregulated by PKC. However, whether PKG can modulate TRPC4 and/or TRPC5 has not been tested. Furthermore, Thr-11 and Ser-263, the two functionally important PKG phosphorylation sites, are not conserved in TRPC4 or TRPC5. On the other hand, human TRPC5 and TRPC4 do contain other potential PKG phosphorylation sites, three sites for TRPC5 and one site for TRPC4. Obviously, future experiments are needed to test whether TRPC4,5 are indeed regulated by PKG and whether PKC can regulate TRPC4,5 indirectly through PKG. Note that the response of TRPC1 to PKC may be very different from that of TRPC3,6,7 or TRPC4,5. It is reported that TRPC1 is activated by direct PKC phosphorylation in vascular endothelial cells (Ahmmed et al., 2004).

In conclusion, PKC and PKG both inhibit the OAG-induced activation of TRPC3 channels. PKC inhibits TRPC3 channels by two independent mechansims, direct phosphorylation on Ser-712 and indirectly through PKG. PKC activates PKG by phosphorylation. Activated PKG then phosphorylates Thr-11 and Ser-263, turning off TRPC3. Taken together, the present study provides a full mechanistic scheme for PKC inhibition on TRPC3.

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