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Role of noradrenaline on the expression of the Na^+/K^+-ATPase α_2 isoform and the contractility of cultured rat vas deferens

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Abstract

Rat vasa deferentia were cultured for 3 days in Dulbecco's modified Eagle's medium in the absence or presence of 1 μM noradrenaline (NA) to investigate if the lack of NA release is the key factor to explain the selective reduction of the Na^+/K^+-ATPase α_2 isoform previously observed after *in vivo* denervation of this organ (Quintas *et al*., Biochem Pharmacol 2000;60:741–7). The lack of effects of the indirect sympathomimetic tyramine and the neuronal amine uptake blocker cocaine on NA curves indicated that cultured organs were denervated completely. Organ culture induced supersensitivity, expressed as a 6.3-fold increase of pD_2 and a 42% elevation of maximal contraction for NA but not for Ba^{2+}. Western blotting indicated that the level of the α_1 isoform of Na^+/K^+-ATPase was unchanged after organ culture, but the α_2 isoform was down-regulated drastically to levels that were barely detectable. The addition of NA to the culture medium did not prevent the reduction of α_2 expression although it did impede NA supersensitivity (in fact a 4-fold decrease of pD_2 and a 32% reduction of maximal response were observed after incubation in the presence of NA). A striking reduction of L-type Ca^{2+} channel expression also was observed, indicated by an 85% decrease of [^{3}H]isradipine binding sites. These data suggest that NA is a trophic factor relevant to the control of muscle contraction, mediated by α_1-adrenoceptors, but not to the expression of either Na^+/K^+-ATPase or the L-type Ca^{2+} channel.

Keywords: Na^+/K^+-exchanging ATPase; L-type Ca^{2+} channel; Vas deferens; Organ culture; Supersensitivity; Noradrenaline

1. Introduction

Chronic suppression of neuromuscular transmission in guinea pig vas deferens leads to adaptive postjunctional supersensitivity, a phenomenon in which the effect of various agonists is potentiated [1]. In addition, a decrease in the density of the Na^+/K^+-ATPase α_2 isoform was also described [2]. Supersensitivity was ascribed to a less negative resting membrane potential of the myocyte that increased excitability to the agonist [2,3]. A similar qualitative pattern of supersensitivity was observed in the rat vas deferens [4,5], but how important Na^+/K^+-ATPase is to this process is still controversial. Although vasa deferentia of rats contain higher Na^+/K^+-ATPase activity than guinea pigs [6], maintenance of membrane potential in rats is less dependent on this activity than in guinea pigs [7]. Nevertheless, we observed that the Na^+/K^+-ATPase α_2 isoform level is also reduced in rats after denervation of their vas deferens [8].

To address the question of what missing factor dictates the reduction of Na^+/K^+-ATPase expression in denervated rat vas deferens, we used an organ culture model developed in the early 1980s by Takeyasu *et al*. [9]. This *in vitro* condition allows one to observe changes that occur in a controlled environment, i.e. it is possible to maintain the organ without the trophic effects of nerves and hormones and to add substances in the culture medium. A few investigators have used the whole vas deferens in culture

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Abbreviations: NA, noradrenaline.

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to study the influence of innervation on smooth muscle properties [10,11], but none have examined the effect of innervation on entities involved in ionic flux, such as Na^+/K^+-ATPase and L-type Ca^{2+} channels, that are essential for excitation–contraction coupling and are reduced after denervation [12].

Our objectives in the present study were to evaluate the expression of Na^+/K^+-ATPase α isoforms and L-type Ca^{2+} channels in cultured adult whole rat vas deferens and to investigate if the presence in culture of the sympathetic neurotransmitter NA is able to change their pattern *in vitro*.

