Does the release of tritiated noradrenaline accurately reflect the release of endogenous noradrenaline from rat vas deferens nerve terminals?

MARCELA BITRAN* and WILDO TAPIA

Departamento de Ciencias Fisiológicas, Facultad de Ciencias Biológicas, Pontificia Universidad Católica de Chile, Santiago, Chile

To determine whether the release of tritiated noradrenaline (NA) from the sympathetic nerve terminals of the rat vas deferens is an accurate reflection of the release of endogenous NA, we compared the electrically-evoked release of tritiated and endogenous NA from the prostatic sections of the vasa deferentia of male rats.

We found that while the release of tritiated NA was completely dependent on the presence of calcium, the release of endogenous NA was not. The overflow of both, tritiated and endogenous NA, was virtually unaffected by blockade of the neuronal uptake mechanism by desipramine. In contrast, blockade of the extraneuronal uptake greatly increased the overflow of endogenous NA, while having no effect on the overflow of tritiated NA. Tritiated NA release, on the other hand, was sensitive to prejunctional regulation, while the release of endogenous NA was not. Increases in stimulus train duration induced a significant increase in the release of endogenous NA, but not in that of tritiated NA. In contrast, the later responded to lower stimulus train frequencies and reached a plateau at lower frequency values as compared to the endogenous NA release.

Our results indicate the existence of marked differences between the release of tritiated and endogenous NA. We conclude that: 1) the assumption that tritiated NA release provides a good marker for endogenous NA release in the rat vas deferens seems unwarranted; 2) the use of endogenous NA to study the release process in the vas deferens requires a re-examination of the experimental conditions used, in order to minimize possible artifacts that may obscure the study of neuronal release; 3) the choice between measuring the release of tritiated or endogenous NA must be evaluated for each tissue in particular, taking into account its cytoarchitecture, as well as the experimental conditions used.

Key-terms: *noradrenaline release, tritiated noradrenaline, vas deferens.*

INTRODUCTION

The release of noradrenaline (NA) is often studied in isolated organs or tissues by means of radioisotopic techniques. These involve prelabeling of the nerve terminals with tritiated NA prior to the induction of release. The assumption that the release of tritiated NA is representative of the endogenous NA release has been questioned by

* Correspondence to: Dr Marcela Bitran, Departamento de Ciencias Fisiológicas, Facultad de Ciencias Biológicas, Pontificia Universidad Católica de Chile, Casilla 114-D, Santiago 1, Chile. Fax: (56-2) 222-5515. E-mail: mbitran@genes.bio.puc.cl

studies showing that the exogenous amine does not distribute homogeneously into the nerve terminals of some tissues.

Due to its dense sympathetic innervation, the rat vas deferens has been used as a model system for the study of noradrenergic neurotransmission. The techniques most commonly employed in these studies involve prelabeling of the tissue by using the neuronal uptake system to introduce radiolabeled NA into the sympathetic nerve terminal prior to release stimuli (Alberts *et al*, 1981; Lara *et al*, 1985; Ross & Kelder, 1979; Schönfeld & Trendelenburg, 1991). Although such techniques are sensitive, it is not known whether tritiated NA release is an accurate reflection of endogenous NA release. In fact, autoradiographic studies indicate that recently incorporated NA is preferentially stored in varicosities close to the surface of the tissue and, in comparison with endogenous NA, is preferentially released from the sympathetic varicosities (Moura *et al*, 1990; Paiva *et al*, 1993).

To determine if the release of tritiated NA is an accurate reflection of the release of endogenous NA, we compared the release of tritiated and endogenous NA evoked simultaneously by transmural electrical stimulation of the prostatic segments of the rat vasa deferentia. Specifically, we studied the requirement for extracellular calcium ions, the importance of reuptake and prejunctional autoreceptor regulatory mechanisms and the dependence of the release on the frequency and duration of the stimulus trains. In addition, to determine whether diffusional barriers may contribute to a non-homogeneous distribution of the labeled NA leading to dissociation in the release of tritiated and endogenous NA, we performed some studies in epithelium-denuded vasa deferentia.

METHODS

Drug sources.

Desipramine, dihydroxybenzylamine, noradrenaline, normetanephrine, pargyline, sodium octyl sulfate and yohimbine were purchased from Sigma Co (St Louis, MO, USA). [^{3}H]NA (levo-[ring-2,5,6-^{3}H], sp act 71.7 Ci/mmol) was purchased from Dupont, NEN (Boston, MA, USA).

Animals and general procedures.

Male Sprague-Dawley rats (200-250 g) were bred in the animal reproduction facilities of this university. The rats were housed under standard conditions (*i.e.*, 25°C and controlled 12:12 h light:dark cycle) and had free access to food and water. The rats were sacrificed by decapitation and the vasa deferentia were carefully dissected free from adipose and connective tissues. The prostatic portions of the two vasa deferentia of each rat were cut and mounted in a 7 ml capacity organ bath containing Tyrode's solution of the following chemical composition (mM): 113 NaCl, 4.7 KCl, 2.5 $CaCl_2$, 2.5 $MgSO_4$, 1 NaH_2PO_4, 16.3 $NaHCO_3$, 7.7 glucose, 0.1 ascorbic acid and 0.003 EDTA, at pH 7.4, bubbled with 95% O_2 plus 5% CO_2, kept at 37°C. The bath was equipped with paired platinum wire electrodes with their axes parallel to the tissue pieces. Transmural neural stimulation was achieved by 1 ms duration pulses.

