Modulation of 5-HT₃ Receptor-mediated Response and Trafficking by Activation of Protein Kinase C

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The serotonin type 3 (5-HT₃) receptor is a member of a ligand-gated ion channel (LGIC) supergene family including γ-aminobutyric acid type A (GABAₐ), glycine, and nicotinic acetylcholine receptors (1). Although molecular studies have identified two 5-HT₃ receptor subunits, 5-HT₃A and 5-HT₃B (1), homomeric 5-HT₃A receptors are thought to be the dominant functional form in the central nervous system (3). The 5-HT₃A receptors are differentially distributed in a number of important brain areas including the hippocampus, nucleus of the solitary tract, nucleus accumbens, substantia nigra, and ventral tegmental area (4–6). In some of these brain regions, 5-HT₃ receptors have been found to modulate the release of neurotransmitters such as dopamine and GABA (7). In addition, 5-HT₃ receptors are thought to be involved in reward mechanisms of some drugs of abuse and have been proposed to be involved in central nervous system phenomena such as anxiety, psychosis, nociception (8), and cognitive function (9).

Modulation of ligand-gated ion channel function by protein kinases has been the focus of a number of previous studies. For example, such studies have shown that activation of PKC can modulate glycine, GABAₐ, and N-methyl-D-aspartate (NMDA) receptors in various types of neurons and in cell lines expressing these receptors (10–15). Regulation of some of these receptors by PKC is thought to be important for synaptic modulation and neuronal plasticity. PKC has been found to induce internalization of GABAₐ receptors (14, 16) and, on the other hand, to promote trafficking of NMDA receptors to the cell surface (15). The regulation of receptor trafficking by activation of PKC is thought, at least in part, to contribute to PKC-induced alteration in the function of these receptors. Although PKC has been shown to regulate the phosphorylation of GABAₐ and NMDA receptor proteins (10, 17), it appears unlikely that the functional modulation of these receptors by PKC results from direct phosphorylation of the receptor proteins (14, 18). Rather, PKC is thought to phosphorylate receptor-associated proteins, which modulate receptor trafficking through intracellular signaling pathways (15, 18). Recent studies indicate that the actin cytoskeleton may play an important role in synaptic modulation and plasticity by anchoring, clustering, and targeting several LGICs (19–23). Activation of PKC by phorbol esters is proposed to disorder the dynamics of the actin filament network by removing a barrier to vesicle trafficking and docking, thereby promoting exocytosis (24–26). Consistent with this hypothesis, potentiation of NMDA receptor-mediated responses by activation of PKC is dependent on dynamic cycling of actin polymerization/despolymerization (27, 28).

Of all the LGICs, modulation of 5-HT₃ receptor function by PKC has received relatively little attention. Previous studies reported the application of phorbol esters, activators of PKC, can potentiate 5-HT-activated current in Xenopus oocytes expressing 5-HT₃A receptors (29), modulate the desensitization of 5-HT-activated current in HEK-293 cells expressing 5-HT₃A receptors (30), and regulate the probability of occurrence of certain conductance levels of 5-HT-activated single channel currents in mouse neuroblastoma, N1E-115 cells (31). In addition, a recent study suggests that a tyrosine kinase may be involved in PMA potentiation of 5-HT₃A receptors expressed in N1E-115 cells and oocytes expressing 5-HT₃A receptors. In this study, we show that PKC modulation of the serotonin type 3 (5-HT₃) receptor, a ligand-gated membrane ion channel that can mediate fast synaptic transmission in the central and peripheral nervous system. Here, we show that PKC potentiated 5-HT₃A receptor-mediated current in Xenopus oocytes expressing 5-HT₃A receptors and mouse N1E-115 neuroblastoma cells. In addition, using a specific antibody directed to the extracellular N-terminal domain of the 5-HT₃A receptor, treatment with the PKC activator, 4β-phorbol 12-myristate 13-acetate (PMA), significantly increased surface immunofluorescence. PKC also increased the amount of 5-HT₃A receptor protein in the cell membrane with the amount affecting the amount receptor protein in the total cell extract. The magnitude of PMA potentiation of 5-HT₃A receptor-mediated response is correlated with the magnitude of PMA enhancement of the receptor abundance in the cell surface membrane. PKM potentiation is unlikely to occur via direct phosphorylation of the 5-HT₃A receptor protein since the potentiation was not affected by point mutation of each of the putative sites for PKC phosphorylation. However, preapplication of phalloidin, which stabilizes the actin polymerization, significantly inhibited PKM potentiation of 5-HT₃A receptors. The observations suggest that PKC can modulate 5-HT₃A receptor function and trafficking through an F-actin-dependent mechanism.

