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Synaptic facilitation by ectopic octopamine and 5-HT receptors in *Aplysia*

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Abstract

The cAMP pathway plays a critical role in synaptic plasticity. We assessed using the ectopic expression of octopamine (OA) receptor, the contribution of the cAMP pathway to short-term facilitation of sensory–motor synapses in *Aplysia*. When synaptic connections were depressed to 20–30% of their initial EPSP amplitude, the application of OA to sensory cells expressing OA receptor showed significant synaptic facilitation, but this was less than the synaptic facilitation shown by 5-HT treatment. We also found that synaptic facilitation was further enhanced when OA was treated in the presence of 5-HT at non-depressed synapses, but not at depressed synapses. These results imply that the role of cAMP in synaptic facilitation is reduced as the synapse becomes depressed due to repeated activity.

Keywords: PKA; Short-term facilitation; Non-depressed synapse; Depressed synapse

1. Introduction

Studies of the signaling pathway involved in short-term facilitation have shown that two protein kinases (PKA and PKC) contribute to several of the processes involved in short-term facilitation [1,3,5,13,17]. Two signaling pathways are activated, presumably by different isotypes of the 5-HT receptor in *Aplysia* [14]. For example, previous studies have shown that 5-HT receptors are dissociated by the antagonist cyproheptadine (CYP) and that the CYP-sensitive and CYP-insensitive isotypes differ with respect to the activation of the PKA and PKC pathways in *Aplysia* sensory neurons [14]. The relative contributions of the two pathways to several processes of synaptic facilitation seem to be time- and state-dependent, although they certainly overlap in terms of their contributions to short-term facilitation [3]. The synaptic facilitation of a non-depressed synapse appears to be mediated primarily by the cAMP/PKA pathway. Such a facilitation is stimulated by activators of PKA, for example, forskolin and cAMP analogs [2], and is blocked by inhibitors of PKA, but unaffected by inhibitors of PKC. The facilitation of non-depressed synapses by PKA involves both spike-duration independent and spike-duration dependent processes [3]. In contrast, the facilitation of depressed synapses, when available neurotransmitters are depleted, relies on a spike-duration independent process [6]. In this situation, PKC is critical for 5-HT-induced facilitation [1,5,13]. At moderate levels of synapse depression, PKA and PKC seem to be equally involved. To date attempts to determine the degree of contribution of each pathway to various aspects of short-term facilitation at depressed synapses have been limited to pharmacological dissection. For example, Ghirardi et al. [5] reported that by using the PKC inhibitor, H-7, in combination with the PKA inhibitor, Rp-cAMPS, the activation of both PKA and PKC is required to facilitate depressed synapses, and that the contribution of PKC becomes more important as the synapse becomes more depressed in a sensory–motor coculture system. However, Liao et al. [12] reported that bath application of Rp-cAMPS is not effective in *Aplysia* sensory neurons of pleural ganglions. In order to complement these pharmacological approaches, we undertook a gene transfer approach to assess the contribution of PKA in the depressed synapses.

It was previously reported that Ap oa_1, cloned in *Aplysia*, can only selectively activate the PKA pathway, and that Ap oa_1 is not endogenously expressed in *Aplysia* sen-

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sory neurons. Octopamine (OA) treatment of sensory cells expressing OA receptor induced the processes associated with 5-HT-induced synaptic facilitation, namely, membrane depolarization, increased membrane excitability, spike broadening, and increased EPSP [4].

In this study, we applied the same approaches to non-depressed and depressed synapses, to examine the differential role of PKA in synaptic facilitation as a function of synapse state.

2. Materials and methods

2.1. Preparation

Adult animals (*Aplysia kurodai*) weighing 150–250 g were obtained from a local supplier in Pusan, Korea. Animals were anesthetized by injecting a volume of isotonic $MgCl_2$ equal to approximately a half of their body weights. Single pleural-pedal ganglia were dissected and bathed for 45–60 s in 0.5% glutaraldehyde diluted in artificial seawater (ASW). The ganglia were then removed surgically and pinned in a Sylgard recording dish containing ASW.

