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Sensitization of Vanilloid Receptor Involves an Increase in the Phosphorylated Form of the Channel

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A vanilloid receptor (VR1, now known as TRPV1) is an ion channel activated by vanilloids, including capsaicin (CAP) and resiniferatoxin (RTX), which are pungent ingredients of plants. Putative endogenous activators (anandamide and metabolites of arachidonic acid) are weak activators of VR1 compared to capsaicin and RTX, and the concentrations of the physiological condition of those activators are not sufficient to induce significant activation of VR1. One way to overcome the weak activation of endogenous activators would be the sensitization of VR1, with the phosphorylation of the channel being one possibility. The phosphorylation of VR1 by several kinases has been reported, mostly by indirect evidence. Here, using an in vivo phosphorylation method, the VR1 channel was shown to be sensitized by phosphorylation of the channel itself by multiple pathways involving PKA, PKC and acid. Also, in sensitizing VR1, BK appeared to show activation of PKC for the sensitization of VR1 by phosphorylation of the channel.

Key words: Vanilloid receptor, Phosphorylation, *In vivo*, Kinase activators and inhibitors, Sensitization

INTRODUCTION

The capsaicin receptor is a non-selective cation channel that is activated by the so-called 'vanilloids', capsaicin (CAP) and its analogues (Oh *et al.*, 1996). The cDNA of the CAP-activated channel, known as vanilloid receptor 1, VR1 or TRPV1, was cloned and characterized as a nonselective ion channel that is activated by noxious temperature, acid and vanilloids (Caterina *et al.*, 1997). Disruption of the VR1 gene reduces the thermal hyperalgesia induced by inflammation (Caterina *et al.*, 2000; Davis *et al.*, 2000), suggesting that VR1 is essential for the thermal hyperalgesia. In addition, several endogenous activators of the channel have been identified, for example, lipoxygenase products, HETES (hydroxyeicosatetraenoic acid) and anandamide (Hwang *et al.*, 2000; Zygmunt *et al.*, 1999; Smart *et al.*, 2000). Even though they are able to activate the channel, the effective concentration to open the channel is far above that inside the cell. For example, anandamide needs a 10 to 100- fold greater concentration than CAP (Smart *et al.*, 2000), and 12-HEPTE needs about an eight fold greater concentration than CAP (Hwang *et al.*, 2000). Therefore, the required sensitization or activation of the channel has been suggested (Lazar *et al.*, 2004).

In this sense, anandamide (ANA) has been reported to be able to stimulate sensory VR1 by the concomitant activation of protein kinase A (PKA) (De Petrocellis *et al.*, 2001). The EC_{50} of the Ca^{++} rise in the cytoplasm mediated by VR1 due to anandamide was significantly decreased by the PKA activators, 8-Br-cAMP and forskolin (FSK) (De Petrocellis *et al.*, 2001). In addition to the response of VR1 to ligands, Rathee *et al.* reported that heat-activated ionic currents were potentiated after exposure to forskolin in rat nociceptive neurons (Rathee *et al.*, 2002).

Conversely, PKC-mediated phosphorylation of VR1 was also suggested to sensitize the proton- and ligand-induced stimulations of VR1. For example, a strong (10-15 fold) enhancement of the anandamide action on VR1-gated currents by PKC has also been reported (Prekumar and

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Ahren, 2000). Stimulation of PKC alone, in the absence of a ligand, elevated temperature or decreased pH, was able to induce VR1 activation (Di Marzo *et al.*, 2001). Tominaga *et al.* also reported that PKC epsilon directly phosphorylated VR1, and reported that two serine residues were involved in the phosphorylation (Numazaki *et al.*, 2002). PKCα was also reported to be involved in enhancing pain during inflammation by activating VR1 (Olah *et al.*, 2002).

We also reported that the VR1 activity was controlled by Ca^{2+}-calmodulin dependent kinase II (CaMKII) and calcineurin through ligand binding (Jung *et al.*, 2004). Therefore, VR1 seems to be controlled by a wide range of excitabilities in response to various adverse stimuli *via* protein kinase A, C and CaMKII.

