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Computer modeling of synapsin I binding to synaptic vesicles and F-actin: Implications for regulation of neurotransmitter release

(protein phosphorylation/cytoskeleton/exocytosis/nerve terminal)

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ABSTRACT **Synapsin I is a neuron-specific phosphoprotein that binds to small synaptic vesicles and actin filaments in a phosphorylation-dependent fashion. It has been hypothesized that dephosphorylated synapsin I inhibits neurotransmitter release either by forming a cage around synaptic vesicles (cage model) or by anchoring them to the F-actin cytoskeleton of the nerve terminal (crosslinking model). Computer modeling was performed with the aim of testing the impact of phosphorylation on the molecular interactions of synapsin I within the nerve terminal. The results of the simulation experiments demonstrate that in the crosslinking model the phosphorylation of synapsin I causes a severalfold increase in the number of vesicles released from the cytoskeleton and that in the cage model the phosphorylation induces a 2-fold increase in the number of vesicles bearing one or more unsaturated synapsin I binding sites. These data are compatible with the view that the function of synapsin I in the short-term regulation of neurotransmitter release is to induce a phosphorylation-dependent transition of synaptic vesicles from a "reserve pool" to a readily "releasable pool" of vesicles.**

Evoked neurotransmitter release occurs in multimolecular packets (quanta) in response to Ca^{2+} influx into the nerve terminal (1, 2). The most widely accepted hypothesis to explain the quantal nature of neurotransmitter release holds that each quantum is confined within one synaptic vesicle and is released by exocytosis when the vesicle membrane fuses with the axolemma (3).

Several manipulations, such as repetitive stimulation of the nerve, the application of neurotoxins, or changes in the ionic composition of the medium, are known to modulate the number of quanta that are released in response to a stimulus. One possible explanation for the modulation of neurotransmitter release upon activity is that this modulation is brought about by variations in the intraterminal levels of second messengers. Second messengers are thought to modulate neurotransmitter release by activating specific enzymes, the protein kinases, which in turn phosphorylate specific protein substrates. Among the phosphoproteins present in nerve terminals, a key role in the process of neurotransmitter release is probably played by those which are located on synaptic vesicle membranes (4).

Synapsin I is a neuron-specific phosphoprotein that associates with the cytoplasmic side of the synaptic vesicle membrane. In *in vitro* systems, synapsin I also interacts with actin filaments (5). Phosphorylation of synapsin I by Ca^{2+}/calmodulin-dependent protein kinase II (CaM kinase II) regulates its interaction both with synaptic vesicles and with F-actin, inducing a 5-fold decrease in the affinity for synaptic vesicles (6) and a 50% reduction in the amount of synapsin I maximally bound to F-actin (7). The observed correlation between phosphorylation of synapsin I and stimulation of neurotransmitter release in various mammalian preparations (4), as well as the inhibition of exocytosis observed after the microinjection of dephosphosynapsin I (but not of phosphosynapsin I) into the squid giant axon (8), suggests that synapsin I plays a regulatory role in the process of neurotransmitter release. The experimental data indicate that the dephosphorylated, but not the phosphorylated, form of synapsin I inhibits the fusion process.

One hypothesis to account for these results (cage model) holds that dephosphosynapsin I forms a cage around synaptic vesicles, preventing them from fusion with the plasma membrane, and that the phosphorylation-induced dissociation of one (or more) synapsin I molecule(s) from the vesicle membrane unmasks fusion site(s). Alternatively, synapsin I might regulate the release of neurotransmitter by reversibly crosslinking synaptic vesicles to the actin filaments within the nerve terminal (crosslinking model). According to this model, the phosphorylation-induced dissociation of synapsin I from F-actin and/or the vesicle membrane causes the disassembly of the ternary complex and the release of the vesicle from the cytoskeleton. With either model, the inhibitory constraint is removed and the vesicle can become part of the pool of synaptic vesicles available for fusion.

