replaced with paraformaldehyde (4%) in isotonic phosphate-buffered saline (PBS) (pH 7.4). After fixation in situ, the carotids were isolated and dissected free of adherent fat and connective tissue. Mid-carotid segments were embedded in paraffin for sectioning.
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12. Injections (six) of purified human PDGF (75 μg) were administered to a goat subcutaneously every 2 weeks for 3 months. The initial injection was in complete Freund's adjuvant with subsequent injections in incomplete Freund's adjuvant. Once a titer was established, plasma was collected by plasmapheresis every week, with periodic boosts of PDGF (300 μg) in incomplete Freund's. The IgG fraction was prepared by sodium sulfate precipitation and DEAE-Sephacel chromatography. The 18% sodium sulfate precipitate was resolubilized and dialyzed to remove excess sodium sulfate, and the dialysate was further purified by passage over a DEAE-Sephacel (Pharmacia, Piscataway, NJ) column. Purified IgG was obtained by elution with 0.01M phosphate buffer (pH 6.8). The pooled void volume and pH 6.8 wash were concentrated by ultrafiltration (PM-10, Amicon Corp., Danvers, MA) and dialyzed against PBS to a protein concentration of 60 to 100 mg/ml as determined by the method of O. H. Lowry, N. J. Rosebrough, A. L. Farr, and R. J. Randall [*J. Biol. Chem.* **193**, 265 (1951)], with bovine serum albumin (BSA) as a standard and after application of a correction factor for immunoglobulin [J. A. Klosse, P. K. De Bree, S. K. Wadman, *Clin. Chim. Acta* **32**, 321 (1971)]. Before use, the IgG was filter-sterilized through a 0.22-μm filter (Millipore, Bedford, MA) and stored at 4°C. A nonimmune goat IgG was prepared by the same method with commercially available goat plasma.
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17. Microtiter plates (96-well, Nunc, Denmark) were coated with PDGF-AB (10 ng per well) for 18 hours at 4°C. Nonspecific protein binding was reduced by blocking with BSA (1%) in Tween 20 (0.2%) and PBS for 1 hour at 37°C. Dilutions of standards and samples were prepared in a 1:16 mixture of rat plasma–derived serum and PTB (0.05% Tween 20 and 0.2% BSA in PBS). Aliquots (100 μl) of sample or standards of known concentrations of anti-PDGF were incubated in wells for 90 min at 37°C. The plates were rinsed five times in wash buffer (0.5% Tween 20, 0.9% saline) and then incubated with 1:1000 biotinylated swine antibody to goat IgG (100 μl per well) (Caltag Laboratories, South San Francisco, CA) in PTB for 60 min at 37°C. Unbound secondary antibody was removed by rinsing in wash buffer and subsequently incubated with avidin and biotinylated peroxidase (Vector Labs, Burlingame, CA) for 30 min at 37°C. After washing, substrate (0.04% *o*-phenylenediamine, Sigma) was dissolved in a mixture of 0.05M citrate and 0.1M Na_2PO_4 (pH 5.0) that contained hydrogen peroxide (10 μl of a 30% solution per 25 ml); 100 μl was added to each well and incubated for 10 min at room temperature. The reaction was terminated by the addition of sulfuric acid (4N), and the absorbance of the reaction product was read at 490 nm.
18. Sections of carotid were deparaffinized and dipped in NTB-2 photograph emulsion (Kodak, Rochester, NY). The autoradiographs were exposed for 14 days at 4°C in a light-tight box and were then developed with Kodak D-19 developer and fixed with Kodak Rapid Fix. Cell nuclei were stained with hematoxylin. Cells with more than five silver grains above their nuclei were considered positive. We examined duplicate sections from two levels of each carotid under oil immersion, using a Zeiss photomicroscope with a ×63 objective (Carl Zeiss Inc., West Germany). Approximately 600 cells per well compartment were counted to determine the proportion of labeled cells.
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28. We acknowledge the help and collaboration of M. Clowes and A. Clowes in a series of early experiments with normal rats that led to the experiments in this paper. We also acknowledge the technical assistance of A. Eng, E. Olson, K. Engel, B. Ashleman, L.-C. Huang, and S. Chao. Supported by NIH grants HL-18645 (R.R.) and HL-03174 (M.A.R.), the American Heart Association, and the British Heart Foundation (G.A.A.F.). The William Harvey Research Institute is funded by a grant from Ono Pharmaceutical Company Ltd., Chuoku, Japan.

