Mechanisms of Homomeric α1 Glycine Receptor Endocytosis†

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ABSTRACT: Little is known regarding the mechanism(s) by which glycine receptors are endocytosed. Here we examined the endocytosis of homomeric α1 glycine receptors expressed in HEK 293 cells using immunofluorescence/confocal microscopy and whole-cell patch-clamp recordings. Our studies demonstrate that constitutive endocytosis of glycine receptors is blocked by the dominant negative dynamin construct K44A and that intracellular dialysis with peptide P4, a dynamin/amphiphysin-disrupting peptide, increased whole-cell glycine-gated chloride currents. To examine whether receptor endocytosis could be regulated by PKC, experiments with the PKC activator PMA (phorbol 12-myristate 13-acetate) were performed. PMA, but not its inactive analogue PMM (phorbol 12-monomyristate), stimulated receptor endocytosis and inhibited glycine-gated chloride currents. Similar to constitutive endocytosis, PKC-stimulated endocytosis was blocked by dynamin K44A. Mutation of a putative AP2 adaptin dileucine motif (L314A, L315A) present in the receptor cytoplasmic loop blocked PMA-stimulated receptor endocytosis and also prevented PMA inhibition of glycine receptor currents. In patch-clamp experiments, intracellular dialysis of a 12-amino acid peptide corresponding to the region of the receptor containing the dileucine motif prevented PKC modulation of wild-type glycine receptors. Unlike PKC modulation of the receptor, constitutive endocytosis was not affected by mutation of this dileucine motif. These results demonstrate that PKC activation stimulates glycine receptor endocytosis, that both constitutive endocytosis and PKC-stimulated endocytosis are dynamin-dependent, and that PKC-stimulated endocytosis, but not constitutive endocytosis, occurs via the dileucine motif (L314A, L315A) within the cytoplasmic loop of the receptor.

Glycine receptors are amino acid neurotransmitter receptors that contain integral anion-selective channels and are primarily responsible for fast synaptic inhibitory neurotransmission in the brainstem and spinal cord (*1*). These receptors are members of the cysteine loop superfamily of ligand-gated ion channels, which also includes nicotinic acetylcholine, γ-aminobutyric acid A ($GABA_A$), serotonin subtype 3 (5-HT3), and $GABA_C$ receptors (*2*−*4*). With four α subunits and one β subunit cloned to date, the receptors exist as homopentamers (α1−4 subunits) or heteropentamers (α1−4 coexpressed with the β subunit) (*1*). Topologically, glycine receptors display a large extracellular N-terminus, four membrane-spanning regions, with a large cytoplasmic loop between the third and fourth membrane-spanning regions, and a short extracellular C-terminus.

To participate in neurotransmission, glycine receptors are localized on the cell surface opposite glycinergic presynaptic terminals. Exocytosis of the receptor to the cell surface occurs primarily as clusters at nonsynaptic sites followed by lateral diffusion to the synapse in the "diffusion retention" model (*5*). The number of receptors per cluster is dynamic, with both stabilized and freely moving receptors contributing to the cluster (*6*). Multiple diffusion domains within extrasynaptic, perisynaptic, and synaptic regions have been noted (*7*). At the cell surface, glycine receptor accumulation and stabilization are promoted by gephyrin, a tubulin-binding protein that functions as a postsynaptic scaffolding protein at inhibitory synapses (*8*−*10*). Interestingly, chronic strychnine treatment decreases surface receptors presumably by preventing the trafficking of newly synthesized receptors to the cell surface (*11*).

Although insertion and stabilization of glycine receptors have been studied in several laboratories, very little is known about the mechanism(s) by which glycine receptors are endocytosed. α1 glycine receptors are extensively ubiquitinated while in the plasma membrane, suggesting that endocytosis of the receptor may be regulated by ubiquitination (*12*). The mechanism(s) by which glycine receptors are endocytosed, however, remains unknown. Since the number of receptors at the cell surface is a determinant of the efficacy of glycinergic transmission, it is important that receptor trafficking to and from the plasma membrane be understood. Here we demonstrate that PKC activation stimulates glycine receptor endocytosis, that both constitutive endocytosis and PKC-stimulated receptor endocytosis proceed in a dynamin-dependent manner, and that PKC-regulated, but not constitutive, endocytosis involves a dileucine motif at L314, L315 within the receptor intracellular loop.

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EXPERIMENTAL PROCEDURES

Materials. Glycine, PMA[1] (phorbol 12-myristate 13-acetate), PMM (phorbol 12-monomyristate), and protein kinase C inhibitory peptide (PKCI 13−19) were from Sigma. Bisindolylmaleimide I was from LC Laboratories (Woburn, MA). PMA, PMM, and bisindolylmaleimide I were dissolved in DMSO, frozen at stock concentrations, and diluted to working concentrations prior to use. Working concentrations of DMSO were ≤0.1%. The dileucine peptide (RQHKELL-RFRRK), P4 peptide (QVPSRPNRAP), and scrambled peptide (PRAPNSRQPV) were synthesized by Genemed Synthesis (San Francisco, CA). All peptides were diluted in a pipet solution to a concentration of 50 μM prior to patch-clamping. cDNAs were obtained from the following sources: human glycine receptor α1 subunit cDNA (Dr. Heinrich Betz, Max-Planck Institute for Brain Research, Frankfurt, Germany), dynamin and dynamin K44A (Dr. Sandra Schmid, Scripps Research Institute). The α1 glycine receptor subunit was subcloned from its original pCIS2 vector into pcDNA3.1 V5-His (Invitrogen, Carlsbad, CA). Mutations of receptor cDNA were performed using a QuickChange site-directed mutagenesis kit (Strategene, La Jolla, CA) using appropriate primers (Integrated DNA Technologies). Mutations were verified by sequencing. HEK 293 cells (American Type Culture Collection, Manassas, VA) were transiently transfected using calcium phosphate precipitation or liposome (Lipofectamine 2000, Invitrogen, CA) transfection methods. Transfected cells were used for experiments approximately 24−48 h following transfection.

Immunofluorescence and Confocal Microscopy. To assess endocytosis, living HEK 293 cells expressing recombinant V5-His-tagged α1 glycine receptors were incubated in serum-free medium for 30 min. The cells were then incubated on ice in 100 μL of HEPES buffer (HEPES (25 mM), NaCl (140 mM), KCl (5.4 mM), $CaCl_2$ (1.8 mM), glucose (15 mM), pH 7.4) containing mouse anti-V5 antibody (1:100 dilution; Invitrogen) for 1 h, followed by a 1 h incubation on ice with a Texas Red-conjugated goat anti-mouse antibody (1:500 dilution; Molecular Probes, Eugene, OR) to label cell surface glycine receptors. The cells were then returned to the incubator (37 °C) for 0−30 min with or without drug treatments (times and treatments as indicated) to allow receptor endocytosis. To identify the Texas Red-labeled receptors that remained on the cell surface during the endocytosis period, the cells were incubated for 1 h on ice in 1 mL of buffer containing an Alexa 488-conjugated rabbit anti-goat antibody (1:1000 dilution; Molecular Probes).