2. Materials and methods

2.1. Rat vas deferens culture

Adult Wistar rats weighting 350–450 g (4- to 6-months-old) from our BAW2 colony [13] were killed under ether anesthesia, and vasa deferentia were isolated aseptically (non-cultured organs were not submitted to the following steps). For cultivation, organs were placed vertically in test tubes containing 50 mL of normal culture medium (Dulbecco's modified Eagle's medium) with 10% fetal bovine serum and antibiotics (100 μg/mL of streptomycin and 100 μg/mL of penicillin). The epididymal portion was fixed with a silk thread to the cap of the tube and the prostatic end to a glass weight. Different weights (0.25, 0.5, or 1.0 g) were used in order to test which one would give the best results. After choosing 0.5 g as the optimal load, the organs were incubated at 37° for 3 days without (control group) or with 1 μM NA (NA group). EGTA (10^{-5} g/L) was added to the NA medium to prevent NA degradation. The rat vas deferens, either immediately after isolation (non-cultured) or after organ culture, was mounted in an organ bath or used for membrane preparation.

2.2. Recording of contractile responses

Non-cultured and 3-day cultured (control or NA) organs were mounted in 10-mL chambers containing modified Tyrode's solution of the following composition: 136 mM NaCl, 5.6 mM KCl, 1.8 mM $CaCl_2$, 0.36 mM $NaHPO_4$, 15 mM $NaHCO_3$, and 5.5 mM dextrose [14]. Air was bubbled continuously through the solution, which was maintained at 30°. Each muscle was equilibrated for 30 min under a resting tension of 1 g before recording isotonic contractions on a kymograph, with tangential levers giving a 6-fold amplification. Tyramine (100 μM) was always added at the beginning of the experiments to ensure that stored NA was absent. After the washout of tyramine, cumulative concentration–response curves were always made for Ba^{2+}, followed, after washout, by a curve for NA. In general, an interval of about 30 min was allowed between consecutive concentration–response curves. In some experiments, cocaine (6 μM) was added to block neuronal uptake of NA.

pD_2 values were calculated as the negative logarithms of EC_{50} obtained experimentally in order to represent apparent affinities. Maximal contractile effects (E_{max}) of agonists also were determined. The maximal effect of Ba^{2+} was measured to indicate the maximal capability of the contractile system [15].

2.3. Homogenate preparation

As described by Noël *et al.* [16], pooled organs were minced and homogenized in sucrose buffer (pH 7.4) plus 0.2 mM phenylmethylsulfonyl fluoride (PMSF) and 2 mM dithiothreitol (DTT) using an Ultraturrax apparatus. After 10 strokes in a glass Potter homogenizer, homogenates were gauze-filtered under vacuum and centrifuged at 108,000 *g* for 1 hr at 4°. The pellet was resuspended in the same buffer (without DTT) and stored at −80°. The method of Lowry *et al.* [17] was employed to determine protein concentration. The method of preparation of the brain hemispheres and kidney homogenates has already been reported [16].

2.4. SDS–PAGE and western blotting

Samples run (without previous heating) on a 7.5% polyacrylamide gel were transferred to nitrocellulose filter papers and sequentially incubated for 1 hr in 5% non-fat dry milk plus 0.1% Tween 20, 1 hr with either monoclonal mouse IgG anti-rat Na^+/K^+-ATPase α_1 (McK1) or the α_2 (McB2) or α_3 (F9-G10) isoforms, and 1 hr with an anti-mouse horseradish peroxidase-conjugated antibody (see details in Quintas *et al.* [8]). Immunoreactivity was detected using enhanced chemiluminescence by exposure to Hyperfilm-ECL (Amersham). 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), as reported previously [8].

2.5. Radioligand binding assays

Under sodium light, [^{3}H]isradipine ((+)-[^{3}H]PN200-110) in different concentrations (0.1 to 1.0 nM) was incubated with diluted homogenates (200 μg protein/mL) at 37° for 90 min in 50 mM Tris–HCl buffer (pH 7.4). After incubation, samples were diluted rapidly with 5 mL of ice-cold Tris–HCl buffer and filtered instantaneously on Whatman glass fiber filters (GF/C) under vacuum; the filters were then washed three times with 5 mL of buffer, dried, and immersed in scintillation fluid, and the radioactivity retained in the filters was measured with a Packard Tri-Carb 1600 TR liquid scintillation analyzer. Non-specific binding was estimated in the presence of 10 μM unlabeled

nifedipine. K_D and B_{max} parameters were calculated from equilibrium binding experiments by non-linear regression analysis, as in a previous report [8].

