Phosphorylation and Modulation of a Kainate Receptor (GluR6) by cAMP-Dependent Protein Kinase

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Ligand-gated ion channels gated by glutamate constitute the major excitatory neurotransmitter system in the mammalian brain. The functional modulation of GluR6, a kainate-activated glutamate receptor, by adenosine 3′,5′-monophosphate–dependent protein kinase A (PKA) was examined with receptors expressed in human embryonic kidney cells. Kainate-evoked currents underwent a rapid desensitization that was blocked by lectins. Kainate currents were potentiated by intracellular perfusion of PKA, and this potentiation was blocked by co-application of an inhibitory peptide. Site-directed mutagenesis was used to identify the site or sites of phosphorylation on GluR6. Although mutagenesis of two serine residues, Ser^{684} and Ser^{666}, was required for complete abolition of the PKA-induced potentiation, Ser^{684} may be the preferred site of phosphorylation in native GluR6 receptor complexes. These results indicate that glutamate receptor function can be directly modulated by protein phosphorylation and suggest that a dynamic regulation of excitatory receptors could be associated with some forms of learning and memory in the mammalian brain.

Second messenger systems function to regulate the excitability of central nervous system neurons in part by modulating the activity of voltage-gated ion channels (*1*). Less is known about the regulation of transmitter-gated channels (*2*–*6*). Glutamate currents in cultured hippocampal neurons are enhanced by the activity of adenosine 3′,5′-monophosphate–dependent protein kinase (PKA) (*4*, *5*). Thus, activation of adenylate cyclase may be an important mechanism for the modulation of excitatory transmission in the hippocampus. Although most cloned glutamate receptor subunits contain potential phosphorylation sites for protein kinase C and Ca^{2+}-calmodulin–dependent protein kinase II, only the GluR6 subunit (*7*, *8*) contains a consensus sequence for phosphorylation by PKA (*2*, *9*). PKA may also modulate combinations of GluR1 and GluR3 glutamate subunits by acting at low-affinity sites (*10*).

We used whole-cell patch-clamp recordings from human embryonic kidney (HEK-293) cells (*11*) that expressed homomeric GluR6 channels to examine the functional consequences of phosphorylation of GluR6 by PKA. The consensus sequence for PKA phosphorylation at Ser^{684} (Arg-Arg-Gln-Ser^{684}-Val) exits between the putative third and fourth transmembrane domains of GluR6, which is probably a cytoplasmic location. We used site-directed mutagenesis (*12*) to investigate PKA-mediated phosphorylation of GluR6 at this site and at one other location.

Applications of kainate (10 μM) to HEK-293 cells that expressed wild-type GluR6 receptors evoked inward currents that rapidly desensitized [time constant of desensitization (τ) = 150 ms] (Fig. 1A). This suggested that we may not have been able to apply kainate rapidly enough to temporally resolve peak currents (*13*). Therefore, we treated the cells with concanavalin A (Con A) or wheat germ agglutinin (WGA) (*7*, *14*) before treating them with kainate. Both of these lectins enhanced currents induced by kainate to approximately the same extent and greatly reduced desensitization (Fig. 1B). The onset of the potentiation of these currents was quite rapid (<3 min) after treatment with WGA but was considerably slower (>20 min) after treatment with Con A (Fig. 1, C and D).

The catalytic subunit of PKA (cPKA) was directly perfused into individual cells through a patch pipette (*5*). Repeated applications of a single dose of kainate (10 μM) were made to Con A– or WGA-treated cells before and during perfusion of cPKA (Fig. 2A). The application of cPKA potentiated kainate-evoked currents, which reached a steady state in about 5 to 15 min (Fig. 2). We tested the specificity of this enhancement by co-injecting the inhibitory peptide of PKA (PKI) (*15*) with cPKA once the potentiation had been established. PKI caused a reversal of this potentiation and caused a depression of kainate currents themselves (Figs. 2B and 3B).

