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Protein kinase C-mediated Ca^{2+} entry in HEK 293 cells transiently expressing human TRPV4

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1 We investigated whether protein kinase C (PKC) activation stimulates Ca^{2+} entry in HEK 293 cells transfected with human TRPV4 cDNA and loaded with fura-2.

2 Phorbol 12-myristate 13-acetate (PMA), a PKC-activating phorbol ester, increased the intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) in a dose-dependent manner, with an EC_{50} value of 11.7 nM. Exposure to a hypotonic solution (HTS) after PMA further increased $[Ca^{2+}]_i$. Two other PKC-activating phorbol esters, phorbol 12,13-didecanoate (PDD) and phorbol 12,13-dibutyrate, also caused $[Ca^{2+}]_i$ to increase.

3 The inactive isomer 4α-PMA was less effective and the peak $[Ca^{2+}]_i$ increase was significantly smaller than that induced by PMA. In contrast, 4α-PDD produced a monophasic or biphasic $[Ca^{2+}]_i$ increase with a different latency, while 4α-phorbol had no effect.

4 The PMA-induced $[Ca^{2+}]_i$ increase was abolished by prior exposure to bisindolylmaleimide (BIM), a PKC-specific inhibitor, and suppressed by the nonspecific PKC inhibitor 1-(5-isoquinolinesulphonyl)-2-methylpiperazine. The $[Ca^{2+}]_i$ increase induced by 4α-PMA, 4α-PDD or HTS was not significantly affected by BIM.

5 These results suggest that both PKC-dependent and -independent mechanisms are involved in the phorbol ester-induced activation of TRPV4, and the PKC-independent pathway is predominant in HTS-induced Ca^{2+} entry.
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Keywords: Calcium entry; phorbol esters; protein kinase C; TRPV channels

Abbreviations: BIM, bisindolylmaleimide X; $[Ca^{2+}]_i$, intracellular Ca^{2+} concentration; GFP, green fluorescence protein; H-7, 1-(5-isoquinolinesulphonyl)-2-methylpiperazine; HTS, hypotonic solution; MAPK, mitogen-activated protein kinase; PDBu, phorbol 12,13-dibutyrate; PDD, phorbol 12,13-didecanoate; PKC, protein kinase C; PMA, phorbol 12-myristate 13-acetate; TRP, transient receptor potential

Introduction

The concentration of intracellular free Ca^{2+} ($[Ca^{2+}]_i$) plays an important role in controlling a wide variety of cellular functions such as muscle contraction, secretion, proliferation, differentiation, gene expression and cell death. In vascular endothelial cells, mechanosensitive Ca^{2+} entry is essential for maintaining a cellular response to the constant mechanical stress caused by blood pressure and pulsatile flow. Although several mechanosensitive cation channels have been identified at the functional level in various types of endothelial cells (Jow & Numann, 1999; Nakao *et al*., 1999; Yoshikawa *et al*., 1999; Yao *et al*., 2000; Brakemeier *et al*., 2002), none has been identified at the molecular level.

Exposing cells to a hypotonic solution (HTS) is a form of mechanical stress that causes cells to swell. This also elicits an increase in $[Ca^{2+}]_i$ in many types of cells including endothelial cells (Oike *et al*., 1994; Chen *et al*., 1996; O'Neil & Leng, 1997; Yu & Sokabe, 1997; Altamirano *et al*., 1998; Viana *et al*., 2001). TRPV4 (also known as OTRPC4/VR-OAC/VRL-2/TRP12), which encodes a nonselective cation channel activated by hyposmotic stimulation, has been cloned in different species (Liedtke *et al*., 2000; Strotmann *et al*., 2000; Wissenbach *et al*., 2000; Delany *et al*., 2001). Since it is widely expressed in the kidneys, lungs, heart, brain and endothelial cells, TRPV4 is thought to contribute to osmo-mechanosensitive Ca^{2+} entry into these tissues. In addition, heat and some phorbol derivatives can induce a $[Ca^{2+}]_i$ increase in TRPV4-transfected cells and in native mouse aortic endothelial cells (Güler *et al*., 2002; Watanabe *et al*., 2002a, b). Although these findings suggest that the TRPV4 channel has functions in addition to responding to hyposmosis, its physiological roles and regulation remain to be determined.

Mechanical stimuli such as shear or cyclic stress produce an increase in protein kinase C (PKC) activity (Cheng *et al*., 2001; Wedgwood *et al*., 2001). Several studies have indicated that PKC is involved in the gene expression induced by mechanical stress in endothelial cells (Morita *et al*., 1994; Morawietz *et al*., 2000). PKC also modulates various ion channel activities. In rabbit renal epithelial cells, osmo-mechanically induced Ca^{2+} influx is augmented by phorbol 12-myristate 19-acetate (PMA), a PKC-stimulating phorbol ester, and is suppressed by staurosporine, a PKC inhibitor (O'Neil & Leng, 1997). In human umbilical vein endothelial cells, the single-channel current density and mechanosensitivity of a Ca^{2+}-permeable stretch-activated cation channel (SAC) is upregulated by

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laminar shear stress, and 1-(5-isoquinolinesulphonyl)-2-methylpiperazine (H-7), a nonselective PKC inhibitor, prevents this upregulation (Brakemeier *et al*., 2002). These results suggest that PKC also modulates mechanosensitive Ca^{2+} entry channels. The TRPV4 channel is activated by PMA or 4α-phorbol 12,13-didecanoate (4α-PDD) and this activation might be independent of PKC (Watanabe *et al*., 2002a), but the involvement of PKC in the PMA-induced activation of TRPV4 remains to be elucidated. In this study, we isolated TRPV4 from cultured human aortic endothelial cells, and investigated whether PKC activation stimulates Ca^{2+} entry into human TRPV4-transfected HEK 293 cells loaded with fura-2.

