Inhibitory Role of Ser-425 of the α1 2.2 Subunit in the Enhancement of CaV 2.2 Currents by Phorbol-12-myristate, 13-Acetate*

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Voltage-gated calcium channels (CaV) 2.2 currents are potentiated by phorbol-12-myristate, 13-acetate (PMA), whereas CaV 2.3 currents are increased by both PMA and acetyl-β-methylcholine (MCh). MCh-selective sites were identified in the α2 2.3 subunit, whereas the identified PMA sites responded to both PMA and MCh (Kamatchi, G. L., Franke, R., Lynch, C., III, and Sando, J. J. (2004) J. Biol. Chem. 279, 4102–4109; Fang, H., Franke, R., Patanavanich, S., Lalvani, A., Powell, N. K., Sando, J. J., and Kamatchi, G. L. (2005) J. Biol. Chem. 280, 23559–23565). The hypothesis that PMA sites in the α2.2 subunit are homologous to the PMA-responsive sites in α2.3 subunit was tested with Ser/Thr → Ala mutations in the α2.2 subunit. WT α2.2 or mutants were expressed in Xenopus oocytes in combination with β1b and α2/β subunits. Inward current (IBa) was recorded using Ba2+ as the charge carrier. T422A, S1757A, S2108A, or S2132A decreased the PMA response. In contrast, S425A increased the response to PMA, and thus, it was considered an inhibitory site. Replacement of each of the identified stimulatory Ser/Thr sites with Asp increased the basal current and decreased the PMA-induced enhancement, consistent with regulation by phosphorylation at these sites. Multiple mutant combinations showed (i) greater inhibition than that caused by the single Ala mutations; (ii) that enhancement observed when Thr-422 and Ser-2108 are available may be inhibited by the presence of Ser-425; and (iii) that the combination of Thr-422, Ser-2108, and either Ser-1757 or Ser-2132 can provide a greater response to PMA when Ser-425 is replaced with Ala. The homologous sites in α2.2 and α2.3 subunits seem to be functionally different. The existence of an inhibitory phosphorylation site in the I-II linker seems to be unique to the α2.2 subunit.

The voltage-gated calcium channels (CaV)3 are complex proteins composed of α, β, α2/δ, and sometimes γ subunits. These channels are divided into three families (CaV 1–3) based on the sequence homology of the α subunits. The α1.0 family encodes the L-type channels; α2.1 encodes P/Q-type channels; α2.2 encodes N-type channels; α2.3 encodes R-type channels; and α3.0 encodes T-type channels (3, 4). The α1 subunit is the largest, and it incorporates the conduction pore, the voltage sensor, and the gating apparatus. The β, α2/δ, and γ subunits are collectively called the auxiliary subunits, and they dramatically influence the surface expression of the channels and the kinetics of the current (5). The intracellular segments of the α1 subunit, viz., the N and C termini and the intracellular loops between domains I and II, II and III, and III and IV, possess the binding/recognition sites for second messengers such as G protein βγ subunits or intracellular Ca2+ ([Ca2+]i) as well as sites that can be phosphorylated by protein kinase C (PKC) (6–9).

CaV channels respond differently to various activators of PKC. In the oocyte expression system, phorbol-12-myristate, 13-acetate (PMA) did not affect CaV 1.2 or 2.1 currents, whereas CaV 2.2 and 2.3 currents were potentiated. In contrast, acetyl-β-methylcholine (MCh) potentiated CaV 2.3 currents, decreased CaV 1.2c currents, and failed to modulate CaV 2.1 or 2.2 currents (1). Involvement of various PKC isozymes was suggested in the potentiation of CaV 2.3 currents (10). Since the auxiliary subunits used in our studies were the same, the source of the differential responses to PMA or MCh may be the α1 subunits. It is possible that PKC phosphorylation sites (Ser/Thr) with varying selectivity to PKC isozymes exist in the α1 subunit.

