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Effects of Reboxetine on Sympathetic Neuroeffector Transmission in Rabbit Carotid Artery [1]

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Running title: Reboxetine and Neuroeffector Transmission

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ABBREVIATIONS: $CaNa_2EDTA$, calcium disodium ethylenediaminetetracetate; COMT, catechol-O-methyltransferase; MAO, monoamine oxidase; NET, norepinephrine transporter; NRI norepinephrine reuptake inhibitor; PSS, physiological salt solution; U-0521, 1-(3,4-dihydroxyphenyl)-2-methyl-1-propanone.

Section

Cardiovascular

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ABSTRACT

The effect of reboxetine on sympathetic neuroeffector transmission in rabbit isolated carotid artery was examined. Reboxetine (10^{-8} – 3 x 10^{-6} M), and cocaine (10^{-6} – 3 x 10^{-5} M), but not desipramine (10^{-8} – 3 x 10^{-7} M), increased contractions evoked by electrical field stimulation. At higher concentrations, reboxetine (10^{-4} M), cocaine (3 x 10^{-4} M) and desipramine (3 x 10^{-7} – 10^{-5} M) inhibited the neurogenic contractions. The enhancement seen with reboxetine and cocaine was partially reversible, while the inhibition was readily reversible. Reboxetine (10^{-7} M) and cocaine (10^{-5} M) prevented the inhibitory action of bretylium (10^{-6}M). Reboxetine (10^{-8} – 10^{-5} M), desipramine (10^{-7} – 10^{-4} M) and cocaine (10^{-6} – 10^{-5} M) increased the stimulation-evoked [^{3}H]norepineprine release. Pargyline (5 x 10^{-4} M) augmented the facilitatory effect of reboxetine (3 x 10^{-9} – 10^{-6} M) and cocaine (10^{-7} – 3 x 10^{-5} M). Reboxetine (10^{-8} – 10^{-6} M), desipramine (10^{-8} – 10^{-6} M) and cocaine (3 x 10^{-8} – 10^{-5} M) reduced the [^{3}H]norepinephrine (10^{-8} M) uptake. Reboxetine (10^{-7} M) and cocaine (10^{-5} – 2 x 10^{-4} M) enhanced the contractions evoked by phenylephrine and norepinephrine. Higher concentrations of reboxetine antagonized the contractions. Reboxetine (10^{-5} – 6 x 10^{-5} M) antagonized the contractions evoked by potassium. The contractions evoked by tyramine (3 x 10^{-6} – 10^{-3} M) was reduced by reboxetine (3 x 10^{-8} – 10^{-6} M) and by cocaine (10^{-7} – 10^{-5} M). We conclude that reboxetine inhibits the membrane amine pump (uptake-1) in the terminals of postganglionic adrenergic neurons in a cocaine-like manner.

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Reboxetine, a racemate of (-)-R,R- and (+)-(S,S)-([2-[α[2-ethoxyphenoxy]benzyl]-morpholine], is a non-tricyclic antidepressant drug (Melloni et al.,1985). It is reputedly clinically effective in the treatment of major depressive illness, well-tolerated and has a wide margin of safety (Burns, 2000; Wong et al., 2000; Scates and Doraiswamy, 2000). Reboxetine is a highly selective inhibitor of the norepinephrine transporter (NET) in rat brain synaptosomes (Melloni et al., 1984; Riva et al., 1989; Wong et al., 2000). The inhibition of norepinephrine reuptake in central neurons is believed to be responsible for its antidepressive activity (Scates and Doraiswamy, 2000). Reboxetine has low affinity to α_1-adrenoceptors, histaminergic and muscarinic receptors (Riva et al., 1986; Wong et al., 2000), which could explain why reboxetine only has mild to moderate cardiovascular side effects (Burrows et al., 1998). The overall frequency of adverse effects of reboxetine was similar with comparative drugs. Reboxetine appears to be relatively well tolerated with a good safety profile (Scates and Doraiswamy, 2000).

Desipramine, a tricyclic antidepressant, is a potent inhibitor of norepinephrine reuptake at central noradrenergic nerve endings (Sánchez and Hyttel, 1999). Desipramine is a potent antagonist at histamine H_1-receptors (Green and Maayani, 1977) and has weak antagonistic actions at α_1- and α_2-adrenoceptor (Hall and Ögren, 1981) and at muscarinic receptors (Golds et al., 1980). Cocaine is an inhibitor of norepinephrine uptake (Maxwell et al., 1969) without any direct effect on α-adrenoceptors and muscarinic receptors.

The action of reboxetine on vascular neuroeffector transmission has not been studied. The aim of the present study was therefore to examine the pre- and postsynaptic actions of reboxetine on sympathetic neuroeffector transmission in the isolated rabbit carotid artery. The actions of reboxetine were compared to those of desipramine and cocaine. A preliminary report of some of the results in this article was presented to the 44th Annual Meeting of the Western Pharmacology Society in Vancouver, British Columbia (Rasmussen and Nedergaard, 2001), and the International Congress of Pharmacology in San Francisco, California (Rasmussen and Nedergaard, 2002).