2.2. Gene transfer into the sensory cells

The mechanosensory neurons in the ventrocaudal clusters of pleural ganglia were microinjected with the DNA construct of pNEXδ-GFP or with a mixture of pNEXδ-GFP and pNEXδ-Ap oa_1 [4]. GFP (enhanced GFP) DNA construct was used as a marker of DNA expression in the living cell. The microinjection solution was composed of 0.5–1.0 mg/ml DNA construct, 10 mM Tris–HCl (pH 7.3), 100 mM KCl, and 0.1% fast green (Sigma, St. Louis, MO). The injected ganglia were maintained at 18 °C for 18–48 h to allow gene expression. The green fluorescence was detected in the microinjected sensory neurons using a Nikon upright microscope (model Optiphot-2, Tokyo, Japan) with a

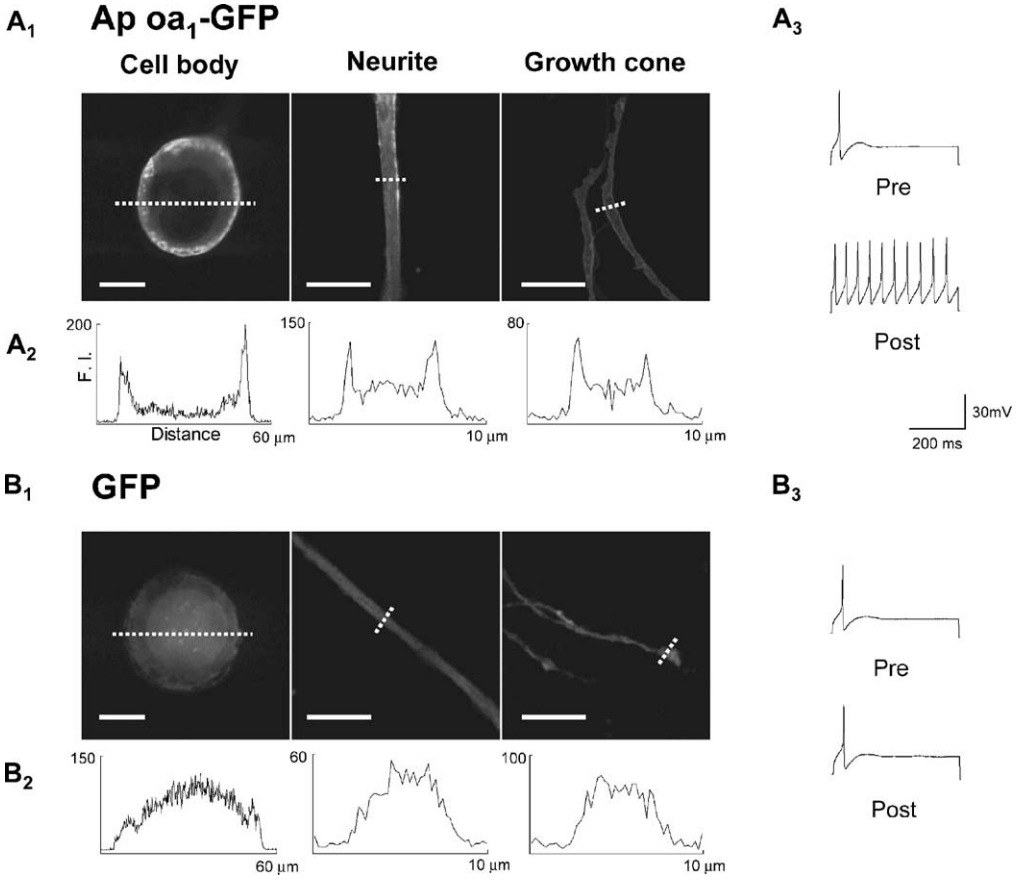

Fig. 1. Localization of Ap oa_1-GFP and GFP expressed in cultured sensory neurons. All images were obtained from cultured sensory neurons 24 h after microinjecting with either Ap oa_1-GFP (A_1, A_2, A_3) or GFP alone (B_1, B_2, B_3). The dashed lines in the confocal fluorescence images (A_1, B_1) indicate the paths along which the fluorescence intensities of the corresponding images were plotted in A_2 and B_2. GFP-fused Ap oa_1 is localized primarily on the plasma membrane, while GFP is localized in the cytosol. No fluorescence was observed in uninjected cells (data not shown). Cross-sections of fluorescence intensity in confocal images were analyzed using LaserPix (BioRad). Scale bars indicate 20 μm in the confocal fluorescence micrographs. F.I., fluorescence intensity in arbitrary units. Scales of the *x*- and *y*-axes in A_2 and B_2 have been appropriately adjusted. (A_3 and B_3) Action potentials were recorded by injecting a depolarizing current for 500 ms, which was calibrated to produce a single spike before OA was treated to sensory cells. Pre and Post indicate the recording traces before and after OA treatment, respectively. The number of spikes was increased by OA treatment of the cell expressing Ap oa_1-GFP.

