Proc. Natl. Acad. Sci. USA
Vol. 95, pp. 11418–11422, September 1998
Neurobiology

Role of the Doc2α–Munc13–1 interaction in the neurotransmitter release process

(C2 domain/neurotransmission/cholinergic synapse)

SUMIKO MOCHIDA*†, SATOSHI ORITA‡, GAKU SAKAGUCHI‡, TAKUYA SASAKI§, AND YOSHIMI TAKAI§

*Department of Physiology, Tokyo Medical College, Tokyo 160-8402, Japan; ‡Sionogi Institute for Medical Science, Settsu 566-0022, Japan; and §Department of Molecular Biology and Biochemistry, Osaka University Medical School, Suita 565-0871, Japan

Communicated by Erwin Neher, Max Planck Institute for Biophysical Chemistry, Goettingen, Germany, July 10, 1998 (received for review November 25, 1997)

ABSTRACT **Doc2α and Munc13–1 proteins are highly concentrated on synaptic vesicles and the presynaptic plasma membrane, respectively, and have been implicated in Ca^{2+}-dependent neurotransmitter release. Doc2α interacts with Munc13–1 through the N-terminal region of Doc2α (the Mid domain; amino acid residues 13–37). Here we examine whether the interaction between Doc2α and Munc13–1 is required for Ca^{2+}-dependent neurotransmitter release from intact neuron. A synthetic Mid peptide (the Mid peptide), but not a control mutated Mid peptide or a scrambled Mid peptide, inhibited the interaction between Doc2α and Munc13–1 *in vitro*. Introduction of the Mid peptide into presynaptic neurons of cholinergic synapses, formed between rat superior cervical ganglion neurons, reversibly inhibited synaptic transmission evoked by action potentials. In contrast, the control peptides did not inhibit synaptic transmission. This inhibitory effect depended on the presynaptic activity and was affected by extracellular Ca^{2+} concentrations. The onset of the Mid peptide effect was shortened when the neuron was stimulated at a higher frequency, and the inhibition was more potent at 1 mM Ca^{2+} than at 5.1 mM Ca^{2+}. These results suggest that the Doc2α–Munc13–1 interaction plays a role in a step before the final fusion step of synaptic vesicles with the presynaptic plasma membrane in the evoked neurotransmitter release process.**

Synaptic vesicles are transported to the presynaptic plasma membrane where Ca^{2+} channels are located. Depolarization induces Ca^{2+} influx into the cytosol of nerve terminals through the Ca^{2+} channels, and this Ca^{2+} influx initiates the fusion of the vesicles with the plasma membrane, finally leading to neurotransmitter release. Recent studies have revealed that this synaptic vesicle exocytosis consists of many complicated steps (1), such as the translocation of vesicles from the reserve pool to the active zone, the docking of vesicles at the active zone, transition from the docking to the priming step, and the fusion step, each of which is regulated by many components.

Genetic and electrophysiological studies suggest that synaptotagmin serves as a Ca^{2+} sensor for neurotransmitter release (2–4). This protein has two C2 domains interacting with Ca^{2+} and phospholipid (5). However, other Ca^{2+} sensors may also be involved in neurotransmitter release. Other candidates for Ca^{2+} sensors are Doc2 (6) and Munc13 (7), which also have two C2 domains and have been implicated in Ca^{2+}-dependent neurotransmitter release (8–10). Doc2 consists of two isoforms, Doc2α and Doc2β (6, 11, 12). Doc2α is specifically expressed in neuronal cells and localized on synaptic vesicles, whereas Doc2β is ubiquitously expressed (6, 11–13). Both isoforms of Doc2 interact with Munc13 (9), a mammalian homologue of *Caenorhabditis elegans unc*-13 (7, 10). Munc13 consists of three isoforms, Munc13–1, -2, and -3. All isoforms of Munc13 have one C1 domain that interacts with phorbol ester or diacylglycerol (14). Munc13 is specifically expressed in neuronal cells and is located at the presynaptic plasma membrane (7). Doc2α and Munc13–1 interact with each other through the N-terminal region of Doc2α (the Mid domain; amino acid residues 13–37) and the C-terminal region of Munc13–1 (the Did domain; amino acid residues 851–1,461). This interaction is induced by binding of diacylglycerol or phorbol ester to Munc13–1 (9).

