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Down-regulation of Na^+/K^+-ATPase α_2 Isoform in Denervated Rat Vas Deferens

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ABSTRACT. In the rat vas deferens, an organ richly innervated by peripheral sympathetic neurons, we have demonstrated recently the expression of α_1 and α_2, but not α_3 isoforms of the α subunit of Na^+/K^+-ATPase (EC 3.6.1.37), a membrane-bound enzyme of vital function for living cells (Noël *et al.*, *Biochem Pharmacol* 55: 1531–1535, 1998). In the present work, we characterized, qualitatively and quantitatively, Na^+/K^+-ATPase α isoforms in denervated rat vasa deferentia. [^{3}H]Ouabain binding at concentrations defined for high-affinity isoforms (α_2 and/or α_3) detected only one class of specific binding sites in control (C) and denervated (D) vas deferens. Although the dissociation constant was similar for both groups [K_d = 138 ± 14 nM (C) and 125 ± 8 nM (D)], a marked decrease in density was observed after denervation [716 ± 81 fmol·mg protein^{-1} (C) and 445 ± 34 fmol·mg protein^{-1} (D), $P < 0.05$]. In addition, western blotting revealed that denervated vasa deferentia produce the α_1 and α_2 isoforms but not α_3, just as we reported for the controls previously (Noël *et al.*, *Biochem Pharmacol* 55: 1531–1535, 1998). Densitometric analysis showed a decrease of the α_2 isoform by about 40% in denervated organs, in very good agreement with what was shown with the [^{3}H]ouabain binding technique, but no significant change in α_1 isoform density. Truncated α_1 (α_1T), an isoform suggested to exist in the guinea pig vas deferens, was not detected. Altogether, our results demonstrated that Na^+/K^+-ATPase α_2 is down-regulated after sympathetic denervation of the rat vas deferens. BIOCHEM PHARMACOL **60**;6:741–747, 2000. © 2000 Elsevier Science Inc.

KEY WORDS. vas deferens; Na^+-K^+-exchanging ATPase; denervation; ouabain; adaptation; physiological; rat

Present in virtually all cells that compose mammalian organisms, the Na^+/K^+-stimulated, Mg^{2+}-dependent adenosine triphosphatase (Na^+/K^+-ATPase; EC 3.6.1.37) is an enzyme responsible for Na^+ and K^+ transport across the plasma membrane [1]. The catalytic α subunit has at least three isoforms (α_1, α_2, and α_3)§ with differences concerning gene origin, amino acid composition, tissue distribution, affinity for cardiac glycosides, and ontogenesis- and environment-dependent expression [4–7].

One example of the dependency of Na^+/K^+-ATPase isozyme expression upon the environment is the suppression of autonomic neurotransmission in richly innervated organs such as the vas deferens. After chronic interruption of neuromuscular adrenergic coupling, this smooth muscle develops adaptive (postjunctional) supersensitivity characterized by a leftward shift of the dose–response curve to several excitatory compounds and ions [8–10]. In some cases, an increase of maximal response also is observed [8]. To investigate the cellular mechanisms responsible for such nonspecific supersensitivity, studies in the rat and guinea pig have identified alterations in the threshold and resting membrane potential, respectively [11]. In the guinea pig vas deferens myocyte, the partial depolarization detected after denervation is comparable to depolarization provoked by the addition of high concentrations of ouabain or low K^+ solution in control vas deferens, suggesting the participation of Na^+/K^+-ATPase in this process [12]. Furthermore, binding assays with [^{3}H]ouabain pointed out a lower maximal number of specific binding sites in the denervated vas deferens of this animal [13]. More recently, identification of two isoforms of the α subunit of Na^+/K^+-ATPase—truncated α_1 (α_1T) and α_2—and subsequent discovery of the reduction of α_2 isoform protein levels [14] and of time–course correlation between this decline and the appearance of supersensitivity [15] have suggested that Na^+/K^+-ATPase is the major cellular component that gives rise to the macroscopic phenomenon in guinea pig vas deferens.