Bradykinin (BK) has long been known as a mediator of inflammatory pain (Cortright and Szallasi, 2004). Recently, BK was suggested to activate VR1 *via* lipoxygenase pathway (Shin *et al.*, 2002). BK has been reported to increase the level of phosphorylation of VR1, most likely via the PKC pathway, as the activator, PMA, increased the phosphorylation of VR1 (Hong and Wiley, 2005). In addition, bisindolylmaleimide (BIM, Toullec *et al.*, 1991), a specific inhibitor of PKC, significantly decreased the level of VR1 phosphorylation induced by BK, suggesting that VR1 is phosphorylated by BK *via* PKC.

VR1 has been identified as being desensitized with the repeated application of CAP (Jung *et al.*, 2004) in a Ca^{++} dependent manner. This desensitization was suggested to involve phosphorylation/dephosphorylation involving calcineurin (Docherty *et al.*, 1996). However, sensitization of the channel by phosphorylation has mostly been suggested by indirect evidence of the channel phosphorylation. Here, using an *in vivo* phosphorylation study, VR1 has been shown to be sensitized by a mechanism of phosphorylation of the channel by multiple pathways involving PKA, PKC and acid. Also, in sensitizing VR1, BK appeared to show activation of PKC in the sensitization of VR1 due to phosphorylation of the channel.

MATERIALS AND METHODS

In vivo phosphorylation

Human embryonic kidney (HEK) 293T cells were transfected with the expression vector of VR1, its derivatives and rat bradykinin receptor 2 (B_2R), using Lipofectamine PLUS (Life Technologies), as suggested by the manufacturer. Rat VR1 was cloned as previously described (Jung *et al.*, 1999). Rat B_2R was cloned into pcDNA3 by RT-PCR from mRNA isolated from the rat brain, using the primers, 5'-cccaagcttgccgccaccatgttcaacatcaccacg-3' and 5'-ccggtacctcactgcttgttccccgc-3'. Approximately 24 h post-transfection, the HEK 293T cells were labeled for 4 h with ^{32}P. After chemical treatment for 10 min, with CAP and various activators and inhibitors of the kinases, the cells were washed with ice-cold PBS, and then lysed with cold immuno-precipitation buffer (IP buffer) containing 0.2 mM sodium ortho-vanadate, 0.2 mM phenylmethylsulfonyl fluoride and 0.5% NP-40, with a protease inhibitor cocktail (Boehringer Mannheim). After removal of the large aggregates, the soluble cell lysates were immuno-precipitated with polyclonal anti-VR1 antibody raised against recombinant NVR1 in mice (Jung *et al.*, 2004) and Protein A-agarose. The precipitates were washed three times with ice-cold IP buffer and incubated in 2X electrophoresis sample buffer for 10 min at 55°C. The samples were centrifuged at 4°C for 10 min, separated by 8% SDS-PAGE and transferred to PVDF membranes for immuno-blotting and autoradiography.

Ca^{++} imaging

Transfected cells are loaded with Fluo-3 AM (2.5 mg/mL, Molecular Probe) for 45-90 min at 37°C, and then washed three times with Krebs-Ringer HBSS (25 mM HEPES, 5 mM KCl, 0.96 mM NaH_2PO_4, 1 mM $MgSO_4$, 2 mM $CaCl_2$, 5 mM glucose, 1 mM probenecid, pH 7.4) containing 1 mM Ca^{++}. Calcium imaging was performed using a Leica confocal fluorescence microscope equipped with a variable filter system. The cells were initially imaged with HBSS buffer, after which the same volume of HBSS, containing test reagents DOG (500 μM) or CZP (10 μM) at twice the desired concentration, was added. After stimulation, the cells were observed for 60-120 sec.

Oocyte recording

Templates were linearized with XbaI and transcribed with SP6 RNA polymerase (Megascript, Ambion). Xenopus laevis oocytes were injected with 5-10 ng of VR1 or VR1 mutant cRNAs in water treated with 50 nL of DEPC. Two to five days after injection, two-electrode voltage-clamp recording was performed (E_{hold} = -60 mV) to find the whole cell currents. The recording chamber was perfused at a rate of 2 mL/min, with a bath solution containing (in mM) 96 NaCl, 5 HEPES, 2 KCl, 1.8 $CaCl_2$, and 1 $MgCl_2$, at pH 7.4 and room temperature.

Oocyte immunoblot

Extracts isolated from oocytes injected with cRNAs of VR1 were loaded onto a SDS-PAGE gel (8%), and the transferred proteins incubated with polyclonal VR1 antibody raised in mice against VR1 residue 1~432 encoding the cytosolic N-terminus (diluted 1:1000, Jung *et al.*, 2004). Proteins were revealed with peroxidase-conjugated secondary antibody (Sigma) and enhanced chemiluminescence (ECL, Amersham).

