Selective Potentiation of N-Methyl-D-aspartate-induced Current by Protein Kinase C in Xenopus Oocytes Injected with Rat Brain RNA*

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Glutamate receptors and protein kinase C (PKC) may play significant roles in long-term potentiation in hippocampus. To clarify the regulatory involvement of PKC in the functions of glutamate receptors, we examined the effects of PKC activation on current response induced by the activation of each subtype of glutamate receptor in Xenopus oocytes injected with rat brain RNA. Treatment with the PKC activator, 12-O-tetradecanoylphorbol-13-acetate (TPA), potentiated N-methyl-D-aspartate (NMDA)-induced current by about 2.5-fold, although it did not affect kainate-induced current at all. Quisqualate-mediated oscillatory current was almost abolished by this treatment. The TPA-induced potentiation of NMDA current was suppressed by staurosporine, an inhibitor of protein kinases. Pretreatment with 4-O-methyl-TPA, an inactive phorbol ester, had no effect on NMDA current. Current response mediated by NMDA receptors would thus appear to be modulated by PKC.

The excitatory amino acid, L-glutamate, is a critical neurotransmitter in the central nervous system and implicated in various brain functions such as learning and memory. Pharmacological and biological studies distinguish glutamate receptors into NMDA, non-NMDA (kainate/[RS]-α-amino-3-hydroxy-5-methyl-4-isoxazolepropionate), and mGlu (metabotropic glutamate) subtypes. The functional involvement of each glutamate receptor subtype in long-term potentiation (LTP), thought to be a model of memory, has been investigated (1). LTP formation in the hippocampus may include activation of protein kinase C (PKC) based on the following reasons: 1) a nonspecific protein kinase inhibitor, H-7, blocks LTP (2); 2) PKC is translocated from cytosol to membranes (3); 3) Pretreatment with 4-O-methyl-TPA, an inactive phorbol ester, antagonized by staurosporine, an inhibitor of protein kinases. Pretreatment with 4-O-methyl-TPA, an inactive phorbol ester, had no effect on NMDA current. Current response mediated by NMDA receptors would thus appear to be modulated by PKC.

The maintenance and dissection of frogs, handling of oocytes, and detection of drug-evoked current in electrophysiological recordings were carried out as previously reported (9). Total RNA was extracted from whole brains of adult male Wistar rats by the cesium chloride method (10) and stored at -80 °C in sterile water until use (5 mg/ml). Oocytes defolliculated by collagenase treatment (1 mg/ml in Ca²⁺-free MBS at 22 °C for 30 min) were injected with 50 ng of total RNA and cultivated at 22 °C in sterile modified Barth's solution (MBS: 88 mM NaCl, 1 mM KCl, 0.4 mM CaCl₂, 0.33 mM Ca(NO₃)₂, 0.82 mM MgSO₄, 2.4 mM NaHCO₃, 7.5 mM Tris-HCl, pH 7.6) for 2-5 days. Following removal of the deteriorated oocytes, vigorous oocytes possessing negative membrane potential exceeding -20 mV were used for electrophysiological recording. In electrophysiological experiments, Mg²⁺-free MBS was used so as to prevent Mg²⁺ blockade of ion channels coupled to NMDA receptors. A single oocyte was placed in a small bath continuously perfused with Mg²⁺-free MBS at room temperature (18-23 °C), impaled with two microelectrodes, and the membrane potential was maintained at -60 mV. Drugs were diluted with Mg²⁺-free MBS and applied to oocytes in the bath at a rate of 1 ml/min. 10-15 s were required to change all the solution in the bath. In quantitative analysis, each oocyte was treated as follows. After application of a voltage clamp, current was evoked in an oocyte by NMDA application as the first response, and the second NMDA response was measured after treatment with phorbol esters or other drugs and expressed as a percentage of the first response. Xenopus laevis were purchased from Hamamatsu Seibutsu Kyouzai (Shizuoka, Japan). Collagenase was from Wako. All other chemicals were obtained from Sigma. The phorbol esters were stored at 10 mM in dimethyl sulfoxide, and staurosporine was stored at 1 mM in dimethyl sulfoxide.

