Phosphorylation of Rabphilin-3A by Ca^{2+} /Calmodulin- and cAMP-Dependent Protein Kinases *in vitro*

Else Marie Fykse,^a Cai Li, and Thomas C. Südhof

Department of Molecular Genetics and Howard Hughes Medical Institute, The University of Texas Southwestern Medical Center, Dallas, Texas 75235

Regulation of neurotransmitter release is thought to involve modulation of the release probability by protein phosphorylation. In order to identify novel targets for such regulatory processes, we have studied the phosphorylation of rabphilin-3A *in vitro*. Rabphilin-3A is a synaptic vesicle protein that interacts with rab3A in a GTP-dependent manner and binds Ca^{2+} in a phospholipid-dependent manner. Here we show that rabphilin-3A is an efficient substrate for Ca^{2+} /calmodulin-dependent protein kinase II, which phosphorylates rat rabphilin-3A at residue 234 and 274, and for cAMP-dependent protein kinase, which phosphorylates rat rabphilin-3A at residue 234. This identifies the middle region of rabphilin-3A situated between the N-terminal rab3A-binding sequences and the C-terminal C_2 -domains involved in Ca^{2+} /phospholipid binding as a regulatory domain. Thus, rabphilin-3A is a second phosphoprotein on synaptic vesicles that, similar to synapsin I, may integrate phosphorylation signals from multiple protein kinase signaling pathways in the cell.

[Key words: synaptic vesicles, synaptic plasticity, exocytosis, protein kinases, Ca^{2+} , rab proteins, phosphorylation]

In nerve terminals, neurotransmitters are stored in synaptic vesicles and secreted by synaptic vesicle exocytosis. Neurotransmitter secretion is acutely regulated by Ca^{2+} , which triggers exocytosis in a rapid (<1 msec) and cooperative manner. On a longer time scale, neurotransmitter release is modulated by a variety of regulatory processes, such as occur during the different forms of short-term synaptic plasticity (Zucker, 1989), modulation by presynaptic receptors (Takeuchi and Takeuchi, 1966; Silinsky, 1984; Scholz and Miller, 1992; Weisskopf et al., 1993), and possibly long-term potentiation (LTP) and long-term depression (LTD) (Bekkers and Stevens, 1990; Malinow and Tsien, 1990; Bolshakov and Siegelbaum, 1994). These regulatory phenomena are thought to involve covalent modifications of proteins, in particular, by protein phosphorylation. In support of this notion, mice carrying a mutant form of the gene for α - Ca^{2+} /calmodulin-

dependent protein kinase II (α -CaMK II) exhibit dramatic changes in short-term synaptic plasticity even in the heterozygous state, which presumably only lowers the levels of enzyme by half (Silva et al., 1992; A. Silva, personal communication). LTP in mossy fiber synapses, a known presynaptic form of synaptic plasticity (Zalutsky and Nicoll, 1990), is related to cAMP-dependent protein kinase (PKA) activation (Frey et al., 1993). Pharmacological experiments using a variety of protein kinase inhibitors and stimulators also suggest a role for protein kinases in synaptic plasticity (Zhuo et al., 1994; reviewed in Stevens, 1993). Thus, there is significant evidence for a major role of protein phosphorylation events in at least some forms of regulation of neurotransmitter release.

At least two possible mechanisms can be envisioned by which protein phosphorylation could regulate neurotransmitter release. First, protein kinases could phosphorylate ion channels and their associated proteins. As a regulatory endpoint, such phosphorylation events could lead to an increase or decrease in the intracellular Ca^{2+} levels achieved after an action potential and thereby regulate release (for a review, see Nicoll et al., 1990). Second, protein kinases could phosphorylate proteins involved in the synaptic vesicle traffic. Thereby, they could modulate the efficiency of synaptic vesicle trafficking as a function of Ca^{2+} . Although there is evidence in support of both mechanisms (Takeuchi and Takeuchi, 1966; Man-Son-Hing et al., 1989; Dale and Kandel, 1990; Scholz and Miller, 1992), the molecular basis for these processes is largely obscure. In particular, for the synaptic release apparatus, the mechanisms underlying the modulation of release and the relevant phosphorylation targets are mostly unknown.

Several protein kinase substrates in the nerve terminal have been extensively studied. The synapsins constitute a family of phosphoproteins that are associated with synaptic vesicles (De Camilli et al., 1983a,b; Südhof et al., 1989) and are substrates for at least four different protein kinases, CaMK I and II, PKA, and proline-directed kinase (reviewed in Jahn and Südhof, 1994). The abundance and regulated phosphorylation of the synapsins suggest that they function in regulating neurotransmitter release, but their exact functions are unknown except for the C-terminal phosphorylation site for CaMK II in synapsin I, which appears to act as a negative regulator during paired-pulse facilitation (Rosahl et al., 1993). GAP-43 (also called B-50) represents an abundant phosphoprotein of the nerve terminal plasmamembrane, with a possible role in exocytosis but no currently known phosphorylation-dependent function (Dekker et al., 1989). Dynamin I is a synaptic phosphoprotein with the interesting property of quantitative dephosphorylation during nerve

Received July 15, 1994; revised Oct. 3, 1994; accepted Oct. 5, 1994.

We thank I. Leznicki, A. Roth, and E. Borowicz for excellent technical assistance, Dr. H. Schulman (Stanford University), Dr. M. Kennedy (California Institute of Technology), and Dr. R. Jahn (Yale University) for supplying us with purified enzymes and with antibodies; and Drs. H. McMahon and B. Davletov for advice.

Correspondence should be addressed to Thomas C. Südhof at the above address.

^aPresent address: Norwegian Defence Research Establishment, Division of Environmental Toxicology, P.B. 25, N-2007 Kjeller, Norway.

Copyright © 1995 Society for Neuroscience 0270-6474/95/152385-11\$05.00/0

Figure 1. Phosphorylation of rabphilin-3A by cAMP- and Ca^{2+} -dependent protein kinases. Immunoprecipitates of rat brain proteins with preimmune serum (PIS, left lane) or with polyclonal antibodies to rabphilin-3A (Anti-Rabphilin) were incubated with or without cytosol as a source of kinases and with Ca^{2+} , cAMP, and cGMP as activators. Samples were analyzed by SDS-PAGE, blotted onto nitrocellulose membranes, and exposed to film for 3 hr at -70°C with a screen.