To assess cell surface vs intracellular receptor expression, living HEK 293 cells expressing V5-His-tagged α1 glycine receptors were incubated in serum-free medium for 30 min. The cells were then incubated on ice in 100 μL of HEPES buffer containing mouse anti-V5 antibody for 1 h, followed by a 1 h incubation on ice with a Texas Red-conjugated goat anti-mouse antibody to label cell surface glycine receptors. The cells were then fixed with 4% paraformaldehyde/4% sucrose, permeabilized with 0.5% Triton X100, and blocked with BSA/10% FBS. Intracellular receptors were immunolabeled with an anti-V5 antibody for 1 h at room temperature followed by incubation with an Alexa 488-conjugated rabbit anti-mouse secondary antibody.

A Bio-Rad MRC-1024 laser scanning system with an argon/krypton laser was used for confocal microscopy (Bio-Rad, Hercules, CA). Alexa 488 was excited at 488 nm and the emission of light collected at 522 nm using a 522/30 emission filter. For Texas Red detection, a 568 nm excitation wavelength was used and the emission of light collected at 605 nm using the 605/32 emission filter. A 60× objective was used to collect images. The laser intensity, photomultiplier gain, and iris were optimized for each set of experiments but kept constant within each "*n*". Images were captured using LaserSharp MRC-1024 software. At least three independent experiments were performed, where independent experiments were defined as experiments performed on different batches of HEK 293 cells transfected at different times. Each independent experiment was performed in triplicate, and at least three cells were quantified for each condition. Data were quantified using Metamorph software (Universal Imaging Corp., Downingtown, PA). Specifically, a region of interest was drawn around the cell perimeter. Signal threshold values were then selected and kept constant for each experiment. Colocalization measurements were performed to determine the percentage of Texas Red signal not colocalized with the Alexa 488 signal. The percentage of receptors endocytosed during a given time period was taken from this measurement. The data from each independent experiment were then averaged and analyzed by an unpaired *t* test.

Whole-Cell Patch-Clamp Recordings. Whole-cell currents activated by glycine were recorded by the whole-cell patch-clamp technique. Patch pipets of borosilicate glass (1B150F, World Precision Instruments, Inc., Sarasota, FL) were pulled (Flaming/Brown, P-87/PC, Sutter Instrument Co., Novato, CA) to a tip resistance of 1−2.5 MΩ. The pipet solution contained 140 mM CsCl, 10 mM EGTA, 10 mM HEPES, and 4 mM Mg-ATP, pH 7.2. Coverslips containing cultured cells were placed in a small chamber (∼1.5 mL) and superfused continuously (7−10 mL/min) with extracellular solution to prevent accumulation of drugs. Recordings made at 35 °C were achieved with an in-line solution heater (TC-324B, Warner Instrument, Hamden, CT). The extracellular solution contained 125 mM NaCl, 5.5 mM KCl, 0.8 mM $MgCl_2$, 3.0 mM $CaCl_2$, 20 mM HEPES, and 10 mM D-glucose, pH 7.3. Glycine-induced chloride currents from the whole-cell patch-clamp recordings were obtained using an Axoclamp 200A amplifier (Axon Instruments, Foster City, CA) equipped with a CV 201A headstage. Glycine currents were low-pass filtered at 5 kHz, monitored on an oscilloscope and a chart recorder (Gould TA240), and stored on a computer (pClamp 6.0 software, Axon Instruments) for subsequent analysis. A 60−80% series resistance compensation was applied at the amplifier. To monitor the possibility that access resistance changed over time, at the initiation of each recording the current response to a 5 mV voltage pulse was measured and then continually referenced throughout the recording period. If a change in access resistance was observed during the recording period, the patch was aborted and the data were not included in the analysis. Cells were voltage-clamped at −60 mV. Glycine was prepared in the extracellular solution and then applied at 5 min intervals from

[1] Abbreviations: PMA, phorbol 12-myristate 13-acetate; PMM, phorbol 12-monomyristate; PKCI 13−19, protein kinase C inhibitory peptide; BIM, bisindolylmaleimide.

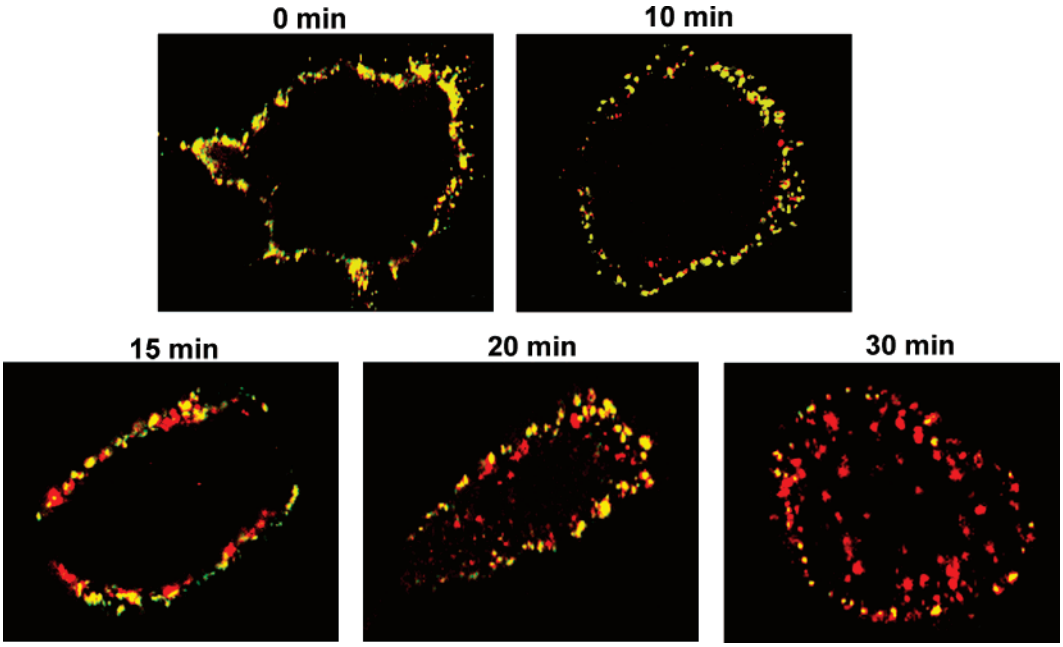

FIGURE 1: Time course of constitutive glycine receptor endocytosis. Endocytosis of homomeric α1 glycine receptors expressed in HEK 293 cells was measured using the protocol described in the Experimental Procedures. Briefly, cell surface receptors were Texas Red-labeled on ice and subsequently incubated at 37 °C for 0, 10, 15, 20, or 30 min. Texas Red-labeled receptors that remained on the surface during the incubation period were then labeled on ice with Alexa 488. The cells were fixed and visualized by confocal microscopy. The Texas Red labeling within the cell interior represents receptors that were originally labeled on the cell surface and were endocytosed during the 37 °C incubation periods. The yellow signal (red + green double label) represents receptors that were on the cell surface during the first labeling and remained on the surface (not endocytosed) during the 37 °C incubation period. Shown is a representative time course from three independent experiments.