Some experiments were carried out in epithelium-denuded vasa. The removal of the epithelium was accomplished mechanically. The ductus was held firmly from its prostatic end with a pair of forceps, a circular cut of the muscular layers was done, taking care not to sever the epithelium and the muscular layers were gently stripped out.

We routinely recorded the muscle contractions evoked by the transmural electrical stimulation in order to evaluate the viability of the preparation and the post-junctional responses. Isometric muscle contractions of the prostatic segments of the two vasa deferentia of each rat were recorded under 1 g basal tension through FT.03 force displacement transducers connected to a model 7 Grass Polygraph. Each experiment was performed 4 to 6 times, each time with tissues from a different rat.

Study design.

To compare the release of [^{3}H]NA with that of endogenous NA from the rat vas deferens, we measured the release (or the over-

flow) of tritium and NA before and after stimulating the prostatic halves of the vasa deferentia, prelabeled with [^{3}H]NA, with two consecutive trains (S1 and S2) of electrical pulses (of 75 V, at 7.5 Hz) delivered 30 min apart. In most studies, each train lasted 20 s.

In control experiments, S1 and S2 were delivered under identical conditions. In experiments designed to evaluate the effects of different drugs on the release of [^{3}H]NA and endogenous NA, these drugs were added to the organ bath 10 min prior to S2 and remained in contact with the tissues for the remainder of the experiment. Since the overflow of NA (both tritiated and endogenous) measured after electrical stimulation was fairly small, in most experiments we measured NA overflow after combined pharmacological blockade of the uptake mechanisms, the prejunctional regulation by α_2 autoreceptors and the oxidation of NA by monoaminooxidase (MAO). This was accomplished by supplementing the Tyrode's solution with 0.6 μM desipramine, 3 μM yohimbine, 10 μM normetanephrine and 1 μM pargyline. We refer as ***NA release*** the overflow of NA obtained in the presence of all the above-mentioned drugs. In some studies, part of the experimental protocol (B2 and S2) was carried out using a calcium-free solution supplemented with 0.1 μM EGTA.

In studies aimed at evaluating the effect of the stimulus duration on NA release, the tissues were stimulated four consecutive times (S1, S2, S3 and S4; at 7.5 Hz with 75 V pulses) every 30 min, with train durations randomly assigned to either 2, 5, 10 or 20 s. Similarly, in studies aimed at evaluating the effect of stimulation frequency on NA release, the tissues were stimulated four consecutive times (S1, S2, S3 and S4; for 20 s with 75 V pulses) every 30 min, with pulses frequencies randomly assigned to either 3.5, 7, 15 or 45 Hz.

Uptake of [^{3}H]NA and release protocol.

The prostatic segments of two vasa deferentia were incubated in Tyrode's solution for an initial period of 30 min. To allow for the neuronal uptake of [^{3}H]NA, the tissues were incubated for 60 min in solution containing [^{3}H]NA (spec act 71.7 Ci/mmol, 28 nM) and 10 μM normetanephrine to minimize the extraneuronal uptake of the tracer. After a 50 min washing period, the tissues were mounted in the organ bath, allowed to equilibrate for 1 h and subjected to electrical stimulation.

The solution in the organ bath was changed every 5 min during the release period and the 7-ml fractions were received in test tubes containing perchloric acid (PCA, final concentration 0.05 N) and sodium metabisulfite (final concentration 0.0036%). The collected fractions were kept at 4°C until assayed for tritiated and endogenous NA. S1 and S2 were delivered at 19 and 49 min, respectively, from the start of the release period. At the end of the release protocol, the tissues were homogenized in 15 vol of 0.4 N PCA. The homogenates were centrifuged (7.000 g x 20 min) and the supernatants were assayed by liquid scintillation spectrometry and high precision liquid chromatography coupled to electrochemical detection (HPLC-EC) to determine the remaining tissue contents of [^{3}H]NA and endogenous NA, respectively. The same protocol was used in experiments carried out in epithelium-denuded vasa deferentia.

Measurement of [^{3}H]NA.

The radioactivity present in 0.5 ml aliquots of the fractions of Tyrode's solution collected during the release protocols, as well as the radioactivity present in 0.5 ml aliquots of the perchloric supernatants originated from the sedimentation of the tissue homogenates, was measured by liquid scintillation spectrometry. Under control conditions, approximately 30% of the basal tritiated compounds released corresponds to NA, whereas NA accounts for approximately 65% of the tritiated compounds released by electrical stimulation (data not shown).

The fractional tritium release was calculated by dividing the tritium released in each fraction by the amount of radioactivity present in the tissue at the time of collection. Stimulation-evoked tritium release was defined as the tritium released during

the period of stimulation plus 3 ensuing collection periods, values to which the basal tritium release had been previously subtracted. Results of these studies are expressed as percentages of fractional tritium release (% FR).

Measurement of endogenous NA.