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Materials and Methods

Drugs. The following drugs were used: bretylium tosylate (Burroughs Wellcome, Research Triangle Park, NC); (-)-cocaine hydrochloride (Merck, Darmstadt, Germany), desipramine hydrochloride (Ciba-Geigy AG, Basel, Switzerland); 1-(3,4-dihydroxyphenyl)-2-methyl-1-propanone (U-0521; The Upjohn Company, Kalamazoo, MI); (-)-norepinephrine hydrochloride (Sigma-Aldrich Chemical Co., St. Louis, MO); (-)-[7-^{3}H] (N)norepinephrine hydrochloride (specific activity, 12.0-14.9 Ci/mmol; New England Nuclear Research Products, Boston, MA); pargyline hydrochloride (Abbott Laboratories, North Chicago, IL); and reboxetine hydrochloride (a racemic mixture of (-)-R,R- and (+)-S,S-(2-[α[2-ethoxyphenoxy]benzyl]-morpholine hydrochloride; synthesized in the Department of Medicin Chemistry, H. Lundbeck A/S, Copenhagen, Denmark).

Stock solutions were prepared in twice-distilled water (bretylium, cocaine, desipramine, norepinephrine, [^{3}H]norepinephrine, and pargyline). The stock solutions were diluted with physiological salt solution (PSS) to the concentration required. Stock solutions were stored at 4°C.

Rabbit isolated carotid artery. New Zealand white rabbits of either sex were obtained from Harlan AD (Horst, The Netherlands). Their weight was 1.8-2.7 kg. The rabbits were sacrificed by cervical dislocation and exsanguinated. All procedures conformed with Danish national guidelines for the care and handling of animals. The common carotid arteries on each side were divided into 4-6 rings (4 mm wide).

In vitro experiments. All isolated tissue experiments were carried out in equipment made from glass rather than plastic. In preliminary experiments we found that rods (containing platinum electrodes), tissue holders, and isolated tissue baths made of plastic were not suitable. In spite of repeated and careful washing with soap and water of these plastic utensils, they retained reboxetine.

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During subsequent experiments, reboxetine leaked from the plastic into the PSS and thereby compromised the experiments. Even when all glass utensils were used, the removal of reboxetine required careful washing and soaking (8-12 h) with 40 % (v/v) ethanol.

Salt solution. The composition of the PSS was: Na^+, 1.442 x 10^{-1} M; K^+, 4.9 x 10^{-3} M; Ca^{2+}, 1.3 x 10^{-3} M; Mg^{2+}, 1.2 x 10^{-3} M; Cl^-, 1.267 x 10^{-1} M; HCO_3^-, 2.5 x 10^{-2} M; SO_4^{2-}, 1.2 x 10^{-3} M; $H_2PO_4^{2-}$, 1.2 x 10^{-3} M; and D-(+)-glucose, 1.11 x 10^{-2} M. The solution also contained calcium disodium EDTA ($CaNa_2EDTA$; 3 x 10^{-5} M) and L-(+)-ascorbic acid (10^{-4} M). The solution was maintained at 37.0°C, equilibrated before and during the experiment with O_2 containing 5% (v/v) CO_2 (pH 7.4).

Electrical field stimulation-evoked release of [^{3}H]norepinephrine. The method described by Jensen and Nedergaard (1999) was used. Each ring was incubated in 6 ml test tubes containing PSS (2.0 ml). After an equilibration period (20 min), the rings were incubated with [^{3}H]norepinephrine (10^{-7} M) for 30 min. They were washed three times for 5 min each with salt solution by transferring them to new test tubes. The rings were then mounted in isolated tissue baths, which were automatically emptied and refilled with PSS (2.0 ml) every 5 min for the remainder of the experiments. The fractions (5-min) were collected 135 min after the onset of washout directly in a counting vial by means of a fraction collector. At the end of each experiment, each ring was treated with Solvable (DuPont de Nemours, Dreieich, Germany) for 16 h at room temperature (18-22°C). The ^{3}H content in each 5-min fraction and tissue was determined by liquid scintillation spectrometry (Tri-Carb 2100TR, Packard Instrument Company, Meriden, CT). The spectrometer automatically corrected for quenching and determined the counting efficiency by mean of an external standard.

Electrical field stimulation was applied to the vessels using a stimulator (model S48, Grass Medical Instruments, Quincy, MA) in connection with a constant current unit. Electrical field

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stimulation was applied at various times (min) after onset of washout: 80 (S_1) and then every 35 min (S_2-S_7). Each period of stimulation consisted of 300 pulses (200 mA, 0.5 ms, 3 Hz). S_1 and S_2 were disregarded, and S_3 used as an initial control value (~ 100%). The ^{3}H overflow evoked by electrical field stimulation was calculated by summation of the ^{3}H overflow in the three fraction (F_3-F_5) which entered in the formation of the peak less the estimated basal ^{3}H outflow during this period. The latter was calculated for each stimulation period (S_3-S_7) by assuming a linear decline of the basal ^{3}H outflow between the two fractions (F_1-F_2) just preceding the stimulation and the fraction (F_6) collected 20 min after the onset of stimulation. The tritium in each 5-min fraction was expressed as a percentage of the ^{3}H content in the tissue at the time of sampling. This calculation was done by summation of the assayed tritium in each 5-min fraction and the ^{3}H content in the tissue at the end of the experiment. The calculated stimulation-evoked ^{3}H overflow was expressed as a percentage of the initial S_3 control stimulation (~100%). In some experiments, the ^{3}H overflow evoked by stimulation (S_3-S_7) was corrected for time-dependent changes. This was done by stimulating untreated tissue in parallel with tissue exposed to the drug being examined.