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Regulation of Kainate Receptors by cAMP-Dependent Protein Kinase and Phosphatases

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In the mammalian central nervous system, receptors for excitatory amino acid neurotransmitters such as the α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA)–kainate receptor mediate a large fraction of excitatory transmission. Currents induced by activation of the AMPA-kainate receptor were potentiated by agents that specifically stimulate adenosine 3′,5′-monophosphate (cAMP)–dependent protein kinase A (PKA) activity or were supported by intracellular application of the catalytic subunit of PKA by itself or in combination with cAMP. Furthermore, depression of these currents by a competitive inhibitor of PKA indicates that AMPA-kainate receptors are regulated by endogenous PKA. Endogenous protein phosphatases also regulate these receptors because an inhibitor of cellular phosphatases enhanced kainate currents. Modulation of PKA and phosphatases may regulate the function of these receptors and thus contribute to synaptic plasticity in hippocampal neurons.

EXCITATORY AMINO ACID RECEPTORS, which contain ion channels, can be broadly classified as *N*-methyl-D-aspartate (NMDA) or non-NMDA receptors on the basis of selective pharmacological antagonism (*1*). Non-NMDA receptors have been further subdivided into AMPA and kainate subtypes although it has been difficult to find a specific antagonist for either subtype. Kainate currents do not desensitize, whereas AMPA currents desensitize rapidly (*2*). However, desensitization is not necessarily indicative of distinct receptor subtypes (*3*).

We have investigated the regulation of AMPA-kainate receptors by protein kinases (*4*) and phosphatases in mammalian neurons (*5*). We used kainate (Fig. 1) to avoid the desensitization that occurs with AMPA (*2, 3*). The amplitude of kainate currents declined exponentially with a time constant of 7 min when electrode solutions without adenosine triphosphate (ATP) were used (Fig. 1, A and C). This "washout" (*6*) of kainate currents was similar to that reported for NMDA currents (7), but it had a slower time course. Introduction of an ATP-regenerating solution (*8*) into the neurons by means of the recording electrode significantly reduced the washout of kainate currents (Fig. 1, B and C). In contrast, when we avoided intracellular dialysis by recording whole-cell currents with the nystatin patch technique (*9*), kainate currents were stable for periods of up to 2 hours after the initial period of patch perforation (Fig. 1D). Such evidence suggests that the magnitude of kainate currents is determined at least in part by the ability of the neuron to maintain intracellular phosphorylation.

In order to examine a possible role of PKA in the regulation of AMPA-kainate receptors, we quantified the effects of cAMP and the catalytic subunit of PKA (Sigma) on the washout of kainate currents. This washout was significantly retarded when the catalytic subunit of PKA (20 μg/ml) was included in the recording pipette. Intracellular applications of cAMP (40 μM) were associated with a transient potentiation of kainate currents. In contrast, cAMP did not enhance NMDA currents (Fig. 1E). A combination of ATP, cAMP, and PKA was the most efficacious in retarding or preventing washout of kainate currents.

The recording electrode was internally perfused with various solutions (*10*). The patch pipette initially contained a control

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recording solution that permitted washout of the kainate currents (Fig. 2, A and B), and washout continued when the patch electrode was perfused with this control solution (Fig. 2A). In contrast, perfusion with a solution containing cAMP and the catalytic subunit of PKA at the concentrations given above caused a partial recovery of the kainate currents. Reperfusion with the control solution was then associated with washout of the currents (Fig. 2B). In other neurons, the partial recovery was maintained as long as the cell was perfused with the cAMP and PKA solution. These results indicate that AMPA-kainate currents are reversibly modulated by PKA.