Figure 2. Phosphorylation of rabphilin-3A by the catalytic subunit of cAMP-dependent protein kinase (PKA) and by Ca^{2+} , calmodulin-dependent protein kinase II (CaMK II). Rabphilin and control immunoprecipitates (see legend to Fig. 1) were incubated with the indicated protein kinases as described in Experimental Procedures. Samples were subjected to SDS-PAGE, blotted onto nitrocellulose membranes, and analyzed by autoradiography (top panel; exposure: 5 hr at -70°C with screen) and by immunoblotting with rabphilin antibodies (bottom panel) to control for the amount of rabphilin-3A immunoprecipitated. The additional phosphoprotein bands observed in the samples incubated with CaMK II and Ca^{2+} are probably due to autophosphorylated CaMK II (see also Fig. 7).

Figure 3. Schematic overview of the bacterial glutathione S-transferase (GST) fusion proteins used to map the phosphorylation sites in rabphilin-3A. The diagram on top depicts the domain structure of rabphilin-3A. The structures of GST and of 10 GST-rabphilin fusion proteins used in the current study are shown in the middle, and the legend is shown on the bottom. The locations of the phosphorylation sites that were identified in the current study are indicated by "P." GST-RaphH contains the entire N-terminal half of rabphilin-3A. Serine-to-alanine mutations were introduced into serine²³⁴ (the putative PKA phosphorylation site) in GST-RaphI, and into serine²³⁴ and serine²⁷⁴ (the putative CaMK II phosphorylation site) in GST-RaphJ. A mutant in which only the putative CaMK II phosphorylation site (serine²⁷⁴) was substituted was also made but the expressed protein was unstable in bacteria and, therefore, not analyzed. However, the corresponding mutant was also expressed as a holo-protein in COS cells (see below).

terminal depolarization that probably plays a role in triggering synaptic vesicle endocytosis (Robinson et al., 1993).

These and other synaptic phosphoproteins (e.g., AP180, synaptotagmin, and others) are candidates for mediating some regulatory functions in the synaptic vesicle cycle. However, the currently known protein kinase targets at the synapse are clearly insufficient to explain most of these synaptic regulatory phenomena. For example, introduction of CaMK II into presynaptic nerve terminals causes major changes in synaptic vesicle exocytosis (Nichols et al., 1990; Llinas et al., 1991). However, the only currently characterized synaptic CaMK II substrate is synapsin I. Although it was originally hypothesized that CaMK II exerts its presynaptic functions in potentiating neurotransmitter release via synapsin I, the genetic experiments clearly showed that this is not the case (Rosahl et al., 1993). Another example is PKA, which appears to modulate neurotransmitter release and to have a major function in mossy fiber LTP in the hippocampus

(Frey et al., 1993; Chavez-Noriega and Stevens, 1994). Again, the only currently known synaptic PKA substrates are synapsins I and II, which do not appear to account for all of the regulatory phenomena observed with PKA. Therefore, it would be of great interest to define additional protein kinase substrates in the nerve terminal that have properties compatible with a role in nerve terminal regulation.

Rabphilin-3A was identified because of its GTP-dependent binding to rab3A (Shirataki et al., 1992). Rabphilin-3A has a distinct domain structure consisting of a conserved N-terminal domain that binds rab3A, a variable middle domain, and a C-terminal region containing two C₂-domains with homology to those of synaptotagmin (Shirataki et al., 1993; Li et al., 1994). In addition to rab3A, rabphilin-3A may also binds Ca²⁺ and phospholipids in a ternary complex (Shirataki et al., 1993; Yamaguchi et al., 1993). Recently we showed that rabphilin-3A is a synaptic vesicle protein, identifying it as a second potential Ca²⁺

Figure 4. Phosphorylation of rabphilin-GST fusion-proteins by endogenous kinases present in rat brain cytosol. Identical amounts of recombinant GST proteins (proteins are identified on top and described in see Fig. 3; approximately 3 μ g protein were loaded per lane) were incubated with rat brain cytosol and γ -³²P-ATP without additions (*top panel*), in the presence of cAMP (*second panel*), or in the presence of Ca²⁺ (*third panel*).

sensor on synaptic vesicles in addition to synaptotagmin (Li et al., 1994). Furthermore, it was found that in mice lacking rab3A, neurons that do not contain rab3C show a defect in the synaptic targeting of rabphilin-3A, indicating that rabphilin-3A is targeted to synaptic vesicles via its N-terminal binding to rab3A (Geppert et al., 1994; Li et al., unpublished observations). Together, these findings implicate rabphilin-3A as a potential regulatory protein in the nerve terminal that functionally interacts with rab3A. This functional interaction was confirmed in experiments with rab3A-deficient mice in which rab3A deficiency leads to a 70% reduction in the levels of rabphilin-3A (Geppert et al., 1994). In the current study, we show that *in vitro*, rabphilin-3A is an excellent substrate for at least two protein kinases that have been shown to regulate synaptic transmission, PKA and CaMK II. The phosphorylation sites for the two kinases were identified in the middle domain of rabphilin-3A, suggesting that this is a regulatory domain. The identification of rabphilin-3A as a substrate for multiple protein kinases suggests that rabphilin-3A, similar to the synapsins, may serve as a signal integration point for multiple protein kinases in the nerve terminal.

Materials and Methods

Construction of eukaryotic expression vectors and expression of proteins in COS cells by transfection. For the construction of the wild-type rabphilin-3A expression vector (pCMV85-4), PCR was performed on the full-length rat rabphilin cDNA (Li et al., 1994) using oligonucleotides A and B (sequences [redundant nucleotides in brackets]: GCCTC-TAGACGGTACCATGACTGACACTGTGGT and GCCGTCGACTA-GTCGGAGGA[C,G]AC[A,G]TG[A,G]TT[T,C]TC[A,G]TT[T,C]TG). The 2.07 kb product was digested with KpnI and SalI and subcloned into the same sites of pCMV5. For construction of the expression vector encoding rabphilin-3A with a serine²³⁴ to alanine substitution (pCMV85-3), PCRs were performed on the full length rat rabphilin-3A cDNA clone with two pairs of oligonucleotides, A and C (sequence: TCC-GGGCCTCCGCGCCTGCGTGTGGAG) and oligonucleotides B and D (sequence: GCAGGCGCGCGAGGCCCGGATGAGTA). The products from the two amplifications were gel purified, cut with SacII, and ligated to each other. They were then reamplified with oligonucleotides A and B, subcloned into pCMV5 as above, and confirmed by restriction enzyme mapping. The expression vector encoding rabphilin-3A with a single point mutation in the putative CaMK II phosphorylation site (pCMV85-5) substituting serine²⁷⁴ to alanine was constructed similarly with two internal oligonucleotides E and F (sequences: TGGT-CTAGAGGCTGGACTGCGTTAGCTCGCCT and GCCTCTAGAC-CAGCTCCAGCCTCCATG), except that the first PCR products were cut with XbaI before ligation and performing PCR again. The double mutant (pCMV85-6) (serine²³⁴ to alanine, serine²⁷⁴ to alanine) was constructed by introducing the CaMK II phosphorylation site mutation as described above into the vector encoding rabphilin-3A with the PKA-site mutation. Expression vectors were transfected into COS cells using the DEAE-Dextran method as described (Gorman, 1985).