independent reservoirs by gravity flow for 10 s to cells using a Y-shaped tube positioned within 100 μm of the cells. With this system, the 10−90% rise time of the junction potential at the open tip was 12−51 ms (*13*). Once a stable baseline glycine response was obtained, the cells were perfused with either 100 nM PMA or 100 nM PMM for 15 min, followed by a return to the extracellular solution lacking phorbol esters. Glycine applications were continued at 5 min intervals throughout the phorbol ester treatment period and for at least 15 min beyond the termination of phorbol ester application. The current activation time was assessed using the time to rise from 10% to 90% of the current amplitude activated by saturating glycine (1 mM). Desensitization is described by the percentage of desensitization using the formula D (%) $= 100(I_{peak} - I_{10s})/I_{peak}$, where I_{peak} is the current at the peak response to saturating glycine and I_{10s} is the residual current at the end of 10 s of glycine application.

RESULTS

To demonstrate that glycine receptors undergo constitutive endocytosis, we performed an antibody-labeling technique in living cells that measures receptor endocytosis (*14*, *15*). For these experiments, cell surface receptors were labeled with Texas Red-conjugated antibody and then incubated at 37 °C for various times to allow endocytosis of the labeled receptor. The cells were then removed from the incubator, and any Texas Red-labeled receptors remaining on the cell surface were then further labeled with Alexa 488. Using this technique, receptors that were endocytosed during the 37 °C incubation period were observed as a red signal within the interior of the cell, whereas those Texas Red-labeled receptors that did not undergo endocytosis were doubly labeled and appear as a yellow signal around the cell perimeter. A time course for constitutive glycine receptor endocytosis is shown in Figure 1. At the zero time point, no receptor endocytosis is visible. By 10 min a very small amount of endocytosis can be observed. At 15 and 20 min, increasing amounts of receptor are endocytosed and appear as red vesicular structures, presumably endosomes. Within 30 min the majority of receptors that were on the cell surface during the initial labeling period had been endocytosed.

Endocytosis can occur via a variety of routes including macropinocytosis, clathrin-coated vesicles, and flask-shaped lipid microdomains called caveolae (*16*). Endocytosis by clathrin and caveola pathways utilizes dynamin, a small GTPase that is involved in the scission event that liberates nascent endocytic vesicles from the plasma membrane. To better define the mechanism by which glycine receptors were constitutively endocytosed, experiments were conducted in cells cotransfected with the receptor and either dynamin or the dominant negative dynamin construct K44A (Figure 2A,B). During a 30 min, 37 °C incubation period, endocytosis of the receptor was nearly complete in cells cotransfected with dynamin. Cotransfection with dynamin K44A, however, significantly blocked receptor endocytosis, indicating the receptor is endocytosed via a dynamin-dependent pathway. To determine whether coexpression of the receptor with dynamin K44A would result in the accumulation of surface receptors, surface vs intracellular receptors were immunolabeled in cells expressing the receptor with empty vector, wild-type dynamin, or dynamin K44A (Figure 2C). Coexpression with dynamin K44A increased glycine receptor cell surface expression.

As a complementary functional approach to our immunofluorescence experiments, whole-cell patch-clamp record-