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§The abbreviations used are: 5-HT, serotonin; LGIC, ligand-gated ion channel; GABAₐ, γ-aminobutyric acid type A (GABAₐ) type A; PKC, 4β-phorbol 12-myristate 13-acetate; PKC, protein kinase C; PKCI, PKC inhibitory peptide; PKA, protein kinase A; PKM, protein kinase C catalytic subunit; CCMD, cytochalasin D; Lat-A, latrunculin-A; Me₂SO, dimethyl sulfoxide; LIL, large intracellular loop; NMDA, N-methyl-D-aspartate; PBS, phosphate-buffered saline; WT, wild type; PLD, phalloidin.
Xenopus oocytes (32). Another recent study indicates that 5-HT₃ᵦA receptors are colocalized and clustered with F-actin in NG108–15 cells, hippocampal neurons and in cells transiently transfected with cloned 5-HT₃ᵦA receptors (33), suggesting that F-actin might be involved in the regulation of 5-HT₃ᵦA receptor targeting and clustering. Nevertheless, the molecular and cellular mechanisms by which PKC modulates 5-HT₃ receptors function have not been determined. To address this question, we have used variane 19 mitochondrial ATPase. In this study we found that PKC can modulate 5-HT₃ receptor function and receptor trafficking in N1E-115 cells and in Xenopus oocytes expressing 5-HT₃ᵦA receptors. We have also found that PKC modulation of 5-HT₃ᵦA receptor function is likely to occur via an F-actin-dependent mechanism.

**EXPERIMENTAL PROCEDURES**

**Site-directed Mutagenesis**—Point mutations of a cloned mouse 5-HT₃ᵦA receptor were introduced using a QuickChange site-directed mutagenesis kit (Stratagene). The authenticity of the DNA fragments that flank the mutation site was confirmed by double-strand DNA sequencing using an ABI Prism 377 automatic DNA sequencer (Applied Biosystems).

**Preparation of Complementary RNAs and Expression of Receptors**—Complementary RNAs were synthesized in vitro from linearized template cDNAs with a mMESSAGE mFACTORY RNA transcription kit from Ambion Inc. The quality and the sizes of synthesized complementary RNAs were confirmed by denatured RNA agarose gels. Mature female Xenopus laevis frogs were anesthetized by submersion in 0.2% 3-aminobenzolic acid ethyl ester (Sigma), and a group of oocytes was surgically excised. The oocytes were separated, and the follicular cell layer was removed by treatment with type I collagenase (Roche Applied Science) for 2 h at room temperature. Each oocyte was injected with a total of 20 ng of RNA in 20 nl of diethyl pyrocarbonate-treated water and was incubated with sucrose. Membrane currents were recorded in the whole-cell patch clamp configuration using an Axopatch 200B amplifier (Axon Instruments, Inc.). The recording microelectrodes were filled with 3 M KCl and were diluted either directly in bathing solution or dissolved in dimethyl sulfoxide (Me₂SO) before the dilution. The final Me₂SO concentration was less than 0.1%, which did not produce detectable effects on the membrane currents. Membrane currents were recorded by two-electrode voltage-clamp at a holding potential of −70 mV using a Gene Clamp 500 amplifier (Axon Instruments, Inc.). The recording microelectrodes were filled with 3 M KCl and had electrical resistances of 0.5–3.0 MΩs. Data were routinely recorded on a chart recorder (Gould 2300S). Average values are expressed as mean ± S.E.

**Recording from Xenopus Oocytes**—After incubation for 2–5 days, the oocytes were studied at room temperature (20–22 °C) in a 90-μl chamber. The oocytes were superfused with MBS at a rate of 6 ml/min. Agonists and chemical agents were diluted in the bathing solution and applied to the oocytes for a specified time using a solenoid valve-controlled superfusion system. The agonist and chemical agents were diluted either directly in bathing solution or dissolved in dimethyl sulfoxide (Me₂SO) before the dilution. The final Me₂SO concentration was less than 0.1%, which did not produce detectable effects on the membrane currents recorded under our experimental condition. Membrane currents were recorded by two-electrode voltage-clamp at a holding potential of −70 mV using a Gene Clamp 500 amplifier (Axon Instruments, Inc.). The recording microelectrodes were filled with 3 M KCl and had electrical resistances of 0.5–3.0 MΩs. Data were routinely recorded on a chart recorder (Gould 2300S). Average values are expressed as mean ± S.E.