This biochemical evidence suggests that Doc2 plays an important role in Ca^{2+}-dependent neurotransmitter release. However, it remains to be clarified whether the Doc2α–Munc13–1 interaction is indeed involved in Ca^{2+}-dependent neurotransmitter release and, if so, which stage in the neurotransmitter release process this interaction regulates. We have attempted here to address these issues. For this purpose, we analyzed nerve impulse-evoked transmitter release between pairs of cultured superior cervical ganglion neurons (SCGNs) in which the synthetic peptide of the Mid domain (the Mid peptide) is introduced into the presynaptic partner. Cultures of SCGNs are favorable for these experiments because peptides or proteins can be introduced into the relatively large presynaptic cell bodies by microinjection, the injected peptides or proteins can rapidly diffuse to nerve terminals forming synapse with adjacent neurons, and the effects of the stimulated release of acetylcholine can be accurately monitored by recording the excitatory postsynaptic potentials (EPSPs) evoked by action potentials in the presynaptic neurons (15, 16).

MATERIALS AND METHODS

Materials. The Mid peptide [the Mid domain of Doc2α (amino acid residues 13–37); IQEHMAINVCPGPIRPIRQISDYFP], the mutated Mid peptide (IYKDWAFNVCPGPIRPIRQISDYFP), and the scrambled Mid peptide (ICPIQRHNSQPDPVGYIFEIRIMAP) were synthesized by a Multiple Peptide Synthesizer (SYRO II, MultiSynTec, Witten, Germany). The cDNA fragment encoding the N-terminal fragment of human Doc2α (amino acid residues 1–90) (6) was inserted into a pGEX-2T plasmid, expressed in *Escherichia coli* as a glutathione *S*-transferase fusion protein, and purified on a glutathione–Sepharose 4B column (Amersham Pharmacia Biotech, Uppsala, Sweden).

Assay for the Doc2α–Munc13–1 Interaction in a Cell-Free System. The Munc13–1 cDNA inserted into pBluescript-myc

The publication costs of this article were defrayed in part by page charge payment. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. §1734 solely to indicate this fact.

Abbreviations: Mid, interacting domain of Doc2α with Munc13–1; SCGNs, superior cervical ganglion neurons; EPSP(s), excitatory postsynaptic potential(s); RT, reverse transcriptase.

†To whom reprint requests should be addressed at: Department of Physiology, Tokyo Medical College, 1–1, Shinjuku-6-chome, Shinjuku-ku, Tokyo 160-8402, Japan. e-mail: mochida@tokyo-med.ac.jp.

was translated *in vitro* by using a TNT T7-coupled reticulocyte lysate system (Promega). One microgram of glutathione *S*-transferase–Doc2α (amino acid residues 1–90) was immobilized onto 20 μl of glutathione–Sepharose 4B beads. The immobilized beads were added to 500 μl of buffer A (50 mM Hepes, pH 7.4/150 mM NaCl/1 mM EGTA) containing *in vitro* translated product of Munc13–1, and gently mixed for 4 hr at 4°C in the presence of the Mid peptide or its control mutated Mid peptide or scrambled Mid peptide. The beads were washed four times with buffer A and the bound proteins were eluted by addition of 100 μl of buffer A containing 20 mM glutathione. The eluates were subjected to SDS/PAGE followed by autoradiography.

Reverse Transcriptase (RT)-PCR Analysis. Total RNA was isolated from rat brain and SCGNs by using TRIzol Reagent (GIBCO/BRL), and reverse-transcribed by using the T-Primed First-Strand kit (Amersham Pharmacia Biotech). PCR was performed by using a Perkin–Elmer PCR kit. Nucleotide sequences for primers were as follows: rat Doc2α (12), 5′-GATGTTAACGGCTACTCTGA-3′ and 5′-ATGTCG-TAGTCCCAGACTGT-3′; and rat Munc13–1 (7), 5′-GAAGAAGCCAAGAGCTTGACC-3′ and 5′-ATGCGT-GAACAGCTCCACGTG-3′. An aliquot of PCR products was electrophoresed and visualized by staining with ethidium bromide. Another aliquot was subcloned by using a pCR-Script AmpSK plasmid (Stratagene), and the authenticity of the products was verified by DNA sequencing.