The fractions of Tyrode's solution collected during the release protocols were processed by adsorption chromatography in alumina (Anton & Sayre, 1962), prior to their injection into the HPLC system. Dihydroxybenzylamine (70 pmol/sample) was added as internal standard to the remaining 6.5 ml of each fraction. Noradrenaline was eluted from the alumina with 0.10 N PCA and its concentration in the perchloric eluates was determined by HPLC-EC, as previously described (Torres *et al*, 1992). Briefly, 100 µl samples of perchloric eluates or tissue supernatants (diluted 20-fold in mobile phase) were injected onto a lichrosphere reverse phase ion-pair column (60 RP Select B, Merck 50981, 5 µm, 124 x 4 mm) using a mobile phase of pH 2.7, containing (mM): 100 NaH_2PO_4, 1.0 Na_2EDTA, 1.15 sodium octyl sulfate and 1.5% acetonitrile. A flow rate of mobile phase of 0.8 ml/min was maintained by a Merck Hitachi L-6200 A pump. The electrochemical detection system (656 ED, Metrohm) consisted of a glassy carbon working electrode and an Ag/AgCl reference electrode, set at a potential of 0.8 V with an amperometric detector (641 VA, Metrohm) connected to a Merck Hitachi D-2500 integrator. The retention time for NA under these conditions ranged between 3.9 and 4.2 min, and the limit for NA detection was approximately 0.2 pmol/injection. The signal-to-noise ratio for detection of basal endogenous NA was approximately 5. Results are expressed as pmol of NA released per ml solution.

Statistical analyses.

Results are expressed as means ± SEM's. Differences between means were evaluated by the paired Student's *t*-test in the case of endogenous NA and by the paired Wilcoxon test in the case of tritiated NA (which is expressed as a relative value). The results of the studies on the effects of stimulus train frequencies or durations were evaluated by one-way ANOVA, followed by *post-hoc* Newman-Keuls multiple range test. Prior to the ANOVA, the proportions of tritiated NA release were subjected to arc-sin transformation. In all cases, significance was set at $P < 0.05$.

RESULTS

Overflow of tritiated and endogenous noradrenaline.

In conditions in which the mechanisms of neuronal and extraneuronal reuptake, enzymatic degradation and prejunctional regulation of NA are operative (*i.e.*, Tyrode's solution without drugs), the electrical stimulation of the tissues (Fig 1, S1) evoked small overflows of tritiated and endogenous NA, comparable to the respective basal overflow values (Fig 1, B1). In fact,

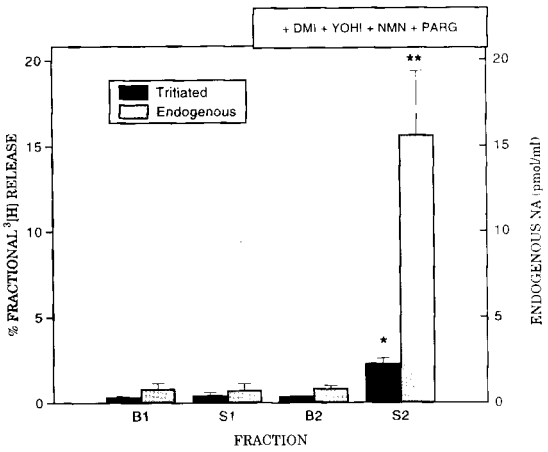

Fig 1. Effect of transmural stimulation on overflow and release of tritiated and endogenous NA. The prostatic segments of the two vasa deferentia of each rat, previously loaded with [^{3}H]NA, were transmurally stimulated with 2 trains of stimuli (S1 and S2; at 7.5 Hz for 20 s; pulses of 70 V, 1 ms) delivered 30 min apart. During first half of release protocol (B1 and S1), tissues were incubated in Tyrode's solution without drugs. During second half of protocol (B2 and S2), tissues were incubated in Tyrode's solution supplemented with 0.6 µM desipramine, 3 µM yohimbine, 10 µM normetanephrine and 1 µM pargyline. Results expressed as means ± SEM's (n = 4-6). ** $P<0.005$; * $P<0.05$ *vs* corresponding values in S1.

in two out of four tissues studied no overflow of NA was detected in response to electrical stimulation, whereas one of 4 tissues failed to display tritiated NA overflow in response to electrical stimulation.

In contrast to the above, in conditions in which the mechanisms of reuptake, degradation and prejunctional regulation of NA were blocked (solution supplemented with desipramine, normetanephrine, pargyline and yohimbine), the electrical stimulation evoked marked releases of tritiated and endogenous NA, which were, respectively, 5 and 20 times larger than the corresponding basal release values (Fig 1). The basal overflow of tritiated and endogenous NA was not affected by the presence of the above-mentioned drugs in the solution.

Effect of blockade of extraneuronal uptake.

Addition of 10 μM normetanephrine to a Tyrode's solution otherwise devoid of drugs increased the stimulated and basal overflows of endogenous NA by 54 and 5 times, respectively (Fig 2). In contrast, normetanephrine had no significant effects on the overflow of tritiated NA (Fig 2).

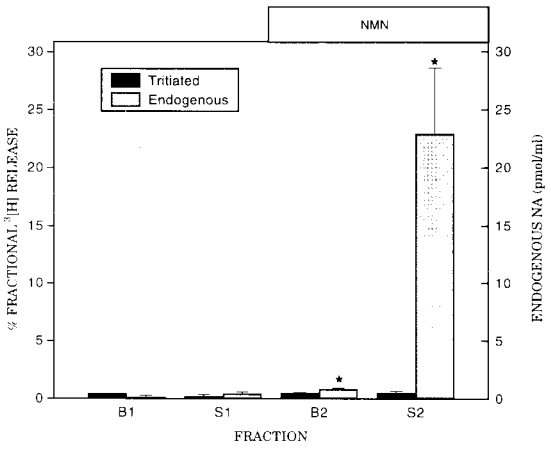

Fig 2. Effect of normetanephrine on overflow of tritiated and endogenous NA. The prostatic segments of the two vasa deferentia of each rat, previously loaded with [^{3}H]NA, were transmurally stimulated with 2 trains of stimuli (S1 and S2; at 7.5 Hz for 20 s; pulses of 70 V, 1 ms) delivered 30 min apart. During first half of release protocol (B1 and S1), tissues were incubated in Tyrode's solution without drugs. During second half of protocol (B2 and S2), tissues were incubated in Tyrode's solution supplemented with 10 μM normetanephrine. Results expressed as means ± SEM's (n = 4-6). * P<0.005 *vs* corresponding values in S1 and B1.

