Indomethacin Activates Protein Kinase C and Potentiates α7 ACh Receptor Responses

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Key Words
Indomethacin • Protein kinase C • α7 ACh receptor • Hippocampal synaptic transmission • Facilitation

Abstract
Background/Aims: We have earlier found that indomethacin activates CaMKII, as a novel action distinct from COX inhibition. To explore further indomethacin actions, the present study focused upon PKC and examined the effect of indomethacin on α7 ACh receptor responses and hippocampal synaptic transmission through PKC. Methods: We recorded currents through α7 ACh receptors expressed in Xenopus oocytes, quantified PKC activity in the in situ and cell-free PKC assay, and monitored field excitatory postsynaptic potentials (fEPSPs) and miniature excitatory postsynaptic currents (mEPSCs) from the CA1 region of rat hippocampal slices. Results: Indomethacin potentiated α7 ACh receptor currents in a bell-shaped concentration (100 nM-1 mM)-dependent manner, and the potentiating effect was inhibited by the PKC inhibitor GF109203X. Indomethacin (100 µM) also increased the rate of nicotine-evoked mEPSCs, and the effect was prevented by α-bungarotoxin or GF109203X. Conclusion: The results of the present study show that indomethacin activates PKC, possibly PKC-ε in the brain, thereby potentiating α7 ACh receptor responses to stimulate presynaptic glutamate release, which in part contributes to facilitation of hippocampal transmission. This extends our knowledge about diverse indomethacin actions.

Introduction

2-{1-[(4-Chlorophenyl)carbonyl]-5-methoxy-2-methyl-1H-indol-3-yl} acetic acid (Indomethacin), a...
nonsteroidal anti-inflammatory drugs (NSAIDs), inhibits cyclooxygenase-1 and -2 (COX-1 and -2) bearing prostaglandin synthesis from arachidonic acid, thereby suppressing fever, inflammation, and pain. Intriguingly, NSAIDs may exhibit the beneficial effects on Alzheimer disease; NSAIDs reduces the risk of developing Alzheimer disease, delay its onset, or amyloid-β peptides accumulation in the brain [1-4].

In our earlier study, indomethacin stimulated presynaptic glutamate release and facilitated hippocampal synaptic transmission by activating Ca\(^{2+}\)/calmodulin-dependent protein kinase II (CaMKII) [5]. Indomethacin ameliorated scopolamine-induced spatial learning and memory impairment for rats or age-related spatial learning and memory deterioration for senescence-accelerated mouse-prone 8 mice [5]. Of particular interest was the finding that indomethacin could still enhance learning and memory potentials for healthy humans and normal rats [5]. These raise the possibility that indomethacin exerts its diverse actions in addition to COX inhibition. In the preliminary study, indomethacin indirectly activated CaMKII, possibly by inhibiting protein phosphatase 1 (PP1) that converts phosphorylated active form of CaMKII to dephosphorylated inactive form. Amazingly, the linoleic acid derivative DCP-LA activates CaMKII in a fashion similar to indomethacin [6]. Moreover, DCP-LA serves as a selective and direct activator of PKC-ε, thereby potentiating presynaptic α7 ACh receptor activity and inducing a long-term facilitation of hippocampal synaptic transmission [7]. Then, we wonder whether indomethacin also potentiates α7 ACh receptor activity and facilitates hippocampal synaptic transmission by activating PKC.

To address this question, we monitored currents through α7 ACh receptors expressed in Xenopus oocytes, assayed PKC activity in cultured rat hippocampal neurons and PKC-ε under the cell-free conditions, and recorded field excitatory postsynaptic potentials (fEPSPs) and miniature excitatory postsynaptic currents (mEPSCs) from the CA1 region in rat hippocampal slices. We show here that indomethacin potentiates α7 ACh receptor responses by activating PKC, possibly PKC-ε in the brain, to stimulate presynaptic glutamate release, in part responsible for indomethacin-induced facilitation of hippocampal synaptic transmission.

Materials and Methods

Animal care
All procedures have been approved by the Animal Care and Use Committee at Hyogo College of Medicine and were in compliance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals.

Indomethacin
Indomethacin was dissolved with dimethyl sulfoxide and stocked at 1 M. In case of experiments, indomethacin stocked was diluted with extracellular solution for each experiment.

In vitro transcription and translation
The rat α7 ACh receptor cDNA was kindly provided from Dr. Patrick (Baylor College of Medicine, USA). The mRNA coding the rat α7 ACh receptor subunit was synthesized by in vitro transcription. Mature Xenopus oocytes were surgically removed from female frogs under ether anesthesia and manually separated from the ovary. Collagenase (0.5 mg/ml) treatment was carried out to remove the follicular cell layer, and 24 h later oocytes were injected with approximately 50 nl of the α7 ACh receptor subunit mRNA (1 mg/ml), and incubated in Barth’s solution [in mM: 88 NaCl, 1 KCl, 2.4 NaHCO\(_3\), 0.82 MgSO\(_4\), 0.33 Ca(NO\(_2\))\(_2\), 0.41 CaCl\(_2\), and 7.5 Tris, pH 7.6] at 18 °C.