Since CaV 2.3 currents are potentiated by PMA or MCh and CaV 2.2 currents are increased by PMA only, we hypothesized that (i) the α2.3 subunit has both MCh-selective (Ser-888, Ser-892, Ser-894, and Ser-1995) and PMA-selective (Thr-365, Ser-369, Thr-879, Ser-1995, and Ser-2011) potential PKC phosphorylation sites; (ii) the α2.2 subunit has MCh-selective sites (Thr-422, Ser-425, Ser-926, Ser-2108, and Ser-2132) only; and (iii) the PMA-selective sites of α2.2 and 2.3 subunits are homologous. Our hypothesis about the MCh-selective sites was supported by the observation that Ala substitution of these sites in the α2.3 subunit inhibited the MCh response while leaving the response to PMA intact (1). In contrast, our subsequent studies revealed that Ala substitution of PMA-selective sites inhibited both MCh-induced and PMA-induced potentiation of CaV 2.3 currents (2). The contribution of individual sites to...
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PMA-stimulated current in the Ca\textsubscript{2.2} channels was examined in this study. These sites were mutated to Ala, the mutant channels were expressed in *Xenopus* oocytes, and the response to PMA was examined. The results revealed that for the most part, these PMA-selective sites of \( \alpha_1 \) 2.2 subunits respond differently from their homologous sites of the \( \alpha_1 \) 2.3 subunits.

**MATERIALS AND METHODS**

*Construction of Mutants—* We used the splice variant 37b of the \( \alpha_1 \) 2.2 subunit from the rat superior cervical ganglion in this study (11). The selected Ser/Thr were mutated to Ala by primer extension employing PCR with Pfu Turbo DNA polymerase (QuickChange XL site-directed mutagenesis kit, Stratagene, La Jolla, CA). Oligonucleotide primers containing the desired mutations were used to extend the template, the wild type \( \alpha_1 \) 2.2 subunit (37b) cDNA subcloned in the pcDNA 6 (Invitrogen). The PCR product was treated with DpnI, a restriction enzyme specific for methylated and hemimethylated DNA to digest the template DNA. The digested PCR product was transformed in *Escherichia coli* using the protocol from the supplier, and the DNA from the selected transformants was processed for sequencing. The whole coding region of the construct was sequenced (Biomolecular Research Facility, University of Virginia) to confirm the planned mutation and the absence of unwarranted mutations contributed by the PCR reaction.

*Harvesting of Oocytes and cDNA Injection—* Mature female *Xenopus laevis* frogs were obtained from *Xenopus* I (Ann Arbor, MI), housed in an established frog colony, and fed regular frog diet. For the removal of oocytes, a frog was anesthetized in 500 ml of 0.2% 3-aminobenzoic acid ethyl ester (Sigma) in water until unresponsive to a painful stimulus. The anesthetized frog was placed supine on ice, and an incision of skin to 1–2 cm was made, indicative of the ovary being exteriorized, and a lobule of oocytes (~500) was removed. The wound was closed in two layers, and the animal was allowed to recover from anesthesia, kept in a separate tank overnight, and returned to the colony the following day. The oocytes were washed twice in calcium-free OR2 solution (in mM: 82.5 NaCl, 2 KCl, 1.8 MgCl\textsubscript{2}, 5 HEPES, pH 7.5) and transferred to OR2 solution containing 1 mg/ml collagenase (type IA; Sigma). The dish containing the oocytes in collagenase solution was agitated for a period of 2–3 h at room temperature to remove the follicular cell layer. Defolliculation was confirmed by microscopic examination. Following this, the oocytes were washed in OR2 solution and transferred to modified Barth’s solution (in mM: 88 NaCl, 1 KCl, 2.4 NaHCO\textsubscript{3}, 0.41 CaCl\textsubscript{2}, 0.82 MgSO\textsubscript{4}, 15 HEPES, pH 7.4) containing 2.5 mM sodium pyruvate and 10 \( \mu \)g/ml gentamycin sulfate. The oocytes were allowed to recover by incubation at 16 °C for 3–10 h before cDNA injection. Nuclear (germinal vesicle) injection was performed (Drummond Nanoinject, Drummond Scientific Co., Broomall, PA) using 3 ng of 1:1:1 mix (molar ratio) of Ca\textsubscript{2.2}, \( \alpha_1 \) 2.2, \( \beta_1 \)B, and \( \alpha_2/\delta \) cDNA subunits. The oocytes were returned to Barth’s solution and incubated at 16 °C for 6–8 days before the recording of current.

