NEW BIOMEDICAL TECHNOLOGIES

Saponin in the *In Vitro* Phosphorylation System

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The possibility of the use of saponin for permeabilization of synaptic vesicles from P_2 fraction is explored. This fraction is isolated from rat brain cortex. Phosphorylation of membrane-bound proteins is performed *in vitro*. At the saponin:protein ratio 1:10 (w/w) phosphorylation of these proteins increases 2-fold due to increased membrane permeability for ATP and cAMP. Treatment of synaptic vesicles with saponin at the same ratio leads to the loss of cytosolic but not membrane-bound protein. This concentration of saponin can be used for permeabilization of other membranes, providing that the protein:cholesterol ratio is taken into consideration.

Key Words: *saponin; protein phosphorylation; synaptic membranes; synaptosomes*

Permeabilization is necessary to transport compounds that cannot cross the plasma membrane (ATP, cAMP, etc.) into the cell. The plasma membrane can be perforated mechanically [5] and with the aid of channel-forming [4] and lysing agents [2,9]. Saponin is a triterpenic glycoside capable of binding to the plasma membrane cholesterol and forming hexagonally oriented pores [1,8] without affecting the endoplasmic reticulum and mitochondrial membranes [3].

The aim of this study was to analyze the ability of saponin to permeabilize the membrane of synaptic vesicles obtained after hypotonic shock of the P_2 fraction which contains synaptic vesicles, myelin and mitochondria. The degree of permeabilization was assessed by incorporation of ^{32}P in membrane-bound proteins upon cAMP-dependent phosphorylation. It was necessary to find out whether saponin causes any changes in the phosphorylation pattern. The effect of saponin on the label incorporation into membrane-bound proteins and release of cytosolic proteins was examined.

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MATERIALS AND METHODS

Male Wistar rats weighing 150-180 g were used. Synaptic vesicles were obtained by 1-h incubation of P_2 fraction in 5 mM Tris buffer (hypotonic shock). The P_2 fraction was isolated from brain hemispheres as described [6] and purified on a discontinuous sucrose gradient [6]. The membranes were suspended in medium containing 130 mM KCl, 1 mM $MgCl_2$, 1 mM EGTA, and 10 mM HEPES (pH 7.0), preincubated for 1 min at 30°C, and phosphorylated for 20 sec in a 50-μl sample. The sample contained (final concentrations) 1 μg/μl protein, 10 mM Mg^{2+}, 10^{-4} M ^{32}P-γ-ATP, 2×10^{-5} cAMP, and 50 mM Tris-HCl (pH 7.4). The saponin concentration varied from 0.32 to 100% (w/w protein). The reaction was initiated by the addition of ^{32}P-γ-ATP and cAMP and terminated by the addition of SDS buffer to the reaction mixture followed by boiling in a water bath for 2 min. The samples were then electrophoresed in 15% polyacrylamide gel in the presence of SDS (SDS-PAAG) and radioautographed. Alterations of the phosphorylation pattern caused by saponin were analyzed as follows: the label incorporation was quantitated by densitometry of the radioautographs of 5 membrane-bound proteins (104, 96, 81, 67 and 25

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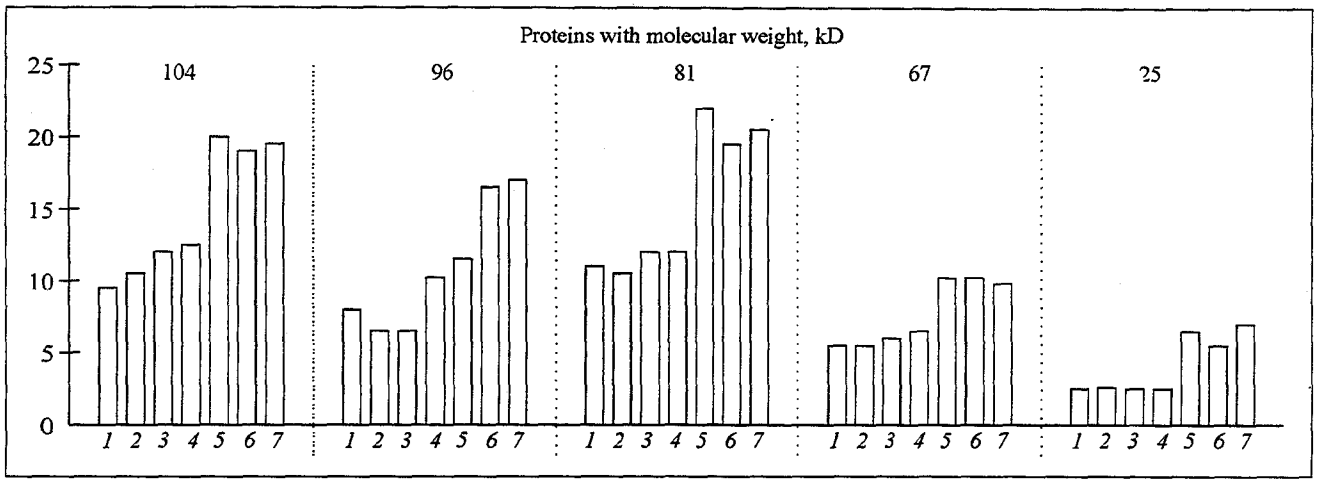