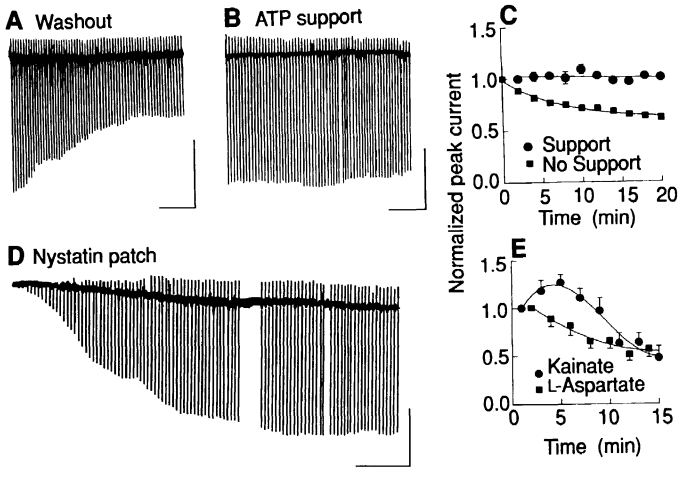

Fig. 1. Stability of peak kainate currents in cultured hippocampal neurons under different patch-clamp configurations and in different intracellular dialysis solutions. (**A**) A typical whole-cell recording of inward kainate currents generated by 15-ms pressure applications of kainate (250 μM, 130 kPa) to the soma. These applications were repeated once every 30 s and commenced immediately after the patch was ruptured. Kainate currents characteristically washed out to approximately 55% of their initial amplitude over the initial 30 min of dialysis (see E). (**B**) Kainate currents in the presence of an ATP-regenerating system (4 mM tris-ATP, 20 mM phosphocreatine, and 50 U/ml creatine phosphokinase) in the patch electrode. (**C**) Results from (A) and (B) illustrated graphically. Mean data (±SEM) from a series of recordings with electrodes with or without the ATP-regenerating system. Each group consisted of 50 neurons. (**D**) A cell-attached patch was initially formed in the absence of nystatin. Records of kainate currents gradually increased in amplitude as nystatin entered the cell membrane and the perforation of the patch formed. Once stable (usually within 30 min), kainate currents remained unaltered for periods of up to 2 hours. In (A), (B), and (D), the transient capacitive currents seen above baseline resulted from hyperpolarizing voltage steps from −60 to −70 mV (duration, 100 ms) just before each application of kainate. We used these steps to monitor capacitance and leak conductance during the development of patch perforation. The holding potential in this and subsequent figures was −60 mV, except where otherwise indicated. (**E**) Supplementing the dialysis solution with cAMP (40 μM) alone did not prevent the washout of kainate currents. However, the responses were transiently potentiated over the first 10 min after rupture of the patch. The washout of NMDA currents that we recorded concurrently from these neurons by applying L-aspartate (250 μM, 15 ms, 130 kPa) was not influenced by the presence of cAMP (n = 4). Calibration bars represent 7 min in all cases; 250 pA for (A) and (D); 750 pA for (B).