standard B-2A filter or through a Leica GFP plus microscope (Heerbrugg, Switzerland). To determine the ectopic expression of Ap oa_1, a mixture of DNA constructs pNEXδ-Ap oa_1-GFP and pNEXδ-Ap oa_1 was microinjected into cultured sensory cells. We empirically found that the addition of pNEXδ-Ap oa_1 to pNEXδ-Ap oa_1-GFP enhanced the targeting of GFP-fused Ap oa_1 to the plasma membrane. GFP fluorescence was determined 24 h after microinjection by using a confocal microscopy (Radiance 2000, BioRad). Cross-sections of fluorescence intensity in confocal images were analyzed using LaserPix (BioRad).

2.3. EPSP recording in the sensory–motor connections

Tail sensory and motor neurons were identified based on their position and size in each ganglion, as previously described [18]. Voltage recording and current injection were carried out in the current clamp mode using an Axoclamp 2B (Axon Instruments, CA). Motor neurons were hyperpolarized by 30 mV. Electrophysiological recording was started 10–20 min after finding a connection between a sensory and a motor neuron. A current pulse of 0.4–0.5 nA was applied for 10–15 ms to generate a single spike in the sensory cells. The resulting EPSP was recorded from the tail motor neuron (the first EPSP recording).

To produce synapse depression, the pleural sensory cell was stimulated at 1 min intervals. When the sensory cell was stimulated at 1 min intervals 40 times, the EPSP was depressed to 20–30% of its initial level. Drugs were applied immediately to a final concentration of 1 μM (OA) or 10 μM (5-HT), respectively, as described previously [4]. Only a single sensory–motor connection per recording chamber was used for the EPSP measurements. The value of each trial was normalized with respect to the EPSP amplitude of the first EPSP recording.

3. Results

3.1. Ectopic expression of Ap oa_1 in sensory neurons

Ectopic expression of Ap oa_1 in sensory neurons was first confirmed by microinjecting a DNA construct encoding Ap oa_1 fused with GFP at its C-terminus into cultured sensory neurons. One day after microinjection, confocal microscopy showed that GFP-fused Ap oa_1 was expressed quite uniformly on the cell membranes of cell body, neurites, and growth cone areas (Fig. 1). Cross-sections of fluorescence images are shown under each confocal image in Fig. 1. Fluorescence intensity near the plasma membrane was signifi-