Electrical field stimulation of isolated carotid artery. Each ring was subjected to electrical-field stimulation using a stimulator (model S48; Grass Medical Instruments, Quincy, MA) connected to a constant current unit. Each period of stimulation consisted of 300 monophasic pulses (200 mA; 3 Hz; 0.5 ms) followed by a 15 min rest period. The contraction evoked by the sixth stimulation was designated the 'control' response. If the stimulation (S_6)-evoked tension was less than 1 g, the artery was discarded. The isometric mechanical tension response was recorded by means of a transducer (type SG 4-180; Swema, Stockholm, Sweden) connected to a data acquisition and analysis unit (Powerlab/800; AD Instruments, Castle Hill, New South Wales) which registered the converted signal in g tension.

Effects of drugs added cumulatively on stimulation-evoked contraction were studied in the following manner. Ten min after the 'control' response, the lowest concentration of the test drug to

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be used was added; the response after 20 min incubation was recorded. The next higher concentration was then added 10 min later and the response again recorded after 20 min. The procedure was continued until a total of six or seven drug concentrations had been tested. In all experiments, the contractions evoked by electrical stimulation (S_6-S_{11}) were corrected for time-dependent changes. This was done by stimulating untreated tissue in parallel with tissue exposed to the test drug being examined. These data were used to correct the former results. The mean 'control' tension (g) for the untreated and drug-treated preparations did not differ ($p > 0.05$).

Effect of reboxetine on the contractions of carotid artery evoked by various agonists and potassium. Rings of carotid artery were mounted suitably in an isolated tissue bath filled with 20 ml of salt solution and a resting tension of 6 g was applied. The rings were washed twice with salt solution during a 1-h equilibrations period. The rings were then primed once with norepinephrine (10^{-6} M). After washout, the respective agonist was added cumulatively. Additions were made whenever a steady contractile response was obtained to the preceding administration. The effect of the agonist was considered to be maximal when at least a 3-fold increase in its concentration failed to cause a further increase in tension. After the maximal response was obtained, the artery was washed repeatedly with PSS until the tension returned to the resting tension value. The tissues were then equilibrated for 30 min before the agonist in question was added cumulatively. The contractions thus evoked were expressed as a percentage of the maximal contraction response developed by the initial addition of the agonist in question. Experiments designed to measure the effect of reboxetine on the contractions evoked by various agonists or K^+ were carried out in the following manner: reboxetine was added to the bath 30 min before the second addition of the lowest concentration of the agonist in question and maintained in the bath for the remainder of the experiment. Only one concentration-response curve determination was made per preparation.

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Uptake of [^{3}H]norepinephrine. The method described by Nedergaard (1989) was used. Four to six rings (each 8 mm wide) of carotid artery were equilibrated for 30 min with PSS and washed once. Monoamine oxidase (MAO) and catechol-O-methyltransferase (COMT) were blocked by pargyline and U-0521, respectively, in the following manner: rings were incubated with pargyline (5×10^{-5} M) for 30 min with subsequent washout of this agent. U-0521 (10^{-4} M) was then added to the bath at least 1 h prior to the incubation with ^{3}H amine and was present throughout the experiment. Each ring was transferred to a separate bath filled with 20 ml PSS. After at least 30 min further equilibration, the tissues were incubated with [^{3}H]norepinephrine (10^{-8} M) for 1 h.
In experiments designed to examine the ability of a drug to alter the uptake of [^{3}H]norepinephrine, the former was added 1 h prior to the latter and maintained in the bath for the remainder of the experiment.

After incubation with [^{3}H]norepinephrine, the rings were cut open into rectangular strips. They were blotted between two pieces of moistened filter paper under pressure (30 g) for 10 s in a standard manner, and their net weight (5.8-12.0 mg) was determined. Each sample was transferred to a 25 ml polyethylene liquid scintillation counting vial and treated with Solvable (DuPont de Nemours, Dreieich, Germany) for 16 h at room temperature (18-22° C) in closed vials. Radioactivity was measured by liquid scintillation spectrometry (Tri-Carb 2100 TR, Packard Instrument Company, Meriden, CT). Aliquots (100 μl) of the bath fluid were counted also. The uptake of [^{3}H]norepinephrine is expressed as millilitres of fluid cleared per gram (ml/g); also referred to as clearance ratio.

