cAMP-Dependent Protein Kinase Regulates Desensitization of the Capsaicin Receptor (VR1) by Direct Phosphorylation

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Summary

The capsaicin receptor, VR1 (also known as TRPV1), is a ligand-gated ion channel expressed on nociceptive sensory neurons that responds to noxious thermal and chemical stimuli. Capsaicin responses in sensory neurons exhibit robust potentiation by cAMP-dependent protein kinase (PKA). In this study, we demonstrate that PKA reduces VR1 desensitization and directly phosphorylates VR1. In vitro phosphorylation, phosphopeptide mapping, and protein sequencing of VR1 cytoplasmic domains delineate several candidate PKA phosphorylation sites. Electrophysiological analysis of phosphorylation site mutants clearly pinpoints Ser116 as the residue responsible for PKA-dependent modulation of VR1. Given the significant roles of VR1 and PKA in inflammatory pain hypersensitivity, VR1 phosphorylation at Ser116 by PKA may represent an important molecular mechanism involved in the regulation of VR1 function after tissue injury.

Introduction

Vanilloid receptor 1 (VR1 or TRPV1), cloned as a capsaicin receptor, is a polymodal ligand-gated ion channel that also responds to heat, protons, anandamide, and leukotrienes (Caterina et al., 1997; Hwang et al., 2000; Tominaga et al., 1998; Zygmunt et al., 1999). Several features of VR1 initially focused attention to a significant role in thermal sensation and hyperalgesia. VR1 is localized to small diameter predominately nociceptive primary afferents and responds to thermal stimuli in the behaviorally noxious range (Caterina et al., 1997). Reminiscent of the response properties of polymodal nociceptive afferents, VR1 is also capable of integrating thermal and chemical stimuli, such as protons or leukotrienes (Hwang et al., 2000; Tominaga et al., 1998). In response to these various stimuli, vanilloid receptors depolarize nociceptive afferents and can initiate inflammatory peptide release from primary afferent nerve terminals (Szallasi and Blumberg, 1999). Thus, the receptor may act as a thermal and chemical transducer and contribute to neurogenic aspects of inflammation. VR1 knockout mice exhibit deficits in strong thermal stimulus detection and inflammatory thermal hypersensitivity, confirming a prominent role for VR1 in thermal transduction, particularly in mediating inflammatory hyperalgesia (Caterina et al., 2000; Davis et al., 2000).

Cyclic AMP-dependent protein kinase (PKA) in primary afferents plays a major role in producing inflammatory hyperalgesia (Levine and Reichling, 1999). Prostaglandins, such as PGE2, produce hyperalgesia by raising intracellular CAMP levels and activating PKA in nociceptive afferents (Taiwo et al., 1989; Taiwo and Levine, 1991). Conversely, mu-opioid agonists mediate peripheral analgesia by diminishing cAMP levels (Levine and Taiwo, 1989). Direct activation of PKA with cAMP analogs also results in behavioral hypersensitivity (Taiwo et al., 1989; Taiwo and Levine, 1991). Mice deficient in a PKA regulatory subunit exhibit reduced PGE2 and formalin-induced inflammatory hypersensitivity (Malmberg et al., 1997).

The prominent roles of VR1 and PKA in inflammatory hyperalgesia suggest that PKA potentiation of VR1 function may be a molecular event involved in the production of thermal hypersensitivity. Consistent with this hypothesis, PKA activation amplifies capsaicin currents and enhances capsaicin-induced peptide release in cultured dorsal root ganglion neurons (Hingtgen et al., 1995; Hu et al., 2002; Lopshire and Nicol, 1998). Recent studies have reported conflicting results concerning PKA modulation of VR1 responses (De Petrocellis et al., 2001; Lee et al., 2000). It remains uncertain whether PKA can modulate and phosphorylate VR1. In this study, we demonstrate that PKA activation phosphorylates VR1 and prevents VR1 desensitization. We identify several candidate PKA phosphorylation sites using in vitro phosphorylation and protein sequencing. Using patch-clamp electrophysiology of putative phosphorylation site mutants, we pinpoint Ser116 as the primary site responsible for PKA-mediated regulation of VR1 desensitization.

Results

PKA Reverses Desensitization and Phosphorylates VR1 in Transfected Cells

Capsaicin activates robust inward currents in CHO-K1 cells expressing wild-type VR1. These responses exhibit dramatic desensitization during agonist application, often termed acute desensitization, as well as upon subsequent reapplication of agonist, commonly denoted as tachyphylaxis (Figure 1A, upper record). These desensitization kinetics mimic native capsaicin responses in rat DRG neurons (Docherty et al., 1996; Koplas et al., 1997). Elevation of cyclic AMP levels in these cells by preincubation (10–30 min) with the membrane permeant analogs CPT-cAMP (10 μM, data not shown) or Br-cAMP (1 mM, Figure 1A lower panel) greatly inhibits desensitization during capsaicin application and with subsequent applications.

