Conserved N-terminal Cysteine Motif Is Essential for Homo- and Heterodimer Formation of Synaptotagmins III, V, VI, and X*

(Received for publication, May 17, 1999, and in revised form, August 5, 1999)

Mitsunori Fukuda‡§, Eiko Kanno‡, and Katsuhiko Mikoshiba¶

From the ²Developmental Neurobiology Laboratory, Brain Science Institute, Institute of Physical and Chemical Research (RIKEN), 2-1 Hirosawa, Wako, Saitama 351-0198, Japan; the ³Department of Molecular Neurobiology, Institute of Medical Science, University of Tokyo, Tokyo 108-8639, Japan; and the ⁴Calciosignal Net Project, Exploratory Research for Advanced Technology, Japan Science and Technology Corporation, 2-28-8 Honkomagome, Bunkyo-ku, Tokyo 113-0021, Japan

The synaptotagmins now constitute a large family of membrane proteins characterized by one transmembrane region and two C2 domains. Dimerization of synaptotagmin (Syt) I, a putative low affinity Ca\textsuperscript{2+} sensor for neurotransmitter release, is thought to be important for expression of function during exocytosis of synaptic vesicles. However, little is known about the self-dimerization properties of other isoforms. In this study, we demonstrate that a subclass of synaptotagmins (III, V, VI, and X) (Ibata, K., Fukuda, M., and Mikoshiba, K. (1998) J. Biol. Chem. 273, 12267–12273) forms \( \beta \)-mercaptoethanol-sensitive homodimers and identify three evolutionarily conserved cysteine residues at the N terminus (N-terminal cysteine motif, at amino acids 10, 21, and 33 of mouse Syt III) that are not conserved in other isoforms. Site-directed mutagenesis of these cysteine residues and co-immunoprecipitation experiments clearly indicate that the first cysteine residue is essential for the stable homodimer formation of Syt III, V, or VI, and heterodimer formation between Syts III, V, VI, and X. We also show that native Syt III from mouse brain forms a \( \beta \)-mercaptoethanol-sensitive homodimer. Our results suggest that the cysteine-based heterodimerization between Syt III and Syt V, VI, or X, which have different biochemical properties, may modulate the proposed function of Syt III as a putative high affinity Ca\textsuperscript{2+} sensor for neurotransmitter release.

Recent genetic and biochemical evidence has indicated that the proteins involved in vesicle traffic are evolutionarily conserved and form a large family, each member of which is thought to regulate constitutive and/or regulated vesicle traffic (1–3). Synaptotagmin is one such protein family and is characterized by a short amino terminus, a single transmembrane region, and two C2 domains (known as the C2A and C2B domains) homologous to the C2 regulatory region of protein kinase C (reviewed in Refs. 4–7). To date, 12 members (synaptotagmins I–X, Syts III–XI, and Srg1) have been described subclass of synaptotagmins (Syts III, V, VI, and X), which is characterized by a lack of inositol 1,3,4,5-tetrakisphosphate binding activity (32). This cysteine motif was shown to be essential for the stable homo- and heterodimer formation through disulfide bonds of Syts III, V, VI, and X by site-directed mutagenesis. On the basis of these results, we discuss the role of cysteine-based heterodimerization properties of Syts III, V, VI, and X.

EXPERIMENTAL PROCEDURES

Materials—ExTaq and AmpliTaq DNA polymerases were obtained from Takara Biomedicals and Perkin-Elmer, respectively. All other chemicals were commercial products of reagent grade. Solutions were made in deionized water.

