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Postsynaptic mechanisms are essential for forskolin-induced potentiation of synaptic transmission

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Abbreviations

AMPA α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid

AP-5 D(-)-2-amino-5-phosphonovaleric acid

cAMP cyclic adenosine monophosphate

eEPSC(s) evoked excitatory postsynaptic current(s)

EP early phase (of the forskolin-induced potentiation)

EPSC(s) excitatory postsynaptic current(s)

LP late phase (of the forskolin-induced potentiation)

LTP long-term potentiation

mEPSC(s) miniature excitatory postsynaptic current(s)

NMDA N-methyl-D-aspartic acid

PKA protein kinase A

PKI protein kinase A inhibitor peptide, 6-22 amide

Rp-cAMPS adenosine 3,5-monoaminophosphorothioate, Rp-isomer

TTX tetrodotoxin

Abstract.

It has been demonstrated that stimulation of protein kinase A (PKA) results in enhanced synaptic transmission in the hippocampus and other brain areas. To investigate mechanisms of the PKA-mediated potentiation of synaptic transmission, we used rat hippocampal embryonic cultures. In low-density cultures, paired recordings under the perforated patch demonstrated that 15 min forskolin treatment produced long-lasting potentiation of evoked EPSCs (eEPSCs) mediated by the cAMP/PKA pathway. eEPSC amplitudes increased to 240±10 % of baseline after 15 min of forskolin treatment (early potentiation). After forskolin washout, eEPSCs declined to a potentiated level. Potentiation was sustained for at least 85 min after forskolin washout and, 60 min after forskolin washout, constituted 152±7% of baseline (late potentiation). Disruption of presynaptic processes with the whole-cell configuration and internal solution containing PKA inhibitor peptide did not affect forskolin-induced potentiation. Disruption of postsynaptic processes, in contrast, impaired early potentiation and abolished late potentiation. Study of mEPSCs confirmed the contribution of postsynaptic mechanisms. Forskolin-induced enhancement of mEPSC frequency observed under the perforated patch was attenuated by the whole-cell configuration. Forskolin also induced an increase of mEPSC amplitudes in the perforated patch, but not in the whole-cell, experiments. Potentiation of eEPSCs was not activity dependent, persisting in the absence of stimulation. NMDA receptor blockade did not abolish forskolin-induced potentiation. In summary, we demonstrate that forskolin-induced potentiation of eEPSCs was mediated by postsynaptic mechanisms, presumably via upregulation of AMPA receptors by phosphorylation.

Key words: protein kinase A, LTP, hippocampus, culture

Introduction.

Long-term potentiation (LTP) is a long-lasting, use-dependent increase of the effectiveness of synaptic transmission. Since LTP is believed to mediate memory formation, activity-induced LTP and its underlying mechanisms have been subjects of intensive research efforts. A substantial body of evidence implicates cAMP-dependent protein kinase (PKA) in the production and maintenance of LTP. For example, LTP induced by repeated tetanic stimulation of hippocampal neurons is accompanied by PKA activation (Huang and Kandel 1994; Roberson and Sweatt 1996). Inhibitors of PKA block or decrease LTP, and activators of PKA facilitate LTP in the CA3 and CA1 areas of the hippocampus (Frey et al. 1993; Huang and Kandel 1994; Huang et al. 1994; Matthies and Reymann 1993; Otmakhova et al. 2000). The role of PKA in LTP and memory has also been demonstrated using genetic approaches (Abel et al. 1997; Huang et al. 1995; Qi et al. 1996).

It is widely known that not only is PKA involved in LTP, but, when directly stimulated, it also produces a marked enhancement of synaptic efficacy (Carroll et al. 1998; Chavez-Noriega and Stevens 1994, 1992; Chen and Roper 2003; Greengard et al. 1991). Because of the involvement of PKA in LTP and memory, numerous attempts have been undertaken to elucidate mechanisms through which PKA might modify the synaptic strength. A large body of evidence suggests that the change of synaptic efficacy might be contributed by modulation of both presynaptic and postsynaptic PKA substrates. Early studies have demonstrated that PKA modulates the AMPA receptor function (Banke et al. 2000; Chavez-Noriega and Stevens 1992; Greengard et al. 1991; Roche et al. 1996; Wang et al. 1991; Wang et al. 1993). Activation of PKA is associated with an increase of

the number of functional release sites in cultured neurons isolated from a dissociated dentate gyrus, and in the CA1 area of the hippocampus (Kohara et al. 2001; Ma et al. 1999). PKA also increased a kainate-sensitive transmitter release in mossy fibers (Goussakov et al. 2000).

Despite a potential involvement of AMPA receptors, electrophysiological studies failed to demonstrate a contribution of postsynaptic mechanisms to the effect of PKA activators on synaptic transmission. For example, Carroll et al. have reported that forskolin dramatically increased the frequency of miniature excitatory postsynaptic currents (mEPSCs) in CA1 pyramidal neurons of the hippocampus but did not change mEPSC amplitudes (Carroll et al. 1998), leading to a conclusion about the presynaptic locus of forskolin action. A similar result has been reported by Chavez-Noriega and Stevens (Chavez-Noriega and Stevens 1994).

Analysis of synaptic currents obtained during electrophysiological recording has being performed using postulates of the classical quantal theory. Classical quantal theory was introduced by Del Castillo and Katz in their study of transmission at the neuromuscular junction (Del Castillo and Katz 1954). According to this theory, the frequency of mEPSCs at each synapse is determined by the probability of releasing a single quantum (interpreted as a vesicle) and the number of releasable quanta. At the neuromuscular junction, studied by Del Castillo and Katz, the number of quanta and active synapses remains unchanged throughout the experiment. Consequently, a change of mEPSC frequency has been thought to reflect a changed probability of neurotransmitter release.

The validity of some postulates on the nature of synaptic transmission, established by Del Castillo and Katz, remains questionable for central synapses. For example, the number of active synapses changes during some forms of LTP (Faber et al. 1991; Isaac et al. 1995). This change, via postsynaptic mechanisms, affects the frequency of mEPSCs, vitiating the conventional view that mEPSC frequency reflects only presynaptic changes. Thus, conclusions about the presynaptic origin of PKA-mediated changes at central synapses, derived from the analysis of mEPSCs, should be re-examined.

To further investigate the locus of expression of PKA-mediated potentiation, we examined the effect of forskolin on eEPSCs and mEPSCs in rat embryonic hippocampal cultured neurons. By manipulating the method used to gain a control over the cell membrane potential in patch clamp experiments (perforated patch or whole-cell voltage clamp), we demonstrate that forskolin-induced potentiation is mediated by postsynaptic mechanisms.

Experimental procedures.

Neuronal culture. Rat hippocampal embryonic cultures were prepared as described (Li et al. 1998). Briefly, hippocampi isolated from Wistar rat embryos at day 18 of gestation (E18) were treated with papain (20mM) for 15 min at 36°C, and then dissociated by trituration and plated on glass coverslips precoated with polyethyleneimine. Cells for mEPSC and paired recordings were plated at a density 200 cells/mm^2 (high-density cultures) and 140 cells/mm^2 (low-density cultures), respectively. Cultures were maintained at 36°C in a 5% CO_2 incubator and were grown in Neurobasal medium (Gibco) supplemented with B27 (Gibco, a complex of vitamins and neurotrophic factors), 0.5 mM Glutamax (Gibco) and glutamate each, and 5% horse serum. Low-density culture medium also contained 1.5 mM more Gln and 15 mM more KCl. All components of the culture medium were purchased from Invitrogen. As glia formed a monolayer 5-6 days after plating, an anti-mitotic agent 1-ß-D-arabinofuranosylcytosine (Sigma) was added to the medium to 0.05% final concentration to inhibit further glial growth. Half of the medium in the dish was then substituted by fresh serum-free medium once every week. Cultures used for paired or mEPSC recordings were 18-20 and 23-25 days old, respectively.