Electrophysiological Recordings. SCGNs were prepared from 7-day postnatal rats as described (15, 16). Conventional intracellular recordings were made from two neighboring neurons and cultured for 4–5 weeks using microelectrodes filled with 1 M potassium acetate (40–70 MΩ). Neurons with nearby cell bodies were selected for recording. EPSPs were recorded from one cell of the neurons when action potentials were generated in the other neuron by passage of current through an intracellular recording electrode. Experiments were carried out at 32–34°C. Neurons were superfused with modified Krebs solution (3 mM Hepes, pH 7.4/136 mM NaCl/5.9 mM KCl/5.1 mM $CaCl_2$/1.2 mM $MgCl_2$/11 mM glucose). Samples for intracellular injection were dissolved in an intracellular solution (10 mM Hepes, pH 7.4/150 mM potassium acetate/5 mM Mg^{2+}-ATP) and introduced into the presynaptic cell body by diffusion from a suction glass pipette (17–20 MΩ tip resistance). Fast Green FCF (5%, Sigma) was introduced in the pipette solution to confirm entry into the presynaptic cell body. EPSPs were recorded once every 5, 20, and 100 sec (0.2, 0.05, and 0.01 Hz). Electrophysiological data were collected and analyzed by using software written by L. Tauc (Centre National de la Recherche Scientifique, Gif-sur-Yvette, France).

RESULTS

Expression of Doc2α and Munc13–1 in SCGNs. To demonstrate the expression of Doc2α and Munc13–1 in SCGNs, RT-PCR was performed by using specific primers. The products derived from the Doc2α and Munc13–1 mRNAs were detected in SCGNs dissected from 7-day postnatal rats (Fig. 1). The sequences of the PCR products were confirmed to be identical with those of Doc2α and Munc13–1 (data not shown). The results indicate that Doc2α and Munc13–1 are indeed expressed in SCGNs, as well as in rat brain.

Inhibition of the *in Vitro* Doc2α–Munc13–1 Interaction by the Mid Peptide. We prepared the Mid peptide, and its control mutated Mid peptide and scrambled Mid peptide, and examined whether they inhibited the *in vitro* interaction between Doc2α and Munc13–1. The Mid peptide inhibited this interaction in a dose-dependent manner, whereas the mutated Mid peptide or the scrambled Mid peptide did not (Fig. 2). These results suggest that an excess amount of the Mid peptide could disrupt the Doc2α–Munc13–1 interaction in intact nerve terminals.

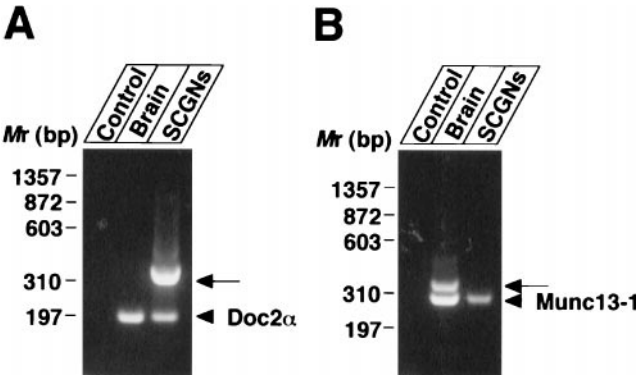

FIG. 1. Expression of Doc2α and Munc13–1 in SCGNs. (*A*) RT-PCR analysis of Doc2α mRNA in rat brain and SCGNs. Control indicates minus RNA control sample. Arrow indicates product derived from genomic DNA. (*B*) RT-PCR analysis of Munc13–1 mRNA in rat brain and SCGNs. Arrow indicates position of an alternatively spliced transcript of Munc13–1 (7, 9). The size of markers is shown at left. Results shown are representative of three independent experiments.