2.6. Statistics

Data are presented as means ± SEM, unless indicated otherwise. The significance of differences of biochemical and functional parameters between two distinct groups was tested by Student's unpaired *t*-test.

3. Results

3.1. General characteristics of cultured rat vas deferens

Our first goal was to choose the appropriate weight to maintain the muscle under a permanent load in the culture medium. We chose 0.5 g because the organ shrank or became excessively extended when using 0.25 or 1.0 g, respectively. As presented in Table 1, the protein content, wet weight, and length of the cultured vasa deferentia, under an applied tension of 0.5 g, were not different from those of non-cultured organs.

3.2. Contractility of cultured vasa deferentia: response to tyramine and Ba^{2+}

None of the 3-day cultured organs responded to 100 μM tyramine, an indirect sympathomimetic agent, denoting the very low level of endogenous catecholamines in nerve terminals probably due to neural degeneration. This possibility was supported further by experiments with cocaine (see below). Under our conditions, the cultured rat vas deferens did not generate intermittent spontaneous contractions (characteristics of postjunctional adaptive supersensitivity), in contrast to what was observed by others using 1-week-old rat vas deferens cultures [11] or after denervation procedures [18]. Concentration–response curves revealed that the cumulative E_{max} of Ba^{2+} (usually obtained with a concentration of 30 mM) was not altered significantly in cultured organs (Table 1), further indicating that there was no atrophy (or hypertrophy) of the cultured organ under our conditions. However, the E_{max} of Ba^{2+} was lower in vas deferens cultured in the presence of NA (Table 1).

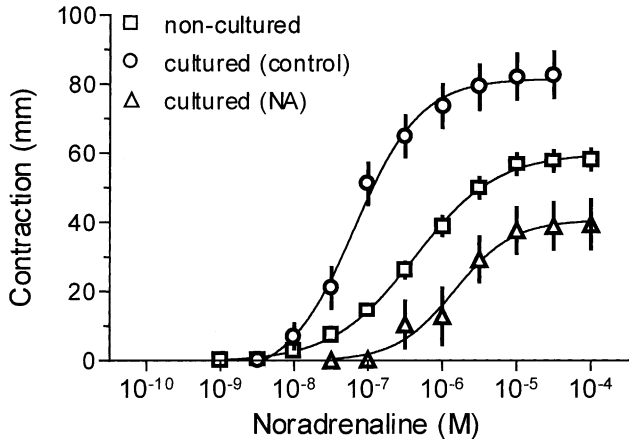

Fig. 1. Concentration–response curves for noradrenaline (NA) in non-cultured and 3-day cultured rat vas deferens without (control) or with NA. Data are the means ± SEM of 5–23 experiments.

3.3. Contractility of cultured vasa deferentia: response to NA

The effect of culture on rat vas deferens sensitivity to NA is depicted in Fig. 1. After 3 days in culture, both potency and maximal (absolute) contraction of the vas deferens were magnified significantly for NA, in such a way that the pD_2 was 0.8 log units higher (corresponding to a 6.3-fold potentiation) and the E_{max} was 42% higher compared with the non-cultured organ (Fig. 1 and Table 2). These results are very similar to what was seen some days following sympathectomy *in vivo* [19,20]. To evaluate the function of prejunctional neuronal catecholamine uptake in such phenomenon, cocaine was used as a classical inhibitor of this process [21]. With the cultured organs (control), cocaine failed to increase the potency of NA, as the NA curve did not shift further to the left (data not shown). In parallel control experiments made with non-cultured vas deferens, cocaine produced a large shift of the NA curve to the left (2.3 log units) without a significant change in E_{max}, as previously shown [21]. Thus, NA supersensitivity (increased pD_2) appears to be due to loss of prejunctional neuronal uptake, while the increase of maximal response

Table 1
Maximal contractile effect (E_{max}) of Ba^{2+}, protein content, wet weight, and length for non-cultured and 3-day cultured rat vas deferens without (control) or with noradrenaline (NA)

Groups	Ba^{2+} E_{max} (mm)	Protein content (mg/g tissue)	Wet weight (mg)	Length (mm)
Non-cultured	108.6 ± 6.4 (14)	53.6 ± 8.6 (3)	61.9 ± 1.8 (5)	56.0 ± 2.9 (16)
Cultured (control)	107.5 ± 5.6 (10)	56.0 ± 3.9 (3)	56.4 ± 5.6 (5)	52.6 ± 2.9 (6)
Cultured (NA)	87.6 ± 4.0* (16)	53.2 ± 6.0 (3)	ND	ND

Data are means ± SEM; the number of samples is given in parentheses. ND: not determined.
* Significantly different from the corresponding non-cultured and cultured (control) values ($P < 0.05$).