Electrophysiology. A glass coverslip with cultured neurons was placed into a perfusion chamber with a solution volume 0.2-0.5 ml. Perfusion rate was set at 0.5-1 ml/min. All experiments were done at room temperature. The following is a composition of the bathing solution used in experiments (in mM): NaCl 145, KCl 5, MgCl 0.5, $CaCl_2$ 2, HEPES 5, glucose 10, pH 7.4. For mEPSC recordings the bath was supplemented with 0.5 μM tetrodotoxin (TTX). When indicated, the superfusion medium contained 50

μmol/l forskolin. The pipette solution used for perforated patch experiments contained (in mM): potassium gluconate 150, NaCl 5, EGTA 0.1, HEPES 5, pH 7.2. Right before the experiment, Amphotericin B (Calbiochem) was dissolved in dimethylsulfoxide at a concentration 60 mg/ml. This Amphotericin B stock was added to the pipette solution to create 1.5 mg/ml final concentration. Electrode resistance was 4-6 MΩ. Access resistance stabilized during the first 10-15 min of experiments and ranged from 18 to 30 MΩ. Cells were voltage-clamped at -60 mV or -65 mV using Axopatch 200 or Axopatch 200A. The contribution of inhibitory currents during recordings of the tetrodotoxin-resistant spontaneous activity, was negligible, since the -80 mV membrane potential (-65 mV with the corrected liquid junction potential) was close to the reversal potential for chloride. In addition, picrotoxin (40 μM) did not significantly change the frequency of tetrodotoxin-resistant spontaneous currents (4 experiments, data not shown). Pipette solution for the whole-cell experiments contained (in mM): potassium gluconate 150, NaCl 5, $MgCl_2$ 2, $CaCl_2$ 0.1, EGTA 5, HEPES 5, PKA inhibitor peptide 7-22 (PKI, Calbiochem) 0.015, pH 7.2. Access resistance in the whole-cell experiments was in the range 15-25 MΩ. Access resistance did not change more than 15% in mEPSC experiments and more than 20 % - during paired recordings.

Whole-cell current in mEPSC experiments was filtered at 2 kHz and digitized at 10 kHz. mEPSCs were analyzed using a custom-made program. Asymmetric events with the rise time shorter than the decay time and amplitudes higher than 4 pA (the threshold for event detection) were chosen for the analysis. mEPSC frequency and amplitudes were established by analyzing a 90 - 300 s long records in the high-density and low-density cultures, respectively. Extending the analysis for longer periods in a number of

experiments did not significantly change the results. mEPSC frequency ranged between 1.3 and 19 Hz in high-density cultures and 0.15 and 2.6 Hz in low-density cultures. The average number of events analyzed for each record was 475 ± 66(SE) in high-density cultures and 118 ± 25(SE) in low-density cultures.

For paired recordings two connected neurons were voltage-clamped using perforated patch configuration. One of the neurons was stimulated at 30 s intervals by applying a 1 ms voltage step to +20 mV. Postsynaptic currents induced within 2-4 ms after the stimulus were recorded for 25-30 min before the forskolin treatment, to establish the baseline level of eEPSC amplitudes. The eEPSC signals were filtered at 1 kHz and digitized at 5 kHz. 80% series resistance compensation was employed in all paired recording protocols. After forskolin washout, paired recordings continued for as long as the gigaseal remained intact. Baseline level was calculated as an average of eEPSC amplitudes during the 10 min preceding the forskolin application. During and after forskolin treatment eEPSC amplitudes in each experiment were normalized to the baseline level. In each group of experiments, results of paired recordings are presented as normalized eEPSCs averaged over a 2 min period.

Western blot. Cells were plated at a density 200 cells/mm^2 in 35 mm Biocoat plastic dishes (BD, Franklin Lakes, NJ). After 24 days in culture cells were incubated with either a solution containing TTX, or a solution with TTX and forskolin, depending on the experiment. Whole-cell lysates were obtained by adding 400 μl lysis buffer to a dish. The following is a composition of the lysis buffer: 62.5 mM Tris pH 6.8, 1% SDS, 5 % mercaptoethanol. PMSF (at a 1 mM final concentration) and Complete Mini Protease inhibitor cocktail tablets (Roche) were added to the lysis buffer just before the

experiments to prevent protein degradation and inhibit phosphatase activity. After short incubation in the lysis buffer, samples were collected and passed through a 26-gauge needle 10 times (to shear genomic DNA). Samples were then boiled for 5 min and placed on ice or stored in a -70°C freezer until use. 25 μl of each sample were loaded onto a precast 4-15% gradient SDS-TrisHCl gel (Bio-Rad Laboratories). Proteins were electrophoretically separated at 120 V for 2 h, and then blotted onto a nitrocellulose membrane (Bio-Rad Laboratories) at 100V for 1h. Membranes were washed with Tris buffered solution (TBS) and blocked with 5% fat free dry milk (Carnation) in TBS for 30 min at room temperature. Blots were then incubated with rabbit anti-phospho-GluR1 (Ser845) (Upstate, 1:4000 dilution) or rabbit anti-GluR1 (Upstate, 1:10,000 dilution) antibodies in 0.1% Tween-TBS for 3 h at room temperature. For negative control, membranes were incubated with the Tween-TBS solution containing anti-phospho(Ser845)-GluR1 antibody and a saturating concentration (1 μM) of phospho-GluR1 immunizing peptide (Upstate). After two 5-min washes in Tween-TBS, blots were subjected to biotinylated anti-rabbit secondary antibodies (Vector Laboratories) for 1 h. After two additional washes HRP-conjugated streptavidin was applied (Vectastain ABC kit, Vector Laboratories) according to vendor instructions. Signal was detected using Vector NovaRED^{TM} substrate kit for peroxidase (Vector Laboratories).

All salts and forskolin were purchased from Sigma.

Results.

Perfusion with forskolin induced a long-lasting PKA-dependent potentiation of eEPSCs.

To investigate the effect of forskolin on eEPSC amplitudes we conducted dual-cell voltage-clamp experiments in isolated pairs of neurons in a low-density culture (see methods), using the perforated patch configuration. Perfusion with forskolin (50 μM) for 15 min substantially increased eEPSC amplitudes. The time course of the forskolin-induced changes of eEPSCs could be divided into three phases. The first, early phase of potentiation (EP) took place during forskolin treatment. It is characterized by a gradual rise of eEPSC amplitudes to 240±10 % of baseline 15 min after the start of forskolin treatment; the change was observed in all 6 pairs tested. The second phase of eEPSC changes consisted of a decline of eEPSC amplitudes during the first 10 min of forskolin washout. eEPSCs then stabilized (at a still potentiated level) marking the third, late phase of potentiation (LP). LP was sustained for at least 85 min after forskolin washout (5 of 6 tested cell pairs). 60 min after forskolin washout, eEPSC amplitudes averaged 152±7 % of baseline. The results of these experiments are summarized in Fig 1a.

To test whether forskolin action was mediated by the adenylyl cyclase/PKA pathway, neuronal cultures were subjected to Rp-cAMPS (50 μM) 1 h prior to forskolin treatment. This drug was continuously present in a perfusion medium during the experiments. Rp-cAMPS is a membrane-permeable non-hydrolyzable cAMP analogue which associates with the cAMP-binding site of PKA, thus blocking PKA activity. Rp-cAMPS abolished the LP (3 cell pairs), indicating that forskolin acted through the PKA

(Fig.1a). However, despite the presence of the PKA inhibitor, there was a 36±17% increase of eEPSC amplitudes during the EP (Fig 1a).

Forskolin increased frequency of mEPSCs.

It has been demonstrated that activation of PKA upregulates mEPSC frequency in very young hippocampal cultures (Greengard et al. 1991) and in pyramidal neurons of the CA1 area of the hippocampus (Carroll et al. 1998; Chavez-Noriega and Stevens 1994). To verify that this is the case in our system, we recorded mEPSCs in high-density cultures using the perforated patch configuration. To establish whether the increase of mEPSC frequency is long-lasting and whether it contributes to the forskolin-induced LP, we performed long-term recordings of mEPSCs. Baseline level of mEPSC frequency was measured just prior to forskolin treatment. 15 min of forskolin treatment (50μmol/l) resulted in an enhancement of mEPSC frequency to 445±47% of the baseline (5 experiments). This enhancement immediately after forskolin treatment was followed by a decline of mEPSC frequency to 163±67% of the baseline during the first 15 min after forskolin washout. Subsequently, mEPSC frequency declined more slowly, and reached the baseline level 45 min after the start of forskolin washout. There was no detectable increase of mEPSC amplitudes (results not shown). The results of these experiments are shown in Fig. 1b.

