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Immobilization of Na,K-ATPase isolated from rat brain synaptic plasma membranes

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Abstract: Rat brain Na,K-ATPase partially purified by SDS from synaptic plasma membranes (SPM) was immobilized by adsorption on nitrocellulose (NC), polyvinylidene fluoride (PVDF) and glass fiber (GF) membranes. Partial SDS solubilization increased the enzyme activity by 40 %. With regard to the preservation of the enzyme activity, nitrocellulose was shown to be the optimal support for the immobilization. The enzyme showed the highest percentage activity (14 %) after 30 min of SPM adsorption, at 20 ºC under the vaccum, with 25 μg of proteins per NC disc filter. In addition, adsorption on NC stabilizes the Na,K-ATPase, since the activity was substantial 72 h after adsorption at 20 ºC. After adsorption, the sensitivity of the enzyme to $HgCl_2$ and $CdCl_2$ inhibition was higher. The results show that immobilized Na,K-ATPase SPM can be used as a practical model for the detection of metal ions in different samples.

Keywords: Na,K-ATPase, synaptic plasma membranes, immobilization, inhibition, cadmium, mercury.

INTRODUCTION

The transmembrane enzyme sodium, potassium (Na, K) dependent adenosinetriphosphatase (Na,K-ATPase) has the function of maintaining sodium and potassium gradients across membranes that subserve cellular activities, such as volume regulation, action potential and secondary active transport.[1] It is ubiquitously expressed in the plasma membranes of almost all animal cells including neurons, but it is especially abundant in synaptic plasma membranes (SPM).[2] The specific inhibitors of Na,K-ATPase are cardiotonic glucosides (ouabain, digoxin and their derivatives),[3] but some toxic organic compounds (pesticides, herbicides)[4] and certain metal ions[5] also show selective effects on this enzyme.

For the investigation of different effects on the enzymatic activity, the enzyme isolated from animal tissue is usually used, but the enzyme immobilized on porous or non-porous

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supports could in many cases serve as a better model. It is well known that enzymes immobilized on porous or non-porous supports show better stability. In addition, immobilization allows operational stability and no contamination of the solution treated by enzymes, because the immobilized enzyme can be easily separated from the solution.[6,7]

A literature survey offers several methods (physical adsorption, covalent bonding, crosslinking, inclusion and encapsulation) for the immobilization of enzymes. Physical adsorption, involving non-covalent interactions, such as ionic, metal bridge and hydrophobic binding, is the most cost effective and simplest of all immobilization methods.[8] In such cases, immobilization can be achieved simply by incubating a given amount of SPM preparation with the support material under specific conditions with respect to temperature, pH, and ionic strength.

Although there is a lack of data in the literature about Na,K-ATPase immobilization, a few publications concerning this problem have appeared recently. A method for the functional immobilization of Na,K-ATPase-rich membrane fragments isolated from pig kidney and from the rectal gland of dogfish, on planar metal oxide waveguides was developed by Pawlak *et al.,* 1998.[9] immobilization of functional membrane fragment in a flat configuration with fully preserved protein activity was achieved. The aspects of sidedness of specific binding sites can be determined under these conditions. Linder *et al.*, 1999[10] immobilized purified membrane fragments containing Na,K-ATPase isolated from kidney tissue by adsorption on uncoated furan polymer substrates. The immobilized membrane fragments were an appropriate substrate for scanning force microscopy, with low surface roughness. The immobilization of SPM Na,K-ATPase on polystyrene microtiter plates was based on the adsorption of membrane fragments.[11] An enlarged time and temperature stability of the enzyme was achieved and the functional characteristics did not change markedly upon immobilization.

In this study the adsorption of SPM Na,K-ATPase on different solid supports, nitrocellulose, glass fiber and polyvinylidene fluoride membranes, was investigated. The aim of the work was to compare the influence of these different supports and conditions of immobilization of SPM Na,K-ATPase with previous results. It was also of interest to investigate the influence of some organic compounds and metal salts on the Na,K-ATPase activity of immobilized SPM.

