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A POSSIBLE ROLE OF THE PHOSPHORYLATION OF SYNAPTIC MEMBRANE PROTEINS IN THE CONTROL OF CALCIUM ION PERMEABILITY

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Summary

Incubation of synaptosomes under conditions which result in complete phosphorylation of membrane bound accepter proteins does not affect the permeability to Na^+ or K^+ as measured by a spectrophotometric method. This technique was not, however, sensitive enough to determine permeability to Ca^{2+} which was thus estimated using $^{45}Ca^{2+}$. It was found that although phosphorylation did not affect the equilibrium binding of ^{45}Ca it did lower the rate of both Ca^{2+} uptake and efflux. The most likely interpretation of these results is that phosphorylation of proteins in the synaptic membrane lowers the permeability of the membrane to Ca^{2+}. This could have a role in the regulation of synaptic transmission.

Introduction

It is well established that synaptosomal plasma membranes and microsomal fragments from brain contain a tightly-bound protein kinase which is stimulated by cyclic AMP, and which catalyses the phosphorylation of endogenous proteins [1—4]. The enzyme appears to be at least partially specific to the proteins of the membrane, since synaptosomal plasma membranes, when correctly prepared, have high intrinsic protein kinase activity but can hardly catalyse the phosphorylation of other proteins such as histones and phosvitin [2,5].

It has been shown that the cyclic AMP-stimulated intrinsic protein kinase is

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concentrated in the region of the synaptic junction [6], suggesting that the reaction may have a role in phenomena associated with synaptic transmission. It has, in fact, been previously suggested that phosphorylation of synaptic membrane proteins may alter the passive permeability of the membrane to certain ions, and thus regulate the passage of nervous impulses [7,8] but before the work described in the present paper there was no experimental evidence for such a reaction.

Proteins in membrane preparations from a number of different tissues apart from brain can be phosphorylated [9] and it has been demonstrated that there is a correlation between the rate of dephosphorylation of membrane proteins in epithelial cells from toad bladder and the rate of increase of Na^+ permeability which occurs on addition of vaspressin to this tissue [10]. Later work, however, demonstrated that much higher concentrations of vasopressin are needed to cause protein dephosphorylation than to change Na^+ permeability indicating that protein phosphorylation is unlikely to be involved in the control of Na^+ permeability in toad bladder epithelial cell membranes [11]. It has also been shown that there is a correlation between the time course of phosphorylation of proteins in the avian erythrocyte membrane and the increase in Na^+ permeability caused by cyclic AMP [12] though similar results could not be obtained with preparations of human erythrocytes [13]. More directly it has been shown that phosphorylation of a membrane protein (rhodopsin) in rod outer segment discs from retina lowers the permeability to Ca^{2+} [14] as does phosphorylation of proteins in the human erythrocyte membrane [13].

In view of these observations experiments were carried out to determine if phosphorylation of proteins in the synaptic membrane alters the permeability of the membrane.

Methods

Preparation of synaptosomes

Synaptosomes were prepared by fractionation of the crude mitochondrial pellet from adult rat brain on discontinuous sucrose-ficoll gradients as previously described [15]. All preparations of synaptosomes were stored at 4° C and used within 24 h during which time there was no detectable loss of intrinsic protein kinase activity or effect on osmotic behaviour (see below).

Preparation of microsomes

Microsomes were prepared by high speed centrifugation of the post mitochondrial pellet used for the preparation of synaptosomes as previously described [5].

Results

Determination of permeability

Before attempting to determine the permeability of the synaptosomes it was first necessary to ensure that the preparation was in the form of sealed vesicles. Such vesicles should behave as osmometers and obey Boyle and Van 't Hoff's law; that is their volume should be inversely proportional to the osmotic

strength of the medium in which they are suspended [16—18]. The volume of particles in suspension is inversely proportional to the optical extinction of the suspension since smaller particles scatter more light [17]. In initial experiments it was observed that E_{520} of synaptosomes suspended in a solution of Na_2SO_4 increased with the strength of the solution (Fig. 1a) and that if $1/E_{520}$ is plotted against $1/Na_2SO_4$ a linear relationship is found (Fig. 1b). These results confirm the observations of Keen and White [16] and show that our preparations of synaptosomes behave as osmometers and so must be in the form of sealed vesicles. Similar experiments with brain microsomes showed no effect of Na_2SO_4 concentration (over the range 50—200 mM) on E_{520} showing that the preparation was not in the form of sealed vesicles.