In control experiments, the time course of changes of mEPSC frequency was monitored in untreated cultures. mEPSC frequency remained unchanged for the first 30 min after being placed in the bathing solution, and then declined to 68±5% of baseline 60

min after the start of experiments (5 experiments, Fig 1b). Because access resistance changes could contribute to the observed decline of mEPSC frequency, in all the analyzed experiments access resistance of perforated patches was monitored and changed ≤ 15% throughout the experiment.

Whole-cell experiments reveal a postsynaptic locus of expression of the forskolin-induced potentiation.

To determine whether presynaptic or postsynaptic events are the main contributing factors to the forskolin-induced potentiation, we uncoupled intracellular events in one of the neurons using the whole-cell patch-clamp configuration. By including PKI (see methods) in an ATP-deficient pipette solution, we attempted both to inhibit events that resulted from cytoplasmic factors, and also to block the activity of the membrane-bound PKA.

In all 6 experiments done under the presynaptic whole-cell configuration, the EP of the forskolin-induced potentiation remained unchanged. During this phase, eEPSC amplitudes rose to 244±21% of baseline. In 4 of these 6 experiments neuronal pairs also displayed the LP at the level similar to the one observed under the perforated patch (153 ± 9 % of baseline 1 h after forskolin washout, 4 experiments, Fig 2a). In the other 2 experiments, the EP was followed by gradual depression of eEPSC amplitudes upon forskolin washout.

As opposed to the forskolin effect under the presynaptic whole-cell, under the postsynaptic whole-cell configuration both EP and LP were either impaired or abolished.

During forskolin perfusion, eEPSCs rose to only 146±11(SE) % of baseline (4 cell pairs) in these experiments. Forskolin washout was associated with a decline of eEPSC amplitudes back to the baseline level soon after the start of the washout. 60 min after forskolin washout, at times corresponding to LP, eEPSC amplitudes constituted 96±6(SE) % of baseline (4 experiments) (Fig 2a).

In control experiments using either the presynaptic or postsynaptic whole-cell configuration, we monitored eEPSCs in untreated cultures (2 experiments in each of configurations). These experiments demonstrated that a disturbance produced by the whole-cell configuration, by itself, does not affect basal synaptic transmission, making it possible to conduct long-term experiments using the whole-cell configuration (Fig. 2b).

Whole-cell configuration attenuated forskolin-induced enhancement of mEPSC frequencies.

To verify if postsynaptic events contributed to the forskolin-induced upregulation of mEPSC frequency, we used the same strategy for disrupting postsynaptic events as in the previous set of experiments. A neuron in a high-density culture was voltage-clamped in the whole-cell configuration. The internal solution was supplemented with PKI. Under these conditions, 15 min forskolin treatment induced an enhancement of mEPSC frequency to 275±18(SE) % of baseline. mEPSC frequency then declined upon forskolin washout, reaching the baseline level 45 min after the start of washout. Thus, the time course of mEPSC frequency changes, produced by forskolin in these experiments, was similar to the one under the perforated patch. However, the increase of mEPSC frequency

during forskolin treatment was significantly lower than in perforated patch experiments ($P<0.01$).

It has been noted in earlier studies that, under the whole-cell configuration, miniature events undergo a substantial rundown, presumably due to the washout of essential cytoplasmic components (Wang et al. 1991). This baseline mEPSC rundown under the whole-cell configuration could contribute to an impairment of the forskolin-induced upregulation of mEPSC frequency. Indeed, control experiments demonstrated that mEPSC frequency decreased by 37±8% of baseline during the first 30 min after formation of the whole-cell configuration (8 experiments). During the next 15 min, however, the decline of mEPSC frequency was negligible (4.5±8 % of baseline, 8 experiments) (Fig 3a). To minimize a contribution of mEPSC rundown under the whole-cell configuration, forskolin was applied 30 min after the whole-cell formation, when the level of AMPA receptor rundown was minimal. The mEPSC frequency just prior to forskolin application was considered a baseline. In these experiments the increase of mEPSC frequency after 15 min of forskolin treatment was 294±25 % (5 experiments), similar to that in the previous set of experiments (275±18 %).

Factors, other than differing patch-clamp configurations, could also contribute to the differing effect of forskolin on mEPSC frequency. Such factors are developmental differences or variability of the cell density between dishes or batches of neurons. To avoid a contribution of these artifacts we examined the effect of forskolin on mEPSCs while recording simultaneously under the perforated patch and whole-cell using dual-electrode voltage-clamp. In these experiments 30 min was allowed between the whole-cell formation and forskolin application. The resulting increase of mEPSC frequency in

each individual experiment was consistently and significantly higher in neurons under the perforated patch compared to those under the whole-cell configuration (1.45±0.11 times, $p<0.05$, 3 experiments). Examples of traces recorded during these experiments are shown in Fig 3b.

Paired-pulse facilitation.

Paired-pulse facilitation (PPF) was not systematically studied, mainly because the double-stimulus protocol occasionally led to the upregulation of baseline eEPSCs. In addition, during baseline recording, presynaptic neurons under the whole-cell configuration did not express PPF. This contrasts with the neurons under the perforated patch, 6 out of 8 of which expressed PPF. The average PPF in the perforated patch experiments was 1.65 ± 0.24 (8 experiments, 50 ms inter-pulse interval). Similarly, in experiments with the postsynaptic neuron under the whole-cell, the average PPF was 1.74 ± 0.22 (8 experiments). None of the 6 presynaptic neurons under the whole-cell configuration expressed PPF. The average ratio of responses to the paired-pulse stimuli in these experiments was significantly lower than in two other sets of experiments (0.85 ± 12, $p<0.01$). Contrasting PPF in experiments using different patch-clamp configurations affirm that presynaptic terminals under the whole-cell were affected by the dialyzing conditions of the experiment, presumably by PKI present in the pipette solution.

Forskolin-induced potentiation was independent of NMDA-receptors but associated with upregulation of AMPA receptors.

To verify whether forskolin treatment produced phosphorylation of the GluR1 subunit of AMPA receptors, we used Western blot analysis. Western blots of the whole-cell protein extracts, electrophoretically separated using SDS-PAGE, were immunostained with the anti-phospho-GluR1 antibody specific for phosphorylated Ser845 (anti-P-GluR1Ser845 antibody). High-density cultures were treated with forskolin for 15 min, and then lysed 0 or 45 min after the treatment. Control sample was obtained from untreated cultures. The 45 min period was chosen because it represents the approximate period of time that the mEPSC frequency, initially up-regulated by forskolin, returned to the baseline level.

The presence of an extra band only in the lane corresponding to cultures lysed immediately after forskolin treatment indicated that forskolin produced a new, short-lived phosphorylation state of the GluR1 (Fig. 4a, top panel). Specificity of the forskolin-induced extra band was demonstrated by incubating the blots with the antibody-containing medium supplemented with the anti-Ser845 phospho-GluR1 immunizing peptide (at a saturating concentration, 1μM). Under these conditions, the forskolin-induced extra band did not appear in the Western blot (Fig 4a, middle panel).

It has been reported that PKA increased the peak open probability of the AMPA receptor channel opening (Banke et al. 2000). This effect is expected to up-regulate macroscopic currents through AMPA receptors, including mEPSCs. Despite an apparent increase of GluR1 phosphorylation as a result of forskolin treatment, the expected increase of mEPSC amplitudes was not observed in dense cultures (data not shown). We hypothesized that the increase of mEPSC amplitudes was undetectable due to the problems of dendritic filtering and a low signal-to-noise ratio in the dense cultures. To

obviate these problems we monitored changes of mEPSC amplitudes in low-density cultures. The filtering effect in such a system must be negligible, since a neuronal dendritic tree in low-density cultures is formed by few short dendrites.

Average mEPSC amplitude in low-density cultures in the perforated patch experiments increased significantly as a result of forskolin treatment (by 29 ± 3 %, $p<0.001$, 3 experiments, two-sample t-test). This effect of forskolin on mEPSC amplitudes was disrupted by the whole-cell patch-clamp configuration. Analysis of mEPSC amplitudes as cumulative probabilities also demonstrated that, under the perforated patch, mEPSC amplitudes were significantly upregulated after 15 min forskolin treatment ($p<0.05$, Kolmogorov-Smirnov test, Fig 4b). The change of mEPSC amplitudes was short-term and reversed upon forskolin washout.