Molecular Cloning of Mouse Synaptotagmin Isoforms—cDNAs encoding the open reading frame of synaptotagmin isoforms (Syts IV, VI, VII, IX, and XI) were amplified by reverse transcriptase-polymerase chain reaction (PCR) from mouse cerebellum cDNAs (10) using the following sets of primers designed on the basis of mouse or rat sequences previously reported (12, 14–16, 18): Syt IV, 5′-ACATGGGTCCTATCCACCC-C-3′ (sense); amino acids 1–6 and 5′-AGTAAACCATCAGAGCAT-3′ (antisense); amino acids 421–425; Syt V, 5′-GATGAGGGGAGTTTG-G-3′ (sense); amino acids 1–5, 5′-CGATTACAGTGCTACACAGTGT-3′ (antisense); amino acids 351–357, 5′-CGATTCGCCGCGCAAA-3′.
Fig. 1. Phylogenetic analysis of the mouse synaptotagmin family (Syts I–XI). The phylogenetic tree was depicted as described under "Experimental Procedures." At least three distinct subclasses of synaptotagmins were observed: Syts IV and XI, which are characterized by a lack of Ca2+−dependent phospholipid (phosphatidylerine (PS)/phosphatidylcholine (PC)) (1:2.5, w/w) binding activity (18, 42); Syts III, V, VI, and X, which are deficient in inositol 1,3,4,5-tetraakisphosphate (IP4) binding activity (32) and showed cysteine-based heterodimerization in this study; and Syts I, II, and IX. Syts I and II are thought to be low affinity Ca2+ sensors for neurotransmitter release (4–7). The sequences of mouse Syts I and II are from Ref. 10; that of mouse Syt III is from Ref. 13; and those of mouse Syts IV–XI are from this study.

Cysteine-based Heterodimerization of Syts III, V, VI, and X

A subclass of Syts deficient in Ca2+−dependent phospholipid (IP4/PC liposome) binding activity

- SytXI
- SytIV
- SytX
- SytVI
- SytV
- SytIII
- Sytl
- SytII
- SytX
- SytVIII
- SytVII

A possible Ca2+−sensor for neurotransmitter release

GAGCTTGGGAAA−3' (sense); amino acids 227–233, and 5′−GAATGAA-
AATCACAAACCG−3' (antisense); amino acids 510–511; Syt VII, 5′−CGG-
AGATCGATCCGAGGGACGGG−3' (sense); amino acids 1–7, 5′−GAATATCCATTGTCCTTCAGGTTCG−3' (antisense); amino acids 381–387, 5′−CGGGATCCCGGAGGACAGGACATGGGCGG−3' (sense); amino acids 132–138, and 5′−GAATATCCATTGTCCTTCAGGTTCG−3' (antisense); amino acids 398–403; Syt IX, 5′−AGCCAGAT-
TCCCCGCGAACG−3' (sense); amino acids 1–5) and 5′−AATCAGGG-
GAAGATCTATGGCTGAGATCACAAATAT−3' (sense; amino acids 1–7) and 5′−CCACCAGAGATA−3' (antisense); amino acids 382–386; and Syt XI, 5′−GAAGATCTATGGGAGATCACAAATAT−3' (sense; amino acids 1–7) and 5′−CCACCAGAGATA−3' (antisense); amino acids 425–430. Reactions were carried out in the presence of Perfect Match PCR Enhancer (Stratagene) for 30 cycles, each consisting of denaturation at 94 °C for 1 min, annealing at 50 °C for 2 min, and extension at 72 °C for 2 min. The PCR products, purified from an agarose gel by a MicroSpin column (Amersham Pharmacia Biotech) were completely sequenced by the ThermoSequenase premixed cycle sequencing kit (Amersham Pharmacia Biotech) using a Hitachi SQ-5500 DNA sequencer.