In the rat, this picture is less clear. Although denervation produces the same qualitative pattern of adaptive supersensitivity exhibited in vasa deferentia of guinea pigs, the role of Na^+/K^+-ATPase in the maintenance of membrane potential of smooth muscle cells is insignificant or absent [12] in spite of the higher enzyme activity [16]. These

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§ A testicular α_4 [2] and an alternative RNA splicing product of the α_1 gene at intron 12, namely α_1T [3], have been reported as well.

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results disproved *a priori* its putative involvement in the mechanism of supersensitivity. We have shown recently that the rat vas deferens expresses two isoforms of Na^+/K^+-ATPase, namely α_1 and α_2 [17]. In the present work, we detected, by distinct but additive methods, a specific decrease of the α_2 isoform following surgical denervation of rat vas deferens, and we discuss the relevance of such findings to overall adaptive events.

MATERIALS AND METHODS

Postganglionic Denervation of Vasa Deferentia

Surgical denervation was performed according to Kasuya *et al.* [8]. Four groups of about 15 male adult Wistar rats (4- to 6-months-old) of the 2BAW colony [18] were anesthetized with ether, and vasa deferentia were denervated bilaterally. Fourteen days after surgery, animals were killed, and vasa deferentia were excised and subjected to a 10^{-4} M tyramine challenge. Organs with a contractile response higher than 5% of that in the control organs were discarded; truly denervated vasa deferentia were stored at −80° until the homogenization procedure was performed. Vasa deferentia from sham-operated animals were used as controls.

Preparation of Vasa Deferentia Homogenates

All steps hereafter were performed at 0–4° essentially as previously described [17]. In brief, organs from normal and denervated rats were minced and homogenized in an Ultraturrax apparatus (20,500 rpm; 1 × 1 min and 2 × 30 sec) in 0.25 M sucrose/5 mM Tris–HCl buffer (pH 7.4) plus 0.2 mM PMSF* and 2 mM DTT. The suspension was subjected to 10 strokes in a glass Potter homogenizer, followed by vacuum filtration through gauze and centrifugation at 108,000 *g* for 1 hr. The pellet was resuspended in the same buffer (without DTT), and stored at −80°. Protein content was determined by the method of Lowry *et al.* [19]. Control preparations of brainstem, brain hemispheres, and kidney homogenates were essentially identical to those in Noël *et al.* [17].

Radioligand Binding Assays

[^{3}H]Ouabain binding experiments were carried out with the incubation of approximately 250 μg protein at 37° for 30 min in 20 mM maleate–Tris buffer, pH 7.4, containing 30 nM [^{3}H]ouabain (New England Nuclear), 3 mM P_i–Tris, 3 mM $MgCl_2$, and 1 mM EGTA. As we previously described, experiments with radiolabeled ouabain utilizing crude preparations of rat vas deferens were unable to give quantitatively precise values for low-affinity binding sites (K_d around 15 μM, supposed to correspond to the rat α_1 subunit isoform of Na^+/K^+-ATPase) [17]. Thus, our assays focused on high-affinity sites, using unlabeled ouabain concentrations ranging from 0 to 500 nM for both groups studied. The nonspecific binding was estimated in the presence of 1 mM unlabeled ouabain. After incubation, samples were diluted rapidly with 5 mL of ice-cold 5 mM Tris–HCl buffer, pH 7.4, and filtered instantaneously on Whatman glass fiber filters (GF/C) under vacuum, and test tubes were washed with 5 mL of Tris–HCl buffer. Filters then were washed twice with 10 mL of the same buffer, dried, and immersed in a scintillation fluid. The radioactivity retained in the filters was counted with a Tri-Carb Packard liquid scintillation analyzer.

SDS–PAGE and Western Blotting

SDS–PAGE was carried out according to Laemmli [20]. Samples were separated without heating on a 6% polyacrylamide gel (or 10% when searching for the ~60-kDa α_1T) and transferred to nitrocellulose filter papers. After incubation for 1 hr in 5% non-fat dry milk dissolved in TBS solution containing 0.1% Tween 20 (TTBS), nitrocellulose sheets were washed with TTBS and then incubated for 1 hr at room temperature in the dark with monoclonal mouse IgG anti-rat Na^+/K^+-ATPase α_1 (McK1) [21], α_2 (McB2) [22], and α_3 (F9-G10) isoforms (diluted 1/1000, 1/750, and 1/900, respectively) supplied by Dr. K. Sweadner. Blots were rinsed and incubated for 1 hr with HRP-conjugated anti-mouse antibodies (1/15,000). Immunoreactivity was detected using enhanced chemiluminescence (ECL, Amersham Corp.) by exposure to Hyperfilm–ECL (Amersham Corp.).

