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Stabilization of trypsin by association to plasma membranes: Implications for tryptic cleavage of membrane-bound Na,K-ATPase

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Abstract

Tryptic cleavage has been a potential method for studying the structure and mechanism of many membrane transport proteins. Here, we report tight association of trypsin to pig kidney plasma membranes enriched in Na,K-ATPase. Trypsin also associated with protein-free vesicles prepared from plasma membrane lipids. Membrane-associated trypsin was found to be highly resistant to autolysis and insensitive to inhibition by PMSF. Na,K-ATPase substrate ions differentially influenced the level of trypsin membrane association. Thus, NaCl significantly increased trypsin membrane association compared to KCl. The ions seem to exert direct effects on the membrane independent of their effects on protein conformation. Bicarbonate anions, which detach peripheral membrane proteins, efficiently released trypsin from the membrane. Trypsin membrane association was found to enhance the cleavage of the Na,K-ATPase γ-subunit. Comparison between membranes from shark rectal gland and pig kidney showed that trypsin association was significantly higher in the former. This was found to be partly due to the presence of higher cholesterol levels in the membrane. In conclusion, the differential membrane association of trypsin may affect the outcome of proteolytic cleavage of membrane-bound proteins.

Keywords: Membrane-bound protein; Trypsin; Membrane; P-type ATPase; Na,K-ATPase

1. Introduction

The Na,K-ATPase is a member of the P2-type ATPases family, which includes gastric H,K-ATPase and sarcoplasmic reticulum Ca-ATPase (see Ref. [1] for review). The Na,K-ATPase is a heterodimeric membrane protein that pumps 3 Na^+ ions out- and 2 K^+ ions into the cell, at the expenditure of the energy derived from hydrolysis of one ATP molecule. It consists of a catalytic α-subunit that undergoes ions- and ATP-dependent conformational transitions coupling ATP hydrolysis to ion transport, and a glycosylated β-subunit that is important for proper expression and function of the overall enzyme complex (see Ref. [2] for review).

Since its discovery [3], the Na,K-ATPase has been a subject of extensive investigations (for review see Ref. [4]). Proteolytic cleavage has been a potential strategy to study the structure and mechanism of this enzyme. Early studies showed that the tryptic cleavage pattern of the α-subunit depends on the presence of substrate ions in the proteolytic assay, which reflects the ion-dependent conformational states of the α-subunit, resulting in exposure of specific trypsin cleavage sites on the protein [5]. Thus, tryptic cleavage of the α-subunit in the presence of Na^+ (where the enzyme adopts an E1 conformation) produces two cleavages at the N-terminal half of the α-subunit whereas cleavage in the presence of K^+ (where the enzyme adopts an E2 conformation) produces a single cleavage in the middle part of the polypeptide [6]. Limited proteolytic digestion has also been successfully used to cleave a specific domain in a functional hetero-oligomeric enzyme complex, aiming at studying the effect of that particular domain on the overall activity of the complex. This could be achieved by trypsin incubation under specific conditions (e.g., varying ionic

Abbreviations: E1, enzyme conformation that has high affinity for Na^+; E2, enzyme conformation that has high affinity for K^+; ECL, enhanced chemiluminescence; PBS, phosphate-buffered saline; PMSF, Phenyl methyl sulfonyl fluoride; PVDF, polyvinylidene fluoride; SDS-PAGE, sodium dodecyl sulphate-poly acrylamide gel electrophoresis; Tricine, N-[2-hydroxy-1,1-bis(hydroxymethyl) ethyl]glycine TCA, trichloroacetic acid; Tween-20, polyoxyethylene sorbitan monolaurate

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strengths [7], treatment with very low trypsin to protein ratios, and/or incubation at low temperatures [8]). This approach was applied to study the functional effects of different integrated domains on the overall activity of P-type ATPases [8–10].

The ion occlusion domain of Na,K-ATPase was characterized by extensive proteolytic cleavage of the membrane-bound protein. Incubation of the enzyme with trypsin in the presence of K^+ (typically 1 h at 37 °C at a trypsin to protein ratio of 1:5, w/w) resulted in removal of cytoplasmic domains of the α-subunit, producing a membrane-bound C-terminal 19 kDa fragment (containing transmembrane domains M7-M10), pairs of transmembrane hairpins (M1M2, M3M4, and M5M6), and an intact or cleaved β-subunit [11], referred to as the "19 kDa membranes". In this preparation, ion occlusion is preserved but ATP-dependent functions are lost. Without occluded K^+, trypsin further degrades components of this preparation.