Statistical analysis. Data are expressed as mean □ ±S.E.M. Log concentration-response curves were plotted. Differences between mean values were evaluated using an unpaired *t* test. In the case of unequal variance between the mean values compared (evaluated with a variance ratio test), an unpaired *t* test for unequal variance was used. When one control value was compared to a set of different concentrations of test drugs, t-test with a Bonferonni-correction was used. When

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multiple comparisons between groups of data were analysed, two-way analysis of variance (ANOVA) was used. Only the overall treatment effect was analysed by two-way ANOVA. Significance was accepted at the 0.05 level of probability. Analysis of data was performed with Excel 97 (Microsoft, Redmond, WA).

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Results

Effect of reboxetine, desipramine and cocaine on stimulation-evoked contractions. Reboxetine (10^{-8} – 3 x 10^{-6} M) and cocaine (10^{-6} – 3 x 10^{-5} M) increased contractions of carotid artery evoked by electrical field stimulation (Fig. 1). Desipramine (10^{-8} – 10^{-7} M) had no effect. At higher concentrations, reboxetine (10^{-4} M), cocaine (3 x 10^{-4} M) and desipramine (3 x 10^{-7} – 10^{-5} M) inhibited the neurogenic contractions (Fig. 1). The enhancement and inhibition of stimulation-evoked contractions seen with 10^{-7} M and 7 x 10^{-7} M, respectively, of reboxetine increased with time (Fig. 2). In contrast, the same effects induced by cocaine had a rapid onset and was maintained unchanged with time (Fig. 2). The inhibition seen with reboxetine (7 x 10^{-5} M) and cocaine (2 x 10^{-4} M) was readily reversed by washing the artery with drug-free PSS (Fig. 2). However, the enhancement caused by reboxetine (10^{-7} M) and cocaine (2 x 10^{-4} M) was only partially reversed; cocaine > reboxetine (Fig. 2).

Effect of reboxetine and cocaine on the inhibitory action of bretylium. Bretylium (10^{-6} M) blocked the stimulation-evoked contractions of carotid artery (Fig. 3). Reboxetine (10^{-7} M) and cocaine (10^{-5} M) prevented the bretylium-induced block (Fig. 3).

Effect of reboxetine, desipramine and cocaine on basal ^{3}H outflow and stimulation-evoked ^{3}H overflow. Reboxetine (10^{-8} – 10^{-5} M), desipramine (10^{-8} – 10^{-5} M) and cocaine (10^{-7} – 3 x 10^{-5} M) had no effect on the basal ^{3}H outflow from carotid artery preincubated with [^{3}H]norepinephrine (data not shown; n=6). Reboxetine (10^{-8} – 10^{-5} M), desipramine (10^{-7} – 10^{-5} M) and cocaine (10^{-6} – 10^{-5} M) concentration-dependently increased the stimulation-evoked ^{3}H overflow from carotid artery preincubated with [^{3}H]norepinephrine (Fig. 4). Emax (%) was: 87 (reboxetine), 45 (desipramine), and 23 (cocaine). Reboxetine (10^{-7} M), desipramine (10^{-7} M), and cocaine (10^{-6} M) rapidly enhanced the stimulation-evoked ^{3}H overflow (Fig. 5). The reboxetine-

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evoked enhancement increased with time, whereas the enhancement seen with desipramine and cocaine remained unchanged (Fig. 5).

Influence of pargyline on the action of reboxetine and cocaine on stimulation-evoked ^{3}H overflow. Pargyline (5 x 10^{-4} M) augmented the facilitatory effect of reboxetine (3 x 10^{-9} - 10^{-6} M) and cocaine (10^{-7} – 3 x 10^{-5} M) (Fig. 6).

Effect of reboxetine, desipramine and cocaine on the uptake of [^{3}H]norepinephrine. Reboxetine (10^{-8} – 10^{-6} M), desipramine (10^{-8} – 10^{-6} M) and cocaine (3 x 10^{-8} – 10^{-5} M) reduced the uptake of ^{3}H by carotid artery incubated with [^{3}H]norepinephrine (10^{-8} M) (Fig. 7). The rank order of inhibitory potency (IC_{50}) was: reboxetine > desipramine > cocaine.

The ^{3}H uptake by carotid artery treated with cocaine (3 x 10^{-5} M) recovered fully after a short washout period (0.5 h). In contrast, after incubation with reboxetine (10^{-6} M) the ^{3}H uptake only recovered partially after longer washout periods: 55 % (1 h); 75 % (2 h) (Fig. 8).

Effect of reboxetine and cocaine on contractions of carotid artery evoked by agonists and potassium. Reboxetine (10^{-7} M) and cocaine (10^{-5} and 2 x 10^{-4} M) enhanced the contractions of carotid artery evoked by either phenylephrine (10^{-7} – 2 x 10^{-6} M) or norepinephrine (10^{-7} – 2 x 10^{-6} M) (Fig. 9). Higher concentrations of reboxetine (3 x 10^{-5} – 6 x 10^{-5} M) antagonized in a non-competitive manner the contractions evoked by either phenylephrine or norepinephrine (Fig. 9). Reboxetine (10^{-5} – 6 x 10^{-5} M) antagonized non-competitively the contractions evoked by potassium (Fig. 10). Cocaine (3 x 10^{-4} M) also reduced the K^{+}-evoked contractions (Fig. 10). However, lower concentrations of cocaine (10^{-5} – 10^{-4} M) had no effect. The contractions of carotid artery evoked by tyramine (3 x 10^{-6} – 10^{-3} M) was markedly reduced by reboxetine (3 x 10^{-8} – 10^{-6} M) and by cocaine (10^{-7} – 10^{-5} M) (Fig. 11).