To evaluate the impact of synapsin I phosphorylation on the regulation of neurotransmitter release, the above models for the proposed function of synapsin I in the nerve terminal have been formalized according to the mass action law. So far it has not been possible to determine experimentally either the total concentrations of binding sites for synapsin I on synaptic vesicles and F-actin within the nerve terminal or the binding constants of the reactions leading to the formation of the putative ternary complex among synapsin I, synaptic vesicles, and F-actin. The following general assumptions were made when modeling the experimental data. (*i*) All reactions reach equilibrium and are fully reversible within the nerve terminal. (*ii*) No significant degradation of synapsin I occurs upon increase of the intraterminal concentration of Ca^{2+}. (*iii*) The total synapsin I concentration (S_T) within the nerve terminal is 12.5 μM; this value was obtained experimentally by measuring the total synapsin I present in purified synaptosomal preparations and assuming a total protein/wet weight ratio of 1:10. (*iv*) The total concentration of binding sites for synapsin I on synaptic vesicles (V_T) within the nerve terminal is equimolar with respect to S_T, as suggested by the experimental observations that in synaptosomes the concentration of free synapsin I is only a small percentage of S_T (9)

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Abbreviations: CaM kinase II, Ca^{2+}/calmodulin-dependent protein kinase II; CF, cooperativity factor.

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and that in highly purified synaptic vesicle preparations the saturation of the vesicle sites by endogenous synapsin I is close to 100% (6). (*v*) The binding sites for synapsin I present on one synaptic vesicle behave independently.

In the simulations, the limiting cases of dephosphorylated synapsin I and of stoichiometrically phosphorylated synapsin I have been considered in order to deal only with one homogeneous type of ligand. In fact, several experimental observations demonstrate that the phosphorylation state of synapsin I in the nerve terminal markedly increases during conditions of enhanced neurotransmitter release. It has been calculated that ≈2% of the total synapsin I is phosphorylated per impulse reaching the nerve terminal and that the changes in the phosphorylation state of synapsin I following sustained electrical stimulation or K^+-induced depolarization can be nearly stoichiometric (10–13).

The Cage Model

In the simple case of synapsin I binding to noncooperative vesicle sites, the interaction is described by the binding isotherm

$$SV = V_T S/(K_d + S), \quad [1]$$

where SV is the concentration of synapsin I bound to vesicles, V_T the concentration of total vesicle sites, K_d the dissociation constant determined experimentally (10 nM and 50 nM for dephospho- and phosphosynapsin I, respectively), and S the free synapsin I concentration. By solving Eq. 1 as a function of S_T (i.e., SV + S; in this model, interactions of synapsin I with F-actin are considered negligible) or by using a computer iterative procedure that minimizes $|S_T - (S + SV)|$, it is possible to calculate the occupancy of synaptic vesicle sites by synapsin I and the free synapsin I concentration in various functional states from the binding constants and total concentrations of ligand and receptor.

The results of such an analysis show that under dephosphorylated conditions synapsin I saturates 96.8% of the synaptic vesicle sites. This value is decreased to 93.3% when synapsin I is stoichiometrically phosphorylated by CaM kinase II. The relatively small effect of synapsin I phosphorylation can be explained by the fact that, in these conditions, S (340 nM and 800 nM for dephospho- and phosphosynapsin I, respectively) becomes >10 times the K_d value. However, this effect could be highly significant functionally if, as assumed by the cage model, the unoccupied synapsin I binding sites were responsible for the interaction with the presynaptic membrane and therefore for the ability of the vesicle to undergo exocytosis. In fact, phosphorylation of synapsin I brings about a >2-fold increase in the free vesicle sites (from 3.2% in dephosphorylated conditions to 6.7% in conditions of stoichiometric phosphorylation).

The average number of vesicle sites unoccupied by synapsin I, *u* (number of unmasked fusion sites), represents a random variable that has to be expressed in terms of probability to assume each possible value between 0 and the total number of synapsin I binding sites per synaptic vesicle. The number of synapsin I copies bound per vesicle at saturation, as calculated from biochemical (6, 14, 15) and immunocytochemical (16) experiments, is relatively small and estimates range from 5 to 15 (mean, 10). A binomial statistics can therefore be used to describe the probability distribution of vesicles bearing unoccupied synapsin I binding sites (17).