EXPERIMENTAL

Chemicals

All the biochemicals were of analytical grade and were purchased from the Sigma Chemicals Co. The nitrocellulose and glass fiber membranes were purchased from Whatman (Maidstone, England), and the polyvinylidene fluoride membrane from Millipore (Bedford, Massachusetts, USA).

Synaptosomal plasma-membrane preparation

The SPM were isolated from the whole brain of 3-month-old male Wistar albino rats according to the method of Cohen *et al.*[12] The preparation procedure and the purity of the SPM preparations have been described previously.[13] The level of mitochondrial contamination, based on both morphological and biochemical markers, was less than 7 %.[14] The SPMs were stored at –70 ºC until use. The protein content was determined by a modified Lowry *et al*. method.[15]

Enzyme purification

The SPM Na,K-ATPase was partially purified by solubilization with sodium dodecyl sulphate (SDS).[16] An amount of 0.2 mg SDS per mg of SPM protein was used.[17] The solubilizied SPMs were stored at 0 ºC until use.

Determination of enzyme activity

The activity of Na,K-ATPase in native and immobilized SPMs was followed. The ATP-ase activity of native SPMs in a final volume of 200 µl was determined in a standard assay. The reaction medium contained 50 mM Tris-HCl (pH 7.4), 100 mM NaCl, 20 mM KCl, 5 mM $MgCl_2$, 2 mM ATP, 20 µg SPM proteins with or without inhibitor (β-methyldigoxin, Cd^{2+} or Hg^{2+} salt in concentration range of 10^{-7} – 10^{-4} M). β-Methyldigoxin was previously dissolved in 0.6 % ethanol. After preincubation for 5 min at 37 ºC, the reaction was initiated by addition of ATP and stopped after 15 min by the addition of 22 µl ice cold 3 M PCA ($HClO_4$) and immediate cooling on ice. The released inorganic orthophosphate (P_i) liberated from the hydrolysis of ATP was measured using a modified spectrophotometric procedure.[18]

The results are expressed as mean % enzyme activity compared to the corresponding control value ± S.E.M, of at least three independent experiments performed in triplicate. The specific activity is expressed in µmol P_i/min/mg protein.

Enzyme immobilization

Nitrocellulose, glass fiber and polyvinylidene fluoride membranes were used as carriers for the adsorption of SPM Na,K-ATPase. The PVDF discs were first activated with methanol. The SPMs were adsorbed on the membrane by incubation and under vacuum. The desired concentration of SPM were applied on the membranes discs (1 cm radius) and after 30 min of incubation at 0 ºC, the filters were washed with 0.5 ml 5 mM Tris, pH 7.4. The SPMs adsorbed under vacuum were spotted directly onto the membranes using a Bio-Rad dot blot apparatus, after which the membranes were washed with 3 ml 5 mM Tris, pH 7.4. The supports were then tested for Na,K-ATPase activity.

RESULTS AND DISCUSSION

Effect of SPM purification on the Na,K-ATPase activity

The Na,K-ATPase was partially purified by solubilization of the SPMs with SDS. The use of SDS at the optimal ratio to the protein (0.2 mg SDS/mg protein)[17] resulted in an increase of the enzyme specific activity by 39.423 ± 0.933 %. The enzyme preparation so obtained was used in the immobilization experiments.

Na,K-ATPase activity of the immobilized SPMs as a function of incubation temperature

The Na,K-ATPase activity of solubilized SPMs immobilized on the different supports was investigated at 0 ºC and 20 ºC. The activities of 25 µg of SPM applied on the three different kinds of supports are presented in Table I.