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Discussion

The ability of reboxetine to interfere with the uptake of [^{3}H]norepinephrine by sympathetic neurons in rabbit carotid artery was examined (Fig. 7). A low concentration (10^{-8} M) of [^{3}H]norepinephrine was chosen so to ensure that the uptake of [^{3}H]norepinephrine primarily represented neuronal uptake via the uptake-1 mechanism (Nedergaard and Bevan, 1971).

Agents which release norepinephrine, even if they do not interact with the amine pump (uptake-1), would appear to be inhibitors of amine uptake and thus erroneously be classified as amine pump inhibitors (Maxwell et al., 1976). Reboxetine at concentrations higher than 10^{-5} M caused an increase in basal ^{3}H outflow from the carotid artery preloaded with [^{3}H]norepinephrine, i.e. reboxetine had no releasing action at 10^{-5} M and at lower concentrations. The maximum inhibition (IC_{100}) of ^{3}H uptake was seen with 10^{-6} M of reboxetine (Fig. 7). Therefore, it is most unlikely that the reboxetine-induced inhibition of ^{3}H uptake is due to a releasing action on norepinephrine stored in the sympathetic neurons.

Reboxetine reduced the uptake of [^{3}H]norepinephrine (Fig. 7.) which confirms findings in rat hypothalamic synaptosomes (Wong et al., 2002) and rat hippocampal synaptosomes (Miller et al., 2002). This reduction is most likely due to an inhibition of the neuronal amine pump (uptake-1 mechanism) since cocaine and desipramine also reduced the uptake (Fig. 7). Furthermore, this in line with the view that reboxetine is a selective NRI (Wong et al., 2000).

The inhibition of [^{3}H]norepinephrine uptake by reboxetine was only partially reversed after a 2 h washout period (Fig. 8). In contrast, the cocaine-induced inhibition was reversed fully after 0.5 h (Fig. 8). This indicates that reboxetine is bound rather tightly to the tissue; perhaps the NET transporter itself. This suggests that there may be a dissociation between plasma concentration of reboxetine and its concentration in the biophase. This is also in line with the observation that there are no defined plasma concentrations of reboxetine that correlate with its therapeutic effect (Scates and Doraiswamy, 2000). Clinical studies have demonstrated that the mean plasma concentration of reboxetine is 3×10^{-7} M (Scates and Doraiswamy, 2000). The correlation between the concentration

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of drugs in salt solution in isolated tissue experiments and their therapeutic plasma concentration is a matter of conjecture. However, our findings with reboxetine in concentrations up to 10^{-6} M can probably be considered therapeutically relevant.

Inactivation of norepinephrine release by sympathetic nerve stimulation in blood vessels is mainly carried out by neuronal (uptake-1) and extraneuronal uptake (uptake-2) of the transmitter and subsequent inactivation by MAO and COMT (Osswald and Guimarães, 1983). The uptake mechanisms for norepinephrine can thus regulate the amount of transmitter in the junctional cleft. Reboxetine in low concentrations (up to 10^{-6} M) enhanced the contractions of carotid artery evoked by electrical field stimulation (Fig. 1). This is probably due to an inhibition of the uptake-1 mechanism. This view is supported by the finding that reboxetine was a potent inhibitor of [^{3}H]norepinephrine uptake (Fig. 7) and that cocaine, a well-known uptake-1 inhibitor, also enhanced the neurogenic contractions (Fig. 1). In contrast to reboxetine and cocaine, desipramine did not enhance the stimulation evoked contraction (Fig. 1). The reason for this is that desipramine, besides being an uptake-1 inhibitor, is a weak α-adrenoceptor antagonist (McCulloch and Story, 1972; Hall and Ögren, 1981). At a high concentration (10^{-4} M), reboxetine markedly reduced the stimulation-evoked contractions (Fig. 1). This is most likely due to a postjunctional non-specific inhibitory action, since reboxetine non-competitively reduced the contractions evoked by phenylephrine, norepinephrine, and K^+ (Figs. 9 and 10).

Reboxetine enhanced the [^{3}H]norepinephrine release evoked by electrical field stimulation (Fig. 4). The enhancement is probably due to an inhibition of the uptake-1 mechanism. This is supported by the finding that reboxetine was a potent inhibitor of [^{3}H]norepinephrine uptake by carotid artery (Fig. 7) (*vide supra*). Furthermore, the uptake-1 inhibitors cocaine and desipramine also enhanced the stimulation-evoked [^{3}H]norepinephrine release (Fig. 4).

