Auto-Positive Feedback Regulation for Nicotinic Acetylcholine Receptors by Protein Kinase C Activation

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Key Words

Nicotinic ACh receptor • Protein kinase C • Two-electrode voltage-clamp • Western blot • *In situ* protein kinase C assay

Abstract

Background/Aims: Protein kinase C (PKC) is well-recognized to modify ligand-gated ion channels such as nicotinic ACh (nACh) receptors by phosphorylating the receptors. The aim of the present study was to obtain direct evidence for PKC activation through nACh receptors. **Methods:** Two-electrode voltage-clamp was made to *Xenopus* oocytes expressing wild-type and mutant *Torpedo* nACh receptors. Western blotting using antibodies against phospho-serine and phospho-threonine was carried out in oocytes expressing *Torpedo* nACh receptors. ***In situ* PKC activation** was monitored in cultured rat skeletal muscle cells expressing nACh receptors. **Results:** In the *Xenopus* oocyte expression systems, ACh-evoked whole-cell membrane currents through wild-type *Torpedo* nACh receptors were depressed by GF109203X, a PKC inhibitor, while currents through mutant receptors lacking PKC phosphorylation sites was not affected. In the Western blot analysis, ACh produced immunoreactive bands against an anti-

phospho-serine or an anti-phospho-threonine antibody in oocytes expressing wild-type *Torpedo* nACh receptors, and those signals were attenuated by α -bungarotoxin, an inhibitor of nACh receptors, or GF109203X. In the *in situ* PKC assay using cultured rat muscle cells that expressed all the mRNAs for muscle nACh receptor subunits such as the α , β , γ , δ , and ϵ subunit, ACh activated PKC in the presence of atropine, an inhibitor of muscarinic ACh receptors, and the activation was abolished by α -bungarotoxin or GF109203X. **Conclusion:** The results of the present study show that ACh activates PKC through nACh receptors and that in turn, activated PKC constantly enhances ACh receptor responses by phosphorylating the receptors. This may represent a new auto-positive feedback regulation for nACh receptors by PKC activation.

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Introduction

Protein kinase C (PKC), a serine/threonine kinase, is ubiquitously present in a wide range of organs and tissues and exerts its diverse signal transductions. PKCs are

activated via several pathways mediated by phospholipase C, phospholipase A₂, phospholipase D, and phosphatidylcholine-specific phospholipase C [1-3]. In the previous study using outside-out patch-clamp techniques, we monitored acetylcholine (ACh)-evoked single-channel currents through *Torpedo* nicotinic ACh (nACh) receptors expressed in *Xenopus* oocytes [4]. Serum, to activate PKC through endogenous serum receptors linked to G_q protein/phospholipase C, increased the slope conductance for ACh (1 μ M)-evoked single-channel currents in oocytes expressing wild-type nACh receptors [4]. Amazingly, a higher concentration of ACh (100 μ M) generated single-channel currents, with the slope conductance similar to that achieved by serum, that was blocked by staurosporine, a PKC inhibitor [4]. Moreover, the slope conductance for ACh (1 μ M)-evoked single-channel currents through mutant *Torpedo* nACh receptors with replacement of Ser³³³ and Ser³⁷⁷ on the α and δ subunit, respectively, by Asp, that mimics PKC phosphorylation, was similar to the slope conductance for single-channel currents through wild-type receptors in oocytes treated with serum or the single-channel currents evoked by a higher concentration of ACh (100 μ M) [4]. In contrast, the slope conductance for ACh (1 μ M)-evoked single-channel currents through mutant *Torpedo* nACh receptors with replacement of Ser³³³ and Ser³⁷⁷ on the α and δ subunit, respectively, by Ala, that mimics PKC dephosphorylation, was lower than the slope conductance for ACh (1 μ M)-evoked single-channel currents through wild-type receptors, reaching a level similar to that obtained with staurosporine or a PKC inhibitor peptide [4]. Notably, similar effects were found with muscle junctional nACh receptors too [4]. Taken together, those results strongly suggest that ACh is capable of activating PKC through nACh receptors. Little, however, is known about PKC activation via ligand-gated ion channels such as nACh receptors, although a study suggests that P2X₂ receptor, a ligand-gated ion channel, is implicated in PKC activation [5].