To assess whether the inhibition of desensitization by cAMP analogs reflected stimulation of PKA-mediated...
phosphorylation, we examined the effect of PKA inhibitors on the phenomenon. The magnitude of desensitization was assessed in the form historically termed tachyphylaxis by measuring the ratio of currents induced by the second of two 20 s applications of capsaicin (1 μM) to those induced by the initial application of capsaicin. As expected, the marked desensitization seen under control conditions for capsaicin (Figure 1B, open bar) was absent following preincubation with 1 mM 8Br-cAMP (Figure 1B, black bar). In contrast, preincubation with 8Br-cAMP in the presence of the PKA inhibitor, H89, resulted in desensitization similar to untreated control (Figure 1B, gray bar). Similar results were observed using the PKA inhibitor Rp-cAMPS (data not shown). These data suggest that VR1 can be maintained in a sensitized functional state and/or rescued from desensitization by PKA-mediated phosphorylation.

Since PKA activation modulates VR1 function, we wanted to determine whether VR1 was directly phosphorylated by PKA. VR1, immunoprecipitated from 32P-labeled, VR1-transfected cells, displayed high levels of basal 32P incorporation (Figure 1C). Surprisingly, 8Br-cAMP had little effect on basal phosphorylation (Figures 1C and 1D). This may indicate that PKA does not phosphorylate VR1, but that VR1 phosphorylation saturates PKA sites on VR1, or that PKA phosphorylation may only become significantly obvious in the desensitized state. Since our physiology experiments suggested that PKA attenuates VR1 desensitization, we pursued the last possibility and examined VR1 phosphorylation after capsaicin treatment. A desensitizing capsaicin application induced robust dephosphorylation, which was significantly blunted by pre-treatment with 8Br-cAMP (Figures 1C and 1D). These data suggest that PKA directly phosphorylates VR1 and regulates desensitization.

**VR1 Cytoplasmic Domains Are In Vitro PKA Substrates**

In order to identify candidate VR1 PKA phosphorylation sites, we produced bacterial, GST fusion proteins of the N- and C-terminal cytoplasmic domains and a synthetic peptide corresponding to the first intracellular loop to serve as in vitro PKA phosphorylation reaction substrates (Figures 2A and 2B). The second intracellular loop was omitted since it does not contain any serines or threonines. All of the engineered proteins acted as functional substrates (Figures 2C–2E). Since PKA activation modulates VR1 function, we wanted to determine whether VR1 was directly phosphorylated by PKA. VR1, immunoprecipitated from 32P-labeled, VR1-transfected cells, displayed high levels of basal 32P incorporation (Figure 1C). Surprisingly, 8Br-cAMP had little effect on basal phosphorylation (Figures 1C and 1D). This may indicate that PKA does not phosphorylate VR1, but that VR1 phosphorylation saturates PKA sites on VR1, or that PKA phosphorylation may only become significantly obvious in the desensitized state. Since our physiology experiments suggested that PKA attenuates VR1 desensitization, we pursued the last possibility and examined VR1 phosphorylation after capsaicin treatment.
Figure 2. The N terminus, C terminus, and First Intracellular Loop of VR1 Are Substrates for PKA Phosphorylation
(A) Schematic representation of VR1 membrane topology with two large N- and C-terminal cytoplasmic domains and two smaller intracellular loops. Three fragment proteins (shaded gray circles) were generated for in vitro phosphorylation analysis. The N- and C-terminal proteins were produced as GST fusion proteins, while the first intracellular loop was manufactured as a peptide with the sequence CYFLQRPSLKLFSLY. The second intracellular loop does not contain any serines or threonines. (B) Coomassie blue stained gel shows successful production of full-length N-terminal (~68 kDa) and C-terminal (~46 kDa) fusion proteins indicated by arrows. (C) Phosphorylation time course for 100 μM VR1 first intracellular loop peptide. (D) Time course of phosphorylation for GST VR1 N-terminal fusion protein. Reactions were incubated for various time points, proteins separated on SDS-PAGE, and 32P transferred quantified using phosphorimaging and densitometry. Examples of phosphorimager bands at different time points shown in inset. Saturating stoichiometry is approximately 6 pmol/μg protein or ~50%. (E) Time course of phosphorylation for GST VR1 C-terminal fusion protein. Examples of bands at different time points shown in inset. Saturating stoichiometry is approximately 6 pmol/μg or ~25% (mean ± SEM, n = 3 for all experiments).