According to this model, we consider *n*, the total number of synapsin I binding sites per vesicle and *p*, the average fraction of binding sites not occupied by synapsin I. The value of *p* can be estimated from the average value of unoccupied sites *u* (calculated above) by using the relation $p = u/n$. $p(k)$, the probability that a vesicle has *k* unmasked sites ($k = 0, 1, 2, \ldots, n$), is defined by

$$p(k) = p^k(1 - p)^{n-k}n!/[(n - k)!k!]. \quad [2]$$

As shown in Fig. 1, the frequency of synaptic vesicles bearing one or more unoccupied sites (i.e., readily releasable vesicles) increases from 27% of the total vesicles in dephosphorylated conditions to 50.1% upon phosphorylation of synapsin I. This means that if synapsin I is stoichiometrically phosphorylated and if a single unoccupied synapsin I binding site is sufficient to allow fusion of the vesicles with the presynaptic membrane, an adequate stimulus leads to exocytosis from a 2-fold higher number of vesicles (i.e., release of a 2-fold higher number of quanta of neurotransmitter) than in basal dephosphorylated conditions. On the other hand, if two or more unoccupied vesicle sites are required for fusion to occur, synapsin I phosphorylation will induce a 3- to 4-fold increase in the number of synaptic vesicles available for exocytosis (see Fig. 1 at $k \geq 2$).

The Crosslinking Model

This hypothetical model is based on the following experimental data: (*i*) synapsin I binds to synaptic vesicles (6) and to F-actin (7); (*ii*) fragment analysis has identified distinct binding sites in the synapsin I molecule for synaptic vesicles and F-actin, consistent with the possibility of forming a ternary complex of F-actin/synapsin I/synaptic vesicle (14, 15, 18). Within the nerve terminal, an equilibrium could be reached among free synapsin I, synapsin I bound to synaptic vesicles, synapsin I bound to F-actin, and synapsin I bound simultaneously to synaptic vesicles and F-actin, as described by the following reactions

$$\begin{array}{ccccc} & & K_1 & & K_2 \\ & & \rightleftharpoons & & \rightleftharpoons \\ S + V + A & & SV + A & & SVA \\ & & \rightleftharpoons & & \rightleftharpoons \\ & & SA + V & & \\ & & K_3 & & K_4 \end{array} \quad [3]$$

SA is the concentration of synapsin I bound to F-actin and SVA is the concentration of synapsin I bound simultaneously to synaptic vesicles and F-actin. While the dissociation constants K_1 and K_3 have been determined experimentally (K_1 = 10 nM and 50 nM for dephospho- and phosphosynapsin I, respectively; K_3 = 1.5 μM for both synapsin I forms; refs. 6 and 7), the unknown dissociation constants K_2 and K_4 are defined as $K_2 = K_3 \cdot CF$ and $K_4 = K_1 \cdot CF$, where CF is a cooperativity factor whose value can be between 0 and 1

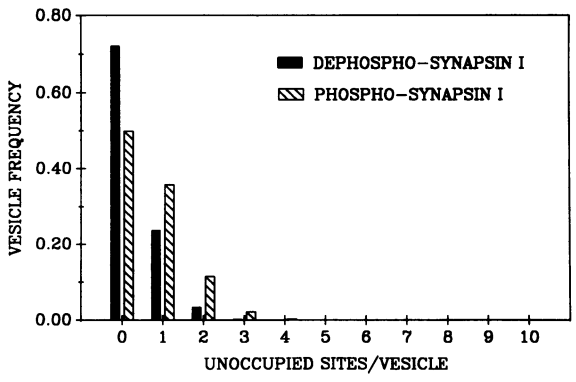

FIG. 1. Frequency of vesicles bearing unoccupied synapsin I binding sites. The average number of unoccupied vesicle sites was calculated by using the simulation procedure described in the text, and the frequency distribution was obtained by applying the binomial distribution statistics, assuming a total of 10 synapsin I binding sites per vesicle.