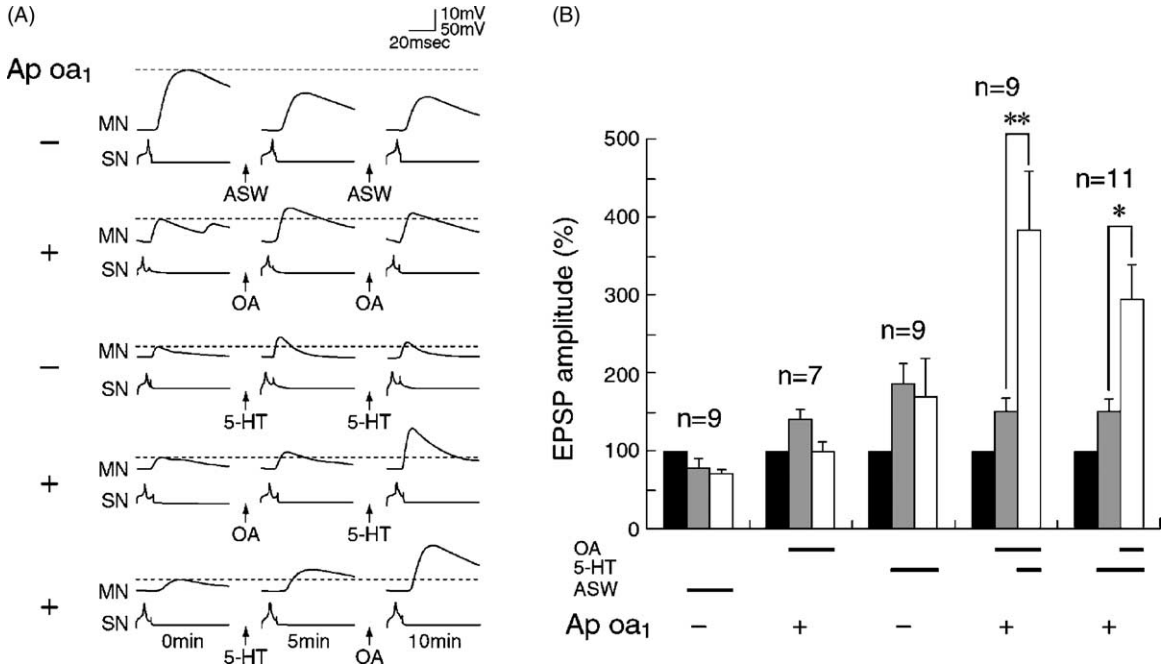

Fig. 2. Synaptic facilitation mediated by different receptors. Synaptic facilitation was augmented by the activation of different receptors in a non-depressed synapse. (A) Representative trace of the monosynaptic EPSPs produced in the motor neuron (MN) of the pedal ganglion and action potential produced in pleural sensory cells (SN) expressing Ap oa_1 (second, fourth, and fifth row) or uninjected sensory cells (first and third row). The sensory cells were stimulated at 5 min intervals. (B) These group data show that the application of 5-HT (10 μM) after OA (1 μM) or the application of OA after 5-HT produced further synaptic facilitation. Data represent the changes in EPSP amplitude (±S.E.M.). EPSP amplitude was normalized to the initial control value (base line). Changes in the EPSP amplitude are represented by black bars (before drug application), gray bars (5 min after the first drug application) and blank bars (5 min after the second drug application). Thick lines illustrate the existence of applied drugs in bath solutions for 10 min (long lines) and 5 min (short lines), respectively. Expression of Ap oa_1 (+); no injection of DNA molecules (−). Comparing the amount of synaptic facilitation induced by treatment of OA or 5-HT for 5 min between four groups except ASW control group reveals no significant difference (one-way ANOVA, $F = 1.24$, d.f. $= 3$, $P > 0.3$). $^{*}P < 0.03$ ($n = 11$); $^{**}P < 0.01$ ($n = 9$) two-tailed paired t-test.

cantly higher than that in the cytoplasm and axoplasm. In contrast, GFP not fused with Ap oa_1 was characteristically expressed in the cytoplasm. These data suggest that the activation of Ap oa_1 by OA is likely to occur in all regions of sensory neurons. Intracellular recording data (the right panels in Fig. 1) showed the electrical responses of DNA-injected sensory neurons in response to a depolarizing step current before (Pre) and after (Post) OA treatment. Membrane excitability was found to be increased in an Ap oa_1 expressing neuron (right upper panel). This effect is due to the activation of the cAMP/PKA pathway through the activation of Ap oa_1 by OA, as was shown by a previous study [4].