Data Analysis

Values from equilibrium binding experiments were represented as Scatchard plots, and binding parameters (dissociation constant, K_d, and maximal number of binding sites, B_{max}) were calculated, assuming the existence of one class of specific binding sites in the range of concentrations used, by means of nonlinear regression analysis (Prism, GraphPad Software Inc.). Student's *t*-test was employed to give the statistical significance of the difference between the means of binding parameters from both groups.

Protein blot images were captured by a Bio-Rad Imaging Densitometer (model GS-700, Bio-Rad) and analyzed using Molecular Analyst imaging system software (Bio-Rad). For comparing the amount of each isoform, 3–4 lanes were loaded with control samples and run side-by-side with denervated samples. After subtracting the background, densitometric values of those 3–4 bands specifically assigned as Na^+/K^+-ATPase α subunits were averaged and subjected to Student's *t*-test. Comparisons were done only between averaged values of bands within the same gel.

RESULTS

Specific [^{3}H]Ouabain Binding

All four control and four denervated groups showed linear Scatchard plots (Fig. 1), indicating that only one class of (high-affinity) binding sites was observed. When binding

* *Abbreviations*: PMSF, phenylmethylsulfonyl fluoride; DTT, dithiothreitol; TBS, Tris-buffered saline; and HRP, horseradish peroxidase.

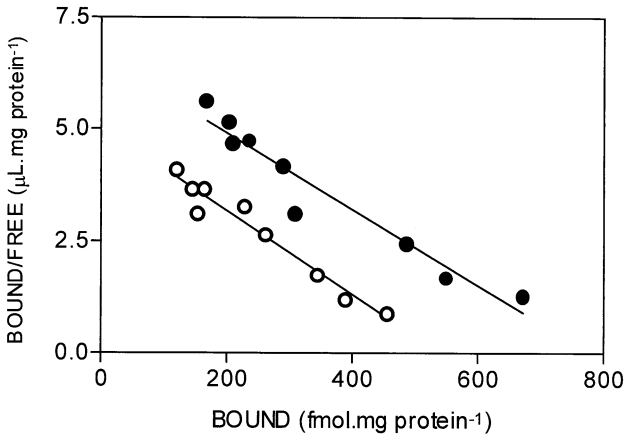

FIG. 1. Typical Scatchard plots for specific binding of [^{3}H]ouabain in crude membrane preparations of control (●) and denervated (○) rat vas deferens. Approximately 250 μg protein was incubated at 37° for 30 min in a medium containing 30 nM [^{3}H]ouabain, 3 mM P_i–Tris, and increasing concentrations of unlabeled ouabain (1 mM unlabeled ouabain was used for nonspecific binding). Each point represents the mean of triplicate determinations. Bound = ouabain specifically bound; Free = free concentration of ouabain.

parameters were compared (experiments were conducted in parallel for each group pair), affinity (K_d) was not affected by denervation, but a reproducible decrease of the density of binding sites (B_{max}) was observed (Table 1). According to these data, after 14 days of denervation there were 38% fewer high-affinity ouabain binding sites—ascribed to the Na^+/K^+-ATPase α_2 isoform (see below)—in rat vasa deferentia. To confirm these results and further extend them to the α_1 isoform, we performed quantitative western blot analysis.

Analysis of Immunoreactivity to Anti-Na^+/K^+-ATPase α_1, α_2, and α_3 Isoform Antibodies

Hybridization of monoclonal antibodies against α_1 and α_2 isoforms was fairly detectable in rat vas deferens preparations, as expected from our previous report [17]. The utilization of positive controls (brainstem or brain hemispheres as the source of α_1, α_2, and α_3 isoforms, and kidney as the source of α_1 isoform) in every experiment performed allowed us to minimize unexpected cross-reactions due to lack of specificity (Fig. 2).

