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Molecules and Cells

State-Dependent Disruption of Short-Term Facilitation Due to Overexpression of the apPDE4 Supershort Form in *Aplysia*

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Phosphodiesterases (PDEs) play important roles in synaptic plasticity by regulating cAMP signaling in various organisms. The supershort, short, and long forms of *Aplysia* PDE4 (apPDE4) have been cloned, and the long form has been shown to play a crucial role in 5- hydroxytryptamine (5-HT)-induced synaptic plasticity in *Aplysia*. To address the role of the supershort form in 5-HT-induced synaptic plasticity in *Aplysia*, we overexpressed the apPDE4 supershort form in *Aplysia* sensory neurons. Consequently, 5-HT-induced hyperexcitability and short-term facilitation in nondepressed synapses were blocked. However, the supershort form did not inhibit 5-HT-induced short-term facilitation in highly depressed synapses. These results show that the supershort form plays an important role in 5-HT-induced synaptic plasticity and disrupts it mainly by impairing cAMP signaling in *Aplysia*.

INTRODUCTION

The second messenger, cAMP is a key signaling molecule modulating synaptic plasticity. For example, cAMP/protein kinase A (PKA) signaling activated by 5-hydroxytryptamine (5-HT) plays a critical role in synaptic plasticity in *Aplysia* (Kandel, 2001; Lee et al., 2008). Phosphodiesterase (PDE) is the only known enzyme that can degrade cAMP, and at least 100 different PDEs have been grouped into 11 families on the basis of substrate specificity and preference for biochemical activators and inhibitors, including PDE4 (Conti and Beavo, 2007). Four different PDE4 genes (*PDE4A*, *PDE4B*, *PDE4C*, and *PDE4D*) generate over 20 isoforms, which are classified into four major categories, including long, short, supershort, and dead-short forms (Houslay et al., 2007). We also recently reported the cloning and characterization of three isoforms of apPDE4, including the long, short, and supershort forms in *Aplysia* (Jang et al., 2010; Park et al., 2005).

The role of PDE4 in synaptic plasticity has been investigated in various organisms, including *Drosophila*, *Aplysia*, and some mammals. A mutation in the *dunce* gene, which encodes a *Drosophila* PDE4 isoform, has been reported to lead to impaired synaptic facilitation and olfactory learning (Byers et al., 1981; Dudai et al., 1976; Zhong and Wu, 1991). In mice, treatment with a PDE4 specific inhibitor rolipram before training increased only long-term retention in freezing to the context, which is a hippocampus-dependent memory task (Barad et al., 1998). In *Aplysia*, the overexpression or knockdown of the apPDE4 long form impaired 5-HT-induced synaptic facilitation (Park et al., 2005). Disruption of synaptic plasticity due to overexpression or knockdown of apPDE4 was considered to mainly occur due to impairment of cAMP dynamics. For example, in *Aplysia*, knockdown of the long form or chronic treatment with rolipram impaired the dynamic change in PKA activation by 5-HT treatment (Park et al., 2005). However, several proteins were affected by the chronic impairment of cAMP signaling. For example, in *dunce* and *rutabaga* mutants, Ca^{2+} dynamics within the neuronal growth cone and K^+ channel regulation were disrupted (Alshuaib and Mathew, 2002; Berke and Wu, 2002).

In the *Aplysia* gill-withdrawal reflex, pharmacological studies of the signaling pathway involved in 5-HT-induced synaptic facilitation have shown that several protein kinases (PKA, protein kinase C (PKC), and Ca^{2+}/calmodulin-dependent protein kinase II) contribute to the processes involved in synaptic facilitation (Braha et al., 1990; Brunelli et al., 1976; Nakanishi et al., 1997). In short-term facilitation, the relative contributions of different signaling pathways to the synaptic facilitation processes seem to be state-dependent (Byrne and Kandel, 1996). For example, synaptic facilitation of nondepressed synapses appears to be mediated primarily by the cAMP/PKA pathway through spike broadening via closure of K^+ channels, including voltage-gated K^+ channels and S-type K^+ channels, and spike duration-independent mechanism. On the other hand, facilitation of depressed synapses on depletion of available neurotransmitters relies mainly on PKCs (Braha et al., 1990; Ghirardi et al., 1992). In particular, PKC Apl II but not PKC Apl I is activated by 5-HT and is involved in synaptic facilitation of depressed synapses (Manseau et al., 2001; Zhao et al., 2006).

