The Journal of Neuroscience, August 1, 2001, *21*(15):5473–5483

Physiological Modulation of Rabphilin Phosphorylation

Davide L. Foletti, Jeremy T. Blitzer, and Richard H. Scheller

Howard Hughes Medical Institute, Department of Molecular and Cellular Physiology, Stanford University School of Medicine, Stanford, California 94305-5428

The dynamic modulation of protein function by phosphorylation plays an important role in regulating synaptic plasticity. Several proteins involved in synaptic transmission have been shown to be targets of protein kinases and phosphatases. A thorough analysis of the physiological role of these modifications has been hampered by the lack of reagents that specifically recognize the phosphorylated states of these proteins. In this study we analyze the physiological modulation of rabphilin using phosphospecific antibodies. We show that phosphorylation on serine-234 and serine-274 of rabphilin is dynamically regulated both under basal and stimulated conditions by the activity of kinases and phosphatases. The two sites are differentially phosphorylated by the stimulation of various kinases, suggesting a possible convergence of different pathways to modulate the function of the protein. Maximal stimulation was observed under plasma membrane-depolarizing conditions that trigger synaptic vesicle exocytosis. The increase in phosphorylation was critically dependent on external Ca^{2+} and on the presence of Rab3a, a small GTPase that recruits rabphilin to synaptic vesicles. The rapid phosphorylation and dephosphorylation during and after stimulation demonstrates the transient nature of the modification. Our results indicate that rabphilin is phosphorylated on synaptic vesicles by Ca^{2+}-dependent kinases that become active in synaptic terminals during exocytosis. We have found that phosphorabphilin has a reduced affinity for membranes; we therefore propose that the modulation of the membrane association of rabphilin has a role in the synaptic vesicle life cycle, perhaps in vesicle mobilization in preparation for subsequent rounds of neurotransmission.

Key words: rabphilin; Rab3a; phosphospecific antibodies; brain acute slices; synaptic transmission; protein kinases

A combination of genetic, biochemical, structural, and functional studies has led to the discovery and characterization of molecules important in the Ca^{2+}-regulated exocytosis of synaptic vesicles, the process that initiates synaptic transmission. The soluble *N*-ethylmaleimide-sensitive factor attachment receptor proteins syntaxin 1, SNAP-25, and vesicle-associated membrane protein-2 (VAMP-2), together with nSec1, Rab proteins and their effectors, play important roles during synaptic transmission and belong to large protein families whose members are implicated in every step of intracellular membrane trafficking in eukaryotic cells (for review, see Jahn and Südhof, 1999; Lin and Scheller, 2000; Bock et al., 2001).

It is well established that protein kinases and phosphatases have an important role in synaptic transmission. At the presynaptic terminal, the interval between an incoming action potential and the fusion of primed synaptic vesicles is likely too short for protein phosphorylation to have a direct effect. It is therefore in subsequent rounds of synaptic vesicle exocytosis that the activity-dependent stimulation of protein kinases and phosphatases becomes manifest. The regulation of synaptic protein function by phosphorylation–dephosphorylation is optimally situated to modulate aspects of synaptic plasticity. Several classes of proteins that function in synaptic transmission have been reported as potential targets for various kinases (for review, see Turner et al., 1999). With few exceptions, most notably the phosphorylation of synapsin (Greengard et al., 1993; Hosaka et al., 1999), the *in vivo* occurrence and physiological relevance of these putative phosphorylation events remains to be fully established. Little is known about the molecular consequences of these phosphorylations, the way they effect protein–protein interactions, and how this translates into changes in synaptic function.

In this report we use phosphospecific antibodies to characterize the modulation of the phosphorylation state of rabphilin, a synaptic protein that has been implicated in the life cycle of synaptic vesicles, but whose function is still unclear (Miyazaki et al., 1994; Chung et al., 1995; Kato et al., 1996; Komuro et al., 1996; Masumoto et al., 1996; Burns et al., 1998; Ohya et al., 1998; Schluter et al., 1999). *In vitro* experiments have shown that serine-234 is the primary phosphorylation site for cAMP-dependent protein kinase (PKA), and both serine-234 and serine-274 are phosphorylated by Ca^{2+}/Calmodulin-dependent kinase II (CaMKII), with serine-274 being the preferred site (Fykse et al., 1995). In hippocampal synaptosomes, activation of PKA and Ca^{2+} influx provoked by high K^+-induced depolarization resulted in a selective increase in rabphilin phosphorylation in mossy fiber CA3 synaptosomes, but not in CA1 synaptosomes (Lonart and Südhof, 1998). In cultured cerebellar granule cells, the overall level of rabphilin phosphorylation was increased approximately twofold after stimulation of protein kinase C (PKC) and high K^+-induced membrane depolarization (Fykse, 1998). Here we study the stimulation-dependent increase in rabphilin phosphorylation and show its dependence on external Ca^{2+} and the interaction with Rab3a. The stimulus-dependent kinetics of phosphorylation and dephosphorylation are rapid and indicate a transient nature of this modification. We found that phosphorabphilin has a reduced affinity for membranes, this suggests a possible role of phosphorylation in modulating its membrane localization during stimulation of synaptic activity.

Received March 6, 2001; revised May 11, 2001; accepted May 17, 2001.

We thank Dr. Michael Finley for help with the preparation of rat brain acute slices and Dr. Cary Austin for critical reading of this manuscript.

Correspondence should be addressed to Richard H. Scheller, Genentech, Inc., 1 DNA Way, South San Francisco, CA 94080-4990. E-mail: scheller@gene.com.

MATERIALS AND METHODS

Animals and reagents. Wild-type (WT; 129/B6; +/+) and Rab3a knockout (KO; 129/B6; −/−) mice were purchased from The Jackson Laboratory (Bar Harbor, ME), 6- to 8-week-old female Sprague Dawley rats were obtained from Simonsen Laboratories (Gilroy, CA) and housed in the Stanford University Animal Facility. Phorbol-12,13-dibutyrate (PDBu; used at 1 μM), forskolin (FO; used at 50 μM), adenosine 3′,5′-cyclic monophosphate 8-(4-chlorophenylthio) (8-CPT-cAMP; used at 500 μM), 3-isobutyl-1-methylxanthine (IBMX; used at 50 μM), 4-aminopyridine (4-AP; used at 100 μM), sphingosine (sphi; used at 30 μM), tetraethylammonium chloride (TEA; used at 25 mM), and okadaic acid (OA; used at 1 μM) were all purchased from Calbiochem (San Diego, CA). Calf intestinal alkaline phosphatase (CIP) was from New England Biolabs (Beverly, MA). Unless otherwise stated, all other reagents were obtained from Sigma (St. Louis, MO) or Fisher Biotech (Pittsburgh, PA).

Rat brain slices and sample preparation. Rat brain slices were prepared essentially as previously described (McQuinston and Madison, 1999). Briefly, 6- to 8-week-old rats were killed under halothane anesthesia by decapitation, and their brains were rapidly removed and placed in cold (4°C) cutting Ringer's solution (in mM: NaCl 119, KCl 2.5, $MgSO_4$ 3.0, $CaCl_2$ 1.0, NaH_2PO_4 1.0, $NaHCO_3$ 26.2, glucose 11, and kynurenic acid 1.0, bubbled with 95% O_2 and 5% CO_2). The brains were then hemisected, and single-hemisphere coronal slices (500 μm thick) were cut on a Vibratome (Lancer). The slices were kept for 15–30 min in an incubation chamber with warm (30°C) cutting Ringer's solution without kynurenic acid. All subsequent experiments were performed at room temperature (~23°C). Incubations with pharmacological agents were performed either in normal Ringer's solution (in mM: NaCl 119, KCl 2.5, $MgSO_4$ 1.3, $CaCl_2$ 2.5, NaH_2PO_4 1.0, $NaHCO_3$ 26.2, and glucose 11, bubbled with 95% O_2 and 5% CO_2) or in high K^+ Ringer's solution (in mM: NaCl 65.5, KCl 56, $MgSO_4$ 1.3, $CaCl_2$ 2.5, NaH_2PO_4 1.0, $NaHCO_3$ 26.2, and glucose 11, bubbled with 95% O_2 and 5% CO_2). In experiments designed to assess the Ca^{2+} dependence of rabphilin phosphorylation, $CaCl_2$ in the normal Ringer's solution and high K^+ Ringer's solution was replaced with EGTA (2 mM). For each experiment, two slices were flash-frozen in liquid nitrogen at the end of each treatment. The slices were homogenized with a glass–Teflon homogenizer in buffer containing 20 mM HEPES-NaOH, pH 7.4, 200 mM NaCl, 1 mM DTT, 2 mM EDTA, 20 mM β-glycerophosphate, 50 mM NaF, 50 mM Na-pyrophosphate, 2 μM Microcystin-LR, 2 μg/ml aprotinin, 2 μg/ml leupeptin, 0.7 μg/ml pepstatin, and 1 mM PMSF. In cases in which no further fractionation was performed, the homogenate was solubilized with 1% Triton X-100 for 1 hr at 4°C. After a centrifugation at 100,000 × *g* for 1 hr to pellet the insoluble fraction, the supernatant was collected and used in quantitative Western blot analysis (see below). For subfractionation into cytosol and membranes, the homogenate was first centrifuged at 1000 × *g* for 15 min. The resulting postnuclear supernatant was further centrifuged at 100,000 × *g* for 1 hr to separate the cytosolic fraction (supernatant) from the membrane fraction (pellet). For the NaCl and Triton X-100 extraction experiments, membranes prepared as above were resuspended in homogenization buffer. NaCl (1 M) or Triton X-100 (1%) were added and after a 1 hr incubation at 4°C the samples were centrifuged at 100,000 × *g* for 1 hr to separate the supernatant (extracted material) from the pellet (nonextracted material). For the preparation of the homogenate in the absence of phosphatase inhibitors, the following reagents were omitted: β-glycerophosphate, NaF, Na-pyrophosphate, and Microcystin-LR; additionally the homogenate was incubated for 30 min with 50 U of CIP.