The experimental conditions, under which tryptic cleavages occur greatly, determine the outcome of the proteolytic degradation. Indeed, a widely variable sensitivity to trypsin of Na,K-ATPase preparations from different sources has been observed: for instance, the C-terminal 19 kDa fragment from shark rectal gland was found to be extremely sensitive to tryptic attack when compared to the pig kidney enzyme [12,13]. Although sequence differences can explain the different cleavage patterns, it does not satisfactorily explain the significant difference in the cleavage rate at the same tryptic site. Another striking observation is the increased rate of tryptic cleavage of the 19 kDa C-terminal fragment of the α-subunit in the presence of Na^+ when compared to K^+ [12]. In this study, we take account of the plasma membrane as a previously unrecognized factor contributing to the outcome of tryptic cleavage of membrane-bound Na,K-ATPase (and possibly other membrane-bound proteins). We found that a substantial fraction of trypsin associates with the membrane. The membrane-associated trypsin is insensitive to phenyl methyl sulfonyl fluoride (PMSF), autolyses at a slow rate, is reversibly inhibited with relatively high concentrations of soybean trypsin inhibitor, and can be efficiently released by a bicarbonate wash, typical of a peripheral membrane protein.

2. Materials and methods

2.1. Materials

TPCK-treated trypsin, PMSF, and cholesterol were purchased from Sigma. Different trypsin preparations were used (catalog number T-1426 (Lot 104K7575) and catalog number T-8642 (Lot 114H7100). Enhanced chemiluminescence (ECL) reagents and polyvinylidene fluoride (PVDF) membranes were from Amersham Biosciences. Phosphatidylcholine (PC) was from Avanti. All other chemicals were of the highest analytical grade commercially available.

2.2. Na,K-ATPase preparation

In this study, pig kidney plasma membranes enriched in Na,K-ATPase were used (in the results presented in Fig. 5, membranes from shark rectal gland were also used). Purification of membrane fragments was as previously described [14]. The membranes were suspended in 20 mM histidine buffer, pH 7.0 containing 25% glycerol, and stored at −20 °C at a protein concentration of 4–5 mg/ml. Protein concentration was determined using Petersen's modification of the Lowry method [15], using bovine serum albumin as a standard.

2.3. Proteolytic cleavage

Membrane-bound enzyme was incubated with trypsin (trypsin to protein ratio of 1:5, w/w) in the presence of 20 mM histidine buffer, pH 7.0, 2 mM EDTA, 5% glycerol, and 20 mM KCl. The proteolytic reactions were started with the addition of trypsin, allowed to proceed for 1 h at 37 °C, and terminated with 5-fold soybean trypsin inhibitor. The mixture was diluted 70-fold in 25 mM imidazole and centrifuged at 200,000×*g* for 45 min (4 °C). The membranes were finally suspended in 20 mM histidine containing 25% glycerol and immediately used in further incubations. Alternatively, the reaction was stopped by 30-fold dilution in 100 mM $KHCO_3$ and centrifuged as above. In some cases, the proteolytic reactions were stopped with SDS sample buffer containing 0.2% TCA, to insure complete inactivation of trypsin.

2.4. Liposome preparation

For preparation of PC/cholesterol liposomes of defined lipid composition, lipids dissolved in chloroform were mixed and liposomes were prepared as previously described [16].

2.5. Gel electrophoresis, staining, and immunoblotting

Proteins were separated using Tricine-based SDS-Polyacrylamide Gel Electrophoresis (SDS-PAGE; 3% stacking gel, 9% intermediate, and 16% resolving gel) as previously described [17,18]. 7–21 μg of the post-tryptic products were loaded onto each lane in the gel and electrophoresis was allowed to proceed overnight. Proteins were either visualized with Coomassie staining or analyzed by Western blotting. For immunoblotting, proteins in SDS gels were transferred to PVDF membranes, washed three times with phosphate-buffered saline (PBS) containing 5% polyoxyethylene sorbitan monolaurate (Tween-20), and incubated over night at room temperature with the primary antibody. The PDVF membranes were washed again with PBS and incubated with goat anti-rabbit antibody for two h. After washing, the protein fragments were detected using ECL reagents. A specific α-subunit antibody, α1002–1016, was used to detect the 19 kDa C-terminal fragment (a generous gift of J. V. Møller). An antibody against the C-terminus of the γ-subunit (a gift of O. Hansen) was used. Scanning and intensity determination of gels and immunoblots was performed using ImageQuant TL image analysis software (Amersham Biosciences, UK).

