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# Negative Regulation of TRPC3 Channels by Protein Kinase C-Mediated Phosphorylation of Serine 712

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## ABSTRACT

TRPC3 is a nonselective cation channel member of the "canonical" transient receptor potential (TRPC) family whose members are activated by phospholipase C-coupled receptors. TRPC3 can be activated by the diacylglycerol analog 1-oleoyl-2-acetyl-*sn*-glycerol (OAG) in a protein kinase C-independent manner. On the other hand, phorbol 12-myristate 13-acetate (PMA) inhibits OAG-mediated TRPC3 channel activation, suggesting that phosphorylation of TRPC3 by protein kinase C is a mechanism of receptor-mediated negative feedback. Here, we show PMA-induced phosphorylation of TRPC3 channels in vivo. We demonstrate by site-directed mutagenesis that a single site containing $Ser^{712}$ and conserved among all members of the TRPC family is essential for protein kinase C-mediated negative regulation of TRPC3. In human embryonic kidney 293 cells expressing a TRPC3 mutant in which $Ser^{712}$ was replaced by alanine (S712A), PMA failed to block channel activation, whereas wild-type TRPC3 activity was completely inhibited. Phosphorylation of the S712A TRPC3 mutant was not stimulated in response to PMA treatment. Furthermore, S712A TRPC3 mutant-mediated $Ca^{2+}$ entry after methacholine activation was significantly greater than that of wild-type TRPC3. These findings demonstrate a dual role for phospholipase C-generated diacylglycerol, which serves as a signal for TRPC3 activation as well as a signal for negative feedback via protein kinase C-mediated phosphorylation.





Control of intracellular $Ca^{2+}$ homeostasis is essential for cell survival and function. By means of $Ca^{2+}$-permeable channels, cells carefully use $Ca^{2+}$ to transduce signals in response to a wide variety of physiological stimuli (Taylor, 2002). In nonexcitable cells, calcium signaling is most commonly initiated through cell-membrane receptors coupled to phospholipase C, resulting in the breakdown of phosphatidylinositol 4,5-bisphosphate ($PIP_2$) and production of inositol 1,4,5-trisphosphate ($IP_3$) (Berridge, 1993), followed by $Ca^{2+}$ release from internal stores and a concomitant $Ca^{2+}$ entry via the capacitative calcium entry (CCE) pathway (Putney, 1986, 1997). Although CCE, or store-operated $Ca^{2+}$ entry, represents a major means of $Ca^{2+}$ entry in nonexcitable cells, there is compelling evidence that it is not the only pathway regulated by phospholipase C-linked receptors. A variety of $Ca^{2+}$-permeable channels are regulated downstream of the phospholipase C pathway by mechanisms not directly related to store depletion and involving a variety of second messengers such as $IP_3$, cytosolic $Ca^{2+}$, and arachidonic acid (Barritt, 1999). Some members of the mammalian transient receptor potential (TRP) superfamily could be regulated by $PIP_2$ or activated by the lipid product of $PIP_2$ hydrolysis, diacylglycerol (Hardie, 2003).

TRPC3, first cloned by Zhu et al. (1996), is a nonselective cation channel belonging to the "canonical" TRP (TRPC) family whose members are activated through phospholipase C-coupled receptors (Zitt et al., 2002; Trebak et al., 2003b). It is clearly established that TRPC3 can be activated by the diacylglycerol analog 1-oleoyl-2-acetyl-*sn*-glycerol (OAG) in a protein kinase C-independent manner providing a possible mechanism of activation of these channels by phospholipase C-coupled receptors (Hofmann et al., 1999). In addition, a recent report from our laboratory suggested that receptor-mediated activation of phospholipase C activates TRPC3 via diacylglycerol production independently of G proteins, pro-

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**ABBREVIATIONS:** $PIP_2$, phosphatidylinositol 4,5-trisphosphate; $IP_3$, inositol 1,4,5-trisphosphate; CCE, capacitative calcium entry; TRP, transient receptor potential; TRPC, canonical transient receptor potential; OAG, 1-oleoyl-2-acetyl-*sn*-glycerol; PMA, phorbol 12-myristate 13-acetate; HEK, human embryonic kidney; GFP, green fluorescent protein; DMEM, Dulbecco's modified Eagle's medium; HA, hemagglutinin; PBS, phosphate-buffered saline; Wt, wild type.

tein kinase C, or $IP_3$ (Trebak et al., 2003a). In the same study, we demonstrated that phorbol 12-myristate 13-acetate (PMA) inhibits OAG-mediated TRPC3 channel activation, suggesting that protein kinase C-mediated phosphorylation of TRPC3, or of a protein regulating TRPC3, may be a mechanism of receptor-mediated negative feedback. This finding was subsequently confirmed by Venkatachalam et al. (2003). In the present study, we investigated the role of protein kinase C in the negative regulation of TRPC3 channels. We demonstrate that TRPC3 can be phosphorylated in vivo upon protein kinase C activation, and we have identified the phosphorylation site on TRPC3 through which protein kinase C exerts its action.