Quantitative Western blotting. The generation and purification of antibodies specific for rabphilin phosphorylated at S234 and S274 (αS234-P and αS274-P, respectively) are described in the accompanying paper. The antibody specific for the nonphosphorylated form of rabphilin at S234 (α-non-P-S234) was generated and purified similarly. Briefly, a peptide corresponding to amino acids 230–239 (TRRASEARMS) of rabphilin was synthesized with an additional cysteine residue at the C terminus for coupling purposes. The peptide was coupled to Imject maleimide-activated keyhole limpet hemocyanin (Pierce, Rockford, IL) and used as immunogen in rabbit. The polyclonal antiserum was preabsorbed over columns carrying a peptide with an unrelated sequence, and the phosphopeptide with same sequence as the peptide used for immunization to remove nonspecific antibodies. Finally the antiserum was affinity-purified by binding and elution from a column carrying the peptide originally used as the immunogen. The mouse monoclonal antibody against total rabphilin (recognizes rabphilin irrespective of its phosphorylation state) was from Transduction Laboratories (Lexington, KY), the monoclonal antibody against SNAP-25 was obtained from Sternberger Monoclonals (Lutherville, MA), and the anti-VAMP-2 antibody was from Synaptic Systems (Goettingen, Germany). Secondary antibodies for quantitative Western Blot analysis were obtained from Amersham Pharmacia Biotech (Arlington, IL) and included anti-rabbit Ig from donkey [^{125}I-labeled F(ab′)$_2$ fragment] and anti-mouse Ig from sheep [^{125}I-labeled F(ab′)$_2$ fragment]. Equal amounts of total protein from each sample were resolved by SDS-PAGE and transferred to nitrocellulose membranes (Hybond ECL; Amersham Pharmacia Biotech, Piscataway, NJ) according to standard protocols. To control and correct for equal loading in each Western blot experiment, the bottom part of the blot was probed and quantified for SNAP-25 and used to normalize the signals obtained with the anti-rabphilin antibodies. Western blots were analyzed by phosphorimaging technology (Molecular Dynamics, Sunnyvale, CA). Recombinant fragments of rabphilin were expressed, purified, and phosphorylated *in vitro* with PKA as described in the accompanying paper.

RESULTS

In the accompanying paper, we have studied the developmental regulation and whole brain and subcellular distributions of rabphilin phosphorylated at serine-234 and serine-274 using phosphospecific antibodies. In this report, we investigated the *in vivo* modulation of the two phosphorylation events to gain insight into their functional significance. We again used the antibodies αS234-P and αS274-P, which recognize rabphilin only when the protein is phosphorylated on serine-234 or serine-274, respectively. The antibodies were used in a series of quantitative Western blot experiments to evaluate changes in phosphorylation levels of rabphilin under a variety of physiological conditions. As a model system, we used rat brain acute coronal slices, a preparation that has been well established and characterized by a large number of electrophysiological experiments (Alger et al., 1984; Madison, 1991). The slices, prepared and maintained in physiological buffers bubbled with 95% O_2 and 5% CO_2, have been shown to be viable for at least 4 hr. The acute slice preparation offers several advantages for the study of synaptic protein phosphorylation: neurons, their synapses, and part of the circuitry in which they are involved, are well preserved and viable. The thickness of the slice (500 μm) allows for efficient entry of most pharmacological reagents. Finally, the amount of nervous tissue allows for quantitative biochemistry.

Stimulation of phosphorylation on S234 and S274 of rabphilin

We first analyzed the effect of various pharmacological agents on the phosphorylation level of rabphilin at serine-234 and serine-274. Slices were incubated in Ringer's solution containing 56 mM K^+ for 2 min, a condition that promotes strong membrane depolarization and Ca^{2+} influx into the nerve terminals, therefore generating a strong burst of synaptic vesicle exocytosis. The other incubations were performed for 30 min and included the following pharmacological reagents: 1 μM PDBu to stimulate the activity of PKC, 50 μM forskolin to activate PKA through stimulation of adenylyl cyclase, a combination of a membrane-permeable analog of cAMP (8-CPT-cAMP, used at 500 μM), and an inhibitor of cAMP phosphodiesterase (IBMX, used at 50 μM) also to stimulate PKA, 100 μM 4-AP, a treatment that delays action potential repolarization and increases firing rate by blocking potassium channels (Wu and Barish, 1992), 30 μM sphingosine to stimulate casein kinase II, and 25 mM TEA to cause depolarization and repetitive action potentials by blocking potassium channels. At the end of each treatment, two slices for each condition were flash-frozen in liquid nitrogen to stop any ongoing phospho-

rylation–dephosphorylation activity. The slices were subsequently homogenized in the presence of phosphatase inhibitors and processed for quantitative Western blotting with αS234-P and αS274-P (see Materials and Methods for details). The increase (or decrease) in rabphilin phosphorylation is expressed as arbitrary units, normalized to the level of phosphorylation detected in unstimulated slices. In Figure 1, the Western blots show representative results, and the graphs summarize the quantitative analysis of four to eight independent experiments (mean and SEM). Rabphilin phosphorylation on serine-234 (Fig. 1*A*) is strongly potentiated to approximately sixfold over the basal level by both the high K^+-induced depolarization ($p < 0.001$; *t* test) and the stimulation of PKA with 8-CPT-cAMP/IBMX ($p < 0.01$; *t* test). Forskolin was somewhat less effective in stimulating rabphilin phosphorylation (threefold increase; $p < 0.001$; *t* test). The phorbol ester PDBu and 4-AP promoted smaller, but still significant increases in phosphorylation (~2.5-fold each; $p < 0.05$; *t* test). Sphingosine and TEA had little or no effect. The amount of total rabphilin, detected with an antibody that recognizes the protein irrespective of its phosphorylation state (αtotal rabphilin), was unaffected by these treatments (data not shown). Our results are consistent with and expand the significance of previous *in vitro* experiments, demonstrating that serine-234 is the primary phosphorylation site for PKA (Fykse et al., 1995). A more modest, but significant, stimulation of phosphorylation at this site is also promoted by PKC activation, in agreement with our *in vitro* phosphorylation experiments (see accompanying paper). Phosphorylation on rabphilin S274 (Fig. 1*B*) was stimulated approximately threefold by high K^+-induced depolarization and activation of PKA and PKC ($p < 0.001$, $p < 0.05$ and $p < 0.05$, respectively; *t* test). Forskolin was again less effective than 8-CPT-cAMP/IBMX in stimulating rabphilin phosphorylation (twofold increase; $p < 0.05$; *t* test), and 4-AP had a similar effect ($p < 0.05$; *t* test). Sphingosine and TEA had little or no effect. Serine-274 of rabphilin has been shown by *in vitro* experiments to constitute a major phosphorylation site for CaMKII (Fykse et al., 1995). Our *in vivo* results are consistent with this view, because CaMKII is likely to become activated during the Ca^{2+} influx promoted by membrane depolarization. Additionally, a phorbol ester was just as effective in stimulating phosphorylation at this site of rabphilin, suggesting that PKC activation might regulate this event as well. Taken together, these results indicate that the phosphorylation of rabphilin can be strongly stimulated *in vivo* by the activity of multiple kinases, particularly under the same depolarizing conditions that lead to Ca^{2+} influx into the nerve terminals and consequent exocytosis.

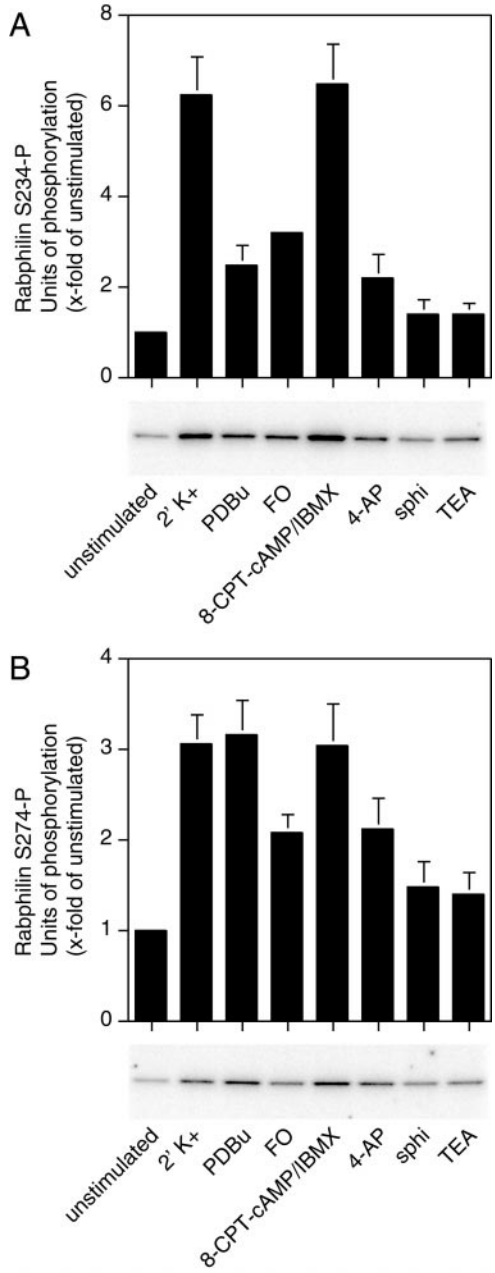

Figure 1. Stimulation of phosphorylation on S234 and S274 of rabphilin. Acute slices prepared from 6- to 8-week-old rats were incubated in normal Ringer's solution supplied with the indicated pharmacological agents. At the end of the incubation, the slices were flash-frozen in liquid nitrogen and processed for quantitative Western blotting with the phosphospecific antibodies against rabphilin S234-P (*A*) and S274-P (*B*). Each panel shows a representative Western blot result and summarizes the quantitative analysis of four to eight experiments (mean and SEM). *PDBu,* Phorbol-12,13-dibutyrate used at 1 μM; *FO,* forskolin used at 50 μM; *8-CPT-cAMP/IBMX*, adenosine 3′,5′-cyclic monophosphate, 8-(4-chlorophenylthio)/3-isobutyl-1-methylxanthine used at 500 or 50 μM, respectively; *4-AP,* 4-aminopyridine used at 100 μM; *sphi,* sphingosine used at 30 μM; *TEA,* tetraethylammonium chloride used at 25 mM. All the above incubations, as well as the unstimulated condition, were for 30 min. *2′K+*, After 28 min in Ringer's solution, the slices were incubated for 2 min in Ringer's solution containing 56 mM K^+.