*Current Recording—* Macroscopic currents, with Ba\textsuperscript{2+} (\( I_{\text{Ba}} \)) as the charge carrier, were recorded employing a two-electrode voltage clamp technique using oocyte clamp OC-725C (Warner Instrument Corp., Hamden, CT). The amplifier was linked to an interface and an IBM-PC-compatible computer equipped with pClamp software (version 8.2; Axon Instruments, Foster City, CA) for data acquisition and analysis. Leak currents were subtracted using the P/4 procedure. Microelectrodes with an agarose cushion were filled with 3 M CsCl; typical resistances were 0.5–2.5 M\text{ohms}. KC1-agar bridges were used as ground electrodes to minimize any junction potential attributable to changes in ionic composition of the bath solution. The oocytes were placed in a recording chamber (~500–μl volume) superfused with the recording solution containing (in mM): 40 Ba(OH)\textsubscript{2}, 50 NaOH, 2 KOH, 5 HEPES, using methanesulfonate as the anion to adjust the pH to 7.4. Niflumic acid (0.4 mM) was included to block intrinsic Cl channels. Oocytes were held at ~80 mV before being depolarized to 0 mV, the test potential for a duration of 850 ms. The current-voltage (I-V) relationship in oocytes expressing the wild type Ca\textsubscript{2.2} channel or the mutant was determined wherever necessary. The I-V was recorded for a duration of 450 ms using step depolarizations from ~50 to 100 mV in 10-mV increments.

*Drug Treatment—* All of the oocytes exhibiting \( I_{\text{Ba}} \) greater than 400 nA underwent control, treatment, and wash protocols. The control \( I_{\text{Ba}} \) was recorded at the 8th min after the oocyte was impaled. Following the recording of the control \( I_{\text{Ba}} \), PMA was perfused for 60 s, and the current was recorded after another 60 s, thus exposing the oocyte to the agonist for a period of 2 min.

*Chemicals—* PMA (Calbiochem) was dissolved in Me\textsubscript{2}SO (0.05%). The concentrated stock solution of PMA was stored frozen at ~20 °C and was diluted to the final concentration in the recording solution on the day of the experiment. To block endogenous Cl\textsuperscript{−} currents, niflumic acid (Sigma) was added to the recording solution, which was stirred overnight in order for it to dissolve.

*Data Analysis—* The data are shown as means ± S.E., unless otherwise indicated. The current represented the maximum amplitude of the inward current. The current inactivation rate was fit by a single exponential function using the equation

\[ y = A \exp(-t/t) + C \]  

(Eq. 1)

where \( A \) is the coefficient of the exponential, \( t \) is time (in ms), \( 1/t \) is the time constant (in ms), and \( C \) is the fraction of residual current. Statistical significance was determined using Student’s \( t \) test or paired \( t \) test, and \( p < 0.05 \) was considered significant.

**RESULTS**

*Selection of Potential PKC Phosphorylation Sites—* The strategy employed for the selection of potential PKC phosphorylation sites in the \( \alpha_1 \) 2.2 subunit was described in detail previously (1). Briefly, the sites were chosen based on the existence of a linear and/or helical motif for PKC recognition and sequence differences between the various Ca\textsubscript{2.2}, \( \alpha_1 \) subunits. These potential PKC phosphorylation sites of the \( \alpha_1 \) 2.2 subunit were chosen from the intracellular regions (a total of 13 regions) only, as they could be expected to have access to PKC. A set of six potential PKC phosphorylation sites (Thr-422 and Ser-425 of I-11
The quintuple mutant resulted in a significantly decreased response to PMA as compared with Ca_{2.2} WT. The effect of PMA in the quintuple mutant was between 40 and 50% of that produced by the single Ala mutation of Thr-422, Ser-1757, Ser-2108, or Ser-2132 (Fig. 1 and Table 1B). However, the PMA response in the double mutation of the I-II linker residues (T422A/S425A) was nearly equivalent to that of the WT (Table 1B). This result suggests two possibilities. (i) Removal of a stimulatory site at Thr-922 may be counteracted by the removal of an inhibitory site at Ser-425, and (ii) the presence of the three selected C-terminal sites (in the double mutant T422A/S425A) is sufficient for the normal PMA response. Conversely, the absence of C-terminal residues in the form of triple mutation (S1757A/S2108A/S2132A) decreased the PMA response significantly as compared with the Ca_{2.2} WT. The PMA response in the triple mutant S1757A/S2108A/S2132A was identical to that of the quintuple mutant and was significantly lower than that produced by the single Ala mutation of C-terminal sites (Table 1B).

