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β-Adrenergic Regulation of Synaptic NMDA Receptors by cAMP-Dependent Protein Kinase

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Summary

To identify the protein kinases regulating synaptic NMDA receptors, as well as the conditions favoring enhancement of NMDA receptor–mediated excitatory postsynaptic currents (EPSCs) by phosphorylation, we studied the effects of kinase activation and inhibition in hippocampal neurons. Inhibition of cAMP-dependent protein kinase (PKA) prevented recovery of NMDA receptors from calcineurin-mediated dephosphorylation induced by synaptic activity, suggesting that tonically active PKA phosphorylates receptors during quiescent periods. Conversely, elevation of PKA activity by forskolin, cAMP analogs, or the β-adrenergic receptor agonists norepinephrine and isoproterenol overcame the ability of calcineurin to depress the amplitude of NMDA EPSCs. Thus, stimulation of β-adrenergic receptors during excitatory synaptic transmission can increase charge transfer and Ca^{2+} influx through NMDA receptors.

Introduction

N-methyl-D-aspartate (NMDA) receptors are implicated in a variety of synaptic and cellular changes, including development, plasticity, and excitotoxicity. These processes are shaped by the rise in intracellular Ca^{2+} resulting from Ca^{2+} influx through NMDA receptors (e.g., Choi and Rothman, 1990; Perkel et al., 1993; Linden, 1994; Scheetz and Constantine-Paton, 1994). Consequently, augmentation or diminution of currents through NMDA receptors is likely to influence these intracellular events. NMDA receptors seem particularly susceptible to modulation, as they contain multiple consensus sites for phosphorylation by several protein kinases (Hollmann and Heinemann, 1994). Protein kinase C (PKC) and tyrosine kinases have been shown to phosphorylate NMDA receptor subunits directly (Tingley et al., 1993; Moon et al., 1994; Lau and Huganir, 1995). Moreover, kinase activation or phosphatase inhibition can increase NMDA receptor–mediated currents measured in membrane patches and whole cells (Chen and Huang, 1991, 1992; Lieberman and Mody, 1994; Tong and Jahr, 1994; Wang et al., 1994; Wang and Salter, 1994).

NMDA receptor–mediated Ca^{2+} flux also triggers processes that reduce currents through NMDA receptors (Vyklicky et al., 1990; Legendre et al., 1993), such as Ca^{2+}-dependent depolymerization of actin filaments (Rosenmund and Westbrook, 1993) and activation of the Ca^{2+}-dependent phosphatase calcineurin (Lieberman and Mody, 1994; Tong and Jahr, 1994). As shown by Tong et al. (1995) in cultured hippocampal neurons, activation of calcineurin also occurs during synaptic stimulation, thereby diminishing the amplitude of subsequent NMDA receptor–mediated excitatory postsynaptic currents (EPSCs). Thus, the flux through NMDA receptors during synaptic activity provides negative feedback that limits the rise in intracellular Ca^{2+}.

The present experiments investigated the possibility that the autoregulation of NMDA receptor conductance might itself be subject to modulation; i.e., given that the decrement in NMDA EPSCs is phosphatase dependent, conditions that elevate the activity of particular kinases, such as the stimulation of second messenger–coupled receptors, might shift an equilibrium toward phosphorylation of NMDA receptors or associated proteins. Such a change might enable more Ca^{2+} to flow through the channels during periods of high synaptic activity.

Initial experiments were directed toward identification of the kinase or kinases that might complement the role of calcineurin in regulating NMDA receptors. Since calcineurin and cAMP-dependent protein kinase (PKA) are colocalized at synaptic densities by anchoring proteins (Coghlan et al., 1995), PKA is a likely candidate for modulation of synaptic receptors, as shown for hippocampal α-amino-3-hydroxy-5-methyl-4-isoxazolepropionate (AMPA)/kainate receptors (Liman et al., 1989; Greengard et al., 1991; Wang et al., 1991; Rosenmund et al., 1995). The present experiments provide evidence that, in the absence of synaptic activity, NMDA receptors are phosphorylated by basally active PKA, thus enhancing NMDA EPSCs after periods of relative quiescence. During excitatory transmission, Ca^{2+} influx stimulates calcineurin, leading to a reduction of NMDA receptor currents. However, β-adrenergic receptor stimulation by the neurotransmitter norepinephrine can overcome the ability of calcineurin to limit Ca^{2+} entry during synaptic activity. Thus, when excitatory synaptic transmission coincides with norepinephrine release, hippocampal neurons may be able to sustain a larger NMDA receptor–mediated Ca^{2+} influx.