(positive cooperativity), 1 (no cooperativity), or >1 (negative cooperativity). At equilibrium, the following equations have to be satisfied:

$$S_T = S + SV + SA + SVA \quad [4]$$

$$V_T = V + SV + SVA \quad [5]$$

$$A_T = A + SA + SVA, \quad [6]$$

where V is the concentration of free vesicle sites, A is the concentration of free F-actin sites, and A_T is the concentration of total F-actin sites, which corresponds to the concentration of polymerized actin molecules ([F-actin]) times the synapsin I/F-actin binding stoichiometry. The experimentally determined stoichiometry is 0.14 mol of dephosphosynapsin I per mol of actin and 0.07 mol of phosphosynapsin I per mol of actin (7). [F-actin] within the nerve terminal is not known and has been tested within the simulation procedure from 0.3 to 1000 μM. To simplify the mathematical modeling, the possibilities of an association between synaptic vesicles and actin not involving synapsin I, as well as of the association of synapsin I with other cytoskeletal components, have not been considered.

By minimizing $|S_T - (S + SV + SA + SVA)|$ with a computer iterative procedure, it has been possible to calculate the values of S, SV, SA, and SVA. This analysis takes into account the transitions between bound and free synapsin I that in turn affect the equilibrium among the various complexes. The results obtained with this procedure (Fig. 2) can be summarized as follows. (*i*) At low [F-actin] (<5 μM), mainly SV and only very little SVA and SA are generated; upon synapsin I phosphorylation, a partial dissociation of the synapsin I/vesicle complex occurs, with a parallel increase in S. This condition is not significantly different from that analyzed for the cage model (see above). (*ii*) At intermediate [F-actin] (5–200 μM), SVA is substantial; its marked decrease induced by synapsin I phosphorylation mainly occurs by release of actin from the ternary complex, inducing an increase in SV. (*iii*) At high [F-actin] (>200 μM), SVA is predominant, but its dissociation upon synapsin I phosphorylation is decreased. Under these conditions, the dissociation of the ternary complex occurs both from the actin sites (increase in SV) and from the vesicle sites (increase in SA).

The simulation procedure was also carried out at various cooperativity states. Positive cooperativity does not markedly affect the formation of ternary complex, whereas the presence of negative cooperativity decreases the amount of ternary complex formed with a parallel increase in SV. Interestingly, phosphorylation of synapsin I induces a selective decrease in SVA and an increase in S independently of the cooperativity state and [F-actin] (i.e., the currently unknown parameters included in the simulation procedure).

As a test of the validity of the cross-linking model, S was experimentally determined in a reconstituted system containing F-actin, synaptic vesicles, and either dephosphorylated or stoichiometrically phosphorylated synapsin I. It was not possible to use total concentrations of the three species similar to those estimated to be present in the nerve terminal. Therefore, the theoretical values were recalculated by running the simulation procedure under the conditions used for the reconstituted system. The experimental values obtained with both dephospho- and phosphosynapsin I closely follow the pattern predicted by the crosslinking model, with a more than 2-fold increase in S when synapsin I is in the phosphorylated state (Fig. 3). However, due to the experimental error in the determinations of free ligand concentrations, it is not possible to evaluate the presence of cooperativity. Interestingly, the concentrations of soluble synapsin I determined in resting synaptosomes after osmotic lysis (9) are in the same range as those predicted by this simulation and obtained in the reconstituted system. Moreover, the relative increase found after depolarization of the synaptosomes (9) parallels that observed in the presence of phosphorylated synapsin I (Fig. 3).

In the case of the crosslinking model, each ternary complex of synaptic vesicle/synapsin I/F-actin represents a cross-bridge linking the vesicle to the F-actin-based cytoskeleton. Thus, binomial distribution statistics were used to obtain the frequency of synaptic vesicles bearing no cross-bridges (free vesicles) from the average number of cross-bridges calculated from the simulation procedure. The probability of occurrence of free vesicles decreases exponentially with the increase in the mean number of cross-bridges, but it represents a significant portion of the total vesicles when the average number of cross-bridges per vesicle is <4 (Fig. 4).