Methods

Cloning TRPV4 from cultured human aortic endothelial cells

Total RNA prepared from cultured human aortic endothelial cells (HAECs, Clonetics) using RNeasy (QIAGEN) was reverse transcribed using a cDNA Cycle® kit (Invitrogen). The full-length coding region of human TRPV4 (hTRPV4) cDNA was amplified using an RT–PCR protocol. The forward and reverse primers were 5′-ctcgagATGGCG-GATTCCAGCGAAGGC-3′ and 5′-ggatccCTAGAGCGGG-GCGTCATCAGTC-3′, respectively, with lower case letters indicating additional *Xho*I and *Bam*HI sites. The PCR sequence consisted of five cycles of 30 s at 95°C, 30 s at 65°C and 3 min at 68°C, followed by 30 cycles of 30 s at 95°C and 3 min at 68°C. The PCR products were confirmed by sequencing (ABI-PRISM-310, Applied Biosystems).

DNA construction, cell culture and transfection

HEK 293 cells, obtained from the Health Science Research Resources Bank (HSRRB, Osaka, Japan; No. JCRB9068), were cultured in Dulbecco's modified Eagle's medium (Invitrogen) containing 10% fetal bovine serum (Sigma) and an antibiotic/antimycotic (Invitrogen), under 5% CO_2 at 37°C. After reaching subconfluence, the cells were detached with 0.25% trypsin–EDTA (Sigma), diluted and reseeded on coverslips ($9 \times 9\,mm^2$) coated with poly-D-lysine (Sigma). For a functional study, human TRPV4 cDNA was subcloned into the pIRES2-EGFP vector (CLONTECH), which contains the internal ribosome entry site (IRES) of the encephalomyocarditis virus (ECMV) between the multiple cloning sites and the EGFP coding region. This permits the TRPV4 gene and the EGFP gene to be translated from a single bicistronic mRNA. Cells were transfected using LIPOFECTAMINE™ PLUS Reagent (Invitrogen), and GFP fluorescence was used as a marker for transfection. Cells transfected with untreated pIRES2-EGFP vector were used as mock controls. Intracellular $[Ca^{2+}]_i$ was measured 36–60 h after transfection.

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

We measured $[Ca^{2+}]_i$ using the fluorescent Ca^{2+} indicator fura 2-acetoxymethyl ester (fura 2-AM) as previously described (Satoh *et al*., 2002). Briefly, cells growing on coverslips were incubated in an isotonic solution containing 5 μM fura 2-AM at 37°C for 30 min in the dark, then washed and incubated for 15 min to hydrolyse internalized fura 2-AM. We measured $[Ca^{2+}]_i$ in single cells that emitted GFP fluorescence, using a spectrofluorometer (CAM-230, Japan Spectroscopic, Tokyo, Japan) at wavelengths of 340 and 380 nm (excitation), and 510 nm (emission). The fluorescence ratio (F_{340}/F_{380}) provided a relative measure of $[Ca^{2+}]_i$. All experiments were conducted at 37°C.

Solutions and drugs

Cells were normally superfused with a solution containing (mM): 88 NaCl, 5 KCl, 5.5 glucose, 1 $CaCl_2$, 10 HEPES and 100 mannitol, adjusted to pH 7.4 with NaOH (300 mosm kg^{-1} H_2O). The HTS was adjusted to 200 mosm kg^{-1} H_2O by omitting mannitol. Osmolarity was confirmed using an advanced osmometer (Model 3W, Advanced Instruments Inc., MA, U.S.A.). Fura 2-AM was purchased from Dojindo Laboratories (Kumamoto, Japan). PMA, PDD, phorbol 12,13-dinbutyrate (PDBu), 4α-PMA, 4α-PDD, 4α-phorbol, bisindolylmaleimidex (BIM) and H-7 were obtained from Sigma (St Louis, MO, U.S.A.). We purchased 2′-amino-3′-methoxyflavone (PD-98059) from Biomol (Plymouth Meeting, PA, U.S.A.). Stock solutions of phorbol esters were initially prepared in dimethyl sulphoxide (DMSO) at a concentration of 1 mM, and stored at −20°C. The final concentration of DMSO in the experimental bath solution containing phorbol esters never exceeded 0.5%.

Statistics

Data are shown as means ± standard error of the mean (s.e.m.). Statistical significance was determined using Student's *t*-test or Welch's *t*-test. When we compared the effects of 4α-PMA with those of PMA, we used the Mann–Whitney test. Values of $P < 0.05$ were considered statistically significant.

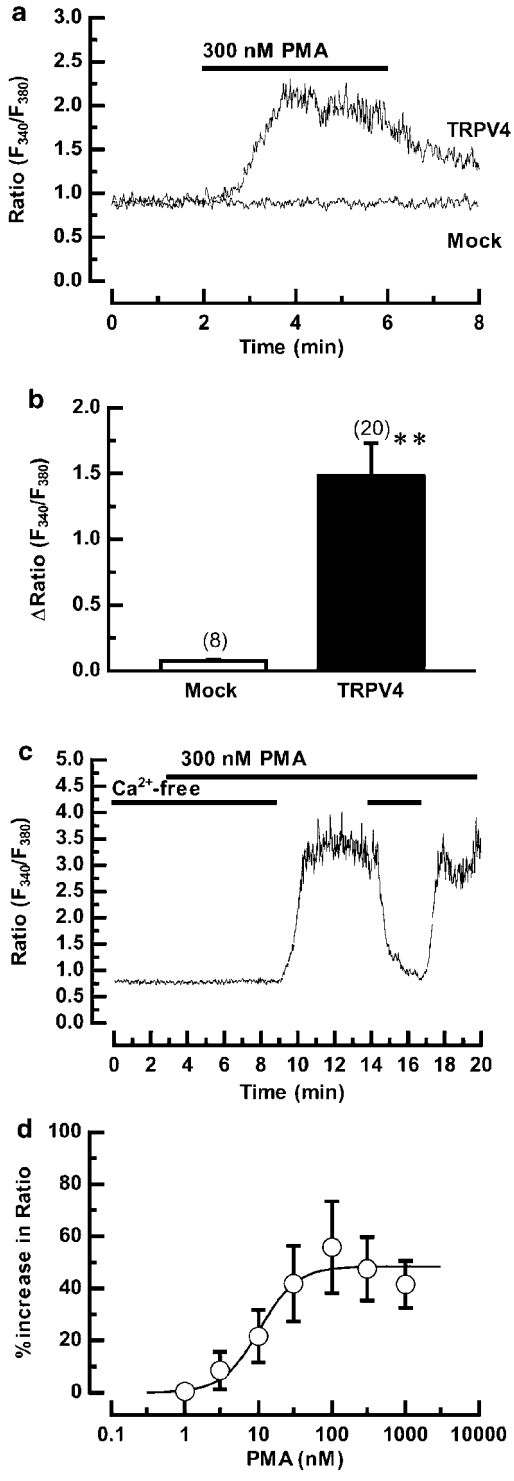
Results

Effects of phorbol esters that activate PKC on $[Ca^{2+}]_i$ in HEK 293 cells expressing hTRPV4