Since the previously reported amino acid sequences of Syts V, VIII, and X were partial (16, 17), we cloned the cDNAs encoding the N termini of Syts V and VIII or the C terminus of Syt X from the Marathon-Ready mouse adult brain cDNA by 5′−rapid amplification of cDNA ends (RACE) (CLONTECH). First 5′− or 3′−RACE reactions were carried out using the internal adapter primer 1 (5′−CTGCTGCTACCTCTGACCTGACCTGTTGCC−3′) and one of the following primers designed on the cDNAs of mouse synaptotagmin isoforms (Syts V–VII and IX–XI) by two independent PCR products of each synaptotagmin. Since we cloned probably not due to PCR-induced errors because they were all found in two independent PCR products of each synaptotagmin. When compared with previously described rat or mouse sequences, amino acid substitutions were found in mouse Syt IV (Q70K, T85A, and G220D), Syt VI (Q36R, P44S, F77L, N90K, I105S, E222D, S471N, C496S, and A498V), Syt VII (P100T, I218V, and I357V), Syt IX (P8L, E14K, and E133Q), Syt X (V93L, T95V, and T347A), Syt XI (E74K, S85P, H86R, L36V, P94R, L104S, E133Q, and T347A), Syt VII (P100T and I218V), Syt IX (P8L, E14K, and E133Q), Syt X (V93L, T95V, and T347A), and Syt XI (E74K, S85P, H86R, L36V, P94R, L104S, E133Q, and T347A). These changes are not probably due to PCR-induced errors because they were all found in two independent PCR products of each synaptotagmin. Since we cloned the cDNA clones of mouse synaptotagmin isoforms (Syt V–VII and IX–XI) by PCR using primers derived from rat sequences, about 20 nucleotide sequences (5 or 6 amino acids) at the N or C terminus reported in this paper originated from rat sequences. Mouse cDNA clones of Syts I–III were described previously (10, 13).

Construction of T7- and FLAG-tagged Synaptotagmin Isoforms—Full-length synaptotagmin isoforms (Syt V–VII and X) were constructed using the appropriate restriction enzyme sites on the pGEM-T Easy vector. Addition of the FLAG tag to the N terminus of each synaptotagmin isomform was done by PCR with primers encoding the FLAG tag sequence (in italics below) because it was identical to the genome sequence previously reported (16) and did not contain any apparent open reading frame. In the shorter, second form, there are three methionine residues just after the 5′−in-frame stop codon, each of which did not correspond well to the Kozak sequence (33). However, expression of Syt VIII proteins from 1, 100, or 118 to 1291 base pairs in COS-7 cells and comparison of their size indicated that the first methionine presented in Fig. 3 is the initiation methionine because this position matches well those of Syts III, VI, and X, the same subclass of synaptotagmins.

Sequence analysis of the 5′−RACE products of Syt VIII showed that there are two forms of cDNA. The shorter form contains a putative retained intron just upstream of the transmembrane region (data not shown) because this position was identical to the sequence previously reported (16) and did not contain any apparent open reading frame. In the shorter, second form, there are three methionine residues just after the 5′−in-frame stop codon, each of which did not correspond well to the Kozak sequence (33). However, expression of Syt VIII proteins from 1, 100, or 118 to 1291 base pairs in COS-7 cells and comparison of their size indicated that the first methionine presented in Fig. 3 is the initiation methionine (data not shown).
Site-directed Mutagenesis—FLAG-tagged Syt III mutants (C10A, C21A, C35A, C10A/C21A, C10A/C35A, C21A/C35A, and C10A/C21A/C35A) were essentially produced by two-step PCR techniques as described previously (13) using the following pairs of oligonucleotides: primer A (5'-CGGGATCCATGTCTGGGGACCTAAGATGTG-3') or mutagenic primer B carrying the C10A mutation (5'-CGCATCAGTCT- GGGGACTCAAGATTACCTCGG-3') and mutagenic primer C carrying the C21A mutation (5'-GAATTCTTGACCACTTACCTCGG- TATGACATCTTGCGGACCCTCGG-3') or mutagenic primer D carrying the C35A mutation (5'-GAATTCTTGACCACTTACCTCGG-3') or mutagenic primer E (5'-GAATTCTGCGGACCCTCGG-3') and primer F (5'-GAATTCTCGGACCCTCGG-3') (right half). The right and left halves of the N terminus of Syt III were separately amplified; and the two resulting PCR fragments were digested with EcoRI (underlined above), ligated to each other, and reamplified by primers A (or B) and F. The PCR fragment obtained encoding the mutant N-terminal domain of Syt III was digested with BamHI, substituted for the BamHI insert of wild-type pGEM-T-FLAG-Syt III, and verified by DNA sequencing. Other plasmids encoding mutant Syts V (C9A), VI (C12A), and X (C13A) were similarly constructed by PCR mutagenic primer B carrying the C10A mutation (5'-CGAATTCTGATCGACCTTACCATCGG-3') (left half); and primer E (5'-CGAATTCTGACCTTACCATCGG-3') and primer F (5'-GAATTCTCGGGATCCGATCCGAGGAACCTCGG-3') (right half).

