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Presynaptic Mechanism Underlying cAMP-Induced Synaptic Potentiation in Medial Prefrontal Cortex Pyramidal Neurons

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Running title: cAMP-dependent synaptic potentiation in the mPFC

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ABBREVIATIONS: mPFC, medial prefrontal cortex; PKA, cAMP-dependent protein kinase; Epac, exchange protein activated by cAMP; MAPK, mitogen-activated protein kinase; HCN, hyperpolarization and cyclic nucleotide-activated; EPSC, excitatory postsynaptic current; mEPSC, miniature excitatory postsynaptic current; aCSF, artificial cerebrospinal fluid; AMPA, α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid; CV, coefficient of variation; PPR, paired pulse ratio; *Nq*, number of release vesicle; PD98059, 2-(2-amino-3-methoxyphenyl)-4*H*-1-benzopyrane-4-one; U0126, 1,4-diamino-2,3-dicyano-1,4-bis[2-aminophenylthio]butadiene; SB203580, 4-[5-(4-fluorophenyl)-2-[4-(methylsulfonyl)phenyl]-1*H*-imidazol-4-yl]pyridine; SP600125, anthrax[1-9-cd]pyrazol-6(2*H*)-one; 8CPT-2Me-cAMP, 8-(4-chlorophrnylthio)-2'-*O*-methyl-cAMP; H-89, N-[2-((*p*-Bromocinnamyl)amino)ethyl]-5-isoquinolinesulfonamide; KT 5720, (9*R*,10*S*,12*S*)-2,3,9,10,11,12-Hexahydro-10-hydroxy-9-methyl-1-oxo-9,12-epoxy-1*H*-diindolo[1,2,3-fg:3',2',1'-kl]pyrrolo[3,4-i][1,6]benzodiazocine-10-carboxylic acid; Sp-cAMPS, Sp-adenosine 3c',5c'-cyclic monophosphorothioate triethylammonium; CNQX, 6-cyano-7-notroquinoxaline-2, 3-dione; ZD7288, 4-ethylphenylamino-1,2-dimethyl-6-methylaminopyrimidinium chloride; D-APV, D-(-)-2-amino-5-phosponopentanoic acid; QX-314, lidocaine N-ethyl bromide

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ABSTRACT

The 3', 5'-cyclic adenosine monophosphate (cAMP), a classic second messenger, has been recently proposed to participate in regulating prefrontal cortical cognitive functions, but yet little is known about how it dose so. In this study, we used forskolin, an adenylyl cyclase activator, to examine the effects of cAMP on excitatory synaptic transmission in the medial prefrontal cortex (mPFC) using whole-cell patch-clamp recordings from visually identified layer II-III or V pyramidal cells *in vitro*. We found that bath application of forskolin significantly increased the amplitude of excitatory postsynaptic currents (EPSCs) in a concentration- and age-dependent manner. This enhancement was completely abolished by coapplication of cAMP-dependent protein kinase (PKA) inhibitor and p42/p44 mitogen-activated protein kinase (MAPK) kinase inhibitor, but not application of either drug alone. The membrane-permeable cAMP analog, Sp-adenosine 3c',5c'-cyclic monophosphorothioate triethylammonium, or activation of β-adrenergic receptor by isoproterenol mimicked the effect of forskolin to potentiate EPSCs. However, neither exchange protein activated by cAMP (Epac) inhibitor, brefeldin A, nor hyperpolarization and cyclic nucleotide-activated channel blocker, ZD7288, affected forskolin response. The augmentation of EPSCs by forskolin was accompanied by a reduction of the synaptic failure rate, coefficient of variation and paired-pulse ratio of EPSCs and an increase in release probability and number of releasable synaptic vesicles. Forskolin also significantly increased the frequency of miniature EPSCs without altering their amplitude distribution. These results indicate that cAMP acts presynaptically to elicit a synaptic potentiation on the layer V pyramidal neurons of mPFC through converging activation of PKA and p42/p44 MAPK signaling pathways.

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Introduction

The prefrontal cortex (PFC) is believed to subserve a wide variety of cognitive processes, including working memory (Goldman-Rakic, 1995), selection and remembering of relevant stimuli (Rainer et al., 1998), and remembering of contextually relevant, cross-modal stimulus association (Fuster et al., 2000). In rodents, the medial aspect of PFC (mPFC), encompassing the infralimbic and prelimbic areas, is considered to be anatomically homologous to the human/primate dorsolateral PFC to execute these functions (Zhart et al., 1997; Birrell and Brown, 2000). Prefrontal dysfunction is commonly found in many psychiatric disorders such as attention-deficient hyperactivity disorder, post-traumatic stress disorder, the affective disorders, schizophrenia, and drug abuse (Heidbreder and Groenewegen, 2003).

The 3', 5'-cyclic adenosine monophosphate (cAMP) is one of the best-studied second messenger. A substantial body of evidence implicates that cAMP activates cAMP-dependent protein kinase (PKA), thereby eliciting a long-lasting increase in transmitter release at many central synapses (Chavez-Noriega and Stevens, 1994; Weisskopf et al., 1994; Colwell and Levine, 1995; Salin et al., 1996). This effect of cAMP, together with that on cAMP response element-binding protein, is also thought to underlie long-term potentiation of synaptic efficacy and memory consolidation (Bailey et al., 1996). Recently, however, PKA-independent actions of cAMP, which enhance release of transmitters and hormones, have been reported. For example, at the crayfish neuromuscular junction, cAMP activates presynaptic exchange protein activated by cAMP (Epac) and hyperpolarization and cyclic nucleotide-activated (HCN) channels, thereby increasing transmitter release (Beaumont and Zucker et al.,

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2000; Zhong and Zucker, 2005). At the calyx of Held, cAMP facilitates transmitter release via activating the Epac pathway in the nerve terminal (Kaneko and Takahashi, 2004). In pancreatic cells, the cAMP-sensitive guanine nucleotide-exchanging factor may interact with Rim2, thereby enhancing insulin secretion (Ozaki et al., 2000; Kashima et al., 2001). In addition, other study has revealed that elevated cAMP levels can potentiate the neuroprotective activity of the noradrenaline on dopamine neurons through activation of MAPK signaling pathway (Troadec et al., 2002). These findings indicate that cAMP may operate a wide variety of targets to exert its cellular functions.

Recent studies support a role for cAMP-regulated signaling in the cognitive function of the PFC. For instance, Aujla and Beninger (2001) have shown that cAMP/PKA inhibition in the PFC immediately before testing impaired working memory performance when long delays were used. However, other study found a dose-dependent impairment of working memory performance on the delayed-alternation task when the cAMP analog, Sp-cAMPS, was infused into the PFC of young rats (Arnsten et al., 2005). A recent study has also described an inhibitory effect of cAMP/PKA signaling on inwardly retifying K^+ conductance of mPFC neurons (Dong and White, 2004). At the present time, however, very little is known about the molecular mechanisms by which cAMP regulates the glutamatergic synaptic transmission in the mPFC. Here, we have demonstrated the first evidence that cAMP presynaptically facilitates synaptic transmission on the layer V pyramidal neurons of mPFC through activating two parallel signaling events, one involving PKA and the other p42/p44 MAPK.

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Materials and Methods

Slice preparation. All experiments were performed according to the guidelines laid down by the Institutional Animal Care and Use Committee of National Cheng Kung University. The coronal brain slices containing the prelimbic area PFC (1.5-2.5 mm anterior to bregma) were prepared from 8- to 23-day-old male Sprague-Dawley rats for whole-cell patch-clamp recordings by procedures described previously (Hirsch and Crepel, 1990). Most experiments (unless otherwise noted) were performed on 14- to 16-day old rats. In brief, rats were killed by decapitation under halothane anaesthesia, and coronal slices (200 μm thick) were prepared using a vibrating microtome (Leica VT1000S; Leica, Nussloch, Germany). The anterior cingulated cortex and the shoulder or frontal area 2 (Fr2) region of the frontal cortex (Paxinos and Watson, 1998) were used for recording. The slices were placed in a storage chamber of artificial CSF (aCSF) oxygenated with 95% O_2-5% CO_2 and kept at room temperature for at least 1 h before recording. The composition of the ACSF solution was (in mM): NaCl 117, KCl 4.7, $CaCl_2$ 2.5, $MgCl_2$ 1.2, $NaHCO_3$ 25, NaH_2PO_4 1.2 and glucose 11 at pH 7.3-7.4 and equilibrated with 95% O_2-5% CO_2.

Electrophysiological recordings. For whole-cell patch-clamp recording, one slice was transferred to a recording chamber of standard design and fixed at the glass bottom of the chamber with a nylon grid on a platinum frame. The chamber consisted of a circular well of low volume (1-2 ml) and was perfused constantly at 32.0 ± 0.5^{o}C with a speed of 2-3 ml/min. Visualized whole-cell patch-clamp recording of synaptically evoked EPSCs and miniature EPSCs (mEPSCs) was conducted using standard methods as described previously (Huang et al., 2002). The layer II-III or V pyramidal neurons were identified by their pyramidal shape, presence

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of a prominent apical dendrites, and distance from the pial surface with an upright microscope (Olympus BX50WI; Olympus, Tokyo, Japan) equipped with a water-immersion ×40 objective and a Nomarski condenser combined with infrared videomicroscopy. Patch pipettes were pulled from borosilicate capillary tubing and heat polished. The electrode resistance was typically 3-6 MΩ. The composition of intracellular solution was (mM): K-gluconate, 115; KCl, 20; HEPES, 10; $MgCl_2$, 2; EGTA, 0.5; Na_2ATP, 3; Na_3GTP, 0.3; QX-314, 5 and sucrose to bring osmolarity to 290-295 mOsM and pH to 7.3.