It is possible to measure the permeability of the outer membrane of vesicles to a substance by measuring the rate of swelling on suspension in a solution of the substance. As the solute enters the osmotic strength of the solution inside the vesicles is raised, water enters to adjust the osmolarity to that of the outside solution, and the particles swell. The rate of swelling, which may be measured by determining the rate of decrease of (E_{520}, is thus proportional to the permeability of the outer membrane of the vesicles.

Keen and White [15] used this technique to measure the permeability of synaptosomes to various ions and we used their method to determine the effect of phosphorylation on the permeability to Na^+, K^+, Mg^{2+} and Ca^{2+}. Since Keen and White [15] observed that acetate ions were more permeable than chloride we used acetate salts.

The effect of phosphorylation of synaptosome membrane proteins on the permeability to various cations

Samples to be phosphorylated were incubated at room temperature for 10 min with 1 mM $MgCl_2$, 1 mM ATP at a protein concentration of about 0.2 mg/ml in 0.3 M sucrose, 10 mM Tris · HCl (pH 7.2). These conditions were chosen

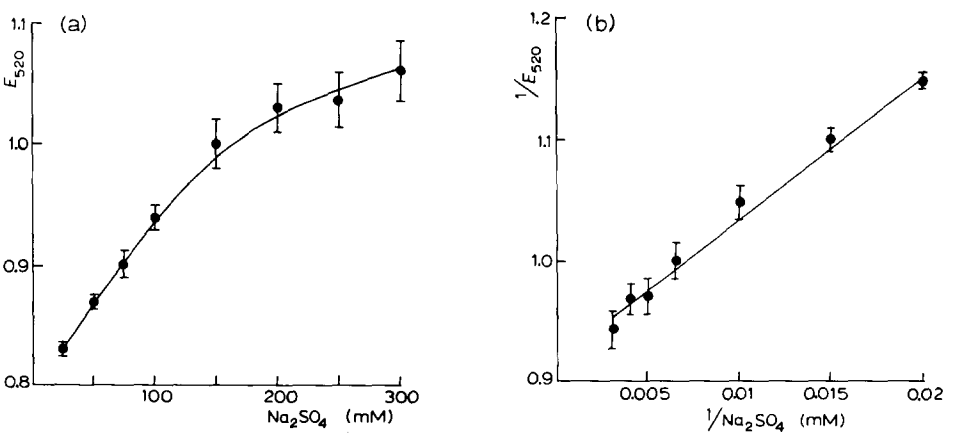

Fig. 1. The effect of Na_2SO_4 concentration on the E_{520} of a suspension of synaptosomes. In (a) the data are plotted normally while in (b) $1/Na_2SO_4$ is plotted against $1/E_{520}$. Results are shown as means ± standard deviations and are taken from 4 observations in two separate experiments.

because they gave complete phosphorylation of all acceptor protein in isolated synaptosomal or microsomal membranes [5]. Separate experiments (not shown) demonstrated that intact synaptosomes were also maximally phosphorylated under these conditions. Samples which were not phosphorylated were incubated for the same time but in the absence of ATP. At the end of the incubation the samples were centrifuged for 10 min at 100 000 × g and the pellets washed once by resuspension in and centrifugation from 0.3 M sucrose, 10 mM Tris · HCl (pH 7.2). All operations were carried out as rapidly as possible at 0—4° C to minimise dephosphorylation by membrane bound protein phosphatases [19]. The pellets were finally suspended in 0.3 M sucrose, 10 mM Tris · HCl (pH 7.2) at a concentration of about 5 mg of protein/ml and 50 µl aliquots added to 1 ml of a 0.2 M solution of the appropriate salt in a spectrophotometer cell. Samples were rapidly mixed with a Pasteur pipette and E_{520} read on a recording spectrophotometer (Beckman SP 1800) within 5 s. As controls samples were added to distilled water and in these cases no change in E_{520} was observed. It was, however, found that there was no effect of phosphorylation on the rate of decrease of E_{520} (proportional to the permeability of the synaptosomal membrane) on suspension in solutions of sodium or potassium acetate (results not shown) from which it may be seen that phosphorylation does not affect the permeability to Na^+ or K^+. There was, however, no detectable decrease in E_{520} on suspension in solutions of calcium or magnesium acetate showing that the permeability to Ca^{2+} and Mg^{2+} was too low to be measured by this technique.

