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Research Report

Suppression of long-term facilitation by Rab3–effector protein interaction

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Abstract

Long-term facilitation (LTF) in *Aplysia* is achieved by the modulation of presynaptic release. However, the underlying mechanism that might be related with the regulation of synaptic vesicle release remains unknown. Since Rab3, a neuronal GTP-binding protein, is known to be a key regulator of synaptic vesicle fusion, we investigated the involvement of Rab3 in LTF. To address this issue, we examined the effect of overexpression of wild type *Aplysia* Rab3 (apRab3) and its mutant forms on LTF. Overexpression of either apRab3 Q80L, a constitutively active apRab3 mutant, or wild type apRab3 completely inhibited LTF. This inhibitory role of apRab3 appears to be mediated by an interaction with an effector molecule(s), possibly Rim. Expression of apRab3 Q80L, V54E double mutant, which do not bind effector molecules such as Rim or Rabphilin, had no effect on LTF. Furthermore, expression of apRab3 Q80L, F18L, D19E triple mutant, which has reduced binding activity with Rim but normally binds with Rabphilin, enhanced evoked basal synaptic release, and the increase in synaptic strength occluded LTF. In conclusion, our data suggest that apRab3 may act as a negative clamp of LTF through the interaction with effector protein(s), possibly Rim.

Theme: Excitable membranes and synaptic transmission
Topic: Presynaptic mechanisms

Keywords: Aplysia; apRab3; 5-HT; Long-term facilitation; Basal synaptic transmission; Microinjection

1. Introduction

The modulation of synaptic release from the presynaptic terminal is an essential mechanism of the changes in synaptic strength, which occur during learning-related synaptic plasticity [1,2,18,22]. Two important interrelated processes, which include (1) the modulation of Ca^{2+} influx into the presynaptic terminal and (2) the modulation of synaptic vesicle machinery involved in docking, priming, fusion, and exocytosis are key mechanisms for the increase in synaptic vesicle release from the presynaptic neuron [33]. Long-term and short-term synaptic facilitation (STF), which is induced by 5-HT (5-hydroxytryptamine or serotonin) treatment, is a well-established cellular basis for learning and memory in *Aplysia*. The synaptic connections between the sensory and motor neurons of *Aplysia* undergo short-term facilitation lasting <2 h and long-term facilitation lasting at least 24 h in in vitro culture in response to either a brief single or repeated pulses of 5-HT [6,9,23,27]. Whereas short-term facilitation requires the modification of pre-existing proteins, long-term facilitation requires a cascade of gene activation [4,23]. Long-term and short-term facilitation are achieved by a change in the efficacy of synaptic transmission at sensory to motor neuron synapse. The quantal analysis of spontaneously released miniature excitatory postsynaptic potential reveals that enhancement of

Abbreviations: LTF, long-term facilitation; STF, short-term facilitation; 5-HT, 5-hydroxytryptamine or serotonin; apRab3, *Aplysia* Rab3; LTP, long-term potentiation; EGFP, enhanced green fluorescent protein; EPSP, excitatory postsynaptic potential; PPF, paired-pulse facilitation; GDI, GDP dissociation inhibitor; GEP, GDP–GTP exchange protein; Rim, Rab3-interacting molecule

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synaptic strength during long-term and short-term facilitation (STF) induced by 5-HT is caused by an increase in the number of neurotransmitter released by the presynaptic sensory neuron [3,8]. These results imply that synaptic vesicle proteins may be essentially involved in 5-HT-induced long-term facilitation in *Aplysia*.

Rab3 is a member of a family of more than 30 low molecular weight GTP-binding proteins. Generally, Rab3 is attached to synaptic vesicles in the GTP-bound state and complexed with effector proteins such as Rim or Rabphilin in a GTP-bound form. Ca^{2+}-triggered membrane fusion of synaptic vesicle leads to hydrolysis of GTP-Rab3 to GDP-Rab3 following the dissociation of Rab3 from effector molecules and synaptic vesicles [32]. Rab3A plays a key role in the regulation of a late step in synaptic vesicle fusion [10]. Emerging evidence suggests that Rab3A and its effector molecules related with synaptic vesicle release are essential for presynaptically expressed long-term potentiation (LTP) in certain mammalian brain areas. In knock-out studies in mice, Rab3A is shown to be critical for presynaptically expressed LTP at hippocampal mossy fiber synapses [5,24]. In addition, PKA-mediated phosphorylation of active zone protein RIM1α (Rab3-interacting molecule), one of Rab3A effector molecules, directly induces presynaptic LTP in cerebellar parallel fiber synapses [21].

Although Rab3 acts as a key factor in synaptic transmission process and synaptic plasticity, the possible role of Rab3 during long-term facilitation in *Aplysia* sensory to motor neuron synapse has not yet been determined. Our objective in this study was to determine whether Rab3 is implicated in the enhancement of synaptic strength during 5-HT-induced LTF. We took advantage of Rab3 mutant forms to clarify the function of Rab3 in LTF. Since Rab3 on the synaptic vesicle is mostly in the GTP-bound state and only in this form, it can be complexed with its effector proteins such as Rim and Rabphilin; we first tested the effect of Rab3 Q80L mutant, which is a GTPase deficient mutant of Rab3 and so is always in the GTP-bound form, overexpression on LTF to focus on the function of Rab3–effector protein interaction in the presynaptic neuron during LTF. By electrophysiologically analyzing the effects of overexpression of various Rab3 recombinant constructs in the presynaptic neuron, we investigated the possible role of Rab3 in LTF formation.

