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Phosphorylation of Proteins in Neuron Terminals: Specificity Depends on Coincidental Signaling

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Abstract We investigate the role of neuronal coincidental signaling mediated by the second messengers, on phosphorylation of three major proteins of neurosecretory vesicles. Our data show that different combinations of coincidental signaling generate specific pattern of phosphoproteins and not strictly additional effects. This suggests that an added phosphate on a site might 'mask' or 'unmask' the next sites for specific kinases and phosphatases action by inducing conformation change or protein association. We show that a function of vesicles such as the uptake of glutamate is highly regulated by coincidental signaling. J. Cell. Biochem. 88: 589–598, 2003. © 2003 Wiley-Liss, Inc.

Key words: coincidental signaling; second messengers; kinases; phosphatases; neurons

The release of transmitters from synaptic vesicles in the synaptic cleft, mediated by fusion of vesicles with pre-synaptic membrane, has been extensively reported [Katz, 1969; Benfenati et al., 1992; Jahn and Südhof, 1993; Hessler et al., 1993; Borst and Sackmann, 1996; Burgoyne and Morgan, 1998; Bennett, 1999; Fernandez-Chacon et al., 2001]. Briefly, the catalog of major proteins in vesicles which are the basic components of the transmitter release machinery have been the topic of many articles [Schwartz, 1991; Südhof and Jahn, 1991; Gotow et al., 1996; Hosaka and Sudhof, 1998; Fernandez-Chacon and Sûdhof, 1999; Hosaka et al., 1999]. The major phosphoproteins (mainly the synapsin group) are phosphorylated by Cam kinase II and at some extent by protein kinase, cAMP dependent (PKA) [Pang et al., 1988; Südhof et al., 1989; Rubenstein et al., 1993; Ceccaldi et al., 1995; Stefani et al., 1997; Hosaka et al., 1999; Janz et al., 1999]. Moreover, sites of phosphorylation by protein kinase C (PKC) are present on the calcium binding protein of annexin family [Regnouf et al., 1995; Rothhut, 1997]. Our goal was to investigate whether the coincidental inputs from different synapses converging on a particular axonal terminal, can influence the specificity of phosphorylation of neurosecretory vesicles in this compartment. We verified therefore whether coincidental inputs acting through seconds messengers produce specific phosphorylation patterns, instead of the addition of the individual effects triggered by each input.

Synaptic connections on axonal terminal, called axo-axonic synapses, are known to depress or enhance the transmitter release by presynaptic inhibition or facilitation [Smith and Augustine, 1988; De Camilli and Jahn, 1990]. This suggests that axo-axonic activity selectively controls the behavior of individual axonal terminals. In other words, neurotransmitters released in synaptic cleft connected to a particular axonal terminal can modulate the transmitter release from this terminal mainly by decreasing or increasing the local concentration of calcium. A local pre-synaptic zone is therefore influenced by the nearby synapses and their combinational inputs, which suggest that local axonal terminals sum the biochemical effects generated by these inputs. This constitutes the

Abbreviations used: PKA, protein kinase, cAMP dependent; PKC, protein kinase C, Cam K II, calcium calmodulin kinase II; Gs, G protein (stimulatory); βγ/*beta gamma* subunits of G protein.

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basis of neuronal network for the synaptic facilitation, which has been extensively studied in invertebrates. These studies also provided models for associative learning and memory, especially in *Aplysia* for learned reflex [Kandel et al., 1983; Sweatt and Kandel, 1989; Thomson, 2000]. In this marine mollusc, a weak touch to siphon is a conditioned stimulus which produces a soft defensive withdrawal response. The unconditioned stimulus like a shock to the tail provokes strong defensive response. By repeating the pairing of the two stimuli, the response to touching siphon is enhanced. The neuronal network which organizes sensory and motor neurons in order to allow the learned facilitation and/or habituation, is anatomically based on axo-axonic synapses. Our initial idea was to evaluate how the different sequential order of phosphorylation by major kinases which 'read' peaks of their respective activating second messenger, triggered by synaptic inputs, could generate specific 'landscape' of phosphoproteins in axonal terminals. If sequential order didn't matter, the final pattern of phosphoproteins should be the same whatever the order we used, which we didn't obtain in our preliminary work. We hypothesize therefore that one phosphorylated site might 'mask' or 'unmask' the next site on the same protein or on the associated proteins, for the same or another kinase, due to induced change in protein conformation, following the previous added phosphate. The aim of this report is to verify whether some sites on crude neuro-secretory vesicles could be phosphorylated or de-phosphorylated depending on coincidental context. We investigate consequently how the different combinations of kinase action affect the uptake of glutamate.

MATERIALS AND METHODS

PKA and PKC were purchased from Sigma (P 2645, P 7956). Rat Cam kinase (mainly II) was purified by affinity chromatography using calmodulin-agarose (Sigma P 4385) according to the procedure described elsewhere [Fong et al., 1989]. Proteins markers for molecular weights labeled with C-14 were purchased from NEN life products (NEC 811). ATP (3000 Ci/mmol, *gamma* phosphate) and C-14 glutamic acid (NEC-290^{E}, 273.3 Ci/mmol) were purchased from NEN life products. PMA (phorbol ester) was obtained from Sigma (P 8139). Syntide 2 (Sigma S 2525) is the peptide substrate for Cam kinase II. Anti-synaptophysin (Tebu, sc-9116), anti-synapsin type I (Sigma, S-193), anti-synapsin IIa (Tebu, sc-8293) and IIIa (Tebu, sc-8292) were used in immunoprecipitation experiments and/or immunoisolation of vesicles.