Inhibition of Synaptic Transmission by the Mid Peptide. Synaptic transmission was monitored between closely spaced (<5 mm) pairs of neurons for 20–30 min, and the samples to be tested were then allowed to diffuse into the presynaptic neurons from a suction pipette for 2–3 min. During this time, the concentration of injected sample inside the presynaptic cell body reached approximately 5% of the concentration in the pipette, as estimated from the intensity of the coinjected Fast Green FCF dye and from correction for the effect of molecular mass diffusion (17). As in the previous functional studies of presynaptic terminal proteins (15, 16, 18, 19), EPSPs were evoked at 0.05 Hz by action potentials elicited by current passage applied into presynaptic cell through a recording microelectrode and were recorded with a second microelectrode in the adjacent postsynaptic cell. The concentration of Ca^{2+} in the external solution was 5.1 mM, instead of the normal concentration of 2.5 mM Ca^{2+}, to produce larger EPSPs (15, 16, 18, 19). After a stable period of control recordings, the Mid peptide was introduced at $t = 0$ with a pipette containing 2.5 mM, which produced a maximum

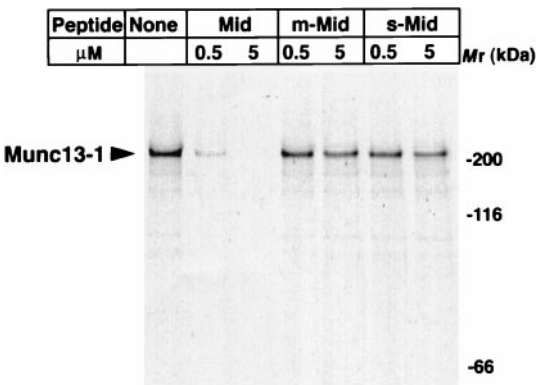

FIG. 2. Effects of the Mid peptide on the Doc2α–Munc13–1 interaction in a cell-free system. Affinity-purified glutathione *S*-transferase–Doc2α (amino acid residues 1–90) immobilized on glutathione–Sepharose beads was incubated with *in vitro* translated, [^{35}S]-methionine-labeled Munc13–1 in the presence of the indicated concentrations of the Mid peptide or the control peptides. Specifically bound proteins were detected by SDS/PAGE followed by autoradiography. Mid, Mid peptide; m-Mid, mutated Mid peptide; s-Mid, scrambled Mid peptide. The protein markers used were myosin (M_r 200,000), β-galactosidase (M_r 116,000), and BSA (M_r 66,200). Results shown are representative of three independent experiments.

concentration of 125 μM in the soma. Injection of the Mid peptide did not affect synaptic transmission for 20–30 min and then gradually reduced the EPSP amplitude (Fig. 3). The maximal decrease in the EPSP amplitude, $-29 \pm 1.8\%$ (n = 7, mean ± SEM, Table 1), was observed 40–50 min after starting the injection. At later times the EPSPs gradually recovered. No significant change in the time courses of the EPSPs induced by single action potential was observed after the injection of the Mid peptide (Fig. 3*A*). Under conditions where the Mid peptide showed an inhibitory effect, the mutated Mid peptide and the scrambled Mid peptide showed little effect on the EPSP amplitude during the 70-min record (n = 6, Fig. 3*B*). This suggests that the inhibitory effect of the Mid peptide is specific. These results provide an additional line of evidence that the Mid domain of Doc2α is involved in the interaction with Munc13–1 and that this interaction plays an important role in the neurotransmitter release process.

Presynaptic Activity-Dependent Effect of the Mid Peptide. To examine whether the effect of the Mid peptide on synaptic transmission depends on presynaptic activity or on presynaptic firing rate, stimulation frequency was varied. When synapses were stimulated at 0.2 Hz, more rapid inhibition of synaptic transmission was observed after the injection of the Mid peptide (n = 5, Fig. 4). The maximal decrease in the EPSP amplitude, $-32 \pm 3.8\%$ (n = 5, Table 1), was observed 25–30 min after the injection, and thereafter the EPSPs recovered slowly. In another series of experiments, stimulation frequency was lowered to 0.01 Hz. The onset of the Mid peptide effect was delayed for 40–50 min after the injection at this lower frequency (n = 5). These results indicate that the Mid peptide produces a presynaptic activity-dependent inhibition of evoked transmitter release.