3.2. Synaptic facilitation by 5-HT is augmented by OA at a non-depressed synapse

Previously it was reported that Ap oa_1, ectopically expressed in pleural sensory neurons, has the ability to produce short-term facilitation in non-depressed synapses, and that an additional increase in spike-duration was induced by OA treatment in the presence of 5-HT or by 5-HT treatment

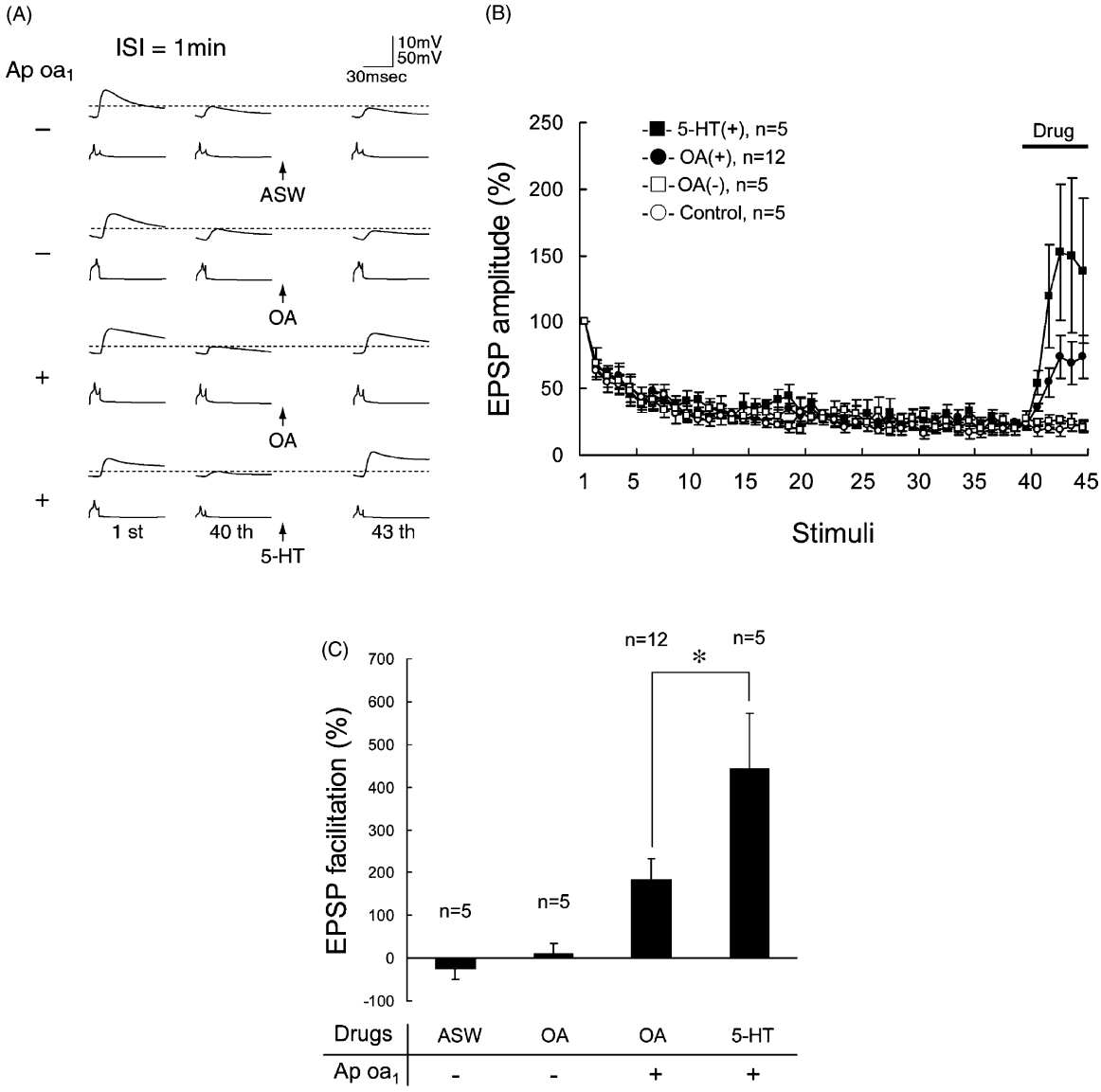

Fig. 3. Homosynaptic depression and synaptic facilitation by receptor activation. The activation of Ap oa_1 is less effective in inducing synaptic facilitation in depressed synapses than the activation of endogenous 5-HT receptors. (A) Representative trace of the monosynaptic EPSPs evoked by stimulating sensory cells (SN) expressing Ap oa_1 (third and fourth row) or uninjected sensory cells (first and second row). The sensory cells were stimulated at 1 min intervals. The 40th EPSP was recorded immediately before drug application and the 43rd EPSP was recorded after drug application. Plus (+) symbol indicates the expression of Ap oa_1 in the sensory cells. (B) Time course of the changes in EPSP amplitude (±S.E.M.). To measure the EPSP of pleural-pedal connections, the sensory cell was stimulated at 1 min intervals. (C) Each bar represents the percent change in the mean amplitudes of the second and the third EPSP evoked after drug application with that of two EPSPs evoked immediately before drug application. The height of each bar shows the mean ± S.E.M. $^*P < 0.05$, two-tailed unpaired *t*-test. ASW, artificial seawater; OA, octopamine; 5-HT, 5-hydroxytryptamine.

in the presence of OA [4]. To examine whether this additional increase in spike-duration produce additional synaptic facilitation in a non-depressed synapse, we treated OA in the presence of 5-HT in pleural sensory cells expressing Ap oa_1. The application of 1 μM OA to sensory–motor synapses that had undergone synaptic facilitation by treatment with 10 μM 5-HT (152.0 ± 13.7%, $n = 11$) for 5 min produced further synaptic facilitation (293.5 ± 46.5%, $P < 