To further investigate the mechanism of the forskolin-induced potentiation, we aimed at establishing whether synaptic activity during and after forskolin treatment was essential for the effect of the drug on eEPSCs. For this purpose, after about half an hour of baseline eEPSC recording, paired stimulation was paused and was not resumed until 40 min after forskolin washout. Under such conditions, eEPSC amplitudes, at times corresponding to LP, were even higher than those in experiments on continuously active neuronal pairs (211±17 % of baseline, 3 experiments, vs. 152±7%, 5 experiments).

To decide whether NMDA receptors contributed to the forskolin-induced potentiation, we treated cultures with the NMDA-receptor blocker D(−)-2-Amino-5-phosphonopentanoic acid (AP-5) (50 μM) for at least 1 h prior to experiments. During the experiments cultures were superfused with forskolin (50 μM) as well as with AP-5 (50 μM). Both the EP and LP of the forskolin-induced potentiation of eEPSCs in these

experiments were unaffected by the NMDA-receptor blocker. With the background of AP-5, eEPSC amplitudes were 162±7% (3 experiments) 60 min after forskolin washout. The results of these experiments are presented in Fig 4c.

Discussion.

In the present study, by comparing patch-clamp results in perforated and whole-cell configurations, we demonstrated that forskolin produced a PKA-dependent LTP of synaptic transmission via postsynaptic mechanisms.

Our strategy was to investigate the effect of forskolin in neurons minimally perturbed by experimental conditions, and to compare this effect to that in neurons dialyzed from the inside using the whole-cell patch-clamp configuration. Under the perforated patch, most of intracellular molecules, including divalent ions, remain intact (Rae et al. 1991). The whole-cell configuration, in contrast, is associated with the washout of intracellular components, including cytoplasmic proteins and ATP, and buffering intracellular calcium by EGTA present in the pipette solution (Marty & Neher, 1995). Such conditions are expected to perturb, if not abolish, intracellular processes relying on cytoplasmic factors. To inhibit the activity of the membrane-bound PKA, which might not be washed out under the whole-cell configuration, we used an ATP-deficient electrode solution supplemented with PKI.

It was essential to demonstrate that long-term changes of eEPSCs under the whole-cell configuration were not associated with the absence of ATP and the presence of PKI in the pipette solution. In a control set of experiments, there was no significant change of eEPSC amplitudes for a prolonged period ($\geq$ 80 min after the whole-cell formation) when either the presynaptic or the postsynaptic cell was dialyzed by the whole-cell configuration (Fig. 2 b). This result does not support the idea that AMPA receptor-mediated currents are stabilized by internal ATP. The role of ATP was emphasized in the study by Wang et al. (1991). That group reported a partial rundown of

the whole-cell currents evoked by pressure application of 250 μM kainate to the soma of hippocampal cultured neurons. This rundown was prevented by supplementing of the internal solution with ATP-regenerating agents. The difference in the stability of evoked currents in our experiments and those done by Wang et al. could be explained by the difference in the nature of the recorded currents. The relative contribution of the extrasynaptic and synaptic receptors in the study by Wang et al. is unknown.

It is worth pointing out the difference between experimental conditions in the present study and other studies involving forskolin. First, 3-isobutyl-1-methylxanthine (IBMX), a phosphodiesterase inhibitor that enhances the effect of forskolin, was omitted from our experiments. Second, the experiments were conducted in the absence of a $GABA_A$ receptor inhibitor, routinely used in other studies (Carroll et al. 1998; Chavez-Noriega and Stevens 1994; Chen and Roper 2003; Duffy and Nguyen 2003). Previously, our group has demonstrated that picrotoxin, presumably acting through the $GABA_A$ receptors, reversed a facilitating effect of Sp-cAMPS on synaptic transmission in the CA1 area of hippocampal cultured slices (Yu et al. 2001). Sp-cAMPS is a membrane-permeable analogue of cAMP which activates PKA by directly binding to its regulatory subunit.

In experiments using the perforated patch, 15 min forskolin treatment (50μM) consistently produced an enhancement of eEPSC amplitudes. Each of the 6 tested neuronal pairs exhibited EP of eEPSCs, and 5 of 6 tested pairs exhibited LP of eEPSCs following drug application. That the LP was mediated by the cAMP/PKA pathway is indicated by the fact that it was blocked by Rp-cAMPS. Rp-cAMPS is a membrane-permeable non-metabolizable analogue of cAMP which prevents PKA activation. In

contrast to LP, forskolin-induced EP was not completely abolished by Rp-cAMPS (36±17% increase of eEPSC amplitudes when both forskolin and Rp-cAMPS were present in the perfusion solution, Fig 1a). Upregulation of eEPSCs during forskolin perfusion, despite the presence of Rp-cAMPS, could be explained by the PKA-independent effect of forskolin on synaptic transmission. Such an effect might be attributed to the ability of forskolin, a lipophilic substance, to incorporate into the plasma membrane and/or directly effect certain types of membrane ion channels (Ono et al. 1995; Su et al. 2001).

Results of experiments with Rp-cAMPS were used to quantify a PKA-mediated component of the forskolin-induced changes of eEPSCs during EP. The PKA-mediated component was determined by subtracting a PKA-independent effect of forskolin from the total effect of the drug. The results of the effect of forskolin on eEPSC amplitudes and mEPSC frequency in experiments using different patch-clamp configurations (summarized in Table 1) indicate that forskolin-induced EP and LP of eEPSCs are contributed by modulation of postsynaptic proteins. Indeed, when presynaptic events were uncoupled by the whole-cell configuration, both EP and LP of eEPSCs were equivalent to those in perforated patch experiments (144± 21% (EP) and 53±9% (LP) vs. 140±10% (EP) and 52±7% (LP) increase of eEPSC amplitudes under the presynaptic whole-cell and perforated patch, respectively). Postsynaptic whole-cell, to the contrary, abolished both the PKA-dependent component of EP, and LP (10±20% (EP) and -4±6% (LP) vs. 104±20% (EP) and 52±7% (LP) PKA-dependent potentiation of eEPSC amplitudes under the postsynaptic whole-cell and perforated patch, respectively). A

similar conclusion about the mechanism of the late potentiation of eEPSCs by forskolin in hippocampal slices was made by Duffy and Nguyen (Duffy and Nguyen 2003).

It is highly improbable that the ineffectiveness of the presynaptic whole-cell configuration in disrupting eEPSC potentiation was due to the inability of PKI to reach presynaptic terminals as a result of its limited diffusion. In the low-density culture used for paired recordings, neuronal network is formed by few closely situated neurons with short processes. 25-30 min allowed from the moment of the whole-cell formation to the start of forskolin application is enough time for dialyzing even extensive axonal arbors or long dendritic trees in slice experiments. That the forskolin effect remained unchanged by the presynaptic whole-cell could not be explained by merely the ineffectiveness of PKI. Even if the drug was not included in the pipette solution, the lack of ATP and substrates for the endogenous ATP-regenerating machinery in the pipette solution is expected to uncouple, if not impair, phosphorylation events. The most convincing evidence of the effectiveness of the presynaptic whole-cell in dialyzing presynaptic terminals is the changed pattern of synaptic responses to the paired-pulse stimulation. Presynaptic whole-cell configuration disrupted PPF, which was consistently present in neuronal pairs under other patch-clamp configurations.

In a minority of experiments (1 of 6 experiments under the perforated patch and 2 of 5 experiments under the presynaptic whole-cell configuration) forskolin failed to produce LP. This variability in the long-term effect of forskolin might be explained by the differential effect of the drug on various types of neurons present in culture. Indeed, similar to the mature hippocampal formation, developed neuronal cultures might contain excitatory neurons of at least two types - pyramidal neurons and granule cells of the

dentate gyrus. Thus, variability of the long-term effect of forskolin on eEPSCs could be ascribed to the differences in regulation by PKA of the synaptic transmission in these two neuronal phenotypes.

The conclusion about the postsynaptic nature of the forskolin-induced EP is supported by experiments on mEPSCs. The increase of mEPSC frequency during perfusion with forskolin was attenuated when the postsynaptic cell was under the whole-cell configuration. We attempted to address the concern that this attenuation could be due to the rundown or loss of sensitivity of AMPA receptors during the whole-cell recordings (Wang et al. 1991), rather than due to a disruption of postsynaptic phosphorylation. To avoid distortion by mEPSC rundown under the whole-cell configuration, forskolin was applied 30 min after the whole-cell formation, at which time the decline of mEPSC frequency was minimal (Fig 3a). The upregulation of mEPSC frequency by forskolin applied 30 min after the whole-cell formation was still less pronounced than that in perforated patch experiments (194±25% vs. 345±47% increase under the whole-cell and perforated patch, respectively, Fig 3a). Such an impairment of the forskolin effect would not be expected if the increase of mEPSC frequency took place exclusively as a result of the presynaptic processes, because presynaptic neurons remained intact during mEPSC recordings. The results of these experiments suggest that almost half the increase of mEPSC frequency was contributed by postsynaptic mechanisms.