Effect of blockade of neuronal uptake.

In the absence or presence of 0.6 μM desipramine the basal and stimulated overflow of tritiated or endogenous NA was below detection limits (not shown). When the experiment was carried out in a Tyrode's solution containing normetanephrine, pargyline and yohimbine, the addition of 0.6 μM desipramine produced no change in either the basal or the stimulated release of tritiated or endogenous NA (Fig 3).

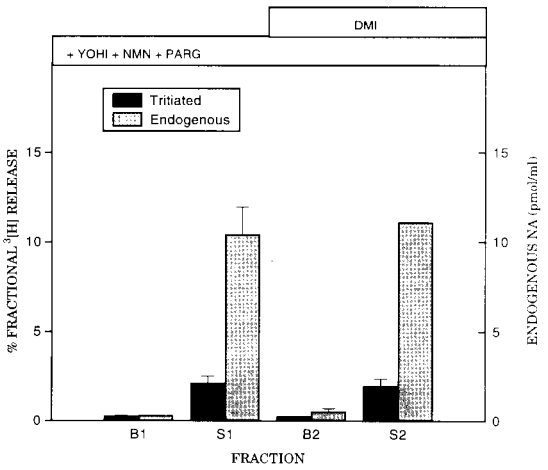

Fig 3. Effect of desipramine on release of tritiated and endogenous NA. The prostatic segments of the two vasa deferentia of each rat, previously loaded with [^{3}H]NA, were transmurally stimulated with 2 trains of stimuli (S1 and S2; at 7.5 Hz for 20 s; pulses of 70 V, 1 ms) delivered 30 min apart. During first half of release protocol (B1 and S1), tissues were incubated in Tyrode's solution containing 3 μM yohimbine, 10 μM normetanephrine and 1 μM pargyline. During second half of protocol (B2 and S2), tissues were incubated in the above-described solution supplemented with 0.6 μM desipramine. Results expressed as means ± SEM's (n = 4-6).

Effect of blockade of prejunctional autoreceptors.

Addition of 3 μM yohimbine to a Tyrode's solution containing normetanephrine, desipramine and pargyline increased the stimulated release of tritiated NA 7.4 times but had no effect on the basal release of tritiated NA. Yohimbine had no effect on either the basal or the stimulated release of endogenous NA (Fig 4).

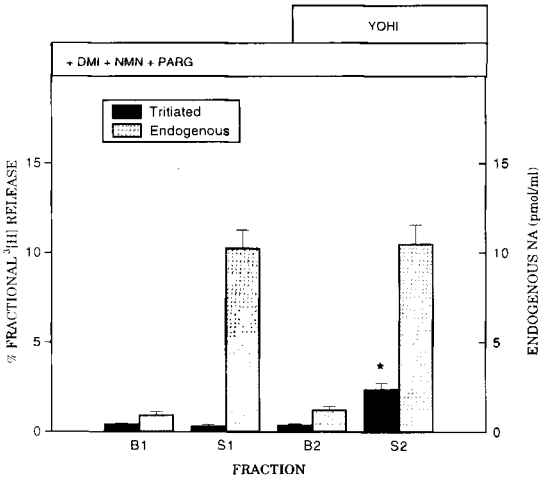

Fig 4. Effect of yohimbine on release of tritiated and endogenous NA. The prostatic segments of the two vasa deferentia of each rat, previously loaded with [^{3}H]NA, were transmurally stimulated with 2 trains of stimuli (S1 and S2; at 7.5 Hz for 20 s; pulses of 70 V, 1 ms) delivered 30 min apart. During first half of release protocol (B1 and S1), tissues were incubated in Tyrode's solution containing 0.6 μM desipramine, 10 μM normetanephrine and 1 μM pargyline. During second half of protocol (B2 and S2), tissues were incubated in the above-described solution supplemented with 3 μM yohimbine. Results expressed as means ± SEM's (n = 4-6). * P<0.005 *vs* corresponding value in S1.