Results

Figure 1A illustrates the protocol used in all experiments. As in Tong et al. (1995), T−, the test EPSC following a train of four conditioning EPSCs was compared to T+, the test EPSC following a train of conditioning stimuli given while the synapse was bathed in the NMDA receptor antagonist D-2-amino-5-phosphonopentanoic acid (D-AP5). D-AP5 was applied for only 200 ms and was completely washed away before the test EPSC (T+) was elicited (see Experimental Procedures). The ratio of the test EPSC amplitudes, T−/T+ (test EPSC ratio), was 0.77 ± 0.10 (mean ± SD; n = 39). Because transmitter release is unaltered by D-AP5 (Tong et al., 1995), differences in the amplitudes of T+ and T− reflect autoregulation of postsynaptic NMDA receptor sensitivity to released transmitter.

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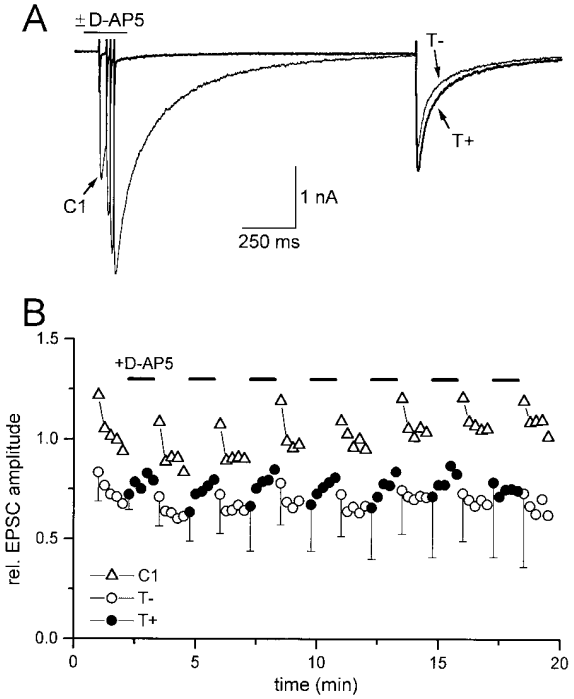

Figure 1. Activity-Dependent Regulation of NMDA EPSCs

(A) Responses of a cell to the stimulation protocol described in Experimental Procedures. Traces are averages of 13 (T+) and 14 (T−) episodes. Test EPSCs with and without D-AP5 application during the conditioning pulses are indicated by T+ and T−, respectively. C1 indicates the first conditioning EPSC. In all figures, the bar indicates the duration of application of 100 μM D-AP5 for the response represented by the thick trace.

(B) Cycling of test EPSC amplitudes for 4 cells during continuous pipette perfusion with K–gluconate. Amplitudes of C1, T−, and T+ in each cell were normalized to the mean C1 amplitude from episodes 2–5. Horizontal bars above the data indicate episodes for which D-AP5 was applied for 200 ms during the conditioning pulses (hence no triangles). Vertical bars are standard errors and are only included on the first T− and T+ of each series for clarity. The increasing amplitudes of the error bars reflect the variable run-up or run-down in the different cells; in all cases, the cycling of test EPSC amplitudes was evident, superimposed on the increasing or declining responses.

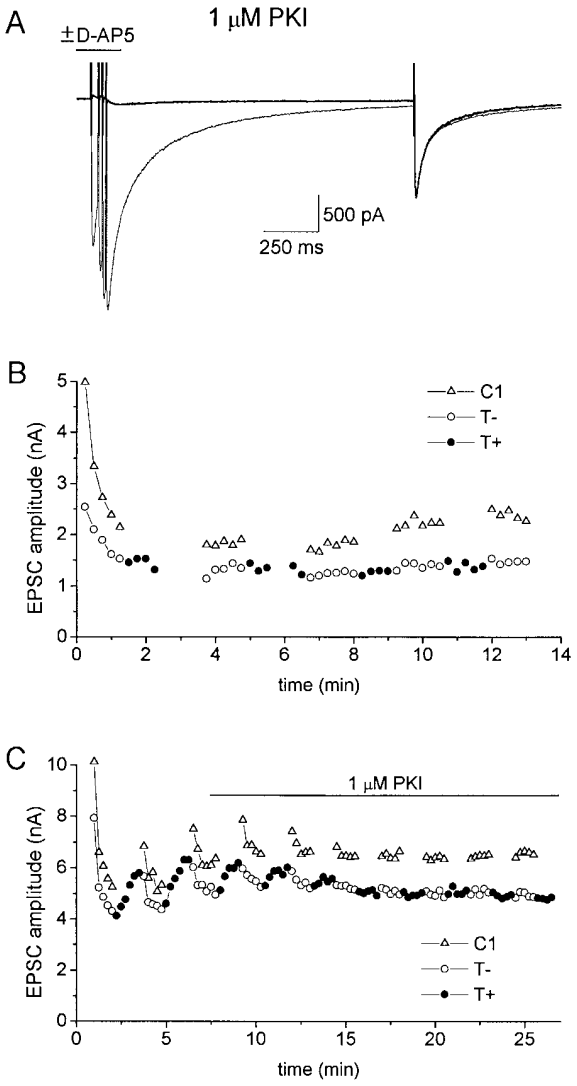

Figure 2. PKA Inhibition Prevents Recovery of Test EPSCs

(A) Including 1 μM PKI in the patch pipette abolished the difference in amplitude between T− and T+. Traces have been averaged.