2.6. Sequence analysis

Mass spectrometry was performed at Alphalyse A/S (http://www.alphalyse.dk). Edman degradation was performed at the University of Nebraska Medical Center (http://www.unms.edu/pscf).

3. Results

We have been using limited trypsin cleavage assays to study the mechanism and regulation of Na,K-ATPase [8,13]. The present study was based on earlier observations in which limited-tryptic membrane preparations (that were prepared, washed twice, and stored at −20 °C) were found to undergo further proteolytic degradation during later handling. Suspension of the post-tryptic membranes in buffers containing PMSF did not prevent enzyme degradation, whereas soybean trypsin inhibitor (1–2 mg/ml) prevented the degradation (see below). However, it was found that the addition of such relatively high concentrations of trypsin inhibitor decreases Na,K-ATPase activity (at 2 mg/ml trypsin inhibitor the ouabain-dependent ATPase activity decreased by 40%). We initially anticipated

that traces of trypsin could be present in the post-tryptic membranes after centrifugation. Thus, post-tryptic membranes were thoroughly washed several times in imidazole buffer to remove these traces. Unexpectedly, several washings did not result in trypsin dissociation from the membrane, indicating tight binding. Indeed, membrane-bound trypsin-like enzymes were previously purified and characterized in different cell types [19–22]. The following experiments were thus conducted to investigate the possibility that the commercially available trypsin from bovine pancreas associates with pig kidney plasma membranes.

3.1. A fraction of trypsin is bound to the plasma membrane

Fig. 1A (lane 1) shows SDS-PAGE of exhaustively trypsinized membrane-bound Na,K-ATPase from pig kidney. Staining with Coomassie revealed the presence of a band running at about 23 kDa in the gel. This protein does not belong to the α-subunit of Na,K-ATPase since the largest fragment produced after extensive proteolysis of the α-subunit has a molecular mass of 19 kDa [11]. This membrane-associated protein was not released after 4 successive washings (each wash involved homogenization of the pellet in histidine/glycerol buffer, 70-fold dilution in cold imidazole followed by high-speed centrifugation in a Ti45 Beckman rotor, at 4 °C).

The gel strip containing the 23-kDa band was cut off the gel and analyzed by mass spectrometry. MALDI-TOF analysis showed unambiguously that the membrane-associated protein is trypsin (Fig. 1B). All identified peptides had sequences identical to cationic beta trypsin from bovine pancreas (E.C.3.4.21.4, GenBank™ accession no. P00760). It should be stressed that the identified peptides are not emerged from the trypsin precursor used for the in-gel digestion of the 23-kDa protein, which appears as a standard hit in the spectrum. Sequence analysis by Edman degradation also confirmed that the 23-kDa protein was trypsin. Thus, a fraction of trypsin associated with the membrane up on incubation of at 37 °C, a routine step in the preparation of "19 kDa membranes" [11,13,23].[1] The membrane-associated trypsin fraction has the same molecular mass as the native trypsin (i.e., it is not an autolyzed trypsin fragment that accumulated during incubation), indicating that it could be functionally active.

In a previous study, a 23-kDa protein was localized in SDS gels after extensive cleavage of membrane-bound Na,K-ATPase and solubilization of the post-tryptic membranes with the non-ionic detergent $C_{12}E_{10}$, this protein was isolated from the soluble fraction by centrifugation and was exclusively present in the insoluble fraction. The identity of that protein was not reported [24]. Similarly, we solubilized the "19 kDa membranes" with the non-ionic detergent $C_{12}E_8$ at 0 °C in the presence of 20 mM K^+ and the soluble and particulate fractions were isolated with high-speed centrifugation and resolved with SDS-PAGE. This showed, as previously reported [24], complete solubilization of the 19 kDa C-terminal fragment (Fig. 1A, lane 2), whereas most of the trypsin remained in the pellet (Fig. 1A, lane 3).