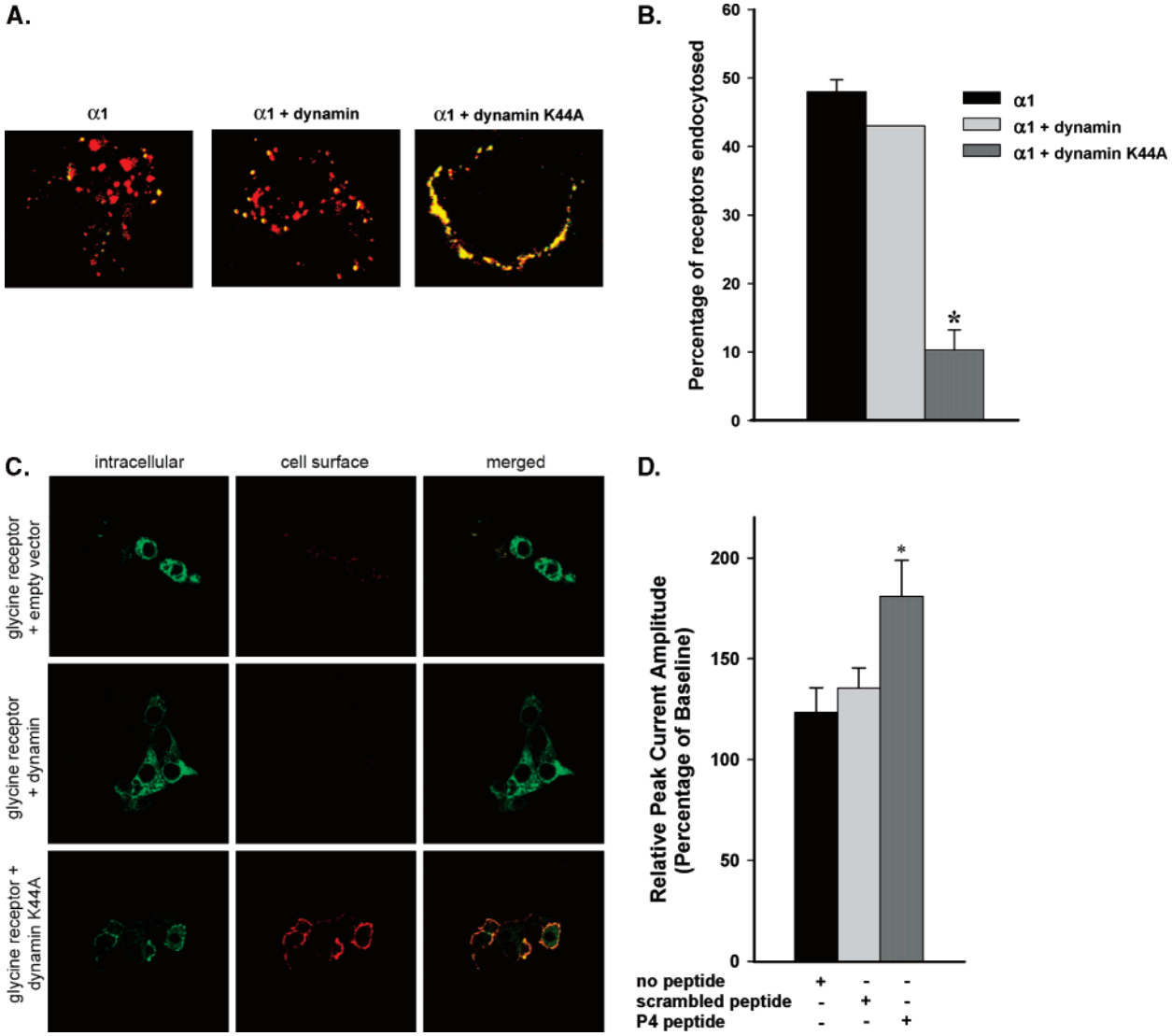

FIGURE 2: Constitutive glycine receptor endocytosis occurs via a dynamin-dependent pathway. (A) The dominant negative dynamin construct K44A inhibits constitutive glycine receptor endocytosis. Receptor endocytosis was assessed in HEK 293 cells expressing α1 glycine receptors alone or coexpressed with either wild-type dynamin or dynamin K44A. Cell surface receptors were immunolabeled with Texas Red and incubated at 37 °C for 30 min to allow endocytosis. Receptors that were not endocytosed and remained on the cell surface were additionally labeled with Alexa 488 (green). The cells were then fixed with 4% paraformaldehyde and visualized by confocal microscopy. Receptors expressed in the absence or presence of wild-type dynamin displayed robust endocytosis. Very little receptor endocytosis was observed when glycine receptors were coexpressed with dynamin K44A. (B) Replicate data for experiments similar to those presented in (A). Endocytosis of glycine receptors coexpressed with dynamin K44A was significantly less than that observed for receptor expressed alone or coexpressed with wild-type dynamin ($p \leq 0.001$, unpaired t test, $n = 3$ for each condition). (C) Coexpression of dynamin K44A increases glycine receptor cell surface expression. Indirect immunofluorescence was used to label cell surface V5-tagged receptors in intact cells (Texas Red). The cells were then permeabilized, and the intracellular receptor pool was immunolabeled with Alexa 488 (green). (D) The dynamin/amphiphysin-interaction-disrupting peptide P4 increases glycine responses in HEK 293 cells expressing α1 glycine receptors. Whole-cell patch-clamp recordings were obtained from HEK 293 cells expressing α1 glycine receptors. The pipet solution contained no peptide, scrambled peptide (50 μM), or P4 peptide (50 μM). Shown are mean results ± SEM of current peak amplitudes at 30 min expressed as a percentage of the initial current at time zero. Current amplitudes in response to glycine (50 μM) displayed some run-up by 30 min in the absence of peptide or with intracellular dialysis of the scrambled peptide. In the presence of P4 peptide, a significant increase in peak amplitude of the glycine response was observed compared to that of either no peptide or scrambled peptide controls (*, $p \leq 0.05$; unpaired t test, $n \geq 8$).

ings were performed in HEK 293 cells expressing the V5-His-tagged α1 glycine receptors. These tagged receptors displayed concentration-dependent glycine responses and Zn^{2+} modulation similar to those of untagged receptors, indicating that addition of V5-His tag did not have adverse functional effects (data not shown). To confirm the involvement of dynamin in constitutive glycine receptor endocytosis, the P4 peptide, which inhibits dynamin/amphiphysin interaction (*17*, *18*), was used in whole-cell patch-clamp experiments (Figure 2D). In these experiments, a 50 μM concentration of either P4 active peptide (QVPSRPNRAP) or scrambled peptide (PRAPNSRQPV) was included in the pipet solution and dialyzed into the cell. Glycine receptor responses for control (no peptide) and scrambled peptide conditions displayed run-up by 30 min consistent with previous studies. In the presence of P4, glycine-gated chloride currents were increased at 30 min compared to currents in cells exposed to the control peptide, suggesting that disruption of the dynamin/amphiphysin interaction results in the accumulation of glycine receptors at the cell surface.

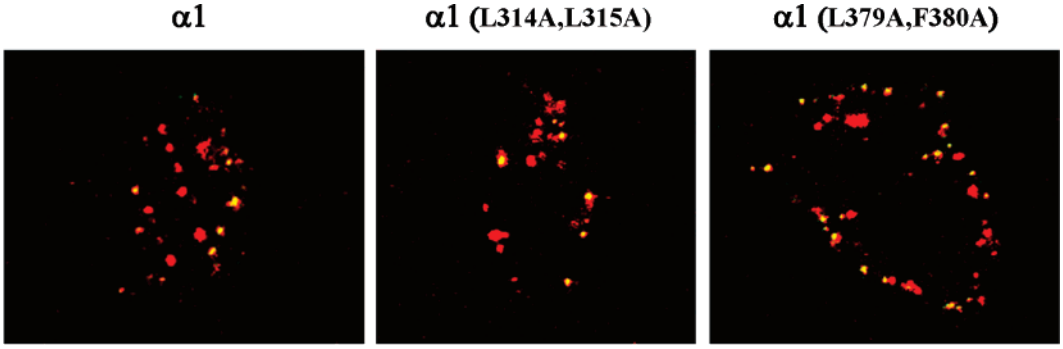

FIGURE 3: Mutation of a dileucine motif on the glycine receptor does not affect constitutive endocytosis. Glycine receptor endocytosis was assessed in HEK 293 cells expressing α1, α1(L314A, L315A), or α1(L379A, F380A) constructs. During a 30 min incubation at 37 °C, the majority of labeled receptors were endocytosed, indicating that the dileucine-type motifs are not required for constitutive glycine receptor endocytosis. Shown are representative images from three independent experiments.

For dynamin-dependent endocytosis, integral plasma membrane proteins contain motifs for recognition by AP2 adaptin, a protein that recruits target proteins for endocytosis (*19*). AP2 adaptin subunits recognize two distinct motifs on target proteins: a dileucine motif ([DE]XXXL[LI]), where X = a polar residue, and a tyrosine-based motif (YXXθ), where θ represents bulky hydrophobic side chain residues. Although no tyrosine-based motifs appeared to be present on the cytoplasmic loop of the glycine receptor α1 subunit, we identified two putative dileucine-type motifs. The first was a leucine pair at residues 314 and 315. A second motif consisted of a leucine/phenylalanine pair at positions 379 and 380. With regard to the latter pair, the presence of two leucines is not an absolute requirement for AP2 binding and bona fide "dileucine" motifs can include non-leucine hydrophobic residues in the second position (*19*). To examine whether these putative dileucine-type motifs affected constitutive receptor endocytosis, the residues were mutated to alanines. As can be seen in Figure 3, receptors containing these mutations, i.e., α1(L314A, L315A) and α1(L379A, F380A), were endocytically competent. As an additional approach, we also determined whether there was a difference in peak amplitude currents between cells expressing the wild-type and dileucine mutant α1(L314A, L315A) receptors. If the dileucine motif were important for constitutive endocytosis, it would be expected that receptors containing the mutant would display larger glycine currents since endocytically incompetent receptors should accumulate at the cell surface (*14*). In whole-cell patch-clamp experiments, similar maximal current densities in response to a saturating glycine concentration were observed for wild-type α1 (96 ± 12 pA/pF, n = 14) and α1(L314A, L315A) (95 ± 11 pA/pF, n = 14) receptors. This result is consistent with the lack of the involvement of the dileucine motif in constitutive endocytosis. Last, the role of the dileucine motif was further investigated using a synthetic peptide (RQHKELLRFRRK) corresponding to the L314, L315 containing region of the receptor. For these experiments, the peptide was included in the pipet to allow intracellular diffusion. Once inside the cell, this peptide would theoretically be available to compete with the dileucine motif on the receptor for binding to AP2 adaptin and thus block receptor endocytosis (*14*, *15*). These experiments also failed to show a role for the dileucine motif in constitutive receptor endocytosis. In the presence of peptide, glycine responses were 103 ± 6% of the initial response at 30 min (n = 8). Thus, both confocal microscopy imaging data and patch-clamp experiments indicate that this dileucine motif does not participate in constitutive glycine receptor endocytosis. This outcome was surprising since a similar dileucine motif on the homologous $GABA_A$ receptor prevents constitutive endocytosis (*14*). To further characterize the dileucine mutant, we also assessed the glycine EC_{50} (α1, 68 ± 8.6; α1(L314A, L315A), 45 ± 4.7; $n \geq 5$), extent of desensitization (α1, 40 ± 3.5%; α1(L314A, L315A), 45 ± 2.8%; n = 14), and channel activation rate (α1, 32 ± 3.3 ms; α1(L314A, L315A), 24 ± 3.4 ms; $n \geq 12$).