Extracellular Ca^{2+}-Dependent Effect of the Mid Peptide. To test whether the Doc2α–Munc13–1 interaction is affected by extracellular Ca^{2+}, the concentration of Ca^{2+} in the external solution was reduced from 5.1 mM to 1 mM. At 1 mM Ca^{2+}, the EPSP amplitude fluctuated but did not significantly decrease during more than 1 hr of recording from synapses stimulated at 0.05 Hz (n = 3, data not shown). This suggests that the size of the readily releasable pool is not affected by superfusion with extracellular solution containing 1 mM Ca^{2+}. The onset of the inhibitory effect of the Mid peptide on synaptic transmission was shortened to 15–20 min after the injection (n = 5, Fig. 5*A*). The maximal decrease in the EPSP amplitude, $-51 \pm 7.1\%$ (n = 5, Table 1), was observed 30–50 min after the injection. These results indicate that the Mid peptide effect was accelerated with a reduction in the Ca^{2+} concentrations of the external solution.

When synapses were stimulated at 0.2 Hz at 1 mM Ca^{2+}, a slight decline in the EPSP amplitude similar to that at 5.1 mM Ca^{2+} was observed after the injection of the control carrier solution (n = 2, data not shown). The onset of the inhibitory effect of the Mid peptide was 5–10 min (n = 5, Fig. 5*B*), similar to that at 5.1 mM Ca^{2+}. This indicates that the time for inducing the peptide effect may be the shortest when synapse is stimulated at 0.2 Hz. As indicated in a previous study (15), EPSPs decreased gradually with time when stimulation was applied at >0.3 Hz, suggesting that such repetitive stimulation causes depletion of vesicles from readily releasable pools. The effect of the Mid peptide on synaptic transmission was stronger and lasted longer in the synapses stimulated at 0.2 Hz at 1 mM Ca^{2+}: the maximal decrease in the EPSP amplitude, $-59 \pm 5.5\%$ (n = 5, Table 1), was observed 40–60 min after the injection. In three of five experiments where the maximal decrease was observed around 40 min, EPSPs increased very slowly and recovered $15 \pm 2.4\%$ (n = 3) at 70 min after the injection. These results indicate that extracellular Ca^{2+} concentration affects the degree and duration of the Mid peptide effect, but not the onset, when stimulation frequency is raised.

DISCUSSION

We have studied here the physiological importance of the Doc2α–Munc13–1 interaction in the neurotransmitter release process. For this purpose, we have injected the Mid peptide into SCGNs and examined its effect on synaptic transmission. We have shown here that the Mid peptide actually disrupts the *in vitro* interaction between Doc2α and Munc13–1. We have