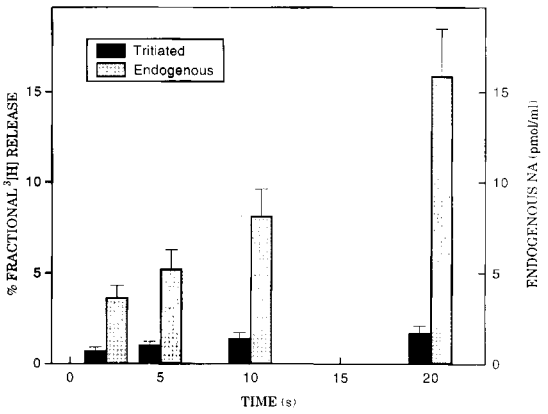

Fig 5. Effect of train duration on release of tritiated and endogenous NA. The prostatic segments of the two vasa deferentia of each rat, previously loaded with [^{3}H]NA, were transmurally stimulated with 4 trains of electrical stimuli (S1, S2, S3 and S4; at 7.5 Hz; pulses of 70 V, 1 ms) delivered 30 min apart. Duration of trains was randomly assigned to either 2, 5, 10 or 20 s. Experiment carried out in Tyrode's solution containing 0.6 μM desipramine, 3 μM yohimbine, 10 μM normetanephrine and 1 μM pargyline. Results expressed as means ± SEM's (n = 4-6). One way ANOVA indicated significant overall difference between means for endogenous NA (F=9.8; P<0.002), but not for tritiated NA (F=1.84; P<0.4).

Effect of duration and frequency of electrical stimulation.

The release of endogenous NA release, unlike that of tritiated NA, increased with the lengthening of the stimulus train duration. One way ANOVA indicated a significant overall difference between means for endogenous NA (F=9.8; P< 0.002), but not for tritiated NA (F=1.84; P<0.4). A *post-hoc* Newman-Keuls multiple range test indicated that the amount of endogenous NA released by a 20 s train was significantly different from that released by 2, 5 or 10 s trains (P<0.05) (Fig 5); whereas the releases evoked by 2, 5 or 10 s trains were not significantly different between themselves. In contrast, the amount of tritiated NA released by a 20 s train was not significantly different from that released by 2, 5 or 10 s trains. In addition, the releases evoked by 2, 5 or 10 s trains were not significantly different between themselves (Fig 5).

The release of both endogenous and tritiated NA increased with the increase in stimulation frequency. One way ANOVA indicated a significant overall difference between means for both, endogenous and tritiated NA (F=11.02; P<0.001; and F=18.16, P<0.001, respectively). A *post-hoc* Newman-Keuls multiple range test indicated that the amount of endogenous NA released by a 45 Hz train was significantly different from that released by 3.5, 7, 15 or 30 Hz trains (P<0.05) (Fig 6), and no significant differences were found between the releases evoked by 3.5, 7 and 15 Hz trains. In contrast, the amounts of tritiated NA released by 3.5 and 15 Hz trains were significantly different from those released by trains at all other frequencies. No significant differences were found between tritiated NA releases evoked by 7, 30 and 45 Hz trains (Fig 6).

Effect of extracellular calcium removal and cadmium addition.

Omission of calcium ions from the incubation solution (Ca^{2+}-free Tyrode's solution supplemented with 0.1 mM EGTA) completely in-

hibited the stimulated release of tritiated NA but had no effect on the stimulated release of endogenous NA (Fig 7). Basal releases of tritiated and endogenous NA were decreased by 21 and 38%, respectively, in the absence of calcium ions (Fig 7).

Addition of 100 μM $CdCl_2$ to a calcium-containing Tyrode's solution completely inhibited the stimulated release of tritiated NA and slightly decreased the basal release of tritiated NA, but had no statistically significant effect on the stimulated or basal release of endogenous NA (Fig 8). The same results were obtained with 500 μM $CdCl_2$ (not shown).

NA release from epithelium-denuded vasa deferentia.

The prostatic segments from epithelium-denuded vasa weighed 28.7 ± 0.9 mg and the prostatic segments from control vasa weighed 33.9 ± 2.5 mg (n=5; P=0.09). The total amount of endogenous NA was similar in control and epithelium-denuded vasa deferentia, but the tissues devoid of epithelium accumulated 50% more [^{3}H]NA than

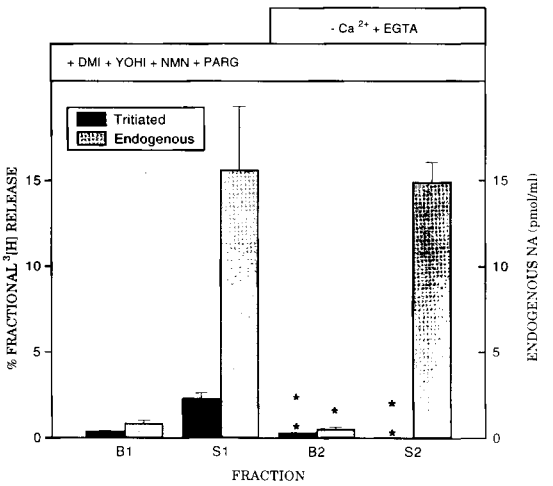

Fig 7. Effect of calcium-free conditions on release of tritiated and endogenous NA. The prostatic segments of the two vasa deferentia of each rat, previously loaded with [^{3}H]NA, were transmurally stimulated with 2 trains of stimuli (S1 and S2; at 7.5 Hz for 20 s; pulses of 70 V, 1 ms) delivered 30 min apart. During first half of release protocol (B1 and S1), tissues were incubated in Tyrode's solution containing 3 μM yohimbine, 10 μM normetanephrine and 1 μM pargyline. During second half of protocol (B2 and S2), tissues were incubated in calcium-free solution containing 0.1 mM EGTA plus drugs indicated above. Results expressed as means ± SEM's (n = 4-6). ** P<0.01 *vs* corresponding values in S1 and B1; * P<0.03 *vs* corresponding values in B1.