To determine whether receptor endocytosis could be regulated by signaling pathways, immunofluorescence experiments were performed with the PKC activator PMA. For these experiments, a 100 nM concentration of either PMA or its inactive analogue PMM was present during a 15 min, 37 °C incubation period (Figure 4). A 15 min incubation period was chosen at which to examine PMA-stimulated endocytosis since at 15 min only a limited degree of constitutive endocytosis is observed. Thus, a PMA-induced increase in endocytosis would be readily detectable. PMA significantly increased glycine receptor endocytosis when compared to its inactive analogue PMM.

To determine whether PKC-stimulated endocytosis proceeded by the dynamin pathway, receptors were expressed alone or coexpressed with either dynamin or dynamin K44A. Endocytosis was then assessed at 15 min in the presence of either PMM or PMA (Figure 5). For these experiments very little constitutive endocytosis occurred in PMM-treated cells for any of the transfection conditions. In contrast, PMA treatment in cells expressing receptor alone or receptor + dynamin displayed robust endocytosis. When the receptor was coexpressed with dynamin K44A, however, PMA treatment failed to promote endocytosis. A graph of replicate data (Figure 5B) shows that no significant difference was observed between PMM and PMA treatments in the presence of dynamin K44A, indicating the requirement for dynamin for PKC-regulated endocytosis.

Although mutation of the α1 L314A, L315A dileucine motif failed to affect constitutive endocytosis, we investigated whether it may be involved in PKC-regulated endocytosis. As shown in Figure 6, PMA treatment significantly stimulated the endocytosis of wild-type receptors; however, it failed to promote receptor endocytosis in cells expressing α1(L314A, L315A).

To correlate our immunofluorescence studies with functional measurements, we performed whole-cell patch-clamp

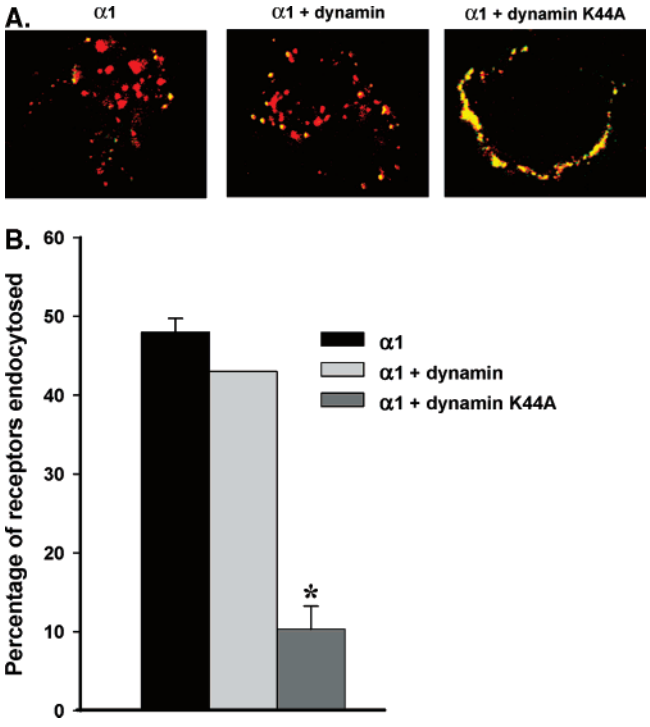

FIGURE 4: PKC activation stimulates endocytosis of glycine receptors. (A) Using the receptor endocytosis immunolabeling protocol, the effect of PMA on the endocytosis of α1 receptors was examined at 15 min. For these experiments either 100 nM PMM or 100 nM PMA was present throughout the 37 °C endocytosis period. Following labeling, the cells were fixed with 4% paraformaldehyde and visualized by confocal microscopy. The Texas Red labeling represents receptors that were labeled at time zero and endocytosed during the 15 min incubation period. The yellow signal represents receptors that were on the cell surface at time zero but not endocytosed. (B) Replicate data for experiments presented in (A). Each experiment was performed in triplicate, and at least three independent experiments were conducted (average ± SEM; *, $p \leq 0.01$; unpaired *t* test).

experiments. Since PMA stimulates receptor endocytosis, it would be expected that PMA treatment would lead to a decrease in the peak amplitude of the glycine response. In patch-clamp experiments, bath perfusion of PMA resulted in a decrease in glycine-gated chloride currents. Fifteen minutes following PMA application, an approximate 25% decrease in peak current amplitude was noted compared to time-matched control recordings (Figure 6C). On the basis of the results obtained in the immunofluorescence experiments, it might be expected that a larger decrease in receptor function would be obtained with PMA. However, the endocytosis protocol does not measure any receptor insertion that may occur concurrently with endocytosis.

Since PMA failed to promote endocytosis of α1(L314A, L315A) glycine receptors, the ability of PMA to inhibit glycine-gated chloride currents of α1(L314A, L315A) receptors was tested (Figure 6C). In cells expressing α1(L314A, L315A) receptors, PMA failed to produce a decrease in glycine-gated chloride currents. Although the dileucine receptor mutant did not exhibit PMA sensitivity, it was possible that mutation of the hydrophobic dileucine residues could alter the conformation of the receptor in a nonspecific manner and render it insensitive to PMA. To rule out this possibility, additional studies were performed on wild-type receptors with a synthetic peptide (RQHKELLRFRRK) that corresponded to the dileucine motif-containing region of the receptor (Figure 7). For these experiments cells expressing wild-type receptors were treated with PMA in the presence of the dileucine peptide in the patch pipet. In the presence of the dileucine peptide, PMA did not inhibit wild-type receptors. Presumably, the ability of this peptide to block the PMA effect is due to the binding of the peptide to AP2 adaptin, thus preventing the recruitment of the receptor for endocytosis by AP2 adaptin (*14*, *15*). This finding is consistent with our above observation that the dileucine motif is required for PMA-stimulated endocytosis.

