Indomethacin Activates Protein Kinase C and Potentiates $\alpha 7$ ACh Receptor Responses

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Key Words

Indomethacin • Protein kinase C • $\alpha 7$ 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 $\alpha 7$ ACh receptor responses and hippocampal synaptic transmission through PKC. **Methods:** We recorded currents through $\alpha 7$ 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 $\alpha 7$ 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 activated PKC in a concentration (1–100 μ M)-dependent manner for

cultured rat hippocampal neurons. Additionally, indomethacin (100 μ M) significantly activated PKC- ϵ under the cell-free conditions. Indomethacin (100 μ M) induced a transient huge increase in the fEPSP slope followed by persistent increase, and the former effect was attenuated by the $\alpha 7$ ACh receptor antagonist α -bungarotoxin or GF109203X. Indomethacin (100 μ M) 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 $\alpha 7$ 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.

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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 $\alpha 7$ ACh receptor activity and inducing a long-term facilitation of hippocampal synaptic transmission [7]. Then, we wonder whether indomethacin also potentiates $\alpha 7$ ACh receptor activity and facilitates hippocampal synaptic transmission by activating PKC.

To address this question, we monitored currents through $\alpha 7$ 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 $\alpha 7$ 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 $\alpha 7$ ACh receptor cDNA was kindly provided from Dr. Patrick (Baylor College of Medicine, USA). The mRNA coding the rat $\alpha 7$ 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 $\alpha 7$ 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 $\text{Ca}(\text{NO}_3)_2$, 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 $\alpha 7$ 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 $\alpha 7$ 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 M Ω , 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 Na₂HPO₄, 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-Pro-Ser-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 and phosphorylated PKC substrate peptide were measured (total area corresponds to the concentration of PKC substrate peptide used here), and the amount of phosphorylated substrate peptide was calculated. Phosphorylated substrate peptide (pmol/min/cell protein weight) was used as an index of PKC activity.

Cell-free PKC-ε assay

Synthetic PKC substrate peptide (Pyr-Lys-Arg-Pro-Ser-Gln-Arg-Ser-Lys-Tyr-Leu) (Peptide Institute Inc.) (10 μM) was reacted with PKC-ε in a medium containing 20 mM Tris-HCl (pH 7.5), 5 mM Mg-acetate, 10 μM ATP, and indomethacin at 30 °C for 5 min. After loading on a reversed phase HPLC (LC-10ATvp, Shimadzu Co.), a substrate peptide peak and a new product peak were detected at an absorbance of 214 nm. Areas for non-phosphorylated and phosphorylated PKC substrate peptide were measured (total area corresponds to concentration of PKC substrate peptide used here), and the amount of phosphorylated substrate peptide was calculated. Phosphorylated substrate peptide (pmol/1 min) was used as an index of PKC-ε activity.

fEPSP recording

Rat hippocampal slices (400 μm in thickness) (male Wistar rat, 6 weeks) were transferred to the recording chamber and superfused in a standard artificial cerebrospinal fluid (ACSF) (117 mM NaCl, 3.6 mM KCl, 1.2 mM NaH₂PO₄, 1.2 mM MgCl₂, 2.5 mM CaCl₂, 25 mM NaHCO₃, and 11.5 mM glucose) oxygenated with 95% O₂ and 5% CO₂ at 34 °C. fEPSPs were recorded from the CA1 region in hippocampal slices by electrically stimulating the Schaffer collateral (0.03 Hz, 0.1 ms in duration).

mEPSC recording

Slice patches were made from CA1 pyramidal neurons in rat hippocampal slices (400 μm in thickness) (male Wistar rat, 6 weeks). Spontaneous mEPSCs were monitored in the standard ACSF oxygenated with 95% O₂ and 5% CO₂, containing tetrodotoxin (TTX) (0.5 μM), DL-2-amino-5-phosphonovaleric acid (100 μM), a selective inhibitor of *N*-methyl-D-aspartate receptor, and bicuculline (20 μM), a selective inhibitor of GABA_A receptors, at 34 °C with an Axopatch-200 A amplifier (Axon Instruments, Inc., Foster City, CA, USA). The patch electrode-filling solution contained 110 mM Cs₂SO₄, 5 mM TEACl, 2 mM MgCl₂, 0.5 mM CaCl₂, 5 mM EGTA, 5 mM HEPES, and 5 mM MgATP. Spontaneous mEPSCs obtained here were eliminated by 6,7-dinitroquinoxaline-2,3-dione (20 μM), a selective inhibitor of α-amino-3-hydroxy-5-methyl-4-isoxazole propionic acid (AMPA) receptor (data not shown), indicating mEPSCs obtained here are produced through AMPA receptor channels (AMPA-mEPSCs).

Statistical analysis

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

Fig. 1. Effect of indomethacin on $\alpha 7$ ACh receptor currents. $\alpha 7$ ACh receptors were expressed in *Xenopus* oocytes, and ACh (100 μ M) was bath-applied to oocytes for 10 s at a 10-min interval before and after 10-min treatment with indomethacin (IM) (100 μ M) in Ca^{2+} -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 $\alpha 7$ ACh receptor currents. $\alpha 7$ ACh receptors were expressed in *Xenopus* oocytes, and ACh (100 μ M) 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 ($\pm$ SEM) percentage of original amplitudes (-10 min) ($n=5-8$ independent experiments).