RESULTS

VR1 channel activity is correlated with the channel phosphorylation

When VR1 is repeatedly treated with CAP, the channel finally becomes insensitive to CAP. This phenomenon, the so-called desensitization, is suggested to involve dephosphorylation of the channel (Jung *et al.*, 2004). That is, the channel is dephosphorylated, and becomes insensitive or much less sensitive to ligands. In order to examine the relationship between the channel sensitivity to ligands and the dephosphorylation of the channel, the phosphorylation state of the channel on CAP treatment was examined. The HEK cells transfected with VR1 alone were treated with 10 μM CAP for 10 min, and a significant decrease of the phosphorylation of the channel was observed, which correlated with the decrease of the channel activity (Fig. 1, 30 ± 12% decrease, n=6). Therefore, it can be concluded that desensitization of the channel was parallel to the dephosphorylation of the channel, the *in vivo* phosphorylation experiment can be used for examination of the channel phosphorylation and the degree of phosphorylation of the channel is roughly correlated with the channel sensitivity to CAP.

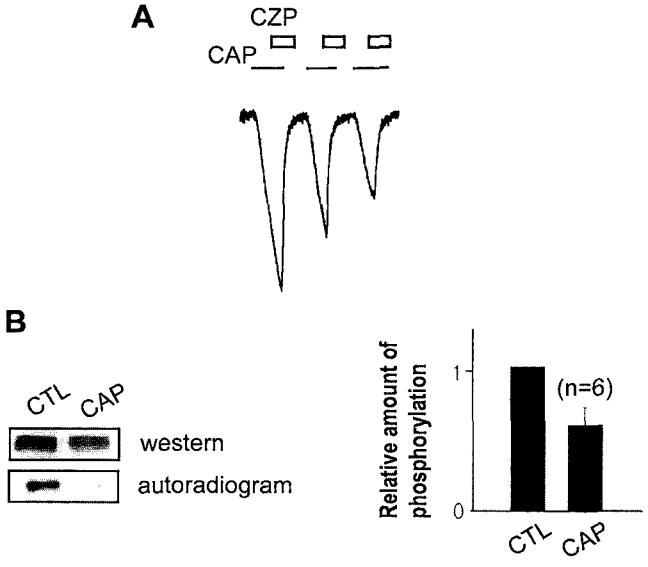

Fig. 1. Capsaicin (CAP) treatment of the vanilloid receptor 1 (VR1) decreased the channel activity (A) and the proportion of the phosphorylated form of the channel (B). A. Whole cell current of VR1 in the oocyte cells expressing VR1 when repeatedly treated with CAP. Capsazepine, a capsaicin antagonist, is designated as CZP. B. HEK293T cells transfected with VR1 were labeled with ^{32}P, the total cell lysate immunoprecipitated with anti-NVR and subjected to SDS-PAGE. The electroblotted membrane was visualized with anti-NVR and AP-conjugated anti-mouse antiserum and NBT/BCIP. The membrane was exposed to X-ray film and developed. The sample treated with CAP (10 μM) and the control are designated as CAP and CTL, respectively.

VR1 sensitization by PKC involves the phosphorylation of VR1

It was reasoned that if dephosphorylation of the channel results in channel insensitivity to ligands, then it would also be related with channel sensitization. So far, it has been suggested that VR1 is sensitized by PKC and/or PKA and/or acid (Prekumar and Ahren, 2000, Di Marzo *et al.*, 2001, Olah *et al.*, 2002, Hong and Wiley, 2005). If this is the case, the sensitization of the channel by PKC, PKA or acid might involve direct phosphorylation of the channel. In order to determine whether VR1 is phosphorylated by factors reported to be involved in sensitization, the phosphorylation of VR1 was examined by an *in vivo* phosphorylation experiment.

In the absence of any reagents, VR1 was identified to be phosphorylated under our experimental conditions. Because phosphorylation of the channel was correlated with the channel activity to CAP, we first wanted to know which kinases were involved in the phosphorylation of the channel, so treated VR1- transfected cells with activators of the various protein kinases.