After a high resistance seal (> 2 GΩ before breaking into whole-cell mode) was obtained, suction was applied lightly through the pipette to break through the membrane. The cell was then maintained at –70 mV for several minutes to allow diffusion of the internal solution into the cell body and dendrites. Recordings were made using an Axopatch 200B (Axon Instruments, Union City, CA) amplifier. Electrical signals were low-pass filtered at 3 kHz, digitized at 10 kHz using a 12 bit analog-to-digital converter (Digidata 1200, Axon Instruments). An Intel Pentium-based computer with pCLAMP software (Version 8.0; Axon Instruments) was used for on-line acquisition and off-line analysis of the data. For measurement of synaptically evoked EPSCs, a bipolar stainless steel stimulating electrode was placed on layer II-III about 150-200 μm away the apical dendrites of the recorded neurons (Fig. 1A) and the superfusate routinely contained bicuculline methiodide (10 μM) to block inhibitory synaptic responses. The strength of synaptic transmission was mostly quantified by measuring the initial rising slope of EPSC (2 ms period from its onset; pA/ms), which contains only a monosynaptic component. In some experiments, synaptic currents were recorded in the presence of NMDA receptor antagonist D-APV (50 μM) or AMPA/kainate receptor antagonist CNQX (20 μM) and

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the amplitude was measured over a 0.5-2 ms window concentrated around the peak. Series resistance (Rs) was calculated according to Rs = 10 mV/I, where I was the peak of transient current (filtered with 10 kHz) evoked by the 10 mV testing pulse when the pipette capacitance was compensated fully. Only cells demonstrating < 25 MΩ series resistance (usually 10-20 MΩ) were used in these experiments.

Miniature EPSCs were recorded from layer V pyramidal neurons held in voltage clamp at a potential of –70 mV in the presence of bicuculline methiodide (10 μM), tetrodotoxin (1 μM), and $CdCl_2$ (100 μM) and analyzed off-line using a commercially available software (Mini Analysis 4.3; Synaptosoft, Leonia, NJ). mEPSCs were abolished by CNQX (20 μM) plus D-APV (50 μM), indicating that these are glutamatergic events. The software detects events on the basis of amplitudes exceeding a threshold set just above the baseline noise of the recording (3 pA). All detected events were reexamined and accepted or rejected on the basis of subjective visual examination. The program then measured amplitudes and intervals between successive detected events. Background current noise was estimated from the baseline with no clear event and was subtracted from signals for analysis. The mEPSC frequencies were calculated by dividing the total number of detected events by the total time sampled. Periods of 5-10 min were analyzed for forskolin treatment. Events were ranked by amplitude and inter-event interval for preparation of cumulative probability distribution. Amplitude histograms were binned in 1 pA intervals.

Drug application. All drugs were applied by manually switching the superfusate. Drugs were diluted from stock solutions just before application. Forskolin, 1,9-dideoxy-forskolin (Dd-forskolin),

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(9*R*,10*S*,12*S*)-2,3,9,10,11,12-Hexahydro-10-hydroxy-9-methyl-1-oxo-9,12-epoxy-1*H*-diindolo[1,2,3-fg:3',2',1'-kl]pyrrolo[3,4-i][1,6]benzodiazocine-10-carboxylic acid (KT5720), N-[2-((*p*-Bromocinnamyl)amino)ethyl]-5-isoquinolinesulfonamide (H-89), 2-(2-amino-3-methoxyphenyl)-4*H*-1-benzopyrane-4-one (PD98059), 1,4-diamino-2,3-dicyano-1,4-bis[2-aminophenylthio]butadiene (U0126), 4-[5-(4-fluorophenyl)-2-[4-(methylsulfonyl)phenyl]-1*H*-imidazol-4-yl]pyridine (SB203580), anthrax[1-9-cd]pyrazol-6(2*H*)-one (SP600125), and brefeldin A were dissolved in dimethylsulfoxide (DMSO) stock solutions and stored at -20 ℃ until the day of experiment. Other drugs used in this study were dissolved in distilled water. The concentration of DMSO in the perfusion medium was 0.1%, which alone had no effect on basal synaptic transmission. Forskolin, isoproterenol, propranolol, 8-(4-chlorophrnylthio)-2'-*O*-methyl-cAMP (8CPT-2Me-cAMP), Sp-adenosine 3c',5c'-cyclic monophosphorothioate triethylammonium (Sp-cAMPS), ZD7288, PD98059, U0126, SB203580, SP600125, CNQX, bicuculline methiodide, and D-APV were purchased from Tocris Cookson (Bristol, UK); Dd-forskolin, brefeldin A, strychnine hydrochloride, and tetrodotoxin were obtained from Sigma (St Louis, MO); H-89 was purchased from Calbiochem (La Jolla, CA).

Statistical analysis. The data for each experiment were normalized relative to baseline, and are presented as means ± S.E.M. Numbers of experiments are indicated by n. The significance of the difference between the mean was calculated by paired or unpaired Student's *t* test. Probability values (*p*) of less than 0.05 were considered to represent significant differences. Comparisons between control and experimental distributions of mEPSCs amplitude and interevent intervals were made by performing Kolmogorov-Smirnov (K-S) test. Distributions were considered

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different using a conservative critical probability level of $p < 0.01$.

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Results

A Photograph of a slice used for recording is shown in Fig. 1A. The area used for recording is outlined by a circle. Figure 1B shows an infrared microscope image of a representative layer V pyramidal neuron. The results of the present study were obtained from 176 pyramidal neurons. These neurons had a mean resting membrane potential, spike height, and input resistance of -68.7 ± 0.8 mV, 93.7 ± 2.3 mV, and 286 ± 29 MΩ (n = 62), respectively, which are comparable with the values reported previously (Wang and O'Donnell, 2001). In all experiments, layer V pyramidal neurons were held at a holding potential of -70 mV and EPSCs were evoked by the stimulation of layer II-III fibers with bipolar stimulating electrodes every 20 sec in the presence of $GABA_A$ receptor antagonist bicuculline methiodide (10 μM). Because EPSCs were completely blocked by CNQX (20 μM) plus D-APV (50 μM) (Fig. 1C), they were predominantly mediated by ionotropic glutamate receptors.

Potentiation of EPSCs by cAMP. We initially examined the effect of the adenylyl cyclase activator, forskolin, on the evoked EPSCs. Typical responses are shown in Fig. 2A. Bath application of forskolin (25 μM) produced a rapid and sustained enhancement of evoked EPSCs on layer V pyramidal neurons. The mean EPSC slope measured 20 min after forskolin application was increased by 53.6 ± 5.8% of the control baseline (n = 8; $p < 0.05$; paired Student's *t* test). No significant recovery was visible after drug washout at least 20 min intervals. The magnitude of EPSC potentiation by forskolin was concentration dependent (Fig. 2B), with an estimated EC_{50} of 21 μM. Because forskolin has been reported to possess many cAMP-independent actions, including the blockade of several types of K^+ conductance, it is possible that the effect of forskolin on EPSCs is caused by its

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nonspecificity (Laurenza et al., 1989). To exclude this possibility, an analog of forskolin, Dd-forskolin, which has no effect on adenylyl cyclase but does mimic many of the cAMP-independent actions of forskolin, was used. As shown in Fig. 2A, Dd-forskolin (25 μM) had no significant effect on EPSCs (4.2 ± 2.3%; n = 4; $p > 0.05$; paired Student's *t* test). As on layer V pyramidal neurons, forskolin (25 μM) also increased the slope of EPSCs of layer II-III pyramidal neurons. The mean EPSC slope 20 min after forskolin application was increased by 48.7 ± 6.9% of the control baseline (n = 5; $p < 0.05$; paired Student's *t* test) (Fig. 2C). Moreover, the facilitatory effect of forskolin on the layer V pyramidal neurons was robust at P8-P10 but became weaker as animal maturated (Fig. 2D), with the magnitude of potentiation by forskolin (25 μM) being 82.5 ± 7.8% at P8-P10 (n = 5), 53.6 ± 5.8% at P14-P16 (n = 10), and 32.8 ± 4.2% at P21-P23 (n = 5), indicating that the enhancement of synaptic transmission by forskolin is age dependent. In the present study, forskolin was not observed to significantly change the holding current under voltage-clamp conditions (16.2 ± 2.2 pA for before and 19.3 ± 3.1 pA for 20 min after forskolin application; n = 35; $p > 0.05$; paired Student's *t* test).

Additional evidence that elevation of cAMP levels can induce a synaptic potentiation of glutamatergic transmission on the layer V pyramidal neurons of mPFC came from experiments using the nonhydrolyzable cAMP analog, Sp-cAMPS. Similar to forskolin, bath application of Sp-cAMPS (100 μM) for 20 min produced a significant enhancement of EPSCs by 42.5 ± 5.7% of the control baseline (n = 5; $p < 0.05$; paired Student's *t* test) (Fig. 3A).

Forskolin Enhances Synaptic Transmission through Converging Activation of PKA and p42/p44 MAPK Signaling Pathways. An established signal transduction

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pathway for forskolin action is the activation of adenylyl cyclase, leading to an increase in intracellular cAMP levels and activating PKA, which in turn modulates the function of a series of cellular substrates by increasing their phosphorylation state. We first examined whether the cAMP-dependent synaptic potentiation is mediated by PKA activation. To test this possibility, two structurally unrelated PKA inhibitors, KT5720 and H-89, were used. Surprisingly, in the presence of either of KT5720 (1 μM) or H-89 (1 μM), forskolin was still able to potentiate EPSCs but the magnitude of potentiation was significantly smaller than that induced in the interleaved control condition ($p < 0.05$; unpaired Student's *t* test). On average, EPSC slope measured 20 min after forskolin application was increased by 38.4 ± 4.2% (n = 8) in the presence of KT5720 and 36.6 ± 4.1% (n = 5) in the presence of H-89 (Fig. 3B). However, neither KT5720 nor H-89 alone had effects on EPSCs (Supplementary Fig. S1A). These results suggest that in addition to PKA signaling cascade, other signal mechanism(s) is necessary for the induction of synaptic potentiation by forskolin.