Earlier reports have also considered the fact of trypsin being 'adsorbed' to pig kidney membranes and it was reported that several incubations at 37 °C in the presence of K^+ (and trypsin

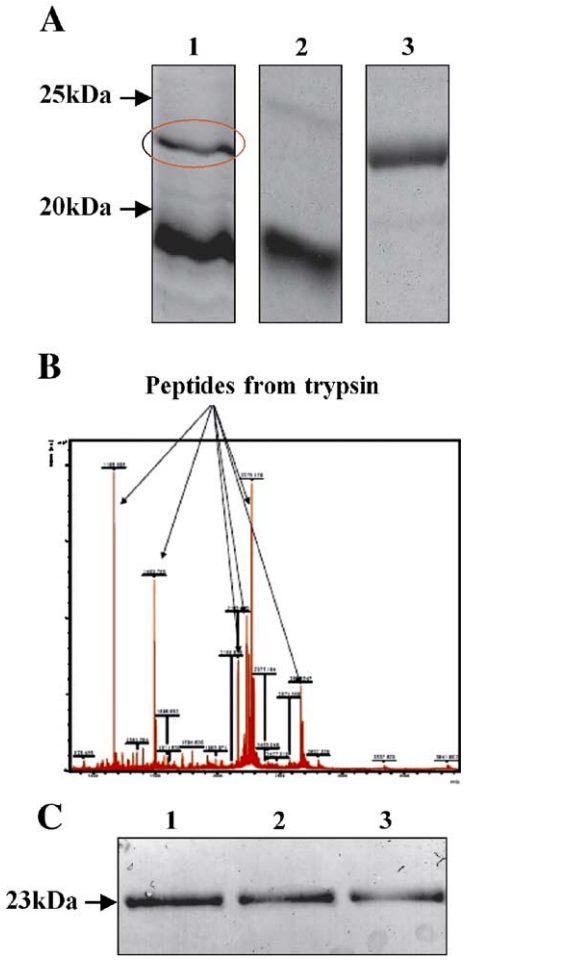

Fig. 1. Membrane association of trypsin. (A) Coomassie-stained SDS-PAGE of post-tryptic Na,K-ATPase preparation, showing the 19-kDa C-terminal fragment of the α-subunit and a 23-kDa protein marked by oval (lane 1). Solubilization of the post-tryptic fragment in the presence of K^+ followed by centrifugation isolated the soluble material containing the 19-kDa C-terminal fragment (lane 2), whereas the 23-kDa protein exclusively remained in the pellet (lane 3). (B) The band representing the 23-kDa protein was cut off the gel, suspended in distilled water, subjected to in-gel trypsin digestion followed by MALDI-TOF analysis, where only one hit was found. Shown is the MALDI-TOF spectrum of the protein. All identified peptides were from the monoisopropylphosphoryl-inhibited trypsin. (C) Post-tryptic membranes containing the membrane-associated 23 kDa protein were further incubated at 37 °C in the presence of 10 mM Tris for 0, 20 (lane 2) or 40 min (lane 3) showing that large fraction of the protein still associates with the membrane. By measuring the area intensities of the bands and data analysis using single exponential decay equation, the rate constant for dissociation of trypsin from the membrane was found to be 0.022 ± 0.01 min^{-1}.

[1] Trypsin–membrane association is clearly seen following preparation of the "19 kDa membranes". This is mainly because of the large trypsin to protein ratio used to prepare the "19 kDa membranes". Thus, the membrane-associated trypsin becomes well above the detection level in SDS-gels after electrophoresis of the post-tryptic membranes and Coomassie staining (e.g., Fig. 1, lane 1). As mentioned above, trypsin membrane association can also be detected after limited tryptic cleavage. Since limited tryptic cleavage involves treatment with substantially lower trypsin concentrations, membrane-associated trypsin is difficult to detect in an SDS-gel with Coomassie staining but its presence is indicated from the effects it has on Na,K-ATPase.

inhibitor) prevented further cleavages of the enzyme [23,25], we fully confirm this. Although this might be helpful in studies on the "19 kDa membranes", however, in studies employing limited tryptic cleavage of the intact enzyme several times incubation of the enzyme at 37 °C is not desirable since it will result in very rapid cleavages at cytoplasmic domains of the α-subunit [8]. The association of trypsin to the membrane was studied by performing experiments in which the washed post-tryptic membranes were incubated at 37 °C followed by centrifugation to isolate the membrane-associated trypsin, showing that incubation for up to 40 min did result in very little dissociation of trypsin from the membrane (Fig. 1C). In contrast, analysis of the time dependence of trypsin autolysis in the absence of membranes showed no intact soluble protease at 20 min incubation (data not shown).