DNA Transfection—Five microliters of pEF-T7-Syt (or pEF-BOS as a control) and pEF-FLAG-Syt were cotransfected into COS-7 cells (5 x 10^5 cells/10-cm dish) by the DEAE-dextran method. Cells were harvested 72 h after transfection and homogenized in 1 ml of buffer containing 50 mM HEPES-KOH, pH 7.2, 250 mM NaCl, 0.1 mM phenylmethylsulfonyl fluoride, 10 μM leupeptin, and 10 μM pepstatin A. After solubilization with 1% Triton X-100 at 4 °C for 1 h, the supernatants (400 μl) were obtained by centrifugation at 15,000 rpm for 10 min. Immunoblotting. Blots were blocked with 1% skim milk and 0.1% Tween 20 in phosphate-buffered saline and then incubated with primary antibody. After the primary antibody incubation, the membrane was washed with phosphate-buffered saline containing 0.1% Tween 20 and then incubated with peroxidase-conjugated goat anti-rabbit or goat anti-mouse IgG. Immunoreactive bands were detected by enhanced chemiluminescence (Amersham Pharmacia Biotech). Polyclonal and monoclonal antibodies (M2) against the FLAG peptide (DYKDDDDK) were obtained from Zymed Laboratories Inc. and Sigma, respectively. Horseradish peroxidase-conjugated anti-T7 tag (MASMTGQGQMQ) antibody and monoclonal antibody against Syt I (SYA148) were from Novagen and Stressgen Biotech Corp., respectively.

**RESULTS**

**Phylogenetic Analysis of Mouse Synaptotagmins I–XI**—To understand the phylogenetic relationship between synaptotagmin family proteins, we cloned the mouse synaptotagmin I–XI cDNAs by PCR on the basis of previously described rat sequences. Since only the partial amino acid sequences of Syts V, VIII, and X have been reported, we first determined the nucleotide sequences of the N terminus of Syts V and VIII or the C terminus of Syt X using the 5'- or 3'-RACE reaction, respectively (see “Experimental Procedures” for details). The predicted amino acid sequences of mouse Syts V, VIII, and X showed that these synaptotagmins consist of 491, 398, and 523 amino acids, respectively (data not shown). Although the sequence similarities among the synaptotagmin family proteins were quite low especially at the N-terminal domain, Syts V and X were highly homologous to Syts III and VI throughout the entire protein, including the N terminus, transmembrane region, C2 domains, and C terminus. Syt V had 43.4, 55.5, and 55.1% identities to Syts III, VI, and X, respectively; and Syt X had 44.7, 55.1, and 60% identities to Syts III, V, and VI at the amino acid level, respectively, indicating that these synaptotagmins constitute a subfamily. This finding was made clearer when a phylogenetic tree of mouse synaptotagmin was constructed (Fig. 1). According to this phylogenetic tree, the synaptotagmin family was checked can be classified into at least four distinct groups: Syts IV and IX, Syts III, V, VI, and X; Syts I, II, and IX; and others (Syt VII or VIII).
To examine whether the SDS-resistant homodimerization properties observed in Syt I or II on SDS-PAGE (10, 31, 35) are also retained in other isoforms, we expressed T7-tagged Syts I–XI in COS-7 cells and analyzed them by SDS-PAGE in the presence or absence of β-mercaptoethanol (Fig. 2). Except for Syts IV, IX, and XI, we could easily detect the minor immunoreactive bands corresponding to the dimer positions predicted from the cDNA sequences in addition to the major monomer bands (Fig. 2A). The dimer band of Syt III was difficult to see on 10% polyacrylamide gel, but could be detected on 7.5% gel (data not shown). Surprisingly, in the absence of β-mercaptoethanol, the majority of the Syt III, V, VI, and X immunoreactive bands were shifted to dimer positions (Fig. 2B, lanes 3, 5, 6, and 10), whereas β-mercaptoethanol had almost no effect on other isoforms’ dimerization properties. This result indicates that certain cysteine residues are involved in the homodimerization of Syt III, V, VI, or X through a disulfide bond. Sequence alignment of Syts III, V, VI, and X revealed seven conserved cysteine residues among these isoforms: three cysteine residues in the N-terminal domain, one cysteine residue within the transmembrane region, one cysteine residue just downstream of the transmembrane region, and two cysteine residues within the C2 domains (Fig. 3 and data not shown). Among these cysteine residues, we initially focused on the N-terminal cysteine residues that probably reside in the intravesicular region (4–7) because there are no cysteine residues in the N-terminal domains of the other isoforms (Fig. 3, asterisks), and some of the cysteine residues around the transmembrane region are thought to be palmitoylated (36, 37) and are therefore unlikely to participate in disulfide bond formation. Two cysteine residues in the cytoplasmic domain are also unlikely to be involved in disulfide bond formation because the cytoplasmic domain of Syts III and VI did not show β-mercaptoethanol-sensitive dimers (data not shown). Furthermore, these N-terminal cysteine residues are also conserved in electric ray synaptotagmin (p65-c), a homologue of mouse Syts III, V, VI, and X, suggesting that these are at least retained in vertebrate evolution (38).