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PKCs activated by 5-HT facilitate the mobilization of neurotransmitter-containing vesicles from a non-releasable pool to the depleted and releasable pool (Byrne and Kandel, 1996; Zhao and Klein, 2002; 2004). Similar to PKCs, Ca^{2+}-activated proteases (calpains) are also involved in vesicle translocation from the non-releasable pool to the depleted and releasable pool in 5-HT-induced synaptic facilitation in depressed synapses (Khoutorsky and Spira, 2005).

We have previously cloned three isoforms of apPDE4, and demonstrated the roles of the long form in 5-HT-induced synaptic plasticity in *Aplysia*. Here, to investigate the function of the supershort form in 5-HT-induced synaptic plasticity, we overexpressed the supershort form in *Aplysia* sensory neurons. Supershort form overexpression blocked 5-HT-induced hyperexcitability and short-term facilitation in nondepressed synapses. However, it did not impair short-term facilitation in highly depressed synapses. These results indicate that the supershort form is involved in 5-HT-induced synaptic plasticity, which is primarily mediated by disruption of cAMP signaling in *Aplysia* sensory neurons.

MATERIALS AND METHODS

DNA constructs

pNEXδ-apPDE4 supershort form-1XFLAG (Park et al., 2005) was digested with *Hin*dIII/*Xba*I and subcloned into the pNEXδ-enhanced green florescent protein (EGFP) vector using *Hin*dIII/*Xba*I restriction sites in order to generate pNEXδ-apPDE4 supershort form-EGFP.

Cell cultures

To obtain a sensory neuron culture, *Aplysia* sensory cells were isolated from the pleural ganglia of adult animals (100-150 g) and were cultured. For a sensory to motor culture, these cells were co-cultured with left F terminal innervating the siphon (LFS) motor neurons from the abdominal ganglia of adult animals, as described previously (Lee et al., 2001; Montarolo et al., 1986). The cultures were maintained for 3-4 days at 18°C, and then used for immunocytochemistry. We obtained and analyzed fluorescence images using a confocal laser-scanning microscope (Radiance 2000; Zeiss, Germany) and NIH Image J software (USA), respectively.

Electrophysiology

Voltage recordings and current injections were carried out as described previously (Chang et al., 2000). For membrane excitability recording, a microelectrode (8-13 MΩ) filled with 0.5 M KCl, 2 M K-acetate, and 10 mM K-HEPES (pH 7.4) was used to impale cultured sensory neurons. The resting potential was measured 5-10 min after impalement. Only cells with a resting potential of less than -40 mV were used. 5-HT (Sigma, USA) was made fresh by dissolving it in L15/artificial seawater (ASW). Before measuring membrane excitability, the resting membrane potential was adjusted to -45 mV through current injection. One min after 10 μM 5-HT treatment, 5-HT-induced hyperexcitability was measured as the number of action potentials elicited during 500 ms by a depolarizing current pulse (0.05-0.3 nA), which produced a single spike prior to drug application.

For membrane excitability recording in pleural ganglia, a single pleural ganglion was dissected and bathed for 45-60 s in 0.5% glutaraldehyde diluted with ASW. The ganglia were then surgically removed and pinned in a Sylgard recording dish containing ASW.