(B) The peak amplitudes of C1, T+, and T− for each episode are plotted against time after onset of recording for the cell represented in (A). Gaps in the plot of T− and T+ are due to verification of D-AP5 washout as described in Experimental Procedures.

(C) Plot of C1, T−, and T+ against time during pipette perfusion. The recording pipette was initially perfused with control K–gluconate solution. At 7.5 min, the intracellular flow was switched to K–gluconate with 1 μM PKI.

Figure 1B illustrates that at least 2 episodes (each composed of conditioning and test stimuli) were required for the increase in T+ or decrease in T− to reach a plateau. Possible reasons for the delay are discussed in Figure 5. Because of this "cycling," the mean amplitudes of T+ and T− in all experiments were obtained by averaging all but the first in a series of 5 episodes (either with or without D-AP5) so that the maximal difference between T+ and T− could be measured. In each series of episodes, the mean T+ is significantly larger than the mean T− in the preceding or following series of episodes ($p < .025$, unpaired t test, one-tailed), indicating that cycling of test EPSC amplitude persists for at least 20 min during pipette perfusion.

The reduction in T− is triggered by the influx of Ca^{2+} through NMDA receptors, which activates calcineurin and shifts an equilibrium toward dephosphorylation of receptors or associated proteins. Conversely, the increment in T+ probably arises from rephosphorylation in the absence of strong synaptic activity. Presynaptic mechanisms are not likely to be involved, since test EPSCs mediated by AMPA receptors were not affected by preceding NMDA receptor–mediated EPSCs (Tong et al., 1995).

To assess whether this phosphorylation is mediated by PKA, the experiment was performed with PKA inhibitory peptide 5–24 (PKI) at 1 μM (n = 4) or 100 nM (n = 7) included in the patch pipette. Under these conditions, the test EPSC ratio was brought to 1.00 ± 0.06 (pooled data), as illustrated in Figure 2A and summarized in Figure 4B. Although the effect of another putative PKA

blocker (Rp cAMPS; 100 μM) was examined, the results were too variable to be interpreted (n = 9; see Figure 4B). Figure 2B shows the time course of the effect of 1 μM PKI on the responses of the cell in Figure 2A. Stimulation began 2 min after obtaining the whole-cell configuration. Initially, the amplitudes of the first conditioning EPSC (C1) and T− were relatively high, and both decreased substantially in the first 1.25 min of recording. With successive episodes, the T+ amplitudes showed no incrementation, and the subsequent T− amplitudes showed no diminution. This pattern was observed in all PKI-containing cells, indicating that blockade of PKA prevented the rephosphorylation of NMDA receptors. To ascertain that PKI could eliminate cycling, 1 μM PKI was perfused into the patch pipette during recording. As the peptide was dialyzed into the cell, the cycling was reduced and then abolished, as shown in Figure 2C. While this cell exhibited moderate run-up initially, the final amplitudes of C1 and of the test EPSCs appear equivalent to the "dephosphorylated" amplitudes of C1 and T−, respectively, just prior to PKI perfusion (at about 7 min).

The robust effect of PKI, even at concentrations as low as 100 nM, suggests that other endogenous kinases such as PKC, Ca^{2+}/calmodulin-dependent protein kinase, or cGMP-dependent protein kinase (PKG) were unable, under these conditions, to reverse the effect of calcineurin. In fact, the difference between T− and T+ persisted for at least 9 min when 10 μM PKC inhibitory peptide (PKC-I) was included in the pipette (n = 6; see Figure 4B). In 3 cells, 9–20 min after stimulation began, the difference decreased or disappeared; the other 3 cells continued to cycle for the length of the recording (5–15 min). Given the rapidity and efficacy of intracellular PKI, it is unlikely that the slow, sporadic effect of PKC-I on the test EPSC ratio can be wholly ascribed to insufficient dialysis. PKC inhibition therefore does not appear to block strongly the cycle of phosphorylation and dephosphorylation.