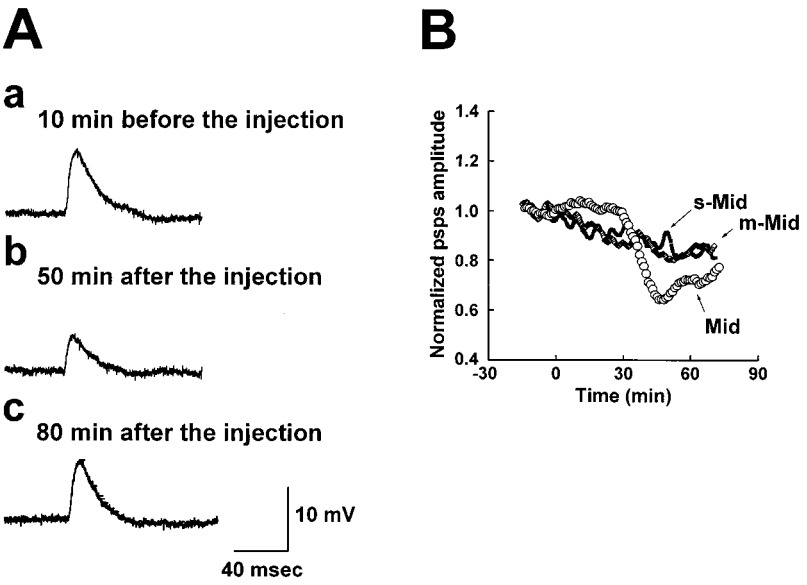

FIG. 3. Effect of the Mid peptide on synaptic transmission. The Mid peptide was introduced into presynaptic neurons, while postsynaptic responses were evoked by presynaptic action potentials elicited at 0.05 Hz at 5.1 mM Ca^{2+}. (*A*) EPSPs induced by single action potential before and after the injection of the Mid peptide from a pipette containing 2.5 mM peptide. Postsynaptic potentials recorded at the indicated times were taken from one representative experiment. (*a*) Ten minutes before the injection, (*b*) 50 min after the injection, (*c*) 80 min after the injection. (*B*) Inhibition of synaptic transmission by the Mid peptide. Normalized average postsynaptic potentials are plotted from experiments with the injection of the Mid peptide (Mid, n = 7), the mutated Mid peptide (m-Mid, n = 6), and the scrambled Mid peptide (s-Mid, n = 6) at 2.5 mM in the pipette. Peak values of the EPSP amplitude were measured and averaged. The resultant values were smoothed by eight-point moving average algorithm and plotted against recording time with t = 0 indicating the beginning of the presynaptic injection. (○), Mid; (◇), m-Mid; (■), s-Mid.

Table 1. Effects of the Mid peptide on evoked EPSPs

Injected reagent	Frequency of stimulation, Hz	Ca^{2+} concentration, mM	No. of experiments	Change in amplitude, %
Mid peptide	0.01	5.1	5	-14 ± 19*
Mid peptide	0.05	5.1	7	-29 ± 1.8†
Mid peptide	0.2	5.1	5	-32 ± 3.8‡
Mid peptide	0.05	1	5	-51 ± 7.1§
Mid peptide	0.2	1	5	-59 ± 5.5¶
Control m-Mid peptide	0.05	5.1	6	-6 ± 11‖
Control s-Mid peptide	0.05	5.1	6	-10 ± 6.8‖
Carrier solution	0.05	5.1	7	-3 ± 2.4‖
Carrier solution	0.2	5.1	5	-8 ± 2.3‖

Samples to be tested were introduced into presynaptic neurons. Concentrations of the Mid peptide and the control peptides in the pipette were 2.5 mM. EPSPs were evoked by presynaptic action potentials elicited at the indicated frequencies of stimulation. Neurons were superfused with modified Krebs solution containing the indicated concentrations of Ca^{2+}. EPSPs were measured when maximum inhibitory effect was observed at 60*, 40–50,† 25–30,‡ 30–50,§ and 40–60¶ min after starting injection.

‖EPSPs were measured at 30 min after the injection of the control peptides or the carrier solution. Changes in amplitude of EPSPs were expressed as percent inhibition of the preinjection value (mean ± SEM).

also shown that the Mid peptide inhibits synaptic transmission induced by presynaptic action potentials. These results indicate that the Doc2α–Munc13–1 system is involved in neurotransmitter release from SCGNs.

It has been shown that injection of the antisynaptotagmin antibody inhibits synaptic transmission at the SCGN synapses, and that this inhibitory effect is rapid (19). These results, together with genetic and biochemical observations (4, 20), suggest that synaptotagmin is involved at least in the final fusion step of synaptic vesicles with the presynaptic plasma membrane. In contrast, the inhibitory effect of the Mid peptide on synaptic transmission is relatively slow. Moreover, the inhibitory effect is dependent on presynaptic activity; the onset of inhibition becomes more rapid and the inhibitory effect becomes more marked during high frequency stimulation. This observation suggests that the inhibition of the Doc2α–Munc13–1 interaction by the Mid peptide may alter the efficiency of synaptic vesicle supply and induce the depletion of fusion-competent vesicles. These results, together with earlier observations (15, 18), suggest that the Doc2α–Munc13–1 interaction is involved in a step before the final fusion step in the neurotransmitter release process.

Moreover, we have shown here that the inhibitory effect of the Mid peptide on synaptic transmission is affected by the Ca^{2+} concentrations of the external solution. The mechanism for this effect of extracellular Ca^{2+} is not known, but our current speculation is that the Doc2α–Munc13–1 interaction is induced by binding of phorbol ester or diacylglycerol to Munc13–1 (9). Diacylglycerol is generated by the phospholipase C-catalyzed hydrolysis of phosphoinositide. Phosphoinositide phospholipase C (type β) requires 10^{-6}–10^{-5} M Ca^{2+} for its activity (21). This Ca^{2+} concentration range is similar to that required for initiation of neurotransmitter release (22). It is assumed that diacylglycerol formation is induced by Ca^{2+} influx through Ca^{2+} channels in presynaptic terminals (23). Therefore, the reduction of extracellular Ca^{2+} may reduce the increase in Ca^{2+} concentrations in nerve terminals, which may cause the reduced formation of diacylglycerol. This may induce weak Doc2α–Munc13–1 interaction and cause a more marked inhibitory effect of the Mid peptide on synaptic transmission. However, it is possible that Ca^{2+} acts on the C2 domains of both Doc2α and Munc13–1, by analogy with synaptotagmin, which has two C2 domains and has been shown to serve as a Ca^{2+} sensor (4, 5).