yet to be reached and that Ser6 is an extremely minor site (Figure 3C). This phosphorylation site may exist farther along in the same peptide or possibly in a low abundance, coeluting peptide. In an attempt to obtain smaller peptides for phosphorylation site identification, we conducted an in-gel trypsin digest followed by ESI mass spectroscopy, but only found Thr144 and Thr370 as phosphorylation sites (data not shown). These data may indicate that Ser6, Thr144, and Thr370 are the only phosphorylation sites. ESI mass spectroscopy, although sensitive, can fail to identify large phosphopeptides that fall outside the dynamic range of the ~79 precursor scan window. Presumably, this was the case with Ser6, which resides in a predicted tryptic peptide of 40 residues. Furthermore, a mutant N-terminal fusion protein with Ser6, Thr144, and Thr370 altered to alanines exhib-
Figure 3. Phosphorylation Site Determination of VR1 Cytoplasmic Domains

(A) Cerenkov counting of HPLC fractions from peptides eluted after a Lys-C in-gel digest of the GST VR1 N-terminal fusion protein. Three larger peaks (indicated by circled numbers) were selected for Edman sequencing. (B) Peak 1 Edman sequencing results correlated with scintillation counting of sequencing cycles point to Thr144 as a phosphorylation site. (C) Peak 2 scintillation counting with Edman sequencing suggest Ser6 as an extremely minor site with most of the counts remaining on the sequencing filter (cycle “F”). Residues indicated in italics were not directly sequenced, but deduced from the predicted peptide. (D) Edman sequencing coupled with scintillation counting of peak 3 identified Thr370 as a phosphorylation site. Residues in italics were deduced from the predicted peptide, while other residues were explicitly sequenced. (E) Cerenkov counting of HPLC fractions from an in-gel Asp-N digest of the GST VR1 N-terminal mutant fusion protein with S6A, T144A, and T370A. A single phosphopeptide peak can be identified. (F) Scintillation counting of Edman sequencing cycles correlated with

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**Graphs:**

- **A:** Cerenkov counting graph with peaks 1, 2, and 3.
- **B:** Thr144 Edman sequencing and scintillation correlation.
- **C:** Ser6 Edman sequencing and scintillation correlation.
- **D:** Thr370 Edman sequencing and scintillation correlation.
- **E:** Phosphorylation of mutants T144A, T370A, S6A, T144A, and T370A.
- **F:** Ser502 Edman sequencing and scintillation correlation.
- **G:** Ser820 and Ser774 m/z peaks and intensity.

**Legend:**

- **WT:** Wild type
- **S774A:** Mutant S774A
- **S820A:** Mutant S820A
Phosphorylation of mutant GST VR1 C-terminal fusion protein with S774A and S820A eliminated 32P incorporation. Mutant phosphorylation MS/MS sequencing of these peptides directly identified Ser774 (peptide m/z 901.3) and Ser 820 (peptide m/z 899.1) as phosphorylation sites.

Peptides from an in-gel trypsin digest of the GST VR1 C-terminal fusion protein identified two major phosphate-associated peptides. Tandem MS/MS sequencing confirmed the phosphopeptides (Figure 3I). Tandem MS/MS sequencing of these peptides identified Ser774 and Ser820 as PKA phosphorylation sites (Figure 3H).

Phosphorylation sites on the C-terminal intracellular domain protein were identified using ESI mass spectrometry. Peptides from an in-gel trypsin digest of the phosphorylated C-terminal protein were subjected to a ~79 precursor ion scan, which delineated two major phosphopeptides (Figure 3I). Tandem MS/MS sequencing of these peptides identified Ser774 and Ser820 as PKA phosphorylation sites. Altering Ser774 and Ser820 to alanines eliminated phosphorylation (mutant phosphorylation was 9.2% of wild-type, n = 3) indicating complete identification of PKA phosphorylation sites (Figure 3J).

Taken together, based on required kinase concentrations and stoichiometry estimates, we can qualitatively identify Ser116 and Ser502 as major in vitro PKA phosphorylation sites and Thr144, Thr370, Ser774, Ser820, and possibly Ser6 as minor sites. Ser502 requires approximately 50- to 100-fold less kinase to achieve saturating phosphorylation, while Ser116 accounts for the largest single site stoichiometry (~35%).