## Materials and Methods

**Cell Culture and Transfection.** HEK293 cells stably expressing TRPC3 with green fluorescent protein fused to its C terminus (TRPC3-GFP) (McKay et al., 2000) were maintained in DMEM containing 10% fetal bovine serum and 0.5 mg/ml G418 as described previously (Trebak et al., 2002). An HEK293 cell line stably expressing the wild-type human TRPC3 (HEK-T3H) (Wedel et al., 2003) and a pool of HEK293 cells stably expressing the S712A TRPC3 mutant version (HEK-SA) generated in the current study, both with the hemagglutinin epitope (HA) fused to their C termini, were used in in vivo phosphorylation studies. For transient transfection in HEK293 cells, we used pcDNA3 vector containing the Topaz coding sequence added in frame either to the C terminus of wild-type TRPC3 (T3T) (Vazquez et al., 2003) or to that of the point mutant S712A TRPC3 (T3T-S712A), in which serine 712 was replaced by alanine. HEK293 cells were transfected at 70 to 80% confluence using the Superfect reagent (QIAGEN, Valencia, CA) as recommended by the vendor. In brief, 10 $\mu$g of plasmid DNA was diluted in growth medium containing no serum or antibiotic to a total volume of 300 $\mu$l. The solution was mixed thoroughly, and 60 $\mu$l of Superfect reagent was added to the DNA solution. After mixing, samples were incubated for 10 min at room temperature to allow transfection complex formation, and then the total volume was brought to 3 ml with growth medium containing serum and antibiotics. Cells in 100-mm dishes were washed once with 5 ml of PBS and then overlaid with 3 ml of transfection medium. The cells were incubated for 6 h; then the transfection medium was removed, and the cells were washed with 5 ml of PBS. Fresh growth medium was added to the cells overnight, and the cells were then harvested and plated onto coverslips. Fura-2 imaging experiments were performed between 24 and 72 h after transfection.

**In Vivo Phosphorylation, Immunoprecipitations, SDS-PAGE, and Western Blots.** Wild-type HEK293 cells, HEK-T3H, and HEK-SA cells were plated at 70 to 80% confluence in polylysine-coated 100-mm dishes. After 24 h, cells were incubated for 2 h in phosphate-free, serum-free DMEM medium at 37°C and then labeled with [$^{32}$P]orthophosphate (300 $\mu$Ci/ml) for 4 h at 37°C. After PMA (Calbiochem, San Diego, CA) stimulation (1 $\mu$M for 10 min at 37°C), the cells were washed twice with ice-cold PBS and then lysed in 1 ml of lysis buffer containing 10 mM Tris-HCl, 150 mM NaCl, 1 mM EGTA, 1 mM $Na_3VO_4$, 1 $\mu$M okadaic acid, 1 mM NaF, 200 $\mu$g/ml phenylmethylsulfonyl fluoride, 1 $\mu$g/ml pepstatin, 4 $\mu$g/ml leupeptin, 10 $\mu$g/ml aprotinin, 1% Nonidet P-40, and 20 mM $\beta$-glycerophosphate, pH 7.5. After 15 min of incubation on ice, cells were detached using a cell-culture scraper, and samples were centrifuged at 4°C for 15 min at maximal speed in a microcentrifuge. Supernatants were precleared with 25-$\mu$l slurry of protein A-Sepharose for 30 min at 4°C with gentle rocking. The supernatants were then incubated with 2 $\mu$g of anti-HA mouse monoclonal antibody and 50 $\mu$l of protein A-Sepharose for 2 h at 4°C with gentle rocking. Immunoprecipitates were washed 6 times with 1.5 ml of lysis buffer, boiled in 2× SDS sample buffer, and separated by SDS-PAGE (10% polyacrylamide). Proteins from gels were transferred onto nitrocellulose membranes and $^{32}$P-detected by autoradiography.

Because recovery of TRPC3 protein after immunoprecipitation varied somewhat from one experiment to the next, we determined the quantity of TRPC3 loaded onto the gels in each experiment by densitometric analysis of the HA-labeled protein. Thus, the same membranes used for determination of $^{32}$P were subsequently subjected to Western blots using an anti-HA antibody and the ECL kit (Amersham Biosciences, Piscataway, NJ) to determine the quantity of TRPC3 protein. Densitometric analysis of $^{32}$P-labeled bands and HA-labeled bands was carried out with a Bio-Rad ChemDoc XRS scanning image analysis apparatus (Bio-Rad, Hercules, CA). Bands were analyzed quantitatively with Quantity One software, version 4.5.0 (Bio-Rad). All data were acquired in the linear range, well below saturation of the instrumentation. Level of phosphorylation was expressed as the ratio of the phosphorylated TRPC3 band to that of the same band detected by anti-HA antibody. These ratios were subsequently normalized to the ratios for control cells to give a value of fold increase in protein phosphorylation.