From the point viewpoint of classical quantal theory, existence of the postsynaptic component of the forskolin-induced enhancement of mEPSC frequency seems paradoxical. Classical quantal theory is based on the assumption that the total number of active synapses in a preparation remains unchanged. Hence, variability of mEPSC

frequency would originate only from a changed probability of transmitter release. The assumption about the constancy of active synapses, however, has been disproven for central synapses. For example, in the hippocampus, one of the mechanisms of synaptic potentiation is induction of postsynaptically silent synapses (Faber et al. 1991; Isaac et al. 1995).

The same mechanism might have contributed to the forskolin-induced potentiation of mEPSC frequency observed under the perforated patch. Although the role of PKA in the induction of silent synapses has not been demonstrated, its role in membrane targeting of AMPA receptors is well established. For example, phosphorylation by PKA of the GluR4 AMPA receptor subunit was necessary and sufficient for its incorporation into synapses (Esteban et al. 2003). In addition, PKA regulates GluR1 recycling between the plasma membrane and endosomal compartments in dissociated hippocampal neurons (Ehlers 2000).

Our data suggest that the enhancement of mEPSC frequency by 194±25%, observed under the whole-cell configuration, was contributed by presynaptic mechanisms. One general class of such mechanism, an upregulation of the probability of spontaneous transmitter release, might result from accelerated vesicle cycling at the ready releasable pool of neurotransmitter. Such an effect of PKA activation has been demonstrated in cerebellar granule cells (Chavis et al. 1998). Another class of presynaptic mechanism, the induction of new or silent release sites, has been reported for hippocampal cultured neurons (Kohara et al. 2001; Ma et al. 1999). This effect of forskolin, however, was demonstrated in young (<14 days) or pharmacologically silenced neurons, and was absent in more mature non-silenced neurons. Thus, induction of new or

silent release sites could not contribute to the enhancement of mEPSC frequency in our experiments.

The conclusion regarding the presynaptic component of the forskolin-induced upregulation of mEPSC frequency should be distinguished from our results with paired recordings, which reveals little or no presynaptic contribution. We note that increased spontaneous, calcium-independent transmitter release, observed during mEPSC recording, does not necessarily contribute to stimulus-induced, calcium-dependent transmitter release, which determines eEPSC amplitudes. For example, enhancement of mEPSC frequency might result from accelerated vesicle cycling and a subsequent increased availability of synaptic vesicles at the ready releasable pool of neurotransmitter. However, only if vesicle availability is a limiting factor in evoked transmitter release will such a mechanism change eEPSC amplitudes. Vesicle availability may not be limiting in our experiments using a moderate rate of stimulation (once every 30 s). The limiting factors for the induced transmitter release during paired recordings might rather be the amplitude of the stimulus-induced calcium transients at the presynaptic release sites, or the effectiveness of these calcium transients to trigger synaptic vesicle exocytosis (reviewed by (Atwood and Karunanithi 2002).

Having established the role of postsynaptic events, we attempted to address whether modulation of glutamate receptors was one of those postsynaptic events contributing to the forskolin-induced upregulation of eEPSCs and mEPSCs. PKA is targeted to AMPA receptors by means of the A kinase anchoring protein and other scaffolding proteins of the postsynaptic density (Colledge et al. 2000). Phosphorylation by PKA of the GluR1 AMPA receptor subunit at Ser 845 increased the peak open

probability of the receptor channel opening, a change expected to increase macroscopic current through these receptors (Banke et al. 2000).

Despite ample evidence that AMPA receptors are targeted by PKA, activation of this enzyme did not increase mEPSC amplitudes in CA1 pyramidal neurons treated with forskolin (Carroll et al. 1998). We also found no detectable increase of mEPSC amplitudes in experiments in high-density cultures (data not shown). We hypothesize that the failure to detect potentiation of mEPSC amplitudes in mature neurons could arise from two factors: low signal-to-noise ratio of mEPSC recordings and dendritic filtering. This hypothesis is supported by the report that, in very young hippocampal cultures (2-7 days *in vitro*), in which the dendritic filtering is minimal, forskolin did potentiate mEPSC amplitudes (Greengard et al. 1991). To avoid the problems of low signal-to-noise ratio and dendritic filtering, we studied the effect of forskolin on mEPSCs in low-density cultures. Neurons in such cultures possess only few, relatively short processes, providing mEPSC records devoid of most dendritic filtering. We have now demonstrated that, in such low-density cultures, PKA activation with forskolin enhanced mEPSC amplitudes. As expected, this effect of forskolin was disrupted by the whole-cell configuration (Fig 4b).

The differential effect of forskolin on mEPSC amplitudes in high- and low-density cultures might arise from developmental differences in these cultures, as well as from the electrophysiological considerations discussed above. For example, GluR1 AMPA receptors may be constitutively and maximally phosphorylated by PKA in a mature hippocampus (Carroll et al. 1998; Mammen et al. 1997). If this also applied to the high-density cultures, it could explain the absence of the forskolin effect on mEPSC

amplitudes. However, our Western blot immunostaining argues against constitutive and maximal level of phosphorylation of GluR1 AMPA receptors. As revealed with the anti-P-GluR1Ser845 antibody, forskolin treatment of high density cultures resulted in an extra band in the Western blot of electrophoretically separated whole-cell lysates indicating increased phosphorylation of the GluR1 subunit (Fig 4a). An increase of the anti-P-GluR1Ser845 signal following forskolin treatment has also been demonstrated in mature rat hippocampal slices (Lee et al. 1998).

It is widely acknowledged that activation of the calcium and calmodulin-dependent protein kinase II (CaMKII), mediated by NMDA receptors, is necessary for activity-induced LTP in the hippocampus (Bliss and Collingridge 1993; Silva et al. 1992). We tested whether forskolin-induced LP of eEPSCs is NMDA receptor dependent. NMDA receptor activation might be triggered by an increase in spontaneous firing which accompanies forskolin treatment of cultures during paired recordings. Such a mechanism for the forskolin-induced late phase of potentiation in the CA1 area of the hippocampus has been recently reported by Otmakhov and colleagues (Otmakhov et al. 2003).

Enhanced spontaneous activity, which manifests itself as an increase of the frequency and amplitudes of spontaneous EPSCs, was also observed in our experiments. We argue, however, that, since during paired recordings the neurons were voltage-clamped at -60 mV, activation of these receptors was hardly possible due to a block of NMDA receptors by magnesium ions. In addition, increase of spontaneous EPSCs was not associated with spontaneous action potential generation. In fact, under no conditions during the experiments in the low-density culture, did neurons spontaneously fire action potentials (some regions of cultured cells typically escape voltage clamp, and such

unclamp action potentials are revealed as transient inward currents). Nevertheless, we could not completely exclude the possibility of NMDA receptor activation during enhanced spontaneous activity, because, at a concentration of external Mg^{2+} used in our experiments (0.5 mM), a small fraction NMDA receptors might remain unblocked even at high negative membrane potentials (Hestrin et al. 1990).

Our data do not confirm a role of NMDA receptors in the forskolin-induced LP in hippocampal cultures. The NMDA receptor blocker AP-5 (50 μM) did not alter forskolin-induced LP (Fig 4c). The observed potentiation of eEPSCs was also stimulus-independent. In fact, LP was even more pronounced if the presynaptic neuron was not stimulated during and 40 min after, forskolin treatment (Fig 4c).

In summary, results presented here challenge the conclusion of previous electrophysiological studies that the forskolin-induced potentiation of synaptic transmission is mediated by presynaptic mechanisms (Carroll et al. 1998, Chavez-Noriega and Stevens 1994). We have demonstrated that postsynaptic mechanisms are essential for the forskolin-induced potentiation of eEPSCs in hippocampal embryonic neuronal cultures. Phosphorylation of the GluR1 subunit of AMPA receptors, subsequent upregulation of their function and/or their membrane targeting are among the possible mechanisms contributing to the effect of forskolin on synaptic currents in hippocampal cultured neurons.