The membrane-associated trypsin digested the 19-kDa C-terminal fragment completely following removal of K^+ from the medium (Fig. 2, lane 1) but addition of trypsin inhibitor protected the fragment (Fig. 2, lane 2). Interestingly, addition of 1 mM PMSF to the incubation mixture did not result in protection of the 19 kDa fragment (Fig. 2, lane 3). This is an important observation because in many tryptic cleavage studies on membrane-bound proteins, addition of trypsin inhibitor is not preferred and PMSF is used instead.

3.2. Ion-dependent membrane association of trypsin to the membrane

It has been well established that incubation of Na,K-ATPase with trypsin in the presence of Na^+ results in much less protection of the pig kidney C-terminal 19 kDa fragment compared to the well-known protection provided by the presence of K^+ [26]: following incubation of the Na^+-occluding Na,K-ATPase with trypsin, the quantity of the resulting 19 kDa fragment was found to be only 30–40% of that produced after tryptic cleavage of the K^+-occluding protein (see Fig. 1 in reference [26]). Furthermore, incubation of enzyme from shark rectal glands with trypsin in the presence of Na^+ results in complete cleavage of the 19 kDa fragment [12], indicating that Na^+ is not as effective as K^+ in protecting the 19 kDa fragment against trypsin attack. This is in contrast to the fact that Na^+ was found even more efficient in protecting the "19 kDa membranes" against thermal inactivation of ion occlusion [27], suggesting that the destabilization of components of the "19 kDa membranes" in the presence of Na^+ occurs during trypsin incubation. Therefore, we compared between K^+ and Na^+ in terms of their ability (1) to protect the 19 kDa fragment and (2) to induce trypsin-release from the membrane. Thus, we first incubated the "19 kDa membranes" with Na^+ or K^+ at 37 °C and then re-incubated it after removal of the ion. Incubation of the post-tryptic "19 kDa membrane" preparation at 37 °C in the presence of K^+ protected the 19 kDa fragment from trypsinolysis (Fig. 3, lane 1) and also resulted in significant trypsin release from the membrane, as evidenced from the lack of cleavage of the 19 kDa fragment up on re-incubation of the membranes in the absence of K^+ (Fig. 3, lane 2, and see also reference [27]). Interestingly, incubation of the "19 kDa membranes" at 37 °C in the presence of 20 mM Na^+, although resulted in partial protection of the 19 kDa fragment against tryptic attack (Fig. 3, lane 3), it did not result in dissociation of trypsin from the membrane, as evidenced from the rapid degradation of the fragment following removal of the ion (Fig. 3, lane 4), strongly indicating that both ions are equally efficient in protecting the 19 kDa fragment, but K^+ seems to be more efficient than Na^+ in inducing trypsin membrane-release.

We further investigated the above possibility by studying whether the ion itself would affect trypsin association to the membrane independent of the presence of protein. We show that the presence of different ions can significantly influence trypsin association to the membrane (Fig. 1, Supplemental Material). In particular, NaCl was found to increase trypsin membrane association significantly compared to KCl. Bicarbonate ions, which detach peripheral membrane proteins, significantly reduced trypsin membrane association.

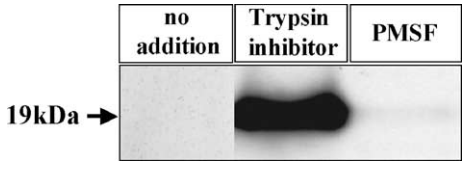

Fig. 2. The membrane-associated trypsin cleaved the 19 kDa C-terminal fragment. The "19 kDa membranes" was incubated for 15 min at 37 °C in the presence of 20 mM Tris, 1 mM EDTA (lane 1), and either trypsin inhibitor (lane 2) or PMSF (lane 3). After incubation, the mixtures were treated with SDS sample buffer, resolved by SDS-PAGE, and immunoblotted with the NKA1002-1016 antibody. Representative of two independent experiments is shown.

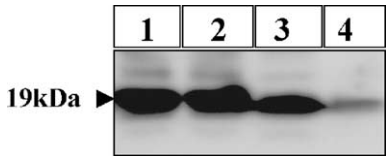

Fig. 3. Effect of K^+ and Na^+ on the stability of the 19 kDa fragment. Immunoblot showing the stability of the 19 kDa fragment after pre-incubation in the presence of either KCl or NaCl. Post-tryptic membranes were incubated for 15 min at 37 °C in the presence of 20 mM KCl and an aliquot was resolved with SDS-PAGE and immunoblotted (lane 1). K^+ was then removed and the membranes were re-incubated in the absence of K^+ for 15 min at 37 °C and proceeded like above (lane 2). Incubation of the membranes with NaCl resulted in somewhat less protection against cleavage with the membrane-associated trypsin (lane 3) but re-incubation of the membranes after removal of Na^+ resulted in complete cleavage of the 19 kDa fragment (lane 4).