In order to see whether PKA and PKC kinase activators increased the phosphorylation of VR1, the VR1-transfected cells were treated with 4α-phorbol 12-myristate 13-acetate (PMA, 10 μM), a PKC activator, and the phosphorylation of VR1 examined. As shown in Fig. 2A, PMA significantly increased the phosphorylation of VR1 compared to the solvent-treated cells (220%). Therefore, it can be concluded that PKC is involved in the phosphorylation of VR1, as the PKC inhibitor and activator decreased and increased the phosphorylation, respectively (see below).

Next, the effect of protein kinase A on the phosphorylation of VR1 was examined using the PKA activators, forskolin (FSK, 10 μM) and 8-bromo-cAMP (Br-cAMP, 10 μM), which increased the phosphorylation by about 70 and 150%, respectively (Fig. 2A. lanes 3 and 4). Consistent with these results, when the VR1-transfected cells were treated with the PKC activator, PMA, the response of the channel to CAP was greatly increased, as shown in Fig. 2B. Similarly, the PKA activator, FSK, also increased the channel activity to CAP. These results were consistent with the recent report of Rathee *et al.*, in that the heat activation of VR1 is mediated by PKA/AKAP signaling (Rathee *et al.*, 2002), even though they did not mention if the activation of VR1 with CAP was due to PKA.

Next, the effects of the inhibitors of kinases on the VR1 channel activity and phosphorylation state were examined. When the VR1 expressed in HEK cells was treated with bisindolylmaleimide (BIM), known as a PKC inhibitor, the phosphorylation signal was significantly decreased (Fig. 3A, about 50% decrease, n=4) compared to the solvent-treated control. When the VR1 transfected cells were treated with an inactive analogue of forskolin, dideoxy-

forskolin (DD-FSK), the signal of phospho-VR1 was also significantly decreased (Fig. 3B). Therefore, it can be concluded that VR1 is phosphorylated *via* PKA and/or PKC-activated pathways.

In summary, the activator and inhibitor of PKC increased and decreased, respectively, the phosphorylation of VR1, which was consistent with the reports of McNaughton's group and Premkumar (Vellani *et al.*, 2001; Premkumar, 2001). Similarly, the PKA activator increased the channel phosphorylation, whereas inactive PKA activators decreased the channel phosphorylation. Therefore, VR1 is phosphorylated due to the PKC and/or PKA.

Ca^{++} imaging experiment

The Ca^{++} imaging experiment also supported the idea that PKC activators sensitize VR1. 1, 2-dioctanoyl-s, *n*-glycerol (DOG, 500 μM), a PKC activator, was able to activate the Ca^{++} uptake by VR1-transfected HEK293 cells, which is blocked by the specific capsaicin channel antagonist, capsazepine, in the absence of VR1 agonists (Fig. 4A). It is speculated that the PKC activator, DOG, activates PKC for the phosphorylation of VR1 and then the temperature threshold to open the channel is greatly lowered for the opening of the VR1, even at room temperature. These results were consistent with the channel activation by PMA, as shown in Fig. 2B, as well as in several recent reports (Numazaki *et al.*, 2002; Hong and Wiley, 2004; Wang *et al.*, 2004).

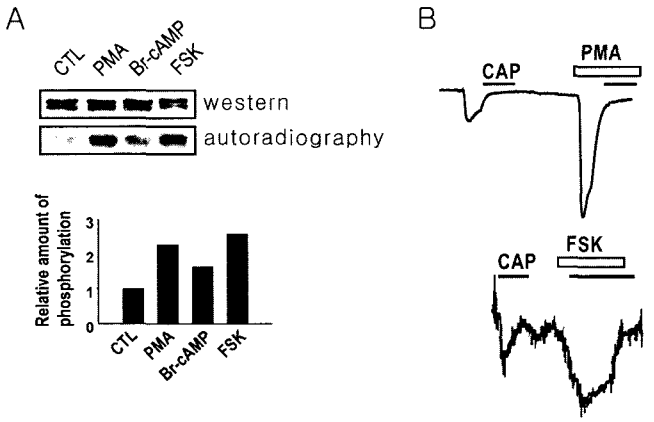

Fig. 2. The effects of kinase activators on the phosphorylation of VR1. A. HEK cells, transfected with VR1, were labeled and treated with the activators (DMSO as control, 10 μM PMA, 10 μM Br-cAMP, and 10 μM FSK) and analyzed with western blotting and autoradiography, as in Fig. 1. B. The whole cell current of the VR1-expressing oocytes when treated with PMA and FSK.

