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A Serum Factor Potentiates ACh and AMPA Receptor Currents via Differential Signal Transduction Pathways

Tomoyuki Nishizaki,*[,1] Toshiyuki Matsuoka,* Tamotsu Nomura,* and Katumi Sumikawa†
Department of Physiology, Kobe University School of Medicine, 7-5-1 Kusunoki-cho, Chuo-ku, Kobe 650, Japan; and †Department of Psychobiology, University of California, Irvine, California 92697-4550

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A serum factor is recognized to interact with a protein kinase C (PKC) pathway. Indeed, treatment with fetal bovine serum enhanced ACh-evoked currents by PKC activation in the neuronal nicotinic ACh receptors (α7) and *Torpedo* ACh receptors expressed in *Xenopus* oocytes. In addition, potentiation of ACh-evoked currents induced by fetal bovine serum was observed also in the mutant *Torpedo* ACh receptors lacking potent PKC phosphorylation sites at Ser^{333} on the α subunit and Ser^{377} on the δ subunit; the potentiation was inhibited by the PKC inhibitor, PKC inhibitor peptide (PKCI), indicating that ACh receptor currents were enhanced by PKC activation but not by PKC phosphorylation of the receptors. On the other hand, fetal bovine serum enhanced kainate-evoked currents in oocytes expressing the α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) receptors, GluR1,3. The enhancement was not affected by the PKC inhibitors, PKCI or GF109203X, and instead, was inhibited by the Ca^{2+}/calmodulin-dependent kinase II (CaMKII) inhibitor, KN-62. These results suggest that serum is not only involved in PKC activation but in CaMKII activation, and that thereby ACh receptor currents and AMPA receptor currents are each potentiated.

Protein kinase C (PKC) exhibits diverse biological functions and appears to have a significant role in signal transmission (1). The potent PKC activator, phorbol esters are widely used to activate PKC. Previous studies have demonstrated that 1-oleyol-2-acetylglycerol, phorbol 12,13-diacetate and phorbol 12,13-dibutyrate accelerate the rate of ACh receptor desensitization in sympathetic ganglion neurons (2) or 12-*O*-tetradecanoylphorbol-13-acetate (TPA) reduces the slope conductance of ACh-elicited single channel currents in cultured embryo myotubes (3), suggesting that the ACh receptor channels are regulated by PKC activation. In earlier studies, 4-β-phorbol-12,13-dibutyrate accelerated the rate of desensitization of the ACh-evoked currents (4) or TPA reduced glycine α1 and α2 receptor currents (5), however these effects were caused by a direct blocking effect on the ACh or glycine receptor channels but not by PKC activation. The data obtained with phorbol esters, thus, does not accurately reflect the consequences of PKC activation. A serum factor is shown to activate the phosphatidylinositol signaling pathway in *Xenopus* oocytes (6); the production of inositol 1,4,5-trisphosphate (IP_3) and diacylglycerol, to release intracellular Ca^{2+} and to activate PKC, respectively, indicating that this pathway enables us to examine the actual effect of PKC activation on the ion channels or neurotransmitter receptors expressed in *Xenopus* oocytes.

The present study was conducted to assess the effects of serum-induced PKC activation on ACh- and kainate-evoked currents in oocytes expressing the nicotinic ACh receptors and the α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) receptors. The present results demonstrate that serum potentiated ACh and AMPA receptor currents; the potentiation of ACh-evoked currents resulted from PKC activation and in contrast, that of kainate-evoked currents was caused by Ca^{2+}/calmodulin-dependent kinase II (CaMKII). We propose here that serum interacts with differential signal transduction pathways involving activation of PKC and CaMKII.

METHODS

In vitro transcription and translation in Xenopus oocytes. mRNAs coding for the rat α7 subunit, the *Torpedo* α,β,γ and δ subunits; and glutamate receptor 1 and 3 subunits were synthesized by *in vitro* transcription as described previously (7). The α and δ subunit mutant lacking PKC phosphorylation sites were constructed using site-directed mutagenesis (8), and Ser^{333} on the α subunit and Ser^{377} on the δ subunit were replaced by Ala. *Xenopus* oocytes were manually separated from the ovary, and incubated overnight 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) after collagenase (0.5 mg/ml) treatment. Oocytes were injected with α7 subunit mRNAs; normal ($\alpha,\beta,\gamma,\delta$) and mutant (m$\alpha\Delta$PKC/Ser333,$\beta,\gamma$,m$\delta\Delta$PKC/

[1] To whom correspondence should be addressed. Fax: +81-78-341-5732.