The absence of Rab3a greatly reduces the stimulus-dependent phosphorylation of rabphilin

Given the interaction between Rab3a and rabphilin, we investigated whether the absence of the small GTP-binding protein has an effect on the high K^+-stimulated phosphorylation of rabphilin. Acute slices were prepared from WT and Rab3a KO mice. Stimulation with high K^+ and processing for Western blotting were as described for rat slices. Figure 2*A* shows representative Western blots probed with αS234-P, αS274-P, and αtotal rabphilin. Figure 2, *B* (rabphilin S234-P) and *C* (rabphilin S274-P), summarizes the quantitative analysis of four independent experiments (mean and SEM). In agreement with the results obtained with rat brain slices, incubation of mouse brain slices in high K^+ Ringer's solution for 2 min produced a strong increase in phosphorylation over the basal state. The effect was observed at both serine-234 (~10-fold increase in phosphorylation; $p < 0.001$; *t* test) and serine-274 (approximately twofold increase in phosphorylation; $p < 0.01$; *t* test). In striking contrast, in the slices

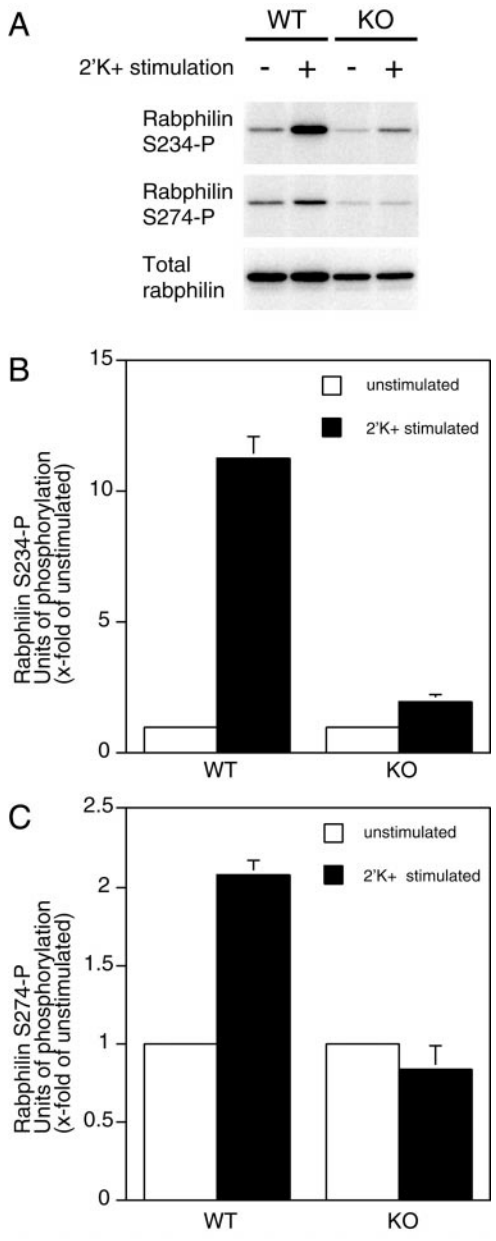

Figure 2. The high K^+-induced increase in rabphilin phosphorylation is severely reduced (S234-P) or completely abolished (S274-P) in slices prepared from Rab3a knock-out mice. Acute slices prepared from wild-type (*WT*) and Rab3a knock-out (*KO*) mice were incubated in normal Ringer's solution. Unstimulated slices and slices that were subjected to a 2 min 56 mM K^+ stimulation were flash-frozen in liquid nitrogen and processed for quantitative Western blotting to detect changes in rabphilin phosphorylation at S234 and S274. *A*, Representative Western blot results. *B, C*, Quantitative analysis of four independent experiments (mean and SEM). In both WT and KO, the level of phosphorylation after stimulation is expressed relative to the level in unstimulated slices.

obtained from the Rab3a KO animals, the high K^+-induced increase in phosphorylation at serine-234 was only approximately twofold ($p < 0.05$; *t* test), whereas the effect on serine-274 was completely abolished (~0.8-fold, statistically not significant). In the Rab3a KO animals, the level of total rabphilin is decreased to 40–50% of that in wild-type animals (Geppert et al., 1994). Moreover, rabphilin was shown to accumulate in the perikarya of neurons lacking Rab3a and was unable to reach its proper localization on synaptic vesicles within nerve terminals (Li et al., 1994). We therefore suggest that rabphilin can be phosphorylated only if the protein is in its correct localization on synaptic vesicles within nerve terminals, a process believed to require interaction with Rab3a (Li et al., 1994; Stahl et al., 1996; Schluter et al., 1999). This suggests that the phosphorylation of rabphilin is linked to its localization at the site of exocytosis.

Both the basal and the high K^+ stimulated levels of phosphorabphilin are strictly dependent on extracellular Ca^{2+}

Because the high K^+-induced depolarization promotes Ca^{2+} influx into nerve terminals, we investigated whether the presence of Ca^{2+} in the external medium is necessary for the observed increase in phosphorylation on rabphilin serine-234 and serine-274. For this experiment, slices were preincubated for 15 min in normal Ringer's solution or in Ringer's solution without Ca^{2+} and then flash frozen, or first stimulated for 2 min in high K^+ Ringer's solution and then flash frozen before processing for quantitative Western blot. In Figure 3, the Western blots show representative results, and the graphs summarize the quantitative analysis of four to seven independent experiments (mean and SEM). As shown in Figure 3*A* for rabphilin S234-P and Figure 3*B* for rabphilin S274-P, depletion of Ca^{2+} completely prevented the increase in phosphorylation induced by high K^+ and also reduced the basal level of phosphorylation of rabphilin (compare *lanes 2–4* and *1–3*, respectively). The high K^+-stimulated increase in phosphorylation was not only completely prevented by the absence of Ca^{2+}, but the level of phosphorylation after the stimulation was even lower than in unstimulated slices incubated in normal Ringer's solution: ~0.5-fold for rabphilin S234-P ($p < 0.02$; *t* test) and ~0.3-fold for rabphilin S274-P ($p < 0.002$; *t* test). The basal level of phosphorylation in the absence of Ca^{2+} was similarly reduced to ~0.5-fold on serine-234 ($p < 0.005$; *t* test) and to ~0.3-fold on serine-274 ($p < 0.002$; *t* test). The preincubation for 15 min in the absence of Ca^{2+} did not just simply damage the slices, because if we re-supplemented them with Ca^{2+} for an additional 15 min, both the basal level of phosphorylation and the increase in phosphorylation induced by high K^+ stimulation were completely restored (compare *lanes 1–5* and *2–6*, respectively). These results indicate that Ca^{2+} is absolutely necessary for both the basal and the high K^+-induced phosphorylation of rabphilin, suggesting that rabphilin is a substrate for Ca^{2+}-dependent protein kinases that are directly or indirectly activated during synaptic vesicle exocytosis. CaMKII could fulfill this role for the phosphorylation of rabphilin at serine-274. For the phosphorylation at serine-234 we acknowledge two possibilities. Although the sequence around serine-234 matches the PKA phosphorylation site consensus motif, and recombinant rabphilin can be efficiently phosphorylated at this site by PKA *in vitro* (Fykse et al., 1995), it is possible that a Ca^{2+}-dependent kinase and not PKA is responsible for the *in vivo* phosphorylation at serine-234 after membrane depolarization. Alternatively, a cascade of events with a Ca^{2+}-dependent component may indirectly activate PKA.

A large proportion of rabphilin is phosphorylated on serine-234 after a 2 min high K^+ stimulation

We next examined the extent of phosphorylation of rabphilin by estimating how much of it becomes phosphorylated after stimulation. While raising the phosphospecific antibodies against rabphilin S234-P and rabphilin S274-P, we also immunized rabbits with the non-phospho version of the peptide used to generate αS234-P. The antibody, intended to be a general αtotal rabphilin

