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Tissue specific membrane association of α1T, a truncated form of the α1 subunit of the Na pump

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Abstract

We have assessed the Na pump α-subunit isoform content utilizing site directed antibodies in two vascular smooth muscle (VSM) preparations known to contain functional Na pump sites, VSM microsomal fractions (Na^+,K^+-ATPase) and intact primary confluent cells (ouabain inhibited ^{86}Rb uptake). A comparison of isoform content was made with kidney microsomes. Both VSM and kidney microsomes contained a full length α1 subunit (~ 100 kDa) as well as a truncated subunit, α1T (~ 66 kDa). SDS treatment of VSM microsomes effected an increase in Na^+,K^+-ATPase and a retention of α1T. SDS treated kidney microsomes retained the α1 isoform and Na^+,K^+-ATPase. Confluent VSM cells showed no detectable α1, only α1T. In the absence of detectable full length α1, the α1T protein may represent a functional Na pump component in canine VSM.

Key words: Na pump; α Subunit isoform; Vascular smooth muscle

1. Introduction

The Na^+,K^+-ATPase, the enzymatic manifestation of the transmembrane Na^+ pump found in virtually all animal tissue has been well characterized in recent years. It is generally thought to consist of at least three isoforms of the larger 100 kDa α-subunit (α1, α2, α3) complexed with one of at least three smaller (~ 50 kDa) β subunit isoforms. However, the specific nature of the complex, its control at all levels, and the function of the isoforms, are still under intense investigation [1,2].

We have recently demonstrated that instead of the expected ~ 100 kDa Na,K-ATPase α1 subunit, canine vascular smooth muscle expresses a ~ 66 kDa α1T (truncated) isoform protein derived from the α1 gene by alternative RNA processing [3]. This protein is identical to the full length α1 through Gly^{554}. There is then an abrupt divergence in sequence, with the encoding of 27 amino acids from the retained intron sequence, completing the carboxy end of the molecule. The truncated protein contains the same key sites as the full length protein: the FITC binding site, phosphorylation sites, and the ouabain binding site. Furthermore, polymerase chain reaction analysis of the same tissue fails to detect mRNA for the α2 or α3 isoforms, although small amounts of α1 mRNA are present [4]. In the absence of a detectable, 'classical' α subunit, these results would suggest that α1T may function as a transport protein in vascular smooth muscle.

There are a variety of approaches available to determine if this α1T can function as a pump protein. We have chosen to utilize existing models of VSM membranes and intact cultured cells to assess this possible role.

Specifically we determined the isoform content of these two well known preparations each of which contains what is accepted as Na pump mediated functions: (i) the Na^+,K^+-ATPase activities of SL membrane fractions [5], and (ii) ouabain inhibited ^{86}Rb uptake of confluent canine VSM cells [6,7]. If the α1T isoform is functional in VSM, it should be readily demonstrable in these two model systems.

2. Methods and materials

2.1. Microsomal preparations

2.1.1. Vascular smooth muscle (carotid artery or saphenous vein). Standard procedures were used. In brief, tissues were cleaned in the cold room (0–4°C), minced with a McIlwain Chopper, and dispersed in a Polytron, 3 times for 15–20 s in approximately 15 ml 0.25 M Sucrose, 10 mM Tris, pH 7.4. The supernatant from a $2{,}000 \times g$ 15 min spin was centrifuged at $100{,}000 \times g$ for 1 h. The resulting pellet was suspended in about 500 μl of homogenizing solution and protein concentration determined by the method of Bradford [9].

2.1.2. Kidney medulla and cortex. Crude microsomes were prepared in a manner similar to the VSM microsomes. Instead of a McIlwain Chopper the tissue was minced with scissors. The initial spin was at $1{,}000 \times g$ 20 min followed by a spin of $100{,}000 \times g$ 60 min.