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Ser377) *Torpedo* ACh receptor subunit mRNAs; and GluR1,3 subunit mRNAs, and incubated at 18°C.

Voltage-clamp recording. The injected oocytes were transferred to the recording chamber 2-7 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 $CaCl_2$, and 5 HEPES, pH 7.0). Ca^{2+}-free extracellular solution consisted of (in mM) 115 NaCl, 2 KCl, 5 $MgCl_2$, 5 HEPES, and 1 EGTA, pH 7.0. Currents evoked were recorded using two-electrode voltage clamp techniques with a GeneClamp-500 amplifier (Axon Instruments, Inc., USA) and analyzed on a microcomputer using pClamp software (Axon Instruments, Inc.; version 6) (5).

RESULTS

Potentiation of α7 receptor-gated channel currents by serum. A serum factor stimulates the phosphatidylinositol/Ca^{2+} second messenger system in *Xenopus* oocytes (6), leading to intracellular Ca^{2+} release and endogenous PKC activation. The cytosolic Ca^{2+} increase induces currents as a result of the activation of the Ca^{2+}-dependent chloride channels expressed in oocytes (9) as shown in Fig. 1A. ACh (100 μM) evoked inward membrane currents at a holding potential of −100 mV and the currents were potentiated after 5-min treatment with fetal bovine serum (0.5 v/v volume%) (Fig. 1A). The action of serum was long-lasting, reaching a maximum (200%) at 10 min after treatment (Fig. 1A). The current potentiation was inhibited by the PKC inhibitor, PKC inhibitor peptide (PKCI) (Fig. 1B), suggesting that serum induced a long-term enhancement of α7 receptor currents by PKC activation.

Effect of serum on ACh-evoked currents through the mutant ACh receptors with PKC phosphorylation site deletion. Fetal bovine serum also enhanced *Torpedo* ACh receptor currents to 147% at 30 min after treatment (Fig. 2). The current enhancement was inhibited by PKCI, whereas the CaMKII inhibitor, KN-62, had no effect (Fig. 2), supporting the idea that serum potentiates ACh-gated channel currents by PKC activation. Furthermore, a same extent of the current potentiation by serum was observed also in Ca^{2+}-free extracellular solution (Fig. 2), suggesting that serum potentiated ACh receptor currents themselves, but Ca^{2+}-dependent Cl^- currents, which contribute to ACh-evoked currents. Interestingly, serum enhanced ACh-evoked currents equally to a same extent (145%) in oocytes expressing the mutant ACh receptors lacking potent PKC phosphorylation sites on the α and δ subunits (mαΔPKC/Ser333 mδΔPKC/Ser377) and this effect was blocked by PKCI (Fig. 2), indicating that ACh receptor currents were enhanced by PKC activation but not by PKC phosphorylation of the receptors.

