Modulation of 5-HT$_3$ Receptor-mediated Response and Trafficking by Activation of Protein Kinase C$^*$

Hui Sun, Xian-Qun Hu, Edgar M. Moradel, Forrest F. Weight, and Li Zhang†

From the Laboratory of Molecular and Cellular Neurobiology, National Institute on Alcohol Abuse and Alcoholism, National Institutes of Health, Bethesda, Maryland 20892-8115

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**Modulation of neurotransmitter-gated membrane ion channels by protein kinase C (PKC) has been the subject of a number of studies. However, less is known about PKC modulation of the serotonin type 3 (5-HT$_3$) 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-HT$_3$ receptor-mediated current in Xenopus oocytes expressing 5-HT$_3A$ receptors and mouse N1E-115 neuroblastoma cells. In addition, using a specific antibody directed to the extracellular N-terminal domain of the 5-HT$_3A$ receptor, treatment with the PKC activator, 4β-phorbol 12-myristate 13-acetate (PMA), significantly increased surface immunofluorescence. PKC also increased the amount of 5-HT$_3A$ receptor protein in the membrane without affecting the amount of receptor protein in the total cell extract. The magnitude of PMA potentiation of 5-HT$_3A$ receptor-mediated responses is correlated with the magnitude of PMA enhancement of receptor abundance in the cell surface membrane. PKC potentiation is unlikely to occur via direct phosphorylation of the 5-HT$_3A$ 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 PKC potentiation of 5-HT$_3$-activated responses in both N1E-115 cells and oocytes expressing 5-HT$_3A$ receptors. On the other hand, latrunculin-A, which destabilizes actin cytoskeleton, enhanced the PKC potentiation of 5-HT$_3A$ receptors. The observations suggest that PKC can modulate 5-HT$_3A$ receptor function and trafficking through an F-actin-dependent mechanism.**

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The serotonin type 3 (5-HT$_3$)$^{1}$ receptor is a member of a ligand-gated ion channel (LGIC) supergene family including γ-aminobutyric acid type A (GABA$_A$), glycine, and nicotinic acetylcholine receptors (1). Although molecular studies have identified two 5-HT$_3$ receptor subunits, 5-HT$_3A$ and 5-HT$_3B$ (2), homomeric 5-HT$_{3A}$ receptors are thought to be the dominant functional form in the central nervous system (3). The 5-HT$_{3A}$ 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-HT$_3$ receptors have been found to modulate the release of neurotransmitters such as dopamine and GABA (7). In addition, 5-HT$_3$ 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, GABA$_A$, 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 GABA$_A$ 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 GABA$_A$ 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/dewpolymerization (27, 28).

Of all the LGICs, modulation of 5-HT$_3$ receptor function by PKC has received relatively little attention. Previous studies reported the application of phorbol esters, activators of PKC, can potentiate 5-HT$_3$-activated current in Xenopus oocytes expressing 5-HT$_3A$ receptors (29), modulate the desensitization of 5-HT$_3$-activated current in HEK-293 cells expressing 5-HT$_3A$ receptors (30), and regulate the probability of occurrence of certain conductance levels of 5-HT$_3$-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-HT$_3A$ receptors expressed in

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† To whom correspondence should be addressed: Laboratory of Molecular and Cellular Neurobiology, National Institute on Alcohol Abuse and Alcoholism, Park Bidg., Rm. 150, Bethesda, MD 20892-8115. Tel.: 301-435-1236; Fax: 301-480-6882; E-mail: lizhang@niaaa.nih.gov.

1 The abbreviations used are: 5-HT, serotonin; LGIC, ligand-gated ion channel; GABA$_A$, γ-aminobutyric acid type A (GABA$_A$) type A; PMA, 4β-phorbol 12-myristate 13-acetate; PKC, protein kinase C; PKC inhibitor, PKC inhibitor peptide; PKA, protein kinase A; PKM, protein kinase C catalytic subunit; CCD, cytochalasin D; Lat-A, latrunculin-A; Me$_2$SO, dimethyl sulfoxide; LIL, large intracellular loop; NMDA, $N$-methyl-$D$-aspartate; PBS, phosphate-buffered saline; WT, wild type; PLD, phalloidin.

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Xenopus oocytes (32). Another recent study indicates that 5-HT3A receptors are colocalized and clustered with F-actin in NG108–15 cells, hippocampal neurons and in cells transiently transfected with cloned 5-HT3A receptors (33), suggesting that F-actin might be involved in the regulation of 5-HT3A receptor targeting and clustering.