The present study was designed to obtain direct evidence for PKC activation through nACh receptors by monitoring nACh receptor responses and protein phosphorylation in *Xenopus* oocytes expressing wild-type and mutant *Torpedo* nACh receptors and by assaying *in situ* PKC activation in cultured rat skeletal muscle cells. We show here that PKC is activated through nACh receptors, thereby phosphorylating the receptors to enhance the receptor responses.

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.

In vitro transcription and translation

In vitro transcription and translation were carried out by the method as previously described [4]. Briefly, mRNAs coding for the wild-type *Torpedo* nACh receptor α , β , γ , and δ subunit and the mutant α (m α S333A) and δ subunit (m δ S377A) with replacement of Ser³³³ and Ser³⁷⁷, respectively, by Ala were synthesized by *in vitro* transcription. Oocytes were manually separated from the ovary, and incubated overnight in Barth's solution (in mM, 88 NaCl, 1 KCl, 2.4 NaHCO₃, 0.82 MgSO₄, 0.33 Ca(NO₃)₂, 0.41 CaCl₂, and 7.5 Tris, pH 7.6) after collagenase (0.5 mg/ml) treatment. Oocytes were injected with a mixture of the $\alpha/\beta/\gamma/\delta$ (1:1:1:1) subunit mRNAs or the m α S333A/ $\beta/\gamma/\delta$ S377A (1:1:1:1) subunit mRNAs, and incubated at 18°C.

Two-electrode voltage-clamp recording

The injected oocytes were transferred to a recording chamber 2 days after incubation and continuously superfused at room temperature (20 to 22°C) in standard frog Ringer's solution (in mM: 115 NaCl, 2 KCl, 1.8 CaCl₂, and 5 HEPES, pH 7.0) containing atropine (1 μ M), an inhibitor of muscarinic ACh receptors. Currents were recorded using two-electrode voltage-clamp techniques with a GeneClamp-500 amplifier (Axon Instruments, Inc., Foster City, USA).

Western blotting

Oocytes expressing wild-type *Torpedo* nACh receptors were treated with ACh (100 μ M) in standard frog Ringer's solution containing atropine (1 μ M) in the presence and absence of α -bungarotoxin (100 nM) or GF109203X (100 nM) for 30 min. Then, oocytes were immediately frozen on dry ice and homogenized with a sonicator in 200 μ l of a lysis buffer [20 mM Tris-HCl pH 7.4, 150 mM NaCl, 50 mM NaF, 5 mM EDTA, 5 mM ethylene glycol-bis(2-aminoethyl ether)-N,N,N',N'-tetraacetic acid (EGTA), 2% (v/v) Triton X-100, cocktail]. The homogenate stood on ice for 20 min, and centrifuged at 8000 $\times$ g for 20 min at 4°C. The pellet was resuspended in 150 μ l of a lysis buffer and 150 μ g protein of lysate was subjected to 10% (w/v) sodium dodecyl sulfate-polyacrylamide gel electrophoresis, which was transferred to a PVDF membrane for immunoblotting. The membrane was reacted to an anti-phospho-serine antibody (1:200 dilution) (QIAGEN, Hilden, Germany) or an anti-phospho-threonine antibody (1:200 dilution) (QIAGEN), followed by an anti-mouse antibody conjugated to biotin (1:4000 dilution) (Zymed, South San Francisco, USA). Immunoreactive bands were detected with a Streptavidine biotin complex peroxidase kit (Nacalai, Kyoto, Japan). Protein was determined using a Protein quantification kit (Dojindo, Kumamoto, Japan).