At the calyx of Held, an increase in cAMP levels in the nerve terminal can facilitate transmitter release via the activation of Epac pathway (Kaneko and Takahashi, 2004). We next examined whether the cAMP-dependent synaptic potentiation seen in this study is mediated by Epac activation. To test this possibility, we used a selective Epac agonist, 8CPT-2Me-cAMP, which has been shown to have little effect on PKA (Enserink et al., 2002). To identify the saturating concentration of 8CPT-2Me-cAMP for activating Epac by external bath application, we treated slices with varying concentrations of 8CPT-2Me-cAMP. As shown in Fig. 3C, 8CPT-2Me-cAMP induced a concentration-dependent potentiation of EPSCs and the magnitudes of potentiation caused by 50 and 100 μM 8CPT-2Me-cAMP were not significantly different (Fig. 3C). Thus, 50 μM 8CPT-2Me-cAMP was chosen to

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examine if forskolin- and 8CPT-2Me-cAMP-induced synaptic potentiation utilize similar induction mechanism. The approach used to address this question is to see if induction of one form of potentiation reduces or occludes induction of the other form of potentiation. As shown in Fig. 3D, after 8CPT-2Me-cAMP-induced potentiation was fully established, application of forskolin still caused synaptic potentiation. On average, EPSC slope measured 20 min after forskolin application was increased by 51.3 ± 4.6% (n = 5), which was not significantly different from that found in slices without receiving 8CPT-2Me-cAMP. As the small G-protein antagonist brefeldin A has recently been shown to effectively antagonize 8CPT-2Me-cAMP's action on synaptic transmission at the crayfish neuromuscular junctions (Zhong and Zucker, 2005), we then used brefeldin A to characterize the role of Epac activation in forskolin-induced synaptic potentiation. Brefeldin A alone (100 μM) had no effect on basal synaptic transmission and did not affect forskolin-induced EPSC potentiation (Fig. 3E). On average, EPSC slope measured 20 min after forskolin application was increased by 49.7 ± 6.5% (n = 5), which was not significantly different from that of potentiation elicited under control condition. These results suggest that activation of Epac is not necessary for forskolin-induced synaptic potentiation in the layer V pyramidal neurons of mPFC.

To clarify the involvement of HCN channels in the induction of forskolin-induced synaptic potentiation, we compared the magnitude of synaptic potentiation in control slices to that obtained in slices preincubated in HCN channel blocker ZD7288. As shown in Fig. 3E, ZD7288 (30 μM) did not affect forskolin-induced EPSC potentiation (Fig. 3E). On average, EPSC slope measured 20 min after forskolin application was increased by 48.4 ± 5.7% (n = 5), which was not significantly different from that of potentiation elicited under control condition.

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Thus, these results rule out an involvement of HCN channels in the forskolin response.

In a number of systems, cAMP-induced MAPK activation independently of PKA has been reported (Iacovelli et al., 2001; Troadec et al., 2002). To determine whether the cAMP-dependent synaptic potentiation is mediated by MAPK activation, forskolin-induced synaptic potentiation was attempted in the presence of multiple types of MAPK inhibitors. As shown in Fig. 3F, forskolin response was strongly reduced by p42/p44 MAPK signaling pathway inhibitors, PD98059 and U0126. On average, EPSC slope measured 20 min after forskolin application was increased by 18.6 ± 3.5% in the presence PD98059 (50 μM) (n = 8; $p < 0.05$ when compared with forskolin alone; unpaired Student's *t* test) and 15.3 ± 3.4% in the presence U0126 (10 μM) (n = 6; $p < 0.05$ when compared with forskolin alone; unpaired Student's *t* test). Neither PD98059 nor U0126 alone had effects on EPSCs (Supplementary Fig. S1B). In contrast, inhibition of p38 MAPK signaling pathway with SB203580 (1 μM) or c-Jun N-terminal kinase inhibitor SP600125 (20 μM) failed to affect the forskolin-induced synaptic potentiation (SB203580, 56.3 ± 4.6%, n = 4; SP600125, 51.6 ± 3.9%, n = 4) (Fig. 3H). In another experiments, we found that forskolin failed to potentiate EPSCs when H-89 and PD98059 or H-89 and U0126 were applied together (H-89 + PD98059, 2.8 ± 1.5%, n = 5; H-89 + U0126, 2.4 ± 1.3%, n = 4) (Fig. 3G and H), suggesting that cAMP elevated by forskolin activates both PKA and p42/p44 MAPK signaling pathways to induced synaptic potentiation.

Presynaptic Expression of Forskolin-induced Synaptic Potentiation. To dissect the synaptic site of action for forskolin, the effects of forskolin on the AMPA and NMDA receptor-mediated component of synaptic currents were examined. If

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forskolin-induced synaptic potentiation was expressed presynaptically, changes in both of the magnitude of AMPA receptor-mediated EPSC ($EPSC_{AMPA}$) and NMDA receptor-mediated EPSC ($EPSC_{NMDA}$) by forskolin would be expected. The $EPSC_{AMPA}$ was recorded from the layer V pyramidal neurons in the presence of NMDA receptor antagonist D-APV (50 μM) at a holding potential of -70 mV, and the $EPSC_{NMDA}$ was recorded in the presence of AMPA/kainate receptor antagonist CNQX (20 μM) and at a holding potential of +40 mV to remove the voltage-dependent block of Mg^{2+}. As illustrated in Fig. 4, A and B, forskolin (25 μM) increased the amplitude of $EPSC_{AMPA}$ by 51.4 ± 5.6% (n = 5) of control baseline. Comparable results were obtained with $EPSC_{NMDA}$ (48.9 ± 5.2% of control baseline, n = 5).

To further test the possibility that forskolin induces synaptic potentiation through a presynaptic mechanism, we examined the effect of forskolin on the failure rate of single-fiber EPSCs evoked by minimal stimulation, which reflects changes in the presynaptic transmitter release (Stevens and Wang, 1994). As a typical example shown in Fig. 4C, the expression of forskolin-induced synaptic potentiation was accompanied by a decrease in synaptic failure rate. On average, the failure rate was decreased from 0.51 ± 0.03 to 0.18 ± 0.03 after forskolin application (n = 6; $p < 0.05$; paired Student's *t* test) (Fig. 4D). We also addressed the synaptic locus of forskolin-induced synaptic potentiation by examining the trial-to-trial amplitude fluctuation in EPSCs with the variance analysis. The value of coefficient of variance (CV) varies with quantal content but is independent of changes in the postsynaptic response to a fixed amount of transmitter, and is a useful measure of changes in presynaptic function (Bekkers and Stevens, 1990). Because variance analysis is best done on unitary synaptic responses, our strategy was to carry out a variance analysis of unitary single-fibre EPSCs evoked by minimal stimulation before and after

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forskolin application. We found that following the induction of synaptic potentiation by forskolin, the value of CV for unitary EPSCs was decreased from 0.85 ± 0.04 to 0.49 ± 0.03 (n = 6; $p < 0.05$; paired Student's *t* test) (Fig. 4D).

When the excitatory afferents to the central neurons are activated twice with a short interval between each stimulus, the response to the second stimulus is generally facilitated in relation to the initial stimulus. This phenomenon is called paired-pulse facilitation (PPF) and is attributed to an increase the amount of transmitter release to the second stimulus (Zucker, 1989). On the other hand, the manipulation of presynaptic transmitter release may result in the change in the magnitude of PPF. If the forskolin-induced synaptic potentiation involves a presynaptic mechanism of action, it will be associated with a decrease in the ratio of paired-pulse (PPR). To test this hypothesis, PPR (using an inter-pulse interval of 50 ms) was determined before and during application of 25 μM forskolin for 20 min. Under control conditions, the ratio of the slope of the second EPSC divided by the first one was 1.48 ± 0.04 (n = 6). We found that forskolin significantly decreased the PPR to 1.15 ± 0.05 (n = 6; $p < 0.05$; paired Student's *t* test) (Fig. 4D), suggesting an increase in glutamate release probability following forskolin application.

Forskolin Enhances Frequency of mEPSCs. To further confirm the possibility that forskolin potentiates synaptic transmission through a presynaptic mechanism, we examined the effects of forskolin on mEPSCs in the presence of tetrodotoxin (1 μM) and $CdCl_2$ (100 μM). mEPSCs in the layer V pyramidal neurons were measured under voltage clamp at -70 mV and were pharmacologically isolated from spontaneous inhibitory currents by the inclusion of 10 μM bicuculline methiodide in the aCSF perfusing the slices. The mEPSCs were totally blocked by bath

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coapplication of CNQX (20 μM) and D-APV (50 μM), confirming them to be true glutamate receptor-mediated events (data not shown). Under control conditions, mEPSCs had a mean amplitude of 5.98 ± 0.23 pA and a variable frequency ranging from 1.9 to 2.7 Hz (mean, 2.13 ± 0.19 Hz; $n = 5$). In five pyramidal neurons tested, forskolin (25 μM) markedly increased the mean frequency of the mEPSCs from 2.13 ± 0.19 to 5.23 ± 0.21 Hz ($p < 0.05$; paired Student's t test) (Fig. 5, A and F). Significant differences in cumulative interevent interval distributions were observed in all five cells tested during forskolin application; i.e., forskolin shifted the interevent interval distribution of mEPSCs to shorter intervals ($p < 0.01$; Kolmogorov-Smirnov test). A typical example of recorded cell is shown in Fig. 5D. However, there was no significant effect of forskolin (25 μM) on the mEPSC amplitude. This can be observed by a lack of effect of forskolin on either the amplitude histogram (Fig. 5B) or the cumulative probability plots (Fig. 5C, $p = 0.94$; Kolmogorov-Smirnov test). The mean amplitude of mEPSCs recorded in the presence of forskolin (25 μM) was 6.21 ± 0.19 pA, which was of comparable amplitude with that of mEPSCs recorded under control conditions (5.98 ± 0.23 pA; $p = 0.78$; paired Student's t test). Therefore, these data further suggest that forskolin may act presynaptically to enhance the amount of glutamate release without changing the postsynaptic sensitivity to glutamate.