Acid also sensitizes VR1 involving phosphorylation

Acids are also suggested to sensitize VR1, because in the presence of acid the activity of the channel to CAP is

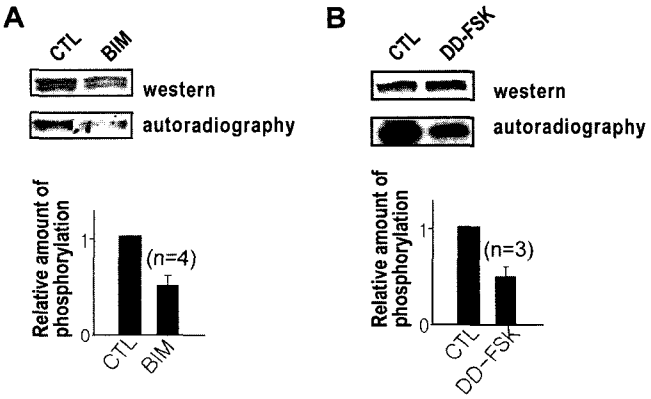

Fig. 3. The effects of kinase inhibitors on the phosphorylation of VR1. A. HEK cells transfected with VR1 were labeled and treated with the inhibitors (DMSO as control, BIM as PKC inhibitor and DD-FSK as inactive homologue of the FSK) and analyzed, as in Fig. 1.

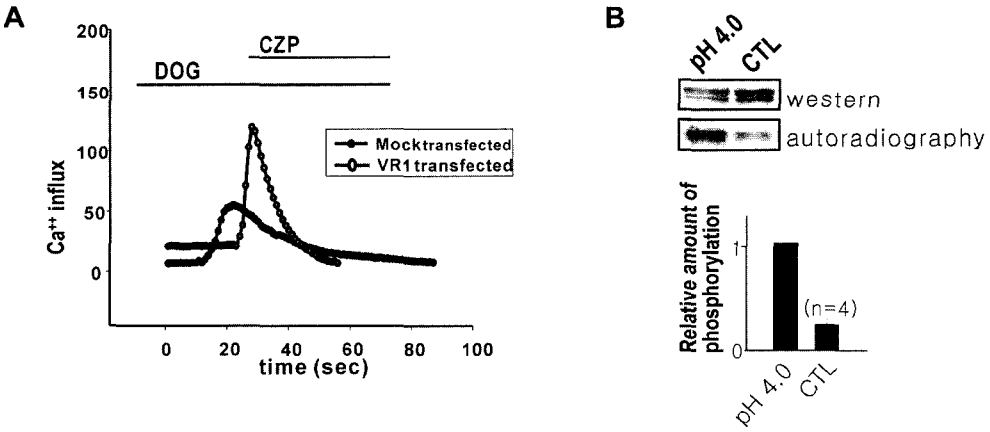

Fig. 4. A. The Ca^{++} imaging of the VR1-transfected cells in response to the PKC activator, 500 μM DOG (1, 2-dioctanoyl-s, n-glycerol). Mock- (filled circle) and VR1-transfected cells (open circle). The VR1-tranfected cells treated with DOG were opened to uptake Ca^{++}. The channel activity was blocked specifically by capsazepine, showing that Ca^{++} influx is through the VR1 channel. B. Acid also increased the amount of the phosphorylated form of VR1. Transfected cells were labeled, treated with acid (pH 4.0) and *in vivo* phosphorylation experiment performed, as in Fig. 1.

greatly increased as reported (Welch *et al.*, 2000, Olah *et al.*, 2001). Therefore, in order to find whether sensitization of VR1 by acid involves the phosphorylation of the channel, the *in vivo* phosphorylation experiment was performed. As shown in Fig. 4B, the treatment of VR1 with acid significantly increased the phosphorylation, suggesting that the sensitization of VR1 by acid also involved phosphorylation of the channel. Therefore, it can be concluded that the sensitization of VR1 by acid, PKC and/or PKA involves phosphorylation of the channel. The phosphorylation of the channel appeared to greatly decrease the threshold temperature of the channel opening and the effective concentration of CAP.

