Identification of Sites Responsible for Potentiation of Type 2.3 Calcium Currents by Acetyl-β-methylcholine*

To address mechanisms for the differential sensitivity of voltage-gated Ca\(^{2+}\) channels (Ca\(_v\)) to agonists, channel activity was compared in Xenopus oocytes coexpressing muscarinic M\(_1\) receptors and different Ca\(_{\alpha1}\) subunits, all with β1/β2/δ subunits. Acetyl-β-methylcholine (MCh) decreased Ca\(_{\alpha1}\) 1.2c currents, did not affect 2.1 or 2.2 currents, but potentiated Ca\(_{\alpha1}\) 2.3 currents. Phorbol 12-myristate 13-acetate (PMA) did not affect Ca\(_{\alpha1}\) 1.2c or 2.1 currents but potentiated 2.2 and 2.3 currents. Comparison of the amino acid sequences of the α1 subunits revealed a set of potential protein kinase C phosphorylation sites in common between the 2.2 and 2.3 channels that respond to PMA and a set of potential sites unique to the α1 2.3 subunits that respond to MCh. Quadruple Ser → Ala mutation of the predicted MCh sites in the α1 2.3 subunit (Ser-888, Ser-892, and Ser-894 in the II–III linker and Ser-1987 in the C terminus) caused loss of the MCh response but not the PMA response. Triple Ser → Ala mutation of just the II–III linker sites gave similar results. Ser-888 or Ser-892 was sufficient for the MCh responsiveness, whereas Ser-894 required the presence of Ser-1987. Ser → Asp substitution of Ser-888, Ser-892, Ser-1987, and Ser-892/Ser-1987 increased the basal current and decreased the MCh response but did not alter the PMA response. These results reveal that sites unique to the II–III linker of α1 2.3 subunits mediate the responsiveness of Ca\(_{\alpha1}\) 2.3 channels to MCh. Because Ca\(_{\alpha1}\) 2.3 channels contribute to action potential-induced Ca\(^{2+}\) influx, these sites may account for M\(_1\) receptor-mediated regulation of neurotransmission at some synapses.

Voltage-gated Ca\(^{2+}\) (Ca\(_v\)) channels comprise a large family of heteromultimeric proteins containing a pore-forming α\(_1\) subunit and auxiliary β, α2/δ, and γ subunits. Electrophysiological and pharmacological characterizations have determined that α\(_1\) 1.1, 1.2, 1.3, and 1.4 subunits encode L-type channels; α\(_1\) 2.1 encodes P/Q-type channels; α\(_1\) 2.2 encodes N-type channels; α\(_1\) 2.3 encodes R-type channels; and α\(_1\) 3.1, 3.2, and 3.3 encode T-type Ca\(_{\alpha}\) channels (1, 2). The α\(_1\) subunit consists of four domains (I–IV), each with six transmembrane segments and is modulated by the binding of auxiliary α2/δ and β subunits (3). The intracellular segments of the α\(_1\) subunit, namely 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 Ca\(^{2+}\) ([(Ca\(^{2+}\)]\(_i\)) as well as sites that can be phosphorylated by protein kinase C (PKC) (4–7).

Members of the Ca\(_{\alpha}\) family are variably influenced by agents that activate PKC. For example, Ca\(_{\alpha1}\) 1.2a (rabbit heart) currents expressed in Xenopus oocytes were potentiated by the PKC activator phorbol 12-myristate 13-acetate (PMA) (8, 9); however, the same channel when expressed in tsA-201 cells, a subclone of the human embryonic kidney cell line HEK-293, was inhibited by PMA (10). In contrast, Ca\(_{\alpha1}\) 1.2c (rat brain and human heart) currents expressed in Xenopus oocytes were not influenced by PMA (11, 12). Among the members of the Ca\(_{\alpha1}\) 2.0 family, Ca\(_{\alpha1}\) 2.1 currents were not affected by PMA, whereas Ca\(_{\alpha1}\) 2.2 and 2.3 currents were potentiated (4–6, 13). Presumably, phosphorylation of certain amino acids alters the gating of channels, leading to greater currents. The sensitivity of Ca\(_{\alpha1}\) channels to PMA suggests that some of these channels may represent potential targets for certain hormones, neurotransmitters, and agonists that activate PKC by a receptor-mediated pathway. For example, stimulation of the odd numbered (M1, M3, and M\(_{\gamma}\)) muscarinic receptors results in the activation of PKC (14). Differential results were obtained when Xenopus oocytes coexpressing M\(_1\) receptors and Ca\(_{\alpha1}\) 1.2c or Ca\(_{\alpha1}\) 2.3 channels were exposed to MCh. Ca\(_{\alpha1}\) 1.2c currents were decreased by MCh (15), but Ca\(_{\alpha1}\) 2.3 currents were potentiated, possibly due to M\(_1\) receptor-induced activation of PKC (13).