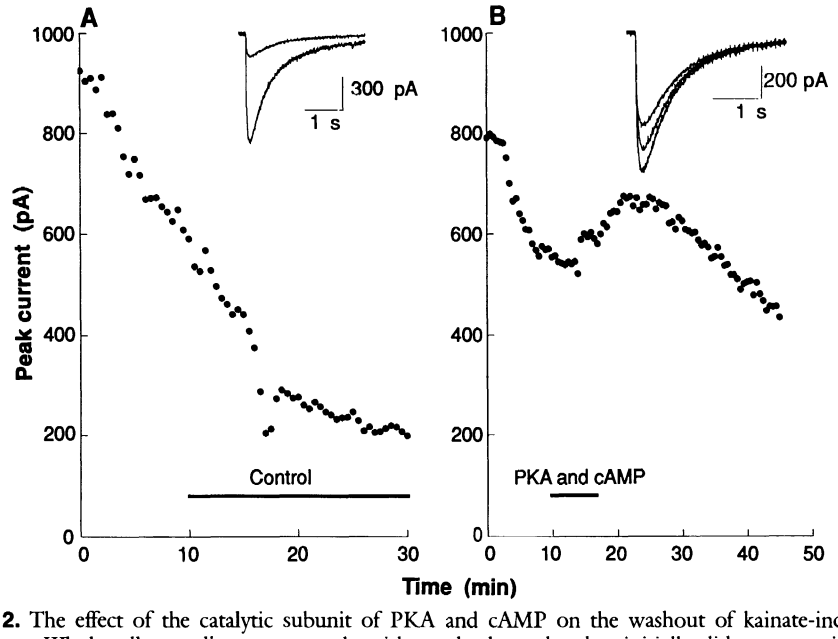

Fig. 2. The effect of the catalytic subunit of PKA and cAMP on the washout of kainate-induced currents. Whole-cell recordings were made with patch electrodes that initially did not contain the ATP-regenerating solution. The recording electrode was perfused during the periods indicated by the horizontal bars. (**A**) We perfused the internal pipette with the same solution used to fill the patch pipette, and this perfusion did not retard washout of kainate currents. A similar result was seen in another two neurons (n = 3). (Inset) The current traces represent responses at time 0 and 30 min. (**B**) The patch pipette was perfused with the PKA and cAMP solution for 6 min, and the kainate currents increased in amplitude. Subsequently, the amplitude of the currents decreased when the pipette was reperfused with the control solution. In other neurons, the recovery was maintained with continued internal perfusion of the electrode with PKA and cAMP (n = 5). (Inset) The current traces represent recordings at time 0, 25, and 45 min.

In order to determine whether endogenous PKA can modulate AMPA-kainate–regulated currents, we used the nystatin perforated patch technique, together with bath application of two membrane permeant analogs of cAMP, Rp- and Sp-cAMPS. These compounds are more membrane permeable than other modulators, such as 8-bromo-cAMP and have much higher specificity and affinity for PKA than does forskolin (*11*). The Rp-cAMPS analog is a particularly potent competitive inhibitor of PKA, whereas Sp-cAMPS is a potent activator. Both are more resistant to phosphodiesterases than other cAMP analogs.

Application of Sp-cAMPS in the bath potentiated kainate currents (Fig. 3A), whereas the inhibitor Rp-cAMPS caused a large depression of kainate currents (Fig. 3B). The inhibitor reversed the potentiation caused by Sp-cAMPS and often decreased the currents below baseline values (Fig. 3C). Application of Rp-cAMPS alone prevented any subsequent potentiation by Sp-cAMPS. Thus, endogenous PKA must be actively modulating kainate receptors in cultured hippocampal neurons. This phosphorylation by PKA might be counterbalanced by dephosphorylation by a protein phosphatase.

We examined this possibility by using okadaic acid, a potent inhibitor of protein phosphatases 1 and 2A (*12*). Bath applications of okadaic acid (250 nM to 1 μM) potentiated kainate currents recorded with the nystatin patch technique (Fig. 4). Okadaic acid had no effect on holding currents. The increase in kainate currents resulted from a significant increase in conductance.

Our results demonstrate that kainate currents are modulated by the activity of PKA and by a protein phosphatase, likely type 1 or 2A. Thus, AMPA-kainate receptors might be directly phosphorylated and dephosphorylated by PKA and phosphatases, respectively. Alternatively, regulation of the receptors by phosphorylation and dephosphorylation could be indirect by means of an intermediate regulatory protein associated with the channels.

A single subtype of receptor may mediate responses to kainate and AMPA because glutamate receptors composed of homomeric subunits express high-affinity AMPA binding and functional responses to both

amino acids. The various subunits of the cloned AMPA-kainate receptors have consensus sequences indicative of at least three phosphorylation sites, but none of these is an ideal consensus sequence for PKA (*3*). Either the receptors we have examined are not identical to cloned AMPA-kainate receptors or PKA can phosphorylate these receptors in spite of a lack of an ideal consensus sequence.

The effect of PKA on kainate currents could result from a recruitment of additional receptors or by the activation of a different type of receptor (*13*). Alternatively, PKA may increase the frequency of channel opening, as has been reported for glycine channels in trigeminal neurons (*14*).

The AMPA-kainate receptor is thought to mediate the fast components of the excitatory postsynaptic potential in hippocampal neurons (*15*). Our results suggest that excitatory transmission could be modulated by a variety of receptors that are coupled to adenylate cyclase and to activation of PKA. An important means of modulating this transmission would be through activation of biochemical pathways capable of affecting protein phosphatase activity. For instance, persistent protein kinase activity is associated with the early postsynaptic events leading to induction of long-term potentiation in the hippocampus (*16*). Regulation of AMPA-kainate receptors by such activity may thus contribute to some aspects of postsynaptic plasticity.