While basal levels of PKA are sufficient to rephosphorylate the receptors when synaptic activity is low, physiological stimulation of PKA activity may shift the balance between phosphorylation and dephosphorylation toward the former. Several neurotransmitters that are released in the hippocampus activate adenylyl cyclase. For example, the locus coeruleus sends a noradrenergic projection to the hippocampus, and norepinephrine acting at β-adrenergic receptors can raise intracellular PKA activity (e.g., Madison and Nicoll, 1986a, 1986b; Pedarzini and Storm, 1993). To investigate whether β-adrenergic stimulation can overcome the effect of calcineurin activation, the test EPSC ratio was examined in the presence of norepinephrine. As illustrated in Figure 3A, application of 1–10 μM norepinephrine brought the test EPSC ratio to 0.98 ± 0.06 (n = 8), significantly greater than the control ratio ($p < .01$, Dunnett's test for multiple comparisons with a control; Dunnett, 1964). In this cell, the amplitude of C1 also increased, as did the ratio of the first two EPSCs, C1/C2. While this apparent potentiation may have resulted from a presynaptic effect of PKA (e.g., Chavez-Noriega and Stevens, 1994), such run-up was inconsistent across cells and was also seen occasionally in control solutions (see Figure 2C) or in the

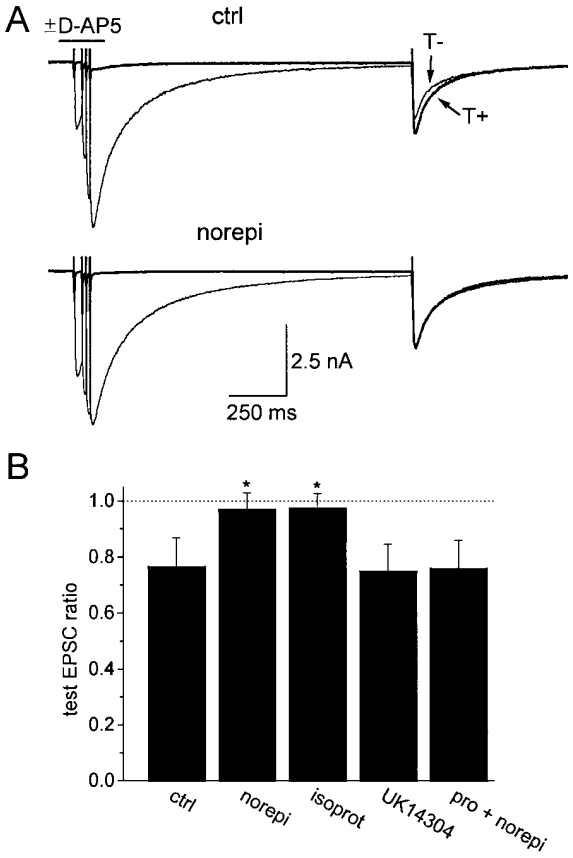

Figure 3. β-Adrenergic Agonists Overcome the Reduction of the Test EPSC by Calcineurin

(A) Averaged records from a single cell, before (top) and after (bottom) application of 10 μM norepinephrine. Scale bars apply to both sets of traces.

(B) Summary of effects of noradrenergic agonists and antagonists on the test EPSC ratio. Bars indicate SD; asterisks indicate that the test EPSC is increased significantly ($p < .01$, Dunnett's test for multiple comparisons with a control; Dunnett, 1964) above controls. The dotted line at a ratio of 1 is drawn to facilitate comparison. isoprot, isoproterenol; norepi, norepinephrine; pro, propranolol.

presence of kinase inhibitors. Figure 3B shows that the β-adrenergic receptor agonist isoproterenol (1 μM) also raised the test EPSC ratio to 0.97 ± 0.05 (n = 6; $p < .01$), while the β-adrenergic receptor antagonist propranolol (300 nM) blocked the effect of 1 μM norepinephrine on the test EPSC ratio (0.76 ± 0.10; n = 4). The α_2-adrenergic agonist UK14304 (1 μM), which is negatively coupled to PKA, did not significantly affect the test EPSC ratio (0.75 ± 0.095; n = 3). None of the noradrenergic agonists produced a detectable change in holding current, and only 1 of the 12 cells exposed to UK14304 or norepinephrine showed evidence of presynaptic inhibition.