Using RT–PCR cloning of cultured HAEC cDNA, we identified TRPV4 corresponding to the Genebank accession number AF258465 and an alternative splice variant (No. AB073669) lacking 180 nucleotides, and which corresponded to the sixth exon of the human TRPV4 genomic clone (No. AC007834). Since the shorter variant did not respond to HTS, we did not characterize this protein further.

The initial studies were designed to examine the effects of phorbol esters that activate PKC on $[Ca^{2+}]_i$ in HEK 293 cells transiently transfected with TRPV4. Figure 1a shows that 300 nM PMA elicited a monophasic increase in $[Ca^{2+}]_i$. The $[Ca^{2+}]_i$ reached a peak 150.8 ± 12.5 s after stimulation with PMA, and the mean maximal Ca^{2+} ratio increase was 1.48 ± 0.25 ($n = 20$, Figure 1c). In contrast, the mock-transfected cells did not respond to PMA (0.074 ± 0.012, $n = 8$). To further determine whether the PMA-induced calcium increase resulted from calcium influx, we examined the effects of PMA in solution depleted of extracellular Ca^{2+}. As shown in Figure 1c, PMA had no effect in Ca^{2+}-free solution, and

readdition of Ca^{2+} caused a robust rise in $[Ca^{2+}]_i$, which depended on extracellular Ca^{2+}. These data suggest that the PMA-induced $[Ca^{2+}]_i$ rise in TRPV4-transfected cells depends on extracellular Ca^{2+}. The responses were concentration-dependent, with an EC_{50} value of 11.7 nM (Figure 1d), which was >100-fold greater than previously reported values (Watanabe *et al*., 2002a). The other two PKC-activating phorbol esters tested, PDD and PDBu (300 nM each), also elicited a monophasic $[Ca^{2+}]_i$ increase (Figure 2a and b). Even at 0.1 nM, PDD obviously increased $[Ca^{2+}]_i$ in cells transfected with TRPV4 (Figure 2aii). The order of potency of these phorbol esters was PDD > PMA > PDBu.



Effects of PKC-inactivating phorbol esters on $[Ca^{2+}]_i$ in HEK *293 cells expressing hTRPV4*

Despite their wide use in studies of PKC, the specific action of phorbol esters is largely unknown. We next compared the effects of the active phorbol esters on TRPV4 channel activation with those of their inactive isomers, 4α-PMA, 4α-PDD and 4α-phorbol. Compared with the PMA-induced $[Ca^{2+}]_i$ response, a longer period was required for 4α-PMA to elicit a response (Figure 3a). As shown in Figure 3ai, 300 nM 4α-PMA failed to elicit a significant increase in $[Ca^{2+}]_i$ in most of the TRPV4-transfected cells (16 out of 27 cells); the response observed in six of the remaining cells was biphasic (typical traces shown in Figure 3aii). The mean maximal $[Ca^{2+}]_i$ (F_{340}/F_{380}) increase elicited by 4α-PMA was 0.67 ± 0.19 ($n = 27$, Figure 3d), which was significantly smaller than that induced by PMA (Figure 3d). In contrast, 4α-PDD caused an obvious $[Ca^{2+}]_i$ increase in all cells, but the time course varied among individual cells (Figure 3b). Figure 3bi shows typical traces indicating that the response was monophasic in five of 19 cells. The response to 4α-PDD in the remaining 14 cells was biphasic (Figure 3bii). $[Ca^{2+}]_i$ reached a peak 286.1 ± 41.8 s after stimulation with 4α-PDD, and the mean maximal $[Ca^{2+}]_i$ (F_{340}/F_{380}) increase was 2.24 ± 0.31 ($n = 19$, Figure 3d). Thus, the mean time-to-peak effect of 4α-PDD was significantly longer than that of PMA ($P < 0.01$). The third phorbol ester tested, 4α-phorbol, did not elicit an increase in $[Ca^{2+}]_i$ (Figure 3c, d). The order of potency of these phorbol esters was 4α-PDD > 4α-PMA > 4α-phorbol. These results suggested that the 4α-isomers of phorbol esters mediate activation of TRPV4 by a different mechanism from that used by the PKC-activating phorbol esters.

Assessment of the involvement of PKC in calcium entry induced by phorbol esters

To gain a better understanding of the role of PKC, we studied the effect of BIM, a selective PKC inhibitor, on Ca^{2+} entry induced by phorbol esters. We compared the effects of the phorbol esters in the presence or absence of BIM. The

Figure 1 Effects of the PKC-activating phorbol ester PMA on intracellular calcium concentration ($[Ca^{2+}]_i$; expressed as F_{340}/F_{380} values) in hTRPV4-transfected HEK 293 cells. (a) Representative trace showing the effect of 300 nM PMA in hTRPV4-transfected or pIRES2-EGFP vector-transfected (mock) HEK 293 cells. (b) Summary of $[Ca^{2+}]_i$ increases caused by PMA. Each column represents mean ± s.e.m.; numbers of cells examined are indicated above each column. $^{**}P < 0.01$ vs mock. (c) Role of extracellular calcium in the PMA-induced calcium increase in hTRPV4-transfected HEK 293 cells. Cells were exposed to 300 nM PMA in the presence or absence of 1 mM extracellular calcium. (d) Concentration–response relationship of the PMA-induced Ca^{2+} influx in HEK 293 cells expressing hTRPV4. Responses were measured as peak increases in fluorescence ratio minus basal level, and are expressed relative to the maximal response evoked by HTS after PMA application. The continuous line was fitted with the Hill equation. Number of observations per point was 6–11.