**Mutational Analysis of the N-terminal Cysteine Residues of Synaptotagmin III**—To identify the cysteine residues respon-
Cysteine-based Heterodimerization of Syts III, V, VI, and X

To further explore the processes of heterodimerization of Syts III, V, VI, and X, we introduced two different tags, the T7 and FLAG tags, into the N terminus of each synaptotagmin and evaluated their association by immunoprecipitation (see “Experimental Procedures” for details). Briefly, T7- and FLAG-tagged synaptotagmins were coexpressed in COS-7 cells, and T7-tagged synaptotagmins were immunoprecipitated by anti-T7 tag antibody-conjugated agarose. Then, the coprecipitated FLAG-tagged synaptotagmins were detected by immunoblotting using anti-FLAG antibody.

As shown in Fig. 5A (lanes 6–10), FLAG-Syt III was efficiently co-immunoprecipitated with T7-Syts V, VI, and X as well as T7-Syt III. In contrast, when FLAG-Syt III and T7-Syts V, VI, or X were separately expressed in COS-7 cells and mixed 1 h before immunoprecipitation, we could not detect the association of FLAG-Syt III with any of the T7-tagged synaptotagmins (Fig. 5A, lanes 1–5). These results indicate that the cysteine-based homo- and heterodimerization only occurred in the living cells.

In the next set of experiments, we examined the possible involvement of cysteine residues in the heterodimerization of Syts III, V, VI, and X using mutant FLAG-Syt III carrying cysteine-to-alanine mutations. Substitution of the first cysteine residue (C10A, C10A/C21A, C10A/C33A, or C10A/C21A/C33A) almost completely abolished the stable dimerization with Syt III, V, VI, or X (Fig. 5A, lanes 6–10), indicating that the disulfide bond mediated by the cysteine at position 10 of Syt III is crucial for the stable homo- and heterodimerization.