PKC isozymes can be divided into three categories: classic PKCs or cPKCs, including PKCα, β, and γ isozymes that require Ca and are stimulated by phosphatidylinositol and diacylglycerol (DAG); novel or nPKCs (δ, ε, η, and θ), which are Ca-independent but still stimulated by phosphatidylinositol and DAG; and atypical or αPKCs (ζ, λ, ι), which are Ca- and DAG-independent. In a recent study, we suggested that αPKCs may be responsible for the action of MCh, whereas nPKCs may contribute to the action of PMA (13). Unique cPKC- and nPKC-selective phosphorylation sites may be present in the α\(_1\) 2.3 subunit.

Here we have compared currents among Ca\(_{\alpha1}\) 1.2c, 2.1, 2.2, and 2.3 channels expressed in Xenopus oocytes also expressing M\(_1\) receptors. Ca\(_{\alpha1}\) 1.2c currents were decreased by MCh (15) but unaffected by PMA. Neither MCh nor PMA affected 2.1 currents. Ca\(_{\alpha1}\) 2.2 and 2.3 currents were potentiated by PMA, but MCh increased only 2.3 currents, as shown before (13, 16). Based on the comparison of amino acid sequences in the channel types, we have mutated unique potential serine/threonine phosphorylation sites in the α\(_1\) 2.3 subunit to alanine and...
coexpressed the mutants with M1 receptors in Xenopus oocytes. Specific MCh-sensitive sites have, thus, been identified.

**EXPERIMENTAL PROCEDURES**

**Construction of Mutants**—Selected serines or threonines were mutated to alanines by using overlap extension PCR mutagenesis method, Stratagene, La Jolla, CA). Mutagenic primers containing the desired mutations were used to extend the template, the wild type rat brain Cav2.3 subunit cDNA subcloned in the pMT2 vector. The PCR product was treated with DpnI, a restriction enzyme specific for methylated DNA and hemi-methylated 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 1 (Ann Arbor, MI), housed in an established frog colony, and fed regular frog brittle on alternate days. For the removal of oocytes, a frog was anesthetized in 500 ml of 0.2% 3-aminobenzoic acid ethyl ester (Sigma, St. Louis, MO) in water until unconscious to a painful stimulus. The anesthetized frog was placed supine on ice, and an incision of ~1.5 cm in length was made through both skin and muscle layers of one lower abdominal quadrant. A section of the ovary was 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 millimolar: NaCl 82.5, KCl 2, MgCl2 1.8, HEPES 5, pH 7.5) and transferred to OR2 solution containing 1 mg/ml collagenase (type 1A, 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 millimolar: NaCl 88, KCl 1, NaHCO3 2.4, CaCl2 0.41, MgSO4 0.82, HEPES 15, pH 7.4) containing 2.5 mM sodium pyruvate and 10 μg/ml gentamicin sulfate. The oocytes were allowed to recover by incubation at 16 °C for 3–10 h before cDNA injection. Nucleo (germinal vesicle) injection was performed (Drummond “Nanoject,” Drummond Scientific Co., Broomall, PA) using a maximum of 4 ng of cDNA containing 3 ng of a 1:1 mix (molar ratio) of Ca2+, α1, β1B, α2/δ, cDNA subunits and 1 ng of rat M1 receptor cDNA in pDE3.1 (Invitrogen, Carlsbad, CA). 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(II) (Ba2+) 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 MΩ. CI-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 millimolar): NaCl 40, NaOH 5, KCl 4, BaCl2 0.8, MgCl2 0.8, HEPES 5, using methanesulfonate as the anion to adjust the pH to 7.4. Niflumic acid (0.4 mM) was included to block endogenous Cl− currents. The drug treatment and the current recording were as described under “Experimental Procedures.” The mean change in peak and late currents are discussed under “Results.”