Table 2
Values of pD_2 and maximal contraction (E_{max}) for NA in non-cultured and 3-day cultured rat vas deferens without (control) or with NA

Group	NA	
	pD_2	E_{max} (mm)
Non-cultured	6.4 ± 0.1 (23)	58.2 ± 3.0 (23)
Cultured (control)	7.2 ± 0.1* (18)	82.7 ± 6.6* (18)
Cultured (NA)	5.8 ± 0.2** (6)	39.5 ± 7.0** (6)

Data are means ± SEM; the number of samples is given in parentheses.
* Significantly different from the corresponding non-cultured value ($P < 0.05$).
** Significantly different from the corresponding non-cultured and cultured (control) values ($P < 0.05$).

appears to be due to a postjunctional, adaptation-derived effect.

3.4. Cultured rat vas deferens: expression of Na^+/K^+-ATPase α isoforms

To correlate changes in the densities of the Na^+/K^+-ATPase α isoforms with contraction, the same amount of protein (40 μg) was loaded for cultured and non-cultured organs. Monoclonal antibodies were able to interact with the α_1 isoform in all samples from the two groups studied and did not exhibit any considerable difference among them (Fig. 2A). Blotting of the α_2 isoform, however, was reduced strikingly in cultured organs (control); there was no immunoreaction or very poor immunoreaction with McB2 antibodies (Fig. 2B). These data indicate that our model of organ culture results in a pattern of expression of α isoforms similar to what is detected *in vivo* after surgical denervation [8].

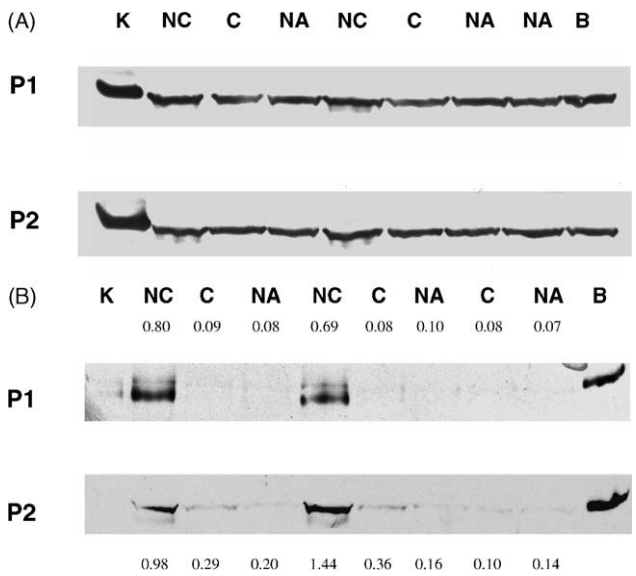

Fig. 2. Western blotting of Na^+/K^+-ATPase α_1 (A) and α_2 (B) isoforms in two different preparations (crude homogenates P1 and P2, 40 μg protein/lane) of non-cultured (NC, in duplicate) and 3-day cultured rat vas deferens without (C, in duplicate in A and in triplicate in B) or with noradrenaline (NA, in triplicate). Rat kidney (K, 15 μg protein/lane) and rat brain hemispheres (B, 10 μg protein/lane) were used as controls for antibody specificity. Numbers above or under the lanes of (B) correspond to the relative optical densities.