Preparation of Rat Crude Synaptic Vesicles and Purified Immunoisolated Vesicles

The method used is fully described elsewhere [Kish and Ueda, 1989]. Briefly, brains were dissected from Sprague–Dawley rats (150 g) and were homogenized in buffer: 0.32 mM sucrose, 1 mM $MgCl_2$, 0.5 mM $CaCl_2$, pH 7.2 with a glass Potter Duall by 11 strokes at 900 g. The extract was centrifuged at 4°C for 15 min at 18,900*g* max. The supernatant was removed and the pellet was gently re-suspended in 20 volumes buffer Tris-glycine (6 mM, pH 8.1) and kept at 4°C for 2 h. The solution was then centrifuged in a Beckman apparatus at 19,000 rpm (43,700*g* max) for 15 min to pellet mitochondrial and synaptosomal membrane. The supernatant was collected and distributed in capped polycarbonate tubes and centrifuged at 45,000 rpm (100,000*g* max) for 55 min (Beckman 70 Ti rotor). The pellet was resuspended in buffer pH 7.2, 0.32 M sucrose, 1 mM $NaHCO_3$, 1 mM dl-dithiothreitol. The synaptic vesicles were stored at −80°C. In parallel experiment, the crude isolated vesicles were incubated with anti-synaptophysin (5 μg of antibody for 250 μl of vesicles solution (1 mg/ml) and with anti-synapsin I (same conditions than above) for 4 h at 4°C. The complex antibody/vesicle was then isolated with 50 μl of protein A agarose. The comparative profile of the major proteins in acrylamide gel electrophoresis was identical with the two preparations. The experiments of phosphorylation with the different kinases as indicated in the figures, didn't give any substantial difference when crude vesicles or immunoisolated vesicles were used. We therefore used mostly crude vesicles in our experiments.

Phosphorylation of Vesicles and Analysis by Acrylamide Electrophoresis

One hundred microliters of vesicles (1 mg/ml) in phosphate buffer 20 mM, pH 7.2, 1 mM dithiothreitol, PMSF (1 μg/ml) was phosphorylated by PKA, Cam kinase II, and PKC with *gamma* 32-P-ATP (2,000,000 cpm) and cold ATP (1–100 μM). PKA (catalytic subunit) was used at 25 U. Ca^{2+} was added to 0.5 mM final and

phorbol ester (PMA) at 1 μg in 100 μl. PKC was used at 0.5 U. The timing and conditions of phosphorylation are indicated for each experience. Isotopic ATP (2,000,000 cpm) for each sample was used to complement the final concentration of 1 or 100 μM of cold ATP depending on the indicated experiment. The phosphorylation was stopped by the addition of 10 μl of trichloroacetic acid (10%) and 10 μl of serum albumin protein (0.1% final) was added as carrier. Samples were kept on ice for 20 min, and then centrifuged for 5 min at 13,000 rpm. The supernatant was removed, the pellet resuspended in loading buffer of electrophoresis and the acidity neutralized by Tris-base buffer. Samples were heated at 90°C and the gels were run, dried, and analyzed by autoradiography. Radioactivity was measured using a *beta* counter Beckman.

Immunoprecipitation of Phosphorylated Proteins

Immunoprecipitation was performed with two antibodies: anti-synaptophysin (Tebu, sc-9116) and anti-synapsin type I (Sigma, S-193). Anti-synapsin IIa and IIIa (Tebu, sc-8293 and sc-8292, respectively) were used to identify the vesicular species labeled with 32-P. Crude vesicles were phosphorylated as indicated above with P-32 ATP and cold ATP at 100 μM for 1 min, then they were solubilized in Triton 100 (1%), diluted five times in phosphate buffer, and finally incubated with the antibodies. The precipitation was performed with agarose-protein A (50 μl) and after brief centrifugation the pellet was washed once and then re-centrifuged. The material isolated was run in electrophoresis gel and the acrylamide band corresponding to the molecular weight of interest was cut with a razor, placed in vial with scintillation liquid, and counted in a *beta* counter Beckman. For the competition experiments using different concentration of peptide Syntide 2, substrate for Cam kinase II, the bands of interest were directly cut with a razor and counted.

Glutamate Uptake by Vesicles

The method is described elsewhere [Kish and Ueda, 1989; Wolosker et al., 1996]. Briefly, the uptake was carried out at 30°C in Tris buffer (20 mM pH 7.4, 1 mM $MgCl_2$, 0.5 mM glutamate, 4 mM KCl, 0.1 mM ATP) and 2,000,000 cpm glutamate (273.3 Ci/mmol) using 50 μg of vesicles. Phosphorylations were carried out with PKA, PKC, and Cam kinase II, in the same conditions than above with 100 μM of cold ATP. Ca^{++} was used at 100 μm. At the indicated time course, tubes were placed on ice and the reaction was stopped by high concentration of KCl (40 mM) followed by filtration on cellulose acetate or nitrocellulose filters (0.2 μm). Filters were counted in scintillation liquid using a *beta* counter Beckman.

RESULTS

Coincidental Phosphorylation by Cam Kinase II/PKA and Cam Kinase II/PKA/PKC

Cam kinase II is known to be physically associated with synaptic vesicles, probably due to the fact that calmodulin is a component of vesicles and furthermore Cam kinase II is a binding protein for synapsin I [Benfenati et al., 1992; Nicol et al., 1997]. We observed that crude and immuno-isolated vesicles, present associated phosphatases and PKC (data not shown). We took advantage of the sub-localized Cam kinase II to phosphorylate vesicles at low concentration of ATP (1 μM) in order to slow down the enzymatic activity and therefore to label only the high affinity sites. Moreover, we designed these experimental conditions to see only the radio-labeled species corresponding to the few abundant and major vesicular proteins. The complexity of the system didn't allow us to perform an exhaustive analysis of all the phosphorylated sites, specially the sites involving low abundance vesicular proteins. The experiment was carried out at 1, 5, and 10 min (see Fig. 1A). Only one band (P-32) was intensively labeled by the addition of calcium to vesicles and this stable phosphorylation, independent of the conditions used, was used as internal control of our experiments. Surprisingly, the coincidental effect of Cam kinase/PKA labels intensively P-38 and less drastically a doublet at 70 kDa. We see clearly that the intensity of the labeling of the doublet at 70 kDa is increased by the combined effect of Ca^{++} (endogenous Cam kinase II) and PKA although Ca^{++} by itself induces weak labeling and PKA has no efficiency in these conditions (data not shown). The combination Cam kinase II and PKA phosphorylates intensively P-38 at 1 min and the phosphorylated band disappears progressively to completely vanish at 10 min. In this protocol, P-38 is poorly phosphorylated by Cam kinase II alone and is also poor substrate for