Construction of bacterial vectors encoding GST-fusion proteins and expression and purification of recombinant proteins. Eleven bacterial expression vectors were constructed for this study using standard recombinant DNA techniques (Sambrook et al., 1989) by subcloning cDNA fragments into the vector pGEX-KG (Guan and Dixon, 1991). The following GST-rabphilin-3A fusion proteins were encoded by the following vectors (vector names and encoded residue numbers of rat rabphilin-3A shown in brackets): GST-RaphA (pGEX85-6, 1–181), constructed by PCR amplification of the corresponding coding region using oligonucleotides A (see above) and G (sequence: GGGGTCGAC-TACGGCTCACCAGCAGGC) and cloning the PCR product cut with XbaI-SalI into the same sites of pGEX-KG; GST-RaphB (pGEX85-3, 67–226), obtained by subcloning 0.48 kb NcoI fragment of rat rabphil-

in-3A cDNA into NcoI site of pGEX-KG; GST-RaphC (pGEX85-11; 199–361), constructed by subcloning 0.48 kb XhoI-SalI fragment of pGEX85-4 (see below) into SalI site of pGEX-KG; GST-RaphD (pGEX85-13; 250–361), constructed by subcloning 0.33 kb NcoI-SalI fragment of pGEX85-4 (see below) into NcoI-SalI sites of pGEX-KG; GST-RaphE (pGEX85-8, 380–684), constructed by PCR amplification of the corresponding coding region using oligonucleotides H (sequence: CGCGTAGCAACCACACTGGGTG) and B (see above), and cloning the PCR product cut with NheI-SalI into the XbaI-SalI sites of pGEX-KG; GST-RaphF (pGEX85-7, 380–531), constructed by PCR amplification of the corresponding coding region using oligonucleotides H and I (sequence: GGGGTCGACTACTGCTCCTCCTCATAGAG), and cloning the PCR product cut with NheI-SalI into the XbaI-SalI sites of pGEX-KG; GST-RaphG (pGEX85-5, 524–684), constructed by PCR amplification of the corresponding coding region using oligonucleotides J (sequence: GCGTCTAGACATGGCTCTCTATGAGGA) and B (see above), and cloning the PCR product cut with XbaI-SalI into the same sites of pGEX-KG; GST-RaphH (pGEX85-4, 1–361), constructed by PCR amplification of the corresponding coding region using oligonucleotides A and K (sequence: GGGGTCGACTACTGGCGGGCAGGG-GCCG), and cloning the PCR product cut with XbaI-SalI into the same sites of pGEX-KG; GST-RaphI (pGEX85-12, 1–361 with a serine²³⁴ to alanine point mutation), constructed by two-stage PCR analogously to pCMV85-3 and pGEX85-4, as described above; GST-RaphJ (pGEX85-15, 1–361 with two point mutations: serine²³⁴ to alanine, and serine²⁷⁴ to alanine), constructed by two-stage PCR with pGEX85-12 as template analogously to pCMV85-6 and pGEX85-4 as described above; GST-RaphK (pGEX85-14, 1–361 with point mutations of serine²⁷⁴ to alanine), constructed by two-stage PCR with pGEX85-4 as template as described above for pCMV85-5 and pGEX85-4, the protein produced by this plasmid in different strains of bacteria was unstable and could not be obtained in sufficient quantities for experiments. Recombinant GST-proteins were expressed in bacteria and purified on glutathione-agarose (Sigma) as described (Smith and Johnson, 1988).

Protein assays, SDS-PAGE, and protein stains. Protein assays were carried out using a Coomassie-blue based assay (Bio-Rad; Bradford, 1976). SDS-PAGE was performed as described (Laemmli, 1970). Gels were either stained with Coomassie blue or transferred to nitrocellulose filter and stained with Ponceau S (Sigma).

Antibodies, immunoblotting, and immunoprecipitations. Previously described polyclonal antibodies against rabphilin (Li et al., 1994) were used in immunoblotting experiments as described (Johnston et al., 1989). For immunoprecipitations, two to four rat brains were homogenized in 15–30 ml 10 mM HEPES-NaOH pH 7.4, 0.25 M NaCl, 2 mM EDTA in the presence of protease inhibitors (PMSF, leupeptin, and pepstatin). Triton X-100 was added to 1% (v/v), the homogenate was rotated at 4°C for 20 min, and subjected to consecutive low and high speed centrifugations (10 min at 20,000 × g and 30 min at 100,000 × g) to obtain a protein fraction containing total rat brain solubilized proteins. Antisera (I734 rabphilin-3A immune or preimmune sera) were added to this fraction at a final concentration of 1% (v/v), incubated overnight at 4°C, and precipitated by incubation with protein G-Sepharose (Pharmacia) for 90 min. Protein G-Sepharose with bound proteins were recovered by centrifugation (2 min at 1000 × g), washed twice in 5 mM HEPES-NaOH pH 7.4, 125 mM NaCl, 0.5% Triton X-100, and once in 10 mM HEPES-NaOH pH 7.4, 150 mM NaCl, and resuspended in the same buffer. Immunoprecipitations of rabphilins expressed by transfection in COS cells were performed similarly except that the transfected COS cells were directly homogenized in Triton X-100 containing buffer and incubated with the antibody only for 2 hr to prevent degradation by the more abundant protease in COS cell homogenates. The immunoprecipitates of rabphilin-3A bound to antibodies on protein G-Sepharose were directly used for phosphorylation experiments.