Cysteine-based Heterodimerization Properties of Synaptotagmins III, V, VI, and X—To further explore the processes of heterodimerization of Syts III, V, VI, and X, we introduced two different tags, the T7 and FLAG tags, into the N terminus of each synaptotagmin and evaluated their association by immunoprecipitation (see “Experimental Procedures” for details). Briefly, T7- and FLAG-tagged synaptotagmins were coexpressed in COS-7 cells, and T7-tagged synaptotagmins were immunoprecipitated by anti-T7 tag antibody-conjugated agarose. Then, the coprecipitated FLAG-tagged synaptotagmins were detected by immunoblotting using anti-FLAG antibody.
In the present study, we first found that this class of synaptotagmins (Syts III, V, VI, and X) based on the inositol 1,3,4,5-tetrakisphosphate binding properties of the C2B domain (32). Among Syts III, V, VI, and X, Syt III is suggested to function as a high affinity Ca\(^{2+}\) sensor for neurotransmitter release because it has a relatively higher affinity for Sr\(^{2+}\) (41). Since all these synaptotagmins are expressed in brain (Syts III, V, and X are neuronal types, and Syt VI is ubiquitous) (11, 16, 17), these isoforms may hetero-oligomerize via disulfide bonding, and Syts V, VI, and X may modulate the Ca\(^{2+}\)-sensing function of Syt III. In addition, the expression of Syt X mRNA in adult brain is known to be regulated by depolarization (17). Thus, it is also possible that the heterodimer between Syts III and X occurs only after some kind of synaptic plasticity.

In conclusion, we first identified, by sequence comparison and mutational analysis, the evolutionarily conserved cysteine residues responsible for intermolecular disulfide bonding at the N terminus of Syts III, V, VI, and X. Our findings suggest that hetero-oligomerization of Syts III, V, VI, and X may produce a variety of Ca\(^{2+}\) sensors that function in neurotransmitter release with different biochemical natures.

Acknowledgments—We thank Dr. Shigekazu Nagata for the expression vector; Dr. Akihiro Mizutani for critical comments on the manuscript; Etsuko Ibaru for technical assistance; and Hiroyuki Kabayama, Keiji Ibata, and Fumiaki Hamazato for valuable discussions.

REFERENCES

1. Bennett, M. K., and Scheller, R. H. (1993) Proc. Natl. Acad. Sci. U. S. A. 90, 2559–2563
2. Ferro-Novik, S., and Jahn, R. (1994) Nature 370, 191–193
3. Söllner, T., and Rothman, J. E. (1994) Trends Neurosci. 17, 344–348
4. Sudhof, T. C., and Rizo, J. (1996) Neuron 17, 379–388
5. Fukuda, M., and Mikoshiba, K. (1997) Bioessays 19, 583–603
6. Linial, M. (1997) J. Neurochem. 69, 1781–1792
7. Schiavo, G., Osborne, S. L., and Sgarro, J. G. (1998) Biochem. Biophys. Res. Commun. 248, 1–8
8. Perin, M. S., Fried, V. A., Mignery, G. A., Jahn, R., and Südhof, T. C. (1990) Nature 345, 260–263
9. Geppert, M., Archer, B. T., III, and Sudhof, T. C. (1991) J. Biol. Chem. 266, 15548–15552
10. Fukuda, M., Aruga, J., Ninobe, M., Aimoto, S., and Mikoshiba, K. (1994) J. Biol. Chem. 269, 29206–29211
11. Mizuta, M., Inagaki, N., Nemoto, Y., Matsukura, S., Takahashi, M., and Seino, S. (1994) J. Biol. Chem. 269, 11675–11678

* M. Fukuda, unpublished observations.
Cysteine-based Heterodimerization of Syts III, V, VI, and X