TABLE I. Na,K-ATPase activity of solubilized adsorbed SPM (aSPM) on different supports at 0 ºC and 20 ºC. The control value is the enzyme activity of solubilized native SPM

Supports	Adsorption of SPM at 0 ºC (% of control)	Adsorption of SPM at 20 ºC (% of control)
NC	12.763 ± 0.800	14.354 ± 0.539
PVDF	8.780 ± 0.748	11.012 ± 0.609
GF	5.632 ± 0.740	9.203 ± 0.515

The results show that the percent of residual activity on all supports is higher after incubation at 20 ºC than at 0 ºC. Also, the SPMs which were immobilized on the NC membrane show a higher activity than those immobilized on the PVDF and GF membranes. These results suggest that nitrocellulose is the most suitable support for Na,K-ATPase immobilization. The mechanism of protein immobilization on nitrocellulose can be ascribed to both charge-charge interactions and weak secondary forces (van der Waals, especially hydrogen interactions).[19] The hydrophobic binding of the enzyme to the PVDF and GF membranes most likely causes the decrease of enzyme activity. It was shown earlier[20] that proteins are more efficiently adsorbed to the PVDF than to the GF membrane.

Nitrocellulose membranes were used in further experiments for the optimization of the immobilization conditions.

Na,K-ATPase activity of immobilized SPMs as a function of the applied amount of protein

The Na,K-ATPase activity after immobilization was followed as a function of the amount of the protein applied on the nitrocelullose membrane discs. The applied amount of SPM ranged from 15 μg to 100 μg per disc. The dependence of the activity on the amount of protein applied onto the support is shown in Fig. 1. The same enzyme activity was obtained with 15 μg, 20 μg and 25 μg of applied protein. The addition of higher quantities of protein resulted in a reduction of the activity, probably due to steric hindrance resulting from the excessive concentration of protein molecules on the carrier surface.[19]

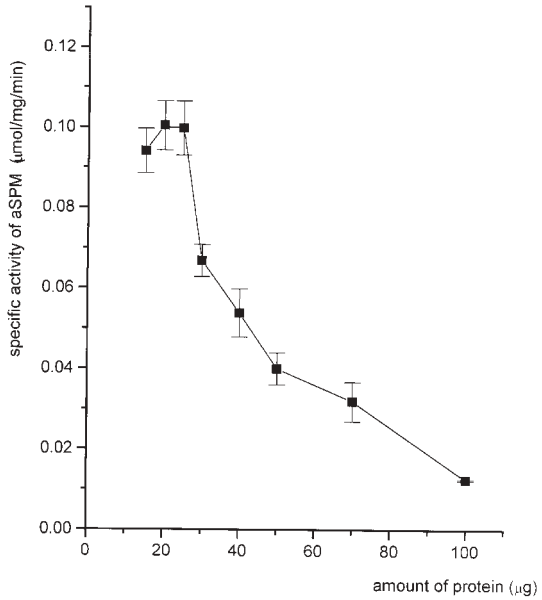

Fig. 1. Na,K-ATPase specific activity of the retained amount of adsorbed SPM (aSPM) on a nitrocellulose membrane at 0 ºC. The reaction conditions are as described in the Experimental. The symbols present the mean ± S.E.M. of at least three experiments performed in triplicate.

Since the results showed optimal binding using 15 μg, 20 μg or 25 μg of protein per disc in the incubation medium, the amount of 25 μg of SPM protein was applied in further experiments.

Stability of the immobilized enzyme as a function of storage duration at 20 ºC

The time stability of Na,K-ATPase immobilized to the different supports was tested at 20 ºC for a total period of 72 h. The influence of the immobilization support on the activity of the SPM is presented in Fig. 2. While Na,K-ATPase in the native preparation (non-adsorbed, nSPM) completely lost its activity after a period of 48 h, the enzyme SPM adsorbed (aSPM) on nitrocellulose showed a loss of 20 % in the first 4 h and a total loss of activity within 72 h. The Na,K-ATPase adsorbed to the GF membrane is stable over the same period of time as the native preparation, but the percent of retained activity was much less. The activity dropped by about 30 % in the first 2 h and then the loss of activity was 70 % within 24 h. The enzyme adsorbed to the PVDF membrane retained about 80 % of its activity during the first 3 h of storage but had lost all its activity after 24 h.