2. Materials and methods

2.1. Cloning of apRab3

We obtained the cDNA sequence of apcRab3 (*Aplysia californica* Rab3) from the NCBI database. Based on the sequences, we cloned the full length of apkRab3 (*Aplysia kurodai* Rab3) from *A. kurodai* using nested PCR strategy. As the primary PCR with a pYESTrp2-based library in *A. kurodai*, we used two primers: *Bco*III, 5′-CGC ACT GCC AGA-3′ and H1-Rab3-Stop-A, 5′-CG GGA TCC TCA GCA GGA GCA GCC AGA-3′. With the product of the primary PCR, we did the second PCR using two primers: D3-Rab3-Start-S, 5′-CCC AAG CTT GCC ACC ACC ATG GCT TCC GCA AAC GAC-3′ and H1-Rab3-Stop-A, 5′-CG GGA TCC TCA GCA GGA GCA GCC AGA-3′. After digestion with *Hin*dIII and *Bam*HI, the second PCR product and pNEXδ were ligated. We confirmed the apkRab3 (apRab3 from now on) sequence by sequencing the ligated clone.

2.2. Plasmid constructions

2.2.1. Flag-tagged wild type apRab3

Full-length clone of apRab3 was obtained from a PCR reaction (sense primer: 5′-CCC*AAGCTT*GCCACCAC-CATG*GACTACAAGGACGACGATGACAAG*GCTTCCG-CAAACGACTCC-3′, *Hin*dIII site and Flag epitope sequences are italicized; anti-sense primer: 5′-CG*GGATCC*-TCAGCAGGAGCAGCCAGA-3′, *Bam*HI site is italicized) and subcloned into pNEXδ, a neuronal expression vector [17] using *Hin*dIII/*Bam*HI digestions.

2.2.2. Flag-tagged apRab3 mutants

To generate various apRab3 mutants, we conducted site-directed mutagenesis by recombinant PCR. We first produced flag-tagged constitutively active apRab3 (apRab3 Q80L) by recombinant PCR using the following primers: for the first fragment, sense primer: 5′-CCCAAGCTTGCCACCAC-CATGGACTACAAGGACGACGATGACAAGGCT-TCCGCAAACGACTCC-3′ and anti-sense primer: 5′-TGCGGTACCGCTCCGGC-3′ and for the second fragment, sense primer: 5′-GCCTGGAGCGGTACCGCA-3′ and anti-sense primer: 5′-CGGGATCCTCAGCAGGAGCAGCC-AGA-3′. For the construction of apRab3 Q80L, V54E mutant, we conducted recombinant PCR from apRab3 Q80L as template DNA using the following primers: for the first fragment, sense primer: 5′-CCCAAGCTTGCCACCACC-ATGGACTACAAGGACGACGATGACAAGGCT-TCCGCAAACGACTCC-3′ and anti-sense primer: 5′-GAAGTCGATGCCCTCCGT-3′ and for the second fragment, sense primer: 5′-TGCCTCCCGTAGCTGAAG-3′ and anti-sense primer: 5′-CGGGATCCTCAGCAGGAGCAGC-CAGA-3′. apRab3 Q80L, F18L, D19E mutant constructs were generated by recombinant PCR reaction from the apRab3 Q80L template DNA using the following primers: for the first fragment, sense primer: 5′-CCCA-AGCTTGCCACCACCATGGACTACAAGGACGACG-ATGACAAGGCTTCCGCAAACGACTCC-3′ and anti-sense primer: 5′-CATGTACTCGAGGTTCTG-3′ and for the second fragment, sense primer: 5′-GTCTTGCTCGAG-TACATG-3′ and anti-sense primer: 5′-CGGGATCCTCAG-CAGGAGCAGCCAGA-3′. All these recombinant PCR products were inserted into the neuronal expression vector pNEXδ using *Hin*dIII/*Bam*HI digestion.

2.3. Cell culture

Cell culture was performed as previously described [28]. *A. kurodai* was purchased from a local supplier in Pusan, South Korea and maintained in recirculating seawater tanks at 14 °C before use. The nervous system of *A. kurodai* is similar to that of *A. californica* [20]. Ganglia were dissected from *A. kurodai* (70–100 g) and incubated at 34 °C for 1.5–2 h in 1% protease (type IX, sigma) dissolved in an equal volume of isotonic L15 and artificial seawater (ASW: 460 mM NaCl, 10 mM KCl, 11 mM $CaCl_2$, 55 mM $MgCl_2$, and 10 mM HEPES, pH 7.6) and washed several times with ASW. Pleural sensory neurons were isolated from the pleural ganglion and co-cultured with identified motor cell LFS isolated from the abdominal ganglia and maintained at 18 °C in an incubator for 4 days.

2.4. Microinjection

The various DNA constructs (1 mg/mL DNA) were dissolved in a buffer containing 0.1% fast green, 10 mM Tris–Cl (pH 7.3), and 100 mM NaCl and microinjected *Aplysia* cultured sensory neurons by applying positive air pressure, as previously described [14–16]. Enhanced Green Fluorescent Protein (EGFP) was co-injected with apRab3 DNA constructs as an expression marker [11]. Microinjected cells were incubated at 18 °C for 18–24 h and used for electrophysiological measurement and immunocytochemistry.

2.5. Electrophysiology

Experiments were performed on culture 4 days after plating. LFS motor neurons co-cultured with sensory neuron were impaled with sharp microelectrodes (10–20 MΩ) filled with 2 M K-acetate, 0.5 M KCl, and 10 mM K-HEPES and hyperpolarized to −80 mV to prevent the cell from firing action potentials. Intracellular signals were amplified using an Axoclamp 2B (Axon instrument). Synaptic potentials were evoked in the LFS motor cell by stimulating each sensory cell with a brief (0.1–0.5 ms) depolarizing pulse using an extracellular electrode placed near the cell body of a sensory neuron. Synaptic potentials were recorded before and 24 h after the onset of five pulses of 5-HT (10 μM) treatment to measure the long-term change. To examine short-term change, EPSP (excitatory postsynaptic potential) was evoked in motor cells with an inter-stimulus interval (ISI) of 5 min. A brief (5 min) pulse of 5-HT (10 μM) was applied to the preparation for 5 min after the initial EPSP measurement. To examine the changes of basal synaptic transmission, the initial EPSP was measured 1 h before DNA microinjection at 4 days after co-culture, and the second EPSP was measured again in EGFP positive cells 24 h after microinjection. Data were stored on VCR recorder tapes using a digital data recorder (Model VR-10B, Instrutech, Corp.).