Cell culture and fluocytochemistry

Hind leg muscles were removed from the embryonic Wistar rats (gestational age, 20 days). Dissociated cells were grown in Dulbecco's modified Eagle's medium (DMEM) containing 10% (v/v) fetal bovine serum, 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. After washing with cold phosphate-buffered saline (PBS), cells were fixed with 4% (w/v) paraformaldehyde. After washing with cold PBS, cells were incubated with α -bungarotoxin conjugated with Alexa Fluor 488 (1:100) (Invitrogen, Carlsbad, CA, USA). The fluorescent signals were detected with a laser scanning microscopes (LSM510, Carl Zeiss Co. Ltd, Germany).

Reverse transcription-polymerase chain reaction (RT-PCR)

Total RNAs of cultured rat skeletal muscle cells were purified by an acid/guanidine/thiocyanate/chloroform extraction method using a Sepasol-RNA I Super kit. After purification, total RNAs were treated with RNase free-DNase I (2 unit) at 37°C for 30 min to remove genomic DNAs, and 10 μ g of RNAs were resuspended in water. Then, oligo dT primers, dNTP, 5 x First Strand buffer, and SuperScript III RNase H-Reverse Transcriptase were added to the RNA solution and incubated at 65°C for 5 min followed by 60°C for 1 min, 56°C for 60 min, 58°C for 60 min, 85°C for 5 min to synthesize the first strand cDNA. Subsequently, 1 μ l of the reaction solution was diluted with water and mixed with 10 x PCR reaction buffer, dNTPs, MgCl₂, oligonucleotide, dimethylsulfoxide [final concentration, 5% (v/v)] and 1 unit of Taq polymerase (final volume, 20 μ l). RT-PCR was carried out with a Takara Thermal cycler Dice programmed as follows: the first one step, 94°C for 2 min and the ensuing 30 cycles, 94°C for 1 s, 62°C for 15 s, and 72°C for 30 s using primers shown in Table 1. PCR products were stained with ethidium bromide and visualized by 2% (w/v) agarose electrophoresis.

In situ PKC assay

PKC activity in cultured rat skeletal muscle cells was assayed by the method as previously described [6]. Cultured rat muscle cells were plated in 96-well plates (1 x 10⁴ cells/well). Cells were treated with ACh (100 μ M) in the presence and absence of α -bungarotoxin (100 nM) or GF109203X (100 nM) together with and without atropine (1 μ M) at 37°C for 10 min in an extracellular solution [137 mM NaCl, 5.4 mM KCl, 10 mM MgCl₂, 5 mM EGTA, 0.3 mM Na₂HPO₄, 0.4 mM K₂HPO₄, and 20 mM 4-(2-hydroxyethyl)-1-piperazine-ethanesulfonic acid, pH 7.2]. Then, cells were rinsed with 100 μ l of Ca²⁺-free phosphate-buffered saline and incubated at 30°C for 15 min in 50 μ l of the extracellular solution containing 50 μ g/ml digitonin, 25 mM glycerol 2-phosphate, 200 μ M ATP, and 100 μ M synthetic PKC substrate peptide (Pyr-Lys-Arg-Pro-Ser-Gln-Arg-Ser-Lys-Tyr-Leu, MW, 1,374) (Peptide Institute Inc., Osaka, Japan). The supernatants were collected and boiled at 100°C for 5 min to terminate the reaction. Aliquot of the solution (20 μ l) was loaded onto a reversed phase 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

Fig. 1. Regulation of nACh receptor responses by PKC. Two-electrode voltage-clamp was made to *Xenopus* oocytes expressing wild-type and mutant (lacking PKC phosphorylation sites) *Torpedo* nACh receptors. ACh (100 μ M) was bath-applied to oocytes for 10 s with 5-min intervals in the presence and absence of GF109203X (100 nM). Currents illustrated were recorded 0 and 5 min at a holding potential of -60 mV. In the graph, each point represents the mean ($\pm$ SEM) percentage of original amplitude (0 min) ($n=8$ independent oocytes for wild-type receptors and 7 independent oocytes for mutant receptors).

detector, Shimadzu Co., Kyoto, Japan). It was confirmed that each peak corresponds to non-phosphorylated and phosphorylated substrate peptide in the analysis of matrix-assisted laser desorption ionization time of flight mass spectrometry (Voyager ST-DER, PE Biosystems Inc., Foster City, USA). 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/cell protein weight was used as an index of PKC activity.