TABLE 1. Values of equilibrium binding parameters of [^{3}H]ouabain binding for control and denervated rat vas deferens

	B_{max} (fmol · mg protein^{-1})	K_d (nM)
Control	716 ± 81	138 ± 14
Denervated	445 ± 34*	125 ± 8

Values indicating capacity (B_{max}) and affinity (K_d) from both groups represent means ± SEM of four different preparations (see Materials and Methods).

*Statistically significant difference ($P < 0.05$) between control and denervated (Student's *t*-test).

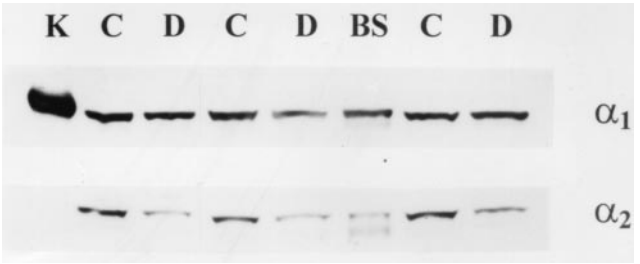

FIG. 2. Na^+/K^+-ATPase α_1 and α_2 isoforms blotted with specific monoclonal antibodies (McK1 and McB2, respectively). Unheated samples were loaded on a 6% polyacrylamide gel, transferred to nitrocellulose filter papers, blocked, incubated with primary antibodies, and finally incubated with anti-mouse secondary antibodies. Immunoreactivity was detected using enhanced chemiluminescence (ECL) by exposure to Hyperfilm-ECL. The same amount (50 μg protein/lane) of control (C) and denervated (D) rat vasa deferentia was used for comparison. Rat kidney (K, 15 μg protein/lane) and rat brainstem (BS, 10 μg protein/lane) served as controls for antibody specificity. After subtracting the background, values of relative optical density of bands C and D were averaged, and the statistical significance of the difference was assessed.

As demonstrated in Fig. 2 (typical western blot) and in Table 2 (mean densitometric values), there was no significant difference between control and denervated organs in relation to the α_1 isoform. In contrast, the relative content of the α_2 isoform in denervated vas deferens was 63% of the control, in very good agreement with the results obtained by the binding technique. It is noteworthy that the amount of protein loaded into the gel (40 or 50 μg/slot) was in a range where a good correlation between optical density versus protein content for both isoforms was seen (Fig. 3).

The presence of the α_3 isoform was not detected in either control or denervated vas deferens, even using another type of specific antibodies (for comparison see Ref. 17) and loading a higher amount of protein (60 μg/slot) (Fig. 4A).

The truncated form of the α_1 subunit, namely α_1T, was not detected in either control or denervated rat vas deferens using McK1, the antibody used for reacting with α_1T in other studies (Fig. 4B).

DISCUSSION

The α subunit of Na^+/K^+-ATPase contains the most important sites that favor enzyme activity and modulation,

TABLE 2. Densitometric values of Na^+/K^+-ATPase α_1 and α_2 isoforms evaluated in control and denervated rat vas deferens

	Relative optical density (arbitrary units)	
	α_1	α_2
Control	1.91 ± 0.39	0.90 ± 0.21
Denervated	1.87 ± 0.45	0.57 ± 0.15*

Values from both groups for each isoform represent means ± SEM of three different preparations (see Materials and Methods).

*Statistically significant difference ($P < 0.05$) between control and denervated (Student's *t*-test).