12. Hilbush, B. S., and Morgan, J. I. (1994) Proc. Natl. Acad. Sci. U. S. A. 91, 8195–8199
13. Fukuda, M., Kojima, T., Aruga, J., Niinobe, M., and Mikoshiba, K. (1995) J. Biol. Chem. 270, 26523–26527
14. Craxton, M., and Goedert, M. (1995) FEBS Lett. 361, 196–200
15. Hudson, A. W., and Birnbaum, M. J. (1995) Proc. Natl. Acad. Sci. U. S. A. 92, 5895–5899
16. Li, C., Ullrich, B., Zhang, J. Z., Anderson, R. G. W., Brose, N., and Südhof, T. C. (1995) Nature 375, 594–599
17. Babity, J. M., Armstrong, J. N., Plumier, J. C., Currie, R. W., and Robertson, H. A. (1997) Proc. Natl. Acad. Sci. U. S. A. 94, 2638–2641
18. von Poser, C., Ichchenko, K., Shao, X., Rizzi, J., and Südhof, T. C. (1997) J. Biol. Chem. 272, 14314–14319
19. Thompson, C. C. (1996) J. Neurosci. 16, 7832–7840
20. Mikoshiba, K., Fukuda, M., Moreira, J. E., Lewis, F. M. T., Sugimori, M., Niinobe, M., and Llinás, R. (1995) Proc. Natl. Acad. Sci. U. S. A. 92, 16703–10707
21. Fukuda, M., Moreira, J. E., Lewis, F. M. T., Sugimori, M., Niinobe, M., Mikoshiba, K., and Llinás, R. (1995) Proc. Natl. Acad. Sci. U. S. A. 92, 16708–10712
22. Mochida, S., Fukuda, M., Niinobe, M., Kobayashi, H., and Mikoshiba, K. (1997) Neuroscience 77, 937–943
23. Ohara-Imaizumi, M., Fukuda, M., Niinobe, M., Misonou, H., Ikeda, K., Murakami, T., Kawasaki, M., Mikoshiba, K., and Kumakura, K. (1997) Proc. Natl. Acad. Sci. U. S. A. 94, 287–291
24. Lang, J., Fukuda, M., Zhang, H., Mikoshiba, K., and Wellheim, C. B. (1997) EMBO J. 16, 5837–5846
25. Littleton, J. T., and Bell, J. H. (1995) Trends Neurosci. 18, 177–183
26. Sugita, S., Hata, Y., and Südhof, T. C. (1996) J. Biol. Chem. 271, 1262–1265
27. Chapman, E. R., An, S., Edwardson, J. M., and Jahn, R. (1996) J. Biol. Chem. 271, 5844–5849
28. Damer, C. K., and Creutz, C. E. (1996) J. Neurochem. 67, 1661–1668
29. Chapman, E. R., Desai, R. C., Davis, A. F., and Tornehl, C. K. (1998) J. Biol. Chem. 273, 32966–32972
30. Osborne, S. L., Herreros, J., Bastiaens, P. I. H., and Schiavo, G. (1999) J. Biol. Chem. 274, 59–66
31. Perin, M. S., Bros, N., Jahn, R., and Südhof, T. C. (1991) J. Biol. Chem. 266, 623–629
32. Ibata, K., Fukuda, M., and Mikoshiba, K. (1998) J. Biol. Chem. 273, 12267–12273
33. Kozak, M. (1984) Nucleic Acids Res. 12, 857–872
34. Mizushima, S., and Nagata, S. (1990) Nucleic Acids Res. 18, 5332
35. Niinobe, M., Yamaguchi, Y., Fukuda, M., and Mikoshiba, K. (1994) Biochem. Biophys. Res. Commun. 205, 1036–1042
36. Veit, M., Sellner, T. H., and Rothman, J. E. (1996) FEBS Lett. 385, 119–123
37. Chapman, S. E., Blasi, J., An, S., Bros, N., Johnston, P. A., Südhof, T. C., and Jahn, R. (1996) Biochem. Biophys. Res. Commun. 225, 326–332
38. Wendland, B., Miller, K. G., Schilling, J., and Scheller, R. H. (1991) Neuron 6, 993–1007
39. Geppert, M., Goda, Y., Hammer, B. E., Li, C., Rosahl, T. W., Stevens, C. F., and Südhof, T. C. (1994) Cell 79, 717–727
40. Fukuda, M., Kojima, T., and Mikoshiba, K. (1997) Biochem. J. 323, 421–425
41. Li, C., Davletov, B. A., and Südhof, T. C. (1995) J. Biol. Chem. 270, 24989–24992
42. Fukuda, M., Kojima, T., and Mikoshiba, K. (1996) J. Biol. Chem. 271, 8430–8434
43. Sugimori, M., Tong, C.-K., Fukuda, M., Moreira, J. E., Kojima, T., Mikoshiba, K., and Llinás, R. (1998) Neuroscience 86, 39–51
44. Fukuda, M., and Mikoshiba, K. (1999) J. Biol. Chem. 274, 31428–31434