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Figure Legends.

Figure 1 a. Effect of forskolin (50 μM) on eEPSCs in perforated patch experiments. 15 min forskolin treatment resulted in a 140±10 % increase of eEPSC amplitudes (6 cell pairs). eEPSC amplitudes declined during the first 10 min upon forskolin washout and then stabilized at a potentiated level, which exceeded the baseline by 52±7% 60 min after forskolin washout (5 of 6 cell pairs). LP was abolished if forskolin was applied with the PKA inhibitor Rp-cAMPS (50 μM), indicating that the forskolin-induced LP was mediated by the cAMP/PKA pathway. An early forskolin-induced potentiation of eEPSCs by 36±17% of baseline (3 cell pairs) on the background of Rp-cAMPS might be explained by the PKA-independent effect of forskolin, presumably on ion channels. In control experiments, long-term eEPSC measurements were done in untreated cultures. Only the last 10 min (of the total 25-30 min prior to the drug application) of baseline recordings are reflected in the Figure.
Insert: representative eEPSCs during baseline recordings, after 15 min in forskolin (EP), and 60 min after forskolin washout (LP).
b. Effect of forskolin on mEPSC frequency in hippocampal cultured neurons under the perforated patch.15 min of forskolin treatment led to an increase of mEPSC frequency to 445±47% of baseline. mEPSC frequency then steadily declined upon forskolin washout reaching the pre-treatment level about 45 min after forskolin washout. Numbers over the data points indicate the number of experiments included in the analysis. There was no detectable effect of forskolin on mEPSC amplitudes (data not shown).
In control experiments, long-term recordings of mEPSCs were done in untreated cultures. mEPSC frequency steadily declined in these cultures, starting at about 30 min after being placed in the bathing solution. mEPSC frequency constituted 68±5% of the baseline (5 cell pairs) 60 min after the start of experiments. The insert shows representative mEPSC traces before and after the forskolin treatment.

Figure 2. a) Effect of forskolin on eEPSCs in experiments with the presynaptic or the postsynaptic neuron under the whole-cell configuration. Under the presynaptic whole-cell configuration, forskolin induced EP comparable to that under the perforated patch (eEPSC increased by 144 ± 21% of baseline, 6 cell pairs). In 4 of the 6 cell pairs forskolin also induced LP (eEPSC increased by 53 ± 9 % of baseline). In contrast to the presynaptic whole-cell, the postsynaptic whole-cell configuration impaired EP and disrupted LP. eEPSC amplitudes increased only by 46±11% of baseline (4 cell pairs) during the EP. As derived from experiments with Rp-cAMPS, most of this increase (by 36±17% of baseline) was contributed by the PKA-independent mechanisms. b) Results of control experiments, in which long-term recordings of eEPSCs were done in untreated cultures. In these experiments, at times corresponding to LP, eEPSC amplitudes constituted 102 ± 6 % and 108 ± 7 % of baseline under the presynaptic and postsynaptic whole-cell configuration, respectively (2 cell pairs in each configuration). These experiments demonstrated that the baseline level of eEPSCs was not affected by conditions of the whole-cell configuration.

Figure 3 a) Effect of forskolin on mEPSC frequency in experiments using the whole-cell configuration. In control experiments (no forskolin), there was a decline of mEPSC frequency to 63±8 % of the initial level during the first 30 min after the whole-cell formation (8 experiments). mEPSC frequency then stabilized; the decline of mEPSC frequency during the next 15 min was only 4.5±8 % of baseline (8 experiments). To avoid a contribution of mEPSC rundown during the whole-cell experiments, forskolin was not applied until 30 min after the whole-cell formation. The rate of mEPSCs prior to forskolin application was considered a baseline. After 15 min of forskolin treatment, the upregulation of mEPSC frequency under the whole-cell configuration was significantly lower than that under the perforated patch (194±25 % of baseline, 5 experiments, vs. 345±47% of baseline, 5 experiments, $p<0.01$). b) Representative current traces recorded during a simultaneous whole-cell and perforated patch experiment. Such experiments using dual-cell voltage-clamp were conducted to avoid a contribution of dish-to-dish variability to the differential effect of forskolin on mEPSCs under the two patch-clamp configurations. Forskolin-induced increase of mEPSC frequency was significantly higher in experiments using the perforated patch than in those using the whole-cell configuration (1.45±0.11 times, $p<0.05$, 3 experiments). These results demonstrate that postsynaptic events contribute to the forskolin-induced potentiation of mEPSC frequency.

Figure 4 a) Western blots of the whole-cell protein extracts from untreated cultures (left lanes), cultures treated with forskolin and lysed immediately (middle lanes), or 45 min after forskolin treatment (right lanes). Equal amounts of the indicated protein extracts were loaded onto three different gels. The first blot was immunostained with the anti-phospho-GluR1 antibody specific for phosphorylated Ser845 (top panel). To confirm the specificity of the anti-phospho-GluR1 (Ser845) antibody staining, the second blot was incubated with the antibody-containing solution supplemented with the anti-phospho-GluR1 immunizing peptide at saturating concentration (1 μM) (middle panel). To demonstrate that approximately equal amounts of GluR1 protein were present in the lanes, the third membrane blot was immunostained with the anti-GluR1 antibody (bottom panel). As indicated by the extra band corresponding to ~ 108 kDa molecular weight, present in the middle lane of the top panel, forskolin treatment induced phosphorylation of the GluR1 subunit of AMPA receptors,. Phosphorylation of GluR1 was negligible 45 min after forskolin washout (top panel, right lane). The intensities of the anti-GluR1 specific bands (lower panel), as well as of the non-specific bands in all three blots, demonstrate that about equal amounts of GluR1 and the total protein were present in the blots.
b) Cumulative probabilities of mEPSC amplitudes in control (filled circles) and after forskolin treatment (blank circles) under the perforated patch (left panel) and the whole-cell configuration (right panel) in low-density cultures. Forskolin significantly increased mEPSC amplitudes in the perforated patch experiments ($p<0.05$, Kolmogorov-Smirnov test, 3 experiments). Under the whole-cell configuration, to the contrary, mEPSC amplitudes experiments were unaffected by forskolin (2 experiments, right panel). There was no detectable effect of forskolin on mEPSC amplitudes in high-density cultures, presumably, due to a combination of the low signal-to-noise ratio and dendritic filtering of mEPSCs in these experiments.
c) Experiments demonstrating that forskolin-induced potentiation of eEPSC amplitudes was independent of neuronal activity (open circles) or NMDA receptor function (filled circles). After baseline recording stimulation of the presynaptic neuron was interrupted until 45 min after forskolin washout. Under these conditions LP constituted 211±17 % (3 experiments) 60 min after forskolin washout. Inhibition of NMDA receptors by AP-5 did not disrupt the effect of forskolin on eEPSC amplitudes, which indicates that potentiation of eEPSCs was not mediated by NMDA receptors.

Table 1. Summary of the results of experiments on the effect of forskolin on eEPSCs and mEPSCs using different patch-clamp configurations.

Type of expe-riment	treatment	configuration		potentiation (% of baseline)			mechanism of potentiation
		pre-synaptic	post-synaptic	EP		LP	
				total	PKA-depend	total	
eEPSC	forskolin	perforated	perforated	140±10	104±20*	52±7	pre- & postsynaptic
	forskolin+ Rp-cAMPS	perforated	perforated	36±17	0	0	pre- & postsynaptic
	forskolin	whole-cell	perforated	144±21	108±27*	53±9	postsynaptic
	forskolin	perforated	whole-cell	46±11	10±20*	-4±6	presynaptic
mEPSC	forskolin+ TTX	intact	perforated	345±47	-	0	pre- & postsynaptic
	forskolin+ TTX	intact	whole-cell	194±25	-	-	presynaptic

* calculated as the difference between the total effect of forskolin and the PKA-independent effect established in experiments using Rp-cAMPS.