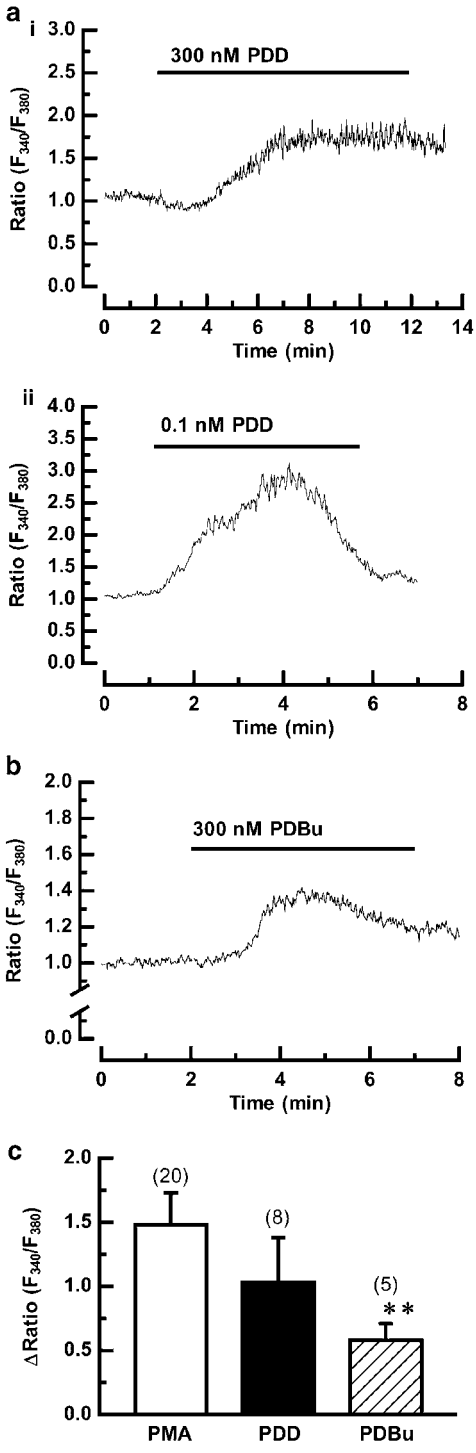


Figure 2 Effects of the PKC-activating phorbol esters PDD and PDBu on $[Ca^{2+}]_i$ in hTRPV4-transfected HEK 293 cells. (a) Representative trace showing the effect of PDD at 300 nM (i) and 0.1 nM (ii). (b) Representative trace showing effect of 300 nM PDBu. (c) Summary of $[Ca^{2+}]_i$ increases caused by the phorbol esters. Each column represents mean $\pm$ s.e.m.; numbers of cells examined are indicated above each column. $^{**}P<0.01$ vs PMA.

experiments were performed on the same day to minimize variation in the parental population of HEK 293 cells used for transient expression. Prior incubation with BIM alone did not affect the basal calcium level, but abolished the PMA-induced Ca^{2+} entry (Figure 4a). In contrast, the 4α-PMA- or 4α-PDD-induced increases in $[Ca^{2+}]_i$ were not significantly affected by BIM (Figure 4b–d), but the mean time-to-peak effect of 4α-PDD was significantly prolonged in the presence of BIM ($P<0.05$; 157.9 ± 16.1 s in control, 314.3 ± 63.5 s in BIM-pretreated cells). The nonselective PKC inhibitor H-7 also suppressed the $[Ca^{2+}]_i$ responses induced by PMA, but not those induced by 4α-PDD (Figure 5). These results indicate that the effect of PMA on the activation of hTRPV4 channels is mainly due to activation of PKC.

Effects of PKC inhibitors on HTS-induced calcium entry into HEK *293 cells expressing hTRPV4*

Since exposure to HTS elicits Ca^{2+} entry *via* TRPV4 channels, we examined whether PKC activation is linked to HTS-induced Ca^{2+} entry. When TRPV4-transfected cells were exposed to HTS, the transient increase in $[Ca^{2+}]_i$ was monophasic, and depended on extracellular Ca^{2+} (Figure 6a, c), and the mean maximal $[Ca^{2+}]_i$ (F_{340}/F_{380}) increase was 1.24 ± 0.19 ($n=23$, Figure 6b). In contrast, the mock-transfected cells did not respond to HTS (0.034 ± 0.008, $n=14$). After the $[Ca^{2+}]_i$ increase induced by 300 nM PMA was saturated, HTS increased $[Ca^{2+}]_i$ further to the maximal level (Figure 6d). BIM failed to inhibit the HTS-induced Ca^{2+} entry (Figure 6e, f). Moreover, HTS also caused $[Ca^{2+}]_i$ to increase in cells that had been preincubated with 1 μM PMA for 18 h to downregulate PKC (data not shown). Preincubation with 3 μM PMA for 6 h abolished the 4α-PDD-induced $[Ca^{2+}]_i$ increase, but did not affect that induced by HTS (Figure 7a). These results suggest that the HTS-induced $[Ca^{2+}]_i$ increase is independent of PKC- or 4α-PDD-sensitive activation of TRPV4 channels. Both PKC and HTS activate mitogen-activated protein kinase (MAPK; Traub *et al*., 1997; Shen *et al*., 2001). We therefore investigated the effect of PD98059, a specific MAPK inhibitor, on PMA- and HTS-induced Ca^{2+} entry (Figure 7b). We found that 30 μM PD98059 had no effect on subsequent Ca^{2+} responses.

Discussion

In the present study, we found that PKC activation stimulates Ca^{2+} entry in HEK 293 cells transiently transfected with human TRPV4. This view is based on the finding that the EC_{50} value for PMA was >100-fold greater than that reported in a previous study by Watanabe *et al*. (2002a), and that, of the phorbol esters tested by us, only the PMA-induced $[Ca^{2+}]_i$ increase was prevented by PKC inhibitors. Moreover, 4α-PMA, which was a negative control for PMA, had little or no effect in 16 of 27 cells when used at the same concentration. Since 4α-PMA is a stereoisomer of PMA with similar lipophilicity and aqueous solubility, our results suggest that PMA increases $[Ca^{2+}]_i$ in a PKC-dependent manner.