RESULTS

NMDA (100 µM) plus glycine (10 µM) showed smooth inward current in brain RNA-injected oocytes. Without glycine, NMDA elicited no detectable current (Fig. 1A). Non-injected oocytes did not respond to NMDA (data not shown). NMDA-induced current following perfusion of 1 µM TPA for 10 min was markedly potentiated compared with the initial NMDA current (Fig. 1A). Potentiated NMDA response was also glycine-dependent (Fig. 1A) and antagonized by 2-amino-5-phosphonovaleric acid (100 µM, data not shown). NMDA-induced current after TPA treatment increased by 264.8 ± 41.1% (n = 4) of the first response, although repeated applications of NMDA every 10 min slightly decreased the response (control, 90.6 ± 8.7% of the first response, Fig. 1B). This potentiation was inhibited by the simultaneous application of staurosporine (1 µM), a protein kinase inhibitor (n = 3, Fig. 1B). Treatment with 4-O-methyl 12-O-tetradecanoylphorbol-13-acetate (4-O-methyl TPA, 1 µM), an inactive phorbol ester, had no effect on NMDA current response (Fig. 1B). Quisqualate (QA, 50 µM)-induced current showed a transient peak.
PKC-potentiated NMDA Responses

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FIG. 1. Potentiation of NMDA-induced currents by phorbol ester in brain mRNA-injected oocytes. Membrane potentials were clamped at −60 mV, and Mg²⁺-free MBS was perfused on oocytes continuously. A, responses to NMDA in the absence or presence of 10 µM glycine were measured before and after 10 min of application of TPA. Agonist stimulation was carried out during the period indicated by closed bars. B, amplitude of NMDA currents after drug treatment (second response) is shown as percent of first current responses before drug application. Current responses were evoked by 100 µM NMDA plus 10 µM glycine. The second responses were measured 10 min after treatment with TPA or vehicle. Each value shows the mean ± S.E. of more than three experiments.

component and slow oscillatory component such as acetylcholine- and serotonin-induced currents in mRNA-injected oocytes (Fig. 2A). Treatment with TPA (1 µM) abolished the transient and oscillatory current, but smooth current could occasionally be detected (Fig. 2A). Kainate is an agonist for non-NMDA receptors and elicits smooth inward current with short latency in mRNA-injected oocytes. The non-NMDA receptor-mediated smooth current was not affected by TPA application (88.9%, n = 2, Fig. 2, A and B).

Fig. 3A shows concentration-response curves for NMDA in the presence of 10 µM glycine before and after TPA treatment. There was potentiation of NMDA current by TPA at all concentrations with the upper shift of the concentration-response curve. The degree of the potentiation increased in a NMDA concentration-dependent manner (175.6–338.8% of the first response, Fig. 3A). Current by 100 µM NMDA increased with the concentration of glycine (Fig. 3B). In contrast to low NMDA concentration, TPA potentiation could not be detected at low glycine concentration (0.1–0.3 µM, Fig. 3B). To determine whether Ca²⁺ is involved in potentiation, the effect of Ba²⁺ replacement on Ca²⁺ in continuously superfused MBS was examined. In Ba²⁺-containing buffer, NMDA current response was observed, but there was no significant TPA effect on NMDA current (106.1 ± 10.4% of the first response, Fig. 4B). However, NMDA-induced current in Ba²⁺ buffer was potentiated by TPA treatment following application of NMDA with glycine in Ca²⁺-containing buffer (155.1 ± 32.1%, n = 4). Fig. 5 shows the voltage dependence of NMDA-activated current before and after TPA treatment. In the absence of Mg²⁺, NMDA current showed a linear I-V curve, and the reversed potential was about 0 mV. The potentiation of NMDA current after TPA treatment was observed at all holding potentials, and the linearity of the I-V curve and reversal potential did not markedly change. In the presence of 100 µM Mg²⁺, the I-V curve for NMDA showed voltage