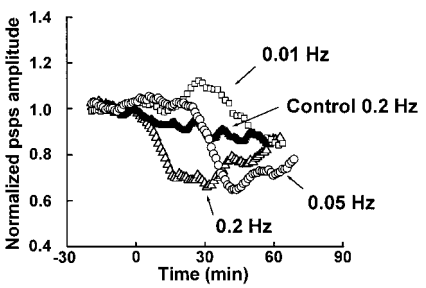

FIG. 4. Activity-dependent effect of the Mid peptide on synaptic transmission. Normalized average postsynaptic potentials are plotted from experiments where the Mid peptide was injected from pipette containing 2.5 mM peptide and presynaptic stimulation was applied at the indicated frequencies. (□), 0.01 Hz (n = 5); (○), 0.05 Hz (n = 7); (△), 0.2 Hz (n = 5); (▲), 0.2 Hz with the injection of the control carrier solution (n = 5).

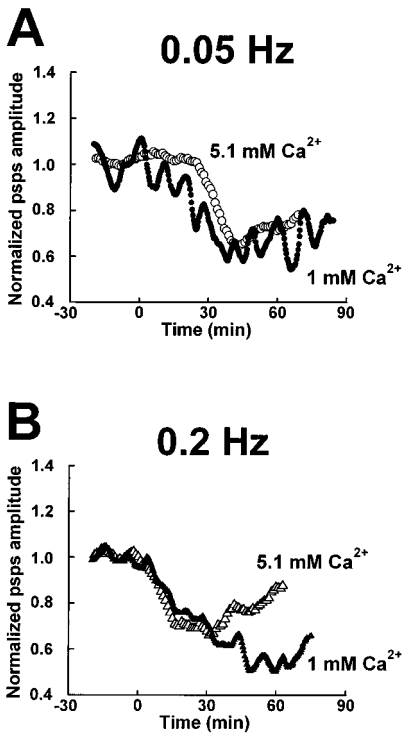

FIG. 5. Extracellular Ca^{2+}-dependent effect of the Mid peptide on synaptic transmission. Normalized average postsynaptic potentials are plotted from experiments where the Mid peptide was injected from pipette containing 2.5 mM peptide and presynaptic stimulation was applied at 0.05 or 0.2 Hz at the indicated concentrations of Ca^{2+}. (*A*) 0.05 Hz. (*B*) 0.2 Hz. (●), 1 mM Ca^{2+} (n = 5); (▲), 1 mM Ca^{2+} (n = 5); (○), 5.1 mM Ca^{2+} (n = 7); (△), 5.1 mM Ca^{2+} (n = 5).

Considering the perturbation of the Doc2α–Munc13–1 interaction by the Mid peptide, it is likely that the Doc2α–Munc13–1 system is involved in the formation of a synaptic vesicle pool from which synaptic vesicle are transported to a releasable pool when nerve impulses arrive repetitively at high frequencies. The Doc2α–Munc13–1 system may function in cooperation with syntaxin and Munc18 in a prior stage of synaptic vesicle docking with the core complex of VAMP/synaptobrevin, SNAP-25, syntaxin, and Munc13–1. Consistent with this possibility, Doc2 and Munc13 have been shown to interact with Munc18 (12) and syntaxin (24), respectively. Further studies are necessary to clarify the role of the Doc2α–Munc13–1 system as a component of the Ca^{2+}-dependent neurotransmitter release machinery.

We are grateful to Drs. Masakazu Hatanaka, Osamu Yoshie, and Hisanaga Igarashi (Shionogi Institute for Medical Science, Osaka) for helpful discussions. We also thank Dr. George J. Augustine (Duke University) for commenting on the manuscript. This work was supported by a grant-in-aid for Scientific Research from the Ministry of Education, Science, Sports, and Culture, Japan (S.M. and Y.T.), by grants-in-aid for Abnormalities in Hormone Receptor Mechanisms and for Aging and Health from the Ministry of Health and Welfare, Japan (Y.T.), grants from the Naito Foundation and the Brain Science Foundation (S.M.), and a grant from the Human Frontier Science Program (S.M. and Y.T.).