Phosphorylation experiments. Phosphorylation reactions were carried out with rat brain cytosol to first screen for Ca²⁺- and cyclic nucleotide-stimulated phosphorylation events, and purified kinases to positively identify the enzymes that phosphorylate rabphilin-3A. Crude rat brain cytosol was prepared fresh for each experiment by lysing crude syn-

Samples were analyzed by SDS-PAGE and autoradiography [exposure times (all at –70°C with screen): *top and second panel*, 90 min; *third panel*, 25 min]. The *bottom panel* depicts a protein stain of an SDS-gel of the samples used for the phosphorylations to demonstrate protein purity. Numbers on the right indicate positions of molecular weight markers.

Figure 5. Identification of the phosphorylation sites for PKA and CaMK II in recombinant rabphilin-GST fusion proteins. **A**, Phosphorylation of wild-type and mutant rabphilin-GST fusion-proteins by endogenous kinases in rat brain cytosol. A GST-rabphilin protein containing the N-terminal half of rabphilin-3A including the phosphorylation sites for PKA and for CaMK II as mapped above (Fig. 4) was analyzed either in wild-type form (GST-RaphH, Fig. 3) or with amino acid substitutions at serine²³⁴ (the putative PKA-site; GST-RaphI) or at serine²⁷⁴ and at serine²⁷⁴ (the putative CaMK II site; GST-RaphJ). Recombinant proteins containing only a mutation in the CaMK II site were unstable and not used. Phosphorylations were carried out in the presence of rat brain cytosol either without additions or after addition of cAMP or of Ca²⁺. The *top panel* shows an autoradiogram (exposure: 2 hr at -70°C with screen) and the *bottom panel* a protein stain with Ponceau S of the same blot to document that all lanes received identical amounts of the proteins. **B**, Wild-type and phosphorylation-site mutant rabphilin-GST fusion-proteins described in **A** were phosphorylated with purified protein kinases to confirm the identity of the protein kinases phosphorylating rabphilin-3A, and analyzed as described for **A**. Numbers on the right indicate positions of molecular weight markers.

Figure 6. Phosphoamino acid analysis of the rabphilin-GST fusion protein phosphorylated by rat brain protein kinases or by PKA. GST-RaphH (Fig. 3) phosphorylated by endogenous protein kinases in rat brain cytosol in the presence or absence of the indicated activators and by PKA (right lane) was studied by acid hydrolysis and phosphoamino acid analysis. The migration positions of inorganic phosphate (Pi) and of phosphoamino acid standards as well as the position of the origin are shown on the right. The arrow points to phosphopeptides. Similar analyses with identical results were performed with phosphorylated rabphilin-3A isolated by immunoprecipitation (data not shown). Autoradiogram was exposed for 30 hr with screen at -70°C .

aptosomes by resuspension in 10 mM HEPES-NaOH pH 7.4 and removal of the membranes by centrifugation at $12,000 \times g$. Purified catalytic subunit of PKA was purchased from Sigma, and purified CaMK II was a generous gift from Dr. H. Schulman (Stanford University) and from Dr. M. Kennedy (CalTech, Pasadena). Immunoprecipitated rabphilin-3A from rat brain or transfected COS cells (100–300 ng estimated by SDS-PAGE and Coomassie-blue staining) bound to protein G-Sepharose with antibodies was phosphorylated in a reaction (50 μl) con-

taining 10 mM HEPES-NaOH pH 7.4, 2 mM MgCl_2 , 1 mM EGTA, 25 μM $\gamma^{32}\text{P}$ -ATP (4000–6000 cpm/pmol) and rat brain cytosol (25 mg/l) with either no additions or with additions of the following activators: 50 μM cAMP, 50 μM cGMP, or 0.4 mM Ca^{2+} /0.1 mM EGTA (instead of 1 mM EGTA). Reactions were incubated at 37°C for 10 min followed by 3 min at room temperature. After the incubations, an equal volume of 5 mM HEPES-NaOH pH 7.4, 125 mM NaCl, 0.5% Triton X-100, and 2.5 mM NaF was added. Protein G-Sepharose beads were centrifuged

Figure 7. Phosphorylation of wild-type rabphilin-3A and rabphilin-3A with point mutations in putative phosphorylation sites expressed by transfection in COS cells. COS cells were transfected with vectors encoding wild-type rat rabphilin-3A (pCMV85-4), rabphilin-3A in which serine²³⁴ was substituted for alanine (pCMV85-3), rabphilin-3A in which serine²⁷⁴ was substituted for alanine (pCMV85-5), and rabphilin-3A with both point mutations (pCMV85-6). Rabphilin-3A was immunoprecipitated from the cytosol of the transfected COS cells and phosphorylated by adding $\gamma^{32}\text{P}$ -ATP, Ca^{2+} , and purified protein kinases as indicated. As a control, COS cells transfected with wild-type rabphilin-3A expression vector were immunoprecipitated with preimmune serum but otherwise analyzed as the other immunoprecipitates (left lanes labeled "Ctrl."). Samples were analyzed by SDS-PAGE and autoradiography [top panel; exposure at -70°C with screen for 8 hr (left half) or for 13 hr (right half)] or immunoblot with a rabphilin-3A antibody (bottom panel) to ensure comparable levels of recombinant rabphilin-3A for the different mutants. All rabphilin-3A immunoblots of transfected material show a doublet except for pCMV85-5. This doublet does not correlate with phosphorylation and is currently unexplained.

Figure 8. Comparative domain models of rabphilin-3A and synaptotagmin. Bar diagrams compare the domain structure of rabphilin-3A (*top panel*) with that of synaptotagmin (Perin et al., 1991). Rabphilin domains are identified above the bar diagram. Positions of phosphorylation sites are indicated by *Ps*. The two C_2 -domains of rabphilin-3A and synaptotagmin are labeled *a* and *b*, and the single transmembrane region in synaptotagmin is identified by *TMR*.

and washed twice in this buffer and once in phosphate-buffered saline before analysis of bound proteins by SDS-PAGE followed by blotting onto nitrocellulose membrane and autoradiography. Phosphorylation of immunoprecipitated rabphilin-3A from rat brain or from transfected COS cells by the catalytic subunit of PKA was performed in an identical manner as described above except that the PKA catalytic subunit was added instead of the rat brain cytosol. For the phosphorylations by purified CaMK II, incubations contained 10 mM $MgCl_2$, 10 mM DTT, 2.5 μ g calmodulin, and 2.5 ng purified CaMK II in addition to or instead of the components listed above, and either 1 mM EGTA (control) or 1 mM Ca^{2+} , 0.1 mM EGTA (test) to stimulate the kinase. All phosphorylation experiments utilizing recombinant bacterial proteins expressed as GST-fusion proteins were performed analogously to those described for immunoprecipitated rabphilin-3As except that fusion proteins bound to glutathione-agarose beads were used as substrates for the phosphorylation reactions instead of immunoprecipitated rabphilin-3A bound to protein G-Sepharose via antibodies. All phosphorylation reactions were started by the addition of protein kinase.