FIG. 2. Effects of TPA on currents activated by each subclass of glutamate receptors. Membrane potentials were clamped at −60 mV, and Mg²⁺-free MBS was perfused. Each agonist was applied twice to the same oocyte before and after 10 min of treatment with TPA. A, the typical current responses induced by each agonist and the influences of TPA treatment; B, effects of TPA on current responses induced by each subclass agonist. Each value shows the mean ± S.E. of the first responses. The experimental number of oocytes is shown in parentheses. KA, kainic acid.

FIG. 3. Concentration-response relationships for NMDA in the presence of 10 µM glycine (A) and for glycine in the presence of 100 µM NMDA (B) before and after TPA treatment. The values show the mean ± S.E. of three to six experiments.
PKC-potentiated NMDA Responses

A) {\( 100 \mu M \text{NMDA} \)}

B) {\( 1 \mu M \text{TPA} \)}

**FIG. 4.** Influence of Ca\(^{2+}\) in TPA-induced potentiation of NMDA currents. 100 \( \mu M \) NMDA, 10 \( \mu M \) glycine-induced currents were measured in Mg\(^{2+}\)-free and 0.75 mM Ca\(^{2+}\)-containing MBS (A) or equimolar Ba\(^{2+}\)-containing MBS instead of Ca\(^{2+}\) (B). Experimental methods are as described in the legend of Fig. 2.

whether Ca\(^{2+}\) influx supports PKC activation or contributes to the activation of other Ca\(^{2+}\)-dependent pathways associated with NMDA potentiation are points to be clarified. That elevation of intracellular Ca\(^{2+}\) following NMDA channel opening may stimulate activation of PKC suggests a positive feedback regulation of NMDA receptors-channels. PKC may regulate NMDA receptor functions through phosphorylation in NMDA receptors-channels or related proteins. Amino acid sequences predicted from the rat NMDA receptor cDNA cloned by Moriyoshi et al. (16) have been shown to contain several consensus phosphorylation sites for Ca\(^{2+}/\text{calmodulin-dependent kinase II}\) and PKC in its putative intracellular domain situated between the third and fourth transmembrane segments. A regulatory mechanism of its channel function through phosphorylation by serine/threonine kinases may thus possibly exist. Inactivation of NMDA receptors in the absence of an energy-rich compound such as ATP, as reported by MacDonald et al. (17), indicates the involvement of phosphorylation in the regulation of NMDA receptors.

Potentiated NMDA response was shown the same properties as normal response with respect to glycine enhancement, inhibition of APV, and Mg\(^{2+}\) blockade. Mg\(^{2+}\) blocks channel activity associated with NMDA receptors in a voltage-dependent manner (18). Activation of NMDA receptors in synaptic transmission under physiological conditions is normally suppressed by extracellular Mg\(^{2+}\). Mg\(^{2+}\) altered the voltage dependence of NMDA-activated current and masked TPA-induced potentiation. TPA-induced potentiation did not prevent the channel blockage of NMDA receptors by potent Mg\(^{2+}\).

The positive regulations of NMDA receptor functions are known to occur through the following: 1) potentiation of electrophysiological response by glycine binding to allosteric sites at NMDA receptor-channel complexes in cortical neu-
rons (19) and in Xenopus oocytes injected with brain mRNA (20); 2) glycine-mediated increase in the binding of [H]MK-801 with channels coupled to NMDA receptors (21); 3) positive and negative regulation of NMDA response by polysaccharides, which are shown to exist in the binding site in NMDA receptor-channel complexes (22); and 4) modification of NMDA response by oxidation and reduction with redox agents such as disulfides in neuronal preparations (23, 24). The potentiation of NMDA-induced current by phorbol esters is apparently similar to that caused by spermine, an endogenous agonist for the polyamine recognition site. Spermine increases the apparent affinity of a binding site for [H]MK-801 even at maximum effective concentrations of L-glutamate and glycine, indicating this effect to possibly be due to the increase in the frequency of channel opening or the increase in the duration at the open channel state (22). The upper shift of the NMDA concentration-response curve after TPA treatment may be due to an enhanced maximum response at effective concentrations of NMDA and glycine. The potentiation of NMDA responses caused by PKC activation would thus appear due to modification of channel activity, similar to the effect of spermine.