Forskolin Increases the Number of Releasable Vesicles and Release Probability. While the above results are consistent with a presynaptic site for the expression of forskolin-induced synaptic potentiation, they may be attributed to a number of presynaptic mechanisms, including an increase in the number of readily releasable quanta (synaptic vesicles) (N) or an increase in release probability (P) (Trudeau et al.,

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1995; Kaneko and Takahashi, 2004). To determine which mechanism underlies the forskolin-induced synaptic potentiation, we first used the approach of high-frequency repetitive stimulation (20 stimuli at 100 Hz) (Schneggenburger et al., 1999), which induced a strong depression of EPSCs (Fig. 6A). In this approach, assuming that depression is largely caused by depletion of readily releasable quanta, *N* multiplied by mean quantal size (*q*) can be estimated from zero time intercept of a line fitted to a cumulative amplitude plot of EPSCs (Fig. 6B), and *P* can be estimated from the first EPSC amplitude divided by *Nq*. During stimulating at 100 Hz, EPSCs underwent a marked depression and reached a steady low level. Forskolin (25 μM) potentiated the first few EPSCs during a train of tetanic stimulation (Fig. 6A). Cumulative amplitude plot of EPSCs before and after forskolin application indicated that forskolin increased *Nq* by 23.2 ± 4.1% (n = 6; $p < 0.05$; paired Student's *t* test) (Fig. 6C) and *P* by 55 ± 11% (n = 6; $p < 0.05$; paired Student's *t* test) (Fig. 6D). Given that forskolin had no significant effect on *q* (Fig. 5E), these results suggest that forskolin increases both the number of readily releasable quanta and release probability.

Another way of assessing the release probability is to evaluate the speed of block of NMDA receptors by irreversible open channel blocker MK-801 (Rosenmund et al. 1993). Repeated activation of synapses in the presence of MK-801 results in a progressive decline in the amplitude of the NMDA receptor-mediated synaptic current and the rate of decay depends on the probability of transmitter release at synapses. If the probability of transmitter release is higher, a larger proportion of postsynaptic NMDA receptors is blocked at any one time, resulting in a faster decline of $EPSC_{NMDA}$ (Rosenmund et al. 1993). The $EPSC_{NMDA}$ was recorded in the presence of CNQX (20 μM) and at a holding potential of +40 mV. After confirming a stable baseline at a basal stimulus frequency of 0.1 Hz, the stimulation was stopped and

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MK-801 (40 μM) was applied. Stimulation was restarted 10 min later and the $EPSC_{NMDA}$ was recorded in the continuous presence of MK-801. From five such experiments, the time course of decline could be fitted by a double exponential function, with a fast time constant of 82 sec and a slow time constant of 186 sec. In the presence of forskolin (25 μM), the fast and slow time constants were 31 and 285 sec, respectively (Fig. 7). Forskolin also apparently increased the proportion of the fast component. Because *P* is inversely proportional to the decay time constant in the MK-801 experiments (Rosenmund et al. 1993), these results further support the proposal that forskolin increases *P*.

β-Adrenergic Receptor Agonist Isoproterenol Potentiates EPSCs. The final test was to determine whether activation of receptors that are positively coupled to elevate cAMP can mimic forskolin to potentiate synaptic transmission on the layer V pyramidal neurons of mPFC. The β-adrenergic receptor agonist isoproterenol has been shown to mimic the enhancement effect of cAMP elevation on synaptic transmission of many brain regions (Huang et al., 1996; Herrero and Sánchez-Prieto, 1996). Although the action of β-adrenergic receptors on the glutamatergic transmission of the mPFC region has not been established, β-adrenergic receptors have been shown to mediate many noradrenaline function in the mPFC (Bing et al., 1992) and noradrenaline has been reported to facilitate the release of glutamate from presynaptic terminals that synapse onto layer V pyramidal neurons of mPFC (Marek and Aghajanian, 1999). Thus, we conducted a series of experiments to test the hypothesis that activation of β-adrenergic receptors would induce a cAMP-mediated synaptic potentiation. As shown in Fig. 8A, bath application of isoproterenol (15 μM) for 20 min induced EPSC potentiation by 43.5 ± 4.3% of the control baseline (n

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= 5; $p < 0.05$; paired Student's *t* test). The response to isoproterenol was completely blocked by propranolol (20 μM), a selective β-adrenergic receptor antagonist, suggesting that this effect is indeed mediated by activation of β-adrenergic receptors (Fig. 8B). In addition, isoproterenol was still able to potentiate EPSCs in the presence of H-89 (1 μM), but the magnitude of potentiation was significantly smaller than that induced in the interleaved control condition ($p < 0.05$; unpaired Student's *t* test) (Fig. 8C). On average, EPSC slope measured 20 min after isoproterenol application was increased by 28.5 ± 3.5% (n = 5) in the presence of H-89. Coapplication of H-89 (1 μM) and PD98059 (50 μM) completely blocked isoproterenol-induced EPSC potentiation (n =5; 2.5 ± 1.2% of pre-isoproterenol baseline) (Fig. 8D). These data are consistent with the hypothesis that the enhancement action of β-adrenergic receptor activation on glutamatergic transmission in the mPFC is mediated by both PKA and p42/p44 MAPK signaling cascades.

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Discussion

We have, for the first time, systemically examined the role of cAMP elevation in the regulation of synaptic transmission on layer V pyramidal neurons of mPFC. Our results indicate that forskolin and membrane permeable cAMP analogs induce synaptic potentiation through presynaptic mechanisms. Most importantly, we provide pharmacological evidence that cAMP acts on both PKA and p42/p44 MAPK signaling pathways to enhance transmitter release from presynaptic nerve terminal. Furthermore, activation of β-adrenergic receptor with isoproterenol mimics forskolin to elicit a cAMP-dependent synaptic potentiation.

Presynaptic Locus of Expression of cAMP-Dependent Synaptic Potentiation. Various approaches were taken to determine the site of action of forskolin in enhancing transmission on layer V pyramidal neurons of mPFC. Based on these experiments, it is likely that forskolin-induced synaptic potentiation is primarily of presynaptic origin. Three lines of evidence support this conclusion. First, forskolin increases equally the AMPA receptor- and NMDA receptor-mediated component of EPSCs (Fig. 4, A and B). Second, the increase in synaptic transmission by forskolin was accompanied by a decrease in the synaptic failure rate, magnitude of CV, and PPR (Fig. 4D), which are generally considered to indicate a presynaptic mode of drug action (Zucker, 1989; Bekkers and Stevens, 1990; Steven and Wang, 1994). Third, forskolin significantly increased the frequency of mEPSCs but did not affect the amplitude of mEPSCs (Fig. 5). A change in the amplitude of mEPSCs is classically interpreted as a postsynaptic modification, whereas a change in their frequency is typically associated with mechanisms that increase the probability of transmitter release. Thus, the lack of effect of forskolin on the amplitude of mEPSCs also

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implies that forskolin-induced synaptic potentiation is not mediated by a change in postsynaptic sensitivity to glutamate.

Forskolin Increases the Number of Releasable Vesicles and Release Probability. Using three independent approaches, the paired-pulse stimulation, high-frequency stimulation and MK-801 protocols, we have shown that forskolin increase the release probability, *P*. The high-frequency stimulation protocol also indicates that forskolin increases the number of releasable vesicles, *N*. In cerebellar parallel-Purkinje cell synapse, the effect of forskolin has been attributed primarily to an increase in *P* (Chen and Regehr, 1997). At the calyx of Held, forskolin has also been proposed to facilitate transmitter release by increasing both *P* and *N* (Kaneko and Takahashi, 2004). cAMP also regulates release from dentate granule cells in the hippocampus by changing both *P* and *N* (Weisskopf et al., 1994). The fit of a double exponential function to the data in MK-801 experiments predicts vesicle populations having different release probability (Rosenmund et al., 1993). The finding that forskolin selectively accelerated the fast decay time constant and increased the relative proportion of the fast decaying component, suggesting that forskolin increase *P* and the proportion of vesicles with high *P* as reported previously (Kaneko and Takahashi, 2004).

Molecular Mechanism of cAMP-Mediated Synaptic Potentiation. Forskolin has been reported to possess many cAMP-independent actions, including the blockade of several types of potassium currents, which could result in prolongation of presynaptic action potentials and consequent increase in transmitter release (Hoshi et al., 1988).

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However, the cAMP-independent action of forskolin could be mimicked by its analog Dd-forskolin, which is unable to activate adenylyl cyclase. In our experiments, Dd-forskolin had no significant effect on EPSCs (Fig. 2, A and C). Thus, the effect of forskolin is not caused by its nonspecificity. This idea was also supported by the finding that nonhydrolyzable cAMP analog Sp-cAMPS mimicked forskolin to potentiate EPSCs (Fig. 3A). Consistent with this idea, we have found that the activation of β-adrenergic receptors that are coupled to Gs-proteins and activation of cAMP-dependent signaling pathways also elicit a cAMP-mediated synaptic potentiation (Fig. 8).

What is the molecular target of cAMP? Previous studies have shown that cAMP-dependent synaptic potentiation is mediated mainly by activating PKA in a variety of brain regions, including the hippocampus, amygdala, cerebellum, and striatum (Chavez-Noriega and Stevens, 1994; Huang et al., 1996; Salin et al., 1996; Chen and Regehr, 1997; Huang et al., 2002). In contrast, at the calyx of Held, presynaptic cAMP is proposed to facilitate synaptic transmission via activating Epac pathway (Kaneko and Takahashi, 2004). The cAMP-dependent potentiation of synaptic transmission at crayfish glutamatergic neuromuscular junctions is mediated by acting on Epac and HCN (Zhong and Zucker, 2005). However, we found that forskolin still caused synaptic potentiation when forskolin was applied in the presence of PKA, Epac or HCN inhibitors. Furthermore, although application of a selective Epac agonist 8CPT-2Me-cAMP potentiated EPSCs, it did not occlude the subsequent forskolin-induced synaptic potentiation. However, antagonists of PKA and p42/p44 MAPK each reduced forskolin-induced synaptic potentiation, and together they almost fully abolished the potentiation. Our findings that the effects of PKA and p42/p44 MAPK activation appear to be additive, suggesting the possibility that

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coincident activation of these two signaling pathway is required for the induction of cAMP-dependent synaptic potentiation on layer V pyramidal neurons of mPFC. The biological step downstream of PKA and p42/p44 MAPK responsible for the cAMP-induced synaptic potentiation remains to be determined. Given that forskolin increases both *N* and *P*, the target of these kinases seems to be in both the vesicular trafficking mechanism and the exocytotic mechanism. Indeed, at many synapses, activation of PKA has been shown to phosphorylate one or more proteins, either associated with, or part of, the protein complex that is necessary for the exocytosis of synaptic vesicles and underlies synaptic facilitation (Nagy et al., 2004). Activation of p42/p44 MPAK was also reported to facilitate glutamate release from rat brain synaptosomes by phosphorylating the synaptic vesicle membrane protein synapsin I, thereby regulating its interaction with the actin cytoskeleton, leading to the recruitment of releasable synaptic vesicles from a distal pool (Jovanovic et al., 2000).