2.6. Immunocytochemistry

The cultures microinjected with various apRab3 DNA constructs were fixed with 4% paraformaldehyde in phosphate-buffered saline. After blocking nonspecific binding by preincubating cells with 3% BSA (Bovine Serum Albumin) in phosphate-buffered saline, the cells were incubated with the anti-Flag Ab (1′ Ab, Sigma), diluted 1:1000 in blocking solution for 1 h. After washing out unbound 1′ Ab, the cells were incubated with the Cy3-conjugated anti-mouse IgG (2′ Ab), diluted 1:100–1:500 in blocking solution for 1 h. Immunofluorescence was observed under a confocal microscope (Radiance 2000, BioRad).

3. Results

3.1. Cloning and sequence analysis of Aplysia kurodai Rab3 (apkRab3)

We cloned the full length of *A. kurodai* Rab3 (apkRab3) by library PCR by using the cDNA sequence of *A. californica* Rab3 (apcRab3) in the NCBI database (Fig. 1). apkRab3 contains 219 amino acids and has an RAB domain from 21 aa to 185 aa. The RAB domain is highly conserved in Rab3 of all other species. The overall identity of amino acid sequence between apkRab3 and apcRab3 is 99% (Fig. 1). In addition, the identity of amino acid sequence between apkRab3 and other species is 77% to human Rab3a, 77% to mouse Rab3a, 81% to *Drosophila* Rab3a, and 82% to *C. elegans* Rab3, respectively (Fig. 1). Moreover, the amino acids from 1 to 192, without the C-terminal variable region, between apkRab3 and human Rab3a, are highly conserved with 85.5% identity and 94.8% similarity. Thus, it appears that apkRab3 is highly conserved with mammalian Rab3A.

3.2. The expression patterns of apRab3 and its various mutants

To investigate the involvement of apRab3 in LTF and the mechanism of apRab3 function, we generated apRab3 mutant constructs (apRab3 Q80L, apRab3 Q80L, V54E double mutant, and apRab3 Q80L, F18L, D19E triple mutant). With a Q80L mutation, apRab3 is GTPase deficient, and so the Rab3 is always in the GTP-bound form and therefore remains active. Thus, apRab3 Q80L acts as a constitutively active form. The apRab3 Q80L, V54E mutant was generated by exchanging Val 54 with Glu at the highly conserved effector domain [26], as previously described [13]. This apRab3 mutant does not bind effector proteins, such as Rim or Rabphilin [30,35]. The apRab3 Q80L, F18L, D19E triple mutant was generated by replacing Phe18 and Asp19 of the apRab3 Q80L construct with Leu and Glu, respectively. This mutation is reported to selectively reduce the interactions of Rab3A with Rim but not with Rabphilin [7]. Before testing the physiological