To investigate the role of Thr-422 versus Ser-425 in combination with the C-terminal sites, quadruple mutants were constructed by including all three C-terminal sites with either Thr-422 or Ser-425. The effect of PMA on the quadruple mutants T422A/S1757A/S2108A/S2132A and S425A/S1757A/S2108A/S2132A revealed differential roles of Thr-422 and Ser-425 as follows. (i) Both of the quadruple mutants showed significant loss of PMA-induced potentiation of Ca_{2.2} currents as compared with Ca_{2.2} WT, (ii) T422A/S1757A/S2108A/S2132A showed a greater loss of the PMA effect than did S425A/S1757A/S2108A/S2132A (Table 1C), (iii) T422A/S1757A/S2108A/S2132A showed a greater loss of the PMA response than did any single Ala substitution (Table 1C), and (iv) the PMA response of S425A/S1757A/S2108A/S2132A was significantly greater than that produced by the triple mutation of the C-terminal sites (S1757A/S2108A/S2132A) (Table 1C).

Since T422A/S1757A/S2108A/S2132A produced greater inhibition of the PMA response than did single Ala substitutions, combined triple and double mutations of these sites were constructed to narrow down the residues required for the action of PMA. The triple mutants constructed were functional, and their response to PMA was as follows. (i) All three triple mutants, T422A/S1757A/S2108A, T422A/S1757A/S2132A, and T422A/S2108A/S2132A showed significant decrease in the response to PMA, and (ii) the response to PMA in T422A/S1757A/S2132A was lower than that of the single Ala mutants and significantly less than that of the other triple mutants (Table 1D). The additional inhibition seen with the triple mutant T422A/S1757A/S2132A suggests that the remaining site Ser-2108 is unable to respond to PMA on its own or that it is selectively blocked by Ser-425. Thus, the quadruple mutant T422A/S425A/S1757A/S2132A was constructed so that the effect of Ser-2108 alone might be examined. The effect of PMA was partially blocked as compared with the WT when Ser-2108 was the only site available (T422A/S425A/S1757A/S2132A); the PMA response in this quadruple mutant was similar to the effect of PMA in the single Ala mutation of Ser-2108 (Table 1D). Thus, Ser-2108 contributes some but not all of the PMA responsiveness.
Ser-425 may be considered an inhibitory site based on the following observations. (i) Ala substitution of Ser-425 increased the response to PMA (Fig. 1 and Table 1); (ii) there was normal PMA response in the double mutant T422A/S425A (Table 1); (iii) the PMA response was greater in the quadruple mutant S425A/S1757A/S2108A/S2132A than in the triple mutation (S1757A/S2108A/S2132A) of C-terminal sites (Table 1, B and C); and (iv) the PMA response of T422A was significantly lower than that of the double mutant T422A/S425A (Fig. 1 and Table 1, A and B).

Based on the above comparisons, it appears that the response of some C-terminal sites in addition to Thr-422 may be inhibited by Ser-425. Thus, several mutants were constructed by combining Ser-425 and the C-terminal sites to identify the sites involved in the interaction with Ser-425.

Three triple mutants, combining S425A and any two C-terminal site mutations (thus leaving Thr-422 and any one C-terminal site), were constructed. Among these triple mutants, S425A/S1757A/S2108A showed small basal currents (100–200 nA). Thus, this construct was considered a poor expressor. Both S425A/S1757A/S2132A and S425A/S2108A/S2132A showed decreased PMA response as compared with the WT (Table 1E). It seems that the availability of Thr-422 and any one potential C-terminal site in these triple mutants is insufficient to reproduce the increased PMA response seen in S425A (Fig. 1 and Table 1A). Two C-terminal sites in addition to Thr-422

### TABLE 1

Effect of mutations of the potential PKC phosphorylation sites on PMA (100 nM)-induced increase in the Cav2.2 current

The currents were expressed with α, 2.2 WT or the indicated mutant with β1B and α2δ subunits in Xenopus oocytes. The parallel WT PMA responses were combined, and the average was normalized to 100%; the PMA data from the mutants were corrected accordingly. The statistical significance was obtained by comparing the mutants with the parallel WT control. The effect of Thr-926 is not shown in this table as it failed to modify the PMA-induced current (Fig. 1).