Presumably, norepinephrine raised the test EPSC ratio to about 1 by increasing the proportion of phosphorylated receptors, via elevation of PKA activity. If so, activators of PKA should also be sufficient to abolish the difference between T− and T+. The experiment was therefore repeated in the presence of various kinase activators, and the resulting test EPSC ratios are displayed in Figure 4B, along with the data from kinase

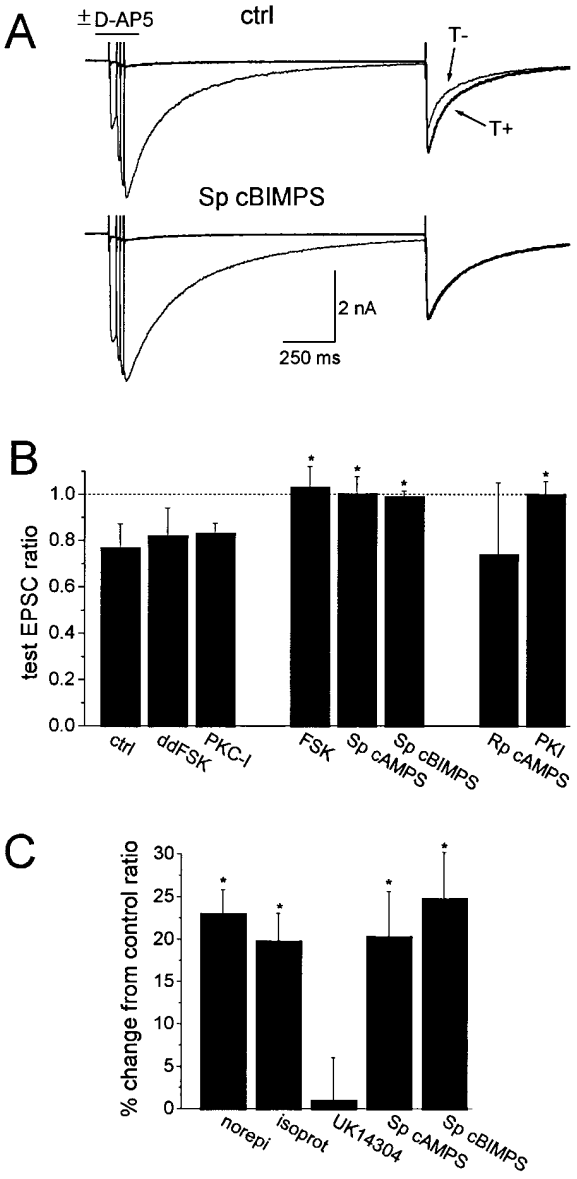

Figure 4. Manipulations That Elevate or Block PKA Can Change the Test EPSC Ratio

(A) Averaged records from a single cell before (top) and after (bottom) application of the cAMP analog Sp cBIMPS (100 μM). Scale bars apply to both sets of traces.

(B) Summary of effects of kinase activators and inhibitors on the test EPSC. Error bars, asterisks, and dotted line are as in Figure 3. Abbreviations as given in text; ddFSK, dideoxyforskolin; FSK, forskolin. The PKC-I ratio was constructed from measurements made during the first 9 min of recording (see text).

(C) The percentage change from the test EPSC ratio in within-cell controls. Asterisks indicate significant differences ($p < .0025$, paired t-test, one-tailed). Abbreviations as in text and Figure 3; concentrations are given in the text, and norepinephrine data were obtained only with 10 μM ($n = 5$).

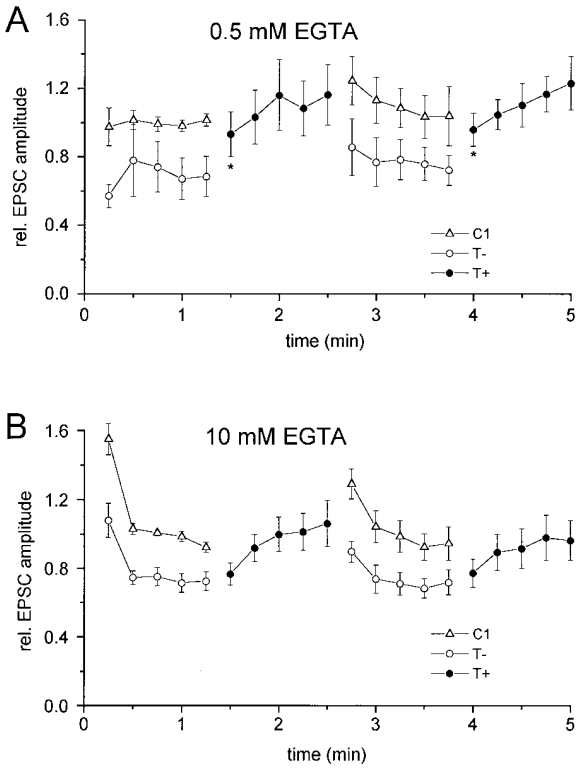

Figure 5. The Rate of Phosphorylation and Dephosphorylation Depends on Intracellular Buffering Capacity

(A) Plots of C1, T−, and T+ against time after onset of recording for 5 cells buffered with 0.5 mM EGTA. Peak currents were normalized as in Figure 1B. Bars indicate SEM. Asterisks indicate that the first T+ is significantly larger that the final T− ($p < .005$, paired t-test, one-tailed).