**Recording from Neuroblastoma Cells**—N1E-115 cells (American Type Culture Collection) were prepared according to a method described previously (31). Cells were continuously superfused with a solution containing 140 mM NaCl, 5 mM KCl, 1.5 mM CaCl₂, 1 mM MgCl₂, 5 mM glucose, and 10 mM Hepes (pH 7.4 with NaOH, −340 mOsm). Membrane current-voltage relationships were recorded in the whole-cell patch clamp configuration using an Axopatch 200B amplifier (Axon) at room temperature. Cells were held at −60 mV. Data were acquired using pCLAMP 8 software (Axon).

**Western Blot of Membrane Surface Proteins**—Immediately after pre-treatment with PMA, Xenopus oocytes expressing 5-HT₃ᵦA receptors were fixed in 4% paraformaldehyde in phosphate-buffered saline (PBS) for 20 min. The oocytes were then washed in PBS and incubated with N-hydroxysuccinimide-SS-biotin (NHS-SS-biotin; Pierce) at a concentration of 1.5 mg/ml in PBS for 30 min at 4 °C under a nonpermeabilized condition, as described previously (15, 34). The oocytes were then washed extensively and homogenized, and the homogenate was centrifuged at 10,000 g for 10 min at 4 °C until all upper granules and melanosomes were pelleted. The final supernatant was incubated with 100 μl of neutravidin-linked beads (Pierce) by end-over-end rotation for 2 h at 4 °C. The beads were centrifuged and washed extensively to isolate bead-bound proteins. Labeled proteins were eluted from the beads with dithiothreitol-containing SDS-PAGE loading buffer and loaded onto 10% SDS-PAGE. After transfer onto a polyvinylidene difluoride membrane (Invitrogen), the surface and intracellular proteins were blocked with PBS, pH 7.5, containing 0.1% Tween 20 (Sigma) and 5% nonfat powdered milk and then incubated for 1 h with a polyclonal antibody (pAb120, 1:1000) directed to the extracellular N-terminal domain of the 5-HT₃ᵦA receptor (5). The proteins were washed, blotted, with a 1:600 dilution of fluorescein-linked anti-rabbit Ig in PBS, and incubated with anti-fluorescein AP conjugate at a 1:2500 dilution in PBS for 1 h. The proteins detected by a Western blotting kit (Amersham Biosciences) were scanned using a Molecular Dynamics Storm Gel and Blot Imaging System with ImageQuant Image Analysis Software (Amersham Biosciences).

**Cell Surface Immunolabeling of Oocytes and N1E-115 Cells—Xenopus oocytes expressing 5-HT₃ᵦA receptors were labeled with pAb120. In a pre-screening, oocytes exhibiting electrophysiological responses to 100 μM 5-HT of 3000–5000 nA were selected for this experiment. These oocytes were then incubated in 3 ml of MBS in the absence or presence of 300 nM PMA for 20 min. After PMA treatment, the oocytes were fixed in 4% paraformaldehyde in calcium and magnesium-free PBS for 10 min, rinsed twice in PBS, and placed in blocking buffer (5% donkey serum, 0.5% bovine serum albumin, PBS, 0.04% Triton X-100) for 30 min. After an incubation with pAb120 at 1:1000 dilution for 1 h, the cells were washed three times in PBS for 5 min and labeled with donkey-anti-rabbit conjugated to fluorescein isothiocyanate secondary antibody (Jackson ImmunoResearch Laboratories) as described previously (15). After mounting the cell on a microscope slide, Fluorescentlabeled oocytes were analyzed using a laser scanning microscope (LSM 5 Pascal, Zeiss), and the intensity was quantified by image analyzing software (Scion Image, Scion Co.). N1E-115 cells were incubated in external recording solution in the absence and presence of PMA (300 nM) and immunolabeled using a method described previously (15).

**Data Analysis**—Statistical analysis of concentration-response data was performed with the use of the nonlinear curve-fitting program ALLFIT (50). Data were fitted to the equation using KaleidaGraph 3.5 (Synergy Software),

\[ Y = \frac{(E_{\text{max}} - E_{\text{min}})(1 + (X/E_{\text{EC}_{50}})^n)}{1 + (X/E_{\text{EC}_{50}})^n} + E_{\text{min}} \]

where \(Y\) and \(X\) are concentration and response, respectively. \(E_{\text{max}}\) and \(E_{\text{min}}\) are the maximal and minimal responses, respectively, \(E_{\text{EC}_{50}}\) is the half-maximal concentration, and \(n\) is the slope factor (apparent Hill coefficient). Data were statistically compared by the paired \(t\) test or analysis of variance, as noted. Average values are expressed as the mean ± S.E.