Reboxetine caused a more marked enhancement of [^{3}H]norepinephrine release than desipramine and cocaine (Fig. 4). The rank order of the maximum effect (Emax) was reboxetine > desipramine > cocaine. The differences in the ability of these three drugs to enhance

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[^{3}H]norepinephrine release may well be related to the strength of their binding to the uptake-1 mechanism. Cocaine and desipramine are both competitive inhibitors of NET (Buck and Amara, 1995). Both of these inhibitors can therefore be displaced by the neurogenic norepinephrine. The more easily they can be displayed by norepinephrine, the less enhancement will be observed. We have presented evidence for the view that reboxetine is bound much more tightly to the uptake-1 mechanism than cocaine (*cf.* Fig. 8). This would therefore result in a more efficient inhibition of uptake-1 with a resultant higher amount of norepinephrine in the junctional gap leading to an increase in the ^{3}H overflow.

All postganglionic sympathetic neurons are endowed with prejunctional inhibitory α_2-adrenoceptors (autoreceptors) which are activated by released norepinephrine (Starke 1977). Inhibition of uptake-1 in carotid artery by reboxetine most likely leads to an increase in the junctional cleft concentration of norepinephrine with a correspondingy increased activation of the autoreceptors. The reboxetine-induced enhancement of stimulation-evoked [^{3}H]norepinephrine release (Fig. 4) may therefore have been dampened. This would probably be more so at high concentrations of reboxetine. The ability of cocaine to modulate the depolarisation-evoked norepinephrine release via prejunctional α_2-adrenoceptors depended *inter alia* on the concentration of cocaine, the stimulation intensity (frequency; length of pulse train) and the geometry of the junctional cleft (Nedergaard 1986). This conclusion was based on a study of the interaction between cocaine and α_2-adrenoceptor antagonists. A similar interaction study using reboxetine instead of cocaine remains to be done. Inhibition of prejunctional α_2-adrenoceptors located on vascular sympathetic neurons by α_2-adrenoceptor antagonists, such as e.g. rauwolscine, enhances the stimulation-evoked [^{3}H]norepinephrine release (Nedergaard, 1986). Since reboxetine has poor affinity to α_2-adrenoceptors (Wong et al., 2000), it is most unlikely that the reboxetine-evoked enhancement of [^{3}H]norepinephrine release (Fig. 4) could be due to α_2-adrenoceptor antagonism.

Desipramine and cocaine rapidly enhanced the stimulation-evoked [^{3}H]norepinephrine release which was then maintained unchanged (Fig. 5). This indicates that the cumulative concentration-

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response curves for each of these 2 drugs (Fig. 4) represent equilibrium responses. In contrast, since the reboxetine-induced enhancement increased with time (Figs. 2 and 5), the cumulative concentration-response curve for reboxetine (Figs. 1 and 4) probably does not represent equilibrium responses.

In series with neuronal and extraneuronal uptake, MAO participates in the metabolism of norepinephrine. Pargyline, a non-selective and irreversible inhibitor of MAO, augmented the facilitatory effect of reboxetine and cocaine (Fig. 6). The simplest explanation for this is that pargyline removed an inactivation pathway for released [^{3}H]norepinephrine, both pre- and postjunctionally, which resulted in an increased ^{3}H overflow. It has been suggested that concomitant therapy with reboxetine and a MAO inhibitor may increase the risk of a hypertensive crisis (Scates and Doraiswamy, 2000). This is supported by the positive interaction between pargyline and reboxetine with regard to norepinephrine release.

The adrenergic neuron blocking agent bretylium reduced the contractions of carotid artery evoked by electrical field stimulation (Fig. 3). Bretylium is taken up into the adrenergic neuron, presumably via the uptake-1 mechanism (Nedergaard and Bevan, 1967; Ross and Gosztong, 1975). The ability of reboxetine and cocaine to prevent the bretylium-induced block (Fig. 3) further supports the view that reboxetine is an uptake-1 inhibitor and has a cocaine-like action.

Reboxetine enhanced the contractions of carotid artery evoked by either phenylephrine or norepinephrine (Fig. 9). The enhancement is most likely due to an inhibition of the uptake-1 mechanism. Both norepinephrine (Iversen, 1967) and phenylephrine (Rawlow et al., 1980) are substrates for this neuronal membrane carrier. Furthermore, cocaine likewise caused an enhancement (Fig. 9). High concentrations of reboxetine antagonized in a non-competitive manner the contractions evoked by phenylephrine and K^+ (Figs. 9 and 10). This suggests that reboxetine in

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high concentrations has a non-specific inhibitory action; the mechanism of which remains to be explored.

Observations in a family with a genetic form of orthostatic intolerance suggest that impairment in norepinephrine clearance can result from NET dysfunction (Shannon et al., 2000). Selective NET blockade by reboxetine in healthy subjects created a phenotype that resembled idiopathic orthostatic intolerance (Schroeder at al., 2002). In this model reboxetine markedly increased sensitivity to phenylephrine; probably as a result of central and peripheral effects of NET inhibition. The sensitivity increase could in part be due a reduced elimination of phenylephrine via the uptake-1 mechanism. This view is supported by our finding that reboxetine enhanced the contractions of carotid artery evoked by phenylephrine (Fig. 9).