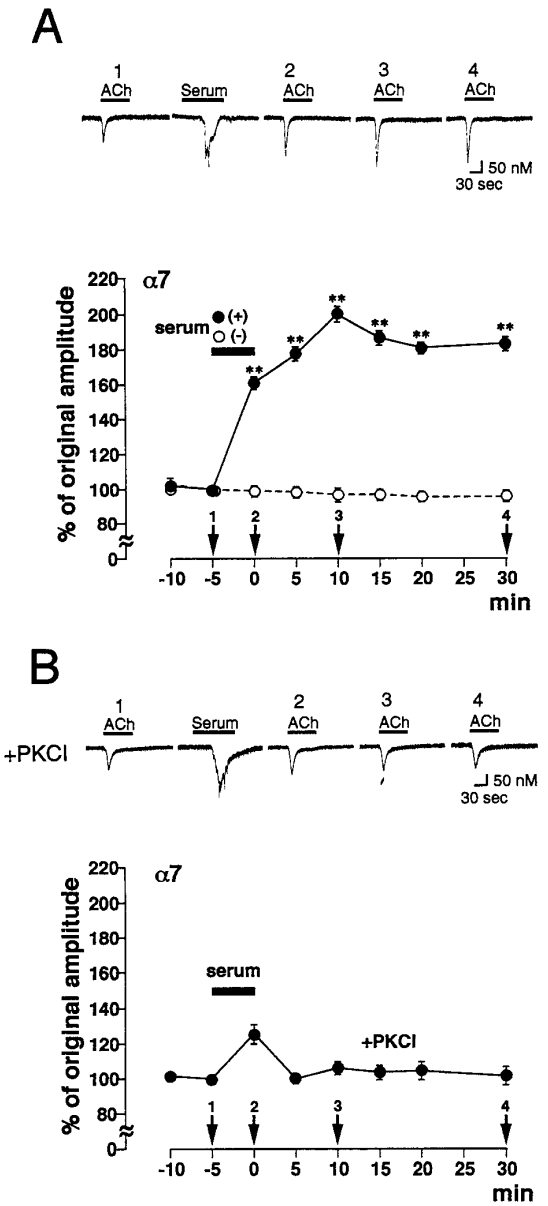

FIG. 1. Effects of serum on α7 receptor currents. (A) ACh (100 μM) was applied to a single oocyte expressing the α7 receptors at 5-min intervals. The oocyte was treated with fetal bovine serum (0.5 v/v volume%) for 5 min. The illustrated currents were recorded at the times indicated. The holding potential was −100 mV. In this and further figures, inward currents correspond to downward deflections. In the graph, each point represents the mean (± SD) percent of the original amplitude (−5 min) from 7 oocytes (closed circle, treatment with serum; open circle, non-treatment with serum). **P<0.01, paired t-test. (B) PKCI (300 μg/ml, final conc.) was injected into the oocytes prior to application of ACh and treatment with serum (n=7).

Potentiation of GluR1,3 currents by serum. Kainate (100 μM) evoked currents through the GluR1,3 channels and the current amplitudes were not affected by repetitive applications (Fig. 3A). Treatment with fetal bovine serum induced a long-lasting enhancement of the currents and the maximal effect was 170% at 20 min after treatment (Fig. 3A). The current potentiation was not affected by the PKC inhibitors, PKCI or GF109203X, but in contrast, clearly inhibited by the CaMKII inhibitor, KN-63 (Fig. 3B), indicating that se-