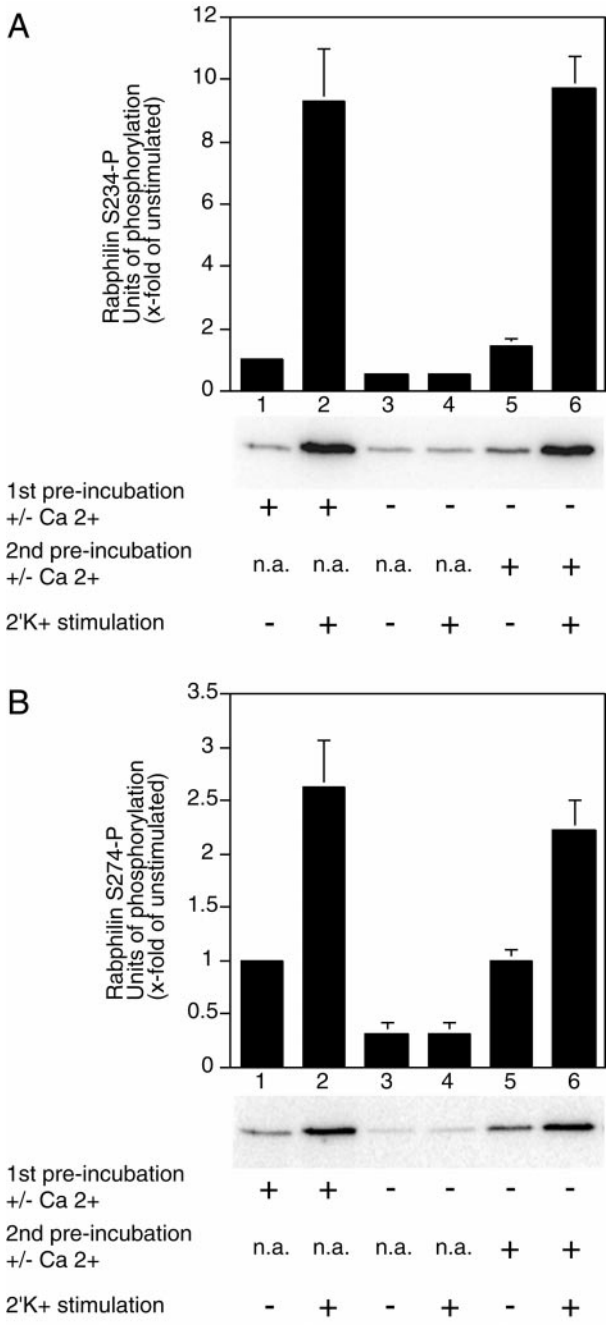

Figure 3. The high K^+-induced increase in rabphilin phosphorylation at S234 and S274 is strictly dependent on extracellular Ca^{2+}. Acute slices from 6- to 8-week-old rats were preincubated for 15 min in Ringer's solution with or without Ca^{2+}, followed by incubation for 2 min in the presence or absence of 56 mM K^+ and subsequent flash freezing. Some slices were first preincubated for 15 min in Ringer's solution without Ca^{2+} and then supplemented with Ca^{2+} for 15 min before a 2 min incubation in the presence or absence of 56 mM K^+ and subsequent flash freezing. Slices were processed for quantitative Western blotting to detect changes in rabphilin phosphorylation at S234 (*A*) and S274 (*B*). Each panel shows a representative Western blot result and summarizes the analysis of four to seven independent experiments (mean and SEM, for each condition the level of phosphorylation is expressed relative to the level observed in unstimulated slices under normal Ca^{2+} conditions).

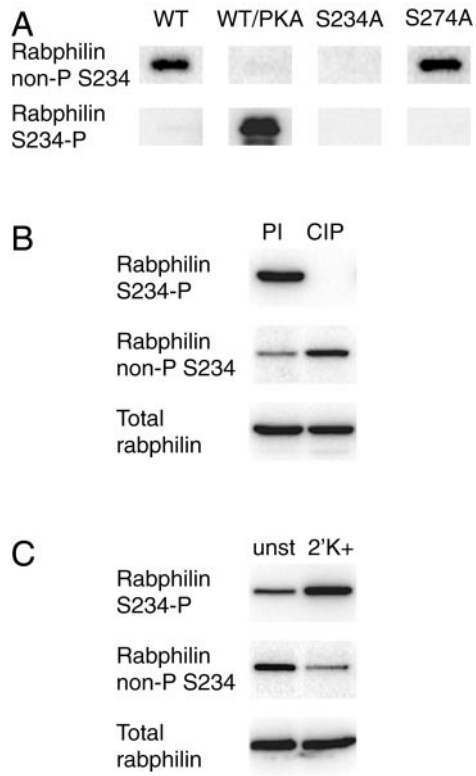

Figure 4. A large proportion of rabphilin is phosphorylated on S234 after a 2 min high K^+ stimulation. *A*, Specificity of the antibody specific for the form of rabphilin that is not phosphorylated at S234 (αnon-P S234). Equal amounts of a recombinant fragment of rabphilin (aa 1–361), wild-type (*WT*), or serine to alanine mutants at the phosphorylation sites (*S234A* and *S274A*), together with the recombinant WT fragment phosphorylated *in vitro* with purified PKA (*WT/PKA*), were resolved by SDS-PAGE and transferred to nitrocellulose membranes. The blots were probed with αnon-P S234 or the antibody specific for the form of rabphilin that is phosphorylated at S234 (αS234-P). *B*, Estimate of the proportion of rabphilin phosphorylated at S234 after a 2 min high K^+ stimulation. Equivalent pools of acute slices prepared from 6- to 8-week-old rats were stimulated for 2 min in Ringer's solution with 56 mM K^+. The slices were flash-frozen in liquid nitrogen, and the two pools were separately homogenized in the presence or absence of phosphatase inhibitors (*PI*). The slices homogenized in the absence of phosphatase inhibitors were additionally treated with calf intestinal phosphatase (*CIP*). Equal amounts of total protein were subjected to Western blotting. The blots were probed with an antibody that recognizes total rabphilin (irrespective of its phosphorylation state) to confirm equal loading, as well as with the antibody that recognizes only S234-phosphorylated rabphilin to confirm the complete dephosphorylation in the CIP-treated sample. In the blot probed with the αnon-P S234 the increase in signal after dephosphorylation (*CIP*), compared with the signal obtained from the sample prepared in the presence of phosphatase inhibitors (*PI*), reflects the proportion of rabphilin phosphorylated on S234 after a 2 min high K^+ stimulation. *C*, After a high K^+ stimulation the increase in rabphilin S234-P is mirrored by a decrease in rabphilin non-P S234. Equal amounts of total protein from unstimulated slices (*unst*) and from slices stimulated for 2 min in Ringer's solution with 56 mM K^+ (*2′K*$^+$) were subjected to Western blotting. The blots were probed with an antibody that recognizes total rabphilin to confirm equal loading, as well as with the antibodies that specifically recognize rabphilin phosphorylated or not phosphorylated at S234. The increase in rabphilin phosphorylated at S234 after high K^+ stimulation is matched by an equivalent decrease in the signal obtained with the antibody that recognizes only S234 nonphosphorylated rabphilin.

antibody to be used in control experiments, proved to recognize only the form of rabphilin that is not phosphorylated at serine-234 (αnon-P-S234; see Materials and Methods for details on its purification). Figure 4*A* shows the specificity of αnon-P-S234 in comparison with its cognate antibody αS234-P. A recombinant fragment of rabphilin [amino acid (aa) 1–361] was expressed and purified from bacteria in its wild-type form (WT), or after the

two phosphorylation sites had been mutated to alanine (S234A and S274A, respectively). The wild-type recombinant fragment was *in vitro* phosphorylated with PKA. Equal amounts of rabphilin wild-type fragment (WT), wild-type fragment phosphorylated by PKA (WT/PKA), and each of the two mutants (S234A and S274A), were resolved by SDS-PAGE and transferred to nitrocellulose. The blots were probed with αnon-P-S234 (Fig. 4*A, top panels*) and αS234-P (Fig. 4*A, bottom panels*). In agreement with results in the accompanying paper, αS234-P recognizes rabphilin only if the protein has been phosphorylated at serine-234 (Fig. 4*A, WT/PKA, bottom panel*). In contrast, αnon-P-S234 recognized the wild-type and S274A recombinant rabphilin, but not the wild-type protein phosphorylated by PKA or the S234A mutant (Fig. 4*A, top panels*). Evidently, the serine at position 234 is critical for epitope recognition by αnon-P-S234, because both mutation of this residue to alanine (S234A) and its modification by phosphorylation (WT/PKA) completely abolished recognition of rabphilin.

We used αnon-P-S234 to quantify the proportion of rabphilin that becomes phosphorylated at the end of a 2 min high K^+-induced depolarization (Fig. 4*B*). Two equivalent pools of acute slices were prepared and stimulated in high K^+ Ringer's solution for 2 min before flash-freezing in liquid nitrogen. The two pools of slices were separately homogenized either in the presence (PI) or absence (CIP) of phosphatase inhibitors with the latter supplemented with calf intestinal phosphatase to achieve maximal dephosphorylation of rabphilin. Equal amounts of total protein from the two samples were resolved by SDS-PAGE and transferred to nitrocellulose. Figure 4*B* shows the results of the Western blots. αTotal rabphilin (Fig. 4*B, bottom panel*) was used to confirm that equal amounts of rabphilin were loaded for each sample, and αS234-P (Fig. 4*B, top panel*) was used to verify that complete dephosphorylation of rabphilin S234-P had occurred in the homogenate treated with CIP. In the blot probed with αnon-P-S234 (Fig. 4*B, middle panel*), we observed a strong increase in signal when we compared the fully dephosphorylated sample (CIP) to the sample prepared under conditions to preserve the phosphorylation on rabphilin (PI). This difference equals the proportion of rabphilin that is phosphorylated after a 2 min high K^+ stimulation. In fact, this pool of rabphilin cannot be detected by αnon-P-S234 in the homogenate prepared in the presence of phosphatase inhibitors (PI), but becomes available for recognition after dephosphorylation (CIP). Quantitative analysis of this experiment showed that 75–80% of rabphilin is phosphorylated at serine-234 at the end of the high K^+ stimulation. However, we have to consider that the depolarization provoked by the incubation in 56 mM K^+ for 2 min is a long stimulation and that these conditions will strongly activate Ca^{2+}-dependent kinases and cause the fusion of a large fraction of the pool of synaptic vesicles at synapses. It seems likely that the magnitude of the increase in phosphorylation of rabphilin reflects the length of the stimulation. Therefore, under the physiological conditions generated by action potentials the level of rabphilin phosphorylation would likely reflect the rate of neuronal firing.

A prediction from this result is that the increase in phosphorylation promoted by high K^+ (detected by αS234-P), must be mirrored by a decrease in rabphilin not phosphorylated at serine-234 (detected by αnon-P-S234). To test this prediction, we compared the relative amounts of phospho- and non-phosphorabphilin in unstimulated (unst) and 2 min high K^+-stimulated slices (2′K^+). Figure 4*C* shows the Western blot results of this experiment. αTotal rabphilin (Fig. 4*C, bottom panel*) was again used to confirm equal loading of the two samples. As expected, the increase in rabphilin S234-P after stimulation (Fig. 4*C, top panel*) was matched by an equivalent decrease in rabphilin not phosphorylated at serine-234 (Fig. 4*C, middle panel*).