(B) Plot as in (A), but cells were buffered with 10 mM EGTA ($n = 9$). In this case, T+ is not at first significantly larger than T− ($p = .1$) but increases gradually.

inhibitors. Extracellularly applied activators of PKA all abolished or significantly reduced the difference between T− and T+ ($p < .01$; Dunnett, 1964). These included forskolin (40 μM; 1.03 ± 0.09; $n = 5$) and the cAMP analogs Sp cAMPS (100 μM; 1.00 ± 0.07; $n = 4$) and Sp cBIMPS (100 μM; 0.99 ± 0.02; $n = 5$). Figure 4A shows the responses of a cell before (top) and after (bottom) application of Sp cBIMPS. In 5 of 6 cells, 40 μM dideoxyforskolin, which should control for nonspecific effects of forskolin, had no significant effect (test EPSC ratio of 0.82 ± 0.12); in the sixth cell, the test EPSC ratio was 1.07. These data are consistent with a PKA-mediated effect of norepinephrine on the test EPSC ratio.

Figure 4C quantifies the effect of the noradrenergic agonists and cAMP analogs, showing the percentage change in the test EPSC ratio from within-cell controls. The percentage change by norepinephrine, isoproterenol, Sp cAMPS, and Sp cBIMPS was significant ($p < .0025$, paired t test, one-tailed) and comparable, while UK14304 had no significant effect ($p = .4$).

Previous work (Tong et al., 1995) associated the reduction of T− with respect to T+ with desensitization of the receptor. However, the present experiments suggest a decoupling of dephosphorylation and desensitization. The earlier experiments used 0.5 mM EGTA to buffer intracellular Ca^{2+}. Under this condition, as illustrated in Figure 5A, C1 and T− were fairly constant in amplitude over sequential episodes of stimulation. T−

stayed relatively small throughout the series of episodes, consistent with activity-dependent dephosphorylation or desensitization that took place within the 1.5 s between C1–C4 and T−. The first T+ was significantly larger ($p < .005$, paired t test, one tailed; $n = 5$) than the T− immediately before it, consistent with phosphorylation and resensitization of receptors during the 15 s interval between episodes.

In contrast, the present experiments used 10 mM EGTA intracellularly. Although the test EPSC ratios under these different buffer conditions were indistinguishable, with high EGTA, activity-dependent diminution of T− occurred over a longer period of time (30–45 s) than in low EGTA (<1.5 s), as shown in Figure 5B (also see Figure 1B). Because of faster buffering, the intracellular concentration of free Ca^{2+} presumably rises more slowly in the high EGTA condition, and more stimulation is required to achieve Ca^{2+} concentrations adequate for activation of calcineurin. Dephosphorylation is therefore slowed, but nevertheless diminishes the amplitude of the NMDA EPSCs. However, the slowed decrease in the NMDA EPSC amplitude suggests that the decrease is not desensitization dependent. This idea is supported by the result that, with successive episodes, the amplitude of the conditioning EPSCs, represented again by C1, decreased in parallel with the amplitude of T−. The parallel decrement in the amplitudes of C1 and T− indicates that the amplitude of T− is not an immediate consequence of the conditioning EPSCs. Rather, dephosphorylation may decrease EPSC amplitudes by favoring a state of the NMDA receptors with a lower maximal open probability (P_o), but probably not a lower single-channel conductance (see Lieberman and Mody, 1994). The gradual (30–45 s) increase in the amplitude of T+ may result from phosphorylation back to a higher P_o state. As further evidence against desensitization as a mechanism of the cycling, the 15 s interval following the final D-AP5-free episode should be sufficient for resensitization, even in the high EGTA condition (Sather et al., 1992; Lester and Jahr, 1992). Instead, T+ was not at first statistically different from the preceding T− ($p = .10$; $n = 9$), but then increased in amplitude over 2–3 successive episodes to reach a level significantly larger than that of T−. Finally, in the experiments using PKI, the dephosphorylated receptors should still desensitize and resensitize; thus, if synaptic desensitization were affecting the amplitude of the test EPSC, the fluctuation in T+ and T− should have still been evident. Instead, T+ and T− reached the same, constant amplitude (see Figures 2B and 2C), consistent with the idea that, in the presence of PKI, NMDA receptors were irreversibly dephosphorylated to a low P_o state.