However, in their study, Watanabe *et al*. (2002a) found that Ca^{2+} entry through TRPV4 was independent of PKC. In our study, PDD and PDBu, as well as PMA, obviously increased $[Ca^{2+}]_i$. Although the reason for this discrepancy is unknown, it might be related to the sensitivity of TRPV4 to heat. Recent studies have shown that TRPV4 can be activated by

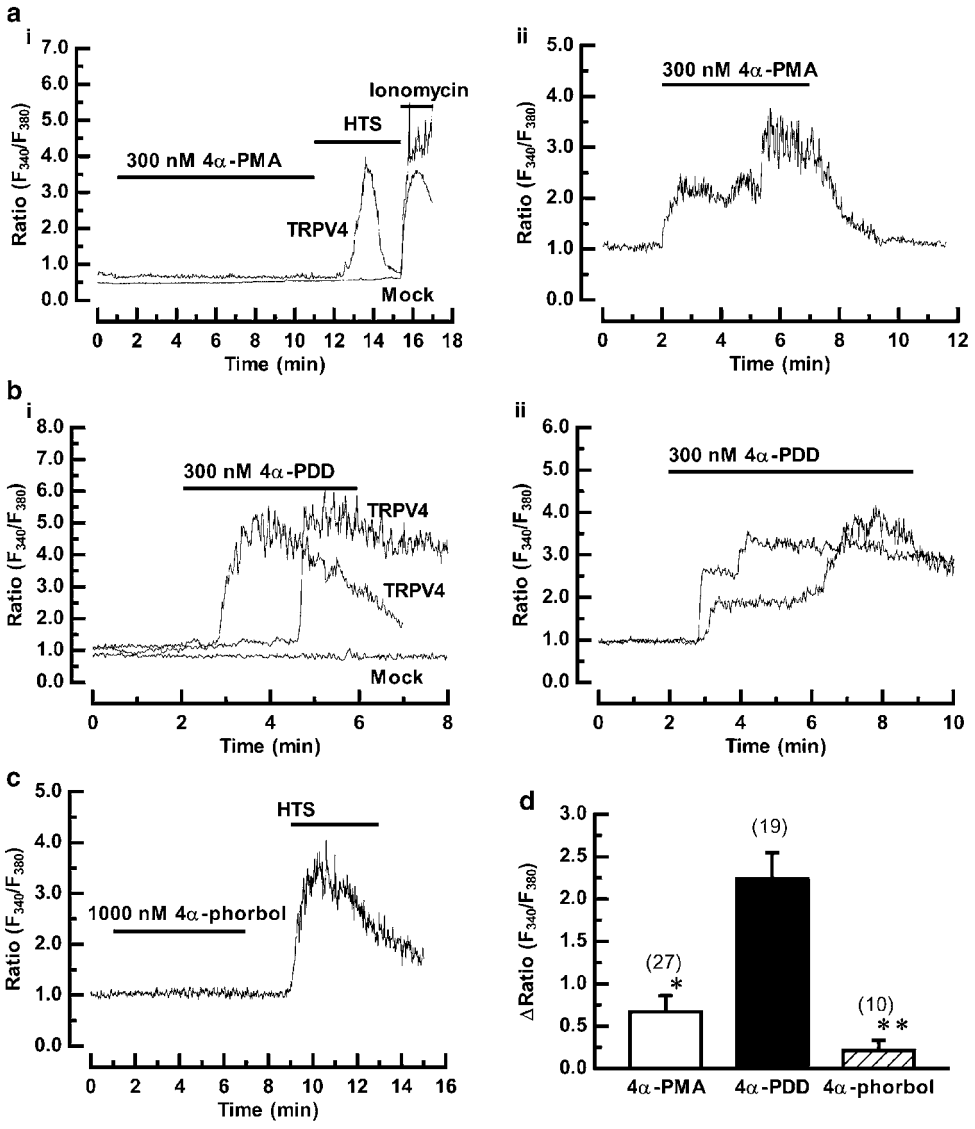
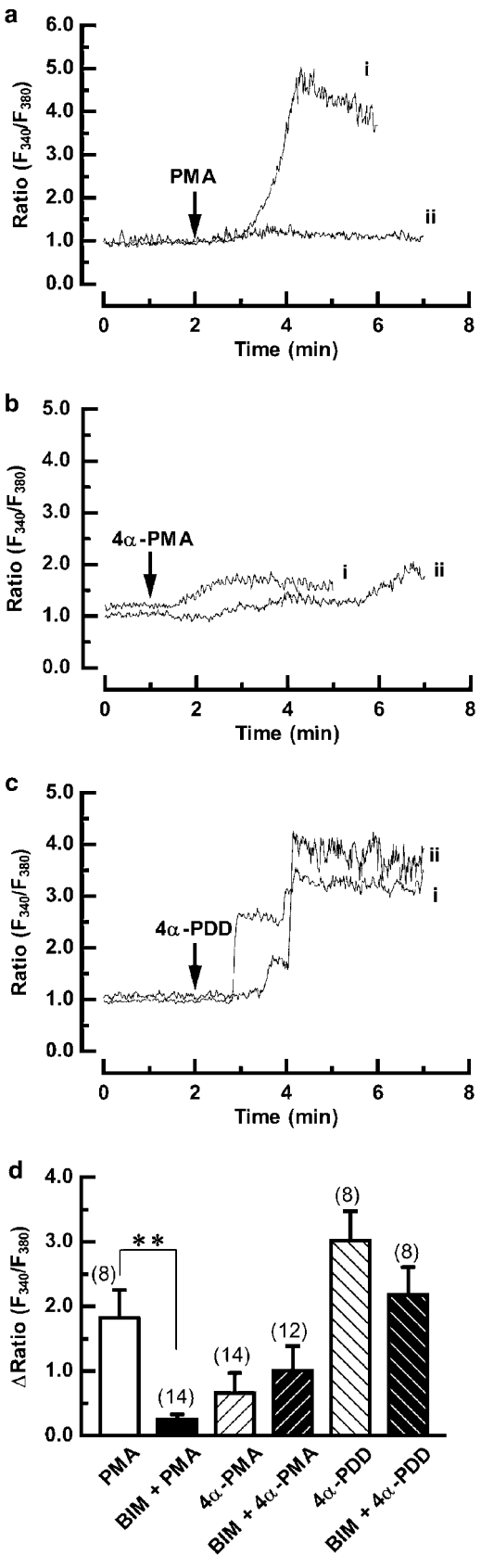