To verify that PMA inhibited glycine receptors by activating PKC, and not through nonspecific actions on the ion channel or activation of other phorbol ester targets (*20*), the effect of the PKC pseudosubstrate inhibitor, PKCI, was examined. When PKCI was included in the patch pipet, PMA failed to inhibit glycine receptor peak amplitude responses (Figure 7), indicating that PMA inhibits the glycine receptor's function via PKC activation. No effect of PKCI on baseline glycine receptor responses was observed (data not shown). The glycine α1 subunit has one serine residue (S391) that has been reported to be a PKC phosphorylation site (*21*). To evaluate whether phosphorylation of S391 may be involved in PKC-stimulated glycine receptor inhibition, the effect of PMA treatment on glycine responses was tested on an S391A mutant receptor. In cells expressing α1(S391A), peak amplitude responses in DMSO or PMA (100 nM) treated cells were 111 ± 9.3% vs 72 ± 5% of the pretreatment values, respectively, at 15 min. These values were significantly different ($p \leq 0.05$, unpaired *t* test), indicating that phosphorylation of S391 is not necessary for the PKC inhibition of glycine receptor function. In patch-clamp experiments, we also assessed whether PMA would affect glycine receptor α1β heteromers. Fifteen minutes following PMA application the currents were 94.7 ± 16.6% of the pre-PMA values, while currents in untreated cells were 101 ± 10.7% of the baseline currents, indicating that α1β heteromeric receptors are insensitive to PMA.

DISCUSSION

Here we demonstrate that homomeric α1 glycine receptors are constitutively endocytosed by an endocytic pathway that utilizes the GTPase dynamin. Endocytosis of the receptor is stimulated by the activation of PKC, and this regulated endocytosis proceeds in a dynamin-dependent fashion. Furthermore, we have identified a dileucine motif that is important for PKC-stimulated receptor endocytosis. Surprisingly, this dileucine motif, while important for PKC-induced endocytosis, does not affect constitutive endocytosis.

Dynamin-dependent endocytosis is a major mechanism by which cells regulate surface levels of integral membrane proteins including neurotransmitter-gated ion channels such as $GABA_A$ receptors (*14*, *15*, *22*) and AMPA and NMDA glutamate receptor subtypes (*23*−*25*). Here we report that the α1 homomeric glycine receptor undergoes dynamin-dependent endocytosis. Inhibition of endogenous dynamin function by the overexpression of the dominant negative dynamin mutant K44A blocked constitutive endocytosis of glycine receptors. Furthermore, a peptide that blocks endocytosis by disrupting the interaction between dynamin and amphiphysin (*17*, *18*) increased glycine currents, consistent with an accumulation of surface glycine receptors. Regulation

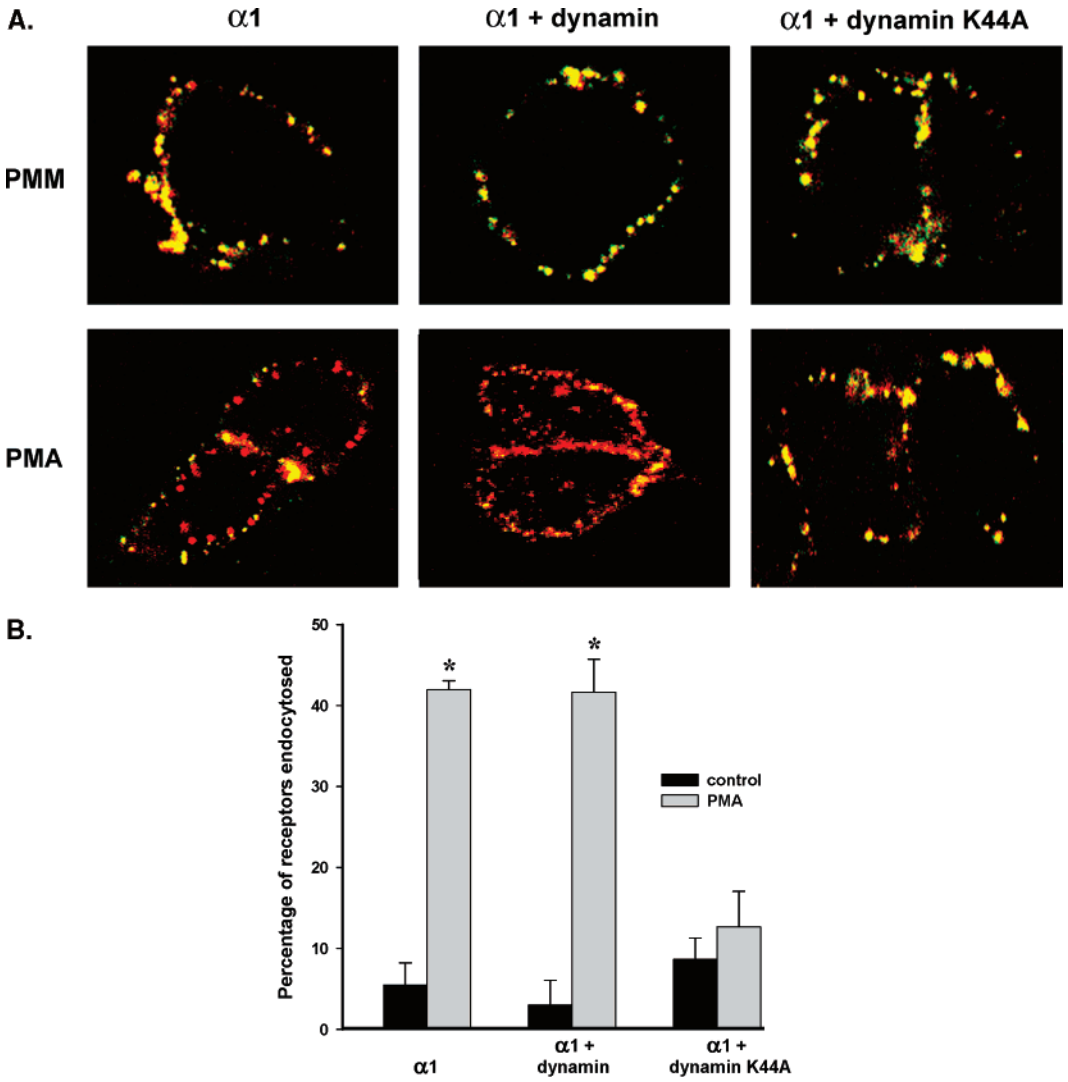

FIGURE 5: PKC-stimulated glycine receptor endocytosis occurs via a dynamin-dependent pathway. (A) α1 glycine receptors were expressed in HEK 293 cells alone or with either wild-type dynamin or dynamin K44A, and immunofluorescence experiments were performed as described in the Experimental Procedures. During the 37 °C, 15 min endocytosis period, the cells were treated with either 100 nM PMM or 100 nM PMA. The cells were then fixed with 4% paraformaldehyde and visualized by confocal microscopy. In the presence of wild-type dynamin, glycine receptor endocytosis was similar to that of α1 expressed alone in both the PMM- and PMA-treated cells. Endocytosis of glycine receptors was inhibited by dynamin K44A in cells treated with PMA. (B) Replicate data for experiments in (A). PMA-stimulated endocytosis of glycine receptors was significantly inhibited by coexpression of dynamin K44A compared to either no dynamin coexpression or wild-type dynamin controls (average ± SEM; *, $p \leq 0.01$; unpaired *t* test, $n = 3$ for each condition).