**Results**

**MCh or PMA Differentially Affect Various Ca2+ Channels Expressed in Xenopus Oocytes**—Currents in oocytes expressing various Ca2+ channels were examined with either 1 μM MCh, the EC50 for MCh observed in our recent study (13), or PMA (100 nM). PMA failed to affect Ca2+ currents (percent change: peak current = −1.1 ± 1.5; late current = −2.6 ± 1.2; mean ± S.E., n = 4) significantly. Similarly, Ca2+ 2.1 currents (percent change: peak current = 0.9 ± 0.5; late current = 1.9 ± 5.8; mean ± S.E., n = 5) were also unaffected by PMA. Under the same conditions, PMA potentiated Ca2+ 2.2 currents (percent change: peak current = 37.2 ± 5.4; late current = 99.9 ± 28.9; mean ± S.E., n = 7) (Fig. 1) and Ca2+ 2.3 currents (see Figs. 1 and 4) significantly.

MCh also modulated the various Ca2+ currents differently. Ca2+ 1.2c currents were significantly decreased (percent change: peak current = −39.0 ± 6.0; late current = −56.0 ± 8.0; mean ± S.E., n = 5) as shown before (15). In contrast, Ca2+ 2.1 (percent change: peak current = 7.9 ± 3.7; late current = 3.6 ± 11.0; mean ± S.E., n = 4) and Ca2+ 2.2 currents (percent change: peak current = 16.5 ± 6.2; late current = 15.4 ± 7.7; mean ± S.E., n =
Sites for $M_1$ Receptor Potentiation of Cav 2.3 Current

4) were not affected by MCh. Ca$_{v}$ 2.3 currents were increased by MCh (see Figs. 2 and 4).

Selection of Possible Phosphorylation Sites—Intracellular regions (a total of thirteen regions) of the $\alpha_2$ 2.3 subunit that could be expected to have access to PKC were examined for potential phosphorylation sites. The sites were selected based on the existence of (a) linear sequence motifs fitting a PKC substrate, (b) sequence differences between the channel subtypes, (c) sites in regions of the channels that are known to possess some regulatory functions, and (d) potential sites fitting a possible helical motif for PKC recognition. There is some evidence that the PKC site in neuregulin is recognized as an $\alpha$-helix rather than as a linear sequence (17).

We identified nine serine/threonine sites in the $\alpha_2$ 2.3 subunit (Fig. 3). Four potential phosphorylation sites (Ser-888, Ser-892, and Ser-894 in the II–III linker and Ser-1987 in the C terminus) are unique to the $\alpha_2$ 2.3 subunit and are considered potential receptor-mediated ($M_1$) PKC phosphorylation sites, because, among the members of the Ca$_{v}$ 2.0 family, only Ca$_{v}$ 2.3 currents were potentiated by MCh application. The other five sites are homologous to the five potential PKC phosphorylation sites we noted in the $\alpha_2$ 2.2 subunit. This second set of potential phosphorylation sites was considered potentially PMA-selective, because both Ca$_{v}$ 2.2 and 2.3 currents were potentiated by PMA.

Ser $\rightarrow$ Ala Substitution of Predicted MCh-selective Serine Residues Inhibits MCh-induced Potentiation of Peak and Late $I_{Ba}$ through Ca$_{v}$ 2.3 Channels—The predicted potential MCh-selective sites of the $\alpha_2$ 2.3 subunit were subjected to mutational analysis. As a first step, a quadruple mutant was constructed by substituting Ser with Ala at all of the predicted potential MCh-selective sites, i.e. S888A/S892A/S894A/S1987A. Oocytes expressing the quadruple mutant or the wild type cDNA were studied in parallel, and the currents were analyzed. The $I_{Ba}$ peaked between −10 and 10 mV in both the wild type (at 81.9 ± 1.08 ms; mean ± S.E.; $n$ = 14) and the quadruple mutant (at 80.3 ± 1.3 ms; mean ± S.E.; $n$ = 6). Analysis of the I-V plot revealed that the $I_{Ba}$ appeared at −30 mV in both the wild type and the quadruple mutant; the $I_{Ba}$ reversed between 50 and 70 mV in the wild type and between 50 and 90 mV in the quadruple mutant. The kinetic properties of the current from the oocytes expressing the quadruple mutation (S888A/S892A/S894A/S1987A) were compared with those of the wild type Ca$_{v}$ 2.3 current. $I_{Ba}$ through the wild type (inactivating current (A1) = 626 ± 60 nA (80.1 ± 1.4% of total current), $\tau$ = 147 ± 12 ms and non-inactivating current (R) = 93 ± 10 nA; mean ± S.E.; $n$ = 14) and the quadruple mutant (A1 = 1447 ± 246 nA (79.7 ± 1.4% of total current), $\tau$ = 140 ± 4 ms and $r$ = 245 ± 53; $n$ = 6) channels showed similar monoexponential inactivation.