Phosphorylation of synapsin I decreases the number of cross-bridges between vesicles and F-actin and thereby increases the number of vesicles released from the cytoskeleton. It seemed of interest to analyze the ratio between the percentage of free vesicles in the presence of stoichiometri-

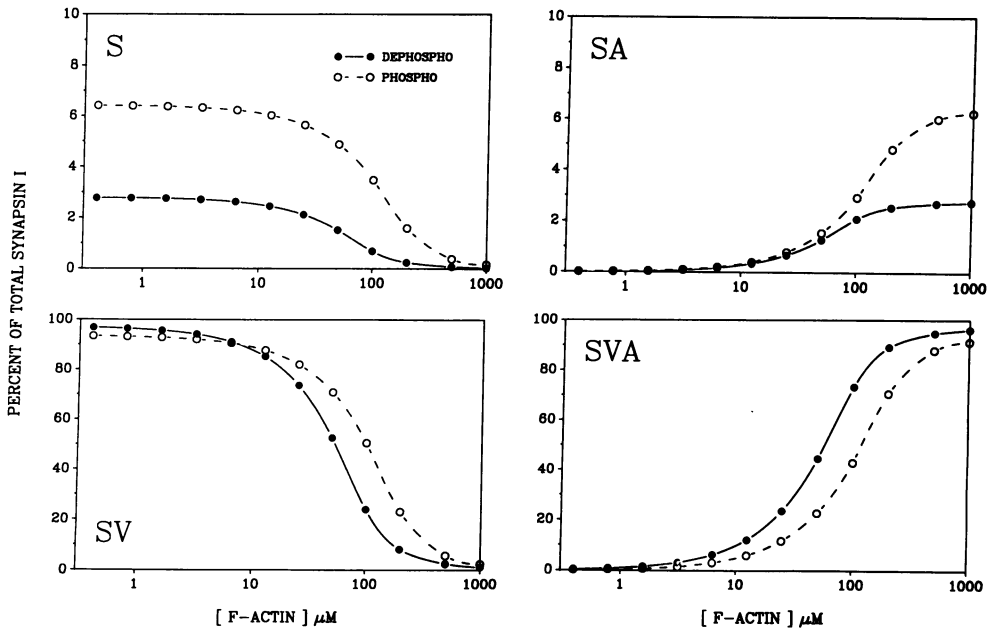

FIG. 2. Relative concentrations of synapsin I free in the medium (S), bound to synaptic vesicles (SV), bound to actin (SA), and bound simultaneously to synaptic vesicles and actin (SVA) as a function of actin concentration in the absence of cooperativity (CF = 1). Values are expressed as percent of total synapsin I concentration (12.5 μM) and were calculated from the solution of Eqs. 3–6 under conditions of complete dephosphorylation (●) and of stoichiometric phosphorylation (○) of synapsin I.