Results

*ACh constantly potentiates currents through *Torpedo* nACh receptors under PKC phosphorylation*

In a two-electrode voltage-clamp configuration, bath-application with ACh (100 μ M) generated inward whole-cell membrane currents at a holding potential of -60 mV in *Xenopus* oocytes expressing wild-type *Torpedo* nACh receptors (Fig. 1). The currents were inhibited by GF109023X (100 nM), an inhibitor of PKC, reaching nearly 60% of original amplitude at 5-min treatment (Fig. 1). For mutant *Torpedo* nicotinic ACh receptors lacking potent PKC phosphorylation sites, no inhibition of the currents was found with GF109203X (100 nM) (Fig. 1). Currents here were recorded in the presence of atropine (1 μ M), an inhibitor of muscarinic ACh receptors, which excludes the implication of muscarinic ACh receptors in PKC activation. These results, accordingly, suggest that ACh activates PKC through nACh receptors, thereby phosphorylating the receptors to potentiate the receptor responses.

*Serine/threonine kinase is activated through *Torpedo* nicotinic ACh receptors*

ACh (100 μ M)-evoked currents through *Torpedo* nACh receptors expressed in *Xenopus* oocytes were abolished by α -bungarotoxin (100 nM) (Fig. 2). This confirms that α -bungarotoxin serves as a blocker of nACh receptors. In the Western blot analysis using oocytes expressing wild-type *Torpedo* nACh receptors, 30-min treatment with ACh (100 μ M) produced immunoreactive bands at 34 kDa against an anti-phospho-serine antibody (Fig. 3A) and at 49 and 92 kDa against an anti-phospho-threonine antibody (Fig. 3B). Those band intensities were attenuated by α -bungarotoxin (100 nM) or GF109023X (100 nM) (Fig. 3A,B). These results interpret that ACh activates a serine/threonine kinase, possibly PKC, in an nACh receptor-dependent manner.

PKC is activated through muscle nicotinic ACh receptors

Our final attempt was conducted to obtain direct evidence for PKC activation through nACh receptors. To address this point, rat skeletal muscle cells were cultured. Cultured cells were reactive to α -bungarotoxin conjugated with Alexa Fluor 488 (Fig. 4), indicating presence of nACh receptors in cultured muscle cells used here. In the RT-PCR analysis, cultured rat muscle cells expressed all the mRNAs for muscle nACh receptor

Fig. 2. Inhibition of nACh receptor responses by α -bungarotoxin (α -BgTX). Two-electrode voltage-clamp was made to *Xenopus* oocytes expressing wild-type *Torpedo* nACh receptors, and ACh (100 μ M) was bath-applied to oocytes before and after 5-min treatment with α -BgTX (100 nM). The holding potential was -60 mV. Note that similar results was obtained with 5 independent oocytes.

Fig. 3. Western blot analysis. *Xenopus* oocytes expressing wild-type *Torpedo* nACh receptors were untreated and treated with ACh (100 μ M) in the presence and absence of α -bungarotoxin (100 nM) or GF109203X (100 nM) for 30 min, followed by Western blotting using an anti-phospho-serine antibody (Anti-P-Ser) (A) or an anti-phospho-threonine antibody (Anti-P-Thr) (B). Lane 1, non-treatment; lane 2, 30-min treatment with ACh alone; lane 3, 30-min treatment with ACh plus α -bungarotoxin; lane 4; 30-min treatment with ACh plus GF109203X. Note that similar results were obtained with 4 independent experiments.