Functional Characterization of PKA Phosphorylation Site Mutants

Given the functional evidence that PKA-mediated phosphorylation can rescue VR1 from agonist-induced desensitization (Figure 1B) and that VR1 itself is dephosphorylated upon agonist stimulation (Figures 1C and 1D), a working hypothesis emerges that functional sensitization and desensitization are related to phosphorylated and dephosphorylated states of the VR1 protein, respectively. Using this hypothesis as a guide, we sought to elucidate the functional roles of the biochemically determined PKA phosphorylation sites in the native VR1 protein. Initially, inactivating or “constitutively dephosphorylated” alanine mutations were created in each of the key serine and threonine residues and capsaicin responses of cells transiently expressing each mutant determined by electrophysiology. Based on our starting hypothesis, we expected two possible results. One possibility was that the dephosphorylated mutant is equivalent to or reproduces the desensitized state. This hypothesis predicts small currents, which fail to desensitize any further. Another possibility was that dephosphorylation enhances the rate of entry or decreases the rate of exit from desensitized states. In this case, a dephosphorylated mutant may show normal currents with exaggerated desensitization.

Both current density (pA/pF) of the initial capsaicin response and the degree of desensitization for the second of two consecutive capsaicin applications were assessed for wild-type, S6A, S116A, T144A, T370A, S502A, S774A, and S820A mutants. Desensitization was significantly altered in several mutants (Figure 4A). S774A and S820A appeared to follow one prediction with enhanced desensitization, while T144A fit the other prediction, exhibiting little desensitization. However, desensitization seemed to be related to initial current density, such that mutants with lower current densities desensitized less than mutants with large current densities (compare Figures 4A and 4B). Even for wild-type VR1, a scatter plot of current density versus desensitization reveals a strong negative correlation for responses with transiently transfected (open circles) or stably transfected (filled circles) cells (Figure 4C). This finding is expected given the numerous studies which have demonstrated a correlation between capsaicin-based calcium influx and desensitization in cultured sensory neurons (Cholewinski et al., 1993; Docherty et al., 1996; Liu and Simon, 1996; Piper et al., 1999). Thus, the apparent differences in desensitization amongst the inactivating mutants probably reflect differences in current density rather than phosphorylation-mediated changes in desensitization. Indeed, when mutant desensitization is plotted against current density, the mutants lie along the regression line determined for wild-type VR1 (Figure 4D).

We hypothesized that differences in current density simply reflected changes in surface expression, thereby corresponding changes in calcium influx. We utilized cell surface biotinylation and streptavidin precipitation to examine surface expression of wild-type VR1 (E) delineates Ser116 as the phosphorylation site. Residues in italics were deduced, while others were directly sequenced. (G) Phosphorylation stoichiometry for mutant GST VR1 N-terminal fusion proteins. Fusion proteins were phosphorylated for 1.5–2 hr and stoichiometry quantified as the percentage of wild-type phosphorylation conducted simultaneously (mean ± SEM, n = 3 for all constructs). (H) Scintillation counting of Edman sequencing cycles for phosphorylated VR1 first intracellular loop peptide. Results clearly delineated Ser502 as the major phosphorylation site rather than Ser505. (I) ESI mass spectrometry ~79 precursor ion scan of peptides from an in-gel trypsin digest of the GST VR1 C-terminal fusion protein identified two major phosphate-associated peptides. Tandem MS/MS sequencing of these peptides directly identified Ser774 (peptide m/z 901.3) and Ser 820 (peptide m/z 899.1) as phosphorylation sites. (J) Phosphorylation of mutant GST VR1 C-terminal fusion protein with S774A and S820A eliminated 32P incorporation. Mutant phosphorylation was 9.2% of wild-type (n = 3).
Figure 4. Current Density and Desensitization in PKA Phosphorylation Site Mutants

Normalized second capsaicin responses (A, see legend for Figure 1B) and current density values for the initial capsaicin response (B) for wild-type VR1 and the indicated PKA site mutants transiently expressed in CHO-K1 cells. Mutants are ordered by increasing current density. Number of cells indicated above each bar. * indicates significant difference from wild-type (ANOVA, post hoc Dunnett’s test, p < 0.05). (C) Correlation plot of normalized second responses versus current density from CHO-K1 cells expressing wild-type VR1 either transiently (open circles, n = 37) or stably (filled circles, n = 21). The straight line is a linear regression fit to all of the wild-type data points with an r² = 0.34. (D) Correlation plot of normalized second capsaicin response (A) versus initial response (B) for the indicated mutant receptors (gray triangles, mean ± SEM). The straight line is the regression fit from panel C. (E) Western blot of total and surface expressed wild-type, T144A, and T144D VR1 using an anti-N-terminal VR1 antibody (1:1000) following surface biotinylation of cells transfected with each receptor or mock transfection control (see Experimental Procedures for details). (F) Relative surface expression of PKA site mutants plotted as the ratio of mutant to wild-type expression in the biotinylated pool normalized for total input loading (mean ± SEM, n = 5).