Phosphoamino acid analysis was performed by blotting phosphorylated proteins separated by SDS-PAGE onto polyvinylidene difluoride membranes, cutting out the appropriate band, and hydrolyzing the protein in 5.7 M HCl at 110°C for 90 min. The hydrolysate was lyophilized, resuspended in pyridine:acetic acid:water (5:50:945), spotted onto cellulose membrane sheets (Kodak) together with phosphoamino acid standards (Sigma), and analyzed by electrophoresis (1 hr at 950 V) followed by autoradiography. Location of standards was identified by ninhydrin treatment.

Results

In order to study the phosphorylation of synaptic proteins implicated in regulating synaptic vesicle traffic, we isolated candidate proteins by immunoprecipitation, mixed them with rat brain cytosol as a protein kinase source, and studied their phosphorylation as a function of cyclic nucleotides and Ca^{2+} . Using this approach, we observed a dramatic increase in the phosphorylation of rabphilin-3A as a function of cyclic nucleotides and of Ca^{2+} (Fig. 1). Immunoprecipitates obtained with preimmune serum as a control exhibited no phosphorylated band at the molecular weight of rabphilin-3A. cAMP, cGMP, and Ca^{2+} triggered phosphorylation of rabphilin-3A, suggesting that it is a substrate for cAMP- and cGMP-dependent protein kinases and for a Ca^{2+} -stimulated kinase. Ca^{2+} -stimulated phosphorylation of rabphilin-3A is approximately twice as effective as that triggered by cyclic nucleotides. Addition of $\gamma^{32}P$ -ATP to immunoprecipitated rabphilin-3A without cytosol also caused rabphilin-3A phosphorylation. However, this background phosphorylation was variable between experiments (compare the second lane in Fig. 1 with third lane in Fig. 2), and recombinant rabphilin-3A showed no phosphorylation in the absence of added kinases (data not shown, see below), suggesting that rabphilin-3A does

not autophosphorylate but that an unidentified protein kinase can coimmunoprecipitate with rabphilin-3A.

To determine which protein kinases are responsible for the cAMP- and Ca^{2+} -stimulated phosphorylation of rabphilin-3A, we incubated immunoprecipitated rabphilin-3A with the catalytic subunit of PKA and with purified CaMK II (Fig. 2). Both phosphorylated rabphilin-3A, with Ca^{2+} -dependent CaMK II phosphorylation, again exceeding PKA phosphorylation. Thus, rabphilin-3A is a likely target for both PKA and CaMK II.

Rabphilin-3A has a distinct domain structure with three overall regions: an N-terminal sequence that binds rab3A, an intermediate region of undetermined function, and a C-terminal region with two C_2 -domains that probably mediate Ca^{2+} /phospholipid binding (Yamaguchi et al., 1993; Li et al., 1994). To localize the phosphorylation sites for PKA and for CaMK II in the structure of rabphilin-3A, we constructed a series of recombinant glutathione S-transferase (GST) fusion proteins with rabphilin-3A that cover rabphilin-3A (Fig. 3). These proteins were expressed in bacteria, purified, and used in phosphorylation reactions with rat brain cytosol in the presence and absence of cAMP and Ca^{2+} as activators (Fig. 4). Low levels of phosphorylation were observed with two of the seven GST-rabphilin fusion proteins (GST-RaphC and D) in the absence of cAMP or Ca^{2+} . Upon addition of cAMP, only GST-RaphC was strongly phosphorylated, whereas addition of Ca^{2+} stimulated phosphorylation of GST-RaphC and GST-RaphD (Fig. 4). Ca^{2+} also stimulated weak phosphorylation of GST-RaphE.

The rabphilin-3A sequences expressed in GST-RaphC and GST-RaphD are from the middle region of rabphilin-3A, with GST-RaphD containing the C-terminal half of the rabphilin-3A insert of GST-RaphC (Fig. 3). The selective phosphorylation of GST-RaphC and GST-RaphD by cAMP- and Ca^{2+} -stimulated protein kinases in rat brain localizes the major phosphorylation sites in rabphilin-3A to the middle region of rabphilin-3A, suggesting that this middle region represents a regulatory domain. The fact that cAMP stimulates phosphorylation of GST-RaphC but not of GST-RaphD suggests that the cAMP-dependent phosphorylation site of rabphilin-3A is localized to the N-terminal part of the rabphilin insert in GST-RaphC. Conversely, efficient Ca^{2+} -stimulated phosphorylation of both GST-RaphC and GST-RaphD indicates that the Ca^{2+} -dependent phosphorylation site of rabphilin-3A is localized in the C-terminal half of the GST-RaphC insert.

We, therefore, analyzed the sequences of rat and bovine rabphilin-3A, in particular, its middle region, for PKA and CaMK

Table 1. Comparison of phosphorylation site sequences of rabphilin-3A with those of the synapsins and of optimal consensus sequences

cAMP-dependent protein kinase	
Optimal consensus sequence ^{1,2}	R-R-x- <u>S/T</u> -x
Synapsin I ³ (6–10)	R-R-L- <u>S</u> -D
Synapsin II ³ (7–11)	R-R-L- <u>S</u> -D
Rabphilin-3A ^{4,6} (231–235)	R-R-A- <u>S</u> -E
Ca ²⁺ /calmodulin-dependent protein kinase II	
Optimal consensus sequence ^{2,5}	R-x-x- <u>S/T</u> -x
Synapsin I ³ (site 1) (563–567)	R-Q-A- <u>S</u> -I
Synapsin I ³ (site 2) (600–604)	R-Q-A- <u>S</u> -Q
Rabphilin-3A ^{4,6} (271–275)	R-A-N- <u>S</u> -V

Sequences are shown in single-letter amino acid code, with the phosphorylated amino acid shown in boldface and underlined. Residue numbers are given after the sequences, with the residue numbers referring to the rat proteins. All of the rabphilin-3A phosphorylation sites are conserved between bovine and rat rabphilin-3A.

