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Adaptive Supersensitivity and the Na^+/K^+ Pump in the Guinea Pig Vas Deferens: Time Course of the Decline in the $\alpha 2$ Subunit

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SUMMARY

Adaptive supersensitivity in the guinea pig vas deferens has been shown previously to be associated with decreases in transmembrane potential, Na^+/K^+-ATPase activity, [^{3}H]ouabain binding sites, and density of the $\alpha 2$ subunit of the pump. One of several procedures that induce adaptive supersensitivity in the guinea pig vas deferens is neurotransmitter depletion by chronic administration of reserpine. Guinea pigs were treated with reserpine (1.0 mg/kg/day, intraperitoneally) for 2, 5, or 8 days. Tissues were homogenized and the concentration of the $\alpha 2$ subunit was quantified by use of the selective antibody McB2, slot blot analysis, enhanced chemiluminescence, and densitometric analysis. As reported previously, the concentration of the $\alpha 2$ protein was reduced 41% after 5 days of pretreatment. The reduction was maintained at 8 days (37%). However, there was no change from control after 2 days of pretreatment with reserpine. Thus, the time course of the decline in the $\alpha 2$ subunit is similar to that of the appearance of supersensitivity, depolarization, and the declines in Na^+/K^+-ATPase and [^{3}H]ouabain binding established earlier. Based upon results in the literature for several different tissues and species, membrane depolarization and decreases in Na^+/K^+ pump sites may represent widely occurring adaptive mechanisms.

Chronic reduction in net stimulus received by muscle and nerve cells induces an adaptation of the cells that is characterized by an increase in sensitivity to excitatory neurotransmitters and drugs (1). In a number of smooth muscles, chronic interruption of transmission from excitatory nerves consistently results in nonspecific supersensitivity (2). That is, the sensitivity to the neurotransmitter and to several agonists pharmacologically unrelated to the transmitter is increased, by equivalent magnitudes. This fact implies that the mechanism responsible for the adaptive supersensitivity in smooth muscle involves a cellular function relevant to multiple receptor systems.

Chronic interruption of noradrenergic neurotransmission (by pre- or postganglionic denervation or reserpine-induced norepinephrine depletion) of the guinea pig vas deferens or the rabbit saphenous artery causes the development of nonspecific adaptive supersensitivity, partial depolarization of the cell membrane, and a reduction in electrogenic Na^+/K^+ pumping (3–5). Additional experiments with the guinea pig vas deferens, using intracellular electrical recording (6), measurements of Na^+/K^+-ATPase activity (4) and [^{3}H]ouabain binding (7), and quantification of the $\alpha 2$ subunit protein (8), combined to suggest that the supersensitivity of the vas deferens is the result of a decrease in the abundance of functional Na^+/K^+ pump sites in the smooth muscle cells.

In the guinea pig vas deferens, the adaptive supersensitivity, membrane depolarization, decrease in Na^+/K^+-ATPase activity, and decline in [^{3}H]ouabain binding all follow a well defined time course (3, 4, 7). The present work was undertaken to determine whether the decline in the abundance of the $\alpha 2$ subunit protein follows a similar time course. Preliminary results of this study have been published in an abstract (9).

Experimental Procedures

The methods used have been presented in considerable detail in previous publications from this laboratory (8, 10). The description that follows is, therefore, abbreviated.

Materials

Monoclonal antibody directed against the $\alpha 2$ subunit of the Na^+/K^+-ATPase (McB2) (11) was the generous gift of Dr. K. Sweadner (Harvard University). The antibodies were diluted in Tris-buffered saline [20 mM Tris·HCl (unbuffered), 137 mM NaCl] and stored at 4°. The antibody to the $\alpha 2$ subunit (McB2) was produced with purified rat brainstem axolemmal Na^+/K^+-ATPase (11) and has been shown to cross-react specifically with the $\alpha 2$ subunit from every mammalian species examined (12). Because the $\alpha 2$ subunit protein in the guinea pig has not been sequenced (to our knowledge), the $\alpha 2$ subunit measured in this paper should properly be considered "$\alpha 2$-like."