Reboxetine markedly reduced the contractions evoked by tyramine (Fig. 11). Tyramine is an indirectly acting sympathomimetic amine which enters the neuron via the uptake-1 mechanism (Trendelenburg, 1972). Reboxetine most likely blocked the ability of tyramine to release norepinephrine from sympathetic neurons by preventing the entry of the latter through the neurilemma, i.e. a cocaine-like action. This view is supported by the finding that cocaine also reduced the tyramine evoked contractions (Fig. 11). This was also the case with rabbit pulmonary artery (Nedergaard 1973).

In summary, the results suggest that reboxetine is a potent NRI of peripheral sympathetic neurons in rabbit carotid artery. Reboxetine probably inhibits the uptake-1 mechanism in the same manner as cocaine.

Acknowledgement

We thank Lundbeck A/S for the generous gift of reboxetine.

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Legends

Fig. 1. Effect of reboxetine, desipramine and cocaine on stimulation-evoked contractions of rabbit carotid artery evoked by electrical field stimulation. Ordinate, mean stimulation-evoked contraction expressed as a percentage of S_6 (100%). The data were corrected for time-dependent changes. Abscissa, concentration (-log M) of drugs added cumulatively. ■, Reboxetine; ●, desipramine; □, cocaine. Vertical lines represent ± S.E.M. (n = 6; * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$; compared to untreated tissue).

Fig. 2. Time-dependence and effect of washout of the effect of reboxetine and cocaine on stimulation-evoked contractions. Ordinate, mean stimulation-evoked contraction expressed as a percentage of S_6 (100%). Abscissa, time (h). The arrow indicates addition of drugs. A: Reboxetine: ▲, 10^{-7} M; ■, 7 x 10^{-5} M. B: Cocaine; ▲, 10^{-5} M; ■, 2 x 10^{-4} M; ○, untreated. C: Reboxetine: ▲, 10^{-7} M; ■, 7 x 10^{-5} M; ○, untreated. D: Cocaine; ▲, 10^{-5} M; ■, 2 x 10^{-4} M; ○, untreated. C,D: W: The preparations were washed twice at 1 min interval with drug-free PSS. Vertical lines represent ± S.E.M. (n = 5-6; A,B *** $p < 0.001$; compared to untreated tissue. C,D: NS $p > 0.05$; *** $p < 0.001$ compared to untreated in the interval 2 – 3h).

Fig. 3. Effect of reboxetine and cocaine on the inhibitory action of bretylium on stimulation-evoked contractions of rabbit carotid artery evoked by electrical-field stimulation. Ordinate, mean stimulation-evoked contraction expressed as a percentage of S_6 (100%). Abscissa, time (h). A, B: ●, bretylium, 10^{-6} M; ○, untreated. The arrow indicates addition of bretylium (10^{-6} M). A: ▲, Pretreated with reboxetine (10^{-7} M). B: ▲, Pretreated with cocaine (10^{-5} M). Vertical lines represent ± S.E.M. (n = 5; *** $p < 0.001$, compared to pretreated tissue).

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Fig. 4. Effect of reboxetine, desipramine and cocaine on stimulation-evoked ^{3}H overflow. Ordinate, mean stimulation-evoked ^{3}H overflow expressed as a percentage of untreated (100%) and corrected for time-dependent changes. Abscissa, concentration (-log M). ●, Reboxetine, ○; desipramine; ■, cocaine. Vertical lines represent ± S.E.M. (n = 6; * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$, compared to untreated tissue).

Fig. 5. Time-dependence of the effect of reboxetine, desipramine and cocaine on stimulation-evoked ^{3}H overflow. Ordinate, mean stimulation-evoked ^{3}H overflow expressed as a percentage of S_3 (100%). Abscissa, time (min). ●, Reboxetine, 10^{-7} M; □, desipramine, 10^{-7} M; ■, cocaine, 10^{-6} M; ○, Untreated. Vertical lines represent ± S.E.M. (n = 5-6; NS $p > 0.05$; * $p < 0.05$; comparison between values at 215 and 320 min).

Fig. 6. Facilitatory action of pargyline on the effects of reboxetine and cocaine on stimulation-evoked ^{3}H overflow from rabbit carotid artery. Ordinate, mean stimulation-evoked ^{3}H overflow expressed as a percentage of S_3 (100%). Abscissa, concentration (-log M). A: □, reboxetine alone; ■, reboxetine in the presence of pargyline (5×10^{-4} M). B: △, cocaine alone; ▲, cocaine in the presence of pargyline (5×10^{-4} M). Vertical lines represent ± S.E.M. (n = 5-8; * $p < 0.05$; *** $p < 0.001$; ANOVA).

Fig. 7. Effect of reboxetine, desipramine and cocaine on the uptake of [^{3}H]norepinephrine by rabbit carotid artery. Ordinate, mean ^{3}H uptake expressed as a clearance ratio (ml/g). Abscissa, concentration (-log M). ●, Reboxetine; ○, desipramine; ■, cocaine. Vertical lines represent ± S.E.M. (n = 4-7; * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$, compared to untreated tissue).