**Mutagenesis.** A point mutation was introduced in the TRPC3 coding sequence of TRPC3-Topaz cloned in the pcDNA3 vector by using specific oligonucleotides and the single site-directed mutagenesis kit (QIAGEN). Sequences of constructs were verified by DNA sequencing.

**Measurement of Divalent Cation Entry.** Coverslips with attached cells were mounted in a Teflon chamber and incubated at 37°C for 30 min in culture media (DMEM with 10% fetal bovine serum) containing 1 $\mu$M Fura-2 AM (Molecular Probes, Eugene, OR). Cells were then washed and bathed in HEPES-buffered saline solution (140 mM NaCl, 4.7 mM KCl, 10 mM $CsCl_2$, 2 mM $CaCl_2$, 1.13 mM $MgCl_2$, 10 mM glucose, and 10 mM HEPES, pH 7.4) for at least 10 min before $Ca^{2+}$ measurements were made. For $Ca^{2+}$ measurements, fluorescence images of several cells were recorded and analyzed with a digital fluorescence imaging system (InCyt Im2; Intracellular Imaging Inc., Cincinnati, OH) as described previously (Trebak et al., 2003a). However, because some of the protocols produced signals that result from a mixture of $Ca^{2+}$ and $Ba^{2+}$, fluorescence ratios without background subtraction rather than calibrated $Ca^{2+}$ concentrations are reported.

## Results

**Nanomolar Concentrations of PMA Inhibit TRPC3.** In a previous study, we showed that PMA inhibits OAG activation of TRPC3 in a HEK293 cell line stably expressing a TRPC3-GFP fusion protein (Trebak et al., 2003a). To determine the sensitivity of this inhibitory process to PMA, we performed dose-response experiments of PMA on HEK-TRPC3-GFP as well as in a HEK293 cell line stably expressing HA-tagged TRPC3 (HEK-T3H; see *Materials and Methods*). Figure 1 shows results obtained with HEK-T3H cells; similar results were obtained with the HEK-TRPC3-GFP cells (data not shown). Nontransfected wild-type HEK293 cells showed no response to OAG (data not shown), whereas HEK-T3H cells responded to OAG with a significant calcium entry (Fig. 1). Concentrations of PMA as low as 25 nM substantially inhibit TRPC3-mediated $Ca^{2+}$ entry in response to the diacylglycerol analog OAG. When concentrations of 500 nM of PMA or higher were used, TRPC3 activity was completely abolished (Fig. 1).

**PMA Induces Phosphorylation of TRPC3 Channels.** Because PMA inhibition of TRPC3 activity is indicative of protein kinase C action, we sought to determine whether TRPC3 channels are phosphorylated after treatment of cells with PMA. For these experiments, we used a HEK293 cell

line stably expressing HA-tagged TRPC3 (HEK-T3H) (Wedel et al., 2003), and an anti-HA antibody was used to immunoprecipitate $^{32}$P-labeled TRPC3 proteins from PMA-treated and untreated HEK-T3H cells. Wild-type HEK293 (HEK-Wt) cells were used as a negative control and showed no detectable phosphorylation (data not shown). Figure 2A shows an autoradiograph reflecting $^{32}$P-incorporation into TRPC3 immunoprecipitates, whereas Fig. 2B represent an immunoblot with the anti-HA antibody. As expected, HEK-T3H cells express significant amounts of TRPC3-HA, as revealed by anti-HA immunoblot (Fig. 2B). TRPC3 proteins seem to be substantially phosphorylated after PMA treatment (Fig. 2A); densitometric measurements indicated a fold increase of 3.01 ± 0.50 ($n$ = 3). This then demonstrates that TRPC3 is directly phosphorylated and suggests an action of protein kinase C in this phosphorylation.