3.3. Cleavage patterns of Na,K-ATPase subunits may depend on trypsin membrane association

Because Na,K-ATPase substrate ions caused differential association of trypsin to the membrane, we sought to determine whether or not trypsin cleavage of subunits of the Na,K-ATPase might be influenced by trypsin membrane association. To do this, we performed limited tryptic cleavage of Na,K-ATPase subunits in the absence or in the presence of bicarbonate, i.e., under conditions favoring or limiting trypsin membrane association, respectively. The kidney-specific γ-

subunit is an ancillary protein associated with Na,K-ATPase and present in two splice variants, γa and γb (for review see reference [28]). Previous studies have shown that the γ-subunit remained intact after formation of the "19 kDa membranes" but addition of 1 mM Mg^{2+} resulted in cleavage of the slow migrating γa protein [29]. Since Mg^{2+} significantly increases trypsin membrane association (see Fig. 1, Supplemental Material) we wondered whether the sensitivity of the γ-subunit to the protease could be decreased by addition of bicarbonate. As seen in Fig. 4, adding 2 mM Mg^{2+} in the tryptic medium resulted in cleavage of both γ variants and the cleavage was largely abrogated by the addition of 20 mM NH_4HCO_3.

3.4. Differences in sensitivity to the protease of different enzyme sources may in part be attributed to differential cholesterol content

Results in Fig. 5A compare the effect of extensive tryptic cleavage of pig kidney and shark rectal gland enzymes in the presence of NaCl, showing that the 19 kDa C-terminal fragment of the shark α-subunit is much more sensitive to tryptic cleavage (labeled "shark") than that of the kidney enzyme (labeled "kidney"). We studied whether the reported sensitivity difference of cleavage of the 19-kDa fragments of the α-subunit might be attributed to differential trypsin membrane association. Thus, membranes from pig kidney or shark rectal gland were incubated with trypsin for 1 h at 37 °C, and the quantity of trypsin associated to both membrane preparations was compared. As seen in Fig. 5B, the amount of trypsin associated to the shark rectal gland membrane was significantly higher than that associated to the pig kidney membranes.

Plasma membranes of shark rectal glands contain 60 mol% cholesterol [30] whereas that of pig kidney contain 16 mol% [31]. To investigate the possibility that cholesterol is responsible for the observed differential trypsin membrane association, we prepared PC vesicles containing different mol% of cholesterol and studied their ability to bind trypsin after incubation at 37 °C. As seen in Fig. 5C, cholesterol induced a significant increase in trypsin membrane association. Nevertheless, trypsin association to liposomes containing 40% cholesterol was much less than that observed with native plasma membranes (trypsin membrane association to PC/cholesterol liposomes with 20 mol% cholesterol was about 15% of that observed with pig kidney lipid vesicles which contain 16 mol% cholesterol), strongly indicating that other membrane component(s) contribute to enhance trypsin membrane association.

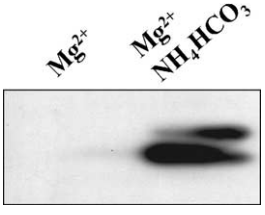

Fig. 4. Cleavage of the γ-subunit is abrogated by addition of bicarbonate to the tryptic assay. The "19 kDa membranes" preparation was incubated with trypsin for 10 min at 37 °C in the presence of 10 mM Tris, pH 7.0, and either 2 mM $MgCl_2$ alone or 2 mM $MgCl_2$ plus 20 mM NH_4HCO_3, as indicated. The reactions were stopped with SDS sample buffer containing 0.2% TCA and aliquots were loaded onto SDS gels, transferred to PVDF membranes, and immunoblotted with γ-specific antibody.