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Pretreatment Schedule

Adult male guinea pigs (300–400 g; Hilltop Lab Animals, Inc.) were treated with reserpine (1.0 mg/kg, daily) by intraperitoneal injection. Reserpine used for injection was prepared in a vehicle composed of benzyl alcohol, citric acid, and Tween 80, as described previously (13). Injectable solutions were derived from this stock solution by appropriate dilution into distilled water. Animals pretreated with reserpine for 2, 5, or 8 days and age-matched controls (no treatment) were killed by exsanguination after stunning. The dosage schedules used have previously been reported to reduce catecholamines in the vas deferens to undetectable levels (14).

Tissue Preparation for Protein Fractionation and Determination

Vasa deferentia were removed, desheathed, and stored in ice-cold protease inhibitor buffer (0.25 M sucrose, 1.0 mM EDTA, 4.0 mM phenylmethylsulfonyl fluoride, 1.0 mM 4-aminobenzamidine, 1 mg/ml bacitracin). Tissues were blotted, weighed, and homogenized in 1.0 ml of protease inhibitor buffer/100 mg of tissue. The homogenate was microcentrifuged for 5 min and the supernatant was stored at −20°. Protein determinations in tissue homogenates were made spectrophotometrically, according to the procedure described by Groves *et al.* (15).

Methods of Quantification

Slot blot analysis and hybridization. Slot blot analysis and hybridization were carried out as described previously (8, 10). The Amersham enhanced chemiluminescence system was used to detect the immobilized antigens complexed with McB2 and horseradish peroxidase-labeled antibodies. The blots were wrapped in plastic wrap and placed in cassettes with X-ray film (X-Omat AR; Kodak). Exposure times varied from 15 sec to 10 min.

Densitometric measurement and statistical analysis. Densitometric analysis was performed on the slot blot X-ray film records by using Bioscan Optimas imaging system software (8, 10). The densitometer reported a real value, extracted from area screen objects, giving the mean gray value of pixels within the area boundary. Densitometric analyses were conducted on films from slot blots in which tissue homogenates from at least one control and one treated animal had been applied. This procedure ensured equivalent treatment of control and treated samples for normalization.

Slot blot replicate gray values were averaged and the mean value was plotted against the amount of protein loaded. Values obtained from the linear portions of both experimental and control curves were normalized to the amount of total protein. The ratio of the normalized experimental values to control values was multiplied by 100 and defined as the percentage of control.

Comparisons of the averaged mean percentage of control values for samples obtained from control animals and from animals pretreated with reserpine were made using Student's *t* test for unpaired samples. Values were considered significantly different at $p \leq 0.05$.

Results

Fig. 1A is an X-ray film record from a slot blot analysis comparing the content of the α2 subunit in vas deferens homogenates from animals treated with reserpine for 5 days with that in a vas deferens homogenate from an age-matched control animal. The volume of crude homogenate loaded into each well of the slot blot manifold is indicated. These three samples had similar total protein concentrations and, therefore, by dilution of samples from 1 to 0.5 μl and from 0.5 to 0.25 μl, each dilution approximately halved the total protein loaded onto the nitrocellulose membrane. Note that the reaction product densities generated at the 1- and 0.5-μl levels for one reserpine-treated sample closely approximate the