**Protein Kinase C Consensus Phosphorylation Site in TRPC3 Mediates the Effect of PMA on TRPC3.** Because protein kinase Cs are the most obvious targets of phorbol esters such as PMA, we next further investigated the involvement of protein kinase C in the negative regulation of TRPC3. There are nine serine or threonine residues that are potential candidate substrates for protein kinase C-dependent phosphorylation in the TRPC3 sequence: Ser$^{6}$, Ser$^{130}$, Ser$^{405}$, Thr$^{485}$, Thr$^{573}$, Ser$^{617}$, Ser$^{712}$, Ser$^{813}$, and Ser$^{837}$. TRPC3 and its close structural relatives TRPC6 and TRPC7 form a subfamily of TRPC channels that are activated by diacylglycerol analogs. The activation of these three channels by OAG is completely inhibited by PMA (Okada et al., 1999; Zhang and Saffen, 2001; Trebak et al., 2003a), suggesting that all members of the TRPC3/6/7 subfamily might be negatively regulated by protein kinase C. By aligning the amino acid sequences of the three members of TRPC3/6/7 subfamily, we found that only three of the nine putative protein kinase C phosphorylation sites were common to all three proteins (Thr$^{573}$, Ser$^{617}$, and Ser$^{712}$). Thr$^{573}$ is located in the predicted intracellular loop between the transmembrane domains 4

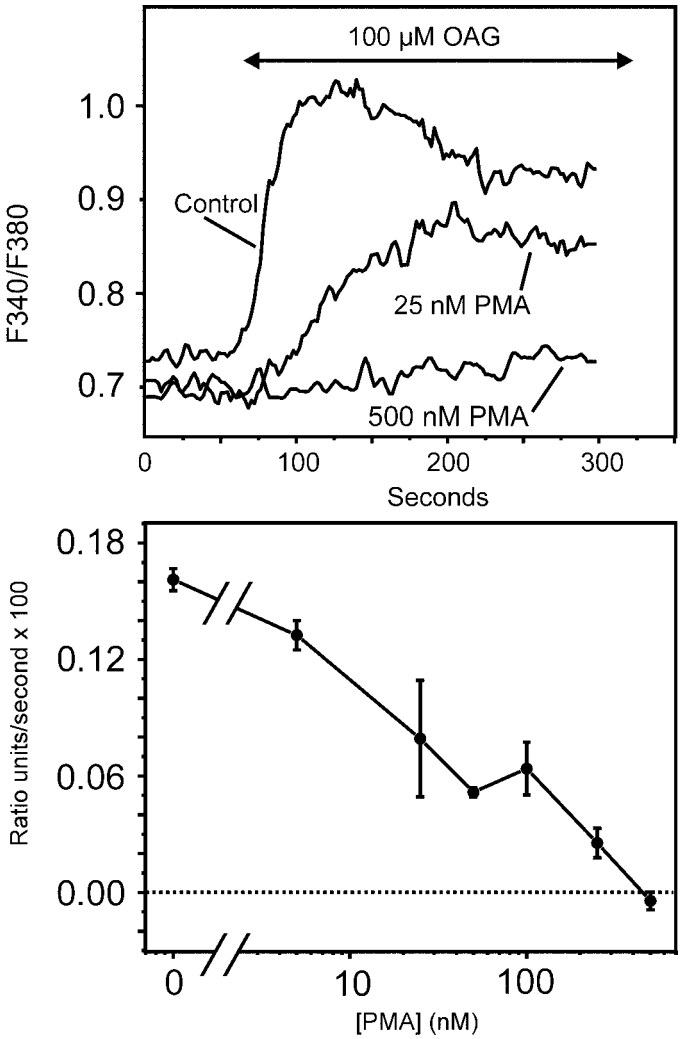


**Fig. 1.** PMA treatment inhibits TRPC3-mediated $Ca^{2+}$ entry. HEK293 cells stably expressing TRPC3-HA fusion protein (HEK-T3H) were treated with different concentrations of PMA 5 min before stimulation with OAG (100 $\mu$M) in the presence of 2 mM external $Ca^{2+}$ and 5 $\mu$M $Gd^{3+}$ to block endogenous CCE. Top, each trace is the average of at least 20 cells from a single experiment representative of three separate experiments. Bottom, average (± S.E.M.) rate of increase of $[Ca^{2+}]_i$ in untreated cells and in cells treated with PMA in concentrations ranging from 5 to 500 nM.