Two-electrode voltage-clamp recording
Oocytes were transferred to a recording chamber 2-3 days after injection of the α7 ACh receptor subunit mRNA and continuously superfused at 22 °C in standard frog Ringer’s solution [in mM: 88 NaCl, 2 KCl, 1.8 CaCl\(_2\), and 5 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), pH 7.0]. ACh (100 µM) was bath-applied to oocytes at 10-min intervals before and after 10-min treatment with indomethacin, and ACh-evoked currents were recorded, i.e., the sampling rate was once per 10 min. It has been established that full recovery of α7 ACh receptor desensitization is obtained with 10-min washing-out of ACh, based upon huge numbers of previous experiments. In a two-electrode voltage-clamp configuration, whole-cell membrane currents were recorded with a GeneClamp-500 amplifier (Axon Instruments, Inc., Foster City, CA, USA), filtered at 20-50 Hz, and analyzed on a microcomputer using pClamp software (version 6.0.3, Axon Instruments, Inc.). The electrode used, with the resistance of 2-3 MΩ, was filled with 2 M KCl.

Cell culture
The hippocampus was removed from the embryonic Wistar rat brain (gestational age, 18 days) under ether anesthesia and dissociated with a Pasteur pipette. Then, cells were seeded on poly-D-lysine-coated 96-well plates and grown in Neurobasal (GIBCO, Carlsbad, CA, USA) supplemented with B27 (GIBCO) (50:1), 2.5 mM glutamine, 50 µM glutamate, penicillin (final concentration, 100 U/ml), and streptomycin (final concentration, 0.1 mg/ml) in a humidified atmosphere of
5% CO₂ and 95% air at 37 °C. Cytosine arabinoside (5 µM) was added to culture medium 2 days after seeding.

In situ PKC assay
PKC activity in cultured rat hippocampal neurons was assayed by the method as previously described [7]. Cultured neurons were treated with indomethacin or phorbol-12-myristate-13-acetate (TPA) in the presence and absence of GF109203X (100 nM) at 37 °C for 10 min in an extracellular solution (137 mM NaCl, 5.4 mM KCl, 2.5 mM CaCl₂, 5 mM MgCl₂, 0.3 mM NaH₂PO₄, 0.4 mM K₂HPO₄, and 20 mM HEPES, pH 7.2). Then, cells were rinsed with 100 µl of Ca²⁺-free phosphate buffered saline (PBS) and incubated at 30 °C for 15 min in 50 µl of the extracellular solution containing 50 µg/ml digitonin, 25 mM glycerol 2-phosphate, 200 µM ATP, and 100 µM synthetic PKC substrate peptide (Pyr-Lys-Arg-ProSer-Gln-Arg-Ser-Lys-Tyr-Leu) (Peptide Institute, Osaka, Japan). The supernatants were collected and boiled at 100 °C for 5 min to terminate the reaction. An aliquot of the solution (20 µl) was loaded onto a reversed phase high performance liquid chromatography (HPLC) (LC-10ATvp; Shimadzu Co., Kyoto, Japan). A substrate peptide peak and a new product peak were detected at an absorbance of 214 nm (SPD-10Avp UV-VIS detector; Shimadzu). Areas for non-phosphorylated peptide peak and a new product peak were detected at an absorbance of 214 nm (SPD-10Avp UV-VIS detector; Shimadzu). Areas for non-phosphorylated peptide peak and a new product peak were detected at an absorbance of 214 nm (SPD-10Avp UV-VIS detector; Shimadzu). Statistical analysis was carried out using Fisher’s Protected Least Significant Difference (PLSD) test, Dunnett’s test, unpaired t-test, and Kolmogorov-Smirnov test.

Results
Indomethacin potentiates α7 ACh receptor responses in a PKC-dependent manner
In Xenopus oocytes expressing α7 ACh receptors, ACh (100 µM) evoked inward whole-cell membrane currents at a holding potential of -60 mV, and indomethacin (100 µM) potentiated the currents (Fig. 1), suggesting that indomethacin has potency to enhance α7 ACh receptor responses. Xenopus oocytes express endogenous Ca²⁺-dependent chloride channels. In the Xenopus oocyte expression systems, therefore, ACh-evoked currents include currents through the chloride channel activated by Ca²⁺ influx through ACh receptor channels. The amplitude of ACh-evoked currents in Ca²⁺-free extracellular solution was lower than that obtained in Ca²⁺-containing extracellular solution, but indomethacin (100 µM) still potentiated the currents in Ca²⁺-free extracellular solution to an extent achieved in Ca²⁺-free extracellular solution (Fig. 1). This confirms that potentiation of ACh-evoked currents induced by indomethacin is due to an enhancement in α7 ACh receptor currents but not in Ca²⁺-dependent chloride channel currents.