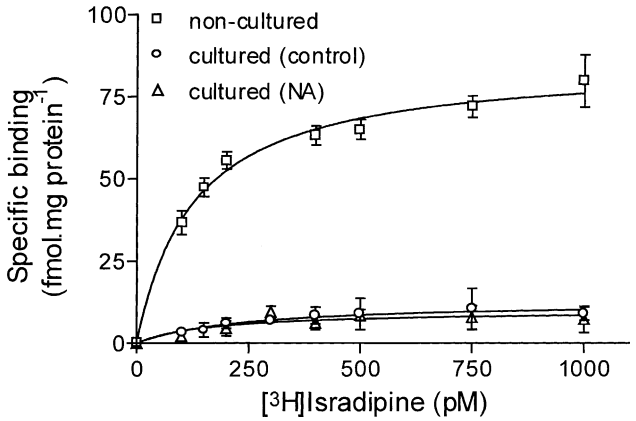

Fig. 3. Saturation curves for specific binding of [^{3}H]isradipine to crude homogenates of non-cultured and 3-day cultured rat vas deferens without (control) or with noradrenaline (NA). Each point represents the mean ± SD of triplicates in a typical experiment. Values of binding parameters are described in the text.

3.5. Cultured rat vas deferens: expression of L-type Ca^{2+} channels

Data obtained from binding experiments with [^{3}H]isradipine at equilibrium were represented as classical saturation curves (Fig. 3). Comparison of binding parameters calculated by non-linear regression demonstrated that the cultivation of rat vas deferens did not change the affinity of [^{3}H]isradipine for these sites ($K_D = 182 \pm 38$ and 188 ± 44 nM for non-cultured and cultured organs, respectively; N = 3). Nevertheless, a decrement of almost 85% in the density of binding sites associated with L-type Ca^{2+}-channels was observed for cultured organs ($B_{max} = 92 \pm 21$ and 14 ± 1 fmol/mg protein, for non-cultured and cultured, respectively; N = 3), similar to that found in the Na^+/K^+-ATPase α_2 isoform.

3.6. Incubation of cultured rat vas deferens with NA: effect on contractility

The persistent presence of 1 μM NA in the culture medium completely changed the concentration–response curve of NA. The pD_2 value for NA in vasa deferentia from the cultured (NA) group was lower than those in the cultured (control) group and the non-cultured group (Fig. 1 and Table 2). Interestingly, the presence of NA in the incubation medium also abolished the increase in the maximal effect of NA observed in cultured organs (E_{max} was almost 32% lower than that for non-cultured organs, Table 2). As observed with the vasa deferentia cultured in the control medium, cocaine failed to increase the potency of NA in these organs incubated with NA (data not shown).

3.7. Incubation of cultured rat vas deferens with NA: effect on Na^+/K^+-ATPase expression

The addition of NA during the incubation period was unable to prevent the down-regulation of the Na^+/K^+-ATPase α_2 isoform, since the cultured treated group (NA) exhibited the same profile as the cultured control group (C), i.e. the presence of α_1 but not α_2 (Fig. 2A and B). As in other reports, brain tissue (source of α_1, α_2, and α_3 isoforms) and kidney (source of α_1 isoform) were always run in parallel to discard cross-reactions due to lack of specificity [8,16]. The α_3 isoform was not detected in any of the groups assessed (data not shown).

3.8. Incubation of cultured rat vas deferens with NA: effect on L-type Ca^{2+} channel expression

Once again, the presence of NA in the culture medium did not interfere with the pattern sustained by cultivation, i.e. few [^{3}H]isradipine binding sites with the same affinity as in non-cultured vas deferens ($K_D = 235 \pm 71$ nM and $B_{max} = 15 \pm 3$ fmol/mg protein, N = 2).

4. Discussion

We report here that the expression of Na^+/K^+-ATPase α isoforms and L-type Ca^{2+} channels in cultured vas deferens behaved as described for *in vivo* denervation. Following 3 days in culture, the Na^+/K^+-ATPase α_2 isoform was markedly down-regulated; in contrast, the level of the α_1 isoform was similar to that of the controls. We reported recently that the rat vas deferens contains α_1 and α_2 isoforms of Na^+/K^+-ATPase [16] and that denervation produces a striking, specific down-regulation of the α_2 isoform [8] in a period when supersensitivity is completely established [19]. The present data are compatible with our recent study, although lower levels of the α_2 isoform were detected when compared with those obtained 14 days after surgical denervation. Other reports have associated the absence of innervation with a decrease of the α_2 isoform in astrocytes [22], oligodendrocytes [23], Schwann cells [24], and also cardiac [25] and skeletal [26] myocytes. Taken together, our results indicate that autonomic innervation modulates the expression of the Na^+/K^+-ATPase α_2 subunit isoform and that this phenomenon is not restricted to just one cellular type. Most interestingly, such a change in the vas deferens of reserpine-treated guinea pigs has been shown to present a temporal correlation with adaptive postjunctional supersensitivity [2,3].