**RESULTS**

**PKC Potentiates 5-HT-activated Current in Oocytes and N1E-115 Cells—Xenopus oocytes have been widely used as an expression system to study functional regulation of recombinant receptors by protein kinases and the underlying molecular mechanisms (11, 35–38). Although pretreatment with 100 nM 4a-PM, an inactive form of PMA, for 10 min did not significantly alter the amplitude of currents activated by 5-HT (Fig. 1A) in an oocyte expressing 5-HT₃ᵦA receptors, in the same cell, treatment with 10 nM PMA for 10 min increased the amplitude of inward current activated by 5-HT (Fig. 1B). The potentiation reached a maximum 10–20 min after the beginning of PMA application, lasted for 30–50 min, and was inhibited by the intracellular injection of a PKC inhibitory peptide 19–31 (PKCI) (Fig. 1C). The graph in Fig. 1D plots average current potentiation after treatment of the oocytes with PMA (solid circles), 4a-PM (open circles), or PMA after the intracellular injection of PKCI (solid triangles). These observations are in accord with previous results from this (29) and other (32) laboratories showing that activation of PKC can potentiate 5-HT₃ᵦA receptors expressed in Xenopus oocytes. To determine whether PKC can modulate 5-HT₃ᵦA receptor-mediated responses in N1E-115 cells, we performed whole cell recording on these cells. The amplitudes of currents evoked by 2 μM 5-HT at 2-min intervals were nearly identical under our experimental conditions (data not shown). Loading cells with 1 μM PKM (the
constitutively active fragment of PKC) through a micropipette for 4 and 6 min increased the amplitude of 5-HT-activated currents (Fig. 2A). In a separate experiment, loading cells with 300 nM PMA (Fig. 2B) for 4 and 6 min increased the amplitude of the 5-HT current, whereas this did not occur using 4α-PMA (Fig. 2C). These findings suggest that enhancement of 5-HT current by PMA in N1E-115 cells is mediated by the activation of PKC. The potentiation appeared to reach the maximal magnitude within 4–6 min after PKM or PMA application. The bar graphs in Fig. 2D plot the average effect of 4α-PMA, PMA, and PKM without or with inclusion of 1 μM PKCI in the pipette. Whereas 4α-PMA was ineffective, PMA or PKM significantly increased the amplitude of 5-HT-activated current. Moreover, inclusion of PKCI significantly reduced the magnitude of potentiation by PMA or PKM from 209 ± 46 and 219 ± 40% of control to 104 ± 1 and 105 ± 5% of control, respectively (p < 0.001, unpaired t test, n = 5). The application of PKCI alone did not significantly alter the amplitude of 5-HT-activated current (data not shown).

**PMA Increases Surface Expression of 5-HT<sub>3</sub> Receptors in Oocytes and N1E-115 Cells**—To determine whether PKC acti-

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**Fig. 1. Effect of 4α-PMA and PMA on 5-HT-activated current in Xenopus oocytes expressing 5-HT<sub>3A</sub> receptors.** A, records of current activated by 0.25 μM 5-HT before (0 min) and 5–30 min after beginning the application of 100 nM 4α-PMA for 10 min. B, records of current activated by 0.25 μM 5-HT before (0 min) and 5–30 min after beginning the application of 10 nM PMA for 10 min. C, records of current activated by 0.25 μM 5-HT before (0 min) and 5–30 min after beginning the application of 10 nM PMA for 10 min in a cell previously injected with 10 μM PKCI. The solid bar above each record indicates the time of 5-HT application. D, graph plotting time-course of average percentage potentiation of 5-HT-activated current after application of PMA (●), 4α-PMA (○), or PKCI plus PMA (▲). The solid bar indicates the time of the application of PMA, 4α-PMA, and PKCI plus PMA. Each data point represents average of 5–7 oocytes. The error bars not visible are smaller than the size of the symbols.