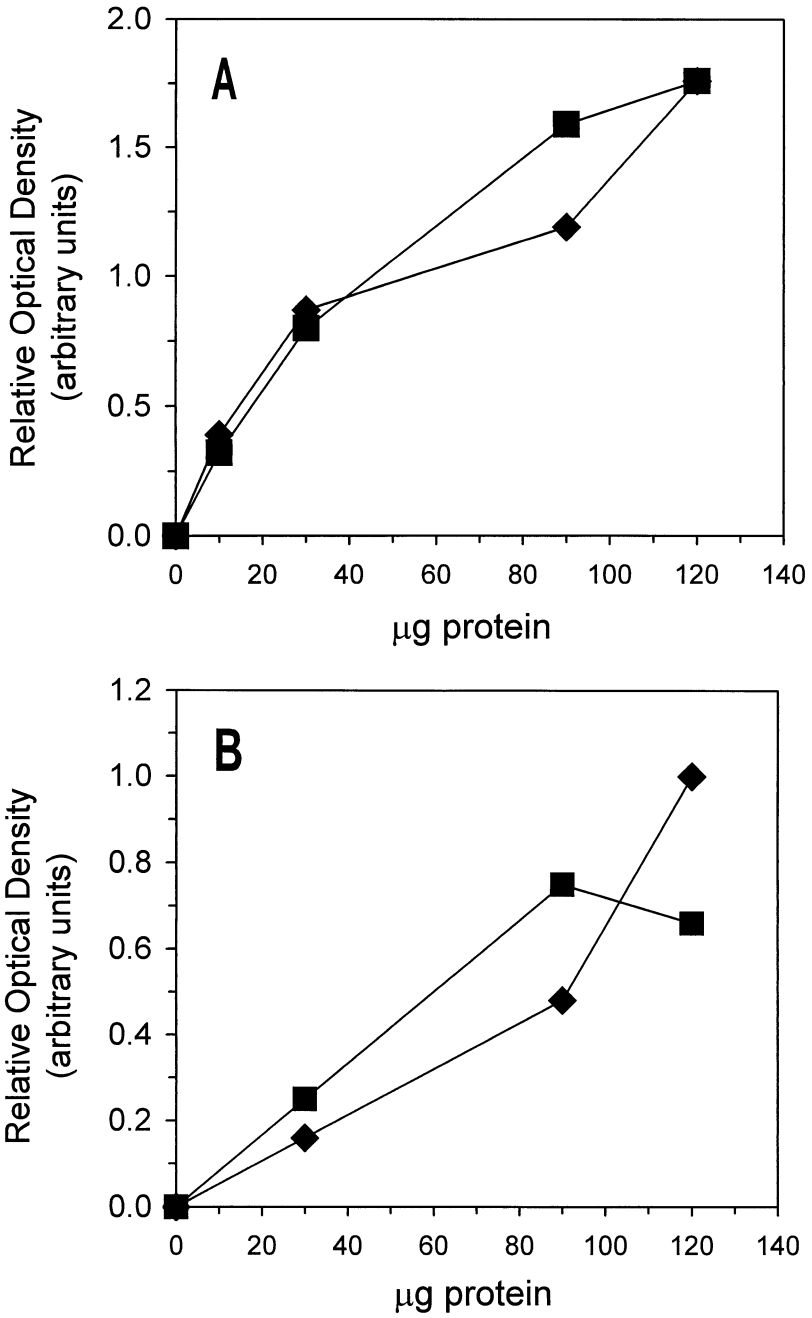

FIG. 3. Relationship between the relative optical density and the amount of total protein loaded into slots. Curves depicted on the left side (A) and the right side (B) concern the McK1-α_1 isoform and McB2-α_2 isoform immunoreactions, respectively. The two different curves (♦ and ■) represent separate experiments where each point is the value of an individual band. Note the good correlation between the two variables in the range of protein used for quantitative analysis (40 or 50 μg).

and its isoforms are regulated differentially by several conditions [5, 7]. In rat vas deferens, two isoforms (namely α_1 and α_2) have been detected previously, using functional (radioligand binding) and molecular biology (western blotting) techniques [17]. The present data indicate that the same isoforms are also present after denervation of this tissue. Relevant to this observation is the kind of antibodies used in the present study. The use of antibodies (in the case of α_2 and α_3 isoforms) against different epitopes than those employed earlier corroborates the presence of solely α_1 and α_2 isoforms. Neither α_3 nor α_1T, a truncated version of native α_1 that was found for the first time in vascular smooth muscle [3] and is able to associate with β subunit and to produce functional pumps [23], could be detected by these specific immunoligands. Interestingly, the truncated form of α_1 subunit, identified in the guinea pig vas deferens (but not the full-length α_1) and likely to be the major isoform in this organ [14], was not found in the rat vas deferens using McK1, the antibody used for reacting with α_1T in other studies.