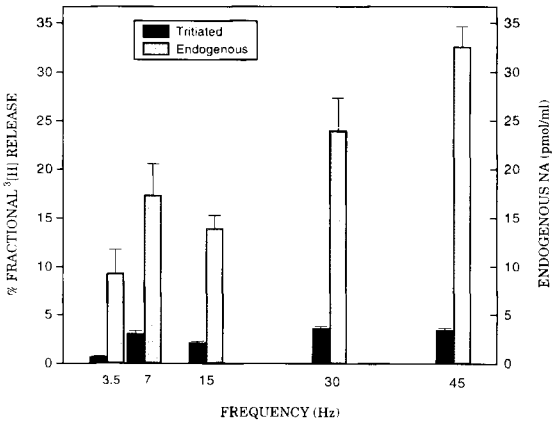

Fig 6. Effect of train frequency on release of tritiated and endogenous NA. The prostatic segments of the two vasa deferentia of each rat, previously loaded with [^{3}H]NA, were transmurally stimulated with 4 trains of electrical stimuli (S1, S2, S3 and S4; for 20 s; pulses of 70 V, 1 ms) delivered 30 min apart. Frequency of trains randomly assigned to either 3.5, 7, 15 or 45 Hz. Experiment carried out in Tyrode's solution containing 0.6 μM desipramine, 3 μM yohimbine, 10 μM normetanephrine and 1 μM pargyline. Results expressed as means ± SEM's (n = 4-6). One way ANOVA indicated significant overall difference between means for both, endogenous NA (F=11.02; P<0.001), and tritiated NA (F=18.16; P<0.001).

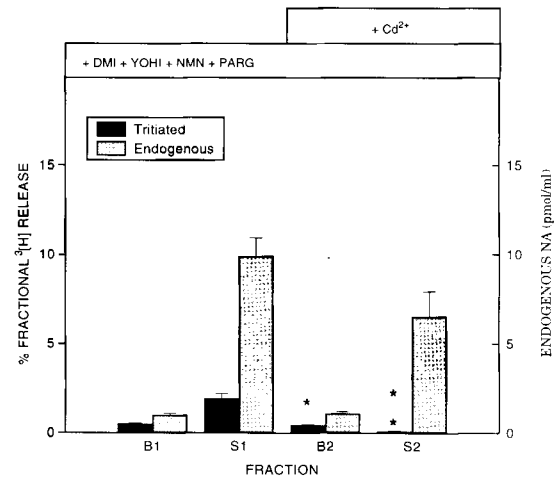

Fig 8. Effect of cadmium on release of tritiated and endogenous NA. The prostatic segments of the two vasa deferentia of each rat, previously loaded with [^{3}H]NA, were transmurally stimulated with 2 trains of stimuli (S1 and S2; at 7.5 Hz for 20 s; pulses of 70 V, 1 ms) delivered 30 min apart. During first half of release protocol (B1 and S1), tissues were incubated in Tyrode's solution containing 0.6 μM desipramine, 3 μM yohimbine, 10 μM normetanephrine and 1 μM pargyline. During second half of protocol (B2 and S2), tissues were incubated in above-described solution supplemented with 100 μM $CdCl_2$. Results expressed as means ± SEM's (n = 4-6). ** P<0.001 *vs* corresponding value in S1; * P<0.01 *vs* corresponding value in B1.

did the control tissues (Table I). Electrical stimulation of epithelium-denuded vasa evoked a sustained contractile response (Fig 9) and the release of both tritiated and endogenous NA (Table I). The responses to S2 were similar to those to S1, indicating that despite the surgical procedure, the mechanical and the release capacities of the tissues were stable in time. The release of endogenous NA from epithelium-denuded vasa in response to electrical stimulation was similar to that of control tissues. However, the release of tritiated NA from epithelium-denuded vasa was approximately 26% that of control tissues (Table I).

In epithelium-denuded vasa deferentia, addition of 100 μM $CdCl_2$ inhibited the basal and stimulated release of tritiated NA by 46 and 72% respectively, but had no effect on the basal or stimulated release of endogenous NA (Fig 10).

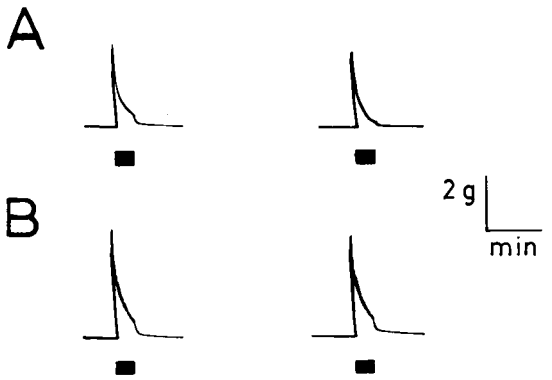

Fig 9. Effect of epithelium removal on mechanical response of the vas deferens to transmural stimulation. Polygraphic recordings of twitch responses of prostatic segments of vasa deferentia to transmural electrical stimulation (at 7.5 Hz for 20 s; pulses of 70 V, 1 ms) with trains delivered 30 min apart (S1 and S2, left and right, respectively). Preparations with intact epithelium (**A**) or epithelium-denuded (**B**).