**Fig. 2. Effect of PKM, PMA, and 4α-PMA on 5-HT-activated currents in N1E-115 cells.** A, records of current activated by 2.0 μM 5-HT during intracellular loading of the cell with 10 μM PKM for 4–6 min. B, records of current activated by 2.0 μM 5-HT during intracellular loading of the cell with 300 nM PMA for 4–6 min. C, records of current activated by 2.0 μM 5-HT during intracellular loading of the cell with 300 nM 4α-PMA for 4–6 min. The bar above each record indicates the time of 5-HT application. D, bar graphs of average 5-HT-activated current by loading the cells for 6 min with 4α-PMA, PMA, and PKM and with PMA and PKM in the presence of PKCI. Bars represent mean ± S.E.
Regulation of 5-HT₃ Receptors by PKC

The surface expression of 5-HT₃A receptors after treatment of the buffer. Fig. 4

SS-biotin (39). The biotinylated surface proteins were separated by labeling cell-surface proteins with sulfo-NHS-biotin (39). The biotinylated surface proteins were separated by labeling cell-surface proteins with sulfo-NHS-biotin (39). The biotinylated surface proteins were separated by labeling cell-surface proteins with sulfo-NHS-biotin (39). The biotinylated surface proteins were separated by labeling cell-surface proteins with sulfo-NHS-biotin (39). The biotinylated surface proteins were separated by labeling cell-surface proteins with sulfo-NHS-biotin (39). The biotinylated surface proteins were separated by labeling cell-surface proteins with sulfo-NHS-biotin (39). The biotinylated surface proteins were separated by labeling cell-surface proteins with sulfo-NHS-biotin (39). The biotinylated surface proteins were separated by labeling cell-surface proteins with sulfo-NHS-biotin (39). The biotinylated surface proteins were separated by labeling cell-surface proteins with sulfo-NHS-biotin (39). The biotinylated surface proteins were separated by labeling cell-surface proteins with sulfo-NHS-biotin (39). The biotinylated surface proteins were separated by labeling cell-surface proteins with sulfo-NHS-biotin (39). The biotinylated surface proteins were separated by labeling cell-surface proteins with sulfo-NHS-biotin (39). The biotinylated surface proteins were separated by labeling cell-surface proteins with sulfo-NHS-biotin (39). The biotinylated surface proteins were separated by labeling cell-surface proteins with sulfo-NHS-biotin (39). The biotinylated surface proteins were separated by labeling cell-surface proteins with sulfo-NHS-biotin (39). The biotinylated surface proteins were separated by labeling cell-surface proteins with sulfo-NHS-biotin (39). The biotinylated surface proteins were separated by labeling cell-surface proteins with sulfo-NHS-biotin (39). The biotinylated surface proteins were separated by labeling cell-surface proteins with sulfo-NHS-biotin (39). The biotinylated surface proteins were separated by labeling cell-surface proteins with sulfo-NHS-biotin (39). The biotinylated surface proteins were separated by labeling cell-surface proteins with sulfo-NHS-biotin (39). The biotinylated surface proteins were separated by labeling cell-surface proteins with sulfo-NHS-biotin (39). The biotinylated surface proteins were separated by labeling cell-surface proteins with sulfo-NHS-biotin (39). The biotinylated surface proteins were separated by labeling cell-surface proteins with sulfo-NHS-biotin (39). The biotinylated surface proteins were separated by labeling cell-surface proteins with sulfo-NHS-biotin (39). The biotinylated surface proteins were separated by labeling cell-surface proteins with sulfo-NHS-biotin (39).

The Magnitude of PMA Enhancement of 5-HT₃A Receptor Surface Expression Is Correlated with the Magnitude of PMA Potentiation of 5-HT₃A Receptor-mediated Current—Next we compared the increase of receptor surface expression with the potentiation of 5-HT₃A receptor-mediated current after treatment with PMA. The bar graphs in Fig. 5A plot the average potentiation of 5-HT₃A receptor-mediated current by various concentrations of PMA from 10 to 1000 nM. The average potentiation induced by 10, 100, 300, and 1000 nM PMA was 152 ± 14% (p < 0.01), 452 ± 24% (p < 0.001, n = 11), 582 ± 30% (p < 0.001, n = 14), and 492 ± 31% (p < 0.001, n = 7) that of control, respectively. Note that the potentiation was maximal at 300 nM PMA and that the potentiation by 1000 nM was significantly less than that by 300 nM (p < 0.01, unpaired t test, n = 7–14).