Surprisingly, we found that the facilitatory effect of forskolin decreases with postnatal development (Fig. 2D). This developmental decline of forskolin-induced synaptic potentiation is not unique to excitatory afferents to layer V pyramidal neurons of mPFC; it was also reported at the calyx of Held (Kaneko and Takahashi, 2004). Although the molecular mechanism underlying this phenomenon remains unclear, a developmental decrease in the molecular target downstream of PKA and/or p42/p44 MAPK seems to contribute to this phenomenon. Further work, involving the use of functional knockout of candidate proteins, is needed to assess this hypothesis.

Recent results suggest that p42/p44 MAPK inhibitors have non-specific effects in modulating glutamate release. Pereira et al. (2002) showed that PD98059

inhibited glutamate release from hippocampal synaptosomes stimulated with KCl when used at concentrations that inhibited p42/p44 MAPK activity. U0126, however, did not significantly affect KCl-induced glutamate release at concentrations shown to inhibit p42/p44 MAPK activity. Thus, the non-specific effects of p42/p44 MAPK inhibitors may mask the forskolin response. This possibility, however, was ruled out by our observations that both PD98059 and U0126 were effectively to block forskolin-induced synaptic potentiation. Additionally, U0126 was also reported to block forskolin-induced increase in p42/p44 MAPK activation in hippocampal area CA1 (Selcher et al., 2003).

Physiological Significance. What is the physiological significance of cAMP-dependent synaptic potentiation in the mPFC? It was recently reported that PKA inhibitor can attenuate the induction of long-term potentiation (LTP) in the mPFC (Huang et al., 2004). Thus, our findings might be implicated in induction of long-term synaptic plasticity in the mPFC. The pyramidal neurons within the deep layers of mPFC integrate multiple excitatory and inhibitory inputs and send projections to many other brain regions (Heidbreder and Groenewegen, 2003). Through this network, the mPFC guides complex cognitive responses, such as working memory and the planning and execution of goal-directed behaviors (Goldman-Rakic et al., 1995; Fuster, 2000). Thus, potentiation of mPFC glutamatergic transmission by elevation of cAMP levels would expect to lead to alter these cognitive functions. Consistent with this prediction, infusion of the cAMP analog Sp-cAMPS into the PFC of young rats has been shown to produce a dose-dependent impairment in working memory performance on the

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delayed-alternation task (Arnsten et al., 2005). However, Aujla and Beninger (2001) have shown that cAMP/PKA inhibition in the PFC impaired working memory performance under condition that requires hippocampal interactions with PFC, suggesting that cAMP/PKA activation might be beneficial for working memory performance. Together these results suggest that the effects of PFC cAMP on working memory performance may follow an inverted-U-shape dose-response relationship (Arnsten et al., 2005). Much work remains to be done to verify this hypothesis. Additionally, other findings support a role for increased cAMP/PKA signaling in cocaine-induced long-lasting neuronal adaptation in PFC pyramidal neurons (Dong et al., 2005), which may be involved in the development of cocaine additive behaviors.

Conclusion. In summary, cAMP acts presynaptically, via activating the PKA and p42/p44 MAPK signaling pathways, to induce synaptic potentiation on layer V pyramidal neurons of mPFC. This enhancement is a result of increase in both release probability and number of releasable vesicles. The finding that β-adrenergic receptor activation mimics the forskolin action provides a major advance in establishing a role for more physiologically relevant stimuli in eliciting such synaptic modification. Given the importance of mPFC for cognitive functions, our findings may provide novel pharmacological strategies to treat human cognitive deficits in the future.

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Footnotes:

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Figure Legends

Fig.1. Pharmacological properties of EPSCs. A, photomicrograph of a coronal slice used for recording. The circle indicates the area typical used for recording. The location of the stimulation (S) and recording electrode (R) is also shown. B, an infrared microscope image of a representative layer V mPFC pyramidal neuron (N) in a slice from a 14-day-old rat. The patch recording pipette (P) can be seen on the right. C, Averaged EPSCs (5 consecutive sweeps) evoked in the layer V pyramidal neurons of mPFC by stimulating the layer II-III at 0.05 Hz in the presence of bicuculline methiodide (10 μM). Perfusing the slices with NMDA receptor antagonist D-APV (50 μM) significantly attenuated the duration and amplitude of EPSCs and further addition of the AMPA/kainate receptor antagonist CNQX (20 μM) completely abolished the synaptic currents.

Fig. 2. Potentiation of EPSCs by forskolin. A, a representative experiment showing time course of the action of forskolin and Dd-forskolin on the slope of evoked EPSCs on layer V mPFC pyramidal neuron. EPSCs were evoked every 20 s by a single pulse and were recording from a holding potential of -70 mV. Each data point represents a single response evoked before, during, and after the application of forskolin or Dd-forskolin. Bath application of forskolin (25 μM; ○) potentiated EPSCs, while the inactive analog Dd-forskolin (25 μM; ●) had no such effect. Sample traces are averages of five consecutive EPSCs recorded at time indicated by the numbers on the graph. Horizontal bars denote the period of delivery of forskolin or Dd-forskolin. B, the concentration-dependence of forskolin

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effect. Ordinate indicates the percentage of EPSC potentiation measured 20 min after forskolin application. Data are derived from 4-10 cells. C, a representative experiment showing time course of the action of forskolin and Dd-forskolin on the slope of evoked EPSCs on layer II-III mPFC pyramidal neuron at a holding potential of -70 mV. Bath application of forskolin (25 μM; ○) potentiated EPSCs, while the inactive analog Dd-forskolin (25 μM; ●) had no such effect. D, EPSC potentiation induced by forskolin (25 μM) at different postnatal ages. Data are derived from 5-10 cells. The potentiation at P21-P23 was significantly smaller than that at P8-P10.

Fig. 3. Coapplication of PKA and p42/p44 MAPK inhibitors blocks the forskolin-induced EPSC potentiation. A, summary of experiments (n = 5) showing that bath application nonhydrolyzable cAMP analog Sp-cAMPS increased EPSCs. B, summary of experiments showing that KT5720 (1 μM; n = 8; ○) or H-89 (1 μM; n = 5; ●) treatment partially blocked the forskolin-induced synaptic potentiation. C, summary of experiments showing that bath application of Epac agonist 8CPT-2Me-cAMP (10-100 μM) enhanced EPSCs in a concentration-dependent manner. Data are derived from 4-6 cells. D, summary of experiments (n = 5) showing that treatment of 8CPT-2Me-cAMP did not affect the forskolin-induced synaptic potentiation. E, summary of experiments showing that forskolin-induced synaptic potentiation was not significantly affected by prior treatment with either brefeldin A (BFA, 100 μM; n = 5; ○) or ZD7288 (30 μM; n = 5; ●). F, summary of experiments showing that PD98059 (50 μM; n = 8; ○) or

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U0126 (10 μM; n = 6; ●) treatment significant reduced the forskolin-induced synaptic potentiation. G, summary of experiments showing that forskolin-induced EPSC potentiation was completely abolished by prior coapplication of H-89 (1 μM) and PD98059 (50 μM) (n = 5; ○) or H-89 (1 μM) and U0126 (10 μM) (n = 4; ●). Sample traces are averages of five consecutive EPSCs recorded at time indicated by the numbers on the graph. H, histogram comparing the effects of different antagonists on the forskolin-induced synaptic potentiation. The magnitude of potentiation was measured 20 min after forskolin application. Data are taken from B, E, F, and G. *Asterisks* represent a significant difference from control group ($p < 0.05$).

Fig. 4. Forskolin increases both AMPA receptor- and NMDA receptor-mediated EPSCs and decreases the failure rate, coefficient of variance (CV), and paired-pulse ratio (PPR). A, average time course of the effect of forskolin (25 μM) on $EPSC_{AMPA}$ amplitude (n = 5). $EPSC_{AMPA}$ was recorded every 20 s by a single pulse stimulation in the presence of NMDA receptor antagonist D-APV (50 μM) at a holding potential of -70 mV. B, average time course of the effect of forskolin (25 μM) on $EPSC_{NMDA}$ amplitude (n = 5). $EPSC_{NMDA}$ was recorded in the presence of AMPA receptor antagonist CNQX (20 μM) at a holding potential of +40 mV. Sample traces are averages of five consecutive EPSCs recorded at time indicated by the numbers on the graph. C, plot of EPSC amplitude recorded at -70 mV holding potential against time, before, during, and after bath application of forskolin for 20 min, evoked at 0.1 Hz by minimal stimulation. It is clear

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that forskolin produced a significant decrease in the number of failures. At the end of experiment, CNQX (20 μM) and D-APV (50 μM) were applied to the bath to make sure that synaptic response was a glutamatergic EPSC. D, changes in failure rate, CV, and PPR associated with forskolin-induced synaptic potentiation (n = 6). PPR was calculated from responses to paired-pulse stimulation with an inter-pulse interval of 50 ms. *Asterisks* represent a significant difference from control group ($p < 0.05$).

Fig. 5. Effects of forskolin on mEPSCs. A, sample traces (three traces superimposed) of mEPSCs before (baseline: in the presence of 1 μM tetrodotoxin to block Na^+ channels and 100 μM $CdCl_2$ to block voltage-dependent Ca^{2+} channels) and after application of forskolin (25 μM). Lower traces are the averaged mEPSCs of 25 events each before and after forskolin application with increasing time resolution, demonstrating the lack of effect on the amplitude and kinetics of mEPSCs. B_1 and B_2, amplitude histograms of mEPSCs. The threshold for peak detection was set at -3 pA. Data were binned in 1 pA intervals. C, cumulative probability plots of mEPSCs before (solid line) and during (dashed line) application of forskolin ($p = 0.94$; Kolmogorov-Smirnov test). D, cumulative interevent interval distribution illustrating a significant decrease in the interevent interval (i.e., increased frequency; $p < 0.01$; Kolmogorov-Smirnov test) during forskolin application. E and F, summary of the effect of forskolin (25 μM) on the average amplitude and frequency of mEPSCs ($n = 5$). Data are presented as means ± S.E.M. *Asterisks* represent a significant difference from baseline ($p < 0.05$). The data shown in A, B, C, and D were taken from the

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same cell. Holding potential, -70 mV.