In work with rod outer segments from retina the effect of protein phosphorylation on Ca^{2+} permeability was estimated using $^{45}Ca^{2+}$ [14]. It was found that the rate of uptake of ^{45}Ca into non-phosphorylated rod outer segment discs was faster than into phosphorylated discs although the same amount was incorporated after incubation to equilibrium. In addition it was found that if rod outer segment discs were loaded with ^{45}Ca and then incubated in a Ca^{2+}-free medium the rate of loss of ^{45}Ca was faster from non-phosphorylated than from phosphorylated discs. It was argued that the most reasonable interpretation of these results is that phosphorylation of proteins in the disc membranes lowered permeability to Ca^{2+}. A similar technique was thus used with preparations of synaptosomes.

Initial experiments showed that phosphorylation of neither synaptosomal nor microsomal preparations changed the binding of ^{45}Ca (Fig. 2) at equilibrium (after 30 min incubation, see Fig. 4). If the results are drawn in the form of Scatchard plots [20] (Fig. 3) it may be seen that both synaptosomal and microsomal preparations have at least two sites of Ca^{2+} binding. Binding constants for the high and low affinity sites of synaptosomes are 0.9 and 0.018 μM^{-1} with capacities of 2.5 and 46 nmol/mg protein, respectively. The comparable figures for microsomal preparations are 2 and 0.004 μM^{-1} and 1.5 and 52 nmol/mg protein for high and low affinity sites, respectively.

In contrast if the time-course of incorporation of ^{45}Ca is measured it may be seen from Fig. 4a that phosphorylated preparations of synaptosomes take up ^{45}Ca more slowly than non-phosphorylated samples although the amount incorporated at equilibrium (after about 25 min) was the same (in agreement with the results shown in Fig. 2). The results obtained with microsomal prepa-

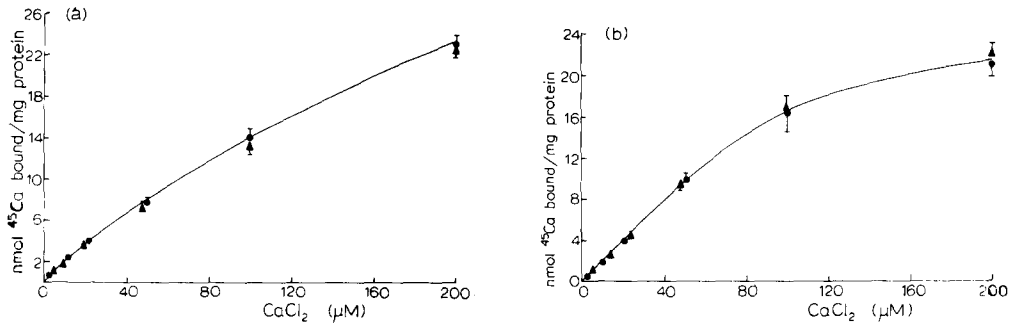

Fig. 2. The effect of phosphorylation of synaptosomal or microsomal preparations on the equilibrium binding of $^{45}Ca^{2+}$. Samples of phosphorylated (●) or non-phosphorylated (▲) synaptosomal (a) or microsomal (b) preparations were prepared as described in the text and incubated at 0°C for 30 min at a protein concentration of about 150 μg/ml in the presence of 10 mM Tris · HCl pH 7.2, 0.3 M sucrose and the stated concentration of [^{45}Ca]$CaCl_2$ (specific radioactivity about 1×10^7 cpm/μmol). Samples were then filtered through Millipore cellulose ester filter discs (0.45 μm pore size), washed twice with 5 ml lots of 10 mM Tris · HCl (pH 7.2) in 0.3 M sucrose, dried, and the bound radioactivity determined as previously described [11]. Results are shown as means ± standard deviations and are taken from 4 observations.

rations were, however, not nearly so clear cut (Fig. 4b). Since the synaptosomes, in contrast to the microsomes, were in the forms of sealed vesicles these observations suggest that phosphorylation has decreased the rate at which Ca^{2+} can enter the synaptosome accross the synaptic membrane, presumably by decreasing Ca^{2+} permeability. The results could not have been due to the presence of traces of ATP in the phosphorylated samples since in separate experiments (not shown) it was found that 100 μM ATP did not affect the rate of uptake of ^{45}Ca into synaptosomes.