Discussion

PKA-Mediated Regulation of NMDA Receptor P_o

These experiments provide evidence that the amplitude of NMDA receptor–mediated EPSCs in hippocampal neurons is regulated by PKA, since cycling of the test EPSC from high amplitude (T+) to low amplitude (T−) can be blocked by inhibiting PKA with PKI. Additionally, directly activating adenylyl cyclase (forskolin), mimicking cAMP (Sp cAMPS and Sp cBIMPS), or applying neurotransmitter receptor agonists that positively couple to adenylyl cyclase (norepinephrine and isoproterenol) can overcome cycling. Thus, PKA can antagonize the effects of calcineurin. Calcineurin and PKA may oppose one another not only by competing for the same site(s) of phosphorylation but also by regulating one another. For instance, calcineurin can reduce PKA activity by dephosphorylating, and thereby stimulating, at least two types of phosphodiesterases (Sharma and Wang, 1985, 1986).

Physiological Control and Effect of P_o Cycling

The signal for decreasing the P_o of NMDA receptors via calcineurin stimulation appears to be synaptic activity (Tong et al., 1995). While tonic levels of PKA apparently rephosphorylate receptors when calcineurin is inactive, phosphorylation can be raised above basal levels by norepinephrine. Thus, NMDA receptors are subject to a complex system of regulation by calcineurin and PKA, which provides negative feedback during activity, primes for a large response after inactivity, and can maintain receptors in a higher P_o state when, for example, norepinephrine shifts the equilibrium toward phosphorylation. In vivo, several variables may influence the level of phosphorylation of NMDA receptors, including the time course and magnitude of Ca^{2+} influx, the proximity of Ca^{2+} influx and effector enzymes, the affinity and kinetics of endogenous Ca^{2+} buffers, as well as the concentration, location, and efficiency of PKA and calcineurin. The buffering capacity of the cell can control the rate at which the NMDA receptors change state after synaptic activity. Experimentally, in low buffering conditions (0.5 mM EGTA), dephosphorylation can occur within 1.5 s, but with higher buffering (10 mM EGTA), the time course of dephosphorylation is prolonged at least 10-fold, possibly owing to a slower rise in free Ca^{2+} and consequent slower activation of calcineurin. Fast buffering with BAPTA prevents dephosphorylation altogether (Tong et al., 1995). However, as long as buffering is relatively slow (e.g., EGTA as opposed to BAPTA), the extent of the effect is the same. The change in the amplitude of NMDA EPSCs may influence firing frequency (see Lanthorn et al., 1984); perhaps more importantly, variations in NMDA receptor–mediated current may have marked intracellular effects as a consequence of modulation of Ca^{2+}-dependent processes.

Modulation of NMDA Receptors by Norepinephrine

Norepinephrine has a variety of effects at α- as well as β-adrenergic receptors in the hippocampus, many of which increase excitability of pyramidal cells (reviewed in Nicoll et al., 1990), consistent with the observation that stimulation of the locus coeruleus increases the amplitude of the evoked population spike in the CA1 of anesthetized rats (Olpe et al., 1986). Activation of β-adrenergic receptors can decrease the resting K^+ conductance and suppresses Ca^{2+}-activated K^+ currents, strongly reducing spike frequency accommodation in CA1 cells (Madison and Nicoll, 1982, 1986a; Dunwiddie et al., 1992; Pedarzini and Storm, 1993). The latter action can be enhanced by interactions with other G protein–coupled neurotransmitters (Andrade, 1993; Gereau and

Conn, 1994). In addition, β-adrenergic receptor stimulation increases voltage-gated Ca^{2+} current in dentate granule cells (Gray and Johnston, 1987). While activation of α-adrenergic receptors can depress transmission at the mossy fiber–CA3 synapse (Scanziani et al., 1993), α-adrenergic effects also include disinhibition of CA1 pyramidal cells, probably by presynaptic inhibition of inhibitory interneurons (Madison and Nicoll, 1988; Doze et al., 1991). The present experiments reveal another means by which norepinephrine can contribute to an enhancement of excitation or synaptic strength in hippocampal CA1 neurons, in this case by increasing the P_o of NMDA receptors and augmenting the total Ca^{2+} flux into CA1 neurons during repetitive stimulation.

Experimental Procedures

Preparation

Autaptic microdot cultures (Segal and Furshpan, 1990; Bekkers and Stevens, 1991; Tong et al., 1995) from the CA1 region of the rat hippocampus were used 6–12 days after plating.