Considering that the high K^+ stimulation promotes a sixfold to eightfold increase in phosphorylation at this site, we can estimate that ~10–15% of rabphilin is phosphorylated under basal unstimulated conditions.

Rapid phosphorylation and dephosphorylation of rabphilin during exocytosis

We next sought to determine if both the phosphorylation and dephosphorylation of rabphilin promoted by membrane depolarization occurs rapidly, because this would imply a tight coupling of this modification to membrane trafficking events mediating exocytosis and endocytosis of synaptic vesicles. First, we investigated the kinetics of phosphorylation during a high K^+-induced depolarization. Figure 5 shows that high K^+-induced phosphorylation at both serine-234 (Fig. 5*A*) and serine-274 (Fig. 5*B*) of rabphilin occurred rapidly with respective maximal increases reached within 2 min. Continued stimulation past this initial phase did not result in any additional phosphorylation; instead the levels of rabphilin S234-P and rabphilin S274-P slowly decreased. In Figure 5, the insets show representative Western blot results of the time courses of phosphorylation, and the graphs summarize the quantitative analysis of five or six independent experiments (mean and SEM). These results demonstrate that the increase in phosphorylation occurs rapidly after stimulation, consistent with the suggestion that kinases activated during exocytosis phosphorylate rabphilin on synaptic vesicles.

We next examined the rate of dephosphorylation after terminating the high K^+-induced depolarization by returning the slices to normal Ringer's solution. Figure 6, *A* (for rabphilin S234-P) and *B* (for rabphilin S274-P), shows the results of the time course of dephosphorylation. The insets are representative Western blot results, and the graphs summarize the quantitative analysis of four independent experiments (mean and SEM). In both graphs, the first point represents the level of phosphorylation in unstimulated slices. The second point is the level of phosphorylation at the end of a 2 min high K^+ stimulation; this point also represents the time = 0 of the dephosphorylation reaction. For both serine-234 and serine-274, we observed a small additional increase in phosphorylation shortly after the end of the stimulation (t = 30 sec), followed by a rapid dephosphorylation reaction. Comparison of these data after normalization to their respective t = 0 levels (Fig. 6*C*) demonstrate that both serines share an initial phase of fast dephosphorylation over the first 2 min, after which rabphilin S234-P continued to become dephosphorylated to ~25% of the initial level after 30 min. In contrast, the phosphorylation level of rabphilin S274-P did not change significantly after the initial drop, and ~65% were still present after 30 min. The slower dephosphorylation of serine-274 suggests that a significant portion of the signal generated by this phosphorylation lasts for quite some time after the initial stimulus. The phosphorylation on serine-234 appears to be more dynamically regulated, and the faster and more robust dephosphorylation suggests that this modification may be linked to a cycle of events that accompany subsequent rounds of exocytosis. To further test the reversibility of this phosphorylation event, we subjected a pool of slices to five rounds of consecutive 2 min high K^+ stimulation followed by 10 min of recovery in normal Ringer's solution to allow for dephos-

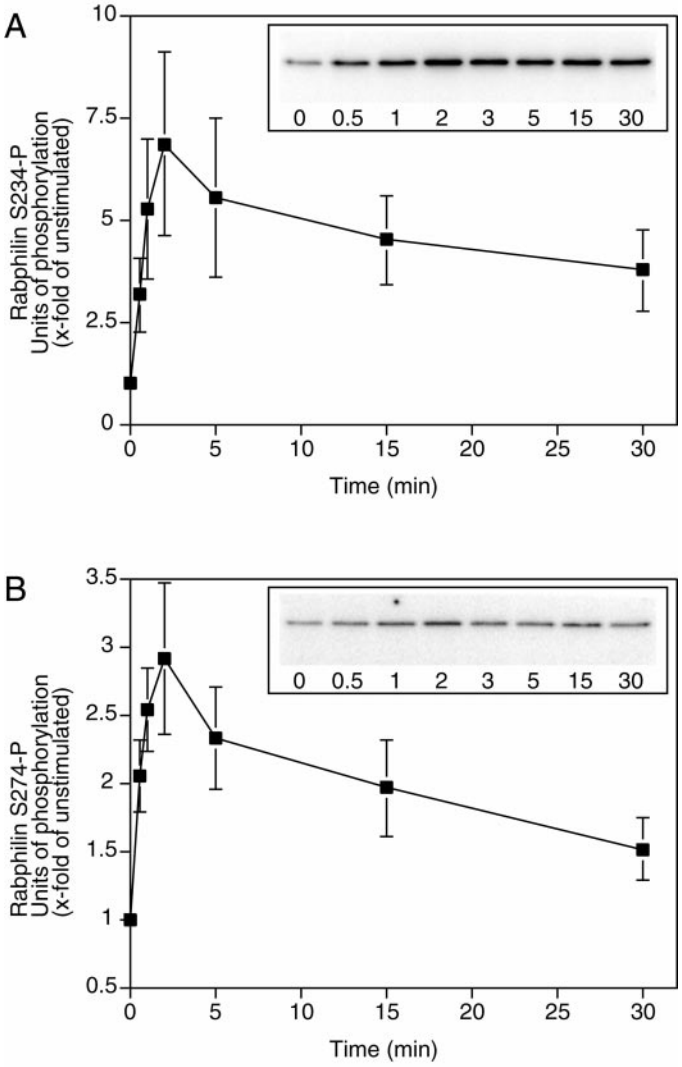

Figure 5. The high K^+ induced increase in rabphilin phosphorylation is maximal within 2 min. Acute slices prepared from 6- to 8-week-old rats were incubated in Ringer's solution containing 56 mM K^+. At the indicated time points, slices were flash-frozen in liquid nitrogen and subsequently processed for quantitative Western blotting to detect changes in rabphilin phosphorylation at S234 (*A*) and S274 (*B*). The *insets* show representative Western blot results of the time course of phosphorylation; the graphs summarize the analysis of six (S234-P) and five (S274-P) independent experiments (mean and SEM).

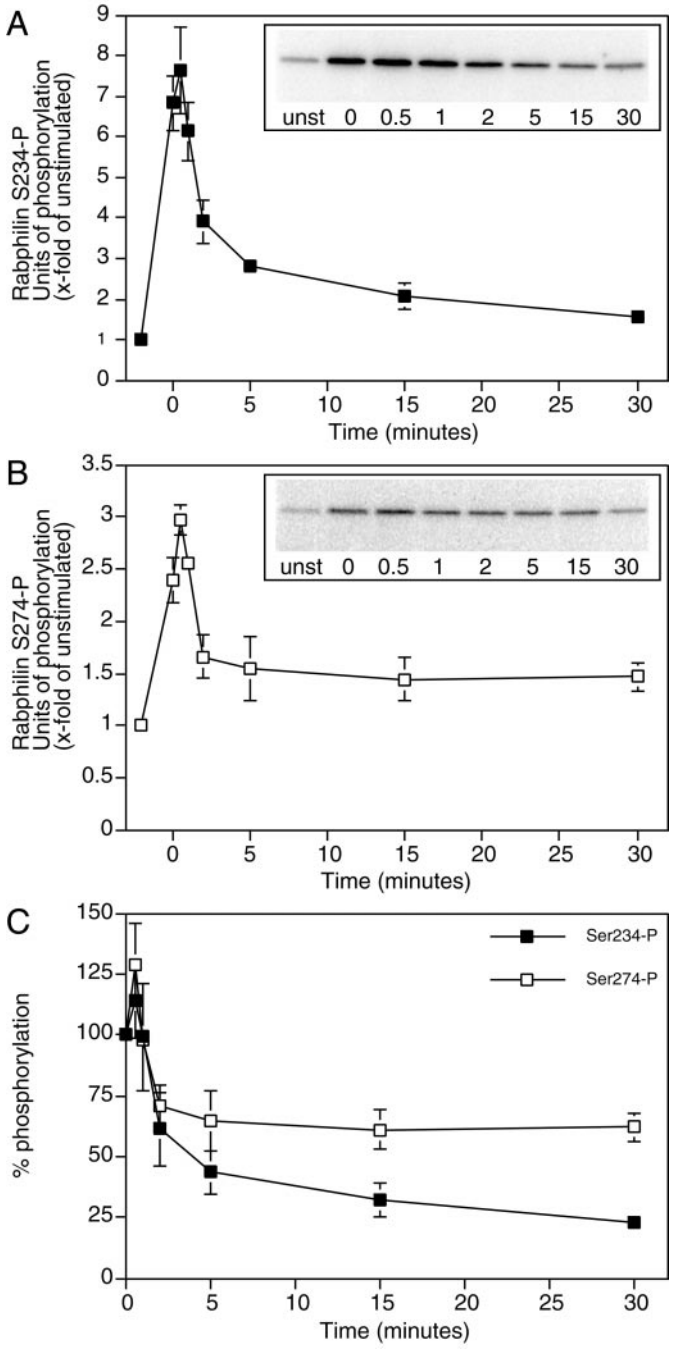

Figure 6. A rapid phosphatase activity dephosphorylates rabphilin S234-P and S274-P. Acute slices prepared from 6- to 8-week-old rats were first subjected to a 2 min incubation in Ringer's solution containing 56 mM K^+ and then rapidly transferred to normal Ringer's solution. Slices were flash-frozen in liquid nitrogen before stimulation (*unst*), at the end of the 2′ high K^+ stimulation ($t = 0$), and at the indicated time points after transfer to normal Ringer's solution. *A* (rabphilin S234-P) and *B* (rabphilin S274-P), *Insets* show representative Western blot results of the time course of dephosphorylation, and the graphs summarize the analysis of four independent experiments (mean and SEM). *C*, The graph shows the extent of dephosphorylation over time relative to the level of phosphorylation at the end of the stimulation (4 independent experiments, mean and SEM).

phorylation. Slices were collected at the end of each round of stimulation and recovery. The Western blot analysis confirmed that rabphilin could be reversibly phosphorylated at serine-234 with each incubation in high K^+ (data not shown). This indicates a rapid turnover of the phosphorylation on serine-234, consistent with the idea that this phosphorylation event generates a signal important in successive rounds of exocytosis.