| S/T/A at C terminus | PMA Increase | n | PMA |
|---------------------|-------------|---|-----|
|                      |             |   |    |
|                      | 20          | 40| 80 | 100| 140|
|                      | WT          |   |    |    |    |
|                      | mutant      |   |    |    |    |
| T S S S S S         | 100 ± 2     | 32|    |    |    |
| A S S S S S         | 49 ± 7      | 11|    |    |    |
| T T S S S S         | 135 ± 12    | 15|    |    |    |
| T T S S S S         | 44 ± 9      | 11|    |    |    |
| T T S S S S         | 60 ± 8      | 13|    |    |    |
| T T S S S S         | 59 ± 5      | 12|    |    |    |
| A A A A A A         | 22 ± 4      | 11|    |    |    |
| A A A A A A         | 85 ± 12     | 10|    |    |    |
| T S A A A A         | 23 ± 11     | 9 |    |    |    |
| A S A A A A         | 21 ± 6      | 9 |    |    |    |
| T A A A A A         | 59 ± 9      | 9 |    |    |    |
| A S A A A A         | 51 ± 6      | 9 |    |    |    |
| A S A A A A         | 16 ± 4      | 9 |    |    |    |
| A S A A A A         | 48 ± 9      | 9 |    |    |    |
| A A A A A A         | 63 ± 2      | 9 |    |    |    |
| T T A A A A S       | 54 ± 5      | 9 |    |    |    |
| T T A A A A S       | 70 ± 12     | 9 |    |    |    |
| T T A A A A S       | 121 ± 6     | 9 |    |    |    |
| T T A A A A S       | 84 ± 10     | 9 |    |    |    |
| T T A A A A S       | 155 ± 15    | 9 |    |    |    |
| T S S S S S         | 60 ± 7      | 9 |    |    |    |
| T T S S S S         | 22 ± 7      | 9 |    |    |    |
| T T S S S S         | 61 ± 8      | 9 |    |    |    |

* p < 0.001, ** p < 0.01, *** p < 0.02, **** p < 0.05 compared with WT, Student’s t test.

1 p < 0.001 as compared with S1757A/S2108A or S2108A/S2132A, Student’s t test.

2 p < 0.01 as compared with T422A/S1757A/S2108A or T422A/S2108A/S2132A, Student’s t test.

3 p < 0.02 as compared with T422A, Student’s t test.

4 p < 0.05 as compared with S1757A/S2108A/S2132A, Student’s t test.

5 p < 0.1 as compared with S425A/S1757A/S2108A/S2132A, Student’s t test.

# p < 0.05 as compared with S425A/S1757A/S2108A/S2132A, Student’s t test.

NS, not significant as compared with single Ala substitutions, paired Student’s t test.
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FIGURE 1. Single and quintuple Ala substitution of potential PKC phosphorylation sites differentially modulate the potentiation of expressed Cav 2.2 currents by PMA (100 nM). The currents were expressed with α, 2.2 WT or the indicated mutant with β1B and α2δ subunits in Xenopus oocytes. The oocytes were incubated at 16 °C for 7–8 days and voltage-clamped using −80 and 0 mM as holding and test potentials, respectively. The top panel shows the current tracings, and the bottom panel shows the averaged peak I_{Ba} in the WT and the mutant. The numbers in parentheses indicate n. **, p < 0.001; *, p < 0.01 as compared with WT; Student’s t test.

FIGURE 2. Combined Ala mutation of Ser-425 with the C-terminal sites differentially affected the potentiation of expressed Cav 2.2 currents by PMA (100 nM). The currents were expressed with α, 2.2 WT or the indicated mutant with β1B and α2δ subunits in Xenopus oocytes. The oocytes were incubated at 16 °C for 7–8 days and voltage-clamped using −80 and 0 mM as holding and test potentials, respectively. The top panel shows the typical current tracings in the WT and the mutants, and the bottom panel shows the averaged peak I_{Ba}. The numbers in parentheses indicate n. *, p < 0.05 as compared with WT; Student’s t test.