Surface expression of the receptor assessed by Western blot analysis exhibited a pattern similar to that of the PMA potentiation of 5-HT₃A receptor-mediated responses (Fig. 5B). The average normalized band density in Western blots after treatment with 10, 100, 300, and 1000 nM PMA was 110 ± 31% (n = 3), 152 ± 18% (n = 4), 192 ± 14% (n = 5), and 162 ± 12% (n = 5) that of control, respectively. These values are significantly different from control (analysis of variance, p < 0.05). In addition, the magnitude of PMA potentiation of 5-HT-activated current is correlated with the magnitude of PMA-induced increase in band density (Fig. 5C, R = 0.98).
phosphorylate the 5-HT3A receptor protein (40). The phosphorylation by PKA is abolished by a point mutation of a putative PKA phosphorylation site in the LIL of the receptor (40). To evaluate if PMA potentiation of the 5-HT3A receptor-mediated response is mediated by the putative PKC or PKA phosphorylation sites in the LIL of the receptor, we sequentially replaced all of the 11 serines (S) or threonines (T) in the LIL of the receptor with alanine (A). The sensitivity of these mutant receptors to both 5-HT and PMA potentiation was examined by two-electrode voltage-clamp in *Xenopus* oocytes previously injected with complementary RNAs of the receptors. Fig. 6A shows the EC_{50} values of the 5-HT concentration-response curves for the WT and mutant receptors in *Xenopus* oocytes expressing the receptors. The EC_{50} values for 5-HT were obtained by fitting the 5-HT concentration-response curves to the Hill equation, as described under “Experimental Procedures.” B, average PMA potentiation of the WT and mutant receptor-mediated responses. Bars represent the mean ± S.E. from 5–6 oocytes. The average potentiation for each of the mutant receptors by PMA was not significantly different from the PMA potentiation of the WT receptor (analysis of variance, p > 0.1). The current was activated by 5-HT at the EC_{5} concentration for each receptor.

**Fig. 5.** Correlation of PMA potentiation of 5-HT-activated current with PMA-induced increase in 5-HT_{3A} receptor surface proteins. A, potentiation of 5-HT-activated current by 10–1000 nM PMA in oocytes expressing 5-HT_{3A} receptors. Bar graphs plot the average percentage potentiation of current activated by 0.25 μM 5-HT 20 min after beginning PMA application. Bars represent the mean ± S.E. (n = 5–9). B, PMA-induced increase in 5-HT_{3A} receptor abundance at the oocyte surface. Western blot gel bands of surface 5-HT_{3A} receptor proteins (see “Experimental Procedures”) 20 min after the application of 0, 100, 300, and 1000 nM PMA. Bar graphs plot the average band densities normalized as a percentage of control. Bars represent the mean ± S.E. (n = 3). C, correlation between the magnitude of PMA potentiation of 5-HT-activated current and the magnitude of PMA enhancement of surface 5-HT_{3A} receptors (r = 0.98, p < 0.01, n = 4); the line is the best fit to a linear regression (Statistica).

all of the 11 serines (S) or threonines (T) in the LIL of the receptor with alanine (A). The sensitivity of these mutant receptors to both 5-HT and PMA potentiation was examined by two-electrode voltage-clamp in *Xenopus* oocytes previously injected with complementary RNAs of the receptors. Fig. 6A shows the EC_{50} values of the 5-HT concentration-response curves for the wild type (WT) and mutant 5-HT_{3A} receptors. The bar graphs in Fig. 6B plot the average PMA potentiation of 5-HT-activated current at the EC_{5} concentration for each receptor. The potentiation by 10 nm PMA was 150 ± 110% for WT, 129 ± 21% for S274A, 151 ± 29% for S326A, 168 ± 27% for T372A, 126 ± 16% for T378A, 130 ± 14% for S412A, and 146 ± 16% for S433A/S434A receptors. These values are not significantly different from the WT (analysis of variance, p > 0.1). In view of sensitivity of S274A and T378A mutants to PMA potentiation, we also examined the sensitivity of these mutant receptors to high concentrations of PMA (300 nm) and found that the S274A or T378A mutation did not significantly alter the sensitivity of 5-HT_{3A} receptor to 300 nm PMA (data not shown).