We next examined the concentration-responsive effect of indomethacin on α7 ACh receptor currents. Indomethacin potentiated the currents in a concentration
(100 nM-100 µM)-dependent manner, the maximum reaching approximately 150% of original amplitude 70 min after 10-min treatment (Fig. 2). In contrast, a higher concentration of 1 mM induced a striking depression of the currents during treatment and then, the effect was reversed to basal levels after washing-out (Fig. 2). It is indicated from these results that indomethacin potentiates α7 ACh receptor currents in a bell-shaped concentration (100 nM-1 mM)-dependent manner.

The potentiating effect of indomethacin (100 µM) on α7 ACh receptor responses was significantly inhibited by GF109203X (100 nM), an inhibitor of PKC, but the effect was not affected by KN-93 (3 µM), an inhibitor of CaMKII (Fig. 3). This accounts for the implication of PKC in indomethacin-induced potentiation of α7 ACh receptor responses. Transient depression of α7 ACh receptor responses induced by 1 mM of indomethacin was not affected by GF109203X or KN-93 (data not shown), suggesting that the depression is due to the non-specific inhibitory effect of indomethacin, independently of activation of PKC or CaMKII.

**Indomethacin activates PKC**

To obtain evidence for indomethacin-induced PKC activation, we assayed PKC activity using a reversed-

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**Fig. 1.** Effect of indomethacin on α7 ACh receptor currents. α7 ACh receptors were expressed in *Xenopus* oocytes, and ACh (100 µM) was bath-applied to oocytes for 10 s at a 10-min interval before and after 10-min treatment with indomethacin (IM) (100 µM) in Ca²⁺-containing and -free extracellular solution. The holding potential was -60 mV. Application with ACh is indicated by bars. Typical currents recorded 10 min before and 70 min after treatment with indomethacin are shown. Note that a similar effect was obtained with 8 independent experiments.

**Fig. 2.** Concentration-responsive effect of indomethacin on α7 ACh receptor currents. α7 ACh receptors were expressed in *Xenopus* oocytes, and ACh (100 µM) was bath-applied to oocytes for 10 s at a 10-min interval before and after 10-min treatment with indomethacin (IM) at concentrations as indicated. The holding potential was -60 mV. In the graphs, each point represents the mean (± SEM) percentage of original amplitudes (-10 min) (n=5-8 independent experiments).

**Fig. 3.** Effect of indomethacin on α7 ACh receptor currents in the presence of a PKC inhibitor. ACh receptors were expressed in *Xenopus* oocytes, and ACh (100 µM) was bath-applied to oocytes for 10 s at a 10-min interval before and after 10-min treatment with indomethacin (IM) (100 µM) in the presence and absence of GF109203X (GF) (100 nM) or KN-93 (KN) (3 µM). The holding potential was -60 mV. Typical whole-cell membrane currents recorded -10 min and 70 min for each treatment are shown in the upper column. Application with ACh is indicated by bars. In the graphs, each point represents the mean (± SEM) percentage of original amplitudes (-10 min) (n=8 independent experiments). *P<0.01, PLSD test.
For cultured rat hippocampal neurons, the potent PKC activator TPA (100 nM) actually enhanced PKC activity and the enhancement was inhibited by GF109203X (100 nM) (Fig. 4A). Indomethacin also enhanced PKC activity in a concentration (1-100 µM)-dependent manner, the maximum reaching 2-fold enhancement at 100 µM, and the enhancement was abolished by GF109203X (100 nM) (Fig. 4A). This provides evidence for indomethacin-induced PKC activation. In the cell-free PKC assay, indomethacin (100 µM) significantly increased PKC-ε activity (Fig. 4B), indicating that indomethacin activates PKC-ε through direct interaction.

Indomethacin facilitates hippocampal synaptic transmission under the control of α7 ACh receptor and PKC

Enhancing α7 ACh receptors under the control of PKC stimulates presynaptic glutamate release, leading to facilitation of hippocampal synaptic transmission [8-12]. Then, we thought that an enhancement in α7 ACh receptor responses through PKC activation could also contribute to indomethacin-induced facilitation of hippocampal synaptic transmission. To address this point, we recorded fEPSPs from the CA1 region in rat hippocampal slices.

Indomethacin (100 µM) induced a huge spike increase in the fEPSP slope, the peak reaching nearly 450% of basal levels, followed by a persistent facilitation with about 200% increase in the fEPSP slope (Fig. 5). The facilitatory effect in the early phase was significantly suppressed by α-bungarotoxin (αBgtX).
an antagonist of $\alpha_7$ ACh receptor, or GF109203X (100 nM), while the effect in the late phase was not affected (Fig. 5).