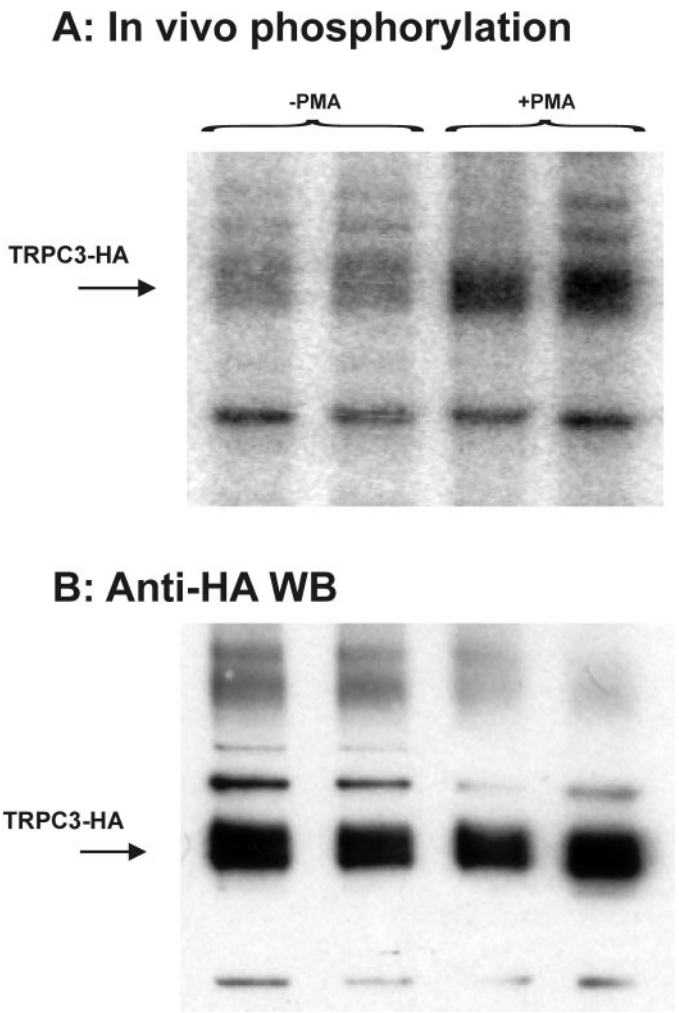


**Fig. 2.** PMA-induced phosphorylation of TRPC3. A, HEK-T3H cells either treated for 10 min with 1 $\mu$M of PMA (+PMA) or not treated (−PMA) were assayed for PMA-induced phosphorylation of TRPC3 as described under *Materials and Methods*. B, immunoblot with anti-HA antibody of the same membrane probed for phosphorylation, showing that similar amounts of T3H proteins were loaded in each lane. The expected molecular mass of T3H protein (~100 kDa) is indicated with an arrow. Data are representative of three separate experiments. For both the +PMA and −PMA condition, duplicate lanes are shown.

and 5. $Ser^{617}$ is located in the putative pore region of TRPC3 and is thus unlikely to be accessible to intracellular kinases. $Ser^{712}$ is located in the C terminus 24 amino acids downstream of the signature EWKFAR sequence (Fig. 3A) and 49 amino acids upstream of the calmodulin/$IP_3$ receptor binding domain (Zhang et al., 2001). Therefore, mutants were constructed in which either $Thr^{573}$ or $Ser^{712}$ was replaced by alanine by using as a template the pcDNA3 vector containing the coding sequence of the TRPC3-Topaz fusion protein (T3T) (Vazquez et al., 2003). The resulting mutant proteins (T3T-T573A and T3T-S712A) and T3T-Wt were transiently transfected into HEK293 cells. Expression was evaluated from detection of the Topaz fusion, and channel activity was assessed by Fura-2 imaging.

The mutant protein T3T-T573A was not expressed (data not shown), indicating that a threonine in this position is important for proper expression and/or targeting. However, T3T-S712A was expressed and was targeted to the plasma membrane as demonstrated by the Topaz fluorescence; the extent of expression and plasma membrane localization seemed similar to that of T3T-Wt (data not shown).

PMA is known to down-regulate the phospholipase C pathway most likely through a protein kinase C-mediated effect, preventing us from using a phospholipase C receptor agonist such as methacholine to study TRPC3 regulation (Orellana et al., 1987). Therefore, we used the diacylglycerol analog OAG to activate TRPC3 channels. The use of a Fura-2 imaging system allowed us to study simultaneously the response to OAG of a large number of cells transiently transfected either with T3T-Wt or the T3T-S712A mutant. Figure 3B shows that T3T-Wt–mediated $Ca^{2+}$ entry in response to OAG (100 $\mu$M) is completely blocked by 1 $\mu$M PMA treatment when T3T-Wt is transiently expressed in HEK293 cells, consistent with results obtained in the HEK293 cell lines stably expressing TRPC3, HEK-T3H, and HEK-TRPC3-GFP (Fig. 1) (Trebak et al., 2003a). When the T3T-S712A mutant was transiently expressed in HEK293 cells, it was similarly activated by OAG (Fig. 3C) and by methacholine (data not shown; Fig. 4). However, PMA treatment (1 $\mu$M) did not inhibit the T3T-S712A mutant activity in response to OAG (Fig. 3C).