Although MCh or PMA potentiated the wild type $I_{Ba}$, differential results were observed with these agents in the oocytes expressing the quadruple mutation S888A/S892A/S894A/S1987A. MCh-induced potentiation of both the peak and the late $I_{Ba}$ was significantly decreased in S888A/S892A/S894A/S1987A compared with the wild type. In contrast, the PMA-induced increase in the $I_{Ba}$ was not affected significantly in the quadruple mutation (Fig. 4, A and B, and Table I).

To determine the relative contribution of the II–III linker serine residues to the MCh response, the triple mutant (S888A/S892A/S894A), three double mutants (S888A/S892A, S888A/S894A, and S892A/S894A), and three single (S888A, S892A, S894A) mutations were constructed. As observed with the quadruple mutation, the MCh-induced increase in the current was significantly inhibited in oocytes expressing the triple mutant S888A/S892A/S894A (Fig. 5). In contrast, none of the single or double mutations of the II–III linker serine residues significantly affected the MCh response when compared with their respective controls (Fig. 6, A and B, and Table I).

The contribution of the C-terminal Ser-1987 in the MCh-induced increase in Ca$_{v}$ 2.3 currents was examined using S $\rightarrow$ A substitution of Ser-1987 failed to affect the MCh response; similarly, S1987A in combination with S888A or S894A also failed to affect the MCh response significantly. However, S1987A in combination with S892A significantly inhibited the effect of MCh (Fig. 7). It appears that both Ser-888 and Ser-894 together, present in the double mutant S892A/S1987A, were not sufficient for the action of MCh.

FIG. 3. Schematic diagram of Ca$_{v}$ channel $\alpha_2$ 2.2 and 2.3 subunits showing the predicted potential PKC phosphorylation sites. I–IV indicates the four transmembrane domains. The numbers with the circles show the position of the amino acids. The sites within the rectangles are homologous. The strategy for selecting these sites is described under "Results."
Ser-888, Ser-892, and Ser-894 May Contribute Differently in the Action of MCh—It appears that one serine residue of the II–III linker region is sufficient for the action of MCh, because any double mutation (S888A/S892A, S888A/S894A, or S892A/S894A) of II–III linker serine residues failed to affect significantly the response to MCh (see Fig. 6B). However, in these double mutants, Ser-1987 in the C terminus was presumed to be available for MCh-induced modulation. Hence, the action of MCh was examined in triple mutants (S888A/S892A/S1987A, S888A/S894A/S1987A, and S892A/S894A/S1987A) that included any two serine residues of the II–III linker and Ser-1987. Application of MCh to the oocytes expressing these triple mutants led to differential results depending on the serine residue that was still available in these triple mutants. The availability of only Ser-894 in the triple mutant (S888A/S892A/S894A/S1987A) failed to affect significantly the response to MCh (see Fig. 6B).

Ser-888A/S892A/S894A mutant of the α1, 2.3 subunits and M1 receptors. The drug treatment and the current recording were as described under “Experimental Procedures.” The top panels show the current traces, and the bottom panels show the averaged peak and late currents in the wild type and the mutant. Numbers in parenthesis indicate “n,” **, p < 0.001; *, p < 0.05, compared with wild type; t test.

**Fig. 4. Effect of MCh (1 μM) or PMA (100 nM) on the Cav 2.3 currents expressed in Xenopus oocytes with the wild type or the quadruple S888A/S892A/S894A/S1987A mutant of the α1, 2.3 subunit.** The currents were coexpressed with α1, 2.3 β1α2δ/β subunits and M1 receptors. The drug treatment and the current recording were as described under “Experimental Procedures.” The top panels show the current traces, and the bottom panels show the averaged peak and late currents in the wild type and the mutant. Numbers in parenthesis indicate “n.” **, p < 0.001; *, p < 0.05, compared with wild type; t test.

**Fig. 5. Effect of MCh (1 μM) on the Cav 2.3 currents expressed with the wild type or the triple mutant, S888A/S892A/S894A of α1, 2.3 subunit in Xenopus oocytes.** The currents were coexpressed with α1, 2.3 β1α2δ/β subunits and M1 receptors. The drug treatment and the current recording were as described under “Experimental Procedures.” The top panel shows the current traces, and the bottom panel shows the averaged peak and late currents in the wild type and the mutant. Numbers in parenthesis indicate “n,” **, p < 0.001; *, p < 0.01, compared with wild type; t test.