Inhibition of phosphatase activity increases both the basal and high K^+-stimulated levels of phosphorabphilin

So far we have provided evidence for a dynamic regulation of the phosphorylation state of rabphilin, a cycle that appears to be linked to the events underlying exocytosis. Robust and rapid changes in the phosphorylation of rabphilin must be coupled to the activation of kinases (Figs. 1, 5) and phosphatases (Fig. 6). We therefore investigated whether the inhibition of phosphatase activity had an effect on both the basal and stimulated levels of phosphorylation of rabphilin. Rat brain slices were preincubated for 30 min in normal Ringer's solution with or without 1 μM OA,

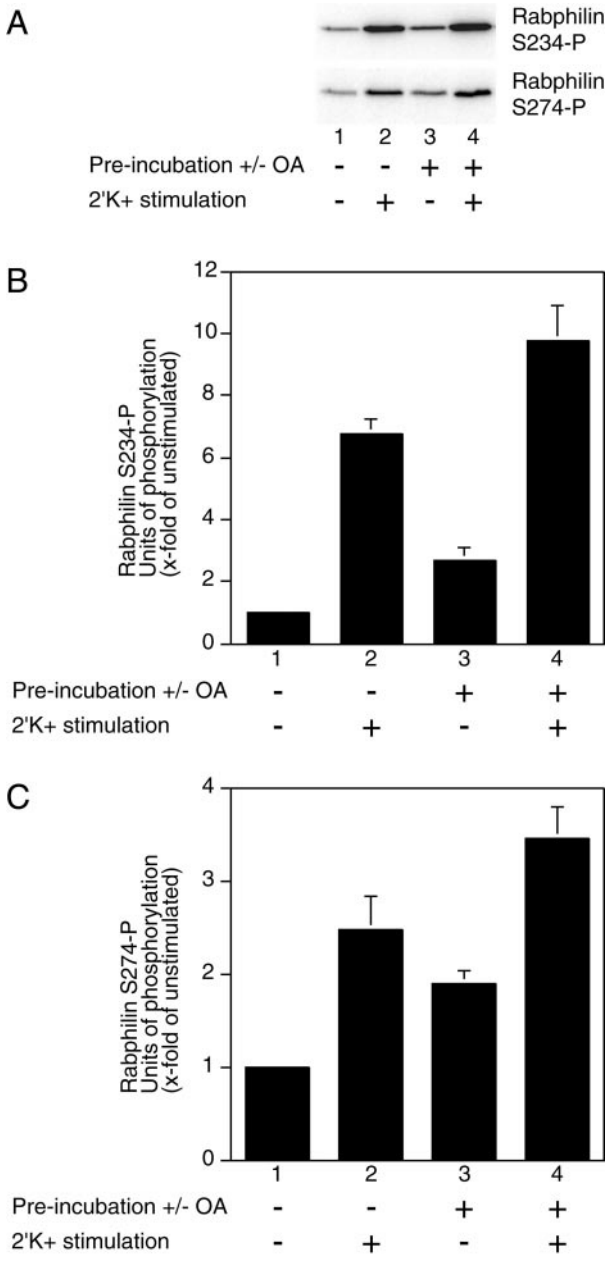

Figure 7. Inhibition of phosphatase activity increases both the basal and the high K^+ stimulated level of rabphilin phosphorylation at S234 and S274. Acute slices prepared from 6- to 8-week-old rats were preincubated for 30 min in normal Ringer's solution with or without the addition of 1 μM OA. Subsequently, slices were either flash-frozen in liquid nitrogen or first stimulated for 2 min in Ringer's solution with 56 mM K^+ and then flash-frozen. Finally, slices were processed for quantitative Western blotting to detect changes in rabphilin phosphorylation. *A*, Representative Western blot results. *B*, *C*, Quantitative analysis of rabphilin phosphorylation on S234 (*B*) and S274 (*C*) of four to eight independent experiments (mean and SEM).

a concentration sufficient to inhibit both protein phosphatase 1 and protein phosphatase 2A. Control slices and slices treated with OA were flash-frozen after incubation in normal or high K^+ Ringer's solution for 2 min and then processed for quantitative Western blot. Figure 7*A* shows a representative Western blot result, and Figure 7, *B* (rabphilin S234-P) and *C* (rabphilin S274-P), summarizes the quantitative analysis of four to eight independent experiments (mean and SEM). Inhibition of phosphatase activity resulted in increased phosphorylation levels on rabphilin serine-234 and serine-274 both under basal unstimulated conditions and after a 2 min high K^+-induced depolarization. The basal level of rabphilin S234-P in the presence of okadaic acid was ~2.7-fold higher than in the absence of the inhibitor (Fig. 7*A, top panel, B*, compare *lanes 1* and *3*; $p < 0.01$; *t* test), and the phosphorylation level after a 2 min high K^+ stimulation increased from ~6.7-fold over unstimulated to ~9.9-fold in the presence of the phosphatase inhibitor (Fig. 7*A, top panel, B*, compare *lanes 2* and *4*) ($p < 0.05$; *t* test). Similarly, for rabphilin S274-P the basal level of phosphorylation went up ~1.8-fold (Fig. 7*A, bottom panel, C*, compare *lanes 1* and *3*) ($p < 0.02$; *t* test), whereas the 2 min high K^+-stimulated level increased from ~2.3-fold to ~3.4-fold (Fig. 7*A, bottom panel, C*, compare *lanes 2* and *4*) ($p < 0.03$; *t* test). These results, together with the kinetic data of Figure 6, implicate a phosphatase in the regulation of the phosphorylation state of rabphilin. The data also suggest that the basal level of rabphilin phosphorylation is regulatable and controlled by the dynamic activity of kinases and phosphatases.

Phosphorabphilin has reduced affinity for membranes

Rabphilin, which does not possess a transmembrane domain, has nevertheless been shown to be mainly membrane-bound through its association with synaptic vesicles. This is mediated, at least in part, through its interaction with the small GTPases Rab3a/c. Therefore, we investigated whether the phosphorylation state of rabphilin alters its membrane association. Rat brain slices were stimulated for 2 min in high K^+ and then flash-frozen. Homogenization of the slices was performed in the presence of phosphatase inhibitors, and the postnuclear supernatant (PNS) was prepared by low-speed centrifugation (1000 × *g*). The PNS was further spun at high speed (100,000 × *g*) to separate the cytosol (C) from the membrane (M) fraction. Membranes were resuspended and extracted for 1 hr at 4°C either with high salt (1 M NaCl) or with 1% Triton X-100 (TX-100). At the end of the incubation, the samples were centrifuged again at 100,000 × *g* to separate the extracted [supernatant (S)] from the nonextracted [pellet (P)] fractions. Figure 8*A* shows a representative set of Western blots probed with αS234-P, αS274-P, αtotal rabphilin, and αVAMP-2. VAMP-2, an integral membrane protein of synaptic vesicles, was detected to confirm the quality of the crude subcellular fractionation and extraction protocols. As expected, VAMP-2 was not found in the cytosolic fraction, indicating that no contamination of synaptic vesicles was present in the supernatant after the high-speed centrifugation of the PNS. Also, consistent with the fact that integral membrane proteins are not extracted from membranes by high salt concentrations, VAMP-2 was not observed in the supernatant of the 1 M NaCl extraction. Finally, the almost complete extraction of VAMP-2 from membranes incubated with 1% Triton X-100 confirmed the efficient solubilization of membranes by this method. Interestingly, both forms of phosphorabphilin appeared to behave differently than total rabphilin in these assays. In the cytosol versus membrane distribution, phosphorabphilin appeared to be more tightly associated with membranes than total rabphilin, as shown in Figure 8, *A* and *B* (quantitative analysis of four to six independent experiments; mean and SEM; $p < 0.05$; *t* test). By contrast, the high salt extraction was able to dissociate almost the entire pool of phosphorabphilin while ~40% of total rabphilin was still on membranes after the extraction, as shown in Figure 8, *A* and *C* (quantitative analysis of four to six independent experiments; mean and SEM; $p < 0.01$; *t* test). To confirm that the form of