Figure 3 Effects of non-PKC-activating phorbol esters on intracellular calcium concentration ($[Ca^{2+}]_i$; expressed as F_{340}/F_{380} values) in hTRPV4- or mock-transfected HEK 293 cells. Representative traces showing the effect of 300 nM 4α-PMA (a), 300 nM 4α-PDD (b) and 1000 nM 4α-phorbol (c) on $[Ca^{2+}]_i$. (ai) Typical trace showing that in 16 out of 27 cells expressing TRPV4, 300 nM 4α-PMA induced no significant $[Ca^{2+}]_i$ increase. (aii) Trace showing that in six cells expressing TRPV4, 300 nM 4α-PMA induced a biphasic $[Ca^{2+}]_i$ increase. HTS used in the present experiment was 200 mOsm/kg H_2O. Ionomycin (1 μM) plus 10 mM $CaCl_2$ was applied to obtain maximal $[Ca^{2+}]_i$. (b) 4α-PDD (300 nM) induced monophasic (bi) and biphasic (bii) $[Ca^{2+}]_i$ increases in cells expressing TRPV4. (d) Summary of $[Ca^{2+}]_i$ increases caused by non-PKC-activating phorbol esters. Each column represents mean ± s.e.m.; numbers of cells examined are indicated above each column. $^*P<0.05$ and $^{**}P<0.01$ vs 300 nM PMA. The comparison between the effects of 4α-PMA and PMA was made using the Mann–Whitney test.

temperatures of 24°C (Watanabe *et al*., 2002b), or above 34°C (Güler *et al*., 2002). All our experiments were conducted at 37°C, whereas the experiments of Watanabe *et al*. (2002a) were performed at 20–23°C. Therefore, TRPV4 channels may be fundamentally active and more susceptible to PKC activation at 37°C. Just recently, during the revision of our manuscript, a paper which shows that PMA activates murine TRPV4 channels *via* a PKC-dependent mechanism at 37°C appeared online (Gao *et al*., 2003). These results provide support for our present observations and our hypothesis that temperature may be a crucial factor for PKC-mediated activation of TRPV4. Another explanation for the difference between our results and those of Watanabe *et al*. (2002a) may be the fact that we used HEK 293 cells transiently transfected with human TRPV4. Using TRPV1 (VR1), another of the TRPV family, Vellani *et al*. (2001) showed that PKC activation caused quite different effects in stable and transient expression systems. PMA alone activated TRPV1 channels in transiently, but not in stably transfected, cells. Watanabe *et al*. (2002a) characterized human TRPV4 (VRL-2) in stably transfected 1321N1 astrocytoma cells; hence, the different transfection system and cells might produce different results. However, they also characterized murine TRPV4 (TRP12) in transiently transfected HEK293 cells, and did not observe any PKC-dependent activation. The reported species difference in pharmacology between rat and human TRPV1 (McIntyre *et al*., 2001) suggests an explanation



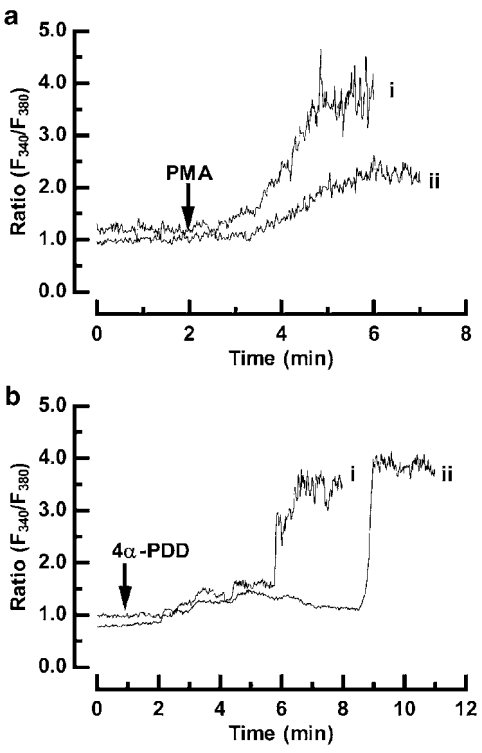


Figure 5 Effect of H-7 on the PMA- and 4α-PDD-induced calcium increases. Traces showing the PMA-induced (a) and 4α-PDD-induced (b) $[Ca^{2+}]_i$ increase without (i) or with (ii) 30 μM H-7. H-7 was applied 60 min before stimulation with phorbol esters, and was present throughout the experiments. Data are representative of three experiments.

for this discrepancy between their results and ours, but this may not hold true for TRPV4, because the predicted human and murine TRPV4 protein sequences have 96% homology (Wissenbach *et al*., 2000).

In agreement with the results of Watanabe *et al*. (2002a), we found that 4α-PDD caused an obvious $[Ca^{2+}]_i$ increase at concentrations above 10 nM, but was at least 100-fold less potent than PDD. Moreover, unlike PMA and PDD, 4α-PDD elicited highly variable $[Ca^{2+}]_i$ responses among cells (Figure 3b). In particular, none of the responses to PMA and PDD was biphasic, whereas 4α-PMA elicited a biphasic $[Ca^{2+}]_i$ increase, and the PKC inhibitor BIM did not affect the peak responses to 4α-PMA or 4α-PDD. These results suggest that the $[Ca^{2+}]_i$ increase induced by 4α-PMA or 4α-PDD is independent of PKC and specific for the 4α-stereoisomer. This notion is supported by the finding that PMA in the presence of BIM caused a biphasic $[Ca^{2+}]_i$ increase (Figure 6e). The order of potency of the 4α-stereoisomers

Figure 4 Effect of inhibition of PKC on the phorbol ester-induced calcium increase. Representative traces showing $[Ca^{2+}]_i$ responses to PMA (a), 4α-PMA (b) and 4α-PDD (c) without (i) or with (ii) 100 nM BIM treatment. BIM was applied 60 min prior to the start of $[Ca^{2+}]_i$ measurement, and was present throughout the experiment. (d) Summary of $[Ca^{2+}]_i$ increases caused by phorbol esters with or without BIM. Each column represents mean ± s.e.m.; numbers of cells examined are indicated above each column. $^{**}P<0.01$.