For excitatory postsynaptic potential (EPSP) recording, a glass microelectrode was used to impale the motor cell intracellularly, and membrane potential was held at -30 mV, below resting value. he EPSP was evoked in LFS by stimulating the sensory neurons with a brief depolarizing stimulus using an extracellular electrode. To examine basal synaptic transmission, EPSP was measured before and 48 h after microinjection of pNEXδ-apPDE4 supershort form-1XFLAG. To investigate the effect of the supershort form on synaptic plasticity, the initial EPSP value was measured 24 h after microinjection. The cultures then received a single pulse of 5-HT to induce short-term facilitation or five spaced pulses of 5-HT for 5 min at 15-min intervals to induce long-term facilitation. The degree of synaptic facilitation was determined based on the percentage change in EPSP amplitude recorded after 5-HT treatment versus the pretreatment value.

To produce synaptic depression, cultured sensory neurons were stimulated at 20 s intervals. When the sensory cell was stimulated 40 times, the EPSP was depressed to 10-20% of its initial level. The drug (5-HT) was applied immediately after the 40th stimulation to a final concentration of 10 μM. The value of each trial was normalized with respect to the amplitude of the first EPSP recording.

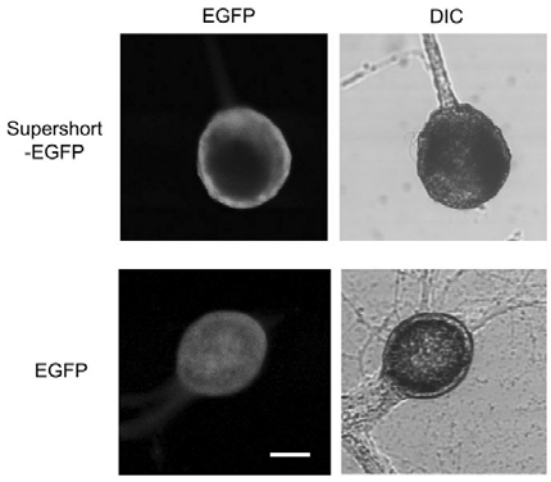

Fig. 1. Cytoplasmic localization of apPDE4 supershort form in cultured sensory neurons. The supershort form of apPDE4-EGFP was expressed in cultured sensory neurons (upper panel). As a control, EGFP was expressed (lower panel). Fluorescent images show the cytoplasmic distribution of the supershort form in sensory cells. Scale bar, 40 μM.

RESULTS

Effects of apPDE4 Supershort form overexpression on 5-HT-induced hyperexcitability in *Aplysia* sensory neurons

First, we examined the localization of the apPDE4 supershort form in *Aplysia* sensory neurons. We fused EGFP into the C-terminus of the supershort form, and consequently observed that the overexpressed apPDE4 supershort form-EGFP was localized to the cytoplasm by diffusion (Fig. 1). As a control, EGFP was expressed in the cytoplasm and nucleus (Fig. 1). Our results showing cytoplasmic expression of the supershort form was similar to a previous report (Jang et al., 2010).

Next, we examined whether overexpression of the supershort form can regulate 5-HT-induced hyperexcitability in *Aplysia* sensory neurons. To do this, we overexpressed the supershort form in pleural sensory neurons within the sensory cluster, as described previously (Chang et al., 2000). Treatment of