type, T144A, and T144D VR1 receptors. The T144 mutants exhibit varying degrees of current amplitude deficits measured as decreased current density (Figure 4B) and reduced proportion of cells responding to 1 μM capsaicin (29/29 for wild-type VR1, 13/34 for T144A, and 6/13 for T144D) or pH 5 (15/15 for wild-type, 3/12 for T144A, and 6/10 for T144D). Correlating well to current density, T144A showed heavily reduced surface expression, while T144D exhibited moderately diminished surface expression compared to wild-type (Figures 4E and 4F). Pilot studies with S6A, T370A, S502A, and S820A showed similar results with surface expression varying between 60% and 130% compared to wild-type (data not shown).

PKA-Dependent Sensitization of VR1 Requires Ser116

Since none of the inactivating mutants exhibited phosphorylation-based changes in desensitization, we hypothesized that PKA phosphorylation of VR1 may prevent desensitization, while dephosphorylation at a particular site may simply be permissive to desensitization. Using this rationale, we constructed aspartate mutants, which mimic a “constitutively phosphorylated” state, and hypothesized that a functionally relevant site would exhibit little desensitization. We returned to results from the in vitro biochemistry and focused our attention on aspartate mutants at the qualitatively significant phosphorylation sites of Ser116 and Ser502.
We again assessed the degree of desensitization by comparing the second of two responses to capsaicin to the initial response for cells transiently transfected with S116D and S502D. While S502D desensitization was indistinguishable from wild-type, S116D exhibited strongly blunted desensitization, mimicking wild-type VR1 after 8Br-cAMP treatment (Figures 5A and 5C). Again, reduced desensitization of S116D may simply reflect lower current density. However, when plotted on the regression curve describing the relationship between desensitization and current density (see Figure 4), S116D shows a significant deviation from wild-type (Figure 5B). Thus, S116D truly mimics nondesensitizing currents seen with wild-type currents following 8Br-cAMP treatment.

Although the use of mimicking mutants is relatively clear in terms of expected results, a positive result as seen with Ser116 may not be specific to phosphorylation by PKA. To confirm the role of Ser116 in PKA-mediated regulation of VR1 desensitization, we returned to the inactivating mutants and pre-treated cells with 8Br-cAMP (1 mM, 30 min) to assess the ability of PKA to reduce desensitization. While 8Br-cAMP treatment prevents normal desensitization of wild-type VR1, it completely fails to alter desensitization of the S116A mutant (Figures 5C and 5D). Conversely, 8Br-cAMP treatment dramatically inhibits desensitization in all of the other desensitizing mutants, including the high current density, heavily desensitizing S774A and S820A mutants. While S502D desensitization was indistinguishable from wild-type, S116D exhibited strongly blunted desensitization, mimicking wild-type VR1 after 8Br-cAMP treatment (Figures 5A and 5C). (Figure 5D). Taken together, these data strongly support an exclusive role for Ser116 in PKA-mediated regulation of VR1 sensitivity to capsaicin.

Given that desensitization and its regulation may be ligand specific, we wanted to verify that our results were generally applicable to other ligands such as protons, which activate VR1 at a different locus (Jordt et al., 2000; Welch et al., 2000) and presumably activate VR1 in vivo. As seen with capsaicin responses, VR1 pH responses also desensitize, and this desensitization is prevented by PKA activation with 8Br-cAMP (Figures 6A and 6B). H89, a PKA inhibitor, blocked 8Br-cAMP-mediated reversal of proton response desensitization (Figure 6B). Furthermore, the S116D mutant exhibited reduced desensitization of proton responses, while the S116A mutation abrogated the effect of 8Br-cAMP on proton responses as seen for capsaicin responses (Figures 6C
Figure 6. PKA Reverses VR1 Proton Response Desensitization by Phosphorylation of Ser116
(A) Inward current responses to consecutive applications of pH 5.0 external solution in CHO-K1 cells transiently expressing wild-type VR1. The upper responses are from a control cell, while the lower responses from a cell preincubated for 30 min in 1 mM 8Br-cAMP. (B) The ratio (mean ± SEM) of the second to the first of two consecutive 60 s applications of pH 5.0 external solution is plotted for CHO-K1 cells expressing wild-type VR1 under control conditions (open bar), following 30 min preincubation with 1 mM 8Br-cAMP (black bar), or following 8Br-cAMP incubation in the presence of 1 μM H-89 (gray bar). Asterisks indicate difference versus control (ANOVA, post hoc Tukey’s comparisons, p < 0.05). (C) Current responses to consecutive pH 5.0 applications in CHO-K1 cells transiently expressing the VR1 mutants S116D (upper recording), S116A (middle recording), and S116A following a 30 min preincubation with 1 mM 8Br-cAMP (lower recording). (D) The ratio (mean ± SEM) of the second to the first of two consecutive applications of pH 5.0 to CHO-K1 cells expressing either wild-type, S116A, or S116D VR1 and with or without 8Br-cAMP preincubation as indicated. Asterisks indicate difference versus wild-type control (ANOVA, post hoc Tukey’s comparisons, p < 0.05).