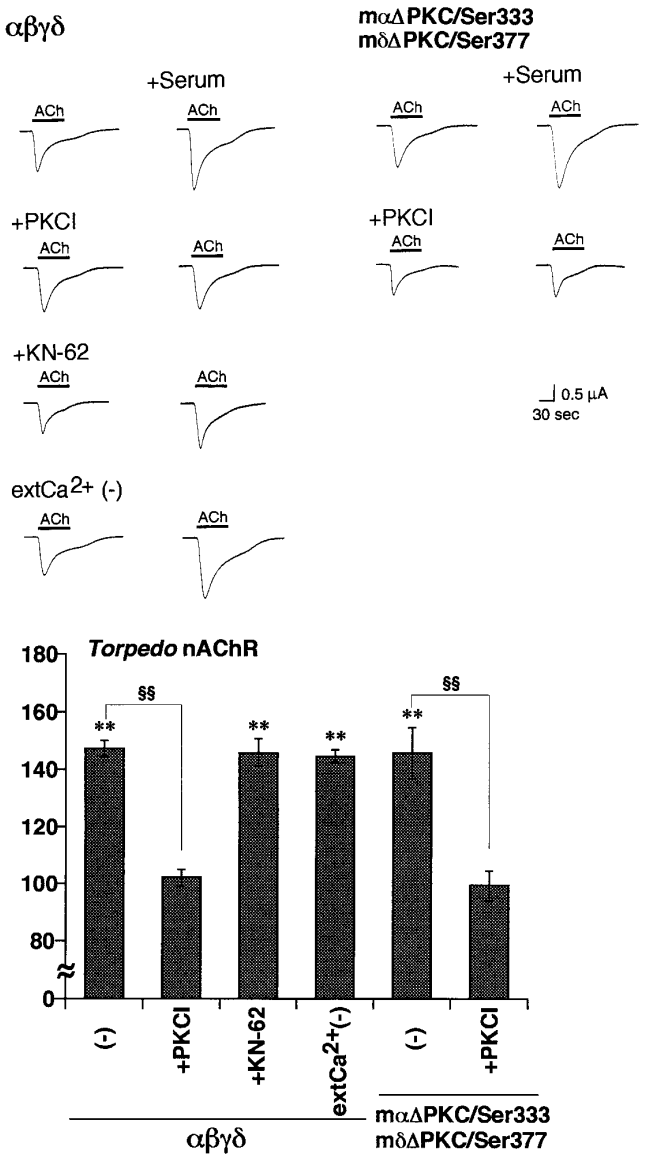

FIG. 2. Effects of serum on normal and mutant *Torpedo* ACh receptor currents. ACh (100 μM) was applied to a single oocyte expressing the normal ($\alpha\beta\gamma\delta$) and mutant ACh receptors (m$\alpha\Delta$PKC/Ser333m$\delta\Delta$PKC/Ser377) in the presence and absence of PKCI (300 μg/ml, final conc.) or KN-62 (1 μM, final conc.), or in Ca^{2+}-free media. The illustrated currents were recorded 5 min before and 30 min after treatment with fetal bovine serum (0.5 v/v volume%). The holding potential was −60 mV. The mean (±SD) percentage of the original amplitude (−5 min) obtained with each experiment is shown in the lower graph (n=7-10). **P<0.01, paired t-test, §§P<0.01, ANOVA with Fisher's least-significance test.

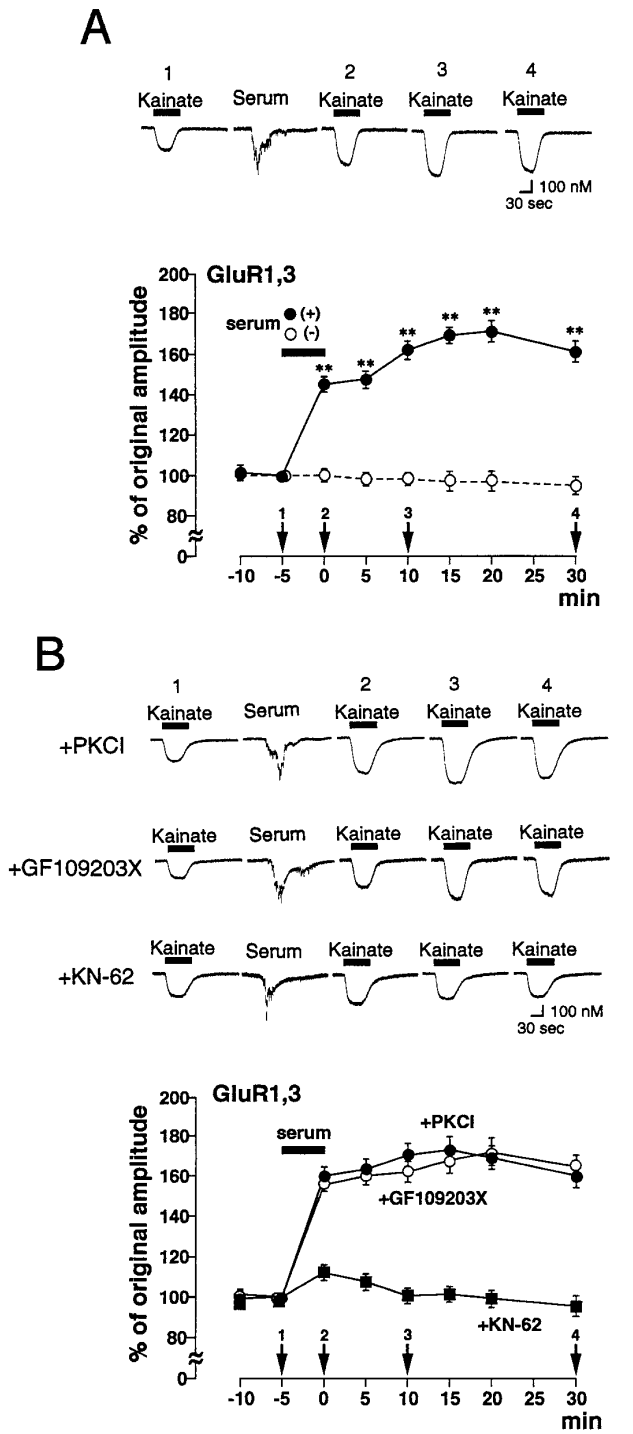

FIG. 3. Effects of serum on GluR1,3 currents. (A) Kainate (100 μM) was applied to a single oocyte expressing the GluR1,3 at 5-min intervals. The currents recorded at the times as indicated are illustrated and the results are summarized in the graph. The holding potential was −80 mV. Each point represents the mean (± SD) percent of the original amplitude from 5 oocytes (closed circle, treatment with serum; open circle, non-treatment with serum). (B) PKCI (300 μg/ml, final conc.) (closed square) or KN-62 (1 μM, final conc.) (closed circle) was injected into oocytes. In some cases, kainate (100 μM) was applied to an oocyte in the presence of GF109203X (100 nM) (open circle). Each point represents the mean percent (± SD) of the original amplitude from 5–7 oocytes.

rum potentiated AMPA receptor currents by CaMKII activation independently of PKC activation.