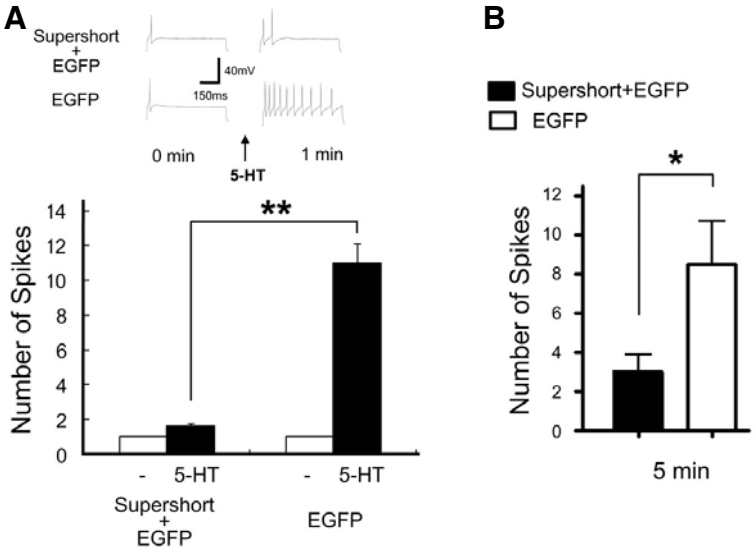

Fig. 2. Effects of apPDE4 supershort form overexpression on membrane excitability in *Aplysia* pleural or cultured sensory neurons. 5-HT-induced hyperexcitability was blocked by supershort form overexpression in pleural (A) or cultured sensory neurons (B). (A) Membrane excitability in pleural sensory neurons was measured before (0 min) and 1 min after treatment. Group data showing that overexpression of the supershort form attenuated 5-HT-induced hyperexcitability in sensory cells compared to EGFP overexpression. (B) Membrane excitability in cultured sensory neurons was measured before (0 min) and 5 min after treatment. Group data showing that overexpression of the supershort form in sensory cells attenuated 5-HT membrane excitability compared to EGFP expression. Membrane excitability is described as the number of spikes (action potentials) produced by a fixed step command over a period of 500 ms. Data are presented as the mean ± SEM. *, P < 0.05; ** P < 0.01, Mann-Whitney *U* test.

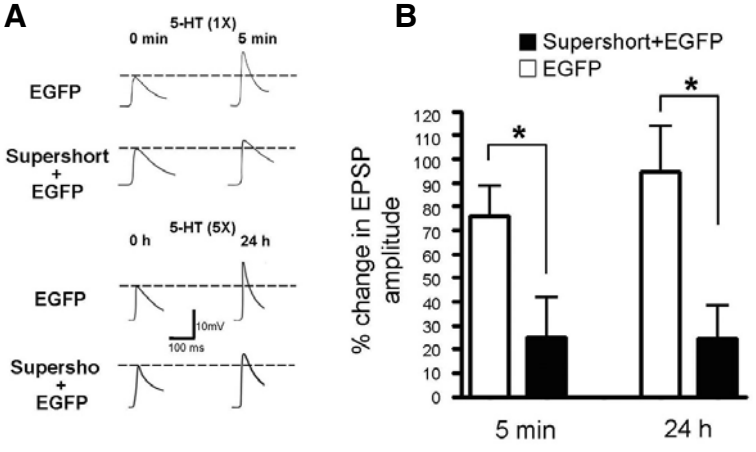

Fig. 3. Effect of apPDE4 supershort form overexpression on synaptic facilitation in nondepressed synapses. (A) Representative mono-synaptic EPSPs evoked by stimulating sensory neurons expressing EGFP or both EGFP and the supershort form. (B) Bar graph representing the effect of supershort form overexpression on short-term and long-term facilitation. Supershort form overexpression by injection of pNEXδ-apPDE4 supershort form-1XFLAG into the sensory cell significantly inhibits 5-HT-induced short-term facilitation and long-term facilitation in nondepressed synapses. As a control, EGFP was expressed. The height of each bar corresponds to the mean percentage ± SEM in EPSP amplitude tested 5 min or 24 h after 5-HT treatment. *, P < 0.05, two-tailed *t*-test.

pleural sensory neurons expressing EGFP with 10 μM 5-HT for 1 min produced an increase in spike number from 1.0 ± 0.0 to 11.0 ± 1.1 (n = 4). However, in the sensory neurons expressing the supershort form, 5-HT-induced hyperexcitability was attenuated significantly (1.6 ± 0.2, n = 7; P < 0.01, Mann-Whitney *U* test, compared to the EGFP-expressing group) (Fig. 2A).