The effect of S425A as PMA-induced current in these mutants was significantly greater than that in the WT. In contrast, there was no increased PMA response in the double mutant S425A/S2108A. Thus, Ser-2108 may be another site required for the increased PMA response but insufficient, by itself, for PMA-stimulated enhancement of current. In addition, one more site, either Ser-1757 or Ser-2132, may be required for the increased current (Fig. 2 and Table 1E).

On the other hand, the stimulatory effects of both Thr-422 and Ser-2108 appear to be blocked when Ser-425 is available (Table 1, B and D). However, none of the mutants constructed so far were suitable to examine the effect of Ser-425 when both Thr-422 and Ser-2108 (and Ser-1757 or Ser-2132) are present together. Thus, double mutants comprising any two C-terminal sites were constructed. These double mutants left both I-II linker residues (Thr-422 and Ser-425) and any one C-terminal site available. All three double mutants S1757A/S2108A, S1757A/S2132A, and S2108A/S2132A showed significant inhibition of the response to PMA as compared with WT (Table 1F). However, the inhibition produced by S1757A/S2132A was markedly greater as compared with that of the other two double mutants, S1757A/S2108A or S2108A/S2132A (Table 1F). Thus, it appears that the effects of both Thr-422 and Ser-2108 but not of Ser-1757 or Ser-2132 are blocked by the presence of Ser-425 (see Fig. 4).

The contribution of Thr-422, Ser-1757, Ser-2108, and Ser-2132 in PMA-induced increase in the Cav 2.2 currents and the inhibitory action of Ser-425 were analyzed by mutating these residues to Asp to mimic the negative charge of a phosphorylated residue. The I_{Ba} through Asp mutants and the Cav 2.2 WT were analyzed in parallel. As compared with the WT, the I_{Ba} through T422D, S1757D, and S2108D were significantly greater. The I_{Ba} with the S2132D was also greater than the WT, although it was not statistically significant (p = 0.104) (Fig. 3A). In contrast, S425D did not cause a significant change in basal current. The PMA-induced potentiation of Cav 2.2 current was significantly reduced in all of these mutants, consistent with the increase in the basal I_{Ba} (Fig. 3B).

DISCUSSION

Potential PKC Phosphorylation Sites in the α, 2.2 Subunit Are Functionally Different from Their Homologues in the α, 2.3 Subunit—We have observed that Cav 2.2 currents are potentiated by PMA. Others have reported similar PMA-induced potentiation of Cav 2.2 currents and suggested that PKC is the mediator of this action (6). The loss or decrease in the action of
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Consistent with our results in the Ca\textsubscript{\(\gamma\)}, 2.2 channels, Ala substitution of \(\alpha\), 2.3 Thr-365, the homologue of \(\alpha\), 2.2 Thr-422, decreased both PMA-induced and MCh-induced increases in Ca\textsubscript{\(\gamma\)}, 2.3 currents. In contrast, Ala substitution of \(\alpha\), 2.3 Thr-879, Ser-1995, and Ser-2111, the sites homologous to \(\alpha\), 2.2 Thr-926, Ser-2108, and Ser-2132, failed to modulate the effect of PMA or MCh on Ca\textsubscript{\(\gamma\)}, 2.3 currents (2).

The increase in the PMA-induced current following the replacement of Ser-425 with Ala suggests that phosphorylation at this site may be inhibitory rather than stimulatory. The inhibitory effect of Ser-425 may be specific to the \(\alpha\), 2.2 subunit since Ala substitution of its homologue, Ser-369, in the \(\alpha\), 2.3 subunit failed to modulate Ca\textsubscript{\(\gamma\)}, 2.3 currents (2). The inhibitory action of this site in the \(\alpha\), 2.2 subunit versus the \(\alpha\), 2.3 subunit may be due to (i) the amino acids adjacent to Ser-425 that may permit the phosphorylation by different PKC isozymes, (ii) a requirement for phosphorylation of other sites not available in the \(\alpha\), 2.3 subunit, and/or (iii) an interaction with other unique regions of the \(\alpha\), 2.2 subunit or associated regulatory factors/subunits distinct from those in Ca\textsubscript{\(\gamma\)}, 2.3 channels. Conversely, a potential inhibitory action of this site of the \(\alpha\), 2.3 homologue may be blocked by other parts of the \(\alpha\), 2.3 subunit or unique channel cofactors/regulatory subunits.