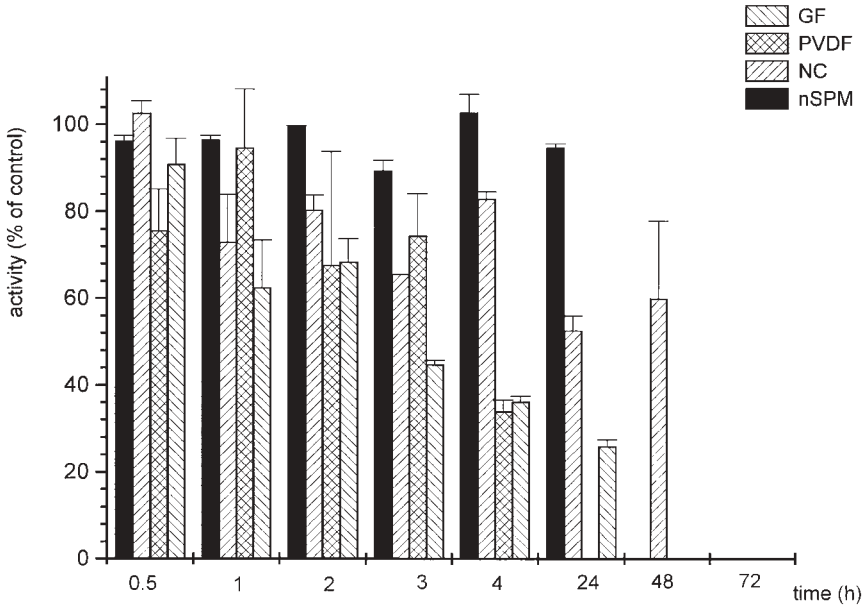

Fig. 2. Stability of the Na,K-ATPase activity of native SPM (nSPM) and adsorbed SPM (aSPM) on different supports at 20 ºC as a function of time after the adsorption (hours). The control was the enzyme activity of native SPM measured in the standard assay as described in the Experimental. The symbols present the mean ± S.E.M. of at least three experiments performed in triplicate.

The best results in terms of enzyme stability were obtained with the nitrocellulose support. The Na,K-ATPase adsorbed to the NC membrane was stable 24 h longer than the native preparation at 20 ºC. The results obtained from these experiments suggest that nitrocellulose is the best support among the investigated supports for Na,K-ATPase immobilization by adsorption.

Effect of Cd^{2+}, Hg^{2+} and β-methyldigoxin on the activity of immobilized enzyme

The activity of immobilized Na,K-ATPase was tested after exposure to solutions containing $CdCl_2$, $HgCl_2$ and β-methyldigoxin in the concentration range from 10^{-10} M to

10^{-4} M. Both metal ions as well as digoxin induced a concentation dependent inhibition of the enzymatic activity. The results presented in Fig. 3 indicate that the immobilized enzyme is more sensitive to Cd^{2+} and Hg^{2+} ions than the native preparation. The unchanged sensitivity of aSPM to β-methyldigoxin is presented in Fig. 4. A possible explanation of this result could be that the adsorption of Na,K-ATPase to NC membrane changes the conformation of the protein. Also, it is possible that orientation of the membrane fragments after adsorption prevents the approach of β-methyldigoxin molecules to the ouabain binding site. All this resulted in a decrease of the sensitivity of the immobilized enzyme to β-methyldigoxin.

The work described in this paper is an attempt to (i) immobilize a partialy purified SPM preparation to three different supports: NC, PVDF and GF membranes, (ii) to examine the stability of immobilized SPM at 20 ºC and (iii) to verify the sensitivity of immobilized SPM.