If phosphorylation has, in fact, lowered the permeability of the synaptosomal membrane to Ca^{2+} then phosphorylation of the membrane proteins should also decrease the rate of loss of ^{45}Ca from preloaded synaptosomes. It

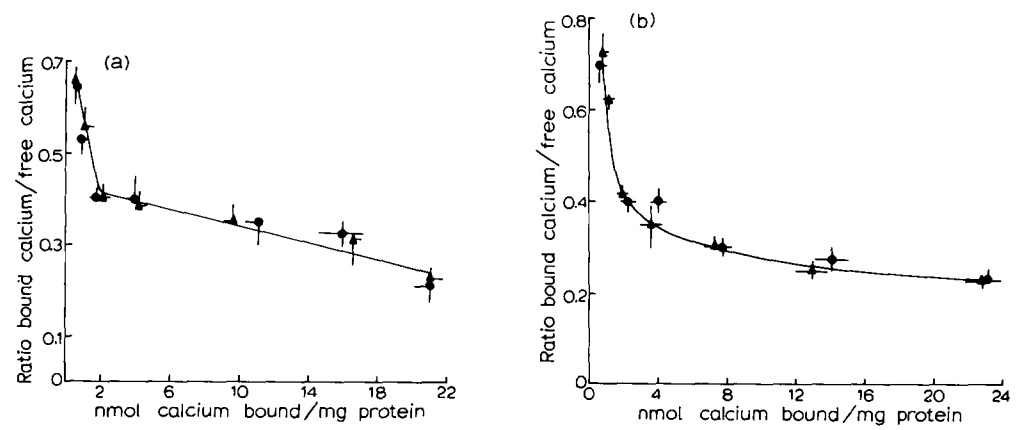

Fig. 3. The data of Fig. 2 are shown as Scatchard plots [14]. (a) Phosphorylated (●) and non-phosphorylated (▲) synaptosomal preparations. (b) Phosphorylated (●) or non-phosphorylated (▲) microsomal preparations.

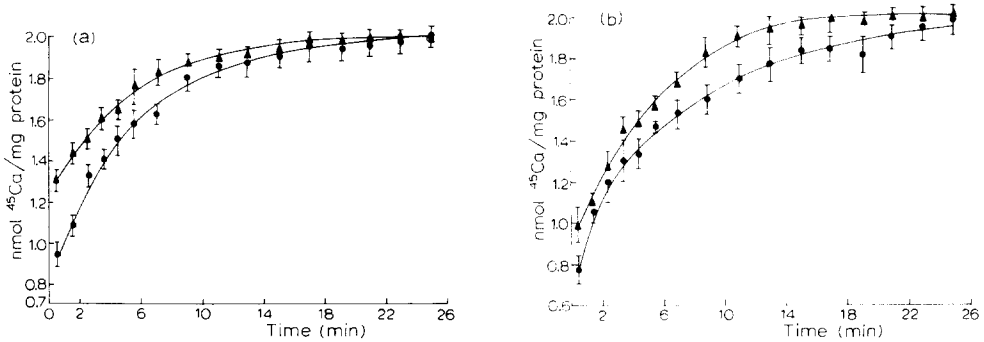

Fig. 4. The effect of phosphorylation of microsomal or synaptosomal preparations on the rate of uptake of $^{45}Ca^{2+}$. Samples of phosphorylated (●) or non-phosphorylated (▲) synaptosomal (a) or microsomal (b) preparations were prepared as described in the text and incubated at a protein concentration of about 150 μg/ml at 0°C with 0.3 M sucrose, 10 mM Tris · HCl pH 7.2 and 10 μM [^{45}Ca]$CaCl_2$ (specific radioactivity about 1 · 10^7 cpm/μmol). Samples were taken at stated times and the amount of bound radioactivity determined after filtration as described in the legend to Fig. 1. Results are shown as means ± standard deviations and are taken from 4 observations.

can be seen from Fig. 5a that phosphorylation of synaptosomal preparations does, in fact, reduce the initial rate at which ^{45}Ca is lost from pre-loaded material although, after about 30-min incubation, the amount of ^{45}Ca in the synaptosomes is reduced to approximately the same level in both phosphorylated and nonphosphorylated samples. By contrast, phosphorylation of microsomal preparations does not affect the loss of ^{45}Ca (Fig. 5b), once again probably due to the lack of a permeability barrier.