In relation to L-type Ca^{2+} channel density, we noted, likewise, a remarkable decrement of [^{3}H]isradipine binding after culture, as we previously reported *in vivo* [12]. Again, the reduction presented here was more pronounced (85% decrease) than that reported earlier (60% decrease [12]). As for the Na^+/K^+-ATPase, dependence of neuromuscular coupling on the cellular expression of L-type Ca^{2+} channels has been documented for skeletal [27,28], cardiac [29], and smooth [30] muscles.

A link between denervation and regulation of expression of these two membrane proteins involved in calcium homeostasis has not been addressed. To investigate whether the lack of release of some neuronal substance is responsible for alterations in protein expression, we incubated the muscle in the presence of NA. Since rat vas deferens is one of the most densely innervated organs of the sympathetic nervous system [31], NA was considered the first logical choice for a putative trophic element to be investigated. Nevertheless, the addition of NA in the culture did not interfere with the down-regulation of the Na^+/K^+-ATPase α_2 isoform, although the neurotransmitter was successful in preventing NA supersensitivity and the augmentation of E_{max}.

In the case of the L-type Ca^{2+} channel experiments, NA was not able to prevent the large decrease in binding provoked by culturing the vas deferens. Data about the effect of NA on L-type Ca^{2+} channels are more confusing because they depend on the adrenergic receptor subtype stimulated [32]. As a first conclusion, our results are in accordance with many works that pointed out that NA has a limited prominence as a trophic factor, and that the existence of another trophic substance(s) may be involved [10,11,20,33,34].

As mentioned above, the presence of NA in the culture medium was not only able to prevent supersensitivity, but the concentration–response curve to NA was even shifted slightly to the right when compared with non-cultured vas deferens. Alterations in sensitivity and E_{max} have already been reported after incubation of the vas deferens with NA [10,35]. *A priori*, the most probable explanation would be a down-regulation of α_1-adrenoceptors due to the permanent presence of neurotransmitter in the medium. This does not seem to be the case, since Takeyasu *et al.* [10] have shown that a decrement of this receptor is seen not only when 1 or 100 μM NA is present, but also when this transmitter is absent from the culture medium (when supersensitivity is achieved). Therefore, assuming that subsensitivity after culture with NA is not due to changes in α_1-receptor density [10], we propose an alternative explanation: the presence of NA in the culture medium may have affected cellular contractile machinery and/or the intracellular pathway that leads to contraction. Indeed, phentolamine and inhibitors of protein synthesis have been shown to prevent subsensitivity [10,36].

Thus, the present results indicate a lack of a cause–effect relationship between changes detected in plasma membrane proteins, especially Na^+/K^+-ATPase, and the development of adaptive postjunctional supersensitivity in rat vas deferens. Under our conditions, we observed a higher maximal response for NA in addition to a shift to the left of the NA concentration–response curve, after culture of the tissue. It is noteworthy, therefore, that the behavior of the

Na^+/K^+-ATPase α isoforms was equal to that seen subsequently to *in vivo* denervation [8]. Moreover, even when subsensitivity appeared, the Na^+/K^+-ATPase α isoforms behaved as when the organ was supersensitive. These data lead one to suggest that the down-regulation of the Na^+/K^+-ATPase α_2 isoform is not needed for the presence of adaptive postjunctional supersensitivity, although a correlation has been demonstrated in the vas deferens of reserpine-treated guinea pigs [3].

In conclusion, our findings support the hypothesis that NA is an important factor for the regulation of drug-induced contractility, but it does not control membrane-bound Na^+/K^+-ATPase or L-type Ca^{2+} channel expression.

Acknowledgments

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