Fig. 6. Forskolin increases both the number of releasable vesicles and release probability. A, depressions of EPSCs during trains (20 stimuli) of 100 Hz stimulation before (control; ○) and after application of forskolin (25 μM; ●). Sample records of the EPSCs before and after forskolin application are shown (superimposed) in the inset. B, cumulative amplitudes of EPSCs during the 100 Hz train before (○) and after forskolin application (●). Amplitudes of EPSCs from sixteen-twentieth were fitted with a linear regression line and extrapolated to time zero for estimating the readily releasable pool size. C, mean number of releasable vesicles (*N*) multiplied by q significantly increased after forskolin application (n = 6). D, the mean release probability (*P*), which was estimated from the ratio of the first EPSC amplitude divided by *Nq*, underwent a significant increase after forskolin application (n = 6). *Asterisks* represent a significant difference from control group ($p < 0.05$).

Fig. 7. Forskolin accelerates the time course of blocking NMDA receptor-mediated EPSC ($EPSC_{NMDA}$) by MK-801. $EPSC_{NMDA}$ was evoked at 0.1 Hz at a holding potential of +40 mV in the presence of MK-801 (40 μM). Data are derived from a different group of cells, one group in the presence of forskolin (25 μM; ●; n = 5) and the other in its absence (○; n = 5). The numbers of sample traces (superimposed) indicate the sequence of stimulation. Ordinate indicates the amplitude of $EPSC_{NMDA}$ normalized to the initial amplitude. Abscissa indicates the time after starting stimulation

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in the presence of MK-801. Mean relative amplitudes derived each from five cells were fitted with double exponential functions.

Fig. 8. β-Adrenergic receptor agonist isoproterenol enhances synaptic transmission. A, summary of experiments (n = 5) showing that bath application isoproterenol (15 μM) for 20 min increased EPSCs. B, summary of experiments (n = 4) showing that isoproterenol-induced EPSC potentiation was completely abolished by prior treatment with β-adrenergic receptor antagonist propanolol (20 μM). C, summary of experiments showing that H-89 (1 μM; n = 5) treatment partially blocked the isoproterenol-induced synaptic potentiation. D, summary of experiments showing that isoproterenol-induced synaptic potentiation was completely abolished by prior coapplication of H-89 (1 μM) and PD98059 (50 μM) (n = 5). Sample traces are averages of five consecutive EPSCs recorded at time indicated by the numbers on the graph.

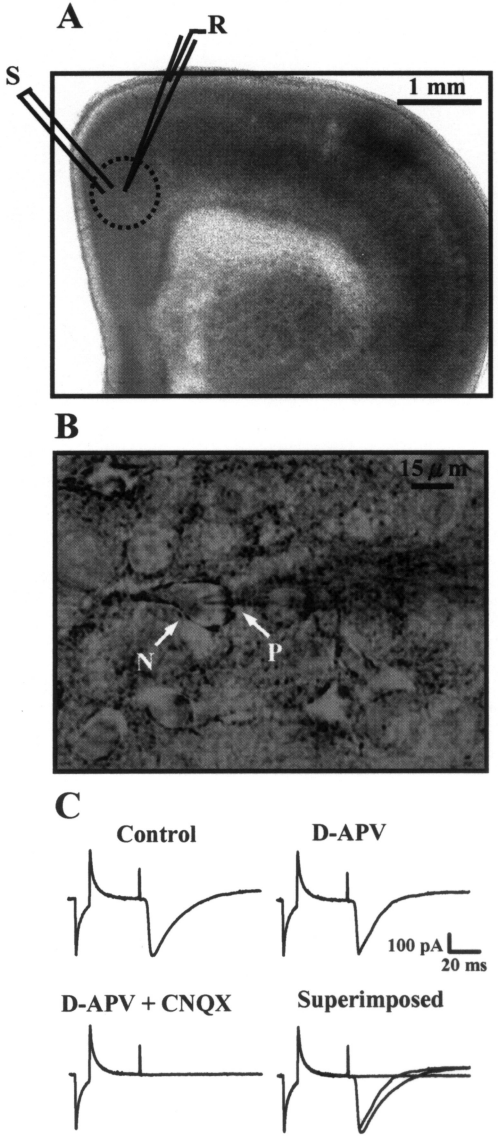
A
R
S
1 mm
B
15 μm
N
P
C
Control
D-APV
100 pA
20 ms
D-APV + CNQX
Superimposed

Figure 1

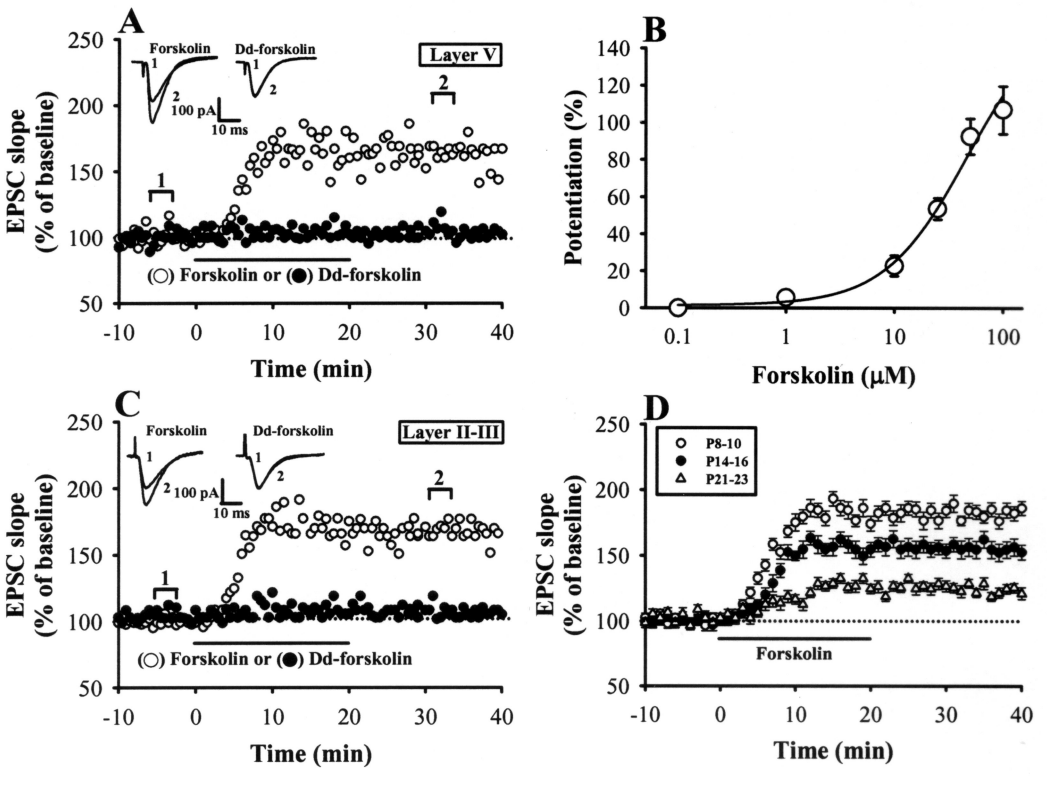
A
Forskolin
Dd-forskolin
Layer V
100 pA
10 ms
EPSC slope
(% of baseline)
(○) Forskolin or (●) Dd-forskolin
Time (min)
B
Potentiation (%)
Forskolin (μM)
C
Layer II-III
D
P8-10
P14-16
P21-23
Forskolin

Figure 2

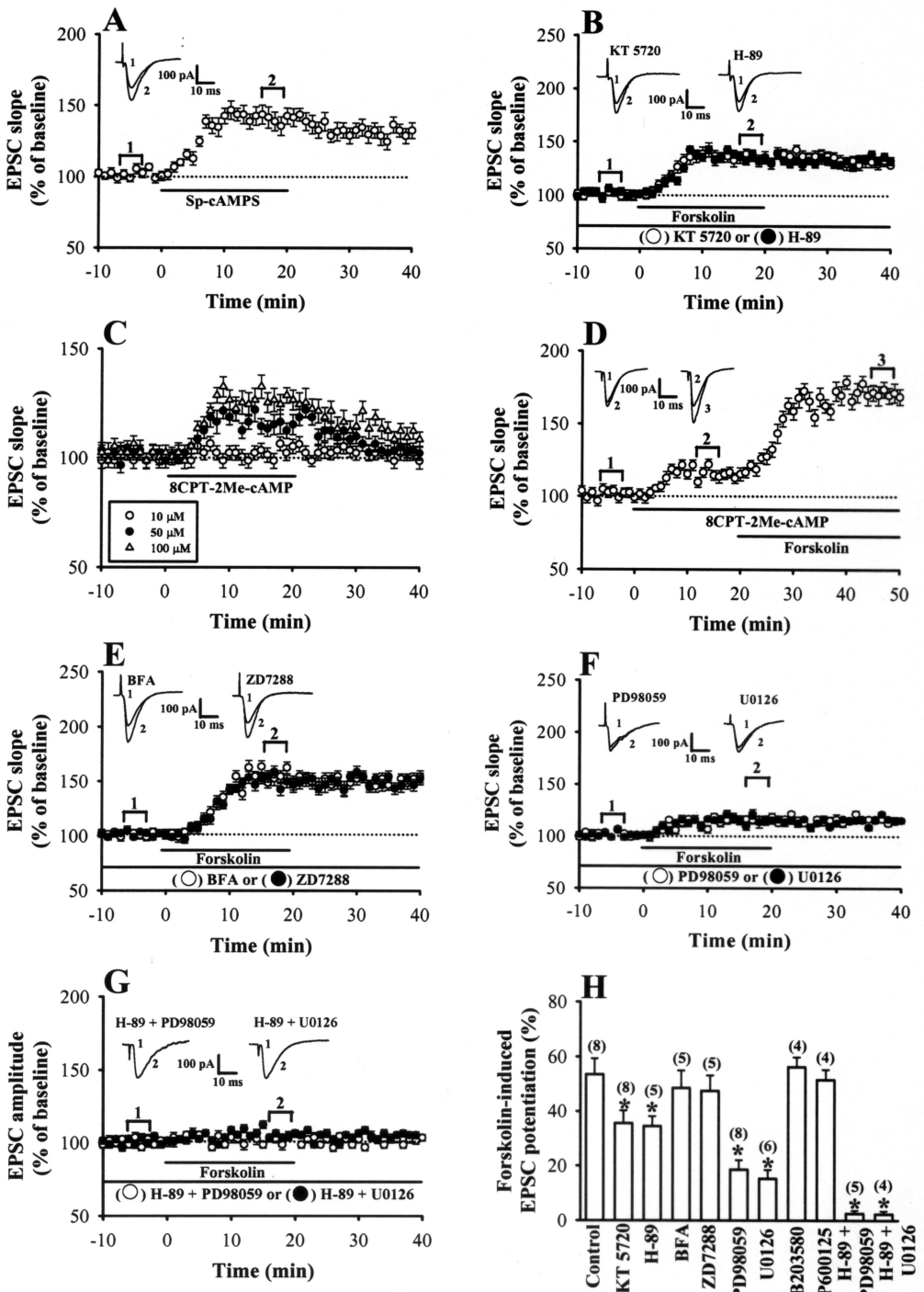
A
EPSC slope (% of baseline)
100 pA
10 ms
Sp-cAMPS
Time (min)
B
KT 5720
H-89
Forskolin
(○) KT 5720 or (●) H-89
C
8CPT-2Me-cAMP
10 μM
50 μM
100 μM
D
8CPT-2Me-cAMP
Forskolin
E
BFA
ZD7288
(○) BFA or (●) ZD7288
F
PD98059
U0126
(○) PD98059 or (●) U0126
G
EPSC amplitude (% of baseline)
H-89 + PD98059
H-89 + U0126
(○) H-89 + PD98059 or (●) H-89 + U0126
H
Forskolin-induced EPSC potentiation (%)
Control
KT 5720
H-89
BFA
ZD7288
PD98059
U0126
SB203580
SP600125
H-89 + PD98059
H-89 + U0126