¹ Kemp et al. (1977).

² Kennelly and Krebs (1991).

³ Südhof et al. (1989).

⁴ Shirataki et al. (1993).

⁵ Hanson and Schulman (1992).

⁶ Li et al. (1994).

II phosphorylation consensus sites (Kennelly and Krebs, 1991). A perfect match with PKA phosphorylation site consensus sequences was found at position 234 for rat rabphilin-3A, and with CaMK II phosphorylation site consensus sequences at position 274 of rat rabphilin-3A (Table 1). The sequences of these sites are similar to the sites for these enzymes observed in synapsins (Südhof et al., 1989) and are completely conserved between rat and bovine rabphilin-3A (Shirataki et al., 1993; Li et al., 1994). Their location corresponds exactly to the phosphorylation pattern with cAMP- and Ca²⁺-stimulated protein kinases observed with the pGEX-Raph proteins (Fig. 4), indicating that they may be used.

To test if the phosphorylation sites identified by the sequence analysis are actually the sites used by CaMK II and PKA in rabphilin-3A, we mutated these sites in a GST-rabphilin fusion protein containing the entire N-terminal half of rabphilin-3A (GST-RaphH, Fig. 3). Both putative phosphorylation sites were mutated individually by changing serine to alanine, and a double mutant containing both amino acid substitutions was also constructed. Expression of the recombinant proteins revealed instability of the protein with the single point mutation in the putative CaMK II site, but the double mutant protein containing point mutations in both CaMK II and PKA sites and the mutant protein with a single mutation in the putative PKA site were stable and could be analyzed (GST-RaphI and J, respectively).

The purified GST-rabphilin proteins were mixed with rat brain cytosol, and phosphorylation was analyzed as a function of activation by cAMP and Ca²⁺ (Fig. 5A). In the absence of activators, cytosol mediated a low degree of phosphorylation of wild-type rabphilin-3A but not of either mutant. cAMP stimulated phosphorylation of wild-type protein but not of the two mutants, both of which carry the mutation in the putative PKA site. Stimulation by Ca²⁺ caused dramatically increased phosphorylation of the wild-type protein and of the mutant protein with a single point mutation in the putative PKA site but not of the double mutant. These results suggest that serine²³⁴ serves as

a target for PKA and for background phosphorylation, and serine²⁷⁴ as a target for a Ca²⁺-stimulated kinase, possibly CaMK II. To test this hypothesis directly, the same wild-type and mutant GST-rabphilin proteins were phosphorylated with purified protein kinases, with the results confirming the assignments (Fig. 5B).

The experiments with recombinant GST-rabphilin fusion proteins suggest that serine²³⁴ and serine²⁷⁴ represent the phosphorylation sites for PKA and CaMK II, respectively, in rabphilin-3A. To provide further evidence for this conclusion, we performed phosphoamino acid analyses on phosphorylated rabphilins. The phosphoamino acid analyses were conducted on immunoprecipitated rabphilin-3A as well as on recombinant GST-rabphilin fusion proteins phosphorylated either with rat brain cytosol as a source of protein kinases or with purified protein kinase A. A representative experiment is shown in Figure 6, revealing that under different stimulation conditions and with rat brain cytosol as a protein kinase source, phosphoserine is the only phosphoamino acid observed.

Our experiment suggests that rabphilin-3A contains phosphorylation sites for PKA and CaMK II that are localized to serine²³⁴ and serine²⁷⁴. However, the localization of these phosphorylation sites was achieved with recombinant bacterial proteins incorporating only parts of rabphilin-3A, and the question arises if these phosphorylation sites are also used in native, full-length rabphilin, and if they are the only phosphorylation sites in rabphilin-3A for these kinases. To address these questions, we constructed eukaryotic expression vectors that encode wild-type and mutant rabphilins. Four vectors were made: a wild-type rabphilin-3A vector (pCMV85-4), vectors encoding mutant rabphilin-3As with serine to alanine substitutions at serine²³⁴ (pCMV85-3) or at serine²⁷⁴ (pCMV85-5), or a vector encoding double mutant rabphilin-3A with both amino acid substitutions (pCMV85-6). The vectors were transfected into COS cells and the expressed rabphilin-3A was immunoprecipitated and phosphorylated by addition of purified protein kinases (Fig. 7).

The results demonstrate that the single amino acid substitution of serine²³⁴ to alanine completely abolishes PKA-mediated phosphorylation of rabphilin-3A, demonstrating that this is the major site in rabphilin-3A recognized by PKA. CaMK II phosphorylation is diminished but not abolished by this mutation and even more strongly affected by the substitution of serine²⁷⁴ to alanine. However, only in the double mutant with both serine substitutions is it almost completely abolished (Fig. 7). Thus, serine²³⁴ represent the primary phosphorylation site for PKA, and both serine²³⁴ and serine²⁷⁴ constitute phosphorylation sites for CaMK II, with serine²⁷⁴ being the major site for CaMK II.

Discussion

Synaptic transmission is highly regulated. One of the fundamental questions in understanding synaptic transmission is what mechanisms mediate different types of regulation. Protein phosphorylation probably plays a major role in modulating a variety of aspects of neurotransmission, including neurotransmitter release by synaptic vesicle exocytosis. However, the mechanisms by which protein kinase might regulate neurotransmitter release are largely unknown. As a first step towards defining potential regulatory targets for protein kinases in the nerve terminal, we have studied the *in vitro* phosphorylation of rabphilin-3A. In an initial screen of synaptic protein kinase substrates, rabphilin-3A was identified as a major substrate for several protein kinases.

Two protein kinases that phosphorylate rabphilin-3A and the sites in rabphilin-3A that they phosphorylate were identified.

The structure of rabphilin-3A predicts the presence of three major regions, of which the N- and C-terminal domains have functions in mediating rab3A binding and Ca^{2+} /phospholipid binding, respectively. However, the function of the middle region, the least conserved of the three regions of rabphilin-3A (Li et al., 1994) is unclear. As indicated in the domain model shown in Figure 8, our studies now reveal that this middle region of rabphilin-3A contains phosphorylation sites for PKA and CaMK II, suggesting that it may act as a regulatory domain.