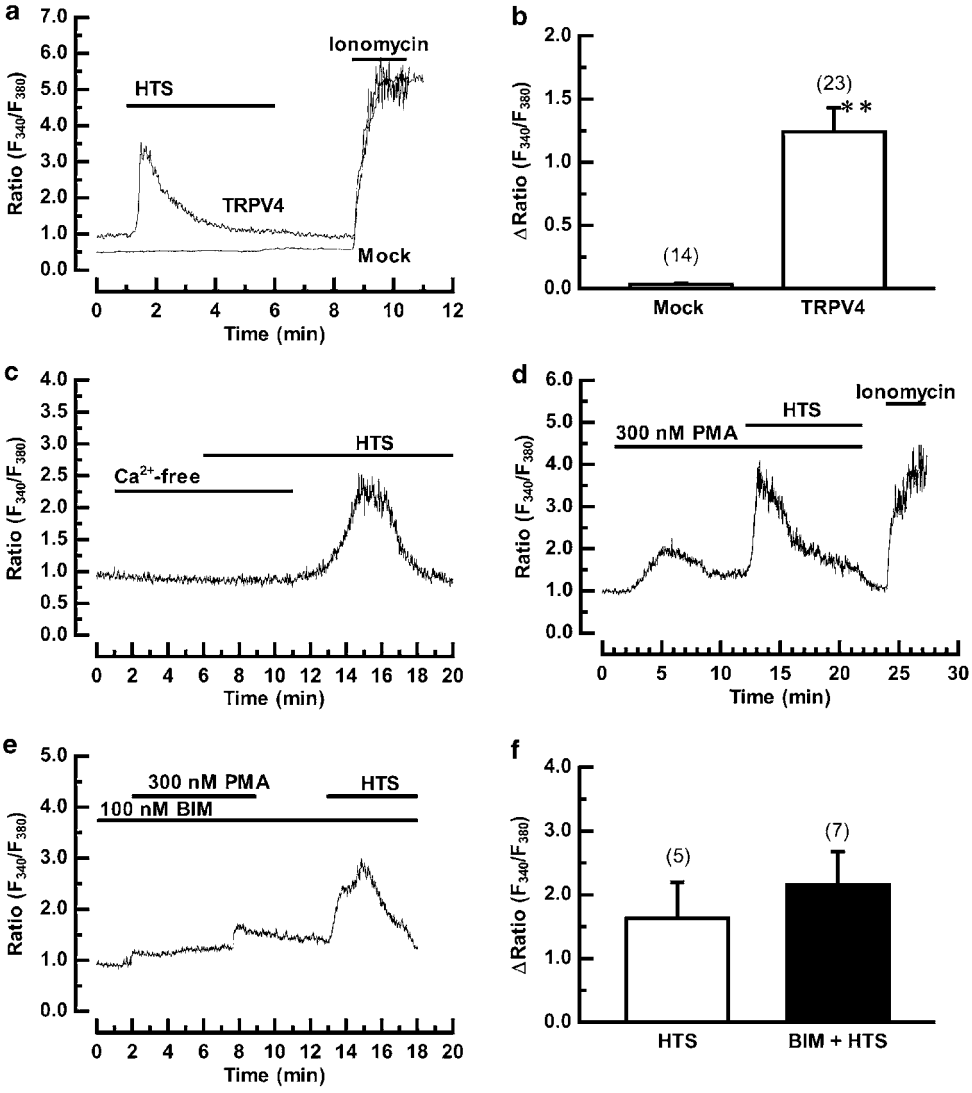


Figure 6 Effect of the specific PKC inhibitor BIM on the hypotonicity-dependent calcium increase. (a) Representative traces showing $[Ca^{2+}]_i$ responses to HTS in hTRPV4- and mock-transfected cells. (b) Summary of $[Ca^{2+}]_i$ increases caused by HTS in TRPV4- and mock-transfected cells. Each column represents mean ± s.e.m.; numbers of cells examined are indicated above each column. (c) Representative trace showing the effect of extracellular Ca^{2+} on the HTS-induced calcium increase in hTRPV4-transfected HEK 293 cells; extracellular Ca^{2+} concentration 1 mM; 1 μM ionomycin plus 10 mM $CaCl_2$ was applied to obtain maximal $[Ca^{2+}]_i$. (d) Whne cells were preincubated with 300 nM PMA, HTS still caused a robust $[Ca^{2+}]_i$ increase. (e) Representative traces showing the PMA- and HTS-induced $[Ca^{2+}]_i$ increases in the presence of BIM applied 60 min before and during stimulation with phorbol esters or HTS. (f) Summary of $[Ca^{2+}]_i$ increases caused by HTS without or with BIM. Each column represents mean ± s.e.m.; numbers of cells examined are indicated above each column.

was 4α-PDD > 4α-PMA > 4α-phorbol, which corresponded to their lipophilicity and water solubility. The Ca^{2+} entry induced by the 4α-stereoisomers might also require an optimal length of fatty acid side chains. Although, at present, the activation mechanism of 4α-PDD remains unclear, PKC stimulation might play a role in the early Ca^{2+} entry mechanism. Firstly, the mean time-to-peak effect of 4α-PDD was significantly prolonged in the presence of BIM. Secondly, long-term incubation with PMA abolished any subsequent response to 4α-PDD. These results might mean that high concentrations of 4α-PDD partly activate PKC, and produce a Ca^{2+} entry response similar to that elicited by PDD.