DISCUSSION

This study shows that the release of tritiated NA from sympathetic nerve terminals of the rat vas deferens is not an accurate reflection of the release of endogenous NA. In fact, major dissociation between labeled and endogenous NA release was observed in relation to the requirement for extracellular calcium ions, the importance of reuptake and regulatory mechanisms and the dependence of the release on the duration of the stimulus train. We found that while the release of tritiated NA was completely dependent on the presence of calcium ions in the extracellular medium, the release of endogenous NA was not. Consistently, blockade of calcium channels by cadmium inhibited the release of tritiated NA but did not inhibit the release of endogenous NA. The lack of effect of cadmium on the release of endogenous NA might be attributed to diffusional problems which may restrict the access of the calcium antagonist to sympathetic varicosities found in the deeper muscular layers of the vas. However, this seems not to be the case as cadmium failed to inhibit the release of endogenous NA in epithelium-denuded vasa deferentia where diffusional barriers must be greatly reduced.

Table I

Effect of epithelium removal on the uptake of [^{3}H]NA and on the stimulated release of tritiated and endogenous NA from vas deferens nerve terminals.

	Tritiated NA			Endogenous NA		
Vasa deferentia	**Uptake** (cpm x 10^3)	**S1** (% FR)	**S2** (% FR)	**Content** (nmol)	**S1** (pmol/ml)	**S2** (pmol/ml)
Control	461 ± 31	2.14 ±0.39	2.00 ± 0.40	3.83 ± 0.21	10.4 ±1.6	11.1 ± 1.0
Epithelium-denuded	698 ± 53 *	0.58 ± 0.11 *	0.51 ± 0.09 *	3.46 ± 0.06	9.5 ± 1.6	10.3 ± 2.0

Control and epithelium-denuded vasa deferentia previously loaded with [^{3}H]NA, transmurally stimulated (S1 and S2; at 7.5 Hz for 20 s; pulses of 70 V, 1 ms). Results expressed as means ± SEM's from 5-6 separate experiments. % FR, percent fractional tritium release. NA content in nmol per prostatic segment. * $P<0.001$ *vs* corresponding values in control vasa deferentia.

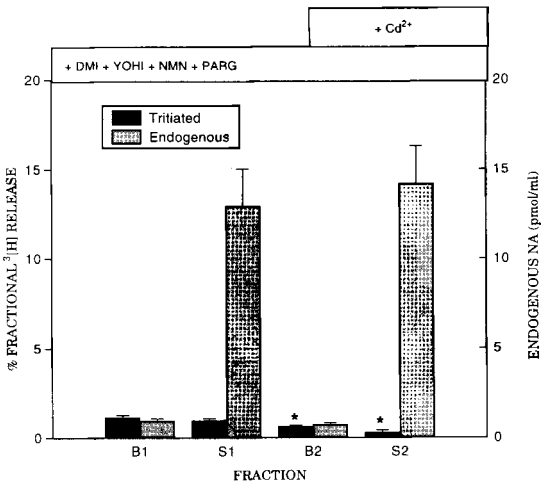

Fig 10. Effect of cadmium on release of tritiated and endogenous NA from epithelium-denuded vasa deferentia. The prostatic segments of vasa deferentia, from which the epithelium had been surgically removed, were loaded with [^{3}H]NA and subsequently stimulated with 2 trains of electrical stimuli (S1 and S2; at 7.5 Hz for 20 s; pulses of 70 V, 1 ms) delivered 30 min apart. During first half of release protocol (B1 and S1), tissues were incubated in Tyrode's solution containing 0.6 μM desipramine, 3 μM yohimbine, 10 μM normetanephrine and 1 μM pargyline. During second half of protocol (B2 and S2), tissues were incubated in above described solution supplemented with 100 μM $CdCl_2$. * $P<0.05$ *vs* corresponding values in S1 and B1.

At present, no easy explanation seems evident for the observation that the electrically-evoked release of endogenous NA does not depend on the presence of extracellular calcium. In fact, this observation is in open contradiction with the widely accepted view that the impulse-propagated release of NA from sympathetic nerve terminals is a calcium-dependent process that occurs through an exocytotic mechanism (Augustine *et al*, 1987; Smith & Winkler, 1972). However, it has been reported that under certain conditions a calcium-independent release of NA occurs from sympathetic nerves from reserpine-pretreated Guinea pig vas deferens and seminal vesicle (Paton, 1973; Rose *et al*, 1994; Ross & Kelder, 1979; Schömig *et al*, 1984; Wakade & Kirpekar, 1974). A similar mechanism has been suggested to participate in the non-exocytotic release of endogenous NA from sympathetic nerves from the ischemic myocardium of rats and dogs (Rose *et al*, 1994; Schömig *et al*, 1984).

In a similar way, the lack of Ca^{2+}-dependence of endogenous NA release observed in this study may indicate rather than an atypical release process, the existence of limitations of the preparation due to the particular cytoarchitecture of the vas and/or to the experimental conditions used. The vas deferens is a thick preparation, the interior of which, especially at 37°C, may well be hypoxic. Under relatively hypoxic conditions, the uptake of endogenous NA by vesicles of the more deeply located axons should be decreased, leading to the accumulation of extravesicular NA during the course of the experiment. The release of this extravesicular NA will be non-exocytotic and therefore independent of Ca^{2+}. In contrast, the more superficial varicosities, preferentially labeled by tritiated NA, are under a higher oxygen tension and display an exocytotic release, markedly dependent on Ca^{2+}. It should also be considered that hypoxic axons may be more susceptible to damage by the electrical stimuli. In this scenario it is conceivable that the "release" of extravesicular NA originated in the more deeply located axons, may occur because of transient breakdown of the plasma membrane, a process less likely to be regulated by Ca^{2+} and prejunctional mechanisms. In fact, α_2 adrenoceptor blockade by yohimbine increased 7.4 times the release of tritiated NA but had no effect on the release of endogenous NA. This implies that inhibition of release by prejunctional adrenergic autoreceptor activation affects selectively the release of tritiated NA and, by inference, the release of NA originated from more superficially located varicosities. This also seems to be the case for other sympathetic prejunctional regulators, such as NPY and cAMP both of which selectively modify the release of tritiated NA but have no effect on the release of endogenous NA from the rat vas deferens (Bitran *et al*, unpublished observations).