Figure 1

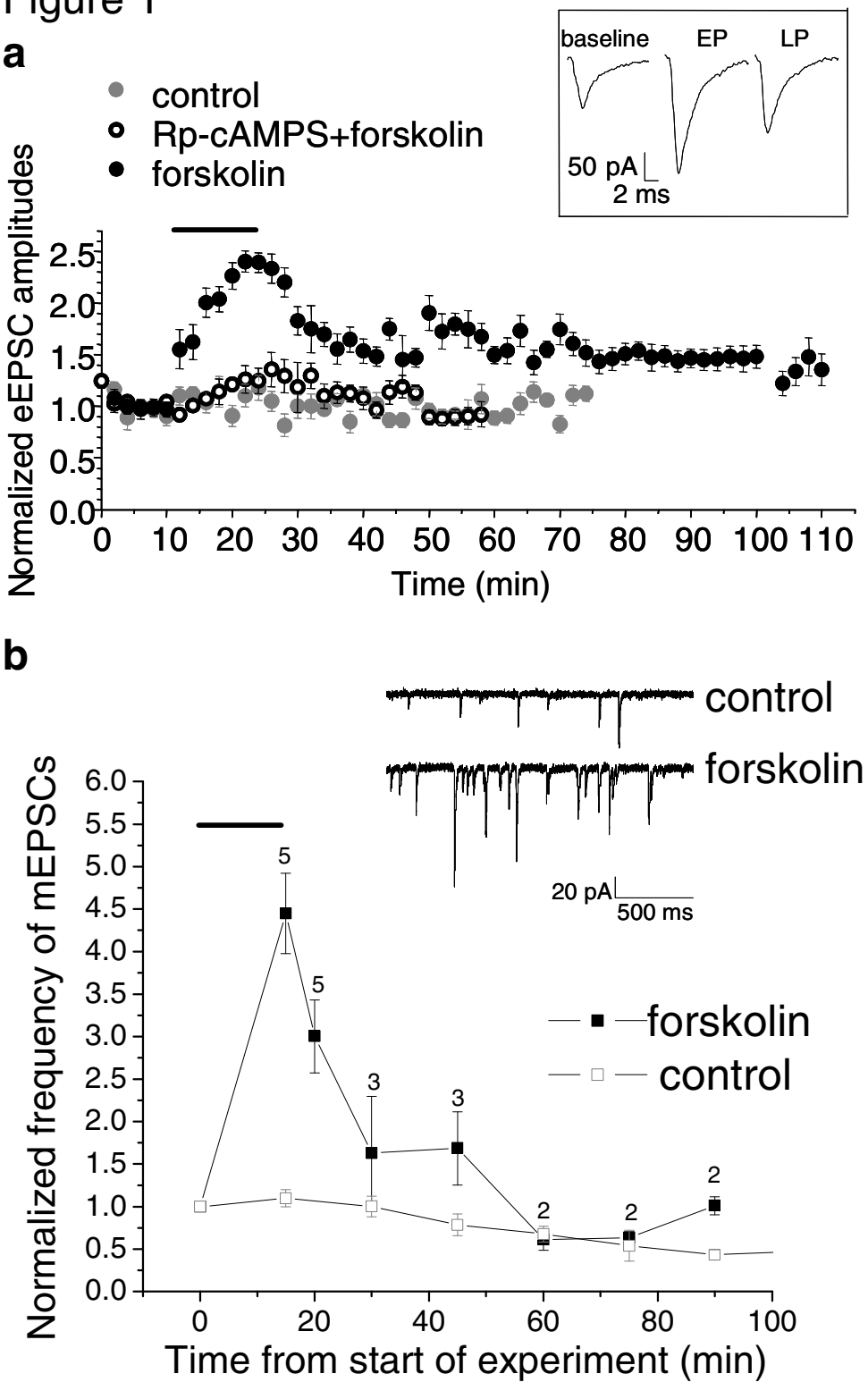

a
control
Rp-cAMPS+forskolin
forskolin
baseline
EP
LP
50 pA
2 ms
Normalized eEPSC amplitudes
Time (min)
b
control
forskolin
20 pA
500 ms
forskolin
control
Normalized frequency of mEPSCs
Time from start of experiment (min)

Figure 2

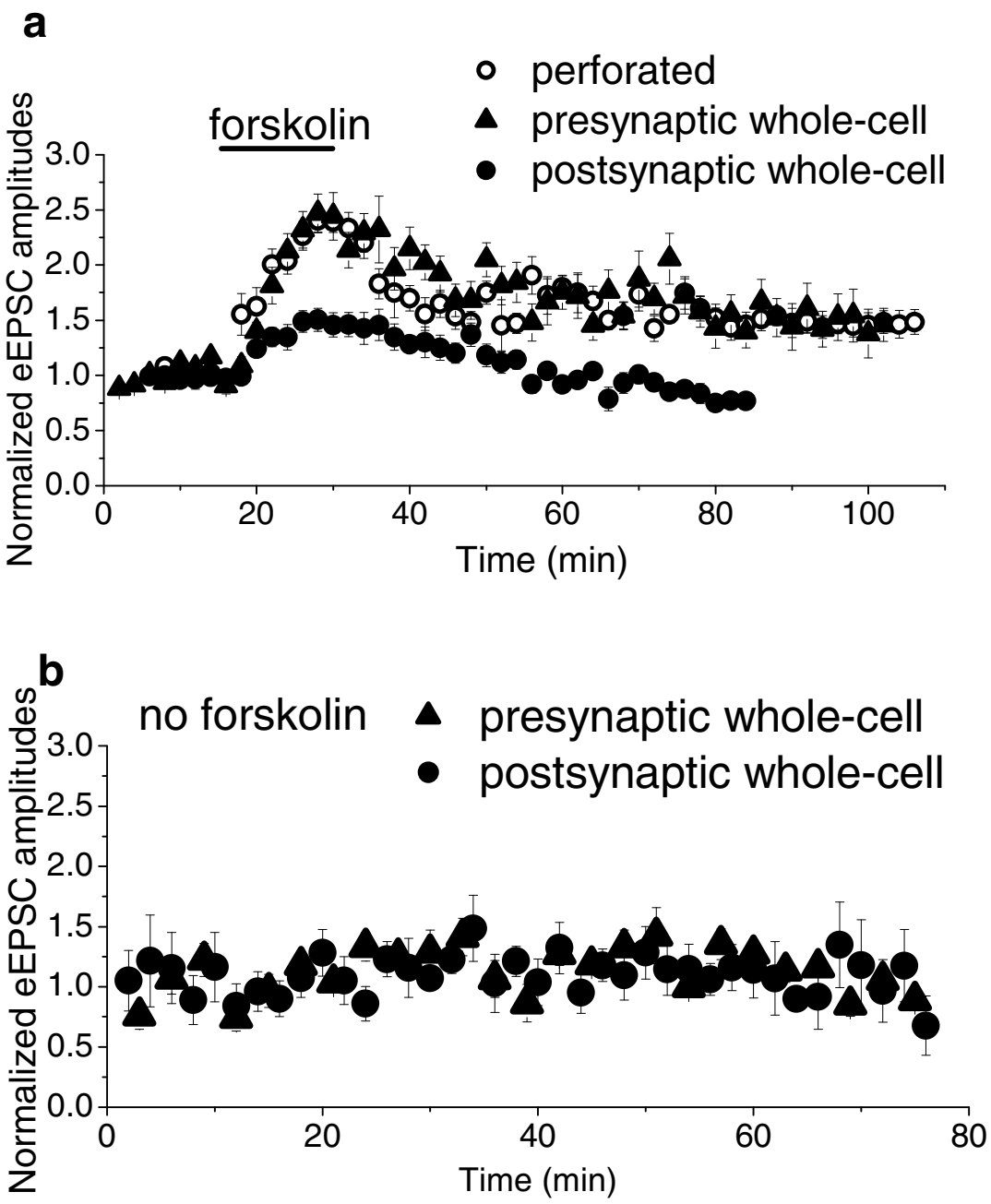

a
perforated
presynaptic whole-cell
postsynaptic whole-cell
forskolin
Normalized eEPSC amplitudes
3.0
2.5
2.0
1.5
1.0
0.5
0.0
0
20
40
60
80
100
Time (min)
b
no forskolin
presynaptic whole-cell
postsynaptic whole-cell
Normalized eEPSC amplitudes
3.0
2.5
2.0
1.5
1.0
0.5
0.0
0
20
40
60
80
Time (min)