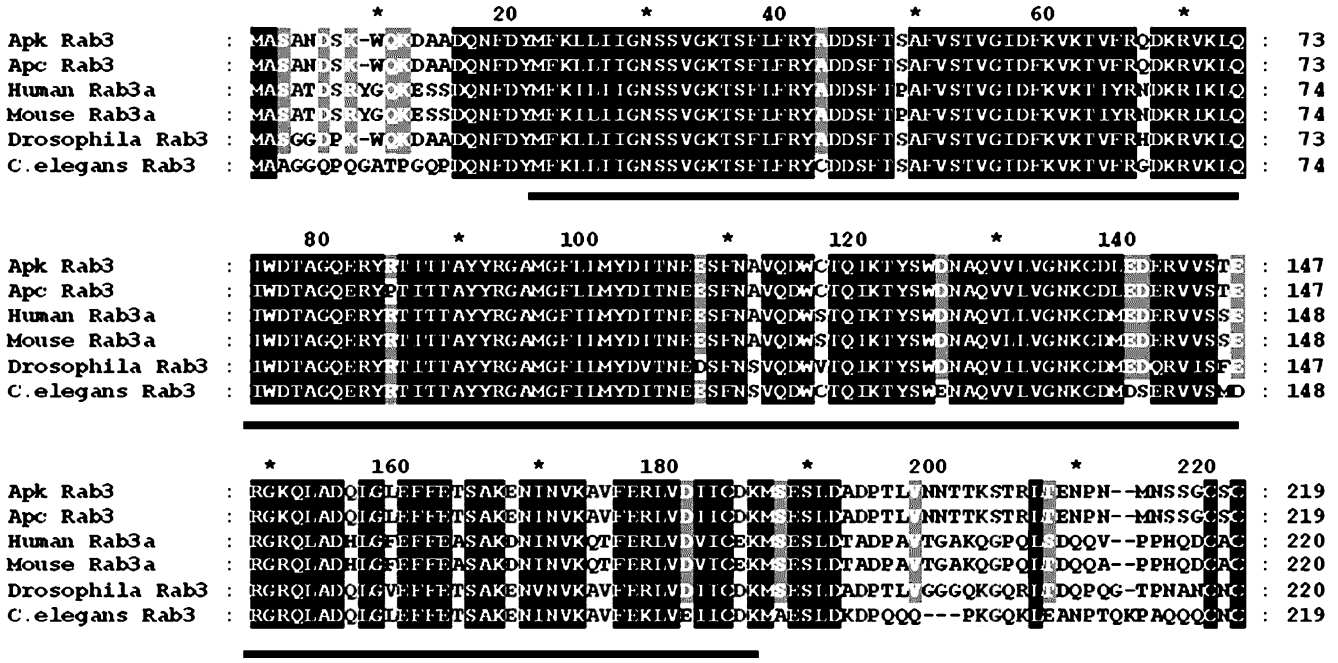

Fig. 1. Alignment of the full-length amino acids of Apk Rab3 (*A. kurodai* Rab3), Apc Rab3 (*A. californica* Rab3), human Rab3a, mouse Rab3a, *Drosophila* Rab3, and *C. elegans* Rab3. We used the CLUSTALW program for alignment. Identical amino acids are marked in black. The difference of either single amino acid or two amino acids is marked in dark gray and light gray, respectively. The black underline from 21 aa to 185 aa indicates a RAB domain, which is highly conserved in all Rab3 of other species. The identity of the amino acid sequence between Apk Rab3 and Apc Rab3 is 99%. The amino acids from 1 to 192, without the C-terminal variable region, between apkRab3 and human Rab3a are conserved with 85.5% identity and 94.8% similarity, indicating that apRab3 is highly conserved with mammalian Rab3A.

effects of various apRab3 constructs overexpression, we examined the expression patterns of these apRab3 constructs. Since apRab3 is targeted to synaptic vesicles at the presynaptic terminal, we co-expressed synaptophysin-EGFP (enhanced green fluorescent protein) [19] as a presynaptic marker to monitor the localization of various apRab3 constructs into synaptic vesicles. The expression of various apRab3 constructs was detected by immunocytochemistry using antibody against flag epitope tags inserted into the N-terminus of apRab3 constructs. apRab3 Q80L and wild type molecules were diffusely expressed as vesicles in the cytosol, near the plasma membrane in the cell body (Figs. 2A1, B1), and at neuritic regions (Figs. 2A2, B2). These constructs showed mostly co-localization with synaptophysin-EGFP, indicating normal targeting into synaptic vesicles. However, apRab3 Q80L, V54E showed more diffuse expression patterns than apRab3 Q80L and wild type at the cytosol in the cell body co-localizing with synaptophysin-EGFP (Fig. 2C1). In addition, apRab3 Q80L, V54E was not expressed at neuritic regions and therefore not co-localized with synaptophysin-EGFP, suggesting that interactions with effector molecule(s) are important for synaptic targeting of apRab3 (Fig. 2C2). apRab3 Q80L, F18L, D19E triple mutant was expressed mostly near the plasma membranes at the cell body showing little co-localization with synaptophysin-EGFP (Fig. 2D1). It also showed similar expression patterns in a vesicular form with apRab3 Q80L and wild type at neuritic regions co-localizing with synaptophysin-EGFP (Fig. 2D2). Taken together, these data indicate that various apRab3 constructs were successfully overexpressed in presynaptic neurons and show that certain apRab3 mutant constructs (apRab3 Q80L, V54E double mutant and apRab3 Q80L, F18L, D19E triple mutant) have differing expression patterns with apRab3 Q80L and apRab3 wild type.

3.3. The overexpression of apRab3 Q80L has no effect on STF

We first examined whether apRab3 overexpression has any effect on 5-HT-induced STF. For this purpose, we used the apRab3 Q80L mutant, a constitutively active form of apRab3. Overexpression of apRab3 Q80L was confirmed by immunocytochemical analysis using an antibody against the flag-epitope tag inserted into the N-terminus of the apRab3 Q80L construct. apRab3 Q80L was expressed specifically in presynaptic varicosities and near the plasma membrane in the cell body (Fig. 3A). EGFP was co-expressed to confirm successful overexpression in all electrophysiological recording experiments. We overexpressed apRab3 Q80L in presynaptic terminal and measured the EPSP changes after a single pulse of 5-HT treatment at these synapses. One pulse of 5-HT treatment for 5 min normally induced the short-term increase of EPSP amplitude at the apRab3 Q80L overexpressing synapse compared to EGFP expressing control synapses (109.1 ± 36.4%, n = 10 and 92.2 ±