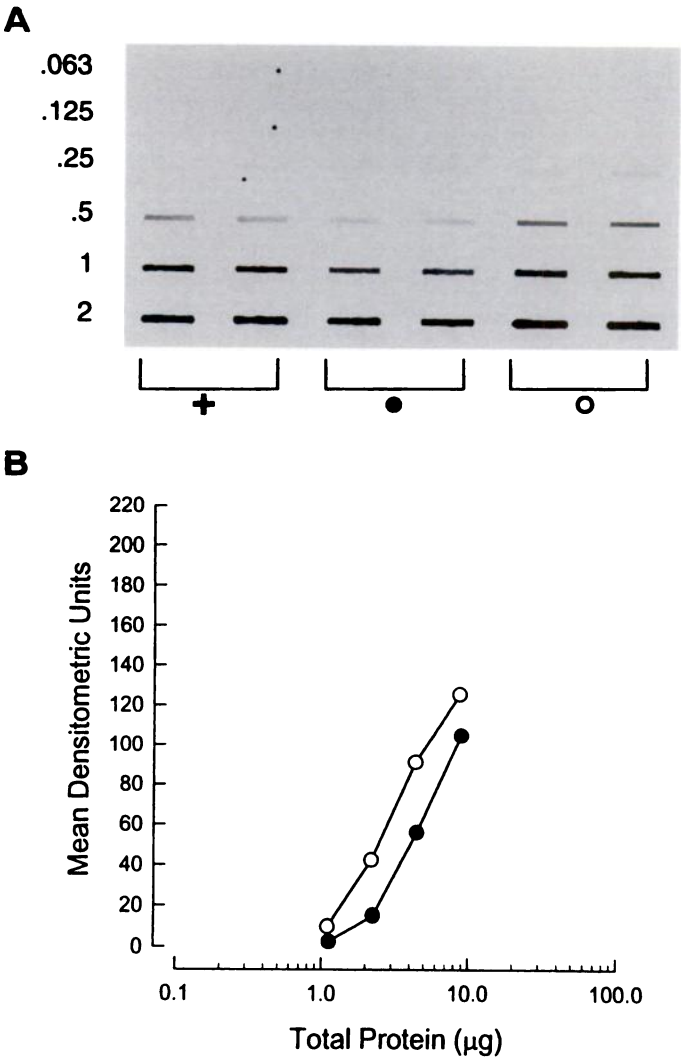

Fig. 1. Quantitative analysis of α2 subunit protein levels. A, Slot blot of tissue homogenates from a control animal (○) and animals pretreated with reserpine for 5 days (●, +). Samples were diluted and loaded in duplicate as described in Experimental Procedures. Blots were exposed to an anti-α2 antibody (McB2) and visualized using a goat anti-mouse IgG conjugated to horseradish peroxidase, in connection with the Amersham enhanced chemiluminescence detection system. *Numbers on the left*, volume (μl) of crude homogenate loaded into each well. B, Graphic presentation of the relationship between protein concentration and signal production (area gray value) in the homogenate from the control animal (○) and one of the preparations from a pretreated animal (●). Each *point* represents the average of the replicate gray values for a given protein concentration and was obtained from the slot blot in A. Note the consistency among replicates and the linear relationship between light intensity and protein concentration, as well as the reduced intensity in homogenates from treated animals, compared with control.

intensities seen at the 0.5- and 0.25-μl levels for the control sample. This same trend was observed for the other reserpine-treated sample, in comparison with the control, but not to the same extent.

When the linear range for the total protein-signal intensity relationship was identified, it was possible to quantitatively compare the amount of α2 subunit among the samples (Fig. 1B). To emphasize the differences in α2 subunit levels typically observed between tissues derived from control and experimental animals, the total protein-signal intensity relationships for the control and one reserpine-treated sample

are presented. The curve for the tissue from the treated animal was shifted to the right of the curve for the tissue from the control animal. This indicated that, for a given total protein concentration, there was less signal produced in the tissue from the animal treated with reserpine, in comparison with tissue from the age-matched control animal. When the data were normalized to total protein and converted to percentage of control values, a statistically significant 41% decrease in the level of $\alpha 2$ subunit in the vasa deferentia from treated guinea pigs was observed (Table 1). Note that the data in Table 1 for the 5-day treatment are taken from a previous publication (8).