Rabphilin-3A has several properties in common with synaptotagmin: both proteins are synaptic vesicle Ca^{2+} -binding proteins with C-terminal Ca^{2+} -binding domains and N-terminal membrane attachment sequences. Furthermore, synaptotagmin is also a phosphoprotein with phosphorylation sites N-terminal to the Ca^{2+} -binding domains. In spite of these similarities illustrated in Figure 8, there are obvious differences: synaptotagmin is an intrinsic membrane protein, whereas rabphilin-3A is only loosely attached to membranes, probably via rab3A, and synaptotagmin is only phosphorylated by casein kinase II, whereas rabphilin-3A is phosphorylated by at least two kinases, PKA and CaMK II, and may also be phosphorylated by cGMP-dependent protein kinases and other Ca^{2+} -dependent protein kinases.

Our studies were restricted to phosphorylation of rabphilin-3A *in vitro*. It is unknown if rabphilin-3A is also phosphorylated *in vivo* but the sites defined in the current study ideally conform to the consensus phosphorylation sites used by these kinases *in vivo* (Table 1). Rabphilin-3A appears to be an excellent substrate for these kinases *in vitro*, suggesting that rabphilin-3A probably also is a substrate for these kinase *in vivo*, although this will have to be investigated in future experiments.

The phosphorylation of rabphilin-3A by two different protein kinases at closely linked sites is intriguing. The biochemical properties of rabphilin-3A are suggestive of a regulatory role in neurotransmission (Yamaguchi et al., 1993; Geppert et al., 1994; Li et al., 1994). As a Ca^{2+} binding protein localized to synaptic vesicles, rabphilin-3A may participate in a Ca^{2+} -regulated aspect of synaptic vesicle function. Indeed, experiments with mice deficient in rab3A demonstrated that in synapses lacking rab3A and rab3C (which may be redundant with rab3A at some synapses but is not present at most synapses), rabphilin-3A is not transported to synapses. Physiological measurements on such synapses revealed a potential defect in synaptic vesicle recycling, suggesting a possible role for rabphilin-3A in a Ca^{2+} -dependent step in synaptic vesicle recycling (Geppert et al., 1994). The regulation of such an activity by protein phosphorylation would be an attractive mechanism to modulate synaptic vesicle exocytosis as a function of Ca^{2+} in nerve terminals.

The phosphorylation of rabphilin-3A by multiple protein kinases is reminiscent of that of synapsin I which is also a peripheral membrane protein of synaptic vesicles that is a substrate for PKA and CaMK II (reviewed in Greengard et al., 1993; Jahn and Südhof, 1994). Currently rabphilin-3A and synapsin I are the only characterized major synaptic CaMK II and PKA substrates. Both are abundant synaptic proteins, suggesting a potential regulatory role for phosphorylation in the synaptic vesicle pathway. CaMK II has demonstrated major effects on neurotransmitter release (Llinas et al., 1991) that, based on the genetics, are clearly not mediated by synapsin I (Rosahl et al., 1993). PKA also has been implicated in several synaptic regulatory processes (Frey et al., 1993; Chavez-Noriega and Stevens,

1994). Our results suggest that rabphilin-3A may be one of the targets by which the regulatory effects of CaMK II and PKA could be mediated. This will have to be addressed in future experiments using mice lacking rab3A (which exhibit a major decrease in rabphilin-3A levels in most synapses but not a complete loss of rabphilin-3A; Geppert et al., 1994; Li et al., 1994) and mice that are completely deficient in rabphilin-3A.

References

- Bekkers JM, Stevens CF (1990) Presynaptic mechanism for long-term potentiation in the hippocampus. *Nature* 346:724–729.
- Bolshakov VY, Siegelbaum SA (1994) Postsynaptic induction and pre-synaptic expression of hippocampal long-term depression. *Science* 264:1148–1152.
- Bradford MM (1976) A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal Biochem* 72:248–254.
- Chavez-Noriega LE, Stevens CF (1994) Increased transmitter release at excitatory synapses produced by direct activation of adenylate cyclase in rat hippocampal slices. *J Neurosci* 14:310–317.
- Dale N, Kandel ER (1990) Facilitory and inhibitory transmitters modulate spontaneous transmitter release at cultured *Aplysia* sensorimotor synapses. *J Physiol (Lond)* 421:203–222.
- De Camilli P, Cameron R, Greengard P (1983a) Synapsin I (Protein I), a nerve terminal-specific phosphoprotein: I. Its general distribution in synapses of the central and peripheral nervous system demonstrated by immunofluorescence in frozen and plastic sections. *J Cell Biol* 96:1337–1354.
- De Camilli P, Harris SM, Huttner WB, Greengard P (1983b) Synapsin I (Protein I), a nerve terminal-specific phosphoprotein: II. Its specific association with synaptic vesicles demonstrated by immunocytochemistry in agarose-embedded synaptosomes. *J Cell Biol* 96:1355–1373.
- Dekker LV, De Graan PNE, Oestreicher AB, Versteeg DHG, Gispen WH (1989) Inhibition of noradrenaline release by antibodies to B-50 (GAP-43). *Nature* 342:74–76.
- Frey Y, Huang Y-Y, Kandel ER (1993) Effects of cAMP simulate a late stage of LTP in hippocampal CA1 neurons. *Science* 260:1661–1664.
- Geppert M, Bolshakov VY, Siegelbaum SA, Takei K, De Camilli P, Hammer RE, Südhof TC (1994) Rab3A function in neurotransmitter release. *Nature* 369:493–497.
- Gorman C (1985) High efficiency gene transfer into mammalian cells. *DNA cloning volume II* (Glover DM, ed), pp 143–190. Oxford: IRL.
- Greengard P, Valtorta F, Czernik AJ, Benfenati F (1993) Synaptic vesicle phosphoproteins and regulation of synaptic function. *Science* 259:780–785.
- Guan KL, Dixon JE (1991) Eukaryotic proteins expressed in *Escherichia coli*: an improved thrombin cleavage and purification procedure of fusion proteins with glutathione S-transferase. *Anal Biochem* 192:262–267.
- Hanson PI, Schulman H (1992) Neuronal Ca^{2+} /calmodulin-dependent protein kinases. *Annu Rev Biochem* 61:559–601.
- Jahn R, Südhof TC (1994) Synaptic vesicles and exocytosis. *Annu Rev Neurosci* 17:219–246.
- Johnston PA, Jahn R, Südhof TC (1989) Transmembrane topography and evolutionary conservation of synaptophysin. *J Biol Chem* 264:1268–1273.
- Kemp BE, Graves DJ, Benjamini E, Krebs EG (1977) *J Biol Chem* 252:4888–4892.
- Kennelly PJ, Krebs EG (1991) Consensus sequences as substrate specificity determinants for protein kinases and protein phosphatases. *J Biol Chem* 266:15555–15558.
- Kishida S, Shirataki H, Sasaki T, Kato M, Kaibuchi K, Takai Y (1993) Rab3A GTPase-activating protein-inhibiting activity of Rabphilin-3A, a putative Rab3A target protein. *J Biol Chem* 268:22259–22261.
- Laemmli UK (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* 227:680–685.
- Li C, Takei K, Geppert M, Daniell L, Stenius K, Chapman ER, Jahn R, De Camilli P, Südhof TC (1994) Synaptic targeting of rabphilin-3A, a synaptic vesicle Ca^{2+} /phospholipid-binding protein, depends on rab3A/3C. *Neuron* 13:885–898.
- Llinas R, Gruner JA, Sugimori M, McGuinness TL, Greengard P