DISCUSSION

The results presented here clearly demonstrate that consistent with the previous report (4) a serum factor is

involved in activation of PKC, leading to a long-lasting potentiation of ACh receptor currents. Lines of evidence indicate that the neurotransmitter receptors are regulated by protein phosphorylation (10). *Torpedo* nicotinic ACh receptor subunits are recognized to be differentially phosphorylated by cAMP-dependent protein kinase, PKC, and protein tyrosine kinase (10,11). The current potentiation here, however, can not be explained by PKC phosphorylation of the ACh receptors in spite of containing PKC phosphorylation sites. This suggests that ACh receptor currents are potentiated by another unknown factor or process activated by PKC activation.

The AMPA receptors are shown to have phosphorylation sites for PKC and protein kinase G (PKG) (12). In addition, AMPA itself leads to transient phosphorylation of the AMPA receptor subunits expressed in Purkinje cells and that persistent phosphorylation is induced by co-treatment with 8-bromo-cGMP, dibutyl-cGMP, and calyculin A (13). These results suggest that the AMPA receptor channels are regulated by PKC activation and following PKC phosphorylation of the receptors. The finding that serum induced a long-term enhancement of GluR1,3 currents and that the potentiation was not inhibited by PKC inhibitors indicates that serum potentiates the currents by a mechanism independent of PKC activation. In contrast, the potentiation was inhibited by a CaMKII inhibitor, suggesting that serum is involved in activation of CaMKII as well as PKC and thereby, potentiates GluR1,3 currents. In optic neurons from *Xenopus* tadpoles, the first glutamatergic transmission is mediated only by the N-methyl-D-aspartate (NMDA) receptors and postsynaptic AMPA responses are induced by CaMKII activation in the process of the maturation of a glutamatergic synapse (14). Taken together with the present findings, the appearance of AMPA responses in maturated neurons may result in part from the potentiation of AMPA receptor currents by CaMKII activation, although, of course, the expression of the AMPA receptors on the plasma membrane can not be ruled out. This may also explain physiological significance of serum for the development of neurons.

In conclusion, the results presented here demonstrate that serum interacts with differential signal transduction pathways responsible for PKC and CaMKII, resulting in a long-lasting potentiation of ACh and AMPA receptor currents.

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REFERENCES

1. Nishizuka, Y. (1995) *FASEB J.* **9,** 484–496.
2. Downing, J. E. G., and Role, L. W. (1987) *Proc. Natl. Acad. Sci. USA* **84,** 7739–7743.
3. Eusebi, F., Grassi, F., Nervi, C., Caporale, C., Adamo, S., Zani, B. M., and Molinaro, M. (1987) *Proc. R. Soc. Lond.* **B230,** 355–365.
4. Nishizaki, T., and Sumikawa, K. (1995) *Neurosci. Lett.* **188,** 129–131.
5. Nishizaki, T., and Ikeuchi, Y. (1995) *Brain Res.* **687,** 214–216.
6. Tigyi, G., Dyer, D., Matute, C., and Miledi, R. (1990) *Proc. Natl. Acad. Sci. USA* **87,** 1521–1525.
7. Sumikawa, K., and Miledi, R. (1989) *Mol. Brain Res.* **5,** 183–192.
8. Gehle, V. M., and Sumikawa, K. (1991) *Mol. Brain Res.* **11,** 17–25.
9. Miledi, R., and Parker, I. (1984) *J. Physiol.* **357,** 173–183.
10. Huganir, R. L. (1990) *Neuron* **5,** 555–567.
11. Huganir, R. L. (1987) *J. Rec. Res.* **7,** 241–256.
12. Nakazawa, K., Tadakuma, T., Nokihara, K., and Ito, M. (1995) *Neurosci. Res.* **24,** 75–86.
13. Nakazawa, K., Mikawa, S., Hashikawa, T., and Ito, M. (1995) *Neuron* **15,** 697–709.
14. Wu, G., Malinow, R., and Cline, H. T. (1996) *Science* **274,** 972–976.