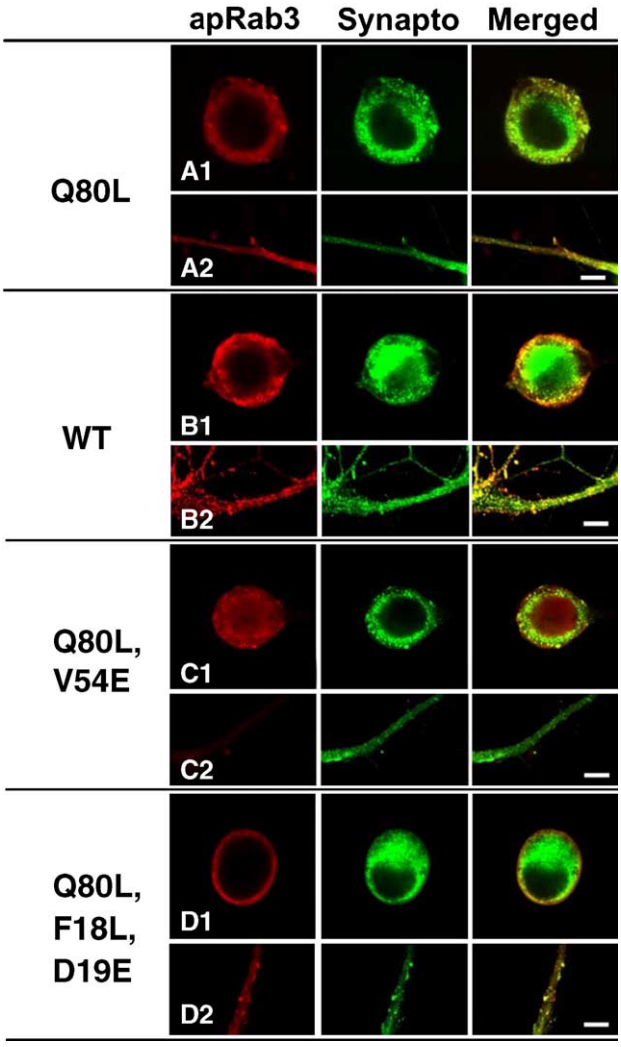

Fig. 2. Expression patterns of various apRab3 constructs used in this study. Confocal microscopic images showing immunocytochemical results using anti-flag antibody for the expression patterns of various apRab3 constructs at the cell body (A1, B1, C1, D1) and neuritic regions (A2, B2, C2, D2). To investigate the localization of overexpressed apRab3 constructs into synaptic vesicles, we co-expressed synaptophysin-EGFP (Synapto), a synaptic vesicle marker, and monitored its colocalization with expression of apRab3 constructs: Q80L, apRab3 Q80L mutant; WT, apRab3 wild type; Q80L, V54E, apRab3 Q80L, V54E double mutant; Q80L, F18L, D19E, apRab3 Q80L, F18L, D19E triple mutant. Scale bar, 20 μm.

22.5%, n = 5, respectively) (Figs. 3B, C). Therefore, these results indicate that GTP-bound apRab3 has no major role in 5-HT-induced STF.

3.4. The interaction of apRab3 with an effector protein, possibly Rim, is critical for the inhibitory role of apRab3 during LTF formation

Since apRab3 Q80L overexpression had no effect on STF, we next investigated the effect of overexpression of various apRab3 constructs on LTF produced by five pulses of 5-HT treatment. We first tested the effect of overexpression of apRab3 Q80L, which is a constitutively active mutant. The overexpression of apRab3 Q80L in the presynaptic terminal completely blocked LTF formation. There was no facilitation of EPSP amplitude 24 h after repetitive 5-HT application at apRab3 Q80L overexpressing synapses (−4.2 ± 18.4%, n = 16; 15 cells among total 16 cells; one-way ANOVA, F = 9.3, df = 4, P < 0.0001) compared with EGFP expressing control synapses (85.3 ± 16.9%, n = 11; 10 cells among total 11 cells) (Figs. 4A, B). In a control experiment, apRab3 Q80L overexpression had no effect on basal synaptic transmission. There were slight decreases in EPSP amplitude (−22.4 ± 5.0%, n = 23), but these changes of EPSP amplitude were not statistically significant compared to that in control cells overexpressing EGFP or injected with buffer solution (−6.3 ± 8.7%, n = 20 and −16.0 ± 5.8%, n = 25, respectively) (Figs. 4A, C) (one-way ANOVA, F = 6.3, df = 5, P < 0.0001).

In addition, apRab3 wild type overexpression, like apRab3 Q80L, also inhibited LTF. When we examined the effect on LTF, 5-HT failed to induce a significant EPSP enhancement at synapses overexpressing apRab3 wild type (−16.4 ± 11.0%, n = 10; one-way ANOVA, F = 9.3, df = 4, P < 0.0001) (Figs. 4A, B). However, apRab3 wild type overexpression had no effect on basal synaptic transmission. The synapses overexpressing apRab3 wild type exhibited no significant differences in the changes of EPSP amplitude before and after overexpression (−17.1 ± 10.5%, n = 10) compared to that in control cells overexpressing EGFP or injected with buffer solution (Figs. 4A, C) (one-way ANOVA, F = 6.3, df = 5, P < 0.0001).

apRab3 in the presynaptic neuron seems to act as a negative clamp of LTF, but it is unclear by what mechanisms apRab3 suppresses LTF formation. We took advantage of the apRab3 mutant constructs to better understand the suppression of LTF formation. Since apRab3 is known to function by interacting with effector proteins, we first examined whether this interaction is important for the inhibition of LTF by apRab3 overexpression in the presynaptic neuron by using the apRab3 Q80L, V54E double mutant. This apRab3 double mutant does not bind effector proteins, such as Rim or Rabphilin. Interestingly, LTF was normally induced at synapses overexpressing apRab3 Q80L, V54E mutant. The EPSP amplitude was markedly enhanced 24 h after 5-HT treatment at synapses overexpressing apRab3 Q80L, V54E (91.4 ± 20.1%, n = 9), thus showing that interaction with effector proteins is essential for inhibition by apRab3 (Figs. 4A, B). As in the case of apRab3 Q80L and apRab3 wild type, apRab3 Q80L, V54E double mutant overexpression had no effect on basal synaptic transmission. The synapses overexpressing apRab3 Q80L, V54E mutant construct exhibited no significant differences in the changes of EPSP amplitude before and after overexpression (−8.7 ± 9.0%, n = 11) compared to that in control cells overexpressing EGFP or injected with buffer solution (Figs. 4A, C) (one-way ANOVA, F = 6.3, df = 5, P < 0.0001).

To specifically investigate the effector protein(s) involved in the inhibitory function of apRab3 on LTF, we