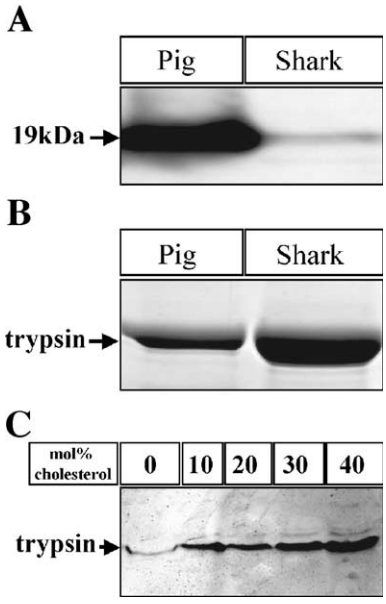

Fig. 5. Sensitivity of different enzymes sources to trypsin may result from different membrane structure. (A) Pig renal (labeled "pig") and shark rectal (labeled "shark") membrane-bound enzymes were incubated with trypsin in the presence of 25 mM NaCl and the membranes were analyzed by SDS-PAGE, transferred to PVDF membrane, and immunoblotted with NKA1002–1016, showing the remaining 19 kDa C-terminal fragment. (B) One mg of plasma membranes prepared from pig kidney (labeled "Pig") or shark rectal gland (labeled "Shark") was incubated with 200 μg trypsin and the membrane-associated trypsin was isolated by SDS-PAGE and visualized with Coomassie, showing that the amount of trypsin associated to the shark rectal membranes is ~2.5 times higher than that associated with the pig kidney membranes. (C) PC/cholesterol vesicles containing the indicated mol% of cholesterol were prepared. Area intensities, expressed as percentage of control, were; 100% (0 mol%), 250.4% (10 mol%), 277% (20 mol%), 382.4% (30 mol%), and 505% (40 mol%).

4. Discussion

Association of a 23-kDa protein to pig kidney plasma membranes was previously observed [24]. The membrane-associated insoluble 23 kDa protein was considered a contaminant not belonging to Na,K-ATPase [24]. In this study, we identified trypsin as a membrane-associated protein that binds to the membrane during tryptic cleavage. The insolubility of trypsin at 0 °C suggests that it rather binds to a specific insoluble lipid component. Further evidence for the specific trypsin–membrane interaction came from a comparison between shark rectal and pig renal plasma membranes in terms of their ability to bind trypsin, showing that trypsin binding to the membrane is an intrinsic property and not due to differences in Na,K-ATPase conformation. This was also evidenced from studies in which the effect of ions on trypsin association to protein free vesicles was measured (see Fig. 1, Supplemental Material).

In proteolytic cleavage experiments of membrane-bound Na,K-ATPase, the presence of NaCl would increase the average life of trypsin by increasing its membrane association. In addition, the presence of Na^+ might increase the probability of tryptic attack at sites close to the plasma membranes, which might, at least in part, explain the faster degradation of the 19 kDa C-terminal fragment following incubation with trypsin in the presence of Na^+ [26]. The presence of Na^+ ions per se does not protect soluble trypsin from autolyses, the protection seems to be indirect by affecting the membrane properties in such away that trypsin membrane association becomes more favorable. On the other hand, addition of ions limiting trypsin membrane association would likely decrease its half-life by increasing the concentration of (the rapidly autolyzed) soluble trypsin.

Biological membranes are surrounded by an aqueous buffer containing di- and monovalent ions, and electrostatic interactions between ions and the lipid molecules are crucial for a variety of membrane-dependent processes such as membrane fusion, phase transition or reorganization of membrane domains. It is possible that trypsin associates with the membrane by electrostatic interactions supported by cations, which would otherwise be reduced in the presence of chelating agents. However, electrostatic interaction alone cannot explain the significant difference between Na^+ and K^+ in inducing trypsin association to the protein-free membranes. In a previous report, monovalent alkali cations were found to produce significant effects on the organization and phase properties of phosphatidylcholine vesicles [32]. Such modulation of membranes properties might be responsible for the observed association of trypsin to the membrane. We may speculate that trypsin membrane association might depend on changes in membrane fluidity, as evidenced from the effect of cholesterol (which strongly modulates membrane fluidity) membrane association (Fig. 5C). However, since cholesterol has many other effects on lipid membranes, further analyses should be performed to uncover the nature of this ion-dependent membrane–protein interaction.

In conclusion, the plasma membrane stabilizes tryptic activity and is a protector against its inhibitor PMSF. In studies involving trypsin cleavage of membrane-bound proteins, a bicarbonate wash should be a useful tool to release trypsin from the membrane.

Acknowledgements

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Appendix A. Supplementary data

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.bbamem.2005.11.001.

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