**TABLE I**

Summary of MCh or PMA effects on the Cav 2.3 WT or Ala mutant channels

| Sites for M1 Receptor Potentiation of Cav 2.3 Current | % Increase (current) | MCh (1 μM) | PMA (100 nM) |
|-----------------------------------------------------|----------------------|------------|--------------|
| S888/892/894 | Ser/Ala at 187 | % | Peak | Late | n | Peak | Late | n |
| SerSerSer | Ser | 49 ± 2 | 104 ± 5 | 116 | 74 ± 6 | 181 ± 21 | 20 | 116 ± 7 | 74 ± 6 | 181 ± 21 | 20 |
| AlaAlaAla | Ala | 22 ± 7 | 19 ± 20 | 6 | 74 ± 10 | 210 ± 39 | 8 | 6 | 74 ± 10 | 210 ± 39 | 8 |
| AlaSerAla | Ser | 14 ± 4 | 31 ± 16 | 7 | 74 ± 10 | 210 ± 39 | 8 | 7 | 74 ± 10 | 210 ± 39 | 8 |
| AlaSerSer | Ser | 55 ± 5 | 130 ± 58 | 4 | 74 ± 10 | 210 ± 39 | 8 | 4 | 74 ± 10 | 210 ± 39 | 8 |
| SerAlaSer | Ser | 52 ± 10 | 131 ± 22 | 5 | 74 ± 10 | 210 ± 39 | 8 | 5 | 74 ± 10 | 210 ± 39 | 8 |
| SerSerAla | Ser | 51 ± 8 | 103 ± 13 | 9 | 74 ± 10 | 210 ± 39 | 8 | 9 | 74 ± 10 | 210 ± 39 | 8 |
| AlaAlaSer | Ser | 54 ± 10 | 120 ± 25 | 8 | 74 ± 10 | 210 ± 39 | 8 | 8 | 74 ± 10 | 210 ± 39 | 8 |
| AlaSerAla | Ser | 50 ± 6 | 82 ± 10 | 5 | 74 ± 10 | 210 ± 39 | 8 | 5 | 74 ± 10 | 210 ± 39 | 8 |
| SerAlaAla | Ser | 65 ± 6 | 138 ± 18 | 7 | 74 ± 10 | 210 ± 39 | 8 | 7 | 74 ± 10 | 210 ± 39 | 8 |
| SerSerAla | Ala | 62 ± 20 | 110 ± 22 | 7 | 74 ± 10 | 210 ± 39 | 8 | 7 | 74 ± 10 | 210 ± 39 | 8 |
| AlaSerAla | Ala | 49 ± 5 | 111 ± 23 | 8 | 74 ± 10 | 210 ± 39 | 8 | 8 | 74 ± 10 | 210 ± 39 | 8 |
| SerSerAla | Ala | 8 ± 3 | 16 ± 10 | 13 | 74 ± 10 | 210 ± 39 | 8 | 13 | 74 ± 10 | 210 ± 39 | 8 |
| AlaSerAla | Ala | 47 ± 6 | 87 ± 13 | 6 | 74 ± 10 | 210 ± 39 | 8 | 6 | 74 ± 10 | 210 ± 39 | 8 |
| AlaAlaSer | Ala | 26 ± 7 | 55 ± 14 | 7 | 74 ± 10 | 210 ± 39 | 8 | 7 | 74 ± 10 | 210 ± 39 | 8 |
| SerAlaSer | Ala | 41 ± 4 | 164 ± 41 | 12 | 74 ± 10 | 210 ± 39 | 8 | 12 | 74 ± 10 | 210 ± 39 | 8 |
| SerSerAla | Ala | 41 ± 5 | 104 ± 16 | 9 | 74 ± 10 | 210 ± 39 | 8 | 9 | 74 ± 10 | 210 ± 39 | 8 |

* p < 0.001; † p < 0.01; ‡ p < 0.05, compared with the respective control; t test.
this triple mutant appears to be insufficient for the MCh response. In contrast, the effect of MCh was not significantly affected in the triple mutants, S888A/S892A, S892A/S894A, S888A/S894A, or double mutation (S888A/S892A, S888A/S894A, S892A/S894A) of II–III linker serine residues of α1, 2.3 subunit. The currents were coexpressed with α2,3β1β2α2β2β2 subunits and M1 receptors. The panels show the averaged peak and late currents in the wild type and the mutants following MCh treatment. Numbers in parenthesis indicate “n.”

The importance played by the selected II–III linker serine residues in the potentiation of Ca v 2.3 currents by MCh was analyzed further by mutating Ser-888, Ser-892, or Ser-894 to aspartate to mimic the negative charge of a phosphorylated residue. Ser → Asp substitution of Ser-888 or Ser-892 in S892A/S894A/S1987A and S888A/S894A/S1987A, respectively, may be sufficient for MCh action.