In contrast to the results shown in Fig. 2A, our previous results showed that overexpression of the supershort form did not significantly attenuate 5-HT-induced hyperexcitability in cultured sensory neurons 1 min after 10 μM 5-HT treatment (Jang et al., 2010). To examine this further, we tested the effect of supershort form overexpression on 5-HT-induced hyperexcitability in cultured sensory neurons 5 min after 10 μM 5-HT treatment. This treatment significantly attenuated 5-HT-induced hyperexcitability compared to the EGFP-expressing group (8.5 ± 2.2, n = 11, EGFP; 3.0 ± 0.8, n = 8, supershort + EGFP; P < 0.05, Mann-Whitney *U* test, compared to the EGFP-expressing group) (Fig. 2B). Overall, although less efficient attenuation of 5-HT-induced hyperexcitability was observed in cultured sensory neurons, overexpression of the supershort form reduced 5-HT-induced hyperexcitability in *Aplysia* sensory neurons.

Effects of apPDE4 supershort form overexpression on short-term and long-term synaptic plasticity in *Aplysia* sensory to motor synapses

We examined the effect of supershort form overexpression on basal synaptic transmission in sensory to motor synapses. Overexpression of the supershort form in sensory neurons had no significant effect on basal synaptic strength (% change, -7.4 ± 4.0%, n = 8), which was comparable to that of EGFP-expressing cells (-3.6 ± 2.9%, n = 14).

To determine the effect of supershort form overexpression at nondepressed synapses, the sensory-motor co-culture was exposed to 1 pulse (5 min) of 10 μM 5-HT, which normally produces a short-term synaptic facilitation, 24 h after supershort form overexpression. Overexpression of the supershort form impaired the short-term synaptic facilitation induced by 1 pulse of 5-HT (25.0 ± 16.9%, n = 8) compared to that in EGFP-expressing cells (76.1 ± 12.4%, n = 14; P < 0.05, two-tailed *t* test, the supershort form + EGFP-expressing group versus EGFP-expressing group) (Fig. 3). These results showed that short-term synaptic facilitation is blocked by supershort form overexpression in nondepressed synapses.

Next, we overexpressed the supershort form in sensory neurons within a sensory to motor co-culture and exposed the co-

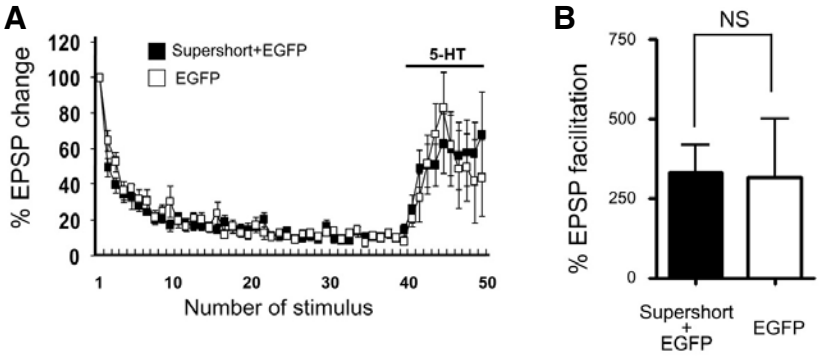

Fig. 4. Effect of apPDE4 supershort form overexpression on synaptic facilitation in highly depressed synapses. (A) Synaptic depression was induced by low frequency stimulus (interstimulus interval: 20 s). EPSP amplitudes were normalized to the first EPSP in the train. (B) Each bar represents the percentage change in the mean amplitudes of the second, third, and fourth EPSP evoked after drug application with that of the three EPSPs evoked immediately before drug application in (A). Overexpression of the supershort form had no effect on short-term facilitation in highly depressed synapses compared to EGFP-expression. The height of each bar represents the mean ± SEM. NS, not significant.