Figure 3

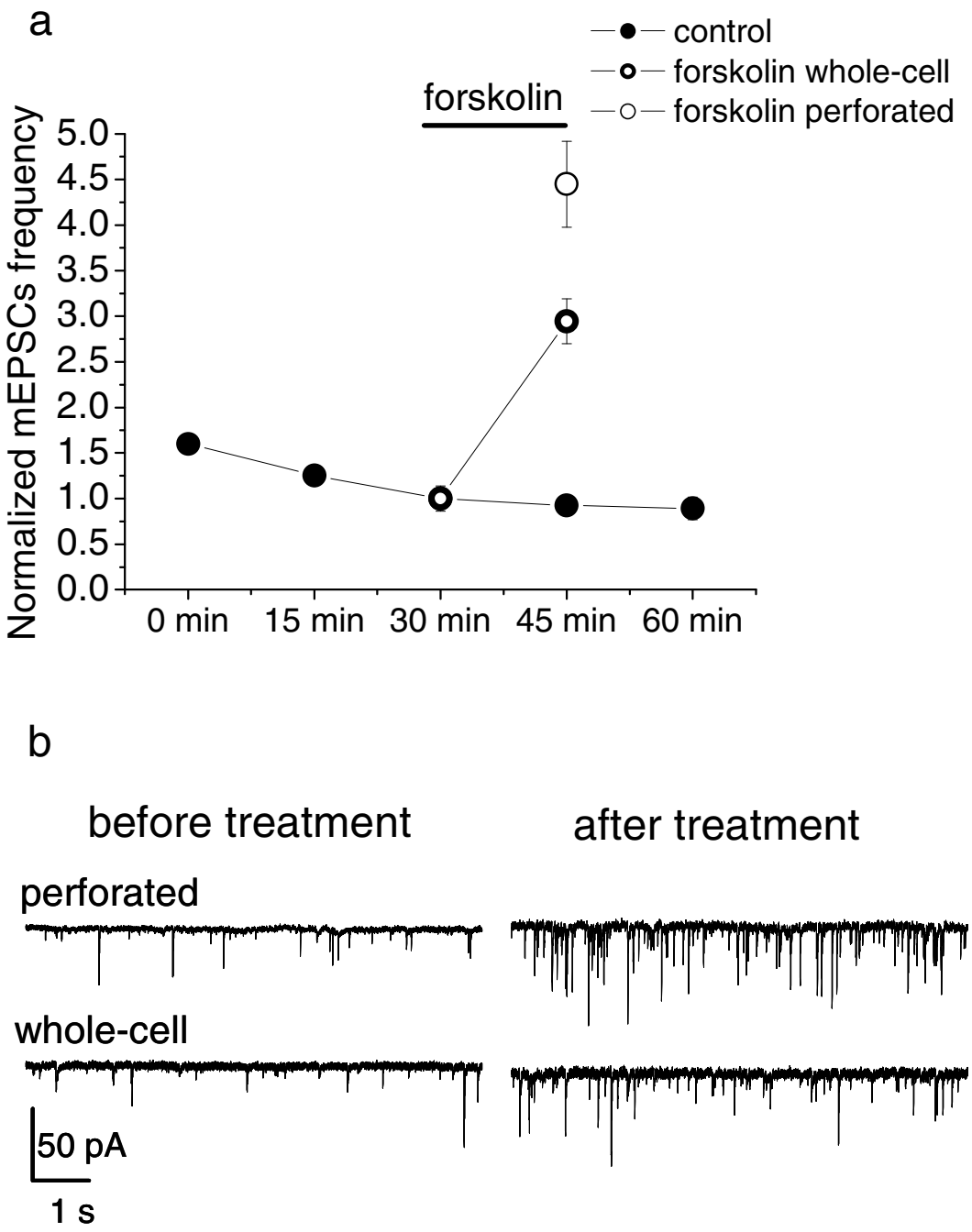
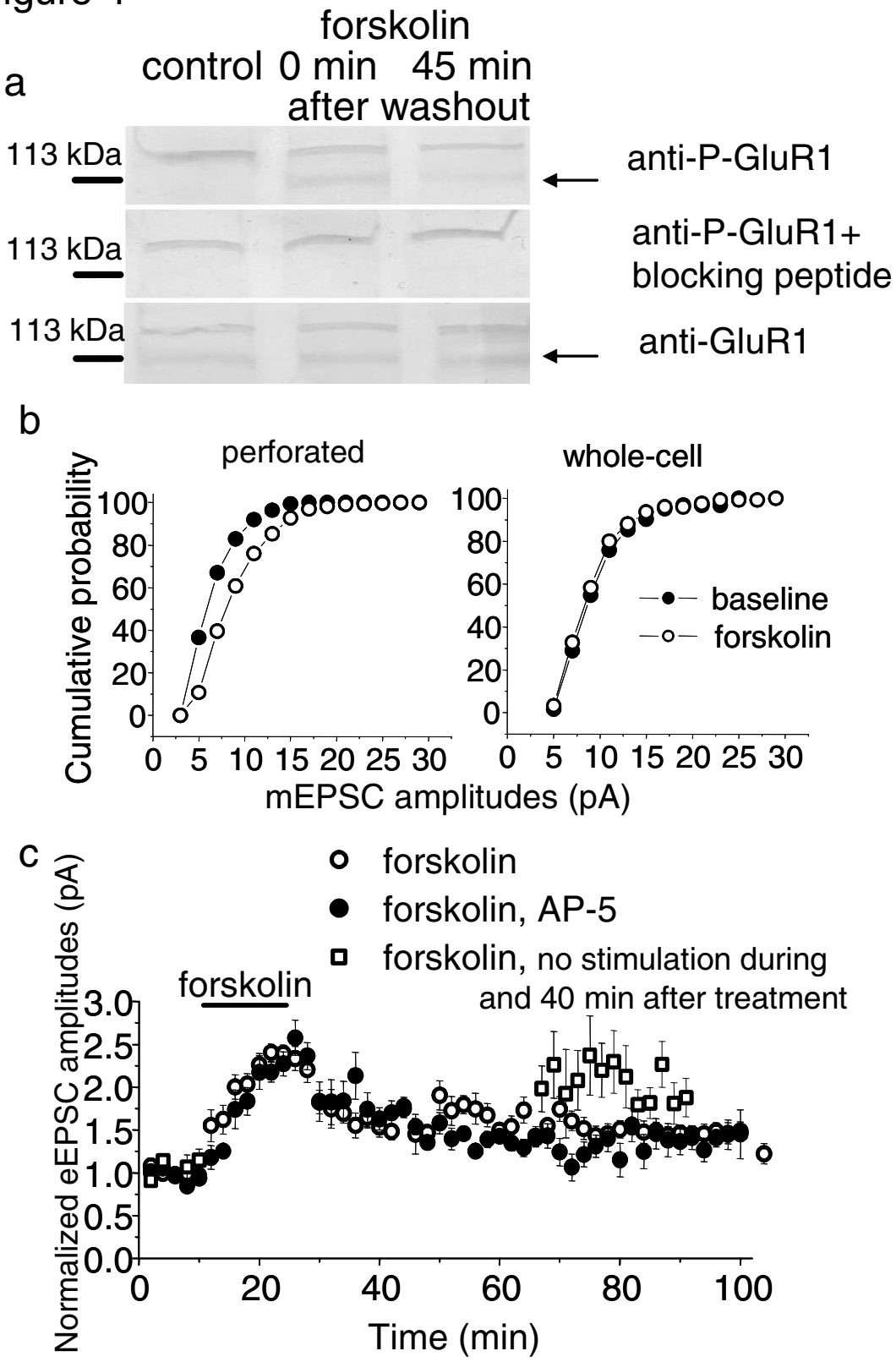

a
control
forskolin whole-cell
forskolin perforated
forskolin
Normalized mEPSCs frequency
5.0
4.5
4.0
3.5
3.0
2.5
2.0
1.5
1.0
0.5
0.0
0 min
15 min
30 min
45 min
60 min
b
before treatment
after treatment
perforated
whole-cell
50 pA
1 s

Figure 4


a
control
forskolin
0 min
45 min
after washout
113 kDa
anti-P-GluR1
113 kDa
anti-P-GluR1+
blocking peptide
113 kDa
anti-GluR1
b
perforated
whole-cell
Cumulative probability
baseline
forskolin
mEPSC amplitudes (pA)
c
forskolin
forskolin, AP-5
forskolin, no stimulation during
and 40 min after treatment
forskolin
Normalized eEPSC amplitudes (pA)
Time (min)

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