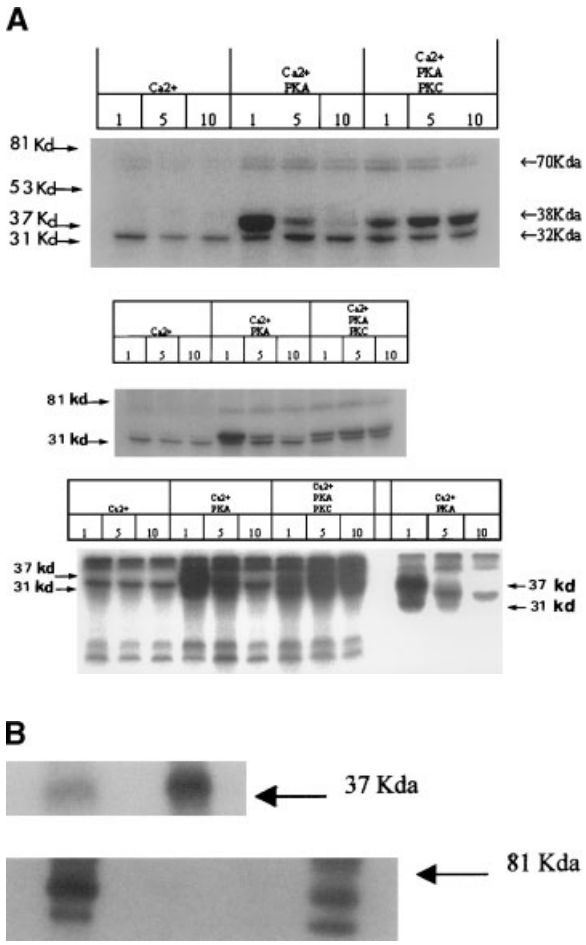

Fig. 1. Time course of vesicles phosphorylation by Cam kinase II, PKA/Cam kinase II, and PKA/Cam kinase II/PKC. The phosphorylation was performed at 1, 5, and 10 min with 32-P ATP and cold ATP (final concentration 1 μM). Cam kinase II is vesicle-associated enzyme activated by calcium. PKC is added enzyme in samples *plus* the vesicle associated form. The catalytic subunit of PKA was added in samples (see Materials and Methods). **A**: *Above*: The material was analyzed by electrophoresis gel 7.5% acrylamide. *Middle*: Gel 12% acrylamide. *Bottom*: A gel at 16% is shown and we observe a fast transient band at 31 kDa induced by the combination Ca^{++}/PKA (see at the right the Ca^{2+}/PKA box at higher magnification). A doublet at about 10 kDa and the band P-32 are non-regulated phosphorylated species in this protocol and constitute internal control. **B**: The band at 38 kDa was precipitated with an anti-synaptophysin antibody from the vesicles homogenate, previously solubilized in non-ionic detergent. The protocol of vesicle phosphorylation was identical to the experimental conditions described above. The **left lane** shows phosphorylation of vesicles with Ca^{++} and PKA after 10 min, the **right lane** shows phosphorylation with Ca^{++} and PKA at 1 min. The doublet at about 70 kDa was precipitated using anti-synapsin IIa (left lane) and synapsin IIIa (right lane). Vesicles were phosphorylated as above and then solubilized in non-ionic detergent. **Middle lanes** show immunoprecipitation control without antibodies. We observed coimmunoprecipitation of these two species due to partial heterodimerization.

PKA in more favorable conditions (see Fig. 3A). Surprisingly, the simultaneous activation PKC/Cam kinase II/PKA stabilizes the phosphorylation of P-38, although PKC, in more favorable conditions, don't induce by itself the phosphorylation of P-38 (see Fig. 3A). When PKC is activated and/or added, the previous band P-32 duplicates in 32 and 31 kDa phosphorylated species. Interestingly, when the vesicles are incubated with Ca^{++}/PKA, the 31 kDa vanishes progressively to disappear after 10 min whereas P-32 appears delayed and progressively increases to a maximum at 10 min. The molecular weight of these species suggests that they are members of the calcium binding/cytoskeleton associated proteins of the vesicles. The arguments to identify P-38 as synpatophysin are: first, this is a major phosphoprotein bound to vesicles, second, the antibody recognition in Western blot and radioactivity transfer are copresent in the same band (data not shown), and finally the immunoprecipitated material with an anti-synatophysin antibody from solubilized radiolabeled vesicles reproduces an identical pattern in electrophoresis analysis (see Fig. 1B). The doublets at 70 kDa are putative synapsin family members according to their molecular weight [Hosaka and Südhof, 1999]. Those molecules are known as major vesicular phosphoproteins and the immunoprecipitations in the same experimental conditions allowed us to identify them as synapsin IIa and IIIa (see Fig. 1B). The immunoprecipitation data show two bands which confirm that the synapsins coat the synaptic vesicles partially as heterodimers [Hosaka and Südhof, 1999].

Coincidental Kinase Action on Vesicles Influences the Resistance of a Specific Phosphorylated Site for Phosphatases

We used the same protocol of phosphorylation than in Figure 1 (1 min of incubation, with low concentration of ATP: 1 μM) with different combinations of kinases action. We observed that the band P-32 is uniformly phosphorylated after 1 min of incubation in the presence of Ca^{2+} independently of the coincidental effect of added kinases, PKA and PKC (see Fig. 1A). P-32 is not PKC substrate (see figure 2 top) or PKA substrate (data not shown), this species is resistant to synaptosomal phosphatases action when the vesicles are treated with the combination PKC *plus* Cam kinase or Cam kinase *plus* PKA *plus* PKC. We used the doublets at about

70 kDa as internal control due to the fact that their phosphorylation state is relatively constant. We noticed the absence of phosphorylated P-38. This suggests that PKC acting on other vesicular targets, modifies the affinity of a particular phospho site for phosphatases (see Fig. 2 bottom).