Dose-response curves for kainate were determined before and during perfusion of cPKA (Fig. 2C). The concentration of kainate required to evoke a half-maximal response (EC_{50}) was the same before and after perfusion of the kinase as were the calculated Hill slopes. Thus, no substantial change in either the apparent affinity or cooperativity of the response had occurred. In contrast, the maximal response to kainate was enhanced by cPKA. Potentiation was not evident with small amounts of agonist (Fig. 2C). Currents recorded from cells transfected with GluR6 had EC_{50} values similar to those reported for GluR6 expressed in *Xenopus laevis* oocytes (*7*) (about 1 μM).

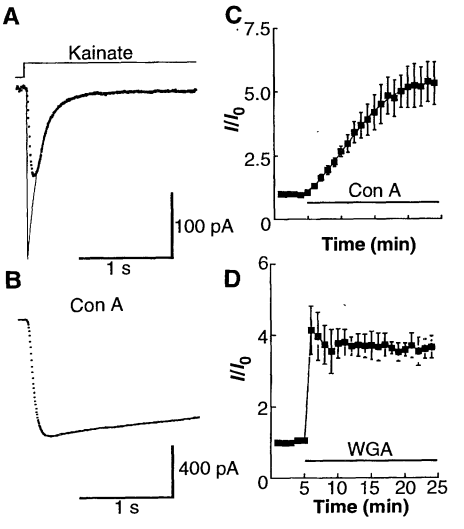

Fig. 1. Ionic currents were recorded from cells and treated with the lectins WGA or Con A. (**A**) Applications of kainate-evoked, whole-cell currents that rose rapidly to a peak and then declined to a steady-state plateau in the continuous presence of the agonist. The average of six consecutive responses to applications of kainate (10 μM) is shown. Averaging greatly increased the signal-to-noise ratio of the response. The holding potential in this and subsequent figures was −60 mV, except where otherwise indicated. Desensitization of GluR6 currents was well characterized by a single exponential function $I(t) = I_p\, e^{(-t/\tau)} + I_{SS}$, where $I(t)$ represents current amplitude at time t and I_p and I_{SS} are the estimated peak and steady-state current amplitudes, respectively. The continuous line shows a typical exponential fitting of this function to the data (τ = 150 ms). (**B**) After exposure to Con A (10 μg/ml) for 20 min, the peak current evoked by kainate was potentiated and desensitization was almost abolished. The data represent the average of six consecutive responses. Note the difference in calibration bars. (**C** and **D**) WGA (10 μg/ml) also enhanced peak currents and eliminated desensitization, but the kinetics of its actions were much faster than those for Con A. The amplitude (I) at any given time was normalized to the amplitude of the first response (I_0), and the ratio was plotted against time after application of the lectins. Each point on the graphs represents the mean response of three cells at the given point in time.

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To determine if this potentiation could be the result of a change in the reversal potential of the response (that is, a change in ionic permeability), we used voltage ramps to generate current-voltage curves both before and during cPKA perfusions. An outward rectification of this relation was observed (Fig. 2D) that was similar to that recorded when GluR6 was expressed in oocytes (*7*). Enhancement by cPKA of currents induced by kainate was not associated with any alteration in either reversal potential or rectification, although the slope of the current-voltage curve was increased, which demonstrates that cPKA had induced an increase in the channel conductance of GluR6 (Fig. 2D).