Discussion
In this study, we identify VR1 as a PKA substrate in vivo and identify Ser116 as a functionally relevant PKA phosphorylation site. Our cellular phosphorylation data

Figure 7. VR1 S116A Exhibits Little Reversal of Capsaicin-Induced Dephosphorylation by 8Br-cAMP
(A) S116A VR1 was immunoprecipitated from transiently transfected, 32P-orthophosphate-labeled COS7 cells and separated by SDS-PAGE. VR1 bands detected with phosphorimaging after various treatments (see Figure 1 legend for details) are shown. (B) Capsaicin-induced dephosphorylation (1 – capsacin treated/vehicle × 100%) is decreased in S116A compared to wild-type (mean ± SEM, n = 4 for wild-type, n = 3 for S116A, * unpaired t test, p < 0.05). (C) No significant difference exists in VR1 32P incorporation between capsacin and 8Br-cAMP treated S116A transfected cells (mean ± SEM, n = 3 for capsacin, n = 4 for Cap + 8Br-cAMP, unpaired t test).
indicated that PKA activation preferentially blunts capsaicin-induced dephosphorylation. This work provides evidence of dephosphorylation of VR1 with desensitizing capsaicin applications at the biochemical level. Previous studies in dorsal root ganglion neurons have shown that calcineurin phosphatase inhibitors reduce acute desensitization, and pipette solutions containing ATP prevent tachyphylaxis of capsaicin responses (Docherty et al., 1996; Koplas et al., 1997; Piper et al., 1999). These data suggest that VR1 dephosphorylation may be mechanistically linked to desensitization. However, capsaicin response desensitization is a complex process with varying kinetic components. A fast component appears to be dependent on intracellular calcium, voltage, and calcineurin activity, while a slower component appears at least to be ATP dependent (Docherty et al., 1996; Koplas et al., 1997; Liu and Simon, 1996; Piper et al., 1999; Tominaga et al., 1998). Further complexity is overlaid by the interactions between these factors, such as voltage-dependent calcium influx and calcium-dependent phosphatase activity. Our data also suggest a relationship between VR1 surface expression and desensitization. While the correlation between current density and desensitization for wild-type VR1 is suggestive, only the direct examination of surface expression afforded by the mutants allowed us to correlate surface expression with current density and desensitization. Considering the several factors affecting VR1 desensitization, it seems most likely that the level of surface expression alters capsaicin-induced calcium influx to indirectly modulate the level of desensitization. These data also underscore the importance of considering the surface expression of VR1 mutants when interpreting their effects on VR1 function.

This study represents a first step in examining the effect of VR1 phosphorylation on desensitization. Our data suggests that Ser116 is dephosphorylated by capsaicin and that PKA regulates VR1 desensitization by Ser116 phosphorylation. However, an inactivating alanine mutant at Ser116 has little effect on desensitization, suggesting that Ser116 dephosphorylation is not rate limiting to desensitization. Dephosphorylation at other sites, which continues to occur in this mutant, as well as calcium- and voltage-dependent steps, may represent rate-limiting steps in the desensitization process. While dephosphorylation at Ser116 is not rate limiting, Ser116 phosphorylation either through PKA activation or through a mimicking aspartate mutant prevents desensitization. Therefore, the Ser116 dephosphorylated and phosphorylated forms of VR1 do not represent desensitized and sensitized states of VR1, respectively, but rather a gate mechanism in which entry into desensitized states is prevented by phosphorylation and allowed to occur with dephosphorylation. Future studies will examine interactions between the phosphorylation state of Ser116, calcium, voltage, and phosphorylation at other sites.