To further examine the effect of indomethacin on hippocampal synaptic transmission, we monitored spontaneous AMPA-mEPSCs from the CA1 region of rat hippocampal slices. Indomethacin (100 µM) increased the rate of nicotine (1 µM)-evoked AMPA-mEPSCs, without affecting the amplitude, and the effect was significantly prevented by $\alpha$BgTX (100 nM) ($P=0.0095$ as compared with the indomethacin effect in the absence of $\alpha$BgTX, Kolmogorov-Smirnov test) (Fig. 6A). Indomethacin (100 µM)-induced increase in the rate of nicotine (1 µM)-evoked AMPA-mEPSCs was also inhibited by GF109203X (100 nM) ($P=0.0074$ as compared with the indomethacin effect in the absence of GF109203X, Kolmogorov-Smirnov test) (Fig. 6B). Collectively, these results indicate that indomethacin stimulates presynaptic glutamate release in an $\alpha_7$ ACh receptor- and PKC-dependent manner.

Indomethacin, thus, appears to facilitate hippocampal synaptic transmission in part by enhancing $\alpha_7$ ACh receptor activity through PKC activation, to stimulate presynaptic glutamate release.

**Discussion**

The results of the present study clearly demonstrate that the COX inhibitor indomethacin activates PKC, thereby potentiating $\alpha_7$ ACh receptor responses and facilitating hippocampal synaptic transmission. To our knowledge, this is the first providing evidence for indomethacin-induced PKC activation.

PKCs are classified into conventional PKCs such as PKC-α, -βI, -βII, and -γ, novel PKCs such as PKC-δ, -ε, -η, -θ, and -µ, and atypical PKCs such as PKC-λ/τ for mouse/human, -ζ and -ν. Novel PKCs including PKC-ε are activated by binding cis-unsaturated free fatty acids such as arachidonic, oleic, linoleic, linolenic, and docosahexaenoic acid, or lysophosphatidylcholine, that are produced by phospholipase A$_2$-catalyzed hydrolysis of phosphatidylcholine, in a Ca$^{2+}$-independent manner [13, 14]. Accumulating evidence has shown that PKC enhances nicotinic ACh receptor responses [8, 15-18]. Of PKC isoforms PKC-ε is enriched in the presynaptic terminals and regulates neurotransmitter release [19]. Amazingly, indomethacin activated PKC-ε under the cell-free conditions. This indicates that indomethacin is capable of activating PKC-ε in the brain, although it is presently unknown about the effect on other PKC isoforms except for PKC-ε. $\alpha_7$ ACh receptors, alternatively, are preferentially localized at presynaptic terminals in the brain and stimulates neurotransmitter release [10, 12, 20, 21]. In the *Xenopus* oocyte expression systems, indomethacin potentiated $\alpha_7$ ACh receptor responses in a PKC-dependent manner. Indomethacin induced a huge spike facilitation of hippocampal synaptic transmission in the early phase and the ensuing persistent facilitation. The facilitation in the early phase was...
prevented by the α7 ACh receptor antagonist αBgTX or the PKC inhibitor GF109203X, although the facilitation in the late phase was not affected. This interprets that indomethacin facilitates hippocampal synaptic transmission in the early phase by targeting α7 ACh receptor under the control of PKC. In addition, indomethacin increased the rate of nicotine-evoked AMPA-mEPSCs monitored from the CA1 region of rat hippocampal slices, and the effect was also inhibited by αBgTX or GF109203X. This indicates that indomethacin stimulates presynaptic glutamate release in an α7 ACh receptor- and PKC-dependent manner. Taken together, indomethacin appear to potentiate activity of presynaptic α7 ACh receptors by activating PKC, possibly PKC-ε in the brain, to stimulate glutamate release from presynaptic terminals, which in part contribute to facilitation of hippocampal synaptic transmission.

We have found that indomethacin activates CaMKII in cultured rat hippocampal neurons [5]. In the cell-free CaMKII assay, indomethacin did not activate CaMKII, but otherwise inhibited protein phosphatase 1 (PP1) (unpublished data). This implies that indomethacin indirectly activates CaMKII by inhibiting PP1 to dephosphorylate an active form of phosphorylated CaMKII. A similar CaMKII activation is found with DCP-LA [6]. Overall, indomethacin may interact with a variety of protein kinases and protein phosphatases.

In summary, the results of the present study show that indomethacin activates PKC, possibly PKC-ε in the brain, causing an enhancement in the activity of α7 ACh receptors to stimulate presynaptic glutamate release, in part responsible for indomethacin-induced facilitation of hippocampal synaptic transmission. This represents a novel action of indomethacin, distinct from COX inhibition.

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