(100 nM-100 μ M)-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 $\alpha 7$ ACh receptor currents in a bell-shaped concentration (100 nM-1 mM)-dependent manner.

The potentiating effect of indomethacin (100 μ M) on $\alpha 7$ ACh receptor responses was significantly inhibited by GF109203X (100 nM), an inhibitor of PKC, but the effect was not affected by KN-93 (3 μ M), an inhibitor of CaMKII (Fig. 3). This accounts for the implication of PKC in indomethacin-induced potentiation of $\alpha 7$ ACh receptor responses. Transient depression of $\alpha 7$ 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-

Fig. 3. Effect of indomethacin on $\alpha 7$ ACh receptor currents in the presence of a PKC inhibitor. ACh receptors were expressed in *Xenopus* oocytes, and ACh (100 μ M) was bath-applied to oocytes for 10 s at a 10-min interval before and after 10-min treatment with indomethacin (IM) (100 μ M) in the presence and absence of GF109203X (GF) (100 nM) or KN-93 (KN) (3 μ M). 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 ($\pm$ SEM) percentage of original amplitudes (-10 min) ($n=8$ independent experiments). * $P<0.01$, PLSD test.

Fig. 4. Effect of indomethacin on PKC activation. (A) Cultured rat hippocampal neurons were untreated and treated with TPA (100 nM) or indomethacin (IM) at concentrations as indicated in the presence and absence of GF109203X (GF)(100 nM), and PKC activity was assayed. In the graph, each column represents the mean ($\pm$ SEM) PKC activity ($n=8$ independent experiments). P values, Dunnett's test. (B) PKC- ϵ activity was assayed in the absence (Control) and presence of indomethacin (IM)(100 μ M). In the graph, each column represents the mean ($\pm$ SEM) PKC- ϵ activity (pmol/min) ($n=6$ independent experiments). P value, unpaired t -test.

phase HPLC. 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 μ M)-dependent manner, the maximum reaching 2-fold enhancement at 100 μ M, 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 μ M) significantly increased PKC- ϵ activity (Fig. 4B), indicating that indomethacin activates PKC- ϵ through direct interaction.

Fig. 5. Effect of indomethacin on fEPSPs. fEPSPs were recorded from the CA1 region in rat hippocampal slices before and after 10-min treatment with indomethacin (IM) (100 μ M) in the absence and presence of α -bungarotoxin (+ α BgTX) (100 nM) or GF109203X (+ GF) (100 nM). fEPSPs superimposed, that were recorded 10 min before (1) and 10 min after treatment with indomethacin (2), are shown in the upper column. In the graph, each point represents the mean ($\pm$ SEM) percentage of basal EPSP slope (-10 min) ($n=5$ independent experiments).

Indomethacin facilitates hippocampal synaptic transmission under the control of $\alpha 7$ ACh receptor and PKC

Enhancing $\alpha 7$ 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 $\alpha 7$ 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 μ M) 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 (α BgTX)

Fig. 6. Effect of indomethacin on AMPA-mEPSCs. AMPA-mEPSCs were monitored from the CA1 region of rat hippocampal slices treated with nicotine (1 μ M) in the absence and presence of indomethacin (100 μ M) together without (+ IM) and with α BgTX (100 nM) (IM + α BgTX)(A) or GF109203X (100 nM) (IM + GF)(B). Typical nicotine-evoked AMPA-mEPSCs are illustrated in the upper columns and graphs of cumulative probability for current amplitude and inter-event intervals are shown in the lower columns (n=6 independent slice patches).

(100 nM), an antagonist of α 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 μ M) increased the rate of nicotine (1 μ M)-evoked AMPA-mEPSCs, without affecting the amplitude, and the effect was significantly prevented by α BgTX (100 nM) ($P=0.0095$ as compared with the indomethacin effect in the

absence of α BgTX, Kolmogorov-Smirnov test) (Fig. 6A). Indomethacin (100 μ M)-induced increase in the rate of nicotine (1 μ M)-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 α 7 ACh receptor- and PKC-dependent manner.

Indomethacin, thus, appears to facilitate hippocampal synaptic transmission in part by enhancing α 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 α 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 , - β I, - β II, and - γ , novel PKCs such as PKC- δ , - ϵ , - η , - θ , and - μ , and atypical PKCs such as PKC- λ /i 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 isozymes 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 isozymes except for PKC- ϵ . α 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 α 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 $\alpha 7$ ACh receptor antagonist α BgTX 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 $\alpha 7$ 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 α BgTX or GF109203X. This indicates that indomethacin stimulates presynaptic glutamate release in an $\alpha 7$ ACh receptor- and PKC-dependent manner. Taken together, indomethacin appear to potentiate activity of presynaptic $\alpha 7$ 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 $\alpha 7$ 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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