Recording

Recording pipettes were pulled from 1.5 mm thin-walled borosilicate glass (WPI) to give an electrode resistance of 1–4 MΩ. Pipettes were filled with an intracellular solution of 140 mM K–gluconate, 6.23 mM $CaCl_2$, 10 mM NaCl, 2 mM $MgCl_2$, 10 mM EGTA, 10 mM HEPES, 2 mM K_2ATP, and 0.1 mM Na_2GTP (buffered to pH 7.4 with KOH), except in the experiments of Figure 5A, which had 0.5 mM EGTA and no added $CaCl_2$. Extracellular solution contained 160 mM NaCl, 3 mM $CaCl_2$, 3 mM KCl, and 5 mM HEPES (buffered to pH 7.4 with NaOH). Recordings were made under whole-cell voltage clamp with an Axopatch 1C amplifier (Axon Instruments), and access resistances were usually <6 MΩ with 60%–80% compensation. Cells were held at −70 mV. In the microdot cultures, cells form synapses onto themselves, and synaptic currents can be evoked and recorded in the same cell. To elicit an EPSC, a 0.5–1.5 ms depolarizing step to 0 mV was applied, and the resulting unclamped axonal action potential triggered transmitter release.

For patch-pipette perfusion, recordings were begun with control K–gluconate solution in the electrode. Both the control K–gluconate solution and the test intracellular solution were in reservoirs connected to the patch pipette with fine tubing. After attaining the whole-cell configuration, flow of the control solution was started and excess solution was withdrawn from the pipette with a Bio-Tek pneumatic transducer. After control records were obtained, the flow was switched to the test solution. Control measurements of the abolition of voltage-gated K^+ currents as Cs^+ was dialyzed into the cells showed that the intracellular solution could be exchanged in 6–10 min.

Perfusion of the synapse was accomplished with a three-barrel array of flow pipes. Gravity-driven flow of solutions was gated by three-way valves. Control solution consisted of 5 μM 6-nitro-7-sulphamoylbenzo(f)quinoxaline-2,3-dione (NBQX) or 6,7-dinitroquinoxaline-2,3-dione (DNQX), 50 μM pictrotoxin, and 20 μM glycine dissolved in extracellular solution. D-AP5 solution was control solution with 100 μM D-AP5. Drug solution was control solution containing agonists, antagonists, or membrane-permeant kinase activators or inhibitors. All drugs were diluted from stock solutions in H_2O except forskolin, dideoxyforskolin, UK14304 (10 mM stock solutions in dimethyl sulfoxide), PKC-I (1 mM stock solution in 5% acetic acid), and isoproterenol, which was prepared daily. Initial experiments used two flow pipes, one with control or drug solution and the other with D-AP5 solution. Later experiments used three pipes, containing control, D-AP5, and drug solution, so that a test EPSC ratio could be established in each cell prior to obtaining the drug effect. For the experiments using propranolol, cells were conditioned with propranolol-containing solution for 30 s.

The stimulation protocol used in all experiments was the same as that used in Tong et al. (1995). A train of 4 conditioning stimuli was given, with interpulse intervals of 35, 20, and 20 ms, followed by a test stimulus 1.5 s later. During the conditioning stimuli, the synapse was bathed either in control solution, in which case conditioning EPSCs were elicited, or in D-AP5 solution, in which case transmitter was released but NMDA receptors were not activated. The D-AP5 solution was applied for 200 ms and washed away well before the test EPSC 1.5 s later. Removal of D-AP5 was monitored by ascertaining that test EPSCs following ±D-AP5 application in the absence of conditioning stimuli were of equal amplitudes; i.e., there was no residual D-AP5 block. In this way, the test EPSCs, called T+ and T− for test following a conditioning train with and without D-AP5, respectively, could be compared for postsynaptic changes while controlling for presynaptic effects of stimulation and drugs. The relative amplitudes of T− and the first conditioning EPSC, C1, varied widely (range T−/C1 = 0.45–0.99), which probably results partly from presynaptic depression. Nevertheless, the amount of presynaptic depression in each cell should be comparable with or without D-AP5, and therefore, differences between T+ and T− are probably postsynaptic in origin (Tong et al., 1995).

Data acquisition and analysis were accomplished with software written in AXOBASIC. An "episode" was defined as a train of conditioning pulses with the following test pulse. The interval between episodes was 15 s. At least 5 episodes were recorded without the D-AP5 application during the conditioning train, followed by 5 episodes with the D-AP5 application. This sequence was repeated 2–10 times. For measurement of the test EPSC ratio, all episodes in either condition (±D-AP5) were averaged while eliminating the first test EPSC of each set of episodes from the average; e.g., in Figure 1B, episodes 2–5, 12–15, 22–25, etc., would be averaged for T−. In this way, the full extent of the difference between T+ and T− could be measured (see Figure 5).

Drugs were obtained from the following sources: CalBiochem (ATP, GTP, forskolin, dideoxyforskolin, Rp cAMPS, Sp cAMPS, and PKC inhibitor peptide 19–36), Biolog (Sp cBIMPS), Sigma (picrotoxin, PKI 5–24, norepinephrine, propranolol, and isoproterenol), Tocris Cookson (D-AP5), RBI (UK14304), and Precision Neurochemicals (DNQX). NBQX was a kind gift from Novo Nordisk.

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