**Stabilizing Actin Cytoskeleton by Pretreatment with Phalloidin (PLD) Inhibits PMA Potentiation**—In the light of recent studies reporting that the dynamics of actin cycling are essential for PKC modulation of NMDA receptor function (27, 28), we examined the effect of agents that disorder the dynamic move-
5). In addition, PLD did not affect either the EC50 value of the reduced the PMA potentiation by 79% of the 5-HT-activated current. On average, injection of PLD reduced 5-HT-activated current in N1E-115 cells. Whereas 10 ^\text{nM} PMA alone was 109% for 4 h abolished the PMA-induced increase in the amplitude of 5-HT-activated current (data not shown). We observed similar results in a study of PMA potentiation of 5-HT-activated current in control oocytes expressing 5-HT3A receptors. Preincubation with 10 ^\text{mM} cytochalasin D (CCD), an agent that disrupts actin cytoskeleton, did not significantly alter the potentiation by 300 ^\text{nM} PMA (Fig. 7B). However, the intracellular injection of PLD (10 ^\text{mM}), which stabilizes F-actin, significantly reduced the magnitude of PMA potentiation of 5-HT-activated current. On average, injection of PLD reduced the PMA potentiation by 79 ± 5% (Fig. 7B, p < 0.01, n = 5). In addition, PLD did not affect either the EC50 value of the 5-HT concentration-response curve or the maximal amplitude of the 5-HT-activated current (data not shown). We observed similar results in a study of PMA potentiation of 5-HT-activated current in N1E-115 cells. Whereas 10 ^\text{mM} CCD did not significantly affect PMA potentiation, preincubation with PLD for 4 h abolished the PMA-induced increase in the amplitude of 5-HT-activated current (Fig. 7C). The percent potentiation by 300 ^\text{nM} PMA alone was 109 ± 8%, after CCD it was 106 ± 12% (p > 0.5, n = 4), and after PLD it was 3 ± 1.2% (p < 0.001, n = 4).

**Desatilizing Actin Cytoskeleton by Latrunculin-A (Lat-A) Enhanced the Potentiation of 5-HT3A Receptor-mediated Current by Low Concentrations of PMA**—In view of reports that PMA by itself can destabilize actin dynamics (24–26), we wondered if pretreatment with Lat-A, a potent disrupter of actin cytoskeleton, can enhance PMA potentiation of 5-HT3A receptor-mediated responses. We found that preincubation with 1 ^\text{mM} Lat-A for 2 h greatly enhanced the potentiation of 5-HT3A receptor-mediated responses by 10 ^\text{nM} PMA (Fig. 8A). On the other hand, pretreatment with Lat-A for 2 h did not appear to affect the magnitude of PMA potentiation when 300 ^\text{nM} PMA was used (Fig. 8A). In cells preincubated with Lat-A, the average magnitude of potentiation by 10 ^\text{nM} PMA increased by 370% (PMA alone versus PMA + Lat A, 150 ± 25% versus 555 ± 39%; p < 0.01, n = 5), whereas Lat-A did not significantly affect the maximal potentiation by 300 ^\text{nM} PMA (Fig. 8B) (p > 0.05, unpaired t test, n = 5). Moreover, the magnitude of 10 ^\text{nM} PMA-induced potentiation after treatment with Lat-A was not significantly different from the maximal potentiation induced by 300 ^\text{nM} PMA alone (555 ± 39% versus 582 ± 33%; p > 0.1, unpaired t test, n = 5).

**DISCUSSION**

In the present study, we confirmed previous reports that activation of PKC can enhance 5-HT-activated current in Xenopus oocytes expressing 5-HT3A receptors (29, 32). We also observed an -2-fold potentiation of 5-HT-activated current in N1E-115 cells by either PKM or PMA. Moreover, the potentiation by either PKM or PMA is likely to be mediated by PKC since 4α-PMA did not affect 5-HT-activated current and the potentiation by PKM or PMA was inhibited by PKCI. Given the fact that N1E-115 cells are neuron-like cells containing both 5-HT3A and 5-HT3B subunits (41) that have been used for cloning and functional characterization of 5-HT3 receptors (42–44), our results also suggest that activation of PKC can modulate the function of native 5-HT3 receptors expressed in N1E-115 cells.

PKC has been found to modulate certain types of LGIC protein trafficking. However, such a study has not been reported for PKC modulation of 5-HT3 receptors. In the present study, we observed that pretreatment with PMA can enhance surface immunolabeling and surface expression of 5-HT3A receptors for both Xenopus oocytes and in N1E-115 cells. The...
increase in surface receptor expression by PKC activation is likely to occur via an increase in receptor trafficking rather than an increase of receptor protein synthesis since the quantity of receptor protein in the total cell extract remained unchanged after PMA treatment. In addition, consistent with previous reports in the studies of PKC regulation of NMDA and GABAA receptor trafficking (15, 45), our observation that the increase in surface expression of the 5-HT3A receptors developed rapidly, within a few minutes after the beginning of PMA application, in both Xenopus oocytes expressing 5-HT3A receptors and in N1E-115 cells also favors PKA modulation of receptor trafficking. An increase in receptor trafficking to the cell membrane may contribute, at least in part, to PKC potentiation of 5-HT-activated current. The solid bar above each bar indicates the time of 5-HT application. B, bar graphs plot the average potentiation of current activated by 0.25 μM 5-HT by 10 and 300 nM PMA with or without preincubation of 1 μM Lat-A (1 μM). Bars represent mean ± S.E. from 5–6 oocytes.