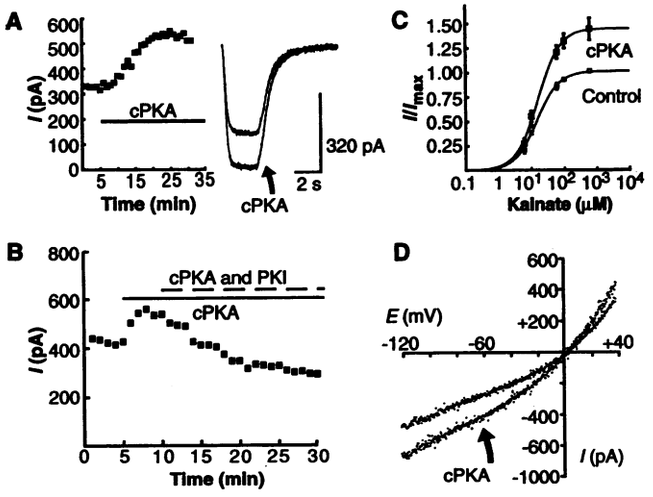

Fig. 2. The effect of cPKA and PKI on kainate-induced currents in transfected HEK-293 cells that expressed GluR6. (**A**) Direct perfusion of cPKA (100 U/ml) into these cells caused a sustained potentiation of currents evoked by repetitive applications of kainate (10 μM). Example currents before (upper) and after (lower) the injection of cPKA are shown to the right of the graph. Each trace is an average of five consecutive responses. (**B**) After the establishment of the cPKA-induced potentiation, subsequent injection of PKI (2 mg/ml) together with cPKA reversed the potentiation and reduced currents evoked by kainate to less than those in the absence of cPKA (Fig. 3B). Bars (solid and dashed) in (A) and (B) indicate the time of internal perfusion of cPKA and PKI. (**C**) Dose-response curves (four cells) constructed before and after cPKA injection were well fit with the logistic equation in the form $I = I_{max}/[1 + (D/EC_{50})^n]$, where I represents the calculated current amplitude at any given kainate concentration (D), I_{max} is the response to a saturating concentration of agonist, EC_{50} is the concentration of agonist that produces 50% of the maximal response, and n is the estimated Hill coefficient. Paired dose-response curves recorded in individual cells were normalized to the maximal response I_{max} observed before injection of cPKA. The application of cPKA increased I_{max} 144.5% ± 11.6% with no effect on the values for n (1.67 ± 0.41, control; 1.41 ± 0.11, cPKA) or for EC_{50} (1.41 ± 0.22, control; 1.63 ± 0.29, cPKA). Each data point is the mean ± SE of four different observations. (**D**) Comparison of current-voltage (*I-V*) curves before and after cPKA perfusion. These *I-V* curves were produced by means of 2-s voltage ramps ranging from −120 to +40 mV. Leak currents were measured in the absence of agonist and were subsequently substracted from those acquired in the presence of kainate (10 μM). Similar results were observed in three other cells. *E*, voltage.

We constructed three mutants to determine whether there was direct phosphorylation of a specific site or sites on the GluR6 subunit by PKA. Initially, Ser^{684} was mutated to Ala (S684A); this single-site mutation decreased the degree of potentiation after intracellular perfusion of cPKA but did not prevent the potentiation altogether (Fig. 3, A and B). Therefore, a second Ser (Phe-Lys-Lys-Ser^{666}-Lys), located within the same putative intracellular loop as Ser^{684}, was also mutated to provide a double mutant (S684A, S666A). Currents evoked by kainate from cells that expressed the double mutant were not enhanced by cPKA (Fig. 3, A and B). A third mutant was then tested with a single mutation at Ser^{666} (S666A). This mutation had no effect on the potentiation produced by infusion of cPKA (Fig. 3B). None of the mutant GluR6 subunits had desensitization kinetics different from that of the wild-type receptor, and all were similarly influenced by Con A and WGA.

These observations indicate that cPKA can phosphorylate GluR6 subunits and modulate channel function. The simplest explanation of our data is that phosphorylation increases the number of active receptors or effectively enhances the gating properties of the channels (that is, increases the probability or frequency of channel opening or both). We have observed a significant enhancement in the frequency of channel opening when cPKA was applied to the cytostolic face of the membrane (*16*).

Native glutamate receptors likely consist of heteromeric combinations of various subunits (*17, 18*). Many of these heteromeric glutamate receptors differ from their homomeric counterparts with respect to agonist affinity, rates of desensitization, and ionic permeability. For instance, co-expression of GluR6 with the kainate receptor subunit KA2 results in a heteromeric channel that is sensitive to AMPA (α-amino-3-hydroxy-5-methyl-4-isoazole propionic acid) even though neither subunit is sensitive on its own (*19*). Our results suggest that the GluR6 subunit may endow native kainate receptor complexes that contain this subunit with the property of functional regulation by PKA. The function of PKA in the modulation of excitatory amino acid transmission as demonstrated by us and by others (*20–22*) is underscored by the suggestion that the dynamic regulation of these receptors by protein kinases is a plausible mechanism