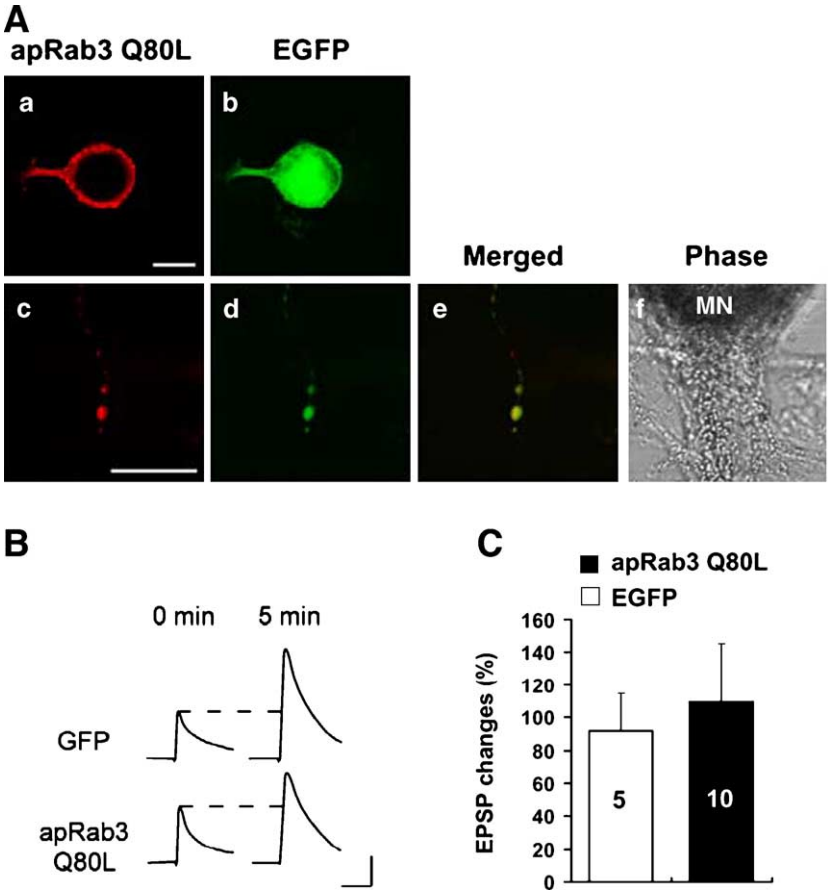

Fig. 3. Effects of the overexpression of apRab3 Q80L, the constitutively active form of apRab3 in the presynaptic neuron on STF. (A) Confocal microscopic images showing immunocytochemical results using anti-flag antibody for the expression patterns of flag-tagged apRab3 Q80L at the cell body (a, b) and synaptic varicosities (c, d, e, f). EGFP expression is used as an expression marker. Phase represents phase contrast image of sensory to motor neuron (MN) co-culture. Scale bar, 20 μm. (B) Representative EPSP recording traces and (C) histograms showing mean percentage changes in EPSP amplitude are shown. Single pulse of 5-HT was applied to cultures after initial EPSP was recorded in cells expressing EGFP or apRab3 Q80L, and the second EPSP was measured 5 min after 5-HT. The height of each bar shows the mean ± SEM. Scale bar, 10 mV, 100 ms.

examined the effects of apRab3 Q80L, F18L, D19E triple mutant overexpression on LTF. We used this apRab3 triple mutant form because this mutation is reported to selectively reduce the interactions of Rab3A with Rim but not with Rabphilin [7], thus enabling us to test the role of specific interactions with Rim. Repetitive 5-HT treatment failed to induce LTF in cells overexpressing the apRab3 Q80L, F18L, D19E mutant. There was no increase of EPSP amplitude after repetitive 5-HT treatment (−13.9 ± 14.0%, n = 9; 8 cells among 9 cells) (Figs. 4A, B). However, the overexpression of apRab3 Q80L, F18L, D19E triple mutant on itself led to the enhancement of basal synaptic transmission. The overexpression of apRab3 Q80L, F18L, D19E elicited statistically significant increases of EPSP amplitude (54.0 ± 22.5%, n = 8; 6 cells among total 8 cells) compared to that at control synapses overexpressing EGFP or injected with buffer solution (−6.3 ± 8.7%, n = 20 and −16.0 ± 5.8%, n = 25, respectively) (Figs. 4A, C) (one-way ANOVA, F = 6.3, df = 5, P < 0.0001). Although apRab3 Q80L, F18L, D19E mutant overexpression on itself elicited the increase of basal EPSP amplitude, there was no further increase of EPSP amplitude by 5-HT. Thus, these data indicate that the enhancement of EPSP amplitude by apRab3 Q80L, F18L, D19E overexpression occluded LTF. In control, LTF was normally induced at EGFP over-expressing control synapses (85.3 ± 16.9%, n = 11). One-way ANOVA indicated a significant effect of the expression of apRab3 Q80L mutant, apRab3 wild type, and apRab3 Q80L, F18L, D19E triple mutant on 5-HT evoked LTF formation (F = 9.3, df = 4, P < 0.0001). Taken together, these results show that GTP-bound apRab3 overexpression inhibits LTF, and this suppression is mediated by the interaction with effector protein(s). We also find that the reduction of the functional interaction of apRab3 with Rim leads to an increase in basal synaptic transmission, and this enhancement of EPSP occludes LTF.

4. Discussion

In the present study, we sought to determine whether Rab3 protein is involved in long-term facilitation induced