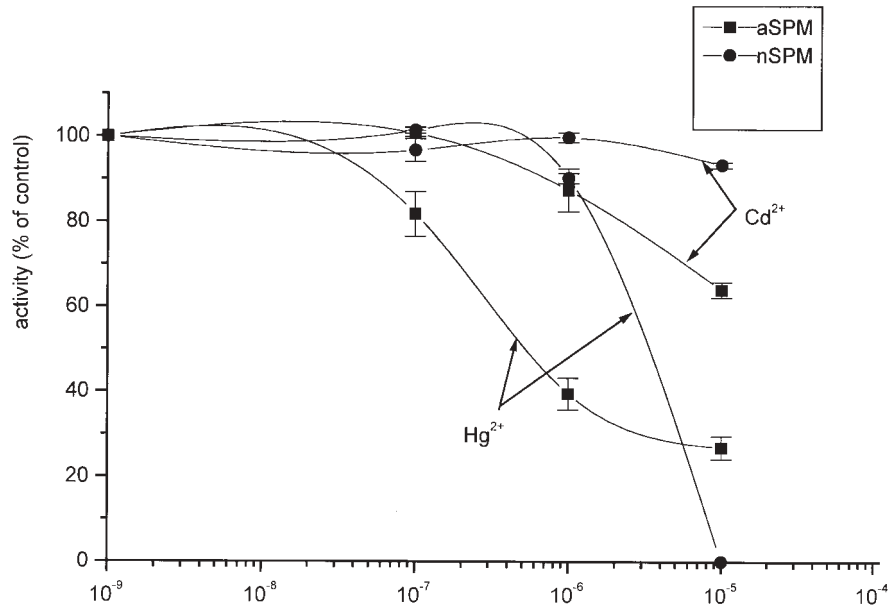

Fig. 3. Inhibition of Na,K-ATPase activity by Cd^{2+} and Hg^{2+} in native (nSPM) and adsorbed SPM (aSPM) on a nitrocellulose membrane. The enzyme activity is expressed as mean % activity relative to the corresponding control value in the absence of inhibitors. The symbols present the mean % value ± S.E.M. of at least three experiments performed in triplicate.

The partial purification of Na,K-ATPase by solubilization of SPM with SDS resulted in an increase of the specific enzyme activity by approximately 40 %, which is comparable with literature data concerning SPM purification.[21] The activation of microsomal Na,K-ATPase with SDS is explained by decreasing the latent activity as a result of an increase in the permeability of the vesicles for the substrate,[22] but it is also accompanied with the removal of a significant amount of, presumably, peripheral membrane proteins.

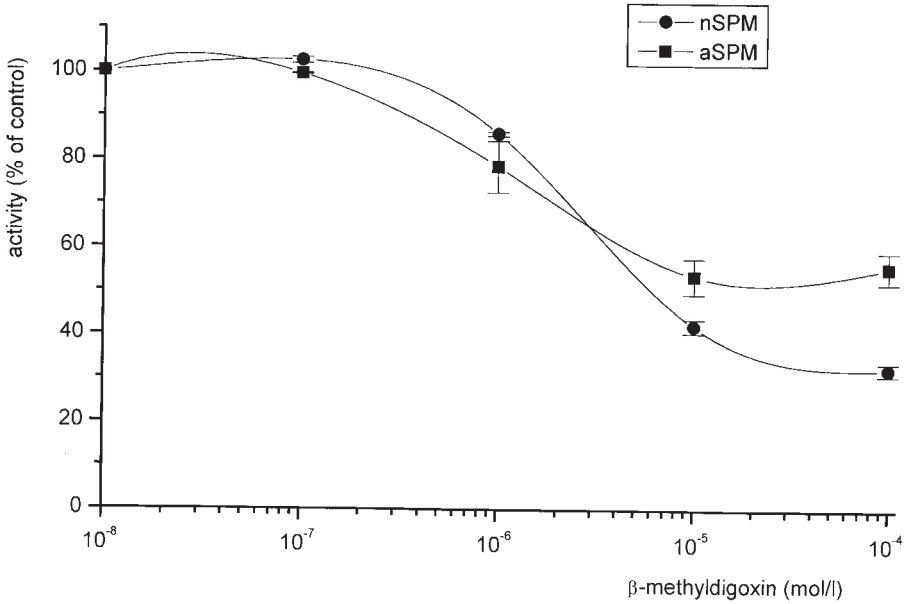

Fig. 4. Inhibition of Na,K-ATPase activity by β-methyldigoxin in native (nSPM) and adsorbed SPM (aSPM) on a nitrocellulose membrane. The enzyme activity is expressed as mean % activity relative to the corresponding control value in the absence of β-methyldigoxin. The symbols present the mean % value ± S.E.M. of at least three experiments performed in triplicate.