1. Südhof, T. C. (1995) *Nature (London)* **375,** 645–653.
2. Nonet, M. L., Grundahl, K., Meyer, B. J. & Rand, J. B. (1993) *Cell* **73,** 1291–1305.
3. DiAntonio, A., Parfitt, K. D. & Schwarz, T. L. (1993) *Cell* **73,** 1281–1290.
4. Geppert, M., Goda, Y., Hammer, R. E., Li, C., Rosahl, T. W., Stevens, C. F. & Südhof, T. C. (1994) *Cell* **79,** 717–727.
5. Perin, M. S., Fried, V. A., Mignery, G. A., Jahn, R. & Südhof, T. C. (1990) *Nature (London)* **345,** 260–263.
6. Orita, S., Sasaki, T., Naito, A., Komuro, R., Ohtsuka, T., Maeda, M., Suzuki, H., Igarashi, H. & Takai, Y. (1995) *Biochem. Biophys. Res. Commun.* **206,** 439–448.
7. Brose, N., Hofmann, K., Hata, Y. & Südhof, T. C. (1995) *J. Biol. Chem.* **270,** 25273–25280.
8. Orita, S., Sasaki, T., Komuro, R., Sakaguchi, G., Maeda, M., Igarashi, H. & Takai, Y. (1996) *J. Biol. Chem.* **271,** 7257–7260.
9. Orita, S., Naito, A., Sakaguchi, G., Maeda, M., Igarashi, H., Sasaki, T. & Takai, Y. (1997) *J. Biol. Chem.* **272,** 16081–16084.
10. Hosono, R. & Kamiya, Y. (1991) *Neurosci. Lett.* **128,** 243–244.
11. Sakaguchi, G., Orita, S., Maeda, M., Igarashi, H. & Takai, Y. (1995) *Biochem. Biophys. Res. Commun.* **217,** 1053–1061.
12. Verhage, M., de Vries, K. J., Røshol, H., Burbach, J. P. H., Gispen, W. H. & Südhof, T. C. (1997) *Neuron* **18,** 453–461.
13. Naito, A., Orita, S., Wanaka, A., Sasaki, T., Sakaguchi, G., Maeda, M., Igarashi, H., Tohyama, M. & Takai, Y. (1997) *Mol. Brain Res.* **44,** 198–204.
14. Kazanietz, M. G., Lewin, N. E., Bruns, J. D. & Blumberg, P. M. (1995) *J. Biol. Chem.* **270,** 10777–10783.
15. Mochida, S., Kobayashi, H., Matsuda, Y., Yuda, Y., Muramoto, K. & Nonomura, Y. (1994) *Neuron* **13,** 1131–1142.
16. Mochida, S., Saisu, H., Kobayashi, H. & Abe, T. (1995) *Neuroscience* **65,** 905–915.
17. Pusch, M. & Neher, E. (1988) *Pflügers Arch.* **411,** 204- 211.
18. Mochida, S., Sheng, Z.-H., Baker, C., Kobayashi, H. & Catterall, W. A. (1996) *Neuron* **17,** 781–788.
19. Mochida, S., Fukuda, M., Niinobe, M., Kobayashi, H. & Mikoshiba, K. (1997) *Neuroscience* **77,** 937–943.
20. Li, C., Ullrich, B., Zhang, J. Z., Anderson, R. G. W., Brose, N. & Südhof, T. C. (1995) *Nature (London)* **375,** 594–599.
21. Taylor, S. J., Chae, H. Z., Rhee, S. G. & Exton, J. H. (1991) *Nature (London)* **350,** 516–518.
22. Zucker, R. S. (1996) *Neuron* **17,** 1049–1055.
23. Wakade, T. D., Bhave, S. V., Bhave, A. S., Malhotra, R. K. & Wakade, A. R. (1991) *J. Biol. Chem.* **266,** 6424–6428.
24. Betz, A., Okamoto, M., Benseler, F. & Brose, N. (1997) *J. Biol. Chem.* **272,** 2520–2526.