Nevertheless, the molecular and cellular mechanisms by which PKC modulates 5-HT3 receptor function have not been determined. To address this question, we have used varanic (or varaz) and found that PKC can modulate 5-HT3 receptor function and receptor trafficking in N1E-115 cells and in Xenopus oocytes expressing 5-HT3A receptors. We have also found that PKC modulation of 5-HT3A receptor function is likely to occur via an F-actin-dependent mechanism.

EXPERIMENTAL PROCEDURES

Site-directed Mutagenesis—Point mutations of a cloned mouse 5-HT3A 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 mMACHINE 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-aminomorphoic 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 20 min. The oocytes were washed in PBS and incubated with 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 3 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). In a holding chamber, each oocyte was placed in a position with the equator perpendicular to the plane of imaging since most foreign receptors have been found to express within the animal pole of oocytes (14). Cross-sectional and tangential images of oocytes were viewed and photographed using a laser scanning microscope (LSM 5 Pascal, Zeiss), and the intensity was quantified by image analyzing software (Scion Image, Synergy Software).

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-HT3A 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-HT3A receptors expressed in Xenopus oocytes. To determine whether PKC can modulate 5-HT3A 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...
PKM without or with inclusion of 1

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. In a separate experiment, loading cells with 300 nM PMA for 4–6 min increased the amplitude of 5-HT-activated current. Moreover, PKM without or with inclusion of 1 μM PKCI significantly increased the amplitude of 5-HT-activated current (data not shown).

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

**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 2.0 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 the 5-HT application. D, bar graphs of average 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.
were then eluted by treatment with dithiothreitol-containing buffer. Fig. 4A illustrates a representative Western blot of 5-HT₃A receptor for un.injected oocytes (left), oocytes expressing 5-HT₃A receptors (5-HT₃AR) without PMA (middle), and oocytes expressing 5-HT₃A receptors 20 min after the beginning of 300 nM PMA application (right). The surface protein was assessed by the biotin surface labeling method as described under “Experimental Procedures.” B, a representative Western blot of total cell 5-HT₃A receptor content for un.injected oocytes (left), oocytes expressing 5-HT₃A receptors without PMA (middle), and oocytes expressing 5-HT₃A receptors 20 min after the beginning of 300 nM PMA application (right). C, average 5-HT₃A receptor band density for surface (left) and total cell (right) receptor. Bars represent the mean ± S.E. of gel band density from 3–5 experiments.

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-HT-activated 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, n = 11), 452 ± 24% (p < 0.001, n = 11), 582 ± 33% (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 ± 4% (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, p < 0.001, n = 4).

PKC and PKA Sites in the Large Intracellular Loop (LIL) of the 5-HT₃AR Receptor Are Not Involved in the PMA Potentiation—It has been reported that protein kinase A (PKA) can
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 EC50 values of the 5-HT concentration-response curves for the wild type (WT) and mutant receptors in *Xenopus* oocytes expressing the receptors. The EC50 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 EC5 concentration for each receptor.

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-
In the present study, we confirmed previous reports that activation of PKC can enhance 5-HT-activated current in Xenopus oocytes expressing 5-HT3_A 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-HT3_A and 5-HT3_B 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-HT3_A receptors for both Xenopus oocytes and in N1E-115 cells. The

![Diagram](http://www.jbc.org/)

Fig. 7. PLD inhibits PMA potentiation of 5-HT-activated currents in oocytes and N1E-115 cells. A, records of potentiation of current activated by 0.25 μM 5-HT by 300 nM PMA before and after the application of CCD or PLD in Xenopus oocytes expressing 5-HT3_A receptors. The solid bar above each record indicates the time of 5-HT application. CCD (10 μM) was preincubated for at least 4 h, and PLD (10 μM) was injected intracellularly for 2 h before testing the effect of PMA. B, bar graphs represent the average percentage potentiation of current activated by 0.25 μM 5-HT by 300 nM PMA before and after the bath application of CCD or intracellular injection of PLD. Each bar represents the mean ± S.E. from 5–6 oocytes. C, representative trace records of inward current activated by 2.0 μM 5-HT in the absence and presence of PMA after preincubation of 10 μM CCD or 10 μM PLD for 4 h in N1E-115 cells. D, bar graphs represent the average percentage potentiation of 5-HT-activated current by PMA after preincubation of CCD (10 μM) or PLD (10 μM) for 4 h. Each bar represents the mean ± S.E. of 4 cells.

*Regulation of 5-HT3 Receptors by PKC*
Regulation of 5-HT₃ Receptors by PKC

The regulation of 5-HT₃ receptors by protein kinase C (PKC) has been studied extensively, with a focus on understanding how PKC can modulate receptor function and trafficking. PKC activation is known to increase receptor trafficking, which can be prevented when the actin cytoskeleton is stabilized (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 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 intermediating 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-HT₃A receptor-mediated phenomena.

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