Fig. 4. Fluorescence microscopy. Rat hind leg muscle cells were cultured. (A) Phase-contrast image. (B) Fluorescent image using α -bungarotoxin conjugated with Alexa Fluor 488. Bars, 20 μ m. Note that similar results were obtained with 4 independent experiments.

subunits such as the α , β , γ , δ , and ϵ subunit (Fig. 5A). This suggests that cultured muscle cells express both the extrajunctional nACh receptors composed of the α , β , γ , and δ subunit and junctional nACh receptors composed of the α , β , ϵ , and δ subunit. Of the PKC isozymes examined here expression of mRNAs for PKC- α , - δ , - ζ , - η , - λ , - μ , and - ν was detected in cultured muscle cells, with much higher expression of the δ and μ mRNAs (Fig. 5B). In the *in situ* PKC assay, basal PKC activation was not affected by atropine (1 μ M) (Fig. 6). ACh (100 μ M) significantly activated PKC in cultured muscle cells, and the activation was eliminated by α -bungarotoxin (100 nM) or GF109023X (100 nM) (Fig. 6). This provides direct evidence that PKC is activated through muscle nACh receptors.

Discussion

PKC as well as other protein kinases modulate properties of ligand-gated ion channels such as nACh receptors [7]. In the present study, the PKC inhibitor GF109203X depressed ACh-evoked currents through wild-type *Torpedo* nACh receptors, but the inhibitor otherwise had no effect on currents through mutant nACh receptors lacking PKC phosphorylation sites. This suggests that PKC is activated through *Torpedo* nACh receptors, to phosphorylate the receptors and potentiate the receptor responses. For oocytes expressing wild-type *Torpedo* nACh receptors, ACh produced immunoreactive signals against an anti-serine antibody or an anti-phosphothreonine antibody, that was suppressed by the nACh receptor inhibitor α -bungarotoxin or GF109203X. This

Fig. 5. RT-PCR analysis in cultured rat skeletal muscle cells. (A) PCR products for the nACh receptor α , β , γ , δ , and ϵ subunit and GAPDH. (B) PCR products for PKC- α , - β I, - γ , - δ , - ϵ , - ζ , - η , - θ , - λ , - μ , and - ν and GAPDH. Note that similar results were obtained with 4 independent experiments.

Fig. 6. *In situ* PKC assay. Cultured rat skeletal muscle cells were untreated or treated with ACh (100 μ M) in the presence and absence of GF109203X (+ GF) (100 nM) or α -bungarotoxin (+ α -BgTX) (100 nM) together with and without atropine (1 μ M) for 10 min, and PKC activity was quantified with a reversed phase HPLC. Each column represents the mean ($\pm$ SEM) PKC activity (pmol/min/ μ g protein) ($n=12$ independent experiments). P value, Dunnett's test.

accounts for activation of a serine/threonine kinase, possibly, PKC, through *Torpedo* nACh receptors. For cultured rat muscle cells, ACh actually activated PKC in

the presence of atropine, an inhibitor of muscarinic ACh receptors, and the activation was inhibited by α -bungarotoxin or GF109203X, providing direct evidence for PKC activation through nACh receptors. Overall, the results of the present study lead to a conclusion that PKC is activated through nACh receptors, thereby phosphorylating the receptors to constantly modulate the receptor properties. ACh, thus, may establish an auto-positive feedback loop for regulation of nACh receptors by PKC.