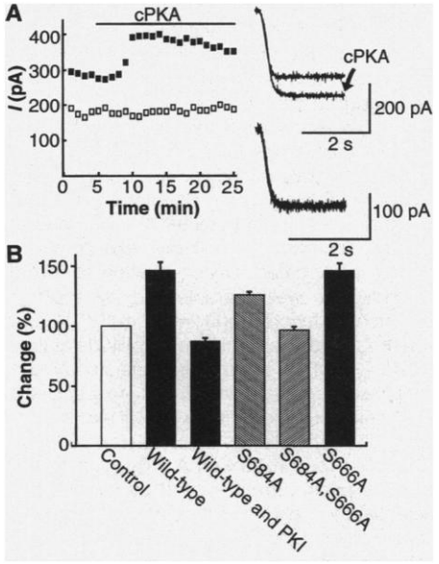

Fig. 3. The effect of mutation of Ser^{684} and Ser^{666} in GluR6 on the potentiation of currents induced by kainate after applications of cPKA. (**A**) The single mutant S684A (filled squares) was potentiated by cPKA, but the double mutant S684A, S666A was not (open squares). The current traces displayed on the right panel of the graph show superimposed responses of the mutant S684A (upper) and the double mutant S666, S684A (lower) before and after the injection of cPKA. (**B**) To summarize the effects of cPKA on the wild-type and mutant GluR6 subunits, we compared an average of five consecutive responses to kainate (10 μM) before and after perfusion of cPKA. Each cell provided its own control (100%). The percentage potentiation for each cell in a group was then pooled and expressed as a mean ± SE. Perfusion of cPKA potentiated the wild-type GluR6 currents by 46.7% ± 7.0% (n = 9), whereas co-applications of cPKA and PKI eliminated this potentiation and reduced currents to values below those for controls (87.1% ± 2.3%, n = 3). The potentiation of the mutant S684A was less than that of the wild-type (26.1% ± 2.5%, n = 9, $P < 0.01$, one-tailed Mann-Whitney U test), whereas no significant potentiation of the double mutant S684A, S666A was detected (96.5% ± 3.2%, n = 5). The single-site mutation S666A was potentiated to the same degree as the wild-type (48.0% ± 6.1%, n = 6).

by which neurons could encode at the molecular level synaptic events associated with learning and memory (*23*).

Note added in proof: It has come to our attention that results very similar to those reported here have recently been found (*24*).

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11. Patch-clamp recordings (Axopatch-1B, Axon, Foster City, CA) were made from monolayer cultures of HEK-293 cells 2 to 3 days after transfection with GluR6 or GluR6 mutants. The intracellular solution contained 140 mM CsCl or KCl, 35 mM CsOH or KOH, 10 mM Hepes, 11 mM EGTA, 1 mM $CaCl_2$, 2 mM $MgCl_2$ and 2 mM tetraethyl ammonium, and 4 mM adenosine triphosphate (ATP) (pH 7.3, 320 to 335 mosM). ATP was added to maintain currents evoked by kainate. This solution was supplemented with cPKA or PKI (Promega and Sigma, respectively), or both. Cells were treated with Con A or WGA (10 μg/ml).
12. Mutations were made with the Mutagene mutagenesis kit from Bio-Rad. Briefly, a Bam HI [nucleotide (nt) 1522]–Bam HI (nt 2478) fragment of GluR6 was subcloned into pBluescript KS+ (Stratagene). The following mutations were made: the oligonucleotide GGAGACAGGCCGTGCTTGT was used to convert Ser^{684} to Ala, and TTAAGAAAGCAAAAATT was used to convert Ser^{666} to Ala. A double mutant (S684A, S666A) was constructed with both oligonucleotides. The mutations were confirmed by restriction analysis and sequencing (Sequenase 2 0 kit, U S. Biochemical, Cleveland, OH) An Nde I (nt 1739)–Bam HI (nt 2478) fragment of the mutated DNA was ligated back in to GluR6 in pBluescript KS+, and the entire mutated GluR6 was subcloned into the Xho I–Not I sites of the pCis expression vector for transfections in HEK-293 cells. Transient transfections were done with a modification of the calcium phosphate method (*25*).
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