Note added in proof: Egebjerg *et al.* (*17*) have cloned glutamate receptor subunits (GluR6) that respond to kainate but not to AMPA.

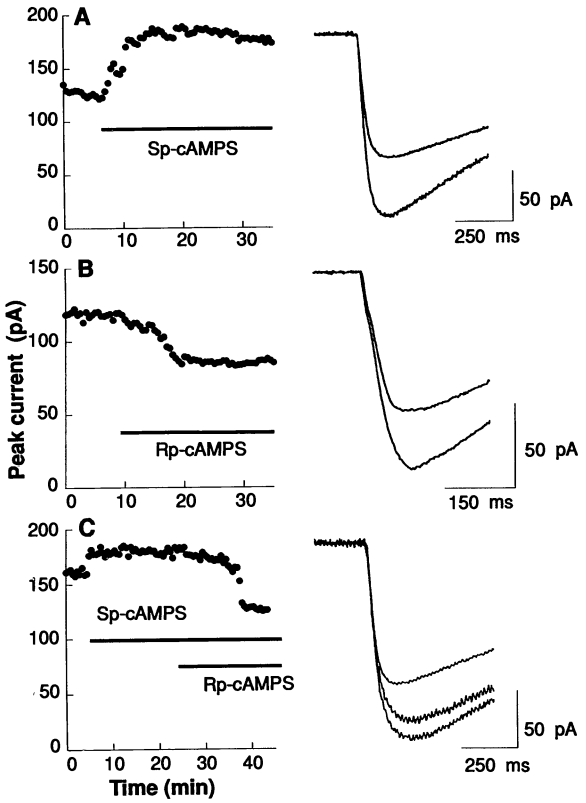

Fig. 3. Kainate currents recorded in perforated patches were modulated by bath applications of Sp-cAMPS and Rp-cAMPS (100 μM, final concentrations) during the periods indicated by the solid bars. Sp-cAMPS consistently potentiated kainate currents (n = 11, range +15 to +80%, mean increase +44 ± 22%, SD). An example of this effect is plotted in (**A**) and example currents before and after treatment with Sp-cAMPS are shown to the right of the graph. (**B**) Applications of Rp-cAMPS (the inhibitor of PKA) substantially depressed kainate currents (n = 3, inhibition to a mean of 58.7 ± 7.1%). Example currents before and after treatment with Rp-cAMPS are shown to the right of the graph. (**C**) Rp-cAMPS reversed the potentiation caused by application of Sp-cAMPS and further depressed the kainate currents below the control level (n = 7, inhibition to 86.1 ± 8.6% of the baseline values). In addition, application of Rp-cAMPS before Sp-cAMPS application prevented any subsequent potentiation by the activator (n = 3; not shown). Example currents before (middle), and after treatment with Sp-cAMPS (lower) and Rp-cAMPS (upper) are shown to the right of the graph. Each current trace is an average of five consecutive responses.