Affinity of Synapsin I and Synaptophysin for Cam Kinase II Depends on PKA and PKC Phosphorylation on Other Vesicle Sites

We analyzed the relative affinity of some major vesicular sites for Cam kinase II depending on the context. We quantified therefore the phosphorylation by measuring the radioactivity in the immunoisolated material using antibodies against synapsin I and synaptophysin. We used synapsin I due to the fact that this species is more abundant than synapsin II or III. For this purpose, experiments were carried out at higher concentration of ATP (100 μM) and for a brief time of incubation (1 min) in order to be as far as possible to the plateau of saturation. Results are shown in Figure 3A. The efficiency of the control of the procedure is shown in Figure 3B. We observe that in these conditions, both molecules in their molecular context in vesicles are poor substrates for PKA and PKC. As expected Cam kinase II (added exogenous kinase) phosphorylates the two molecules and the simultaneous kinase activities (Cam kinase II *plus* PKA or Cam kinase II *plus* PKA *plus* PKC) increase the incorporation of radioactivity in synapsin I and synaptophysin. Although PKC don't recognize these proteins and PKA shows poor efficiency, the PKC and PKA action on vesicles, increase the Cam kinase efficiency to phosphorylate synapsin I and synaptophysin. Altogether, these data suggest that PKA and/or PKC trigger inhibitory effect on endogenous phosphatase or induces conformation change through protein association, which might increase the affinity (decrease the km) of these sites for Cam kinase II. To confirm this hypothesis, the previous experiment was carried out with different concentrations of peptide substrate of Cam kinase II, as competitor for vesicular Cam kinase II sites. We observed that PKA and PKC induce a shift in IC50, for the incorporation of radioactivity in synapsin I and synaptophysin. This suggests an increase of affinity of these two targets for Cam kinase II, induced by PKA and/or PKC phosphorylation on other vesicular targets.

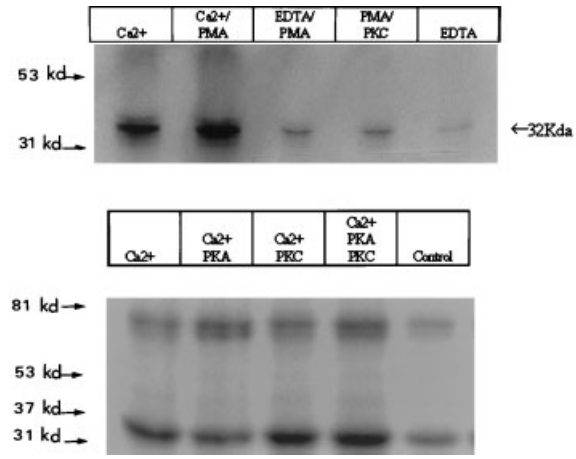

Fig. 2. Phosphatase assay with phosphorylated vesicles. Vesicles were phosphorylated with 32-P ATP for 1 min and at a low concentration of ATP (1 μM) in the conditions described in Figure 1, using the vesicle associated Ca^{2+}/calmodulin kinase II and added purified PKC or PKA (see Materials and Methods). **Top:** Control phosphorylation of P-32 induced by Cam kinase II (endogenous) and PKC. We observe that this species is not target for PKC. **Bottom:** The phosphorylated vesicles with different combinations of kinases were directly incubated with synaptosomal membrane as a source of phosphatase (50 μl, 1 mg/ml) for 5 min in THE presence of large excess of cold ATP (1 mM) to prevent any kinase activity with residual 32-P-ATP. The material was precipitated and analyzed in acrylamide gel electrophoresis (7.5%). We notice the absence of the P-38 band and the increased intensity of P-32 induced by PKC.

Relationship Between Coincidental Signaling and Uptake of Glutamate

We performed an analysis of glutamate uptake as functional test in order to test our hypothesis. We investigated therefore the uptake of glutamate in different conditions of phosphorylation. Results are shown in Figure 4. We observed that Ca^{++} diminishes the uptake although after 1 min the results were similar. This suggests that phosphorylation and/or its molecular consequences such as change in protein conformation or in protein association are responsible for this effect rather than a direct effect of calcium. PKA and PKC seem to elevate the level of glutamate uptake in a biphasic mode. The maximum increase is seen at 5 min whereas the levels of activity after 1 and 10 min are little changed compared to the control. The combination of Ca^{++}/PKA shows substantial inversion of the effect induced by calcium alone. This effect is also seen with the combination Ca^{++}/PKA/PKC.

DISCUSSION

Brain is deeply invested in detecting coincident phenomena at many levels of complexity including conditioning of reflexes, associative competence or performance, learning and memory. It should not be a surprise that highly conserved molecular machines accounting for the bewildering complexity of diverse animal behaviors from *Aplysia*, *C-elegans*, *Drosophila* to mammals and humans, are part of the pre- and post-synaptic compartments. We might summarize that every synaptic event involves a limited number of proteins that receive