In pinpointing the locus of PKA action, in vitro phosphorylation of engineered cytoplasmic domains and protein sequencing revealed several PKA phosphorylation sites. However, only Ser116 mutants demonstrated functional effects completely consistent with phosphorylation. A loss-of-function alanine mutant exhibited no PKA-dependent modulation, while a negative charge conferring aspartate mutant mimicked PKA modulation. While we can confidently attribute Ser116 phosphorylation to prevention of VR1 desensitization, we cannot completely rule out that the other identified sites may mediate unidentified functions. Conversely, these sites may represent artifacts restricted to in vitro phosphorylation and unseen in the native receptor, a potential issue when utilizing this approach for the study of receptor phosphorylation (Mammen et al., 1999).

This work establishes VR1 as a substrate for PKA. Given the prominent roles for PKA and VR1 in inflammatory hyperalgesia, VR1 may act as a PKA substrate during inflammatory pain states. Specifically, PKA phosphorylation at Ser116 may maintain VR1 function in the midst of desensitizing, chronic stimulation from protons released after tissue injury. This can contribute directly to changes in thermal sensitivity or maintain neurogenic components of inflammation, such as peptide release from primary afferent terminals. Future studies will focus on the functional consequences of Ser116 phosphorylation at the cellular and behavioral level in physiologic and pathologic pain states.

Experimental Procedures

**32P Metabolic Labeling and VR1 Immunoprecipitation**

COS7 cells maintained in Dulbecco’s Minimal Essential Media (DMEM) + 10% FBS were plated onto 6 well plates and transfected with pCDNA3 VR1 using Lipofectamine 2000 as per manufacturer’s protocol (Invitrogen, Carlsbad, CA). About 36 hr after transfection, cells were rinsed with phosphate-free Minimal Essential Media with Earle’s salts (P-EMEM; Sigma, St. Louis, MO) and incubated with P-EMEM containing 250 μCi/ml 32P-orthophosphate for 4 hr. Either 10 mM Tris (pH 8) vehicle or 1 mM 8Br-cAMP was added to the media and incubated for 15 min. Thereafter, 0.1% ETOH vehicle, 1 mM capsaicin alone, or capsaicin plus 8Br-cAMP dissolved in patch-clamp external solution (see below) were applied for 5 min. Cells were lysed in 500 μl lysis buffer (LYB—50 mM NaCl, 50 mM NaF, 25 mM Na-phosphate [pH 7.4], 2.5 mM EDTA, 1% Triton-X, 10 mM Na-pyro-phosphate, 2 mM Na-orthovanadate, 0.1 mM PMSF, 10 μg/ml pepstatin, 10 μg/ml aprotinin, 10 μg/ml leupeptin, 5 μg/ml aprotonin, 10 μg/ml cystin-LR) and centrifuged at 14,000 rpm in a microcentrifuge for 15 min at 4°C. The supernatant was pre-cleared with 25 μl protein A Sepharose for 3 hr. Beads were rinsed three times with 1 ml LYB, twice with high ionic strength buffer (LYB but with 500 mM NaCl), and twice with low ionic strength buffer (LYB without NaCl and Na-phosphate replaced with 20 mM Tris [pH 6.8]).

Immunoprecipitated VR1 was released with SDS sample buffer containing 25 mM DTT and alkylated with subsequent addition of 50 mM lodoacetamide. Samples were electrophoresed on 8% SDS-PAGE gels and radiolabeled VR1 was detected by phosphoimaging (Packard Instruments, Meridian, CT). Densities were normalized to total 32P incorporation.

GST Fusion Protein Production

The cytoplasmic N- and C- terminal domains (amino acids 1–432 and 686–838, respectively) were cloned into pGex-4T1 at the XhoI site using PCR. GST fusion proteins were produced in the BL21 R E. coli bacterial strain (Stratagene, La Jolla, CA) by inducing protein production with 0.1 mM IPTG at room temperature for 4–5 hr. Bacteria were lysed using lysozyme treatment (Witholt et al., 1976) and sonication, and fusion proteins were purified using GSTrap FF 1 ml columns according to manufacturer’s instructions (Amersham-Phar-macia, Piscataway, NJ). After elution with 10 mM reduced glutathione, fusion proteins were concentrated to ~0.5–3 mg/ml using Centricon ultrafiltration devices (Millipore, Bedford, MA) and centrifuged at 100,000 × g for 60 min to pellet aggregated protein and debris.
In Vitro Phosphorylation of Fusion Proteins and Synthetic Peptide

Phosphorylation reactions consisted of 100 µM peptide or 1.8 µM fusion protein (−0.1 mg/mL), 50 mM Tris-Cl (pH 7.5), 1 mM EDTA (pH 8), 12 mM Mg-acetate, 0.25 mM ATP, 1 µCi/µL γ−32P-ATP (−4000–8000 cpm/pmol ATP), and 0.01 U/µl (peptide) or 1 U/µl (fusion proteins) bovine heart protein kinase A catalytic subunit (Sigma, St Louis, MO), and were incubated at 30°C. Reactions were stopped at various time points by spotting onto P81 phosphocellulose-lose paper (peptide) or by adding an equal volume of 2× sample buffer and boiling for 5 min (fusion proteins). P81 squares with blotted peptides were washed several times in 75 mM phosphoric acid, dried with methanol, and placed into 10 ml scintillation fluid. Fusion proteins were electrophoresed on SDS-PAGE gels, stained with Coomassie blue, and quantified using nearby BSA standards. 