Figure 3

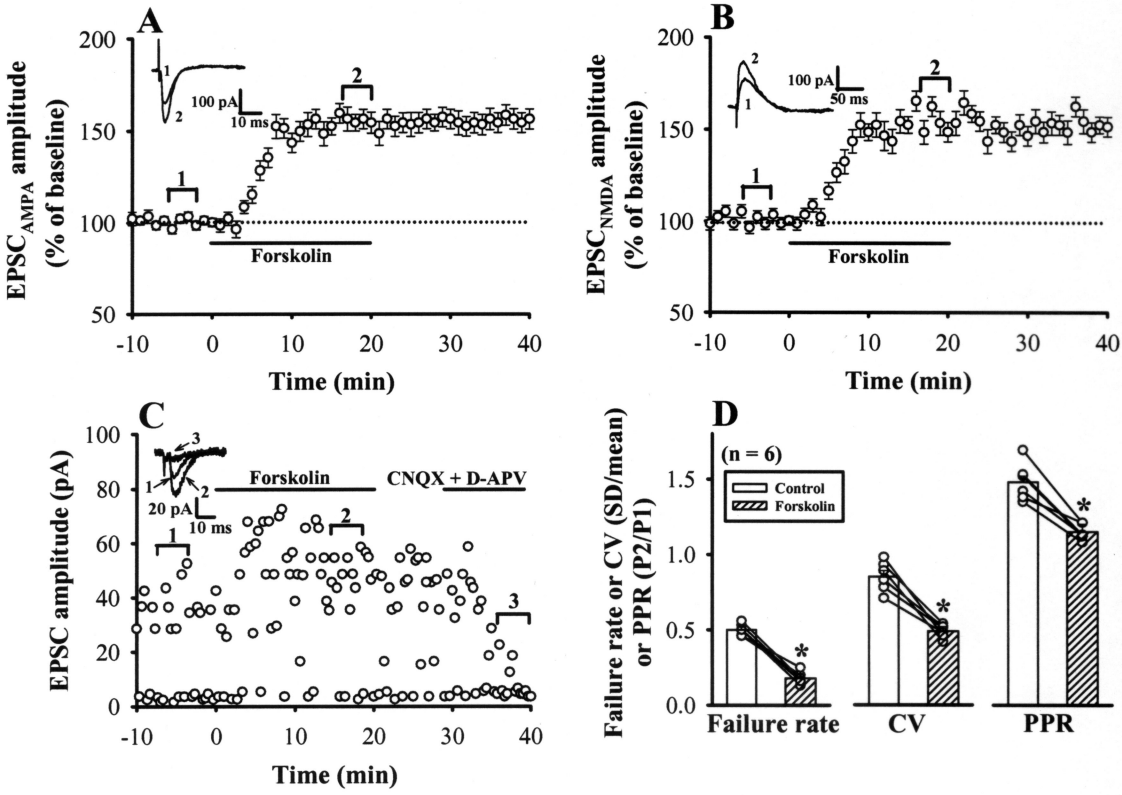
A
B
C
D
EPSC$_{AMPA}$ amplitude (% of baseline)
EPSC$_{NMDA}$ amplitude (% of baseline)
EPSC amplitude (pA)
Time (min)
Forskolin
CNQX + D-APV
100 pA
10 ms
50 ms
20 pA
(n = 6)
Control
Forskolin
Failure rate or CV (SD/mean) or PPR (P2/P1)
Failure rate
CV
PPR

Figure 4

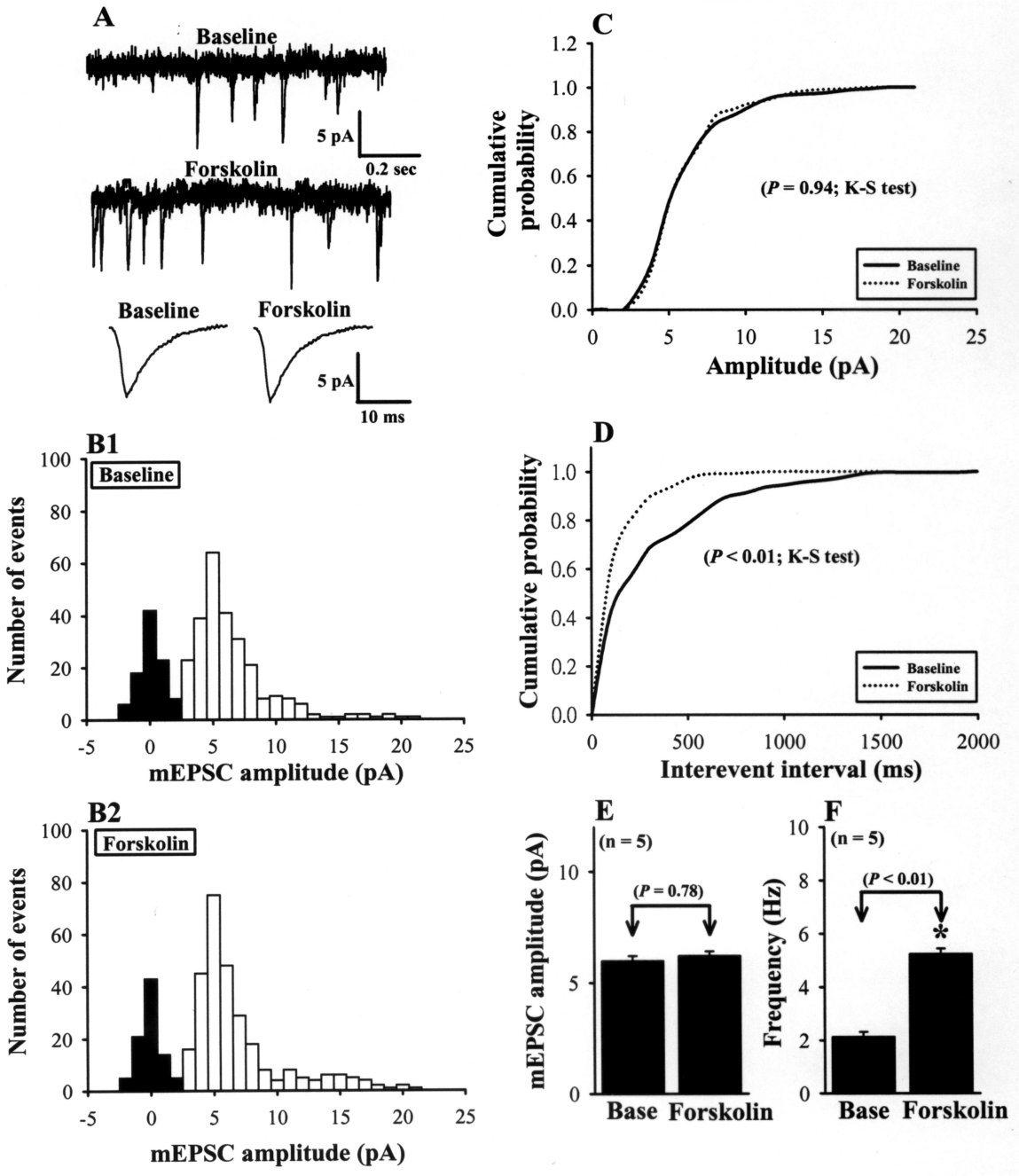
A
Baseline
5 pA
0.2 sec
Forskolin
Baseline
Forskolin
5 pA
10 ms
C
Cumulative probability
(P = 0.94; K-S test)
Baseline
Forskolin
Amplitude (pA)
B1
Baseline
Number of events
mEPSC amplitude (pA)
D
Cumulative probability
(P < 0.01; K-S test)
Baseline
Forskolin
Interevent interval (ms)
B2
Forskolin
Number of events
mEPSC amplitude (pA)
E
(n = 5)
(P = 0.78)
mEPSC amplitude (pA)
Base
Forskolin
F
(n = 5)
(P < 0.01)
*
Frequency (Hz)
Base
Forskolin