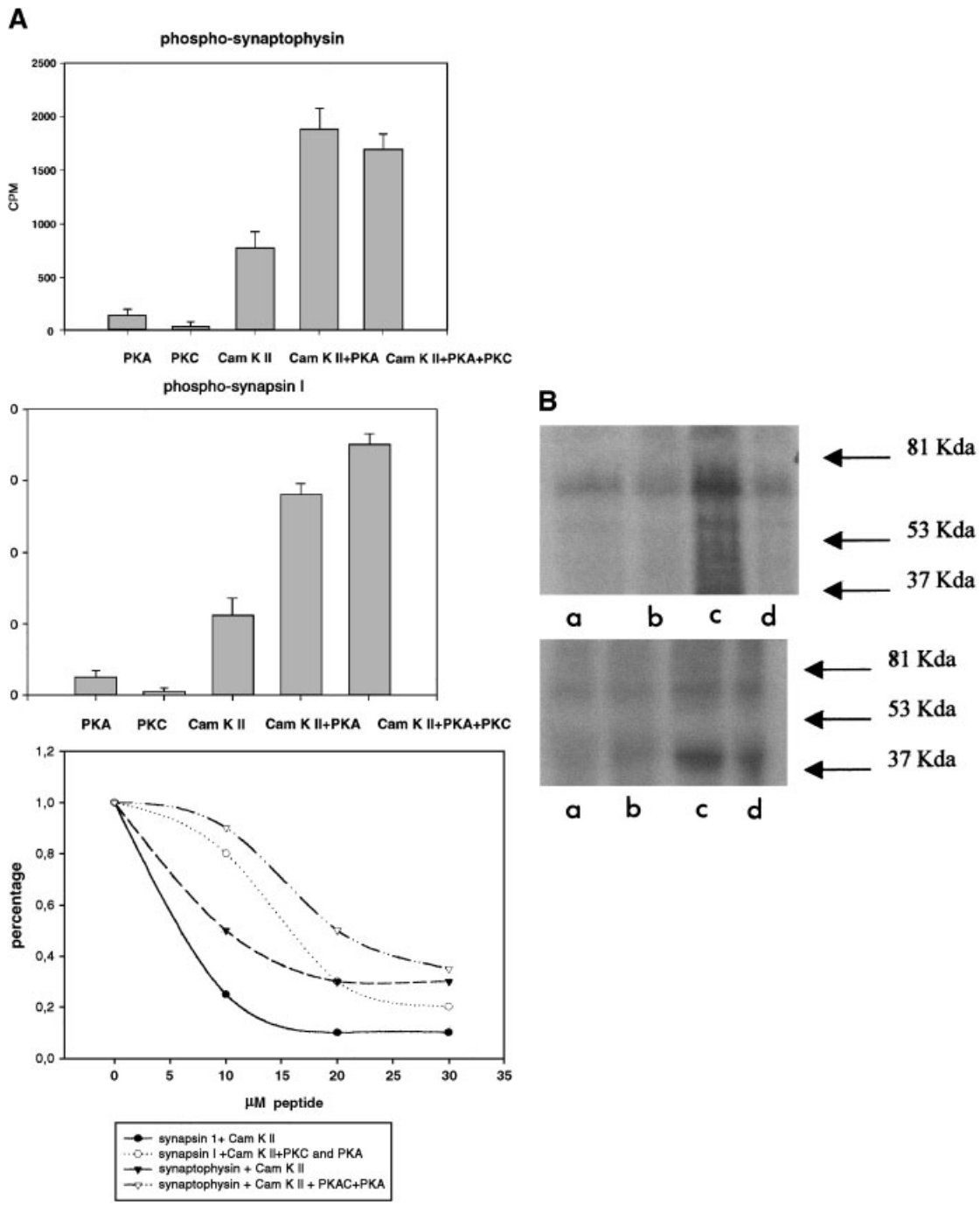

Fig. 3.

signals and relay them in order to amplify or inhibit the synaptic transmission. The highly complex neuronal network supposes that one particular neuron receives signals from many neurons simultaneously and integrate the summed informations. This also supposes that a particular neuron is possibly connected to hundreds and may have thousands of pre- or postsynaptic endings. In other words, behavioral choices of vertebrates and invertebrates are the resulting interpretation of olfactory, gustatory, and visual cues, which suggests that the association of simultaneous signals converge on and are integrated by neurons acting as detectors of coincidence. In this report we analyzed the phosphorylation of proteins which might respond specifically to convergent signaling pathways. The mechanisms of few molecular machines which detect two or more signals and will respond differently when exposed to a single signal, have been well described [see Bourne and Nicoll, 1993 for review]. One of these molecules defined as detector of coincidence was the adenylyl cyclase type I in classic conditioning of the defensive withdrawal reflex of *Aplysia* [Abrams et al., 1991]. This cyclase is under control of calcium/calmodulin and G protein (stimulatory) (Gs). This enzyme is stimulated separately by Ca^{++}/calmodulin and by GTPγS (which acts by binding on Gs). However, Ca^{++}/calmodulin increases the GTPγS effect by one third, but GTPγS amplifies the level of Ca^{++}/calmodulin activation by 17-fold [Bourne and Nicoll, 1993 for review]. The conditioned stimulus is associated with an increase of calcium. The unconditioned stimulus is associated with the release onto the same cell of 5-hydroxytryptamine which translocates the Gs protein in order to activate the cyclase type I [Kandel et al., 1983; Yovell and Abrams, 1992]. The second example is the adenylyl cyclase type II activated by *beta gamma* subunits of G protein (βγ) and Gs. The complex βγ cyclase II is inactive by itself, the tetramer Gs, βγ cyclase II is highly active, the cAMP levels produced by this complex is five fold that produced by Gs alone. Surprisingly, authors demonstrate that βγ may come from GABA binding, while Gs is activated by a neuro-transmitter like norepinephrine, bound on the same neuron [Tang and Gilman, 1991]. This coincidental biochemistry supposes that synapses from different neurons coalesce on the same micro zone of the neuron detector, which is the absolute condition to "read" the coincidence. We therefore decide to investigate in vitro the role of neurosecretory vesicles as a complex system which might be the sensors and targets of converging coincidental signals from different neuronal inputs. We though that converging inputs on an axonal terminal, each of them activating a specific pathway, might lead to different phosphorylated states of vesicles depending on the combination of the seconds messengers involved in the coincidence.

Briefly, the two major classes of phosphoproteins in neurosecretory vesicles are the synapsin family and the synaptophysin. They constitute high affinity substrate for Cam kinase II [Südhof et al., 1989; Benfenati et al.,

Fig. 3. Relative quantification of phosphate in immunoisolated synapsin 1 and synaptophysin. **A**: Vesicles were phosphorylated as described in Materials and Methods at 100 μM ATP with 32-P ATP for 1 min. All the kinases used in the experiment are purified enzymes (see Materials and Methods). The material was then solubilized and submitted to the immunoisolation procedure, then to electrophoresis gel to dissociate putative associated proteins. The acrylamide bands corresponding to the respective molecular weight were cut and counted. Each column is the average of three experiments and the bars represent standard errors. **Bottom:** Vesicles were incubated with the kinases (Cam kinase II or Cam kinase II +PKA +PKC) and 100 μM ATP (+32P ATP) with different concentrations of Cam kinase peptide substrate (syntide 2) for 1 min in a competition experiment (see Materials and Methods). The bands corresponding to synapsin I and synaptophysin were directly cut from acrylamide gel and counted. Each point is the average of three determinations and represents a percentage of the 32-P incorporated in bands without peptide competitor. **B**: Controls of the procedure. The conditions of phosphorylation were the same than above except one aliquot (5 μl) of the immunoprecipitated material was analyzed in electrophoresis gel. **Top:** The precipitation of proteins using anti-synapsin I was assayed on the solubilized vesicular homogenate with protein A-agarose. Vesicles were previously treated as follows: (**lane a**) vesicles phosphorylated with Ca^{++}, (**lane b)** the soluble homogenate of vesicles, previously phosphorylated with Ca^{++} and PKA, was depleted with the antibody/protein A-agarose, then the supernatant was assayed for synapsin I precipitation; (**lane c)** vesicles phosphorylated with Ca^{++} and PKA, (**lane d**) control of precipitation without antibody with vesicles treated with Ca^{++} and PKA. Bottom: Procedure of protein precipitation using anti-synaptophysin. Vesicles were treated as follows and then proteins were precipitated from the homogenate using anti-synaptophysin. Lane a: Control without antibody. Lane b: The soluble homogenate from vesicles, treated with Ca^{++} and PKA, was depleted with the antibody/protein A-agarose and then the supernatant was submitted to a second run of precipitation. Lane c: Vesicles were treated with Ca^{++} and PKA. Lane d: Same protocol than in **c,** except vesicles were treated with Ca^{++}, PKA, and PKC.