FIG. 8. Lat-A increases the sensitivity of 5-HT3A receptors to low concentration PMA. A, records of potentiation of current activated by 0.25 μM 5-HT by 10 and 300 nM PMA before and after application of 1 μM Lat-A for 2 h. The solid bar above each record indicates the time of 5-HT application. B, bar graphs plot the average potentiation of current activated by 0.25 μM 5-HT by 10 and 300 μM PMA with or without preincubation of 1 μM Lat-A (1 μM). Bars represent mean ± S.E. from 5–6 oocytes.

Our results show that PMA potentiation of 5-HT-activated currents can be prevented when actin cytoskeleton is stabilized by treatment with PLD in both Xenopus oocytes expressing 5-HT3A receptors and N1E-115 cells. The observation that disruption of actin polymerization by Lat-A enhanced potentiation by low concentrations of PMA, whereas Lat-A did not alter maximal PMA potentiation, suggests that PMA modulation of 5-HT3A receptor function may involve F-actin. It has been well documented that the F-actin cytoskeleton can serve as a barrier to restrain the transport of vesicles to the cell membrane (25). It has also been reported that PKC isoforms can bind to actin after PMA treatment (46). As a result, the activation of PKC by phorbol esters could cause a rearrangement of actin filaments, which removes a negative clamp that prevents the exocytosis of proteins. In this regard, it seems likely that pretreatment with PMA may result in disassembly of actin cytoskeleton, thereby promoting transport of 5-HT3A receptors to the cell membrane. This hypothesis is supported by our observations that PLD inhibited PMA potentiation of 5-HT-activated responses and Lat-A increased the sensitivity of 5-HT3A receptors to PMA potentiation. This hypothesis is also consistent with a very recent study indicating that F-actin plays an important role in the regulation of 5-HT3A receptor targeting and clustering at cell membranes (33).

It should be noted that PMA may also modulate gating of the 5-HT3A receptor channel, given the observations that the potentiation of 5-HT-activated current by PMA is dependent on agonist concentration in Xenopus oocytes expressing 5-HT3A receptors (29) and PMA modulates subconductance states of 5-HT-activated single channel currents in N1E-115 cells (31). Such a scenario occurs in PKC modulation of NMDA receptor function in which PKC has been found to modulate gating of the receptor channels (15, 47). However, it is also thought that the alteration of NMDA receptor channel gating by PKC results at least in part from PKC modulation of the receptor trafficking such as insertion of new channels into cell surface membranes (15). It should also be pointed out that there are a number of other questions that remain to be determined. For instance, whether F-actin can either directly bind to the 5-HT3A receptor protein or whether it can interact with the receptor through intermediate proteins is not known. Moreover, the mechanisms by which PKC promotes an increase of 5-HT3A receptors in the cell membrane might also involve other mechanisms, such as a reduction in internalization of the receptor channels. In addition, a recent study suggests that tyrosine kinase may also be involved in regulation of 5-HT3A receptor function (32); in this regard, whether tyrosine kinase is involved in 5-HT3A receptor trafficking remains to be determined. These issues will need to be addressed in future studies.

For some LGICs, regulation of protein trafficking by PKC is thought to be critical for synaptic modulation and plasticity (48). Given the observation that 5-HT3A receptors are largely localized in the cytosol of central neurons (3), our observations that PKC can regulate 5-HT3A receptor function and trafficking through actin-dependent pathways raise the possibility that 5-HT3A receptors may be dynamically moved in and out of the cell membranes by PKC and other types of kinases. In light of observations reporting that neurotransmitter release can be regulated through an actin-dependent mechanism in the central nervous system (49) and that 5-HT3A receptors can modulate the release of dopamine and GABA in some important brain areas, it seems possible that enhancement of 5-HT3 receptor function and trafficking by PKC activation may play an important role in modulating the efficacy of serotonergic synaptic transmission, the release of neurotransmitters, and other 5-HT3 receptor-mediated phenomena.

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