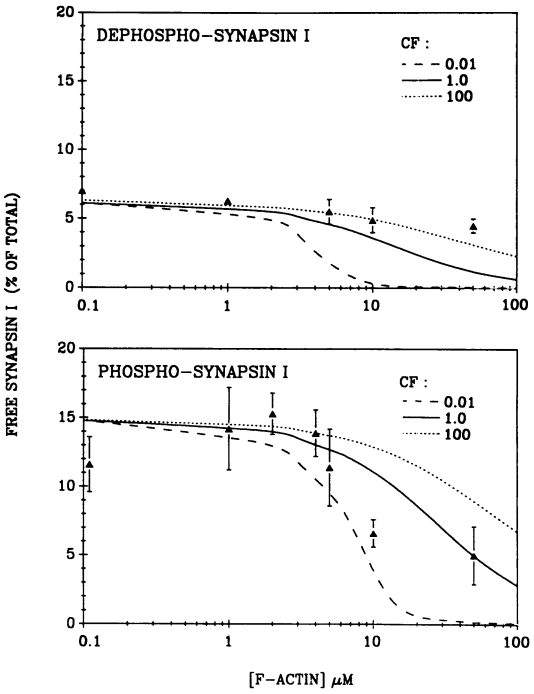

FIG. 3. Experimental determination of free synapsin I concentration in a reconstituted system containing synaptic vesicles, synapsin I, and actin filaments. The incubation mixture (100 μl) contained 1 μM synaptic vesicle sites, equivalent to 100 μg protein of synaptic vesicles purified from rat brain cortex (19) and depleted of endogenous synapsin I (6); various concentrations of polymerized actin (0–100 μM) purified from rabbit skeletal muscle (7); and 1 μM synapsin I purified from bovine brain and either nonphosphorylated or phosphorylated *in vitro* to near stoichiometry by CaM kinase II (6). The incubation buffer was 50 mM KCl/10 mM NaCl/1 mM $MgCl_2$/0.5 mM ATP/2.5 mM Hepes/1.25 mM Tris/150 mM glycine/0.01% NaN_3, pH 7.4. Samples were incubated at room temperature for 30 min and then spun in a Beckman TL-100 ultracentrifuge at 400,000 × *g* for 30 min. Aliquots from the supernatants (free synapsin I) and from the incubation mixture before centrifugation (total synapsin I) were assayed for synapsin I immunoreactivity by dot immunobinding (20). The experimentally determined concentration of free synapsin I (▲, mean ± SEM of three experiments) is expressed as percent of the total synapsin I concentration. Curves represent the theoretical free synapsin I concentrations in three cooperativity states (CF = 0.01, 1, and 100), obtained by running the computer simulation at the concentrations of synapsin I, vesicle binding sites for synapsin I, and actin used in the experiment.

cally phosphorylated synapsin I and the percentage of free vesicles in the presence of totally dephosphorylated synapsin I under various cooperativity states and F-actin concentrations. The increase in free vesicles caused by synapsin I phosphorylation is proportional to the estimated total F-actin concentration, is scarcely affected by the presence of positive cooperativity, and is depressed by the presence of negative cooperativity (Fig. 5).

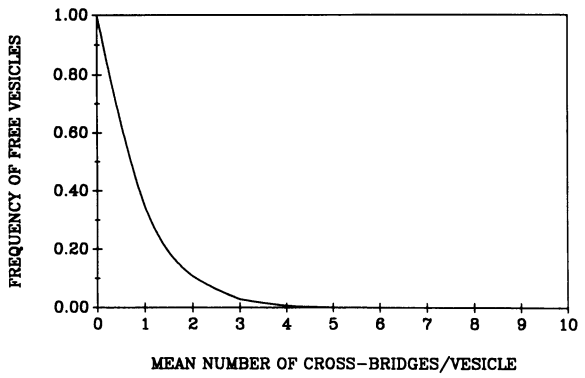

FIG. 4. Frequency of synaptic vesicles that are not crosslinked to F-actin, calculated as a function of the average number of cross-bridges per vesicle. Binomial distribution statistics were applied using an average of 10 synapsin I binding sites per vesicle.

Implications for Regulation of Neurotransmitter Release

The release of neurotransmitter is probabilistic in nature, since repeated identical stimuli lead to responses that exhibit random amplitude fluctuations (1). These fluctuations occur in quantal steps and are due to the release of more or fewer quanta. The mean number of quanta released following a stimulus (m = mean quantum content) can be described as $m = n \cdot p$, where n represents the number of quanta available for release and p the average probability for a quantum to be released (21). The analysis of electrophysiological recordings allows m to be calculated (m = mean response amplitude/mean spontaneous potential amplitude), but n and p cannot be measured independently.

It is widely accepted that m corresponds to the number of synaptic vesicles undergoing exocytosis (3). According to this view, n represents the number of synaptic vesicles available for fusion and p the average probability for a vesicle to fuse with the axolemma.

Quantal release is a dynamic phenomenon, since it is influenced at any moment by previous activity; i.e., the number of quanta released following a stimulus depends on the frequency and duration of previous stimulation. Under physiological conditions, repetitive stimulation usually leads first to an increase and then to a decrease (synaptic depression) in m (22).

Based mainly on the analysis of synaptic depression, it has been suggested that, within the nerve terminal, n corresponds to a subpopulation of synaptic vesicles—i.e., the synaptic vesicles immediately available for release (23). This fraction ("available pool") differs from nerve terminal to nerve terminal and can be as low as 1% of the total releasable pool (24). The majority of the vesicles present in the nerve terminal belong to the so-called "reserve pool," which can be mobilized to refill the available pool after its partial or total