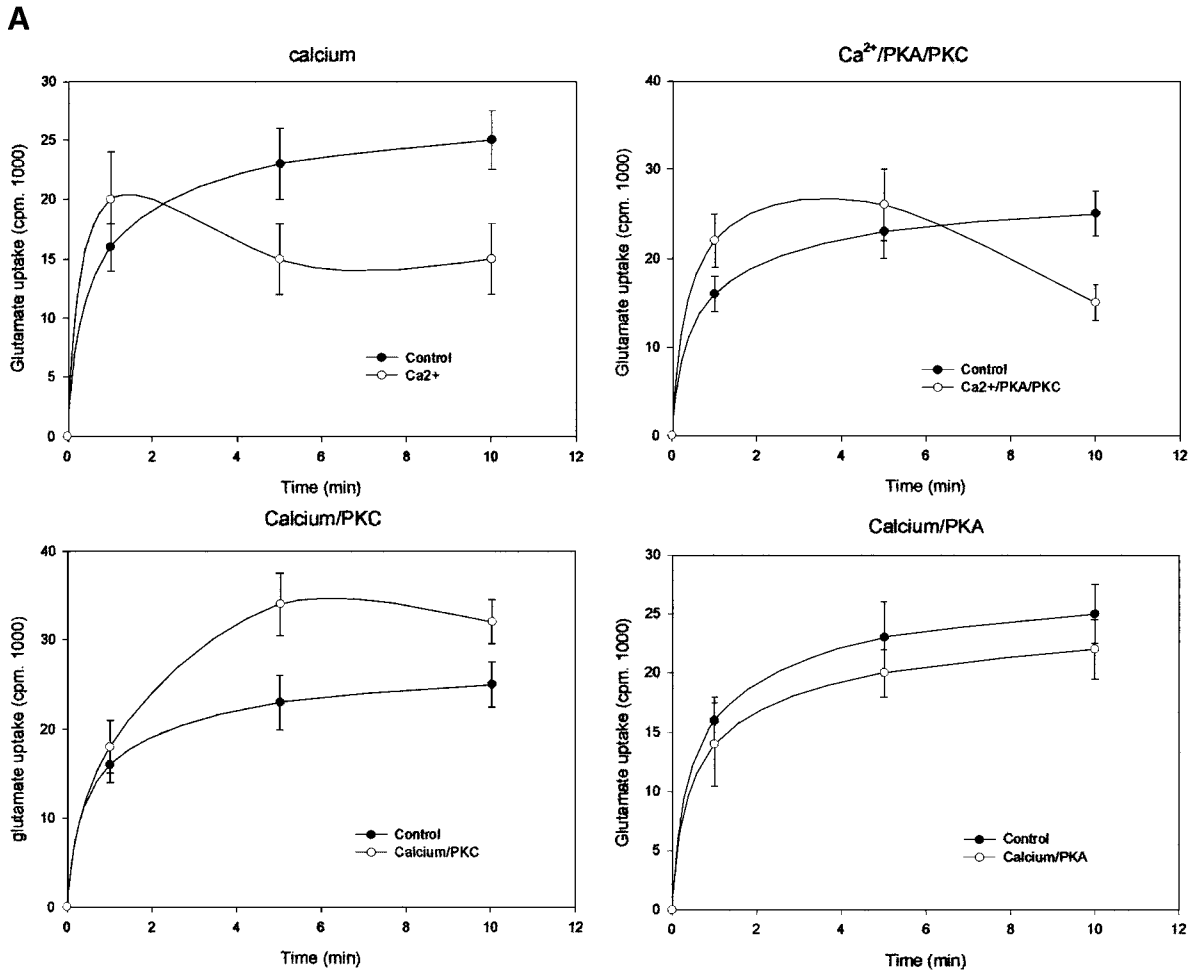

Fig. 4. Glutamate accumulation into synaptic vesicles: effect of coincidental phosphorylation. The uptake of glutamate was measured at the indicated timing (see Materials and Methods). The vesicles were submitted to different combination of kinases in the presence of ATP (100 μM) plus Ca^{++} at 100 μM as indicated in figures and simultaneously the uptake of glutamate was measured. A control test is carried out using synaptic vesicles alone without calcium. The data are expressed as the mean ± SE ($n = 5$). The comparative data are summarized in the diagram (below) and are expressed as a percentage of the control for each time (1, 5, and 10 min of incubation). The statistical analysis was performed by the unpaired Student's t test compared with the control. For Ca^{++}: at 5 and 10 min, $P < 0.05$. For Ca^{++}/PKC at 5 min, $P < 0.01$and at 10 min $P < 0.05$. For Ca^{++}/PKA/PKC at 10 min, $P < 0.01$. For PKA, at 5 min, $P < 0.01$.