Quantitation of the relative protein amount of α_1 and α_2 isoforms by means of densitometric analysis of western blotting bands showed that there is a decrement of almost 40% of specific anti-α_2 immunoreaction in denervated organs. In contrast, α_1 expression was not altered significantly by denervation. Moreover, the density of high-affinity ouabain binding sites, which are inferred to be α_2 isoforms in the case of rat vas deferens, was equally

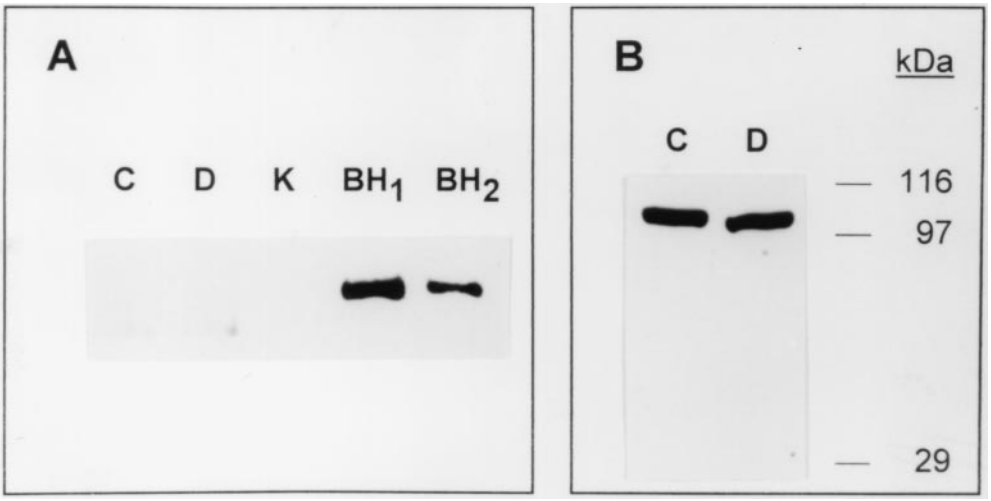

FIG. 4. Search for Na^+/K^+-ATPase α_1T and α_3 isoforms using specific monoclonal antibodies (McK1 and F9-G10, respectively). Unheated samples were loaded on a 6% (or 10% in the case of α_1T) polyacrylamide gel, transferred to nitrocellulose filter papers, blocked, incubated with primary antibodies, and finally incubated with anti-mouse secondary antibodies. Immunoreactivity was detected using enhanced chemiluminescence (ECL) by exposure to Hyperfilm-ECL. (A) α_3: samples used were control (C) and denervated (D) rat vasa deferentia (60 μg protein/lane). Rat kidney (K, 15 μg protein/lane) and rat brain hemispheres (BH_1, 15 μg protein/lane; BH_2, 7.5 μg protein/lane) served as controls for antibody specificity; (B) α_1T: samples used were control (C) and denervated (D) rat vasa deferentia (50 μg protein/lane). The position of molecular mass markers is indicated on the right. Note that no bands were found except the native α_1 in both lanes (molecular mass in the range of 100 kDa).

depressed in denervated rats, in a quantitatively strikingly similar way to that seen in protein blotting. Binding experiments also verified that no alteration in ouabain affinity occurred for these high-affinity binding sites after denervation.

A simple explanation for the decrease of the α_2 isoform level after denervation could be that the quantity of the depleted isoform would correspond to the loss of neuronal α_2. However, some important observations argue against this hypothesis: (i) α_3 isoform, the one expressed in central [24] and peripheral neurons [25–27] and suggested to be a marker for these cells, was not detected in control, innervated vas deferens, in spite of its detection in heart homogenates [17] where α_3 is found only in the cardiac conducting system, a very tiny portion of the heart [28]; (ii) although α_1 isoform is fairly localized in peripheral nerves (and may represent the predominant isoform of sciatic nerve terminals [27]) and associated Schwann cells [25, 27], no decrease of α_1 levels is observed after denervation; and (iii), recent work of Kawai *et al.* [27] described an *enhanced* expression of α_2 isoform and cleavage products in nerve segments distal to the site of transection or crushing of the sciatic nerve, instead of a *decrement* as seen in the present work. Nevertheless, a distinct composition of α isoforms in the hypogastric nerve (e.g. undetectable levels of α_3, high levels of α_2, and low levels of α_1) cannot be discounted completely at the moment, especially due to intrinsic differences between autonomic and somatic motor nerves.