of neurotransmitter receptor endocytosis through this pathway is a key regulator of synaptic plasticity and a critical factor in controlling synaptic efficacy (*25*, *26*).

Receptors that are endocytosed through the dynamin pathway often contain either dileucine or tyrosine-based AP2 adaptin binding motifs that allow the receptor to be recruited into clathrin-coated pits prior to endocytosis (*27*). We recently identified an AP2 adaptin dileucine binding motif on the $GABA_A$ receptor $\beta 2$ subunit cytoplasmic loop that is involved in both constitutive and regulated $GABA_A$ receptor endocytosis (*14*, *15*). Although no tyrosine-based motifs were evident in the α1 glycine receptor cytoplasmic loop, two dileucine-like motifs were present. Neither of these putative dileucine binding motifs appeared to be involved in constitutive endocytosis despite the trafficking of the receptor through the dynamin pathway. It is possible that an atypical motif that is recognized by adaptin may be present as identified for other integral membrane proteins that undergo dynamin-dependent endocytosis (*28*, *29*). Alternatively, it is possible the adaptor protein AP2 may not directly recognize the α1 subunit, but instead may interact with a protein that binds the α1 subunit. Another possible signal for constitutive glycine receptor endocytosis is modification by ubiquitin. In this regard, ubiquitin mediates internalization of G-protein-coupled receptors (*30*) and growth hormone receptors (*31*), the latter in a dynamin-dependent fashion. Interestingly, Buttner et al. (*12*) have shown that recombinant glycine α1 receptors expressed in *Xenopus* oocytes are ubiquitinated prior to internalization, but it is unknown whether this posttranslational modification is necessary for endocytosis.

Previous electrophysiological studies in *Xenopus* oocytes and neurons have demonstrated that PKC activation inhibits glycine receptor peak amplitudes (*32*−*35*); however, the mechanism of this inhibition remains unknown. In the present study we identify the mechanism by which PKC activation decreases glycine receptor responses, namely, an increase in receptor endocytosis. Importantly, we conducted our electrophysiological studies at 35 °C. This recording tem-