1992; Nicol et al., 1997; Hosaka and Sudhof, 1998]. However, the annexin group of proteins binds calcium and some are known to be substrate for PKC [Regnouf et al., 1995; Rothhut, 1997]. We show in this report that some phosphorylation sites are sensors of coincidental signaling. P-38, identified as synptophysin, is intensively phosphorylated after exposure of both calcium and PKA. Strong P-38 labeling was not seen when Cam kinase II (endogenous or added) was used alone. The phosphate group disappears quickly suggesting that synpatophysin is also high affinity substrate for some vesicle associated phosphatase. Surprisingly, PMA activated PKC, eliminates the transient strong labeling of P-38 induced by calcium/PKA, but prolongs the phosphorylated state of the protein. This suggests that discrete PKC phosphorylation might rearrange protein interactions making some phosphosites inaccessible for phosphatase(s) or alternatively that the putative endogenous phosphatase(s) might be inhibited by PKC phosphorylation. Interestingly, although less visible than the well-known role of Cam kinase II, authors reported the role of PKC in early steps of the vesicle fusion [Hilfiker et al., 1999; Yawo, 1999]. The immunoisolation of synapsin I or synaptophysin after phosphorylation of vesicles on brief time scale, confirmed the specific effect induced by coincidentally

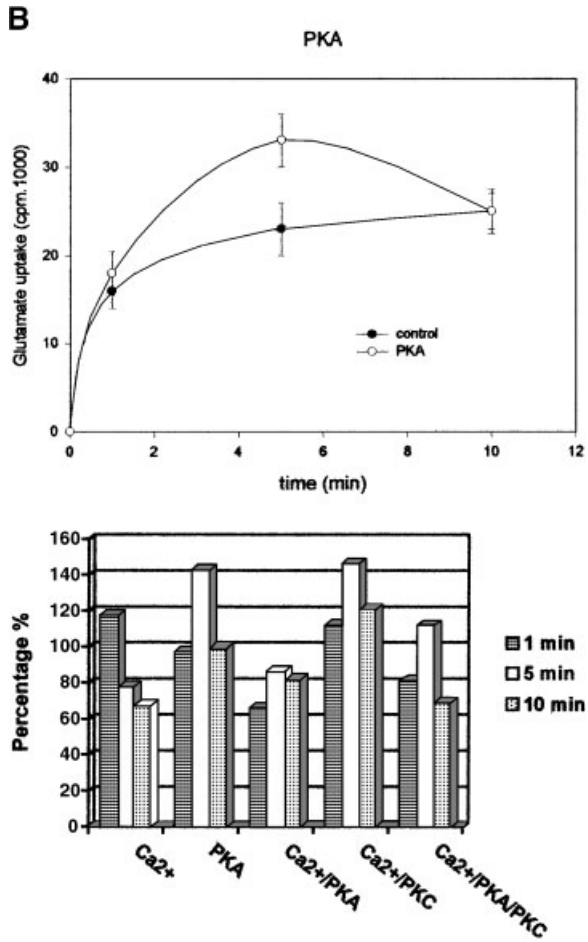

Fig. 4. (*Continued*)

activated kinases, observed in the experiment carried out with low concentration of ATP. Furthermore, the use of different concentration of peptide Syntide 2 in competition experiment with vesicle targets shows that PKA and PKC action on other vesicular targets increases the affinity of synapsin I and synaptophysin for Cam kinase II. This emphasizes the hypothesis that multiple phosphorylations on a complex system like neurosecretory vesicles in neuronal presynaptic space, induce protein association or conformation changes, allowing more efficient phosphorylation of a specific site for a specific kinase. Regarding the uptake of glutamate, we see clearly that calcium diminishes the activity. This effect is not immediate, which suggests that the calcium role is mediated by kinase activities. The other aspect resides in the fact that biphasic curves of uptake of glutamate, obtained with vesicles phosphorylated by PKA or by the combination Cam kinase II/PKC and Cam kinase II/PKC/PKA are observed. The mechanisms of glutamate uptake are well documented and show complexity due to different modes of transport [Maycox et al., 1988; Liu et al., 1994, 1996; Xu et al., 1996; Bellochio et al., 2000; Waites et al., 2001]. Our data underline the role of coincidental signaling to regulate in highly sophisticated way, some major functions of synaptic communication.

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REFERENCES

Abrams TW, Karl KA, Kandel ER. 1991. Biochemical studies of stimulus convergence during classical conditioning in *Aplysia*: Dual regulation of adenylate cyclase by Ca^{++}/calmodulin and transmitter. J Neurosci 11: 2655–2665.

Bellocchio EE, Reimer RJ, Fremeau RT, Edwards RH. 2000. Uptake of glutamate into synaptic vesicles by an inorganic phosphate transporter. Science 289:847–849.

Benfenati F, Valtorta F, Rubenstein JL, Gorelick FS, Greengard P, Czernik AJ. 1992. Synaptic vesicle associated Ca^{++}/calmodulin dependent protein kinase II is a binding protein for synapsin I. Nature 359:417–420.

Bennett MR. 1999. The concept of a calcium sensor in transmitter release. Prog Neurobiol 59:243–277.

Borst JG, Sackmann B. 1996. Calcium influx and transmitter release in a fast CNS synapse. Nature 383:431–434.

Bourne HR, Nicoll R. 1993. Molecular machines integrate coincident synaptic signals. Cell/neuron 10(Suppl):65–75.

Burgoyne RD, Morgan A. 1998. Calcium sensors in regulated exocytosis. Cell calcium 24:367–376.

Ceccaldi PE, Grohovaz F, Benfenati F, Chieregatti E, Greengard P, Valtorta F. 1995. Dephosphorylated synapsin I anchors synaptic vesicles to actin cytoskeleton: An analysis by videomicroscopy. J Cell Biol 128:905–912.

De Camilli P, Jahn R. 1990. Pathways to regulated exocytosis in neurons. Annu Rev Physiol 52:624–645.

Fernandez-Chacon R, Sûdhof TC. 1999. Genetics of synaptic vesicle function: Toward the complete functional anatomy of an organelle. Annu Rev Physiol 61:753–776.

Fernandez-Chacon R, Königstorfer A, Gerber SH, Garcia J, Matos MF, Stevens CF, Brose N, Rizo J, Rosenmund C, Südhof TC. 2001. Synatotagmin I functions as a calcium regulator of release probability. Nature 410:41–49.