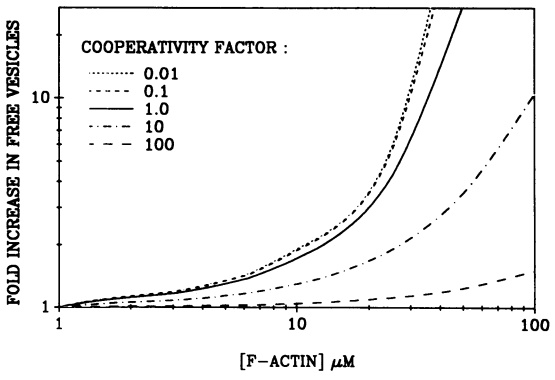

FIG. 5. Increase in synaptic vesicles released from the cytoskeleton (i.e., in the number of synaptic vesicles bearing no cross-bridges) induced by stoichiometric phosphorylation of synapsin I. The average number of cross-bridges per vesicle was calculated as a function of the actin concentration at five cooperativity states for either dephosphorylated or fully phosphorylated synapsin I. The frequency of vesicles devoid of cross-bridges was then calculated from the average number of cross-bridges per vesicle by using the binomial statistics. The ratio between the frequency of free vesicles obtained under conditions of stoichiometric phosphorylation of synapsin I and that obtained under conditions of complete dephosphorylation was plotted.

depletion. The vesicles in the reserve pool may correspond to the vesicles that are linked to the nerve terminal cytomatrix.

Phenomena that result in increased neurotransmitter release may involve recruitment of vesicles from the reserve pool into the available pool, leading to a fractional increase in the available pool and therefore to an increase in n. An increase in p could be ascribed to an increase in the number of vesicles within the available pool that are closely apposed to the plasma membrane.

We suggest that synapsin I, by undergoing cycles of phosphorylation and dephosphorylation, is implicated in the transition of synaptic vesicles from the reserve to the available pool, providing a biochemical basis for the changes in the functional properties of the nerve terminal. Assuming a binding stoichiometry of 10 mol of synapsin I per mol of vesicle, we suggest that, if the cage model is true, synaptic vesicles are almost completely saturated by synapsin I whether it is in the dephosphorylated or in the phosphorylated state. The number of synaptic vesicles bearing at least one unmasked fusion site is low, and phosphorylation of synapsin I to full stoichiometry increases it ≈2-fold. Moreover, the population of vesicles bearing more than one unoccupied fusion site, almost negligible when synapsin I is dephosphorylated, is increased 3- to 4-fold by its stoichiometric phosphorylation (see above; Fig. 1). In the crosslinking model, the transition of synaptic vesicles from the reserve pool to the available pool can be explained in terms of a marked effect of synapsin I phosphorylation on the ternary complex dissociation, mainly due to the reduction in the F-actin binding sites for synapsin I. Our simulation has shown that in dephospho conditions, the relative sizes of the reserve and available pools depend on the actual actin concentration within the nerve terminal and on the cooperativity factor (see Fig. 5). It appears that the increase in the amount of free vesicles (vesicles that are not tethered to actin filaments, i.e., vesicles in the available pool) in response to stoichiometric phosphorylation of synapsin I is more pronounced the higher the actin concentration and the lower the cooperativity factor.

After microinjection of CaM kinase II into the preterminal digit of the squid giant synapse, Llinas *et al.* (8) observed a 3- to 7-fold increase in the magnitude of the postsynaptic potential generated by a presynaptic depolarizing step. This effect seems likely to be due to phosphorylation of a synapsin I-like protein, since synapsin I is by far the most efficient and most abundant substrate for this enzyme in the mammalian nerve terminal (13). According to our simulations, a similar increase in neurotransmitter release upon phosphorylation of synapsin I could be achieved in the crosslinking model, with actin concentrations within the nerve terminal in the range of 20–25 μM in the absence of negative cooperativity, or in the cage model, if multiple unoccupied synapsin I binding sites are necessary for fusion.

Both the cage and crosslinking models are compatible with the hypothesis that synapsin I phosphorylation modulates neurotransmitter release by causing variations in n (i.e., in the number of synaptic vesicles available for fusion). In two experimental systems, the goldfish Mauthner neurons (25) and the squid giant synapse (26), fluctuation analysis has shown that synapsin I phosphorylation–dephosphorylation reactions affect n but not p. In conclusion, it appears that phosphorylation of synapsin I is part of a machinery that finely regulates the state of responsiveness of the nerve terminal to a stimulus.

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