Bradykinin (BK) activates VR1 by phosphorylation *via* PKC pathway

Recently, BK has been suggested to be involved in the activation of VR1 through the lipoxygenase pathway (Shin *et al.*, 2002). In order to observe the involvement of PKC in the signaling pathway from bradykinin receptor 2 (B_2R) to VR1, we cotransfected HEK293T cells with VR1 and B_2R, and then treated with BK. As shown in Fig. 5, when bradykinin (BK, 2 μM) was applied to the cotransfected cells, the VR1 channel activity was enhanced (Fig. 5A) and the VR1 phosphorylation was significantly increased compared to the control group (compare lanes 4 and 2 in Fig. 5B). The increased phosphorylation of VR1 was significantly decreased, to basal level, when the PKC inhibitor, BIM (20 μM), was simultaneously applied with BK (lane 1 in Fig. 5B). This strongly suggests that VR1 phosphorylation *via* the BK pathway is due to PKC. Therefore, as a pain-causing substance, BK can activate VR1 *via* PKC by phosphorylation.

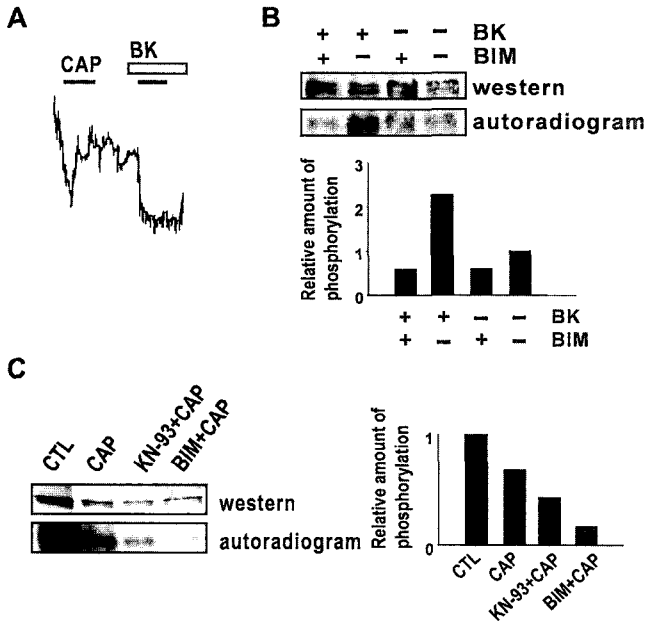

Fig. 5. VR1 is phosphorylated by BK via the PKC pathway. A. BK potentiated the channel activity of the VR1 channel. The whole cell current of the oocytes expressing VR1 and B_2R. B. *in vivo* phosphorylation of VR1 when the channel was treated with BK alone or/and the PKC inhibitor, BIM. C. *in vivo* phosphorylation of VR1 when the channel was treated with CAP and/or KN-93 and/or BIM.

When the VR1-transfected cells were pretreated with PMA, the concentration of CAP needed for the opening of the channel was significantly decreased (data not shown), for example, the cells not pretreated with PMA were mostly not opened with 5 nM CAP, whereas a significant portion of the PMA-pretreated cells were opened with 5 nM of CAP. Even without agonists, a small portion of the cells were already open, possibly due to heat sensitization of VR1 by PMA, supporting the idea that PKC sensitizes VR1 enabling its opening at a lower temperature.

Resensitization after desensitization of the channel

In order to find which kinases are potentially involved in the rephosphorylation of VR1 after channel desensitization, the *in vivo* phosphorylation state of VR1 was tested. VR1-transfected cells were treated with CAP alone or CAP and kinase inhibitor(s), for example, CamKII inhibitor, and the KN-93 or PKC inhibitor, BIM, and the *in vivo* phosphorylation experiment was performed. If BIM treatment decreased the phosphorylation state, then it could be concluded that after desensitization, PKC phosphorylates the channel, but if KN-93 decreases the amount of phosphorylation of VR1, CamKII phosphorylates VR1. In this study, the cotreatment with CAP and KN-93 or BIM was found to decrease the phosphorylation of VR1. Therefore, the *in vivo* phosphorylation experiment showed that rephosphorylation after desensitization involved both PKC and CamKII (Fig. 5C).