Fong YL, Taylor WL, Means AR, Soderling TR. 1989. Studies of the regulatory mechanism of Ca^{2+}/calmodulin dependent protein kinse II. J Biol Chem 264:16759–16763.

Gotow T, Sakata M, Funakoshi T, Uchiyama Y. 1996. Preferential localization of annexin V to the axon terminal. Neuroscience 75:507–521.

Hessler NA, Shirke AM, Malinow R. 1993. The probability of transmitter release at a mammalian central synapse. Nature 366:569–572.

Hilfiker S, Augustine GJ. 1999. Regulation of synaptic vesicle fusion by protein kinase C. J Physiol 515(pt 1): 169–180.

Hosaka M, Sudhof TC. 1998. Synapsin III, a novel synapsin with an unusual regulation by Ca^{++}. J Biol Chem 273:13371–13374.

Hosaka M, Südhof TC. 1999. Homo and heterodimerization of synapsins. J Biol Chem 274:16747–16753.

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

Jahn R, Südhof TC. 1993. Synaptic vesicle traffic: Rush hour at the nerve terminal. J Neurochem 61:12–21.

Janz R, Sudhof TC, Hammer RE, Unni V, Siegelbaum SA, Bolshakov VY. 1999. Essential roles in synaptic plasticity for synaptogyrin I and synaptophysin I. Neuron 24:687–700.

Kandel ER, Abrams T, Bernier L, Carew TJ, Hawkins RD, Schwartz JH. 1983. Classical conditioning and sensitization share aspects of the same molecular cascade in *Aplysia*. Cold-Spring Harbor Symp Quant Biol 48:821–830.

Katz B. 1969. The release of neural transmitter substances. Liverpool University Press, liverpool.

Kish PE, Ueda T. 1989. Glutamate accumulation into synaptic vesicles. Methods Enzymol 104:9–25.

Liu Y, Schweitzer ES, Nirenberg MJ, Pickel VM, Evans CJ, Edwards RH. 1994. Preferential localization of a vesicular monoamine transporter to dense core vesicles in PC 12 cells. J Cell Biol 127:1419–1433.

Liu Y, Waites C, Krantz D, Tan P, Edwards RH. 1996. Molecular analysis of neurotransmitter transport into secretory vesicles. Cold Spring Harb Symp Quant Biol 61:747–758.

Maycox PR, Declewerth T, Hell JW, Jahn R. 1988. Glutamate uptake by brain synaptic vesicles: Energy dependence of transport and functional reconstitution in proteoliposomes. J Biol Chem 263:15423–15428.

Nicol S, Rahman D, Baines AJ. 1997. Ca^{++} dependent interaction with calmodulin is conserved in the synapsin family: Identification of a high affinity site. Biochemistry 36:11487–11495.

Onofri SG, Valtorta F, Vaccaro P, Greengard P, Benfenati F. 1997. Kinetic analysis of the phosphorylation dependent interactions of synapsin I with rat brain vesicles. J Physiol 504:501–515.

Pang DT, Wang JK, Valorta F, Benfenati F, Greengard P. 1988. Protein tyrosine phosphorylation in synaptic vesicles. Proc Natl Acad Sci USA 85:762–766.

Regnouf F, Sagot I, Delouche B, Devilliers G, Cartaud J, Henry JP. 1995. In vitro phosphorylation of annexin 2 heterotetramer by kinase C. Comparative properties of the unphosphorylated/phosphorylated annexin 2 on the aggregation and fusion of chromaffin granule membranes. J Biol Chem 270:27143–27150.

Rothhut B. 1997. Participation of annexins in protein phosphorylation. Cell Mol Life Sci 53:522–526.

Rubenstein JL, Greengard P, Czernik AJ. 1993. Calcium dependent serine phosphorylation of synaptophysin. Synapse 13:161–172.

Sabatini BL, Regehr WG. 1999. Timing of synaptic transmission. Annu Rev Physiol 61:521–542.

Schwartz JH. 1991. Principles of neural science. In: kandel E, Schwartz JH, Jessel TM, editors. Synaptic vesicles. Prentice-Hall International, Inc. 225–243.

Smith SJ, Augustine GJ. 1988. Calcium ions, active zones and synaptic transmitter release. Trends Neurosci 11:458–464.

Stefani G, Onofri F, Valtorta P, Vaccaro P, Greengard P, Benfenati F. 1997. Kinetic analysis of the phosphorylation dependent interactions of synapsin I with rat brain synaptic vesicles. J Physiol 504:501–515.

Sweatt JD, Kandel ER. 1989. Persistent and transcriptionally dependent increase in protein phosphorylation in long term facilitation of *Aplysia* sensory neurons. Nature 339:51–54.

Südhof TC, Jahn R. 1991. Proteins of synaptic vesicles involved in exocytosis and membrane recycling. Neuron 6:665–677.

Südhof TC, Czernik AJ, Kao HT, 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.

Tang WJ, Gilman AG. 1991. Type-specific regulation of adenyl cyclase by G protein $\beta\gamma$ subunits. Science 254: 1500–1503.

Thomson AM. 2000. Facilitation, augmentation and potentiation at central synapses. Trends Neurosci 23:305–312.

Waites CL, Mehta A, Tan PK, Thomas G, Edwards RH, Krantz DE. 2001. An acidic motif retains vesicular monoamine transporter 2 on large dense core vesicles. J Cell Biol 152:1159–1168.

Wolosker H, de Souza D, de Meis L. 1996. Regulation of glutamate transport into synaptic vesicles by chloride and proton gradient. J Biol Chem 271:11726–11731.

Xu CJ, Klunk WE, kanfer JN, Xiong Q, Miller G, Pettegrew JW. 1996. Phosphocreatine dependent glutamate uptake by synaptic vesicles. A comparison with ATP dependent glutamate. J Biol Chem 271:13435–13440.

Yawo H. 1999. Protein kinase C potentiates transmitter release from the chick ciliary presynaptic terminal by increasing the exocytotic fusion probability. J Physiol 515:169–180.

Yovell Y, Abrams TW. 1992. Temporal asymmetry in activation of *Aplysia* adenyl cyclase by calcium and transmitter may explain temporal requirements of conditioning. Proc Natl Acad Sci USA 89:6526–6530.