Guinea pigs were also treated with reserpine for either 2 or 8 days, according to the regimen that produced significant changes in $\alpha 2$ subunit levels after 5 days of drug treatment. There was no significant difference and no trend toward depression of the level of the $\alpha 2$ subunit protein in the vasa deferentia from guinea pigs treated with reserpine for 2 days, in comparison with the tissues from age-matched control animals (Table 1). Although there was a trend toward an increase in the level, there was considerable variability, with values both below and above 100% and a p value between 0.1 and 0.2. In contrast to the short term treatment with reserpine, when animals were treated with reserpine for 8 days a difference similar to that seen at 5 days was observed. The mean result from the 8-day treatment group indicated a 37% decrease in the $\alpha 2$ subunit abundance in the reserpine-treated group, relative to control (Table 1).

Discussion

Previous work (9) established the existence of both a modified $\alpha 1$ subunit and an $\alpha 2$ subunit of Na^+/K^+-ATPase in the guinea pig vas deferens, using the specific antibodies McK1 and McB2. Size fractionation of homogenates of rat brainstem and guinea pig vas deferens, using electrophoresis, resulted in molecular mass estimates for the $\alpha 2$ isoform that were virtually identical (~100 kDa). In contrast, the predominate $\alpha 1$ isoform migrated with greater mobility, similarly to the truncated (55–60-kDa) form ($\alpha 1$T) that had previously been identified only in vascular smooth muscle (16).

The α subunit is the catalytic portion of the enzyme and incorporates the cardiac glycoside and sodium binding sites in addition to the ATPase activity (12). Relevant to the purpose of the present work is the fact that the $\alpha 2$ isoform has been found to have the highest affinity for cardiac glycosides (≤ 1 μM) among α subunits, in contrast to the regular $\alpha 1$ subunit, which has lower affinity (≥ 50 μM) (17, 18). Given that the IC_{50} of ouabain in inhibiting Na^+/K^+-ATPase activity (4) and the K_d for [^{3}H]ouabain binding (7) in guinea pig vas deferens are in the 0.1–1.0 μM range, it is probable that the $\alpha 2$ subunit is responsible for the ouabain-sensitive, Na^+/K^+ pump activity in this tissue. At this time, neither the affinity for glycosides nor the physiological function of the truncated $\alpha 1$ subunit has been determined.

A relationship between adaptive supersensitivity in the guinea pig vas deferens and changes in membrane potential was established in the 1970s. A variety of procedures (postganglionic denervation, preganglionic denervation, or chronic administration of reserpine to deplete the neurotransmitter) that chronically interrupt the excitatory (adrenergic) pathway to the vas deferens produce supersensitivity (3, 19, 20). The sensitivity to adrenoceptor agonists, cholinoceptor agonists, histamine, and potassium ions is nonspecifically increased. All of the aforementioned procedures produce supersensitivity that is qualitatively and quantitatively the same. The supersensitivity is not associated with changes in ligand binding to adrenoceptors (21) but is correlated with a partial depolarization of the smooth muscle membrane (3). The close association of membrane potential and adaptive supersensitivity has been found not only for the smooth muscle of the guinea pig vas deferens but also for rabbit vascular smooth muscle (5) and myenteric S neurons of the guinea pig ileum (22, 23).

The supersensitivity and partial depolarization of the smooth muscle of the vas deferens are both dependent upon a marked decrease in electrogenic sodium pumping (4) and are associated with a 25–40% reduction in Na^+/K^+-ATPase activity (4), [^{3}H]ouabain binding (7), and the abundance of the $\alpha 2$ subunit of the pump (8). The supersensitivity, depolarization, decline in ATPase activity, and decrease in ouabain binding all follow the same time course (3, 4, 7). None of the changes appear during the first 2 days after the innervation is interrupted but are fully developed by day 4, remaining as long as the events have been followed thereafter. The present experiments confirm that the time course for the decline in the $\alpha 2$ subunit, after catecholamine depletion, also fits that pattern (Table 2). In the interest of space, only one agonist (potassium ion) is included in Table 2. Although the shifts of the dose-response curves produced by K^+ are somewhat less than those produced by other agonists, the shifts are very reproducible and experiments have been done with K^+ at all time points.