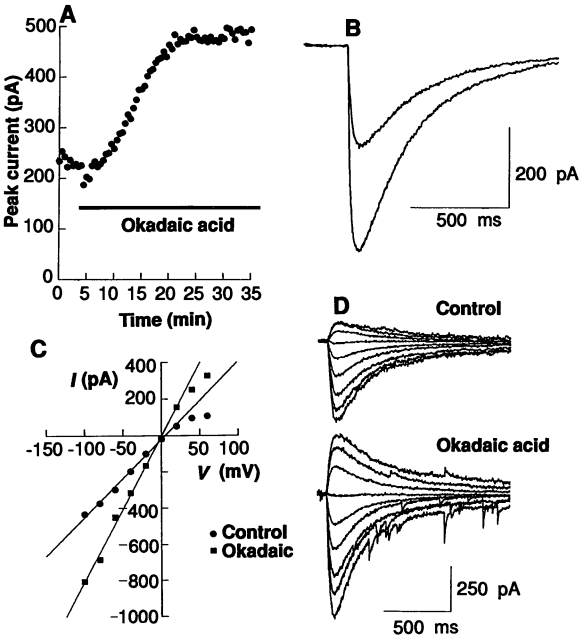

Fig. 4. The effect of the phosphatase inhibitor okadaic acid on kainate currents recorded with the perforated patch technique. (**A**) An example of the increase in amplitude of kainate currents induced by okadaic acid (0.5 μM). Bar indicates the period of application of okadaic acid. (**B**) Actual currents before and after treatment with okadaic acid. (**C**) The reversal potential of kainate currents determined before and after application of okadaic acid (0.5 μM). The holding potential was varied from −100 mV to +60 mV. In the illustrated recording, the conductance to kainate was increased from 4.3 to 8.0 nS, as determined from the slope of the current (I)-voltage (V) relation. (**D**) Individual kainate currents we used to construct the plot in (C). The steady-state current associated with each holding potential was subtracted. Okadaic acid potentiated kainate currents in each of the nine recordings examined and was added to the bath to give final concentrations of 0.25 (n = 1, +28%), 0.50 (n = 5, +58 ± 14%), or 1.0 μM (n = 3, +56 ± 27%).

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4. Regulaton of kainate currents in white perch horizontal neurons by PKA has been reported [A. G. Knapp and J. E. Dowling, *Nature* **325**, 437 (1987)].
5. Hippocampal neurons from fetal mice were grown as monolayer cultures with conventional techniques. Neurons were used after 7 to 14 days in culture. Patch-clamp recordings (Axopatch-1B, Axon Instruments, Foster City, CA) were made with electrodes filled with 140 mM CsCl, 35 mM CsOH, 10 mM Hepes, 11 mM EGTA, 1 mM $CaCl_2$, 2 mM $MgCl_2$, and 2 mM tetraethylammonium (pH 7.3), 320 to 335 mos$M$. This solution was supplemented with various components indicated in the text. Cultures were bathed in an extracellular solution containing 140 mM NaCl, 1.3 mM $CaCl_2$, 5.4 mM KCl, 25 mM Hepes, and 33 mM glucose (pH 7.4), 320 to 335 mos$M$, and 1 μM tetrodotoxin. In experiments where NMDA currents were examined, glycine (1 μM) was included. We elevated extracellular Ca^{2+} concentrations to 13 mM for nystatin patch recordings to accentuate calcium currents. We lowered the concentration of glucose to maintain the osmolarity of the solutions. Amino acids were applied with a Picospritzer (General Valve, East Hanover, NJ), and the drug pipette was placed 20 to 50 μm from the soma.
6. In our experiments, the soma is well dialyzed by the whole-cell pipette, in comparison to the processes. Ca^{2+} currents in the soma rapidly washed out during such recordings, but Ca^{2+} action potentials could still be triggered at remote sites. This suggests that the processes of a neuron likely act as a source for ATP or that Ca^{2+} channels in the processes are resistant to depletion of ATP.
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cell-attached patch recording is made with an electrode containing nystatin. The nystatin pores permit electrical continuity with the cell but do not allow exchange of larger substances (for example, ATP) between the pipette and the cell. We monitored calcium currents to ensure that the nystatin patch did not rupture during the recording.

10. A glass pipette (outer diameter, 0.75 mm) was inserted into the tip of the patch electrode (perfusion rate, 0.01 to 0.02 ml/min).
11. Adenosine 3',5'-monophosphothioate (cAMPS) is an analog of cAMP that includes a sulfur in the phosphate moiety, and the phosphate can exist in one of two (*R* and *S*) diastereomeric forms (Sp-cAMPS and Rp-cAMPS; BioLog Life Science Institute, Bremen, Federal Republic of Germany). The Sp isomer is an agonist of cAMP-dependent protein kinases type I and II, and the Rp isomer is a specific inhibitor [T. Braumann and B. Jastorff, *J. Chromatog.* **350**, 105 (1985); B. Yusta *et al.*, *J. Neurochem.* **51**, 1808 (1988)].
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Enhancement of the Glutamate Response by cAMP-Dependent Protein Kinase in Hippocampal Neurons

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Receptor channels activated by glutamate, an excitatory neurotransmitter in the mammalian brain, are involved in processes such as long-term potentiation and excitotoxicity. Studies of glutamate receptor channels expressed in cultured hippocampal pyramidal neurons reveal that these channels are subject to neuromodulatory regulation through the adenylate cyclase cascade. The whole-cell current response to glutamate and kainate [a non-NMDA (*N*-methyl-D-aspartate) receptor agonist] was enhanced by forskolin, an activator of adenylate cyclase. Single-channel analysis revealed that an adenosine 3',5'-monophosphate–dependent protein kinase (PKA) increases the opening frequency and the mean open time of the non-NMDA–type glutamate receptor channels. Analysis of synaptic events indicated that forskolin, acting through PKA, increased the amplitude and decay time of spontaneous excitatory postsynaptic currents.