0.03$, $n = 11$). Next, we reversed the order of drug application. Treatment with OA for 5 min increased the amplitude of the EPSP by 151.3±15.6% ($n = 9$), and the application of 5-HT for an additional 5 min to the bath containing OA further increased the amplitude of the EPSP to 389.1±62.1% ($n = 9$, $P < 0.01$) (Fig. 2). The effects of the prolonged application of either OA or 5-HT upon synaptic facilitation were tested in control experiments. These prolonged applications produced no further synaptic facilitation when measured 5 and 10 min after drug application. These results show that synaptic facilitation is enhanced through the activation of different receptors in the case of non-depressed synapses.

3.3. Short-term facilitation of depressed synapses

How much could PKA contribute to the synaptic facilitation in a depressed synapse? To assess the role of PKA activated by Ap oa_1 in depressed synapses, synaptic depression was induced using repetitive stimulation (ISI = 1 min). To compare the experiments upon depressed synapses statistically, facilitation was evaluated by comparing the percentage changes in the mean amplitudes of the second and the third EPSP evoked after drug application with that of two EPSPs evoked immediately before drug application.

When the synaptic connections were depressed to 20–30% of the control level, OA treatment in sensory cells expressing Ap oa_1 produced a statistically significant synaptic facilitation (181.6±50.7%, $n = 12$). However, this facilitation was only 43% of the 5-HT-induced facilitation (443.1±129.3%, $n = 5$) in sensory cells expressing Ap oa_1 (Fig. 3). In control experiments, the treatment of OA or ASW in uninjected cells had no effect on EPSP amplitude (10.4±23.4%, $n = 5$ (OA); −25.2±25.7%, $n = 5$ (ASW)). These results showed that the application of OA in Ap oa_1-expressing sensory cells facilitates their synaptic connections to motor neurons in a depressed synapse. However, the level of this OA-induced facilitation is lower than that of a 5-HT-induced facilitation.