**Mutation of $Ser^{712}$ of TRPC3 to Ala Prevents Phosphorylation upon PMA Stimulation.** We sought to determine whether the point mutant S712A TRPC3 channel loses the ability to become phosphorylated after treatment of cells with PMA. For these experiments, we used two HEK293 cell lines stably expressing either HA-tagged Wt TRPC3 (HEK-T3H) (Fig. 2) or HA-tagged S712A TRPC3 (HEK-SA). An anti-HA antibody was used to immunoprecipitate $^{32}P$-labeled TRPC3 proteins from PMA-treated and untreated HEK-T3H and HEK-SA cells. HEK-Wt cells were used as a negative control (data not shown). Figure 4A shows an autoradiograph reflecting $^{32}P$-incorporation into TRPC3 immunoprecipitates, whereas Fig. 4B represents an immunoblot with the anti-HA antibody. In this experiment, a greater amount of protein was loaded onto the gels from the S712A TRPC3-expressing cells to increase the likelihood of detecting small increases. The basal levels of phosphorylation ($^{32}P$/HA density ratios) were comparable in Wt and S712 cells (Wt, 0.78 $\pm$ 0.05; S712A, 0.71 $\pm$ 0.04; $n = 4$). As shown in Fig. 2, Wt TRPC3 proteins seem to be substantially phosphorylated after PMA treatment, whereas S712A TRPC3 proteins were significantly less phosphorylated in response to PMA stimulation (Fig. 4A); quantitative densitometric analysis indi-

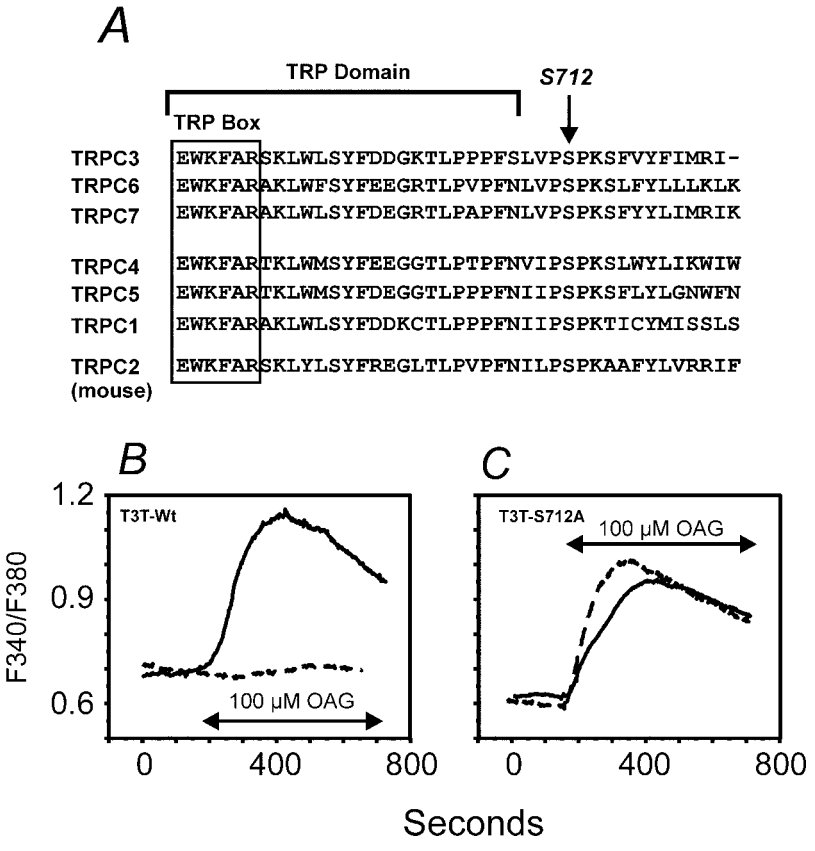


**Fig. 3.** Serine 712 is required for inhibition of TRPC3 by PMA. A, position of serine 712 residue in TRPC3 channel as well as homologous sites in other TRPC family members. B and C, pcDNA3 vectors containing either the TRPC3-Topaz construct (T3T-Wt) or a mutant variant in which serine 712 was replaced by alanine (T3T-S712A) were transiently transfected in HEK293 cells. OAG-induced $Ca^{2+}$ entry in HEK-T3T-Wt cells (B) and HEK-T3T-S712A cells (C) with (broken traces) and without (solid traces) preincubation with 1 $\mu$M PMA. Cells were incubated in HEPES-buffered saline solution containing 2 mM $Ca^{2+}$ and 5 $\mu$M $Gd^{3+}$. Experiments in B and C show averages of at least 20 cells from a single imaging experiment; results from four other experiments were similar.

cated a fold increase of 2.9 ± 0.36 ($n$ = 4) for Wt TRPC3 versus a fold increase of 1.3 ± 0.18 for S712A TRPC3 ($n$ = 4). The latter value is not significantly different from unity, indicating a lack of significantly stimulated phosphorylation in the S712A mutant.