Fig. 1. Increased incorporation of ^{32}P after treatment with saponin. Ordinate: ^{32}P incorporation, relative units. Saponin concentration (weight of saponin/weight of protein)×100%: *1*) 0 (control); *2*) 0.32; *3*) 1; *4*) 3.2; *5*) 10; *6*) 32; *7*) 100%.

kD) which were identified by electrophoretic mobility. The results were expressed as a percent ratio of the area under the peak of a given protein to the total area. The curves for ^{32}P incorporation at various concentrations of saponin were constructed for each protein, after which correlation analysis was performed. The release of proteins from perforated synaptosomes was assessed as follows: saponin (2-20% w/w protein in 10 μl of the medium) was added to the synaptosome suspension (60 μl) and centrifuged at 10,000*g* for 1 h at 4°C. The protein concentration in the pellet and supernatant was determined by the method of Bradford. The supernatant proteins were electrophoresed in SDS-PAAG.

RESULTS

When placed in a hypotonic solution, the synaptic vesicles of P_2 fraction burst, and their membranes form liposome-like structures (membrane vesicles). About 50% of these vesicles expose proteins located on the internal surface of the membrane, and the protein kinase—substrate complexes become available for ATP. The other 50% of vesicles preserve normal orientation of membrane-bound proteins, which prevents the access of ATP to the complexes. Incorporation of ^{32}P in this preparation reflects the phosphorylation of proteins on the "inverse" vesicles (Fig. 1). There were no appreciable changes in the label incorporation at a saponin concentration lower than 10%; however, it increased with an increase in saponin concentration and remained practically constant up to 100% saponin (Fig. 1). It can be concluded that 10% is the threshold concentration for saponin, at which this compound forms pores sufficient for the entry of cAMP and ATP into vesicles with normally oriented proteins. This accounts for the absence of increase in the label incorporation at higher concentrations of saponin. Correlation analysis showed that the spectrum of ^{32}P incorporation in membrane-bound proteins (Mr 104, 96, 81, 67, and 25 kD) does not change in the entire range of saponin concentrations.

Thus, the optimal concentration of saponin for phosphorylation of synaptic membrane-bound pro-

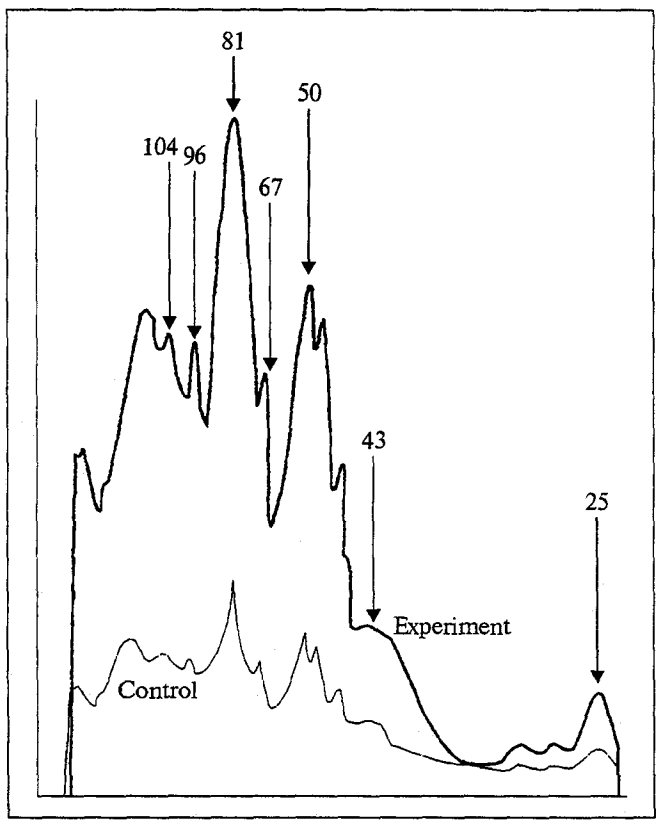

Fig. 2. Increased incorporation of ^{32}P in synaptosomal proteins at 10% saponin. Molecular weighs of proteins (kD) are indicated above the arrows.