2.2. SDS treatment

The microsomes were incubated for 45 min at room temperature with

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3 mg protein/mg SDS in a buffer containing 50 mM Tris, 2 mM EDTA, and 2 mM Na_2ATP with gentle stirring [8], and then centrifuged at 100,000 × g for 1 h. The resulting pellet was suspended in ~ 400–500 μl Tris-EDTA buffer and assayed for protein concentration. 'Control' microsomes were treated in exactly the same manner, but without SDS.

2 3.

Na^+,K^+-ATPase assay was performed by the linked enzyme PK-LDH method [5,12]. The activity of the Na^+,K^+-ATPase was estimated from the difference between the rate of ATP hydrolysis in the absence and presence of 10^{-4} ouabain, and was standardized against the amount of protein.

2 4

Immunoblotting and analysis were performed using conventional techniques. Antigen–antibody interactions were detected with peroxidase-conjugated goat anti-rabbit IgA, IgM, IgG, followed by chemiluminescence and visualization on Hyperfilm-ECL film (Amersham).

Two site-directed oligopeptide polyclonal antibodies were constructed as follows: 'anti-LEAVE' was directed against the Lys^{354} to Glu^{365} residues of the α1 subunit, thought to be present in the α1T subunit and was characterized as described [10]; 'anti-HSL' corresponded to the first 13 amino acids encoded by α1 intron 12, found only in the α1T subunit, and was characterized as previously described [3].

2.5. VSM cultured cells

The protocol is the same as previously published [6,7]. Vessels were cleaned, digested with collagenase and elastase, two times, and plated at 10^5 cells/cm^2 on 35 mm plates, in DMEM containing 10% fetal bovine serum. Cells reached confluence in 5–7 days.

3. Results

3.1. VSM microsomes

In order to evaluate the expression of α1T in VSM, we first determined the isoform content of VSM microsomal fractions. Previous work has demonstrated [5] that such fractions contain minimal ouabain inhibited ATPase activity. It has also been shown that SDS treatment is necessary to effect a consistently measurable ouabain inhibited activity. The isoform content of VSM microsomes with and without SDS treatment, using an antibody that reveals both α1 and α1T is shown in Fig. 1, lanes 1 and 2. Native microsomes revealed bands with mobilities appropriate for both α1 (100 kDa) and α1T (66 kDa) isoforms, while those treated with SDS showed only one band corresponding to α1T.

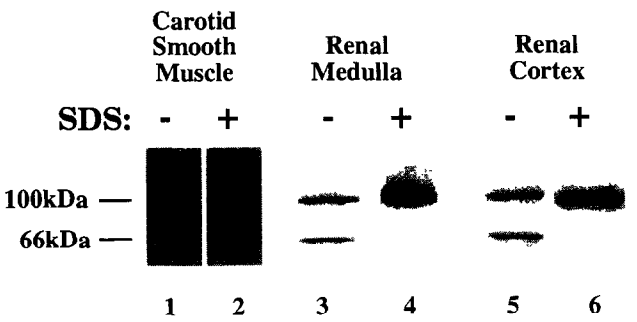

Fig. 1. Immunoblot of microsomes isolated from canine kidney, cortex, medulla, and carotid artery. Each lane contains 20 μg of protein. + = SDS treatment; − = no SDS treatment. Microsomes were isolated as described, treated with SDS for 45 min at room temperature, and centrifuged for 1 h at 100,000 × g. Control microsomes were treated exactly the same, with buffer and ATP, without SDS. Pellets were suspended in Tris buffer, and protein concentration determined. The gel contained 10.0% polyacrylamide, and was run at 200 V for 45 min. Transfer to PVDF was for 1 h at 80 V. The first antibody (anti-LEAVE) was used in a 1:1,000 dilution, for 2 h. Secondary antibody (horseradish peroxidase) was used at 1:3,000 and incubated for 1 h, followed by one 15 min wash with 0.05% Tween/TBS, and one with TBS alone. The PVDF was transferred to a new tray, reagents were added as detailed in the instructions from Amersham, incubated for 1 min, blotted dry with tissue paper, and developed in a stainless steel cassette for 30 s.