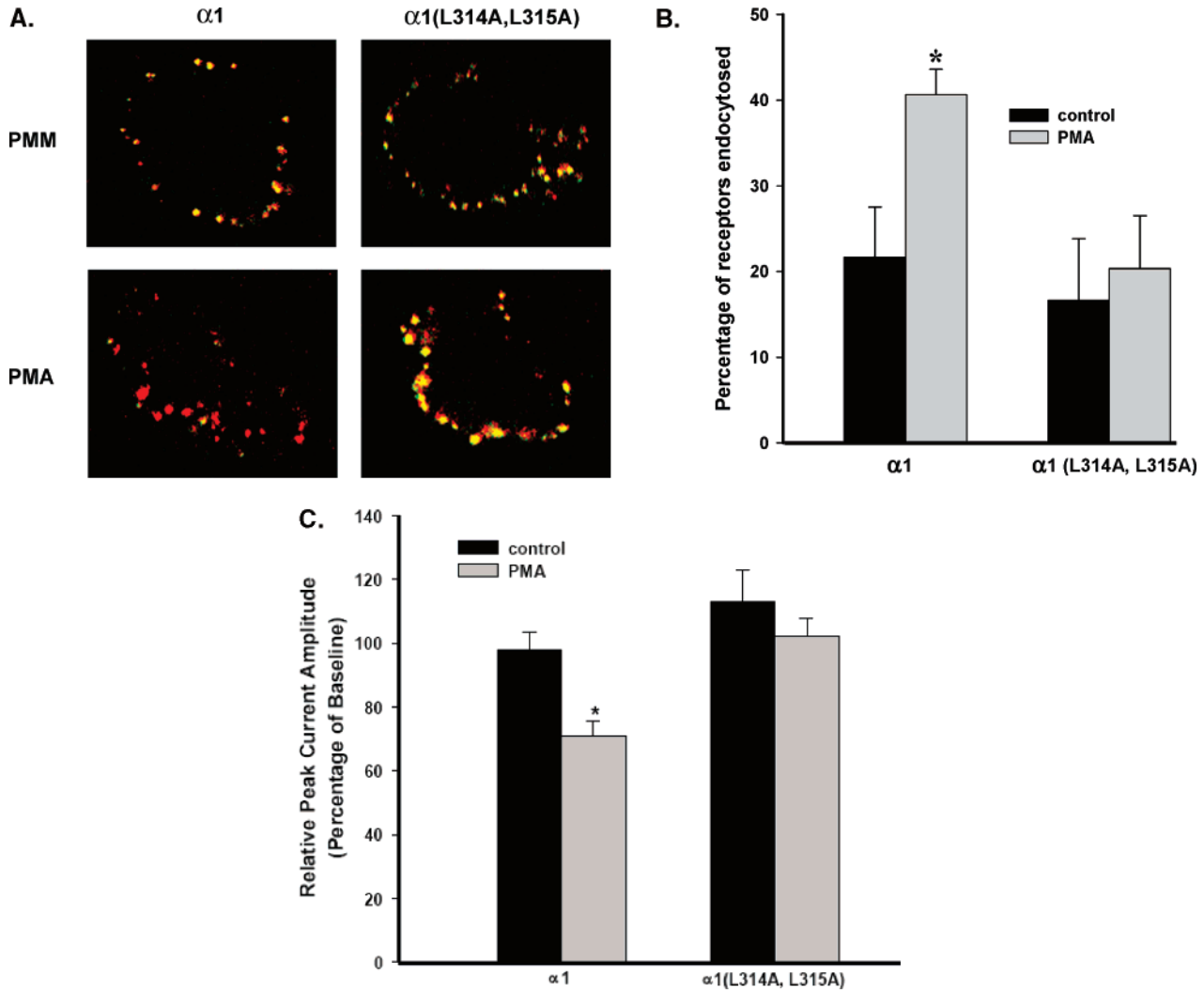

FIGURE 6: Mutation of the dileucine motif on the glycine receptor blocks both PKC-stimulated receptor endocytosis and PMA inhibition of receptor function. (A) Glycine receptors were expressed in HEK 293 cells, and immunofluorescence experiments were performed to measure receptor endocytosis. During the 37 °C, 15 min endocytosis period the cells were treated with either PMM or PMA (100 nM). Receptor endocytosis is apparent in cells expressing wild-type receptors but is inhibited in cells expressing α1(L314A, L315A) mutant receptors that lack the dileucine motif. (B) Replicate data for experiments in (A). PMA-stimulated receptor endocytosis was inhibited in cells expressing α1(L314A, L315A) mutant receptors compared to wild-type receptors. Values are the average ± SEM (*, $p \leq 0.005$; unpaired *t* test, $n = 4$ for each condition). (C) PMA inhibition of glycine-gated chloride currents is prevented by mutation of the dileucine AP2 recognition motif. Whole-cell patch-clamp recordings were obtained from HEK 293 cells expressing α1 or α1(L314A, L315A) receptors. Bath perfusion of PMA (100 nM), but not DMSO vehicle (control), elicited a marked decrease of glycine-gated current in cells expressing wild-type receptors, but had no effect on receptors in which the dileucine motif had been mutated. Shown are peak current amplitude data collected 15 min after the start of PMA treatment (average ± SEM; *, $p \leq 0.05$; unpaired *t* test, $n \geq 4$ for each condition).

perature was chosen since we recently discovered that PKC activation attenuates glycine currents in HEK 293 cells at 35 °C but not at room temperature (*36*). The temperature dependence of the PMA effect is consistent with our present findings that PMA promotes receptor endocytosis since the process of endocytosis does not occur efficiently at room temperature in mammalian cells (*37*, *38*). In addition to inhibiting glycine currents, PKC treatment/activation has also been shown to stimulate glycine receptor function (*39*−*41*), suggesting that, under some circumstances, PKC affects glycine receptors by an additional mechanism. It is difficult, however, to integrate these findings into the present study since these studies were conducted at room temperature or at temperatures that were not specified.

We also examined the effect of PKC activation on αβ heteromers and found that β-subunit-containing receptors are not sensitive to PKC modulation electrophysiologically. Thus, our α homomeric results with PMA may be most pertinent to early developmental stages when α homomers predominate (*1*). The lack of sensitivity of αβ heteromers to PMA in HEK 293 cells is particularly interesting since these cells lack, or have a low abundance of, neuronal inhibitory postsynaptic density proteins. This implies that the β subunit may have some inherent surface-stabilizing property beyond that imparted by the binding of the β subunit to gephyrin at synapses.

Previously, we and others have shown that PKC can regulate $GABA_A$ receptor function by stimulating receptor endocytosis but that the known PKC phosphorylation sites on the receptor were not involved (*42*, *43*). Similarly, our present data show that mutation of the known PKC phosphorylation site S391 on the glycine receptor (*21*) did not prevent PMA-induced inhibition of receptor function. These data suggest that the direct phosphorylation of glycine

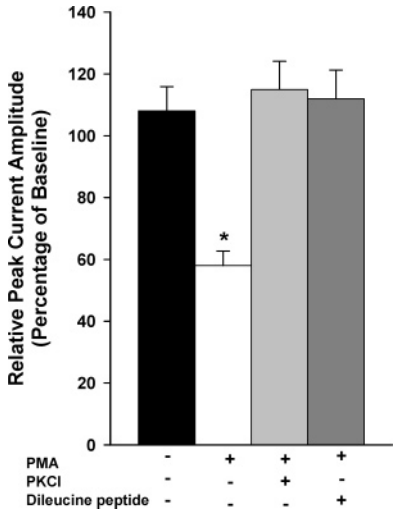

FIGURE 7: Both the PKC inhibitory peptide PKCI and the dileucine motif peptide block the inhibitory effects of PMA on glycine receptor function. Whole-cell recordings of glycine-gated chloride currents in HEK 293 cells demonstrate an inhibition in response to PMA (100 nM) treatment. The inclusion of either PKCI (50 μM) or the dileucine peptide (50 μM) in the patch pipet blocked PMA inhibition of glycine-gated currents (average ± SEM; *, $p \leq 0.01$; PMA alone vs PMA + PKCI or PMA + dileucine peptide, unpaired *t* test, $n \geq 5$ for each condition).

receptors does not underlie the PKC effect; however, we cannot rule out the possibility that there are other noncanonical phosphorylation sites on the α1 subunit that play a role in the PKC-dependent modulation of glycine currents. Thus, the pertinent PKC substrate remains to be identified. It is possible that a key element of the endocytic machinery, such as dynamin, may be the critical PKC substrate since it is phosphorylated by PKC (*44*, *45*). Although the known PKC phosphorylation site on the receptor was not found to be important for PKC modulation of receptor function, our results show that PKC regulation depends on a dileucine motif (L314, L315) in the intracellular loop. It is interesting that this motif is involved in PKC-regulated but not constitutive endocytosis. This selective involvement of the dileucine motif is in contrast to $GABA_A$ receptors in which both constitutive endocytosis and PKC-regulated receptor endocytosis depend on the same dileucine motif on the $GABA_A$ receptor β2 subunit (*14*, *15*). Thus, the dileucine motif on the $GABA_A$ receptor appears to perform a gatekeeper function for endocytosis in general, while the dileucine motif on the glycine receptor highlights a mechanistic distinction between constitutive and PKC-regulated endocytosis. It is not known whether PKC affects endocytosis of other glycine receptor α subunit isoforms; however, the dileucine motif is also present on the α3 subunit.

In addition to affecting glycine receptor endocytosis, PKC promotes internalization of other ligand-gated ion channels including $GABA_A$ (*15*, *43*, *46*), $GABA_C$ (*47*), and AMPA (*48*) receptors. The physiological pathway by which PKC becomes activated to affect glycine receptor endocytosis remains to be identified. Activation of G-protein-coupled receptors that are linked to Gq and subsequently to PKC activation may provide a mechanism for receptor cross talk that ultimately affects the efficacy of glycine at the synapse.

Several studies have provided evidence that glycine receptors may be a therapeutic target for seizure disorders (*49*−*51*) and play a role in inflammatory pain sensitization (*52*, *53*). Mutations in the gene that encodes the α1 subunit have been shown to give rise to hyperekplexia (*54*), a condition characterized by muscle spasms in response to an unexpected stimulus. The mutations responsible for this disorder adversely affect glycinergic receptor function by altering channel gating kinetics and cell surface expression (*55*, *56*). Recently, a β subunit mutant that prevents receptor clustering was found in the aberrant touch response zebrafish bandoneon mutant (*57*). Thus, glycine receptors play a role in certain neurological disorders and pain states, and alterations in glycine receptor cell surface expression and/or clustering is an emerging phenomenon that may underlie some pathologies. Our results further the understanding of glycine receptor surface expression and may ultimately be important for understanding disease processes which are associated with aberrant glycine receptor trafficking.

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