Adaptive adjustments in cellular sensitivity represent a homeostatic mechanism by which excitable cells adapt to

TABLE 1

Time-dependent changes in guinea pig vas deferens $\alpha 2$ subunit protein levels associated with chronic reserpine treatment

	2 Day	5 Day[a]	8 Day
$\alpha 2$ subunit protein level (% of control)[b]	157 (83–231)	59[c] (40–77)	63[c] (22–104)
n	9	7	5

[a] Data from previous work (8).

[b] Slot blot replicate gray values were initially averaged. The mean values were plotted against the amount of protein loaded. Representative points from the linear part of both experimental and control curves were normalized to the amount of protein. The mean ratios (×100) of the normalized experimental to control values are given as mean percentage of control values. Values in parentheses represent 95% confidence intervals.

[c] Unpaired t test, $p < 0.05$ as compared with control (100%).

TABLE 2

Time course of cellular changes (means) with adaptive supersensitivity in smooth muscle of the guinea pig vas deferens

	Days 1–2	Days 4–5	Days 7–8
Supersensitivity to K^+ (ratio of EC_{50} values) (3, 20)	None	1.4	1.5–2.3[a]
Decreases in			
Transmembrane potential (mV) (3)	None	7.9	8.5
ATPase activity (%) (4)	None	25	25–35[a]
[^{3}H]Ouabain binding (%) (7)	None	29	25–40[a]
$\alpha 2$ subunit protein (%)[b]	None	41	37

[a] Ranges of values indicate that results have been obtained in more than one experimental group, yielding slightly different mean values.

[b] Present results.

prolonged (days/weeks) changes in net stimulus/inhibition received (1). The results of this study are consistent with the general hypothesis that the cellular mechanism underlying any adaptative change in sensitivity involves cellular processing of proteins. The particular characteristics of the change in sensitivity are determined by which proteins are modified by the cell. For example, supersensitivity in rat skeletal muscle is highly specific for nicotinic cholinomimetics and is due primarily to up-regulation of nicotinic receptors [see review by Fleming (24)]. Atrial muscle from guinea pigs, in contrast, becomes supersensitive only to agonists that utilize the adenylyl cyclase cascade as a second messenger system, suggesting that the underlying mechanism is a change in adenylyl cyclase (25, 26).

An important related study has been presented by Rogers *et al.* (27). Those investigators found that organ cultures of canine colonic smooth muscle develop an adaptive supersensitivity to acetylcholine within 3 days, presumably due to the extrinsic denervation. This supersensitivity is accompanied by membrane depolarization (from −82 to −55 mV) and a loss of electrogenic Na^+/K^+ pumping. Northern analysis indicated a sharp decline in the mRNA for the α2 subunit, but not the α1 subunit, within 1 day, which was maintained through 6 days.

Adaptive supersensitivity to excitatory agents and/or subsensitivity to inhibitory agents has now been shown to be associated with membrane depolarization in four different tissues in three different species [guinea pig vas deferens (3), rabbit saphenous artery (5), guinea pig myenteric neurons (23), and canine colonic muscle (27)]. In three of these [vas deferens (4), saphenous artery (5), and colonic muscle (27)] there is evidence that decreased electrogenic Na^+ pumping is a factor, and in two [vas deferens (Ref. 9 and the present work) and colonic muscle (27)] there is now evidence of reduced presence or transcription of the α2 subunit of the Na^+/K^+ pump. This growing body of evidence suggests that alterations in resting membrane potential and regulation of the Na^+/K^+ pump may represent widely occurring adaptive mechanisms in smooth muscle and neurons.

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