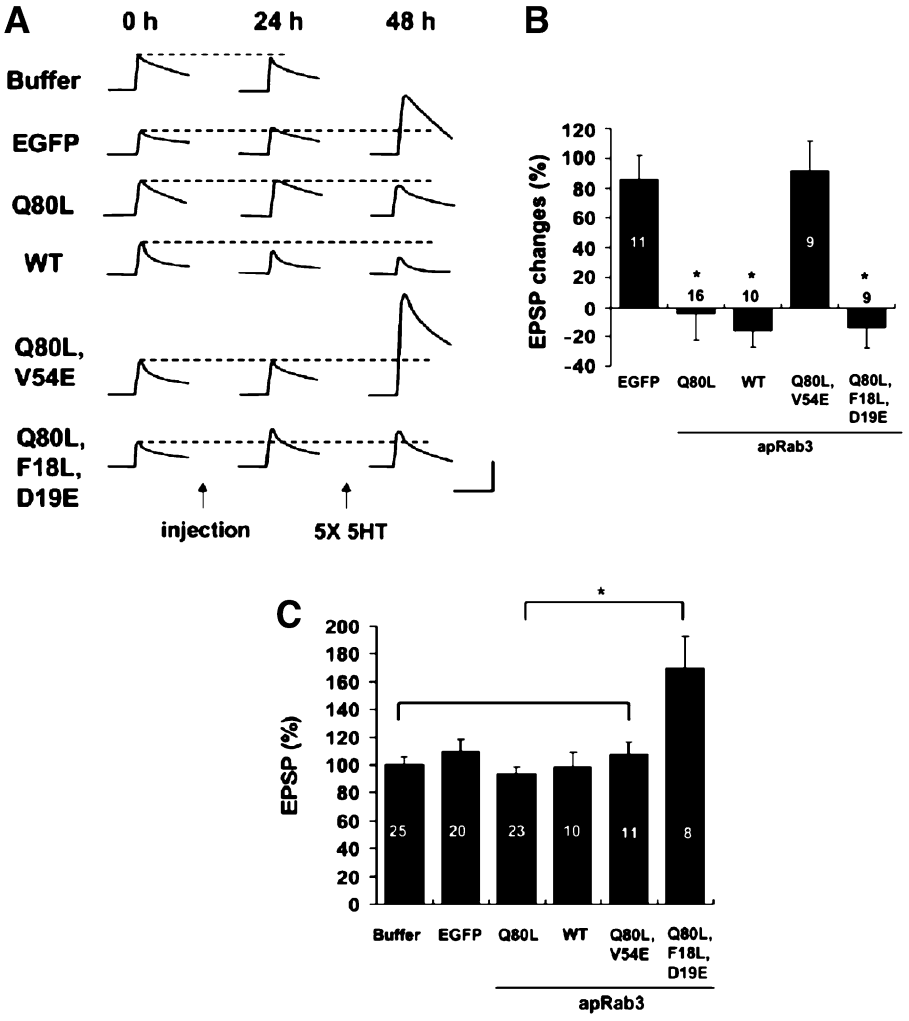

Fig. 4. Effects of the overexpression of various apRab3 constructs in presynaptic neurons on basal synaptic transmission and LTF. (A) Representative EPSP recording traces showing the effects of overexpression of apRab3 wild type and mutant constructs on basal synaptic transmission and 5-HT-induced LTF. Each injection solution was injected after initial EPSP measurements (0 h), and the second EPSP was measured again approximately 24 h after microinjection (24 h). After the second EPSP recording, 5-HT was repeatedly applied for five times to cultures to induce LTF. The same cells were recorded again 24 h after 5-HT (48 h). Buffer: injection buffer alone, EGFP: EGFP alone, Q80L: apRab3 Q80L mutant, WT: apRab3 wild type, Q80L, V54E: apRab3 Q80L, V54E double mutant, Q80L, F18L, D19E: apRab3 Q80L, F18L, D19E triple mutant. Scale bar, 10 mV, 100 ms. (B) Mean percentage changes of EPSP amplitude by repetitive 5-HT treatment recorded in cells expressing apRab3 constructs. Overexpression of apRab3 Q80L and apRab3 wild type inhibits LTF formation. However, V54E mutation neutralized this inhibitory effect on LTF. In addition, there is no further increase of EPSP amplitude in cells overexpressing apRab3 Q80L, F18L, D19E triple mutant in the presence of 5-HT, indicating the occlusion of LTF. One-way ANOVA indicates a significant effect of the expression of apRab3 Q80L mutant, apRab3 wild type, and apRab3 Q80L, F18L, D19E triple mutant on 5-HT evoked LTF formation ($F = 9.3$, $df = 4$, $P < 0.0001$). The height of each bar shows the mean ± SEM. (C) Histogram indicating the effect of overexpression of apRab3 constructs on evoked basal synaptic transmission. Expression of apRab3 Q80L, F18L, D19E mutant on itself enhances synaptic strength. The EPSP changes in cells injected with buffer only were set as 100%. One-way ANOVA indicates a significant effect of apRab3 Q80L, F18L, D19E expression on evoked basal synaptic transmission ($F = 6.3$, $df = 5$, $P < 0.0001$). The height of each bar shows the mean ± SEM.

by 5-HT. The *Aplysia* homologue of Rab3 (apRab3) in *A. kurodai* was cloned, and the effects of overexpression of apRab3 wild type and various mutant forms on LTF were investigated. From these experiments, we demonstrated that (1) GTP-bound apRab3 in presynaptic neurons can suppress LTF, (2) apRab3 has an inhibitory role in LTF by interacting with an effector protein, (3) apRab3–Rim complex may act as a negative clamp in basal synaptic transmission, (4) the modulation of apRab3–Rim interaction may be one of essential mechanisms responsible for LTF, and (5) the inhibitory role of apRab3 is specific for LTF but not STF.

Our study shows that overexpression of the GTP-bound form of apRab3 can inhibit LTF. This inhibitory effect on LTF is specific because apRab3 Q80L or apRab3 wild type overexpression has no effect on STF and basal synaptic transmission. These results also verify that the inhibition of LTF by apRab3 overexpression is not due to nonspecific sequestration of regulatory proteins, for instance, GEP (GTP/GDP exchange proteins) or GDI (GDP dissociation