DISCUSSION

In this study, the phosphorylation state of VR1 was studied *in vivo*. VR1-transfected HEK cells were treated with various activators and inhibitors of protein kinases, and the portions of the phosphorylated form of VR1 examined by an *in vivo* phosphorylation assay. The activators of PKA and PKC were found to increase the phosphorylation of VR1, whereas the inhibitors of protein kinases decreased the phosphorylation. Also, the increase and decrease of phosphorylation of VR1 were correlated with the CAP sensitivity of the channel. Therefore, it can be concluded that the phosphorylation of VR1 is a way of sensitizing the channel, similarly to the cystic fibrosis transmembrane conductance regulator chloride channel (CFTR) (Cheng *et al.*, 1991). The sensitization of VR1 by PKA, PKC and acid involves the direct phosphorylation of VR1. This result was consistent with the

recent reports regarding the phosphorylation of VR1 by PKC (Numazaki *et al.*, 2002; Hong and Wiley, 2005) and PKA (Parvinder *et al.*, 2002). Therefore, VR1 can be concluded to be phosphorylated and then sensitized, so that the thresholds of the ligand concentration and the temperature for opening the channel can both be lowered. Sensitization involving phosphorylation of VR1 can explain why the putative endogenous VR1 ligands identified to date are weak agonists (Hwang *et al.*, 2000; Huang *et al.*, 2002; Smart *et al.*, 2000; Zygmunt *et al.*, 2002). The sensitization of VR1 allows for endogenous ligands to induce a sizable response at concentrations normally found in tissues (Premkumar, 2001; Szolcsanyi, 2000).

The lowered threshold of the channel was observed by potentiation with NGF (Chuang *et al.*, 2001). The channel has also been observed to be opened, even at room temperature, when the channel was activated with NGF. In this sense, we found that PMA-treated cells, transfected with VR1, were permeable to Ca^{++} even without agonists. This might result from the potentiation of the VR1 channel, so that the temperature threshold of the channel is lowered. That is, the sensitivity of VR1 to temperature decreased; therefore, when the conditions are appropriate, even normal body temperature could act as a primary stimulus.

Julius *et al.* suggested that PMA interacted directly with the channel (Chuang *et al.*, 2001). However, the report by Vellani *et al.* that indicated 1 mM of PMA did not activate the VR1 current in the stably transfected hVR1-HEK293 cells lines and DRG, whereas transiently transfected VR1 was activated by PMA (Vellani *et al.*, 2001). Therefore, we used 10 mM of PMA, at which small portions of DRG showed CAP activity in order to activate the VR1 current. This study of *in vivo* phosphorylation supports the idea that PMA does not activate the channel by direct interaction, but rather that PMA sensitize the channel by activating PKC to phosphorylate the channel. It is also possible that PMA activates the channel in a PKC-independent manner, as accumulating lines of evidence indicate that in addition to activating PKC, PMA also produces some regulatory effects in a PKC-independent manner (Samuvel *et al.*, 2005; Xu *et al.*, 2003; Nakamura *et al.*, 2001). The possibility that BIM, in addition to PKC, might inhibit PKA and other Ser/Thr kinases can not be ruled out, as previously reported (Roberts *et al.*, 2004; Davies *et al.*, 2000; Amos *et al.*, 2005).

The nature of the sensitization by phosphorylation of the channel remains to be fully understood, but we can presume that the phosphorylation of the channel increases the susceptibility to ligands, as previously reported (Jung *et al.*, 2004).

In this study, the channel was also shown to have a basal level of phosphorylation. That is, VR1 is ready to respond to subtle changes in the concentrations of the endogenous ligands normally found in tissues. We also found that the channel was dephosphorylated by repeated application of ligands at the molecular level, resulting in desensitization of the channel.

BK is a known mediator of inflammatory pain that works on VR1. In this study, the activation of VR1 by BK was found to appear to involve the activation of PKC and, therefore, the phosphorylation of VR1 is very likely due to direct phosphorylation and sensitization of the channel. PKC-mediated phosphorylation directly activates VR1 (Premkumar and Ahern, 2000) and strongly potentiates the CAP- or heat-induced responses (Cesare *et al.*, 1999; Cesare and McNaughton, 1996). PKC-epsilon has been identified as an important contributor to mechanisms of peripheral hyperalgesia (Khasar *et al.*, 1999). Our results also support that PKC activation enhances the phosphorylation of VR1. In addition, Smith *et al.* reported that bradykinin-evoked responses are mediated by cAMP-dependent protein kinase A (Smith *et al.*, 2000). However, the possibility that PKC activation may induce the release of agonists able to activate VR1 can not be ruled out.

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