3.4. Synaptic facilitation by 5-HT is not augmented by OA at a depressed synapse

Next, we examined whether treatment with additional drug augments synaptic facilitation at a depressed synapse as it does at non-depressed synapses. Synapses were depressed by eliciting 40 EPSPs with a 1-min interstimulus interval. Synaptic facilitation was first induced by applying OA in depressed synapses. The application of 5-HT in the presence of OA showed further synaptic facilitation in the same synapses. However, in the case of the reverse order of drug application, the synaptic facilitation was not further enhanced (Fig. 4). This result is in contrast to the observation that further facilitation was induced by the application of OA in addition to 5-HT in a non-depressed synapse. Therefore, this result indicated that the OA-activated PKA pathway was completely occluded by the 5-HT-activated signal pathways in depressed synapses, whereas the OA-activated PKA pathway was available in non-depressed synapses.

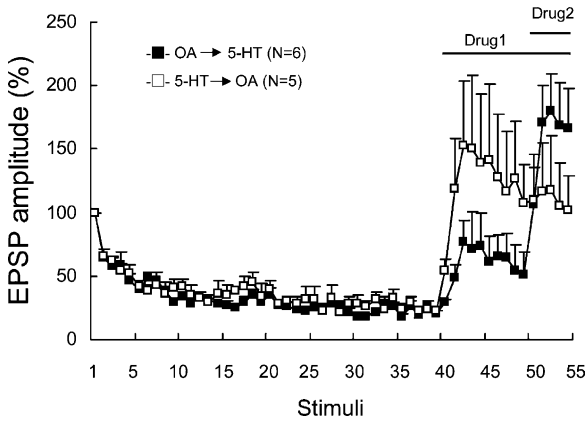

Fig. 4. Synaptic facilitation by OA is not augmented by 5-HT in a depressed synapse. The graph shows the time course of the changes in EPSPs amplitude evoked by stimulating the sensory cells expressing Ap oa_1 at 1 min intervals. The first drug (drug 1) was applied after the 40th stimuli and the second drug (drug 2) after the 50th stimuli. The height of each bar shows the mean ± S.E.M.

4. Discussion

Traditionally, studies on signal transduction pathway in short-term facilitation have relied mostly on pharmacological inhibitors or activators of specific enzymes. Recently it has become possible to transfect *Aplysia* neurons in intact ganglions using a microinjection technique [4,8,9,11]. Using this technique, Ap oa_1 coupled to the cAMP pathway, was ectopically expressed in the sensory neurons of the pleural ganglion to determine the roles of the cAMP and cAMP-dependent protein kinase (PKA) pathway at a depressed synapse. It was previously shown that PKA activation by Ap oa_1 for 5 min produced synaptic facilitation in non-depressed synapses. This facilitation was not significantly different from that of 5-HT receptor-mediated facilitation. It was previously shown that when 5-HT was applied in the presence of OA, or when OA was applied in the presence of 5-HT, additional spike broadening, a feature of short-term facilitation, occurred [4]. This result suggests that the signal transduction pathways involved in short-term facilitation could be additionally activated by different OA or 5-HT receptors. In this study, we extended this gene transfer technique to specifically examine the role of PKA on synaptic facilitation in both non-depressed and depressed synapses.

Our results show that synaptic facilitation can be further augmented by activating different ectopic Ap oa_1 and endogenous 5-HT receptors in non-depressed synapses. Both receptors can activate PKA. Therefore, PKA activation through Ap oa_1 can still produce synaptic facilitation in non-depressed synapses, as long as the PKA substrates have not been saturated by 5-HT receptor activation, even after the same synapses have been facilitated through endogenous 5-HT receptor activation. It has been widely reported that PKA plays a dominant role in the 5-HT-induced synaptic facilitation of non-depressed synapses [2,3,5,10]. Moreover, it has also been reported that 7B-forskolin or 5-HT alone caused comparable increases of intracellular cAMP. The simultaneous application of forskolin and 5-HT produced 2–10-fold increase of intracellular cAMP [10]. Combined with our results, these results show that PKA activity and PKA substrates are not saturated by the activation of endogenous 5-HT receptors, and that they can be further activated by exogenous receptors such as Ap oa_1 in non-depressed synapses.