culture to 5 pulses of 5-HT to induce long-term synaptic facilitation. Twenty-four hours after 5-HT treatment, EGFP-expressing cells showed a normal increase in synaptic potential amplitude (94.9 ± 19.0%, n = 14). On the other hand, in cells expressing the supershort form, no significant increase was observed in the amplitude of synaptic potential (24.5 ± 13.9%, n = 7; $P < 0.05$, compared to the EGFP-expressing group, two-tailed *t* test) (Fig. 3). These results suggest that disruption of cAMP regulation by supershort form overexpression impaired 5-HT-induced long-term synaptic facilitation as well as short-term synaptic facilitation of nondepressed synapses in *Aplysia* sensory to motor synapses.

Effects of supershort form overexpression on short-term synaptic plasticity at depressed synapses

It is known that PKC instead of PKA plays the major role in 5-HT-induced synaptic facilitation in depressed synapses (Byrne and Kandel, 1996). To elucidate the signaling pathway affecting short-term facilitation by overexpression of the supershort form, we examined the effect of supershort form overexpression in depressed synapses. To do this, we depressed the synapse highly (8.17 ± 2.02% of the basal EPSP amplitude) by stimulating the sensory neuron every 20 s for 800 s (Fig. 4A). As soon as the 40th stimulation was applied, 10 μM 5-HT was added to the media to induce short-term facilitation. As shown in Fig. 4B, short-term facilitation of supershort form-expressing synapses was induced as much as EGFP-expressing synapses (Supershort + EGFP, 332.0 ± 88.0%, n = 7; EGFP, 316.7 ± 184.5%, n = 6; $P > 0.9$, two-tailed *t* test). These data indicate that the overexpression of the supershort form does not affect short-term facilitation in highly depressed synapses. Taken together, this result showed that the signaling pathway involved in 5-HT-induced synaptic facilitation in depressed synapses and vesicle release was not affected by supershort form overexpression.

DISCUSSION

The main observation of our study is that overexpression of the apPDE4 supershort form disrupts 5-HT-induced hyperexcitability and synaptic facilitation in nondepressed synapses, but not in highly depressed synapses. In previous reports, we showed that overexpression or knockdown of the long form impaired 5-HT-induced synaptic facilitation in nondepressed synapses. Therefore, taken together with the present results, it is likely that disruption of 5-HT-induced synaptic facilitation by overexpression of apPDE4 is mediated primarily by impairment of cAMP/PKA signaling in *Aplysia* sensory neurons.

Cytoplasmic expression of the supershort form

Previously, we demonstrated the cloning and characterization of three apPDE4 isoforms: the long, short, and supershort form. The supershort, short, and long forms were localized to the cytoplasm, the plasma membrane, and both the plasma membrane and presynaptic terminal, respectively (Jang et al., 2010). The present result, showing cytoplasmic localization of the supershort form, was consistent with a previous report (Jang et al., 2010). Therefore, considering the previous report of supershort form expression in sensory clusters (Jang et al., 2010), in the cytoplasm, they may play a role in the regulation of cAMP signaling in *Aplysia* sensory neurons.

In mammals, three PDE4 supershort forms have been cloned including PDE4A1, PDE4B5, and PDE4D6 (Cheung et al., 2007; Shakur et al., 1993; Wang et al., 2003). Among them, PDE4B5 is a brain-specific form that is distributed in the cytoplasm (Cheung et al., 2007). On the other hand, PDE4A1 is also brain-specific, but it is associated with the membrane via a 25 amino acid N-terminal segment (Huston et al., 2006). In addition, the N-terminal regions of PDE4B5 and PDE4D6 show 81% identity, whereas the N-terminal region of the apPDE4 supershort form did not show any similarity to mammalian PDE4. However, considering the molecular structure and cellular localization of the supershort form, PDE4B5 in mammals is the form of PDE4 corresponding to the apPDE4 supershort form in *Aplysia*.