32P incorporation was quantified using scintillation counting of blotted peptides or phosphorimaging and densitometry of fusion protein bands (Packard Instruments, Meridian, CT).

Initial reactions with and without 0.04 U/µl PKA were incubated for 30 min to determine whether peptide or fusion proteins were in vitro substrates (data not shown). Subsequent preliminary experiments focused on obtaining optimal phosphorylation reaction conditions. A set of experiments was conducted to determine ATP and kinase limitations due to time-dependent consumption or degradation using fusion proteins on beads and adding fresh reaction mix at various time points. These experiments found that reaction components were sufficient for up to 2–3 hr, indicating that reactions should be limited to 2 hr (data not shown). Kinase concentrations were modified according to the criterion of obtaining saturation within 1 hr. Saturation between time points was simply defined as a lack of statistical difference using ANOVA followed by Tukey’s post hoc comparison. If saturation occurred between time points earlier than 1 hr, kinase concentrations were reduced. Conversely, if saturation was not reached within 1 hr, kinase concentrations were increased (data not shown). These optimization experiments attempted to maximize the sensitivity and specificity of the in vitro kinase reactions and established the conditions outlined above.

Phosphorylation Site Determination

In-gel digests, HPLC, and Edman protein sequencing of fusion proteins were essentially conducted as described previously (Anderson et al., 2000). For mass spectroscopy, gel-eluted peptides were desalted using a Zip-Tip C18 column (Millipore, Bedford, MA) and analyzed using electrospray ionization (ESI) mass spectroscopy (FIESCIEX API 3000). Phosphopeptides were delineated using a 79 precursor ion scan and then sequenced using tandem MS/MS.

Phosphorylation Site Mutagenesis

VR1 mutants were constructed using PCR to generate mutant DNA and DpnI to digest methylated template as we have described previously (Gereau and Heinemann, 1998). The entire open reading frame of all mutants was sequenced.

Patch-Clamp Electrophysiology of Transfected Cell Lines

CHO-K1 cells were maintained in Harlan's F12 media with 10% FBS at 37°C, 5% CO2. Cells were cotransfected with pCDNA3 VR1 or various mutants along with pEGFP in a 5:1 ratio using Lipofectamine as per manufacturer’s instructions (Invitrogen, Carlsbad, CA). Whole cell patch-clamp recordings using standard techniques (see Kuzhi-kandathil et al., 2001 for details) were conducted 48–72 hr post-transfection. The external solution consisted of (in mM): 145 NaCl, 5 KCl, 2 CaCl2, 1 MgCl2, 10 HEPES, 10 glucose, pH 7.4; while the internal solution contained the following (in mM): 130 K-aspartate, 20 KCl, 1 EGTA, 1 MgCl2, 10 HEPES, 10 glucose, pH 7.4. Current amplitudes were measured at the peak amplitude recorded in response to capsaicin application and normalized to cell size by dividing by cell capacitance (pA/PF). The kinetics of current responses to capsaicin vary widely, particularly in the second of a pair of capsaicin applications. However, we always measured the peak amplitude, and longer capsaicin applications did not result in larger currents (not shown).

Surface Biotinylation Assay

CHO-K1 cells expressing wild-type or mutant VR1 were grown in 35 mm dishes, rinsed in PBS, and incubated with NHS-SS-biotin (Pierce, 1.5 mg/ml) for 1 hr at 4°C. The biotinylation reaction was quenched with 100 mM glycine in PBS, then the cells were washed, lysed in RIPA buffer, and centrifuged (20,000 × g) for 30 min. The supernatant was removed and incubated with streptavidin beads (Sigma, St Louis, MO) for 1 hr at room temperature. The beads were then spun down to separate proteins associated with the surface membrane from those in the cytosol or intracellular vesicles (supernatant). After washing the beads three times in RIPA buffer, biotinylated proteins were eluted with sample buffer and both supernatant and eluted proteins were subjected to SDS-PAGE and Western blot with a VR1 N-terminal antibody.

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