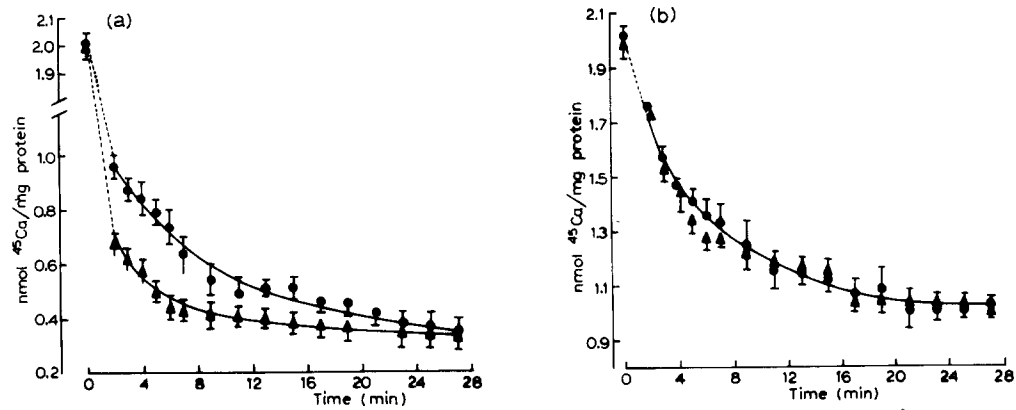

Fig. 5. The effect of phosphorylation of microsomal or synaptosomal preparations on the rate of loss of ^{45}Ca from preloaded samples. Aliquots of phosphorylated (●) or non-phosphorylated (▲) synaptosomal (a) or microsomal (b) preparations prepared as described in the text were incubated at a protein concentration of about 150 μg/ml for 30 min at 0°C with 10 mM Tris · HCl pH 7.2, 0.3 M sucrose and 10 μM [^{45}Ca]$CaCl_2$ (specific radioactivity about 1 × 10^7 cpm/μmol). Two 0.5-ml aliquots were filtered to determine bound radioactivity as described in the legend to Fig. 1 and the remaining material centrifuged at 100 000 × *g* for 15 min. The pellet was rapidly resuspended in ice-cold 10 mM Tris · HCl pH 7.2, 0.3 M sucrose at a protein concentration of about 150 μg/ml. Samples were taken and filtered at the stated times and the amount of bound radioactivity determined as described in the caption of Fig. 1. The time between addition of the suspension medium and the taking of the first sample was always 2 min. Results are shown as means ± standard deviations and are taken from 4 observations.

Discussion

In our experiments we have not been able to directly demonstrate that Ca^{2+} enters synaptosomes. We have, however, observed that phosphorylated synaptosomes incorporate Ca^{2+} less rapidly than non-phosphorylated material while the loss of Ca^{2+} from preloaded synaptosomes is also reduced by phosphorylation although the equilibrium binding of Ca^{2+} is not affected. It is difficult to explain these results unless one assumes that Ca^{2+} enters the synaptosomes and that phosphorylation of proteins in the synaptic membrane reduces the permeability to Ca^{2+}, in effect closing "Ca^{2+} pores". This interpretation is supported by the relative lack of effect of phosphorylation on the Ca^{2+} movement into and out of microsomal fragments since such preparations are not in the form of sealed vesicles and thus contain no permeability barrier.

The decrease in permeability of the synaptic membrane could change, albeit transiently, the Ca^{2+} concentration in the synaptic terminal which could then alter the ease of neurotransmitter release. The dependence of transmitter release upon Ca^{2+} influx is a classical phenomenon (for review see Hubbard, ref. 21), and this type of dependence has been noted with synaptosomes in vitro [22]. In addition long-term changes in membrane permeability towards calcium could change the potential gradient across the membrane leading to hyperpolarisation [23]. In either case, whether by rendering the nerve terminal refractory to depolarization, or by preventing the Ca^{2+} influx which is necessary for co-ordinated release of transmitter, phosphorylation of the presynaptic membrane could serve to regulate the ease of neurotransmitter release.

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