With regard to the mechanisms responsible for the clearance of the released NA, we found that both labeled and endogenous NA were subject to efficient clearance mechanisms. In fact, in the absence of inhibitors of the uptake, degradation and

prejunctional regulation of NA, the overflow of both tritiated and endogenous NA was barely detectable. Blockade of extraneuronal uptake by normetanephrine greatly enhanced the electrically stimulated overflow of endogenous NA while affecting to a lesser extent the overflow of tritiated NA. The results of this study are consistent with reports that indicate the existence of profound differences in the distribution in and release from vas deferens sympathetic nerve endings of endogenous NA and recently incorporated tritiated NA (Azevedo *et al*, 1990; Moura *et al*, 1990; Paiva *et al*, 1993; Schömig *et al*, 1990, 1991, 1992). Specifically, autoradiographic evidence shows a preferential labeling of the nerve terminals located in the outer layers of the vas deferens wall. Since the sympathetic nerves are distributed throughout the entire wall, it follows that the exogenous amine penetrates only poorly to the deeper layers of the vas. The decreased output of tritiated NA observed by us in epithelium-denuded vasa in response to electrical stimulation is also in line with the idea that in vasa with intact epithelium the labeled NA distributes nonhomogeneously into the sympathetic varicosities. Therefore, unlike the tritiated NA that derives from more superficial varicosities, the endogenous NA released from varicosities located across the entire vas wall has a greater chance of being taken up into muscle fibers during its diffusion.

Surprisingly, the neuronal uptake mechanism did not seem to play a main role in the clearance of either tritiated or endogenous NA released by electrical stimulation. In our studies, addition of 0.6 μM desipramine (a concentration that inhibits by 70% the incorporation of exogenously administered tritiated NA) had no effect on either the overflow or the release of tritiated or endogenous NA evoked by electrical stimulation. The lack of effect of desipramine is still puzzling and a complete dose/response study will be required to fundament the present observation.

We found that the release of endogenous NA was more sensitive to increases in stimulus duration than was the release of tritiated NA. This may result from an often overlooked difference between the pools of endogenous and labeled NA with respect to NA synthesis. It is obvious that newly synthesized molecules of NA can contribute to increase the pool size of endogenous NA but not that of tritiated NA. In fact, an increased rate of NA synthesis, often associated with nerve stimulation (Delanoy *et al*, 1982; Schwarz *et al*, 1980), will have opposite effects on the endogenous and labeled NA pools. An increased synthesis may contribute to an enhanced release of NA from the endogenous pool, leading at the same time to a decreased release of tritiated NA as a result of the dilution of the labeled pool by the unlabeled amine. Another factor that contributes to the dissociation between labeled and endogenous NA release derives from the fact that the recently incorporated amine is preferentially released, whatever stimulus is used (Moura *et al*, 1990).

It can not be ignored the tritiated noradrenaline reported to be released in this study is actually a mixture of [^{3}H]-NA, [^{3}H]-metabolites and [^{3}H]-$H_2$0. In a strict sense, and in order to specifically compare the release of tritiated and endogenous NA, the tritiated compounds should be separated in order to know accurately to which extent the release of radioactive NA is changing relative to the release of [^{3}H]-metabolites and [^{3}H]-$H_2$0. However, since in most studies this separation is not performed and the aim of this study was to determine if the currently used radioisotopic techniques can reliably measure the release of endogenous NA, we measured the tritium release, without further separation. The only exemption was a control experiment in which we determined that approximately 30% of the basal tritium release corresponded to NA, whereas NA accounted for approximately 65% of the tritium released by electrical stimulation (data not shown).

Taken together, our results indicate the existence of marked differences between the release of tritiated an endogenous NA. However, the interpretation of theses differences in terms of their physiological significance and the choice between the use of radioisotopic techniques and HPLC-measurement of endogenous NA is a difficult one. On the one hand, evidence

presented here and in previous reports suggests that tritiated NA is accumulated in superficially located axons and at its best, reflects part of the mechanisms and stages involved in endogenous NA release. On the other hand, our results indicate that the release of endogenous NA is Ca^{2+}-independent and not subjected to prejunctional regulation. These observations are quite in contradiction with the accepted view of the regulatory properties of neuronal neurotransmitter release and may indicate rather than an atypical release process, the existence of limitations of the preparation related to the particular cytoarchitecture of the tissue and/or the experimental conditions used in this study. We conclude that: 1) the assumption that tritiated NA release provides a good marker for endogenous NA release in the rat vas deferens seems unwarranted; 2) the use of endogenous NA to study the release process in the vas deferens requires a re-examination of the experimental conditions used, in order to minimize possible artifacts that may obscure the study of neuronal release; 3) the choice between measuring the release of tritiated or endogenous NA must be evaluated for each tissue in particular, taking into account its cytoarchitecture, as well as the experimental conditions used.

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