The importance played by the selected II–III linker serine residues in the potentiation of Ca v 2.3 currents by MCh was analyzed further by mutating Ser-888, Ser-892, or Ser-894 to aspartate to mimic the negative charge of a phosphorylated residue. Ser → Asp substitution of Ser-888 or Ser-892 generated mutants that expressed Ca v 2.3 currents, whereas S894D failed to express the current. In general, the basal $I_{Ba}$ in the oxyoices expressing these Ser → Asp substitutions was significantly larger than that of the respective wild type controls (Figs. 9A and 10A). This increase in the basal $I_{Ba}$ was greater in S888D than in S892D. Coincident with this increase in the size of the basal current, the effect of MCh on both the peak and late $I_{Ba}$ was decreased significantly in S888D. Only the late $I_{Ba}$ was significantly decreased in the case of S892D (Fig. 9B). In parallel, we examined the effect of PMA in oocytes expressing the Ser → Asp substitution of II–III linker serine residues. In contrast to the generalized decrease in the MCh-induced potentiation of Ca v 2.3 currents in these Ser → Asp substitutions, the effect of PMA was intact in all of these aspartate mutants (Fig. 10B).

Based on the loss of the MCh response when Ser-892 and
Ser-1987 were both mutated to Ala (Fig. 7), we constructed Ser → Asp substitution of Ser-1987 and double Ser → Asp substitution of S892D/S1987D. Ser → Asp substitution of Ser-1987 increased the basal peak current significantly; however, the effect of MCh was not affected (Fig. 9, A and B). There was a pronounced increase in the basal current in the double Ser → Asp substitution of S892D/S1987D (Figs. 9 A and 10 A) with a coincident decrease in the effect of MCh (Fig. 9 B). However, the enhancement of current by PMA was not affected in S1987D or in S892D/S1987D (Fig. 10 B).

**DISCUSSION**

**Differential Sensitivity of Ca v Channels to Modulation Related to Various α1 Subunits**—Different Ca v currents vary in their responses to several hormones, neurotransmitters, and agonists that can activate PKC. The PKC activator PMA failed to modulate Ca v 1.2c currents, whereas M 1 receptor stimulation, which can activate PKC, decreased the currents through Ca v 1.2c channels. Neither PMA nor M 1 receptor activation modulated Ca v 2.1 currents. In contrast, PMA and M 1 receptor activation each potentiated Ca v 2.3 currents, whereas Ca v 2.2 currents were increased by PMA only (see Figs. 1 and 2). Because all of these Ca v channels were expressed in the same oocyte expression system, the same PKC isozymes should be available in all cases. The selective action of MCh on the Ca v 2.3 currents may be related to unique PKC sites in the Ca v 2.3 channels. It is likely that the channel-specific effects of MCh or PMA observed here were contributed by the α1 subunit of these channels, because the auxiliary subunits (β1 and β2/δ) used in the expression of these channels were the same. Hence, the selective action of MCh on Ca v 2.3 current may be due to the presence of unique phosphorylation sites in the α1 2.3 subunit. In agreement with this hypothesis, potential phosphorylation sites of Ser at 888, 892, 894, and 1987 were identified in the α1 2.3 subunit, and their quadruple mutation to Ala inhibited the effect of MCh but left the effect of PMA intact under the same conditions (Fig. 4).

**Potential PKC Sites in the II/III Linker of the α1 2.3 Subunit Are Required for Enhancement of Current by MCh**—Serine sites unique to the II/III linker of the α1 2.3 subunit seem to be of critical importance for the MCh responsiveness of these channels. Triple Ser → Ala mutation of all three of these sites caused loss of the MCh response (see Fig. 5). The observation that double or single mutations in this region did not eliminate the response suggests that any one of the sites, Ser-888, Ser-892, or Ser-894, is sufficient for altered channel gating by MCh receptor activation (see Fig. 6). However, the sites are not equivalent. If Ser-1987 in the C terminus is mutated to Ala, the
II–III linker sites Ser-888 or Ser-892 are still sufficient for the MCh response, but Ser-894 is not (see Fig. 8 and Table I). The basal current of the channel was significantly increased (at least 2-fold) following the Ser → Asp substitution of 888 or 892. In the face of this high basal activity, MCh caused less activation of the current, suggesting that the major effect of the Asp mutations was to enhance channel gating and opening rather than to increase channel expression. In the latter case, MCh stimulation similar to control would have been expected. These results are consistent with the hypothesis that the Ca_{2.3} channel-specific response of MCh is mediated via these unique sites in the II/III linker.