Table 1A
ATPase activities of canine carotid artery microsomes treated with SDS

	Canine carotid artery (μmol P_i/mg pro/h (n = 3)	
	− SDS	+ SDS
Total ATPase	44.6 ± 18	51.0 ± 20
Na^+,K^+-ATPase	3.6	18.0

Table 1B
ATPase activities of canine kidney medulla microsomes

	Canine kidney cortex (μmol P_i/mg pro/h (n = 4)	
	− SDS	+ SDS
Total ATPase	51 ± 8	178 ± 16
Na^+,K^+-ATPase	14	177

Microsomes were treated as detailed in Section 2, and resuspended in Tris buffer. Assay for ATPase activities was performed in a Gilford Recording Spectrophotometer, using the linked PK-LDH system, which monitors NADH oxidation at 340 nm [5]. Each cuvette contained identical material (protein 2–20 μg) except for 1 mM ouabain in alternate cuvettes. Mg-ATPase activity is defined as that activity remaining after ouabain inhibition.

Measurement of ouabain-inhibited ATPase activity in both control and SDS treated VSM microsomes is shown in Table 1 (top). Very little ouabain inhibition was present without SDS treatment. Treatment with the detergent enhanced Na^+,K^+-ATPase activity in the absence of any immunologically detectable α1 protein (see Fig. 1).

To determine if this SDS response was peculiar to VSM, we assessed canine kidney microsomes in the same manner. We chose this tissue because Na^+,K^+-ATPase is readily demonstrable, and the specific methods for enzyme purification have been extensively documented [8,14]. Na^+,K^+-ATPase activity of control and SDS treated microsomes from canine kidney are shown in Table 1 (bottom). As expected, SDS treatment significantly enhanced ouabain inhibited activity. Immunoblots of kidney microsomes treated in the same manner and probed with 'anti-LEAVE' show that such treatment eliminated the 66 kDa band corresponding to α1T and retained the 100 kDa band, corresponding to α1 in microsomes from both cortex and medulla (Fig. 1, lanes 3, 4, 5, 6).

In order to confirm the presence of α1T in VSM, we

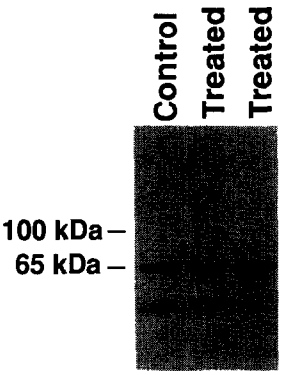

Fig. 2. Immunoblot of vascular microsomes, using anti-HSL antibody. Vascular microsomes were isolated and treated identically to the microsomes as outlined in Fig. 1.

assessed its content with 'anti-HSL', an antibody that reveals only that isoform, and not α1 full length. Fig. 2 shows an immunoblot of control and SDS-treated VSM microsomes using 'anti-HSL'. No 100 kDa (α1 full length) band is present, and SDS treatment does not eliminate the band corresponding to α1T.

Thus detergent treatment of VSM eliminates α1 protein, while retaining α1T. The opposite is true for kidney microsomes which retain α1 and eliminate α1T upon detergent treatment.

3.2. VSM cell culture

Since VSM microsomes retained the α1T and Na^+,K^+-ATPase activity after SDS treatment, we wished to determine the major isoform expressed in cells that clearly demonstrate Na pump function. We have shown that [^{3}H]ouabain binding and ouabain inhibited ^{86}Rb uptake are present in a variety of cell cultures of canine VSM [6,7] and presume these to be representative of the Na pump. Fig. 3 shows an immunoblot of total protein from saphenous vein smooth muscle cells grown for 1, 4 and 5 days probed with 'anti-LEAVE' using canine kidney medulla for comparison. It can be seen that while both α1 and α1T were present in kidney microsomes (see Fig. 1), only α1T was detectable in proliferating VSM cells.

4. Discussion

The data contained in this paper strongly suggest but do not prove that the 66 kDa, α1T protein may serve a transport function in VSM. We showed that SDS treatment of VSM microsomes enhanced the Na^+,K^+-ATPase activity of the preparation, eliminated the α1 protein, and retained α1T protein. This is in sharp contrast to similar SDS treatment of kidney microsomes, which resulted in elimination of α1T and retention of the α1 and Na^+,K^+-ATPase. Thus SDS treated VSM microsomes show Na^+,K^+-ATPase activity, ouabain binding, no detectable full-length α1 protein, and α1T protein.