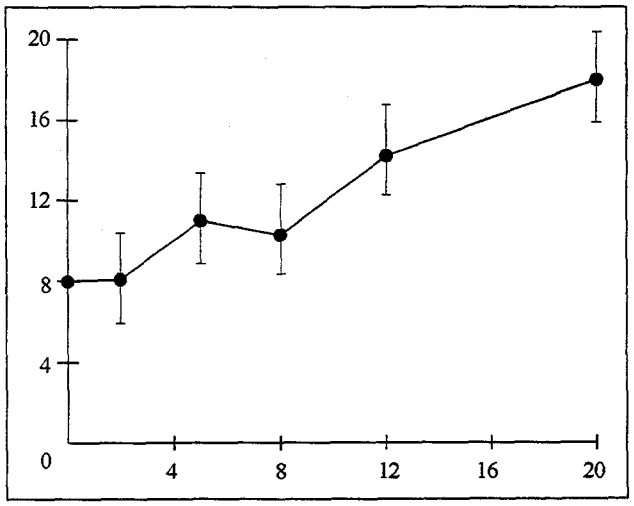

Fig. 3. Relationship between the release of proteins from synaptic vesicles and the concentration of saponin. Abscissa: concentration of saponin (weight of saponin/weight of protein)×100%; ordinate: percent of released protein.

teins in P_2 fraction is 10%. After treatment of membranes with 10% saponin, the sensitivity of the method increased 2-fold. This is consistent with the fact that in a hypotonic solution only 50% of vesicles expose the internal surface of the membrane.

Saponin has been used for perforation of synaptic vesicles [7]. In our experiments, the incorporation of ^{32}P in phosphoproteins increased by 3.1±0.2 times compared with the control after the addition of 10% saponin to the suspension of synaptic vesicles, which is explained by a low basal phosphorylation of these proteins (Fig. 2). Treatment with saponin may be preferable compared with hypotonic shock, since enzymes are not subjected to the effect of a hypotonic medium, the maximum incorporation of ^{32}P is achieved, and the procedure is 1 h shorter. Both hypotonic shock and saponin perforation result in the loss of the vesicle contents. Quantitative and qualitative analysis of the contents revealed the predominance of protein with Mr 33 kD (molecular weight of proteins was calculated by electrophoretic mobility in PAAG). We believe that this protein is the subunit of lactate dehydrogenase (36 kD). The holoenzyme consists of four subunits and dissociates upon electrophoresis in SDS-PAAG. However, the loss of the 33 kD protein did not affect the phosphorylation pattern because this protein probably does not serve as the substrate of cAMP-dependent protein kinase. The relationship between the amount of released proteins and saponin concentration is linear (Fig. 3), which can be used in experiments where cytosol removal is necessary. It should be mentioned that saponin is a mild detergent capable of solubilizing membrane proteins when applied at high concentrations [1].

Some researchers express saponin concentration as weight of saponin per volume of sample [3,9] or as a percentage of weight of saponin per weight of sample [2], which does not seem to be correct. We propose to express it as a percentage of weight of saponin per weight of protein in the sample to standardize the amount of saponin for the studied object. Methodologically, the weight of saponin should be related to the weight of cholesterol in the sample. However, in our experiments the cholesterol/protein ratio remains constant, suggesting that the protein concentration reflects the cholesterol concentration in the sample. The advantage of the proposed unit for the saponin concentration is that the optimal working concentration can be calculated for any object from the cholesterol/protein ratio in its plasma membrane.

Thus, saponin can be used to remove cytoplasmic proteins and intracellular substances, deliver necessary agents to the internal side of the plasma membrane, create an artificial medium with a given ionic composition, reduce the amount of radiolabeled ATP required to achieve the level of ^{32}P incorporation sufficient for radioautography, and reduce the protein content in a sample.

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