Leonard and Kelso (25) consider NMDA-activated current in mRNA-injected oocytes to involve Ca²⁺-activated Cl⁻ current. That is, Ca²⁺ inflow through NMDA receptors-channels activates Ca²⁺-dependent Cl⁻ channels, and Cl⁻ current is one component of current flow induced by NMDA. They also reported in abstract form (32) that NMDA response is enhanced by phorbol ester in oocytes. It is a possible interpretation that the TPA-induced potentiation of NMDA current results from up-regulation of Ca²⁺-dependent Cl⁻ channels by PKC activation. Nevertheless, detection of NMDA potentiation at −20 mV, the reversal potential of Ca²⁺-dependent Cl⁻ current in oocytes, suggests NMDA channel activity is intrinsically facilitated in a TPA-caused event. Ca²⁺-activated Cl⁻ current was previously reported to be potentiated by the calmodulin-dependent pathway but not by PKC (12).

Another explanation for potentiation by PKC activation is the increase in the number of NMDA receptors-channels. Ca²⁺ influx caused by channel opening following NMDA receptor activation stimulates the Ca²⁺-dependent protease, calpain, to elicit the proteolysis of fodrin (26). Glutamate binding stimulated by Ca²⁺ has been shown to be regulated by fodrin (27). If an increment in the number of NMDA receptor-channel complexes causes this phenomena, NMDA potentiation should be evident at low glycine concentration, under the physiological circumstances in order to reconstitute the increase in the number of NMDA receptors-channels.

PKC is believed to be involved in LTP cascade, but its roles and action site remain unclear (2, 4, 29, 30). The present findings using brain mRNA-injected oocytes support the involvement of PKC in postsynaptic activation but do not exclude its presynaptic action. However, it may be difficult to discuss the findings described here about the significance under the physiological circumstances in order to reconstitute all pre- and postsynaptic components in oocytes. Activation of NMDA receptors has been shown responsible for inducing LTP, and non-NMDA receptors mediate the enhanced component of an excitatory postsynaptic potential in synaptic transmission following LTP formation in the CA1 area of the hippocampus (6, 8, 31). The present results are at variance with conventional theory that enhancement of the non-NMDA component in LTP is generated by activation of Ca²⁺-dependent enzymes including PKC by Ca²⁺ influx through the NMDA receptor-channel complex. PKC activation by TPA selectively enhanced the NMDA response but not the kainic acid-induced response. Postsynaptic PKC activity in the induction of LTP would thus not directly modulate the non-NMDA component in synaptic transmission. It is generally considered that the transient potentiation of the NMDA-sensitive current observed in post-tetanic potentiation caused by LTP-inducing stimuli is due to increased presynaptic release (6), but a postsynaptic mechanism as up-regulation of NMDA receptor may contribute to the mechanism for post-tetanic potentiation. PKC activity may control NMDA-sensitive and -insensitive aspects of synaptic transmission in the induction of LTP (30). At an early stage in the induction of LTP, some activation of NMDA receptors during tetanus stimulation may cause transition from NMDA receptor with "normal" channel activity to "potentiated" activity by an increase in phosphorylation by PKC. Increased NMDA receptors with "potentiated" activity may cause still more Ca²⁺ influx to activate Ca²⁺-dependent enzymes which trigger the subsequent steps of LTP.

REFERENCES