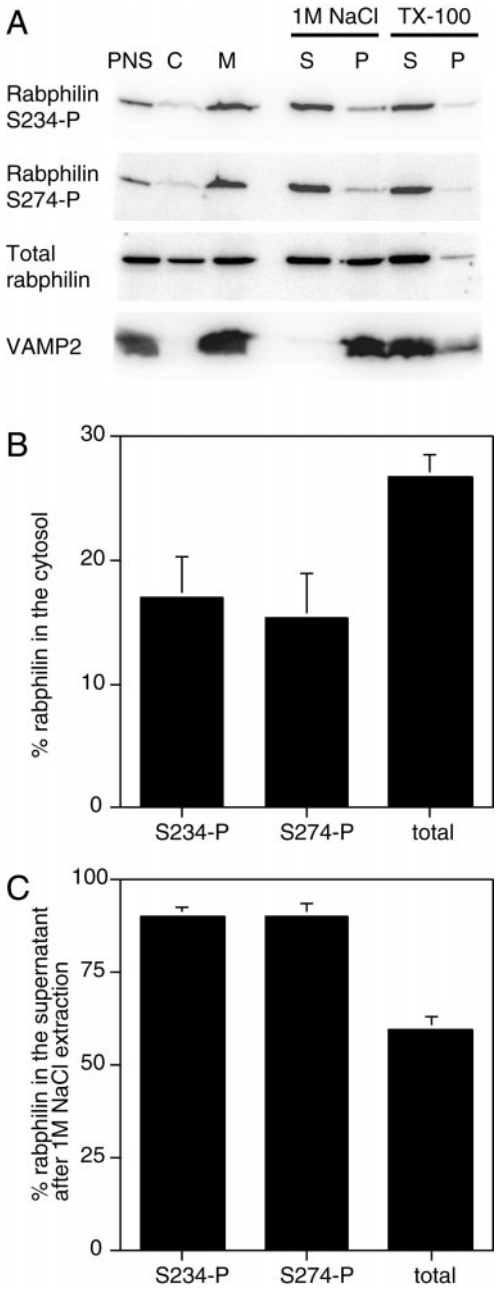

Figure 8. Phosphorabphilin has reduced affinity for membranes. Acute slices were prepared from 6- to 8-week-old rats and stimulated for 2 min in Ringer's solution with 56 mM K^+. The slices were homogenized, and the homogenate was spun at low speed (1000 × *g*) to make postnuclear supernatant (*PNS*). The PNS was further centrifuged at high speed (100,000 × *g*) to separate the cytosol (*C*) and membrane (*M*) fractions. Equal amounts of total protein for PNS, cytosol, and membranes were used for Western blotting. The membrane fraction was resuspended and extracted with either 1 M NaCl or 1% Triton X-100 (*TX-100*). After a high-speed spin to separate the supernatant (*S*, extracted proteins) from the pellet (*P*, not extracted proteins), equal volumes of the two samples were subjected to Western blotting. *A*, Representative Western blot results with samples probed with the two antibodies specific for the S234 and S274 phosphorylated forms of rabphilin, the antibody to detect total rabphilin, and an antibody against the integral synaptic vesicle protein VAMP-2 to control for the fractionation and membrane extraction protocols. *B*, Quantitative analysis of cytosol and membrane distributions of phosphorabphilin compared with total rabphilin (4–6 independent experiments, mean and SEM). *C*, Quantitative analysis of the relative amounts of phosphorabphilin and total rabphilin released into the supernatants after membrane extraction with 1 M NaCl (4–6 independent experiments, mean and SEM).

rabphilin still membrane bound after 1 M NaCl incubation is mostly the nonphosphorylated form, we repeated the extraction experiment and probed the Western blots with the antibody αnon-P-S234 that recognizes only rabphilin not phosphorylated at S234. As expected, and complementing the results shown in Figure 8 for rabphilin S234-P, >85% of rabphilin non-P-S234 was still associated with membranes after salt extraction (data not shown). Taken together, these results indicate that phosphorylation of rabphilin alters its ability to interact with membranes in a way that makes the binding more sensitive to the ionic strength of the medium, suggesting a decreased affinity of rabphilin for a membrane binding site on synaptic vesicles.

DISCUSSION

A large body of work has implicated the activity of various kinases and phosphatases in the regulation of synaptic transmission by controlling the phosphorylation state of several synaptic proteins (for review, see Turner et al., 1999). To further understand the physiological significance of these modifications and to gain insights into their role in modulating synaptic strength and plasticity, we have generated a set of antibodies that specifically recognize synaptic proteins only in their phosphorylated form. In this study we report the biochemical characterization of the modulation of phosphorylation of rabphilin, a synaptic protein implicated in exocytosis, using phosphospecific antibodies directed against the two major phosphorylation sites of rabphilin, serine-234 and serine-274.

Our results show that the phosphorylation of rabphilin at serine-234 is greatly stimulated (about sevenfold over basal) by activation of PKA and high K^+-induced membrane depolarization, a condition that mimics the events underlying synaptic vesicle exocytosis. Activation of PKC elicits a more modest but still significant (~2.5-fold) increase in phosphorylation. The activity-dependent modification is strictly dependent on external Ca^{2+}, occurs rapidly, and is followed by an equally rapid and efficient dephosphorylation step mediated by phosphatases. The depolarization-promoted increase in phosphorylation at serine-234 is also critically dependent on the presence of Rab3a, because the effect is nearly abolished in acute slices prepared from Rab3a knock-out animals. An estimate of the relative proportion of rabphilin S234-P revealed that ~10–15% of rabphilin is phosphorylated at this site under basal conditions and that this value can reach 75–80% after the extensive stimulation of exocytosis by the 2 min incubation in high K^+ Ringer's solution. Taken together, these results indicate that rabphilin undergoes a strong, dynamic, and activity-dependent phosphorylation at serine-234. We do not observe an increase in phosphorabphilin immunostaining in stimulated brain slices. Because phosphorabphilin is not detected by preimmune sera, and the immunoreactivity is blocked by phospho- but not nonphosphopeptides and is absent in Rab3A knock-out mice, our staining for phosphorylated rabphilin is clearly specific. Therefore, we propose that the sites that become phosphorylated after stimulation are blocked from recognition by the antibodies, perhaps because of interactions with other proteins.

The dependence on Rab3a and our detection of phosphorabphilin at synapses (see accompanying paper) suggest that phosphorylation of rabphilin occurs on synaptic vesicles and is initiated by the influx of Ca^{2+} triggered either by high K^+ or action potential depolarization. The sequence around serine-234 (RRA*S*E) matches the PKA phosphorylation site consensus motif (RRx*S*/*T*x) (Fykse et al., 1995), suggesting that this phosphor-

ylation event might be a direct or indirect consequence of PKA activation. Our findings are consistent with previous work on hippocampal synaptosomes, in which rabphilin phosphorylation was shown to be selectively increased in CA3 but not in CA1 synaptosomes after stimulation of PKA or high K^+-induced membrane depolarization (Lonart and Südhof, 1998). Interestingly, a study performed with cerebellar granule cells in culture also found an increase in phosphorylation after high K^+-induced depolarization, but PKC and not PKA was implicated in the process (Fykse, 1998). In both studies, no information was available for the individual contribution of the two phosphorylation sites, and the maximal increase in phosphorylation observed was only 2.5–3.5-fold.

The phosphorylation at serine-274 of rabphilin is stimulated approximately threefold by activation of PKA, PKC, and by high K^+-induced membrane depolarization. As for the phosphorylation on serine-234, the activity-dependent increase in serine-274 phosphorylation is strictly dependent on external Ca^{2+} and on the presence of Rab3a. The kinetics of phosphorylation are fast and comparable to those for serine-234. Although phosphatases clearly regulate the phosphorylation state of serine-274, the dephosphorylation kinetics show that a first phase of rapid dephosphorylation is followed by a plateau with ~65% of the initial rabphilin S274-P still present for at least 30 min. The dephosphorylation kinetics of rabphilin S274-P are therefore significantly different from those of rabphilin S234-P, for which dephosphorylation proceeds steadily, resulting in only ~25% of it remaining after 30 min. CaMKII has been suggested by *in vitro* experiments to be the kinase that phosphorylates serine-274 *in vivo,* and the region around serine-274 (RAN*S*V) matches the consensus sequence for CaMKII phosphorylation (Rxx*S*/*T*x) (Fykse et al., 1995). Our *in vivo* data suggest that in addition to CaMKII, which could be activated during the Ca^{2+}-influx promoted by high K^+ depolarization, PKA and PKC can phosphorylate this site equally well. Although the two modifications share common modes of regulation, they do show distinct features concerning the kinases involved, the extent of the increase in phosphorylation, and the kinetics of dephosphorylation after stimulus removal. We suggest that the phosphorylation of rabphilin on serine-234 and serine-274 may be regulated by the convergence of various kinases in a synapse-specific and activity-dependent manner. We speculate that the phosphorylation on both sites is complementary and synergistic in generating a modulatory influence on the function of the protein.

The domain structure of rabphilin consists of an N-terminal region containing the Rab3a binding site (Yamaguchi et al., 1993; Li et al., 1994; Ostermeier and Brunger, 1999) and a C-terminal region characterized by the presence of two C2 domains that have been shown to bind phospholipids in a Ca^{2+}-dependent manner (Yamaguchi et al., 1993; Oishi et al., 1996; Chung et al., 1998; Ubach et al., 1999). The two phosphorylation sites are located in the middle region of the molecule, and phosphorylation of rabphilin could either generate a new binding site for an as yet unidentified binding partner, or exert an effect based on conformational changes on the other two domains of the protein.

Whereas there is clear evidence supporting a critical role for Rab3a in assuring the stability of rabphilin and its recruitment to synaptic vesicles, several experiments strongly suggest that rabphilin may have independent activities and not mediate the effects of Rab3a in exocytosis. In Rab3a knock-out mice, the levels of rabphilin are reduced to 40–50% (Geppert et al., 1994), and the protein is unable to localize correctly at synapses, accumulating instead in the cell body of neurons (Li et al., 1994). Furthermore, cross-linking and coimmunoprecipitation experiments have confirmed that rabphilin and Rab3a are present in a complex on synaptic vesicles, and *in vitro* incubation of synaptic vesicles with GDP and Rab GDI removes both Rab3a and rabphilin from membranes (Stahl et al., 1996). Finally, it has been shown that exocytosis is accompanied by GTP hydrolysis of vesicle-associated Rab3a (Stahl et al., 1994) and coordinated dissociation of both Rab3a and rabphilin from synaptic vesicles (Fischer von Mollard et al., 1991; Stahl et al., 1996). In agreement with these findings, we show that the activity-dependent phosphorylation of rabphilin is nearly abolished in the absence of Rab3a, despite the fact that 40–50% of the wild-type level of rabphilin is still present in Rab3a knock-out animals. The most straightforward interpretation of these results is that Rab3a is required to recruit rabphilin to synaptic vesicles where it can be phosphorylated during Ca^{2+} influx stimulated by membrane depolarization.