Several observations suggest that the MCh enhancement may be mediated via phosphorylation of the sites in the II/III linker: 1) The implicated sites were selected because they fit a PKC phosphorylation motif. 2) Replacement of the potentially phosphorylated Ser with Ala eliminates the MCh effect. 3) Single mutation of any of these sites to Asp (to mimic the negative charge of a phosphate) increases basal activity greatly, and there is less stimulation by MCh. 4) The MCh-induced increase in the Ca_{2.3} currents was inhibited by PKC inhibitors (13).

Ser-1987 in the C Terminus of the α_{2.3} Subunit Plays a Minor Role in the Enhancement by MCh—The lack of MCh enhancement, when Ser-1987 is available but when all the II/III linker serines were mutated to alanine (see Fig. 5), indicates that Ser-1987 is not sufficient for the MCh response. The presence of an intact MCh response in the Ser to Ala or Ser to Asp substitution of Ser-1987 (Figs. 7 and 9) indicates that Ser-1987 is not necessary for the MCh response. However, the presence of a serine at position 1987 could enable Ser-894 to serve as the sole available Ser site in the II–III linker (when residues 888 and 892 were mutated to Asp). When residue 1987 also was mutated to Ala, Ser-894 was insufficient alone and Ser-888 was insufficient in the presence of Ser-894. It is possible that Ser-888 and Ser-894 are incompatible when Ser-892 and Ser-1987 are mutated to Ala (see Fig. 7 and Table I). Attempts to make mutant α_{2.3} subunits with Ser → Asp substitutions at 894 were unsuccessful due to lack of expression of the mutant constructs (data not shown). Collectively, this information suggests that a constitutive negative charge at residue 894 may be detrimental for protein expression. Ser-892 seems to be capable of mediating MCh responsiveness independent of the availability of serine residues at the other sites examined.

The Ca_{2.3} II–III Linker Sites Are Not Required for Responsiveness to PMA—In contrast to MCh, PMA still increased the Ca_{2.3} currents when potential PKC sites unique to Ca_{2.3} channels (Ser-888, Ser-892, and Ser-894 in the II–III linker and Ser-1987 in the C terminus) were mutated to Ala (Fig. 4 and Table I) or to Asp (Fig. 10). The PMA responsiveness may be conferred by different sites such as Ser-369 in the I–II linker, Thr-879 in the II–III linker, and Ser-1995 and Ser-2011 in the C-terminal segment that are homologous to sites Thr-422, Ser-425, Thr-926, Ser-2108, and Ser-2132 in the PMA-responsive Ca_{2.2} channels. Thr-422 and Ser-425 have been identified as PMA-responsive sites in the α_{2.1} subunit of Ca_{2.2} channels (5).

Non-selective PKC inhibitors (a PKC β pseudosubstrate and high concentrations of Ro-31-8425) blocked both PMA and MCh effects on Ca_{2.3} channels suggesting involvement of PKCs in responses to both agonists (13). However, inhibitors more selective for calcium-dependent PKC isozymes (a PKC β translocation inhibitor or low concentrations of Ro-31-8425) or agents that prevent release or action of intracellular calcium blocked only the MCh response (13). These results are consistent with the hypothesis that MCh activates calcium-dependent PKCs via phospholipase activation to generate diacylglycerol and release calcium from intracellular stores. These calcium-dependent PKCs may phosphorylate specifically the II–III linker sites unique to Ca_{2.3} channels. PMA can bind to both cPKCs and nPKCs with much greater affinity than do diacylglycerols;
but, because it does not generate directly an increase in intracellular calcium, this action may be inadequate to activate cPKCs. PMA alone may activate predominantly nPKCs.

The Cav 2.3 channels contribute, along with Cav 2.1 and Cav 2.2 channels, to action potential-induced Ca influx in the central nervous system and at many peripheral synapses (18–21). The II/III linker in CaV 2.1 and CaV 2.2 channels is a site of interaction with the soluble N-ethylmaleimide-sensitive attachment factor receptor proteins required for the release of neurotransmitters (22–24). Receptor-induced phosphorylation of the II/III linker in Cav 2.3 channels may provide a rapidly reversible mechanism for regulating just the CaV 2.3 channel subtype, either directly, by altering intramolecular interactions between channel domains to promote channel opening, or possibly via effects on interaction with other regulatory proteins (G proteins or channel subunits). Thus M1 receptor-mediated regulation of these II/III linker sites may contribute to regulation of neurotransmission in both the central and peripheral nervous systems.