**T3T-S712A Mutant Shows Higher Agonist-Activated $Ca^{2+}$ Entry than T3T-Wt.** We next sought to determine whether the negative regulation of TRPC3 by protein kinase C plays a significant role in determining the magnitude of signaling through a physiological, phospholipase C-linked receptor. A potential complication is that reagents which block or augment protein kinase C activity will affect signaling at a site or sites upstream of TRPC3 activation (i.e., at the level of phospholipase C activation) (Orellana et al., 1987). Therefore, to determine whether protein kinase C-mediated inhibition of TRPC3 via serine phosphorylation is functionally significant during physiological stimulation by an agonist, we compared the extent and kinetics of methacholine-activated $Ca^{2+}$ entry in HEK293 cells transiently expressing T3T-Wt and T3T-S712A proteins. With this strategy, the mutation of $Ser^{712}$ disrupts only protein kinase C regulation of TRPC3 without affecting other protein kinase C-sensitive processes. For the transient transfections, we used the Topaz fusion constructs and verified by flow cytometry that both the wild-type protein and the S712A protein were expressed at similar levels (data not shown). As shown in Fig. 5, top, the sustained $[Ca^{2+}]_i$ elevation after addition of maximal concentrations of methacholine (300 $\mu$M) was higher in cells transfected with the T3T-S712A mutant than in cells transfected with wild-type T3T. We compared the initial rate of rise of $[Ca^{2+}]_i$ (initial slopes, fluorescence ratio units per minute) in the two groups: T3T-Wt, 0.206 ± 0.014, $n$ = 119; and T3T-S712A, 0.299 ± 0.018, $n$ = 72 ($p < 0.0001$). These data suggest that diacylglycerol produced through phospholipase C-coupled receptors activation assumes a dual role in both activation and negative modulation of TRPC3 channels. To ensure that the differences did not result from alterations in $Ca^{2+}$ pumping or sequestration, we also carried out experiments using $Ba^{2+}$ as a surrogate for $Ca^{2+}$ (Fig. 5b). The average rates of $Ba^{2+}$ entry (initial slopes, fluorescence ratio units per minute) were: T3T-Wt, 0.261 ± 0.029, $n$ = 34; and T3T-S712A, 0.416 ± 0.033, $n$ = 23 ($p < 0.001$). For both the $Ca^{2+}$ experiments and the $Ba^{2+}$ experiments, the peak $Ca^{2+}$ release was not significantly different between the two cell types.

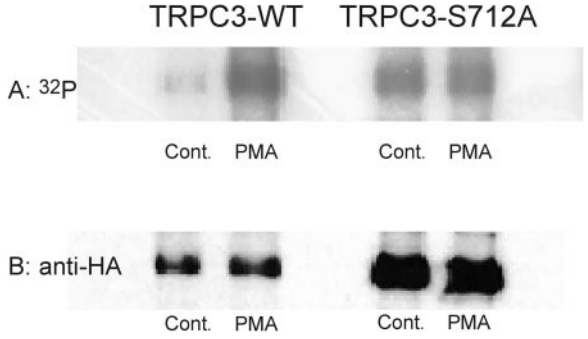


**Fig. 4.** PMA does not significantly increase phosphorylation of S712A TRPC3. A, HEK-T3H and HEK-SA cells either treated for 10 min with 1 $\mu$M of PMA (PMA) or not treated (Cont.) were assayed for PMA-induced phosphorylation of TRPC3 proteins as described under *Materials and Methods*. B, Immunoblot with anti-HA antibody of the same membrane probed for phosphorylation, showing that equal amounts of TRPC3 proteins from control and PMA-treated cells were loaded in each lane. However, larger amounts of the TRPC3-S712A material were loaded to detect possible small increases in phosphorylation. Data are representative of four separate experiments.

## Discussion

To the best of our knowledge, this is the first report of protein kinase C-dependent phosphorylation of a TRPC channel. There is evidence for protein kinase C phosphorylation of TRPV channels; in this case, protein kinase C seems to exert a positive influence on channel activity (Niemeyer et al., 2001; Numazaki et al., 2002; Olah et al., 2002). Hassock et al. (2002) reported cyclic AMP-mediated phosphorylation of TRPC6 in platelets, but the physiological consequence of this phosphorylation was not assessed. Kwan et al. (2004) reported that protein kinase G phosphorylated TRPC3, and this inhibits channel activity. In this study, we have shown that protein kinase C activation (i.e., by PMA) induces TRPC3 phosphorylation and completely inhibits TRPC3 channel activity. A highly conserved protein kinase C consensus site in the C terminus of TRPC3 was identified and was shown to mediate protein kinase C inhibition of TRPC3.