However, there is evidence suggesting that rabphilin can interact with membranes in a manner independent of Rab3a, possibly through its two C2 domains. Shirataki et al. (1994) have shown that exogenous rabphilin can bind to synaptic vesicles from which Rab3a had been removed by the action of Rab GDI. In chromaffin and pheochromocytoma 12 (PC12) cells, in which overexpression of rabphilin enhances regulated secretion (Chung et al., 1995; Komuro et al., 1996), deletion of the two C2 domains reduced membrane association of rabphilin, whereas disruption of the Zn^{2+}-finger in the Rab3a-binding domain had no effect (McKiernan et al., 1996). Moreover, a rabphilin deletion mutant, containing both C2 domains but defective in binding to Rab3a, not only localized to chromaffin granules, but also stimulated secretion as effectively as the wild-type protein (Chung et al., 1997). Similarly, single point mutants of rabphilin that do not bind to Rab3 fully maintain the stimulatory effect on exocytosis when overexpressed in PC12 and insulin-secreting cells (Joberty et al., 1999). Finally, overexpression of a transmembrane-anchored form of rabphilin that lacked the Rab3 binding region stimulated secretion in pancreatic β cells even more effectively than the wild-type protein (Arribas et al., 1997).

Our membrane extraction experiments indicate that phosphorabphilin has a lower affinity for membranes. *In vitro* studies have shown that both PKA- and CaMKII-catalyzed phosphorylation of rabphilin do not affect its interaction with Rab3a (Kato et al., 1994; Numata et al., 1994). These findings have not been confirmed *in vivo,* and it is therefore possible that phosphorabphilin interacts less strongly with Rab3a on synaptic vesicles. Alternatively, the phosphorylation could influence the interaction of rabphilin with membranes through its C-terminal domain. Although the function of rabphilin remains controversial, the modulation of its membrane association by phosphorylation seems strategically placed to have a role in the synaptic vesicle life cycle, perhaps in the events that regulate the mobilization, docking, fusion, or recycling of vesicles. It is unlikely that the docked and primed vesicles that are rapidly fused in response to the action potential are affected by rabphilin phosphorylation. The kinetics of the phosphorylation suggest a transient event that may, for example, reorganize vesicles after a release event. The kinetics of phosphorylation and dephosphorylation also suggest that rabphilin may be important in short-term plasticity events that could be critical in formation of memories.

REFERENCES

Alger BE, Dhanjal SS, Dingledine R, Garthwaite J, Henderson G, King GL, Lipton P, North A, Schwartzkroin PA, Sears TA, Segal M, Whittingham TS, Williams J (1984) In: Brain slices (Dingledine R, ed), pp 381–437. Plenum: New York.

Arribas M, Regazzi R, Garcia E, Wollheim CB, De Camilli P (1997) The stimulatory effect of rabphilin 3a on regulated exocytosis from insulin-secreting cells does not require an association-dissociation cycle with membranes mediated by Rab 3. Eur J Cell Biol 74:209–216.

Bock JB, Matern HT, Peden AA, Scheller RH (2001) A genomic perspective on membrane compartment organization. Nature 409:839–841.

Burns ME, Sasaki T, Takai Y, Augustine GJ (1998) Rabphilin-3A: a multifunctional regulator of synaptic vesicle traffic. J Gen Physiol 111:243–255.

Chung SH, Takai Y, Holz RW (1995) Evidence that the Rab3a-binding protein, rabphilin3a, enhances regulated secretion. Studies in adrenal chromaffin cells. J Biol Chem 270:16714–16718.

Chung SH, Stabila P, Macara IG, Holz RW (1997) Importance of the Rab3a-GTP binding domain for the intracellular stability and function of Rabphilin3a in secretion. J Neurochem 69:164–173.

Chung SH, Song WJ, Kim K, Bednarski JJ, Chen J, Prestwich GD, Holz RW (1998) The C2 domains of Rabphilin3A specifically bind phosphatidylinositol 4,5-bisphosphate containing vesicles in a Ca^{2+}-dependent manner. In vitro characteristics and possible significance. J Biol Chem 273:10240–10248.

Fischer von Mollard G, Südhof TC, Jahn R (1991) A small GTP-binding protein dissociates from synaptic vesicles during exocytosis. Nature 349:79–81.

Fykse EM (1998) Depolarization of cerebellar granule cells increases phosphorylation of rabphilin-3A. J Neurochem 71:1661–1669.

Fykse EM, Li C, Südhof TC (1995) Phosphorylation of rabphilin-3A by Ca^{2+}/calmodulin- and cAMP-dependent protein kinases *in vitro*. J Neurosci 15:2385–2395.

Geppert M, Bolshakov VY, Siegelbaum SA, Takei K, De Camilli P, Hammer RE, Südhof TC (1994) The role of Rab3a in neurotransmitter release. Nature 369:493–497.

Greengard P, Valtorta F, Czernik AJ, Benfanti F (1993) Synaptic vesicle phosphoproteins and regulation of synaptic function. Science 259:780–785.

Hosaka M, Hammer RE, Südhof TC (1999) A phospho-switch controls the dynamic association of synapsins with synaptic vesicles. Neuron 24:377–387.

Jahn R, Südhof TC (1999) Membrane fusion and exocytosis. Annu Rev Biochem 68:863–911.

Joberty G, Stabila PF, Coppola T, Macara IG, Regazzi R (1999) High affinity Rab3 binding is dispensable for Rabphilin-dependent potentiation of stimulated secretion. J Cell Sci 112:3579–3587.

Kato M, Sasaki T, Imazumi K, Takahashi K, Araki K, Shirataki H, Matsuura Y, Ishida A, Fujisawa H, Takai Y (1994) Phosphorylation of Rabphilin-3A by calmodulin-dependent protein kinase II. Biochem Biophys Res Commun 205:1776–1784.

Kato M, Sasaki T, Ohya T, Nakanishi H, Nishioka H, Imamura M, Takai Y (1996) Physical and functional interaction of rabphilin-3A with alpha-actinin. J Biol Chem 271:31775–31778.

Komuro R, Sasaki T, Orita S, Maeda M, Takai Y (1996) Involvement of rabphilin-3A in Ca^{2+}-dependent exocytosis from PC12 cells. Biochem Biophys Res Commun 219:435–440.

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.

Lin RC, Scheller RH (2000) Mechanisms of synaptic vesicle exocytosis. Annu Rev Cell Dev Biol 16:19–49.

Lonart G, Südhof TC (1998) Region-specific phosphorylation of rabphilin in mossy fiber nerve terminals of the hippocampus. J Neurosci 18:634–640.

Madison D (1991) Whole-cell voltage-clamp techniques applied to the study of synaptic function in hippocampal slices. In: Cellular neurobiology: a practical approach (Wheal H, Chad J, eds), pp 137–149. Oxford: Oxford UP.

Masumoto N, Sasaki T, Tahara M, Mammoto A, Ikebuchi Y, Tasaka K, Tokunaga M, Takai Y, Miyake A (1996) Involvement of Rabphilin-3A in cortical granule exocytosis in mouse eggs. J Cell Biol 135:1741–1747.

McQuinston AR, Madison DV (1999) Nicotinic receptor activation excites distinct subtypes of interneurons in the rat hippocampus. J Neurosci 19:2887–2896.

Miyazaki M, Shirataki H, Kohno H, Kaibuchi K, Tsugita A, Takai Y (1994) Identification as beta-adducin of a protein interacting with rabphilin-3A in the presence of Ca^{2+} and phosphatidylserine. Biochem Biophys Res Commun 205:460–466.

McKiernan CJ, Stabila PF, Macara IG (1996) Role of the Rab3A-binding domain in targeting of rabphilin-3A to vesicle membranes of PC12 cells. Mol Cell Biol 16:4985–4995.

Numata S, Shirataki H, Hagi S, Yamamoto T, Takai Y (1994) Phosphorylation of Rabphilin-3A, a putative target protein for Rab3A, by cyclic AMP-dependent protein kinase. Biochem Biophys Res Commun 203:1927–1934.

Ohya T, Sasaki T, Kato M, Takai Y (1998) Involvement of Rabphilin3 in endocytosis through interaction with Rabaptin5. J Biol Chem 273:613–617.

Oishi H, Sasaki T, Takai Y (1996) Interaction of both the C2A and C2B domains of rabphilin3 with Ca^{2+} and phospholipid. Biochem Biophys Res Commun 229:498–503.

Ostermeier C, Brunger AT (1999) Structural basis of Rab effector specificity: crystal structure of the small G protein Rab3A complexed with the effector domain of rabphilin-3A. Cell 1999 96:363–374.

Schluter OM, Schnell E, Verhage M, Tzonopoulos T, Nicoll RA, Janz R, Malenka RC, Geppert M, Südhof TC (1999) Rabphilin knock-out mice reveal that rabphilin is not required for rab3 function in regulating neurotransmitter release. J Neurosci 19:5834–5846.

Shirataki H, Yamamoto T, Hagi S, Miura H, Oishi H, Jin-no Y, Senbonmatsu T, Takai Y (1994) Rabphilin-3A is associated with synaptic vesicles through a vesicle protein in a manner independent of Rab3A. J Biol Chem 269:32717–32720.

Stahl B, Fischer von Mollard G, Walch-Solimena C, Jahn R (1994) GTP cleavage by the small GTP-binding protein Rab3A is associated with exocytosis of synaptic vesicles induced by alpha-latrotoxin. J Biol Chem 269:24770–24776.

Stahl B, Chou JH, Li C, Südhof TC, Jahn R (1996) Rab3 reversibly recruits rabphilin to synaptic vesicles by a mechanism analogous to raf recruitment by ras. EMBO J 15:1799–1809.

Turner KM, Burgoyne RD, Morgan A (1999) Protein phosphorylation and the regulation of synaptic membrane traffic. Trends Neurosci 22:459–464.

Ubach J, Garcia J, Nittler MP, Südhof TC, Rizo J (1999) Structure of the Janus-faced C2B domain of rabphilin. Nat Cell Biol 1:106–112.

Wu RL, Barish ME (1992) Two pharmacologically and kinetically distinct transient potassium currents in cultured embryonic mouse hippocampal neurons. J Neurosci 12:2235–2246.

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.