We isolated full-length TRPV4 from cultured human aortic endothelial cells. Since Watanabe *et al.* (2002a, b) have identified TRPV4-like channels that are activated by 4α-PDD or heat in mouse aortic endothelial cells, it seems probable that TRPV4 channels are involved in the physiological functions of these cells. Originally, TRPV4 channels were considered to be Ca^{2+}-permeable sensors of cell volume. The supposed heat sensitivity of these channels has been implicated in the maintenance of normal endothelial function (Watanabe *et al.*, 2002b). The present results indicate that TRPV4 can be activated by PKC stimulation. Since PKC is involved in mechanical stress-induced cellular responses, TRPV4 probably contributes to mechanosensitive Ca^{2+} entry in endothelial cells. For instance, TRPV4-mediated Ca^{2+} entry might increase nitric oxide (NO) synthesis in response to shear stress, because endothelial NO synthase (eNOS) gene expression is induced by shear stress *via* the PKC signalling pathway (Wedgwood *et al.*, 2001).

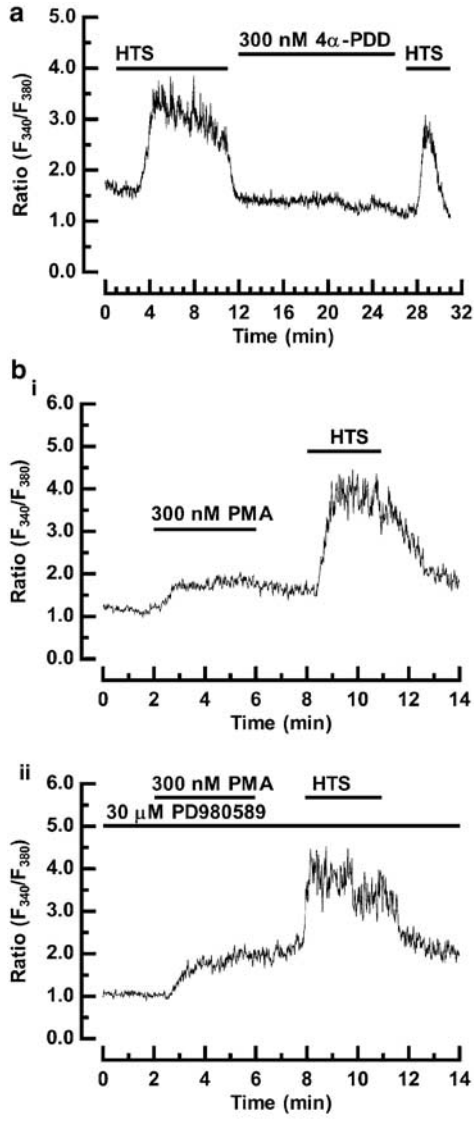


Figure 7 (a) Traces showing the effects of preincubation with 3 μM PMA on HTS- and 4α-PDD-induced calcium entry in hTRPV4-transfected cells. Only the 4α-PDD-induced calcium increase was abolished. (b) Traces showing the $[Ca^{2+}]_i$ increase induced in the absence (i) or presence of PD98059 (ii) applied 60 min before and during stimulation with PMA and HTS. Results are representative of three experiments.

Signalling by HTS has been well studied in diverse models, but the sensor for hypotonic stimuli and the corresponding gating mechanisms still remain elusive. In rabbit renal epithelial cells, HTS caused an increase in $[Ca^{2+}]_i$ that was augmented by PMA and suppressed by staurosporine, a PKC inhibitor (O'Neil & Leng, 1997). These findings suggest that osmo-mechanosensitive Ca^{2+} entry is predominantly regulated *via* the PKC signalling pathway in these cells. However, this mechanism is unlikely to operate with TRPV4 channels. Although in the present study PMA similarly augmented the HTS-induced $[Ca^{2+}]_i$ increase (Figure 6d), neither BIM nor long-term prior exposure to PMA inhibited the HTS-induced $[Ca^{2+}]_i$ increase. In TRPV1 channels, PKC activation has also been reported to enhance the currents activated by diverse stimuli including capsaicin, heat, anandamide and proton (Premkumar & Ahern, 2000; Vellani *et al*., 2001). Thus, PKC may modulate TRPV4 channels or sensitize them to hypotonic stimulation. The lack of dependence of the HTS-induced Ca^{2+} entry on PKC raised the question of how HTS leads to a $[Ca^{2+}]_i$ increase in TRPV4-transfected HEK cells. One might speculate that HTS caused a $[Ca^{2+}]_i$ increase *via* a mechanism similar to 4α-PDD. However, we showed that, unlike 4α-PDD, HTS increased $[Ca^{2+}]_i$ with a short latency. In addition, HTS caused a $[Ca^{2+}]_i$ increase in 4α-PDD-insensitive cells (Figure 7a). These results indicate that 4α-PDD and HTS do not induce Ca^{2+} entry *via* a common mechanism. As the other signalling pathway, it has been reported that mechanical stress and PKC stimulation activate MAPK (Traub *et al*., 1997; Shen *et al*., 2001). We, therefore, investigated the possible involvement of MAPK in HTS-induced Ca^{2+} entry using the specific MAPK inhibitor PD98059, and found that PD98059 had no effect on the HTS- and PMA-induced Ca^{2+} responses. Thus, MAPK is probably not involved in activation of the TRPV4 channel. In a recent paper, Xu *et al*. (2003) showed that Src family tyrosine kinase-dependent tyrosine phosphorylation is implicated in HTS-induced Ca^{2+} entry *via* TRPV4. Therefore, a pathway involving tyrosine kinase may be important in HTS-induced channel activity.

In conclusion, we have shown that activation of hTRPV4 channels by phorbol esters can be either PKC-dependent or -independent. The present results suggest that TRPV4, like TRPV1, can be modulated by diverse stimuli which act *via* different mechanisms. Although the physiological role of TRPV4 channels in native cells and the interconnection between the various signalling pathways remain obscure, PKC activation may be involved in a range of signalling pathways during the TRPV4 gating process.

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