Established pathways for PKC activation are relevant to hydrolysis of the membrane phospholipids catalyzed by phospholipase C, phospholipase A₂, phospholipase D, and phosphatidylcholine-specific phospholipase C [1-3]. PKC isozymes are classified as 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 / ι for mouse/human, - ζ , and - ν [1, 2]. Phospholipase C hydrolyzes phosphatidylinositol 4,5-bisphosphate into diacylglycerol and inositol 1,4,5-trisphosphate (IP₃), the latter activating IP₃ receptors to release Ca²⁺ from intracellular calcium stores, and conventional PKCs are activated by diacylglycerol and intracellular Ca²⁺ rise [1, 2]. Phosphatidylcholine-specific phospholipase C produces diacylglycerol by hydrolysis of phosphatidylcholine, thereby activating PKC [3]. Phospholipase A₂ hydrolyzes phosphatidylcholine into *cis*-unsaturated free fatty acids such as arachidonic, oleic, linoleic, linolenic, and docosahexaenoic acid, bearing activation of novel PKCs in a Ca²⁺-independent manner, and lysophosphatidylcholine [1, 2]. The free fatty acids, alternatively, synergistically activate conventional PKCs or sustain activity of conventional PKCs activated [1, 2]. Phospholipase D hydrolyzes phosphatidylcholine into

choline and phosphatidic acid. Phosphatidic acid is converted to diacylglycerol, bearing activation of conventional PKCs, or further hydrolyzed into *cis*-unsaturated free fatty acids, bearing activation of novel PKCs, and lysophosphatidic acid, that participates in activation of conventional PKCs through lysophosphatidic acid receptors linked to G_q protein involving phospholipase C activation [1, 2].

What mechanism underlies nACh receptor-mediated PKC activation remains to be explored. We have earlier found that PKC might be activated through nACh receptors still in outside-out patches, where there is only a particularly small piece of the excised plasma membrane expressing the receptors under the intracellular Ca²⁺-free conditions, without cytosolic components [4]. This suggests that PKC might be activated by interacting with cytoskeletal proteins including nACh receptor-associated proteins. There is presently no direct evidence for phosphorylation of nACh receptors under the Ca²⁺-free condition in intact cells. In the present study, mRNAs of novel PKCs such as PKC- δ and - μ , with much higher expression, and PKC- η , with lower expression, was found with cultured muscle cells. This raises the possibility that nACh receptors here might activate novel PKCs in a Ca²⁺-independent manner, thereby phosphorylating the receptors. To address this question, we are currently carrying out further experiments.

In conclusion, the results of the present study show that PKC is activated through nACh receptors and that in turn, activated PKC constantly potentiates the receptor responses by phosphorylating the receptors. This may represent a fresh insight into regulation of nACh receptors by PKC as mediated via an auto-positive feedback loop.

References

- 1 Nishizuka Y: Intracellular signaling by hydrolysis of phospholipids and activation of protein kinase C. *Science* 1992;258:607-614.
- 2 Nishizuka Y: Protein kinase C and lipid signaling for sustained cellular responses. *FASEB J* 1995;9:484-496.
- 3 Isakov N: Activation of murine lymphocytes by exogenous phosphatidylethanolamine- and phosphatidylcholine-specific phospholipase C. *Cell Immunol* 1993;152:72-81.
- 4 Nishizaki T, Sumikawa K: Nicotinic receptors are regulated by protein kinase C activated via a nicotinic receptors-mediated signaling pathway. *Mol Brain Res* 1998;61:211-218.
- 5 Bradford MD, Soltoff SP: P2X₂ receptors activate protein kinase D and p42/p44 mitogen-activated protein kinase (MAPK) downstream of protein kinase C. *Biochem J* 2002;366:745-755.
- 6 Kanno T, Yamamoto H, Yaguchi T, Hi R, Mukasa T, Fujikawa H, Nagata T, Yamamoto S, Tanaka A, Nishizaki T: The linoleic acid derivative DCP-LA selectively activates PKC- ϵ , possibly binding to the phosphatidylserine binding site. *J Lipid Res* 2006;47:1146-1156.
- 7 Swope SL, Moss SJ, Raymond LA, Huganir RL: Regulation of ligand-gated ion channels by protein phosphorylation. *Adv Second Messenger Phosphoprotein Res* 1999;33:49-78.