Figure 5

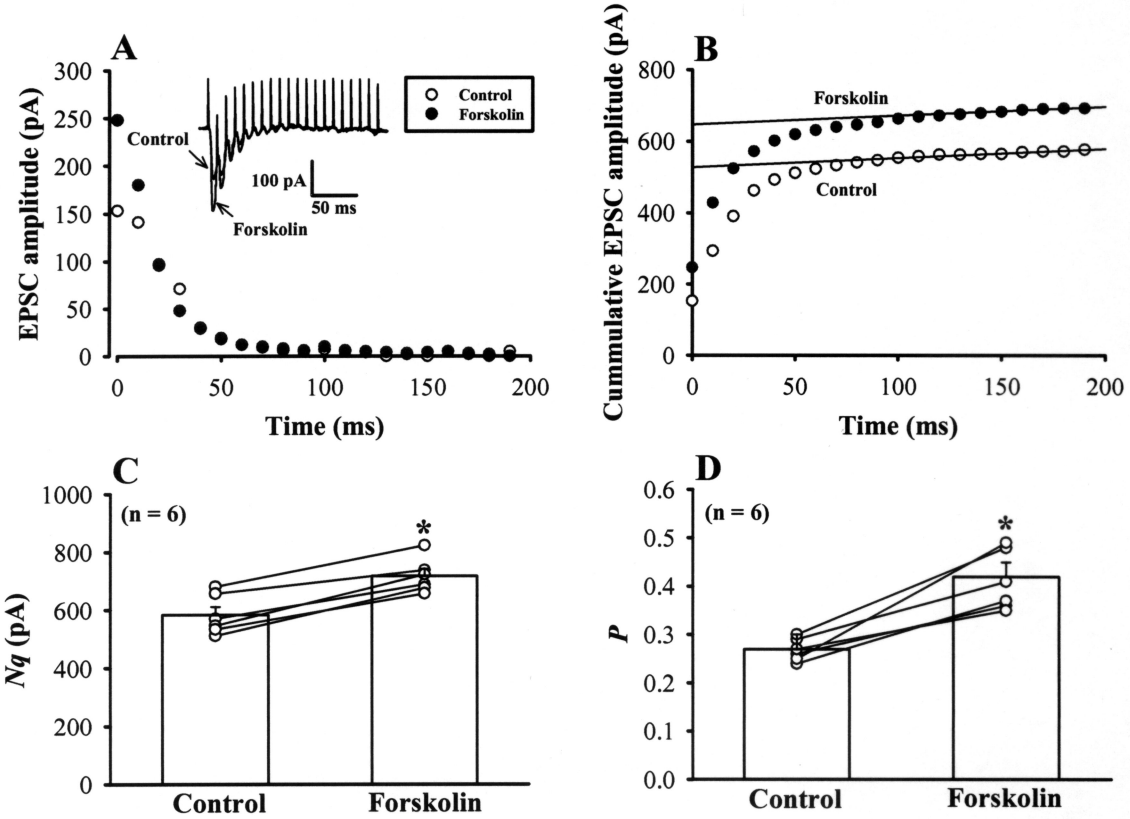
A
EPSC amplitude (pA)
300
250
200
150
100
50
0
Control
Forskolin
100 pA
50 ms
0
50
100
150
200
Time (ms)
B
Cummulative EPSC amplitude (pA)
800
600
400
200
0
Forskolin
Control
0
50
100
150
200
Time (ms)
C
(n = 6)
*
Nq (pA)
1000
800
600
400
200
0
Control
Forskolin
D
(n = 6)
*
P
0.6
0.5
0.4
0.3
0.2
0.1
0.0
Control
Forskolin

Figure 6

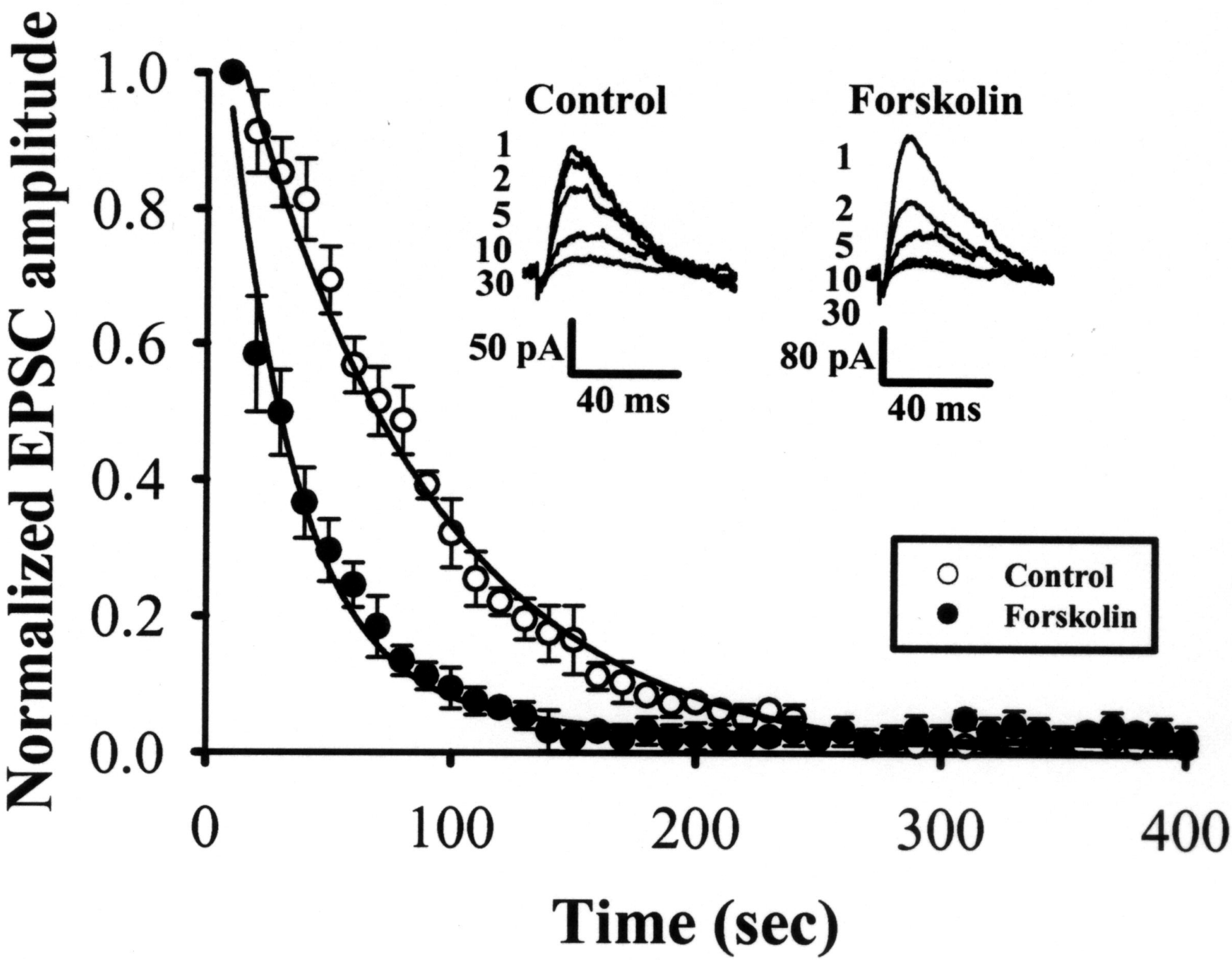
Normalized EPSC amplitude
1.0
0.8
0.6
0.4
0.2
0.0
0
100
200
300
400
Time (sec)
Control
Forskolin
1
2
5
10
30
50 pA
40 ms
80 pA
Control
Forskolin

Figure 7

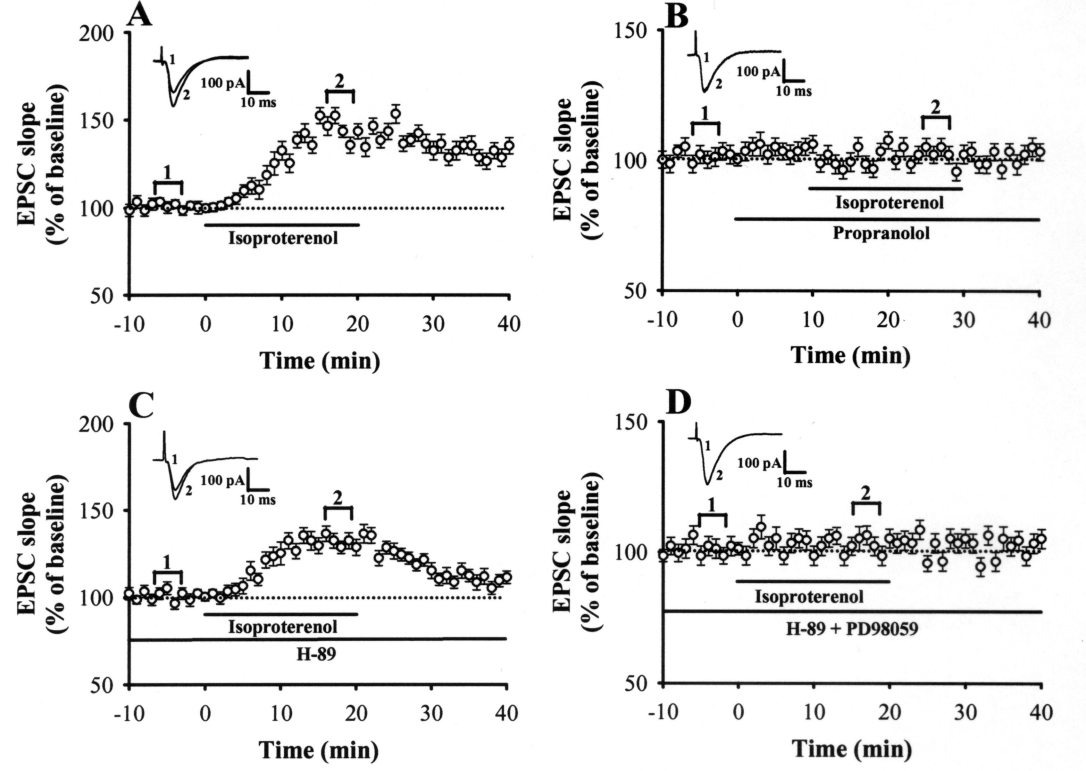
A
B
C
D
EPSC slope
(% of baseline)
Time (min)
100 pA
10 ms
1
2
Isoproterenol
Propranolol
H-89
H-89 + PD98059

Figure 8