- (1991) Regulation by synapsin I and Ca^{2+} -calmodulin-dependent protein kinase II of the transmitter release in squid giant synapse. *J Physiol (Lond)* 436:257–282.
- Malinow R, Tsien RW (1990) Presynaptic enhancement shown by whole-cell recordings of long-term potentiation in hippocampal slices. *Nature* 346:177–180.
- Man-Son-Hing H, Zoran MJ, Lukowiak K, Haydon PG (1989) A neuromodulator of synaptic transmission acts on the secretory apparatus as well as on ion channels. *Nature* 341:237–239.
- Nichols RA, Sihra TS, Czernik AJ, Nairn AC, Greengard P (1990) Calcium/calmodulin-dependent protein kinase II increases glutamate and noradrenaline release from synaptosomes. *Nature* 343:647–651.
- Nicoll RA, Malenka RC, Kauer JA (1990) Functional comparison of neurotransmitter receptor subtypes in mammalian central nervous system. *Physiol Rev* 70:513–565.
- Perin MS, Brose N, Jahn R, Südhof TC (1991) Domain structure of synaptotagmin (p65). *J Biol Chem* 266:623–629.
- Robinson PJ, Sontag JM, Liu J-P, Fykse EM, Slaughter C, McMahon H, Südhof TC (1993) Dynamin GTPase regulated by protein kinase C phosphorylation in nerve terminals. *Nature* 365:163–166.
- Rosahl T, Geppert M, Spillane D, Herz J, Hammer RE, Malenka RC, Südhof TC (1993) Short term synaptic plasticity is altered in mice lacking synapsin I. *Cell* 75:661–670.
- Sambrook J, Fritsch EF, Maniatis T (1989) Molecular cloning. A laboratory manual, 2d ed. Cold Spring Harbor, NY: Cold Spring Harbor Laboratory.
- Sasaki T, Kikuchi A, Araki S, Hata Y, Isomura M, Kuroda S, Takai Y (1990) Purification and characterization from bovine brain cytosol of a protein that inhibits the dissociation of GDP from and the subsequent binding of GTP to smg p25A, a ras p21-like GTP-binding protein. *J Biol Chem* 265:2333–2337.
- Scholz KP, Miller RJ (1992) Inhibition of quantal transmitter release in the absence of calcium influx by a G protein-linked adenosine receptor at hippocampal synapses. *Neuron* 8:1139–1150.
- Shirataki H, Kaibuchi K, Yamaguchi T, Wada K, Horiuchi H, Takai Y (1992) A possible target protein for smg-25A/rab3A small GTP-binding protein. *J Biol Chem* 267:10946–10949.
- Shirataki H, Kaibuchi K, Sakoda T, Kishida S, Yamaguchi T, Wada K, Miyazaki M, Takai Y (1993) Rabphilin-3A, a putative target protein for smg p25A/rab3A small GTP-binding protein related to synaptotagmin. *Mol Cell Biol* 13:2061–2068.
- Silinsky EM (1984) On the mechanism by which adenosine receptor activation inhibits the release of acetylcholine from motor nerve endings. *J Physiol (Lond)* 346:243–256.
- Silva AJ, Stevens CF, Tonegawa S, Wang Y (1992) Deficient hippocampal long-term potentiation in α -calcium, calmodulin kinase II mutant mice. *Science* 257:201–206.
- Smith DB, Johnson KS (1988) Single-step purification of polypeptides expressed in *Escherichia coli* as fusions with glutathione S-transferase. *Gene* 67:31–40.
- Stevens CF (1993) Going down the way you came up. *Curr Biol* 3:891–892.
- Südhof TC, Czernik AJ, Kao H, Takei K, Johnston PA, Horiuchi A, Kanazir SD, Wagner MA, Perin MS, De Camilli P, Greengard P (1989) Synapsins: mosaics of shared and individual domains in a family of synaptic vesicle phosphoproteins. *Science* 245:1474–1480.
- Südhof TC, Jahn R (1991) Proteins of synaptic vesicles involved in exocytosis and membrane recycling. *Neuron* 6:665–677.
- Takeuchi A, Takeuchi N (1966) A study of the inhibitory action of gamma-aminobutyric acid on neuromuscular transmission in the crayfish. *J Physiol (Lond)* 183:418–432.
- Weisskopf MG, Zalutsky RA, Nicoll RA (1993) The opioid peptide dynorphin mediates heterosynaptic depression of hippocampal mossy fiber synapses and modulates long-term potentiation. *Nature* 362:423–427.
- Yamaguchi T, Shirataki H, Kishida S, Miyazaki M, Nishikawa J, Wada K, Numata S, Kaibuchi K, Takai Y (1993) Two functionally different domains of Rabphilin-3A, Rab3A p25/smg p25A-binding and phospholipid- and Ca^{2+} binding domains. *J Biol Chem* 268:27164–27170.
- Zalutsky RA, Nicoll RA (1990) Comparison of two forms of long-term potentiation in single hippocampal neurons. *Science* 248:1619–1624.
- Zhuo M, Hu Y, Schultz C, Kandel ER, Hawkins RD (1994) Role of guanylyl cyclase and cGMP-dependent protein kinase in long-term potentiation. *Nature* 368:635–639.
- Zucker RS (1989) Short-term synaptic plasticity. *Annu Rev Neurosci* 12:13–31.