The mechanism by which this phosphorylation inhibits TRPC3 activity is not known. The serine phosphorylated by protein kinase C, $Ser^{712}$, lies just downstream of the highly conserved TRP domain (Montell, 2001), a proline-rich sequence that is involved in the interaction of TRP proteins with regulatory proteins such as the immunophilin FK506

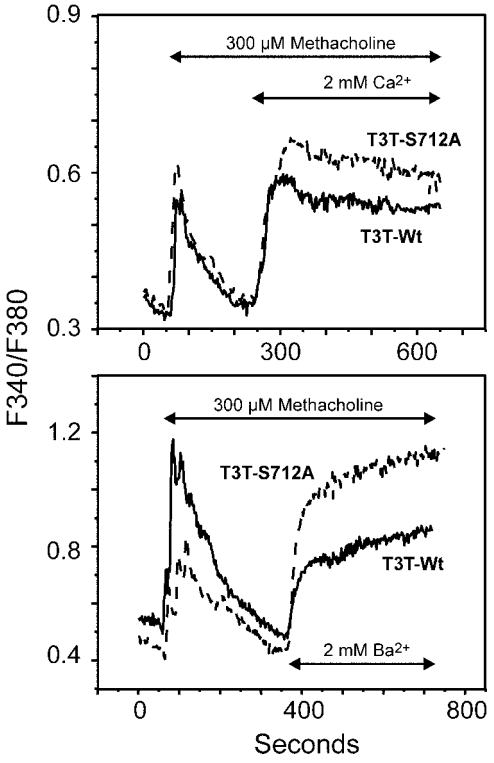


**Fig. 5.** Comparison of $Ca^{2+}$ entry after addition of methacholine to HEK-T3T-Wt and HEK-T3T-S712A cells. HEK-T3T-WT (solid trace) or HEK-T3T-S712A (broken trace) cells were incubated in the absence of added $Ca^{2+}$. Here, 5 $\mu$M $Gd^{3+}$ was present throughout the experiments to inhibit endogenous CCE. Methacholine (300 $\mu$M), and 2 mM $Ca^{2+}$ (top) or 2 mM $Ba^{2+}$ (bottom) was added where indicated. See text for statistical data.

(Goel et al., 2001; Sinkins et al., 2004) and the scaffolding protein, Homer (Yuan et al., 2003) (Fig. 3A). Recently published studies have suggested that trafficking of TRP channels is an important aspect of their regulation (Bezzerides et al., 2004; Cayouette et al., 2004; Morenilla-Palao et al., 2004; Singh et al., 2004). Thus, we considered the possibility that phosphorylation at this locus could prevent proper targeting to the plasma membrane or could cause channels to be internalized. However, we found no evidence of protein kinase C-mediated internalization or redistribution of fluorescently labeled TRPC3 channels, examined either by confocal microscopy or by total internal reflection fluorescence microscopy (data not shown).

Our data also suggest that the negative regulation of TRPC3 channel represents a physiological feedback mechanism associated with G protein-coupled receptor stimulation and that protein kinase C plays an important role in this regulatory mechanism. TRPC3 can be activated by exogenous application of diacylglycerol analogs (Hofmann et al., 1999) and its activation via phospholipase C-coupled receptors is probably caused by diacylglycerol production independently of $IP_3$, protein kinase C, or G proteins (Trebak et al., 2003a). After physiological stimulation of phospholipase C-coupled receptors, it is clearly established that the diacylglycerol produced from $PIP_2$ breakdown activates protein kinase C through binding to the C1 domain of protein kinase C (Nishizuka, 1992). TRPC3 represents an example of a cation channel that can be both positively and negatively regulated by the same second messenger (i.e., diacylglycerol) during physiological stimulation. The positive limb of this dual regulation of TRPC3 by diacylglycerol might occur as a direct action of diacylglycerol on the channel or through an intermediary effector protein, whereas the negative part would be mediated by protein kinase C. Protein kinase C activators as well as compounds interfering with the sequence containing $Ser^{712}$ might be useful in modulating TRPC3 activity and controlling $Ca^{2+}$ entry into cells. Of course, identifying cell types in which TRPC3 is a component of native channels activated via specific physiological stimuli would be very useful in strategies aimed at selective control of $Ca^{2+}$ homeostasis during disease. Understanding how the phosphorylation of $Ser^{712}$ interferes with TRPC3 activation will help to unravel the molecular mechanisms of TRPC3 activation and shed light on how TRPC3 contributes to $Ca^{2+}$ signaling in cells during physiological functions.

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