Our study also shows that the role of PKA activated by Ap oa_1 is reduced as the sensory–motor synapses are depressed by repeated activity. PKA-induced synaptic facilitation by Ap oa_1 was much lower than 5-HT receptor-mediated synaptic facilitation in a depressed synapse. Furthermore, PKA-induced synaptic facilitation by Ap oa_1 could not augment 5-HT receptor-mediated synaptic facilitation in depressed synapses. Homosynaptic depression in *Aplysia* sensory–motor synapses is caused by a progressive reduction in neurotransmitter release. The recovery of the depressed release process could be a key factor in the facilitation of depressed synapses. One simple explanation for the relative incompetency of ectopic Ap oa_1 in facilitating depressed synapses, is that it might not be located, like 5-HT receptors, near the synapse site. Therefore, Ap oa_1 may be less effective than 5-HT receptors at activating PKA, and therefore, at facilitating transmitter release in depressed synaptic sites. This possibility is supported by previous reports [7,16], which showed that small cardioactive peptide (SCP), which may activate only the PKA pathway in sensory neurons, could efficiently produce synaptic facilitation in non-depressed synapses, but not in depressed synapses [16]. However, SCP in the presence of a phosphodiesterase inhibitor IBMX produced significant facilitation in depressed synapses [16]. These results suggested that SCP did not produce levels of cAMP within synaptic terminals high enough to mobilize transmitter release, because of either the low effectiveness of SCP receptors in stimulating cyclase [7] or the low expression level of SCP receptors near the synaptic terminals [16]. However, other reports do not support this possibility of the inefficient activation of PKA through Ap oa_1 within the presynaptic terminals. Firstly, whereas SCP produced lower spike broadening than 5-HT [7], OA produced a level of the spike broadening similar to that induced by 5-HT in pleural sensory neurons expressing Ap oa_1 [4], indicating that Ap oa_1 may be as effective as 5-HT at activating cyclase. Secondly, our confocal data showed that the ectopic expression of Ap oa_1-GFP was more or less uniformly distributed in the axonal membranes, including the axon terminals, suggesting the possibility of the appropriate expression of ectopic Ap oa_1 near the synaptic release site. Another report by Klein [10] showed that in depressed synapses, spike broadening, one element of synaptic facilitation, was synergistically increased by the simultaneous application of forskolin and 5-HT, in addition to a 2–10-fold increase of intracellular cAMP. However, synaptic facilitation was not augmented by this simultaneous application.

A second explanation of the inefficient facilitation by OA at depressed synapses is that Ap oa_1 cannot activate PKC, unlike 5-HT [15]. Many reports showed that the inhibitors of PKC were more effective in blocking 5-HT-induced synaptic facilitation in depressed synapses than in non-depressed synapses (for a review, see [3]). Increased contribution of PKC in depressed synapse indicates that PKC may stimulate the recovery of the depressed release process by phosphorylating specific molecules involved in the process of synaptic vesicle mobilization and therefore replenishing the depleted vesicle stores. The release process, the downstream of action potential, is the rate-limiting factors of synaptic facilitation in depressed synapses. Therefore, the process affected by PKC may lie downstream from that by PKA in depressed synapses.

Finally, it is also possible that the PKA substrates or PKA-mediated processes may saturate at a lower concentration of cAMP in depressed synapses.

In this study, we demonstrated that the contribution of PKA became less effective in 5-HT-induced synaptic facilitation as the sensory–motor synapse was depressed by repeated activity. The gene transfer experiments conducted provide data complementary to that produced by previous pharmacological studies, which showed the differential roles of the PKA pathway in non-depressed and depressed sensory-motor synapses in *Aplysia*. It becomes critical that we identify the molecular substrates of these kinases in order to understand the molecular mechanisms underlying short-term synaptic facilitation.

Acknowledgements

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