PMA with Ala substitution of potential PKC phosphorylation sites in various Ca\textsubscript{\(\gamma\)}, channels further supports the contribution of PKC to the increased current (1, 2, 7). Similarly, in this study, Ala substitution of Thr-422, Ser-1757, Ser-2108, and Ser-2132 led to a decrease in the PMA-induced potentiation of Ca\textsubscript{\(\gamma\)}, 2.2 current. The contribution of these sites to phosphorylation-induced modulation of the current is further supported by the observation that Ala substitution of these sites to mimic the negative charge of a phosphate group led to an increase in the basal current as well as a decrease in the effect of PMA (Fig. 3).

It was suggested that Thr-422 and Ser-425 were the potential PKC phosphorylation sites in an earlier study (using rat brain \(\alpha\), 2.2, \(\beta\)1B, and \(\alpha\)2\(/\beta\)2 cDNA subunits) that employed patch clamp and a mammalian expression system (TSA 201 cells) (7). In that study, single Ala substitution of either Thr-422 or Ser-425 failed to modulate PMA-induced increase in the Ca\textsubscript{\(\gamma\)}, 2.2 currents, whereas double Ala substitution of Thr-422 and Ser-425 almost totally blocked the effect of PMA. The different results seen in our study may be due to the use of the \(\alpha\), 2.2 subunit from the superior cervical ganglion and the different expression system employed.

\textbf{FIGURE 3.} Single Asp substitution differentially affected the basal current (A) and the potentiation of expressed Ca\textsubscript{\(\gamma\)}, 2.2 currents by PMA (100 nM) (B). The currents were expressed with \(\alpha\), 2.2 WT or the indicated mutant with \(\beta\)1B and \(\alpha\)2\(/\beta\)2 subunits in \textit{Xenopus} oocytes. The oocytes were incubated at 16 °C for 7–8 days and voltage-clamped using –80 and 0 mV as holding and test potentials, respectively. The bars represent the averaged peak \(i_{\text{p}}\). The numbers in parentheses indicate \(n\), a, \(p < 0.001\); b, \(p < 0.01\); c, \(p < 0.05\) as compared with WT; Student’s t test. NS, not significant.

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Ca$_2+$, 2.2 channels are critical for pain transduction. The contribution of Ser-1757 to the increased PMA response may be biologically significant. Ser-1757 is present in exon 37b, a splice variant of $\alpha_1$, 2.2 from superior cervical ganglion. Ser-1757 is substituted by Ala in the exon 37a, the dorsal root ganglia-specific $\alpha_1$, 2.2 subunit splice variant preferentially present in neurons that contain nociceptive markers, vanilloid receptor 1, and voltage-gated sodium (Na$_v$, 1.8) channels (12). The differential localization of these two $\alpha_2$, 2.2 subunit splice variants and the modulation of one of them by PKC phosphorylation may be relevant in the regulation of the signal transduction for pain.

Thr-422, Ser-425, and Ser-2108 are homologous to Thr-365, Ser-369, and Ser-1995 of $\alpha_2$, 2.3 subunit, and the latter set of Thr/Ser were responsible for PMA- or MCh-induced potentiation of Ca$_2+$, 2.3 currents (2). The regulation at these sites appears to be complex. The availability or phosphorylation state of one site may determine the availability or role of another, i.e. the phosphorylation events may be ordered. It is possible that in the WT, Ser-425 becomes phosphorylated and blocks the phosphorylation of Thr-422 and the C-terminal sites leading to a limited response to PMA. The observation that T422A or S2108A showed significant decrease in the response to PMA is supportive of this interpretation. The decrease in the PMA response following the Asp substitution of Ser-425 is consistent with this possibility (Fig. 3B). However, the existence of any ordered phosphorylation of the potential PKC phosphorylation sites in the $\alpha_1$, 2.2 subunit is yet to be established. The combined action of Thr-422 or Ser-425 of the I-II linker with Ser-2108 and not Ser-1757 or Ser-2132 of the C terminus suggests specific functional interaction, either direct or indirect, between the I-II linker and the C terminus (Fig. 4). Such an action is analogous to the proposed interaction between the II-III linker and the C terminus in the $\alpha_2$, 2.3 subunit (1).

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