BRAIN SECOND-MESSENGER SYSTEMS often alter nerve cell activity by modifying characteristics of voltage-gated channels (*1–3*). Although the regulation of neuronal excitability by phosphorylation of voltage-gated channels is widespread, the neuromodulation of ligand-gated channels, crucial for the control of synaptic strength, has been reported in only a few instances (*4–6*). Because the modification of synaptic strength through the action of second-messenger systems should be an effective way to regulate the computational capabilities of neuronal circuits, this form of neuromodulation could be as common as the ones that affect electrical excitability. We anticipated that the hippocampus might be a favorable site to search for synaptic neuromodulation; this brain region is well known to regulate synaptic strength by long-term potentiation (*7*), and the abundantly represented glutamate receptor channels in hippocampus have been implicated in memory, epilepsy, excitotoxicity, and some neurodegenerative diseases (*8*).

We used both the whole-cell recording and the outside-out mode of the standard patch-clamp method (*9*) to study responses to glutamate in 2- to 7-day-old cultured hippocampal pyramidal neurons from newborn Long Evans rats (*10*). We included 5 μM magnesium adenosine trisphosphate (MgATP) in all the whole-cell recordings to prevent "run-down" of glutamate-induced responses (*11*).

Because adenylate cyclase is commonly involved in neuromodulatory systems, we examined responses to glutamate in whole-cell recordings before and after treatment with forskolin (FSK), an activator of adenylate cyclase (*12*). In the continual presence of 50 μM FSK, glutamate responses increased in all cells tested (+91% ± 27% at 15 min after glutamate application; $n = 4$; mean ± SEM) (Fig. 1). For comparison, we recorded the responses in another set of cells not treated with FSK. The responses to glutamate in this control group of cells generally decreased over the same time interval (−30% ± 10% at 15 min after glutamate application; $n = 4$) (Fig. 1B). Thus, FSK produces a marked neuromodulation of glutamate receptors.

FSK can sometimes interact directly with ion channels without the intermediary action of the adenosine 3',5'-monophosphate (cAMP) cascade (*13, 14*), and a similar direct mechanism might conceivably account for the FSK effect on glutamate receptor channels. In an effort to determine if FSK acts directly, we treated our cells with 1,9-dideoxyforskolin, a structural analog. Dideoxyforskolin does not stimulate adenylate cyclase but does mimic other actions of FSK. Of the three cells treated with 50 μM dideoxyforskolin, none showed an increased response to glutamate (−23% ± 10%; $n = 3$) (Fig. 1B).

If the increased glutamate response involves the cAMP-dependent protein kinase (PKA), then blocking kinase activity should prevent the neuromodulatory effect of FSK. To test this, we added IP_{20}-amide, a 20-amino acid synthetic peptide that specifically inhibits PKA (*15*), to the solution in the recording pipette. IP_{20}-amide (1 μM) in the electrode blocked the neuronal response to FSK (−28% ± 5%; $n = 6$) (Fig. 1B). In addition, with IP_{20}-amide alone in the recording pipette (and no FSK) the response to glutamate became smaller than the control within 20 min (−50% ± 9%; $n = 4$) (Fig. 1B), suggesting a basal PKA activity.

The increased macroscopic response to glutamate after FSK treatment could have resulted from changes in various biophysical properties of the glutamate channels, such as a more frequent entry into the open state, a lengthened stay in the open state, or an increased unitary conductance. Furthermore, glutamate and its structural analogs activate a mixture of receptor subtypes. *N*-methyl-D-aspartate (NMDA) preferentially activates large conductance (40 to 50 pS) channels, whereas non-NMDA channels—kainate, quisqualate, and α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid—preferentially activate small conductance (typically less than 30 pS) channels (*16*). The non-NMDA channels are difficult to distinguish from each other but are distinct from the NMDA class (*16, 17*). Changes in either or both types of channels could have led to an increased whole-cell current response to glutamate.

To determine the basis for the increased

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