The results of the present study show that nitrocellulose is the best support for Na,K-ATPase SPM adsorption. Adsorption harbors about 14 % of the SPM control activity, which is comparable with literature data concerning protein adsorption on other support materials. It has been reported that the immobilization efficiency ranges from 0.1 to 40 % depending on the chemical nature of the support and applied immobilization method.[23,24] Although PVDF membranes are often used for the adsorption of the enzymes, usually in immunoblotting applications, the results presented here show that nitrocellulose is the support which harbors higher enzyme activity. It can be assumed that the reason for this finding lies in the hydrophilic nature of nitrocelullose, because a lower percent activity is preserved after adsorption of the enzyme on hydrophobic supports, PVDF and GF membranes.

The higher stability of the enzyme in SPM adsorbed to nitrocellulose compared to that adsorbed to PVDF membranes was a confirmation that nitrocellulose is the optimal support for immobilization. The finding that the enzyme is more stable in the adsorbed SPM than in the native SPM at 20 ºC indicates that the increased stability is probably simply due to a lack of microbial and proteolytic degradation, although stabilization of the enzyme conformation can not be excluded.

The significant advantage that have been demonstrated in the use of aSPM is the changed enzyme sensitivity in comparison with nSPM. The affinity of Na,K-ATPase for inhibitors such as Hg^{2+} and Cd^{2+} after adsorption was higher. It is well known that cadmium and mercury ions inhibit the Na,K-ATPase activity by binding with cystein, because

they have a great affinity for sulfhydryl (SH) groups.[25] It is possible that because of the conformation changes introduced by adsorption, the SH groups are more exposed to the inhibitory ions. The reason for the lower sensitivity of aSPM compared with nSPM for to β-methyldigoxin could be the changed conformation of the ouabain binding site to which β-methyldigoxin is binding. It is possible that orientation of the SPM fragments during adsorption prevents the approach of large inhibitory molecules, such as β-methyldigoxin, to the binding site.

CONCLUSION

The adsorption of SPM to nitrocellulose membranes is a satisfactory immobilization method which improves the stability and sensitivity of Na,K-ATPase to divalent heavy metal ions, such as Cd^{2+} and Hg^{2+}. The use of a dot blot apparatus for enzyme immobilization enables a large number of samples of aSPM to be obtained in a short period of time. These results suggest the possibility of using immobilized Na,K-ATPase SPM for the detection of these ions in different samples.

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ИЗВОД

ИМОБИЛИЗАЦИЈА Na,K-АТРазе ИЗОЛОВАНЕ ИЗ СИНАПТИЧКИХ ПЛАЗМА– МЕМБРАНА МОЗГА ПАЦОВА

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Делимично пречишћена Na,K-АТРаза синаптичких плазма–мембрана (SPM) мозга пацова имобилизована је адсорпцијом на нитроцелулозне (NC), поливинилиден-флуорид (PVDF) мембране и мембране од стаклених влакана (CB). Активност ензима делимично пречишћеног солубилизацијом SDS-ом повећана је око 40 %. Највећи проценат активности (14 %) ензим задржава после 30 минута адсорпције SPM на 20 ºC, под вакуумом, са 25 μg протеина по нитроцелулозном диску. Na,K-АТРаза имобилизована на нитроцелулозној мембрани стабилна је 72 сата на 20 ºC. Адсорпцијом, осетљивост ензима на инхибицију Hg^{2+} и Cd^{2+} се повећава. Резултати показују да се имобилизована Na,K-АТРаза SPM може користити за детекцију токсичних металних јона у различитим узорцима.

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