Acknowledgments—We are grateful to Dr. T. P. Snutch (University of British Columbia, Vancouver, British Columbia, Canada) for the clones of Ca channels and Dr. T. I. Bonner (Laboratory of Cell Biology, National Institute of Mental Health, Bethesda, MD) for the muscarinic M1 receptor clone. The technical assistance of Jacqueline Washington is gratefully appreciated.

REFERENCES
1. Jones, S. (1998) J. Bioenerg. Biomembr. 30, 299–312
2. Ertel, E., Campbell, K., Harpold, M., Hofmann, F., Mori, Y., Perez-Reyes, E., Schwartz, A., Snutch, T., Tanabe, T., Birnbaumer, L., Tsien, R., and Catterall, W. (2000) Neuron 25, 533–535
3. Walker, D., and De Waard, M. (1998) Trends Neurosci. 21, 148–154
4. Stea, A., Soug, T. W., and Snutch, T. P. (1995) Neuron 15, 929–940
5. Hamid, J., Nelson, D., Spaethgens, R., Dubel, S., Snutch, T., and Zamponi, G. (1999) J. Biol. Chem. 274, 6195–6202
6. Melliti, K., Meza, U., and Adams, B. (2000) J. Neurosci. 20, 7167–7173
7. Zuhlke, R., and Reuter, H. (1998) Proc. Natl. Acad. Sci. U. S. A. 95, 3287–3294
8. Shistik, E., Ivanina, T., Blumenstein, Y., and Dascal, N. (1998) J. Biol. Chem. 273, 17901–17909
9. Shistik, E., Keren-Raifman, T., Idelson, G., Blumenstein, N., and Ivanina, T. (1999) J. Biol. Chem. 274, 31145–31149
10. McHugh, D., Sharp, E., Scheuer, T., and Catterall, W. (2000) Proc. Natl. Acad. Sci. U. S. A. 97, 12334–12338
11. Snutch, T. P., Tomlinson, W. J., Leonard, J. P., and Gilbert, M. M. (1991) Neuron 7, 45–57
12. Bouron, A., Soldatov, N., and Reuter, H. (1995) FEBS Lett. 377, 159–165
13. Kamatchi, G., Tiwari, S., Chan, C., Chen, D., Do, S.-H., Durieux, M., and Lynch, C., III (2003) Brain Res. 968, 227–237
14. Caulfield, M., and Birdsall, N. (1998) Pharmacol. Rev. 50, 279–290
15. Kamatchi, G., Durieux, M., and Lynch, C. (2001) J. Pharmacol. Exp. Ther. 297, 981–990
16. Kamatchi, G., Tiwari, S., Durieux, M., and Lynch, C. I. (2000) J. Pharmacol. Exp. Ther. 293, 360–369
17. Vinton, B., Wertz, S., Jacob, J., Steere, J., Grisham, C., Cafiso, D., and Sando, J. (1998) Biochem. J. 330, 1433–1442
18. Artaidej, C. R., Adams, M. E., and Fox, A. P. (1994) Nature 367, 72–76
19. Takahashi, T., and Moniymaya, A. (1998) Nature 366, 156–158
20. Wheeler, D. B., Randall, A., and Tsien, R. W. (1994) Science 264, 107–111
21. Wu, L.-G., Borst, J. G. G., and Sakmann, B. (1998) Proc. Natl. Acad. Sci. U. S. A. 95, 4729–4735
22. Rettig, J., Sheng, Z.-H., Kim, D. K., Hodson, C. D., and Snutch, T. P. (1996) Proc. Natl. Acad. Sci. U. S. A. 93, 7363–7368
23. Sheng, Z.-H., Rettig, J., Takahashi, M., and Catterall, W. A. (1994) Neuron 13, 1303–1313
24. Sheng, Z.-H., Rettig, J., Cook, T., and Catterall, W. (1996) Nature 379, 451–454
Identification of Sites Responsible for Potentiation of Type 2.3 Calcium Currents by Acetyl-β-methylcholine
Ganesan L. Kamatchi, Ruthie Franke, Carl Lynch III and Julianne J. Sando

J. Biol. Chem. 2004, 279:4102-4109.
doi: 10.1074/jbc.M308606200 originally published online November 18, 2003

Access the most updated version of this article at doi: 10.1074/jbc.M308606200

Alerts:
• When this article is cited
• When a correction for this article is posted

Click here to choose from all of JBC's e-mail alerts

This article cites 23 references, 12 of which can be accessed free at http://www.jbc.org/content/279/6/4102.full.html#ref-list-1