Regulation of α subunit expression, especially of the α_2 isoform, has been observed in diverse physiologic and pathologic conditions such as ontogenesis [6, 29], diabetes [26], hypertension [30], and cardiac hypertrophy [31], so that it can be considered a widespread adaptive phenomenon that probably depends on a large number of factors. In regard to the neural control of muscular Na^+/K^+-ATPase, Arystarkhova and Sweadner [32], utilizing neonatal rat cardiomyocytes, recently demonstrated that the expression of the Na^+/K^+-ATPase α_2 isoform was induced when cocultured with ganglionic sympathetic neurons. Thus, autonomic innervation seems to coordinate the genetic expression of the α_2 subunit isoform in muscle cells.

The reason why the α_2 isoform is specifically reduced after rat vas deferens denervation and what its role is in the course of adaptation are not simple questions. Interest in the study of events and mechanisms that arise after the blockade of neuromuscular interaction is not new, and many excitable organs have been subjected to investigation [9, 33]. The first fact associated with such interruption is an increase of sensitivity of the innervated organ to (usually) unrelated agonists [8, 10, 34]. Depending on the organ, however, the microscopic molecular mechanisms responsible for nonspecific supersensitivity may be different. For organs innervated by the sympathetic nervous system, a partial explanation for supersensitivity is a lack of neuronal uptake of catecholamines when sites of neurotransmitter release are absent (also known as prejunctional, deviation supersensitivity) [9]. On the other hand, "true," nondeviation, postjunctional supersensitivity accounts for mechanisms developed on the target organ [9]. Characteristic modifications, from up-regulation of receptors for agonistic substances to increased efficacy of the intracellular transducing cascade, have been identified and may represent a way of homeostatic adaptation [33, 35].

Particularly for the vas deferens, species is thought to play an important role in mechanistic adjustments. Rats and guinea pigs present similar nonspecific, postjunctional

supersensitivity after various surgical and chemical sympatholytic procedures [8, 10]. For the guinea pig, this process has been very well correlated with a partial depolarization of the smooth myocyte membrane, and the molecular entity responsible for this alteration seems to be the electrogenic Na^+/K^+-ATPase (cf. Hershman *et al.* [15]). Progressive studies first detected a slight but significant decrease of the resting plasma membrane electric potential (without changes in threshold membrane potential) of denervated, decentralized, or reserpinized guinea pig vas deferens [11, 36]; these studies were followed by data showing a decrement of Na^+/K^+-ATPase activity [37] and [^{3}H]ouabain binding [13] and, more recently, a specific fall in Na^+/K^+-ATPase α_2 isoform expression at the protein level [14]. Together with a straight time–course correlation of all these findings [15], Na^+/K^+-ATPase is thought to be the main protein involved in the nonspecific supersensitivity found in the guinea pig vas deferens.

The molecular mechanism in the case of rat vas deferens is far from being elucidated. In contrast to the guinea pig, Goto *et al.* [11] demonstrated that resting membrane potential of rat vas deferens smooth muscle cells is not altered even when supersensitivity reaches its maximum; threshold membrane potential, however, becomes more negative, which could at least partially explain supersensitivity, i.e. the magnitude of depolarization able to trigger a muscular action potential would be around 25% less (similar to the 40% less accounted for in the guinea pig). In relation to resting membrane potential, subsequent work has indicated definitively the presence of Na^+/K^+-ATPase in the rat vas deferens [16], but resting potential is unaltered by either adding high concentrations of ouabain or lowering external K^+ concentration [12], two established criteria to support the existence of electrogenic activity associated with the Na^+/K^+-pump. As a consequence, the authors inferred that Na^+/K^+-ATPase of rat vasa deferentia either is not electrogenic or fails to contribute significantly to the maintenance of resting membrane potential.

In conclusion, we report here a selective down-regulation of the Na^+/K^+-ATPase α_2 isoform after denervation of the rat vas deferens. Whether or not the present demonstration is related to the adaptive supersensitivity phenomenon is still unclear.

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