Attenuation of 5-HT-induced hyperexcitability by overexpression of the supershort form

Overexpression of the supershort form attenuated 5-HT-induced hyperexcitability (Fig. 2). However, the impairment of 5-HT-activated cAMP/PKA signaling by the supershort form was not efficient compared to other forms. With the long and the short forms, 5-HT-induced hyperexcitability in cultured sensory neurons was attenuated at 1 min after 10 μM 5-HT treatment (Jang et al., 2010). Meanwhile, the supershort form blocked the hyperexcitability in cultured sensory neurons by 5 min, but not at 1 min after 10 μM 5-HT treatment. On the other hand, 5-HT-induced hyperexcitability was completely blocked in sensory neurons expressing the supershort form in sensory clusters at 1 min after 10 μM 5-HT treatment (Fig. 2A). Therefore, although the supershort form less efficiently reduced 5-HT-activated cAMP/PKA signaling within sensory neurons, it was enough to eventually attenuate 5-HT-induced hyperexcitability in *Aplysia* sensory neurons.

In *Aplysia*, it is well known that the regulation of membrane excitability by 5-HT is fully dependent on S-type K^+ channel activity, which can be modulated by PKA (Shuster et al., 1985; Siegelbaum et al., 1982). Closure of the S-type K^+ channels by

activated PKA induces hyperexcitability in *Aplysia* sensory neurons. Therefore, disruption of the cAMP/PKA pathway by overexpression of the supershort form may explain the impairment of 5-HT-induced hyperexcitability. Overexpression of the supershort form may reduce basal PKA activity and expression of the S-type K^+ channels in sensory neurons. However, this might not be possible since 5-HT-induced hyperexcitability was normal in cultured sensory neurons expressing the supershort form at 1 min after 5-HT treatment. It was previously reported that PKA activity in pleural ganglion declined slightly between 1 min and 5 min after 50 μM 5-HT treatment (Muller and Carew, 1998). This might be the reason that supershort form overexpression disrupted 5-HT-induced hyperexcitability at 5 min but not at 1 min after 5-HT treatment. Therefore, disruption of the cAMP/PKA pathway by supershort form overexpression may explain the impairment of 5-HT-induced hyperexcitability.

State-dependent disruption of synaptic plasticity due to overexpression of the supershort form

Our results showed that overexpression of the supershort form in sensory neurons impaired 5-HT-induced synaptic facilitation in nondepressed synapses (Fig. 3). It is well known that 5-HT-induced hyperexcitability and short-term synaptic facilitation in nondepressed (resting) synapses are also primarily dependent on activation of the cAMP/PKA pathway (Byrne and Kandel, 1996; Ghirardi et al., 1992). Therefore, disruption of the cAMP/PKA pathway by supershort form overexpression may explain the impairment of both 5-HT-induced hyperexcitability and synaptic facilitation in nondepressed synapses. This conclusion is consistent with previous studies showing that disruption of the long form in sensory neurons impaired PKA activation (Park et al., 2005). However, these results could not exclude other possibilities including the regulation of exocytotic machinery, Ca^{2+} channels, or K^+ channels. In addition, overexpression of the supershort form has no effect on 5-HT-induced synaptic facilitation in highly depressed synapses (Fig. 4). In highly depressed synapses, which are almost completely depleted of the releasable pool, PKCs instead of PKAs play a key role in 5-HT-induced synaptic facilitation (Ghirardi et al., 1992; Manseau et al., 2001). Activated PKCs modulate Ca^{2+} channels localized to the release sites, directly stimulate vesicle translocation from the non-releasable to the releasable pool and exocytosis in depressed synapses (Byrne and Kandel, 1996; Zhao and Klein, 2002). Overexpression of the supershort form most likely had no effect on either the Ca^{2+} channels near the release sites or exocytotic machinery in *Aplysia*. Therefore, disruption of the cAMP/PKA pathway by overexpression of the supershort form may explain the impairment of 5-HT-induced synaptic facilitation.

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