inhibitors). Our results indicate that interactions with effectors are required for apRab3 to suppress 5-HT-induced LTF since overexpression of apRab3 Q80L, V54E double mutant, which has a mutation in effector domain and so does not bind effector proteins, has no effect on LTF. These results imply that the interactions of apRab3 with Rim or Rabphilin are required for the inhibition of LTF. In addition, the localization of apRab3 at the right place, neurite or presynaptic terminal, may be critical for the inhibition of LTF because apRab3 Q80L, V54E double mutant has a localization defect, showing reduced expression at neurite and synaptic sites and mostly accumulating in the cell body. However, the localization defect is less likely to result from the lack of binding to Rim or Rabphilin, considering the following findings. apRab3 Q80L, F18L, D19E triple mutant, which has reduced binding affinity for Rim, is normally targeted to neurite and synaptic sites. And, Rabphilin is known not to affect Rab3A localization in mammalian nervous system [29]. A previous study [12] in *C. elegans* reported a similar localization defect of Rab3 protein in *aex-3* mutant. Aex-3 is a putative Rab3 GDP–GTP exchange protein. Thus, it is believed that if no GDP–GTP exchange happens on Rab3 because of defective aex-3 mutation, the protein is constitutively bound to GDI in a soluble complex and cannot be attached to synaptic vesicles. Thus, it is likely that the mislocalization of apRab3 Q80L, V54E mutant may be caused by a defect of the GDP–GTP exchange due to the lack of binding to Rab3 GEP (GDP–GTP exchange protein). Nevertheless, we cannot completely rule out the possible role of the interactions with Rim or Rabphilin in the localization of apRab3. In this experiment using Rab3 Q80L, V54E double mutant, since this Rab3 double mutant is not targeted to synaptic terminal, it is not clear whether no effect on LTF in cells expressing Rab3 Q80L, V54E results from the lack of functional interactions with effector molecules at synaptic site or localization defect or both. However, synaptic localization of Rab3 is also dependent on the interactions with effector molecules. Thus, at least, it is reasonable to conclude that the interaction of Rab3 with effector molecules is important for inhibition of LTF by Rab3 overexpression.

apRab3–Rim interaction appears to act as a negative clamp in basal synaptic transmission at sensory to motor neuron synapses. Replacement of endogenous apRab3 with overexpressed apRab3 Q80L, F18L, D19E mutant, which has reduced binding affinity specifically to Rim, led to the increase of synaptic strength. Rab3 triple mutant has reduced binding affinity specifically to Rim rather than binds selectively only some of the effectors. Using this construct, we sought to determine the importance of Rab3–Rim interaction in basal synaptic transmission and synaptic plasticity by weakening specific interaction. Considering that other Rab3 constructs, Rab3 wild type and Rab3 Q80L mutant which are transported to the terminals and also bind effectors, do not affect basal synaptic transmission, endogenous Rab3–Rim interaction seems to be already sufficient for the negative clamping function in basal synaptic transmission, and so expression of Rab3 wild type or Rab3 Q80L may have no further negative effect such as decrease of EPSP amplitude on basal synaptic transmission. Although several lines of evidence have showed that the GTP-bound form of Rab3A generally acts as a negative modulator in evoked basal synaptic release in neurons and neuroendocrine cells [10,13,34], only mild behavioral defects were observed in Rab3 knock-out *C. elegans*, in which a single Rab3 gene exists [25]. Therefore, Rab3 may have divergent functions in basal synaptic release depending on the kinds of synapses such as glutamatergic or cholinergic synapse in different animal systems. In addition, this result is consistent with previous study in mammalian nervous system showing the clamping role of Rab3A–Rim complex for Ca^{2+}-dependent exocytosis [35]. In their study, co-transfection of N-terminal Rab3 binding domain of Rim with growth hormone in PC12 cells results in the elimination of functional interactions of Rab3 with Rim and elicits large increases in calcium-dependent secretion, but this increase is neutralized by co-transfection of Rab3A.

Interestingly, the increase of synaptic strength by overexpression of apRab3 Q80L, F18L, D19E triple mutant occluded LTF. 5-HT failed to induce further increases of EPSP amplitude at synapses overexpressing this apRab3 triple mutant. Thus, these results allow us to propose a notion that apRab3–Rim interaction may act as a negative clamp on the enhancement of presynaptic release during LTF and that the regulation of this interaction may be one of critical mechanisms responsible for LTF. Similarly, a previous study in mammalian nervous system [29] shows that Rabphilin is not required for Rab3 function. In contrast to the Rab3A knock-out mice study [5], Schlüter et al. [29] found that mossy fiber LTP is normally induced in Rabphilin knock-out mice. In contrast, Rim is important for cerebellar parallel fiber LTP. LTP is absent in neurons from RIM1α KO mice [21]. Thus, it is more likely that the specific modulation of interactions of apRab3 with Rim, but not Rabphilin, may be a critical component of mechanisms for the increase in synaptic strength during LTF. However, it is not demonstrated in our study how the interaction between apRab3 and Rim specifically can be regulated by repetitive 5-HT treatment. One possibility is that Rab3 itself may be modulated by repetitive 5-HT application and subsequently affect the interactions with effectors. However, the mRNA level of apRab3 shows no significant changes by 5-HT ([31], our result (data not shown)). In addition, the direct phosphorylation of RIM1α by PKA is shown to be an essential mechanism underlying mossy fiber presynaptic LTP, although the role of this phosphorylation remains unknown. Thus, another more plausible possibility is that the changes of expression level or phosphorylation state of Rim are induced by PKA signaling activated by 5-HT and this leads to the reduction of binding affinity of Rim to Rab3. Since Rab3–Rim complex appears to act as a negative clamp in synaptic release in our study, the

weakening of Rab3–Rim interaction by 5-HT signaling may contribute to the increase of EPSP amplitude during long-term facilitation. Direct biochemical analysis of the interaction between the apRab3 Q80L, F18L, D19E triple mutant used in this study and Rim protein or electrophysiological analysis of the function of Rim during LTF would provide further evidence for the role of Rim in LTF. Future studies would be needed to clone the *Aplysia* homologue of Rim and to clarify the role of apRab3–Rim interaction during LTF. It will be very interesting to examine whether the expression level or phosphorylation state of RIM or Rab3 is regulated by LTF induction, and this regulation indeed affect the interaction between Rab3 and Rim in the future study.

In conclusion, interaction of apRab3 with effector molecule(s), possibly Rim, may act as a negative clamp on LTF, and thus the regulation of apRab3–Rim interaction by 5-HT may be one of the essential mechanisms responsible for LTF formation.

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