It appears that despite the presence of both α1 and α1T isoforms in crude microsomal fractions from kidney and VSM, the proteins have differing membrane-associative characteristics. This is not surprising in view of their large structural differences and may also reflect different lipid profiles of the membranes of different tissues as well. Regardless of the reasons for such tissue-specific differences, we suggest that selective retention in the cell membrane of either of the α subunits reflects interaction with the β subunit necessary to form a functional pump site. Since no detectable full-length α1 subunit is retained in VSM fractions this argues that the α1T subunit functions as a pump protein in this tissue and that α1 does not associate with β.

Furthermore, these data suggest that this truncated protein, lacking 40% of the -COOH terminal sequence, can be targeted to the membrane and fulfill a transport function. Some workers have suggested that glutamic acid residues 955 and 956 are important in the ion transport function of the full length protein [16,17]. Since α1T lacks this region, this would seem to preclude a transport role for this protein. However, more recent evidence utilizing site-directed mutagenesis of these predicted cation binding sites argues against the contribution of these sites and the carboxy terminus [18]. Thus specific designation of ion transport residues remains unclear and α1T may yet prove to retain the necessary motifs for Na^+ and K^+ transport.

Since intact tissues (or at least microsomes) demonstrate both α1 and α1T proteins, suggestions that α1T serves a transport function cannot be made without cytochemical localization studies, or the determination of the actual molecular nature of the pump complex (i.e. (α1)(β) or (α1T)(β)). For these reasons, we assessed the protein components contained in confluent cultures of canine vascular smooth muscle cells, which we know express functional Na pump sites [6,7]. These cells show a large component of α1T and no detectable α1. Clearly since microsomes show an α1 full-length protein before SDS treatment, the lack of detectable α1 in confluent

Fig. 3. Immunoblot of saphenous vein smooth muscle cells, and canine kidney medullary microsomes. Saphenous vein cells were grown for indicated time on 100 mm plastic dishes, lysed with NP 40, and Western analysis performed as in Fig. 1 and Section 2. Canine kidney microsomes were assessed as described above.

cells may reflect the up-regulation of the α1T, and down-regulation of α1. Such modulation is currently under investigation. Freshly dispersed cells do show a small amount of α1, which seems to disappear during proliferation (data not shown).

Thus strong circumstantial evidence suggests that α1T may serve a transport function in VSM. We have shown that in the absence of detectable α1, VSM microsomes show a Na^+,K^+-ATPase activity and [^{3}H]ouabain binding. In addition, in VSM confluent cells, containing apparently normal Na pump function, α1 was undetectable and α1T was clearly demonstrable.

We have shown earlier that canine VSM contains both β1 mRNA [4] and protein [15], but have not yet determined its associative characteristics with the α1T in the sarcolemma. It does, however, remain attached to the sarcolemma after extensive purification attempts with detergents.

Furthermore, the SDS data in this paper reveal nothing about the nature of the association of α1T with the different membranes, or the reasons for such different associative characteristics. More extensive characterization with additional detergents is necessary to more clearly define these phenomena.

Despite these limitations we can conclude that both α1 and α1T exist in both canine kidney and VSM canine tissues but their membrane associative characteristics vary greatly. The α1 is resistant to SDS treatment in the kidney membrane, and the α1T is more tightly retained in VSM. Since there is no detectable α1 in confluent cultured VSM, it is possible that α1T serves as a pump subunit in VSM. In the absence of α1 in VSM, α1T may complex with an appropriate β, insert in the membrane and may form functional pump sites in the SL of this tissue. This would account for the Na^+,K^+-ATPase activity in VSM SL preparations. The manifestations of α1T not associated with a β subunit, and not inserted in the membrane could be significantly different from α1T complexed with β, and bound to a membrane.

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