Fig. 8. Effect of washout on the inhibitory action of reboxetine and cocaine on uptake of [^{3}H]norepinephrine by rabbit carotid artery. Ordinate, mean ^{3}H uptake expressed as a clearance

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ratio (ml/g). Abscissa, duration of washout period (h). Rings were either untreated (A,B; open columns) or pretreated (shaded columns, A: Cocaine (3×10^{-5} M); B: Reboxetine (10^{-6} M)) followed by a washout period (0.5; 1 or 2 h) prior to incubation (30 min) with [^{3}H]norepinephrine (10^{-8} M). Vertical lines represent ± S.E.M. (n = 6; NS $p > 0.05$; * $p < 0.05$; ** $p < 0.01$, compared to untreated tissue).

Fig. 9. Effect of reboxetine and cocaine on contractions of rabbit carotid artery evoked by either phenylephrine or norepinephrine. Ordinate, mean agonist-evoked contraction expressed as a percentage of E_{max}. Abscissa, agonist concentration (-log M). A, B: Phenylephrine. C, D: Norepinephrine. A-D: ○, Untreated (control). A, C: Agonist in the presence of reboxetine: ●, 10^{-7} M; □, 3×10^{-5} M; ▲, 6×10^{-5} M. B, D: agonist in the presence of cocaine: ■, 10^{-5} M; △, 2×10^{-4} M. Vertical lines represent ± S.E.M. (n = 5-6; *** $p < 0.001$, compared to untreated tissue).

Fig. 10. Effect of reboxetine and cocaine on contractions of rabbit carotid artery evoked by potassium (K^+). Ordinate, mean K^+-induced contraction expressed as a percentage of E_{max}. Abscissa, K^+ concentration (-log M). A, B: ○, Untreated. A: Reboxetine: ■, 10^{-5} M; □, 3×10^{-5} M; ▲, 6×10^{-5} M. B: Cocaine: ▲, 10^{-5} M; ●, 10^{-4} M; △, 3×10^{-4} M. Vertical lines represent ± S.E.M. (n = 5-6; *** $p < 0.001$, compared to untreated tissue).

Fig. 11. Effect of reboxetine and cocaine on contractions by rabbit carotid artery evoked by tyramine. Ordinate, mean tyramine-induced contraction expressed as a percentage of E_{max}. Abscissa, tyramine concentration (-log M). A, B: ○, Untreated. A: Reboxetine: △, 3×10^{-8} M; ●, 10^{-6} M. B: Cocaine: ■, 10^{-5} M; △, 2×10^{-4} M; ●, 3×10^{-4} M. Vertical lines represent ± S.E.M. (n = 6; * $p < 0.05$; *** $p < 0.001$, compared to untreated tissue).

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Figure 1.

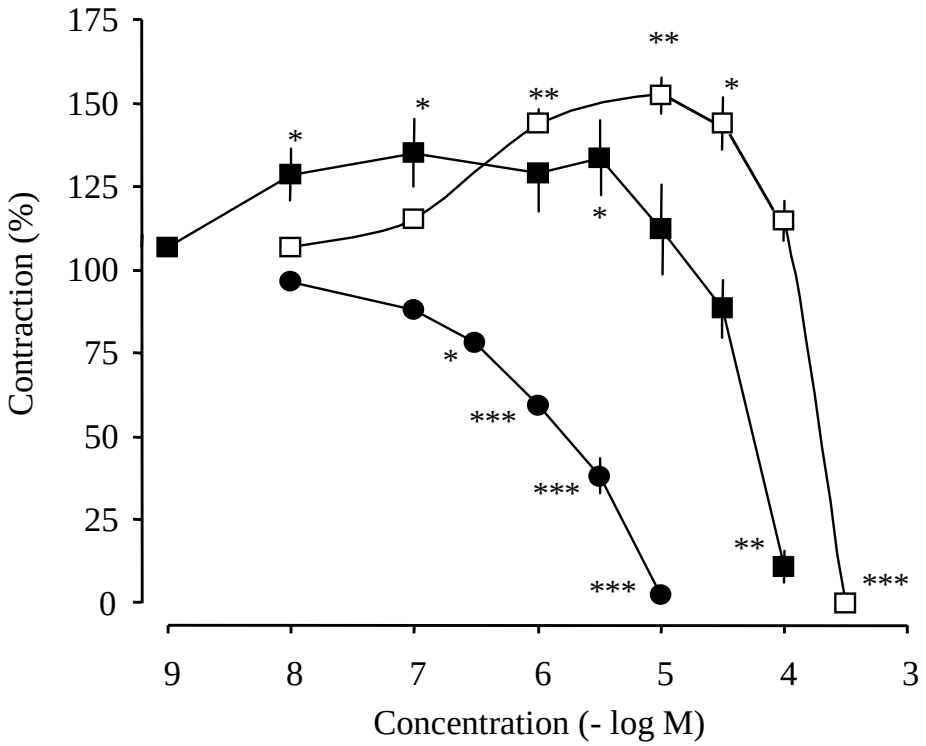

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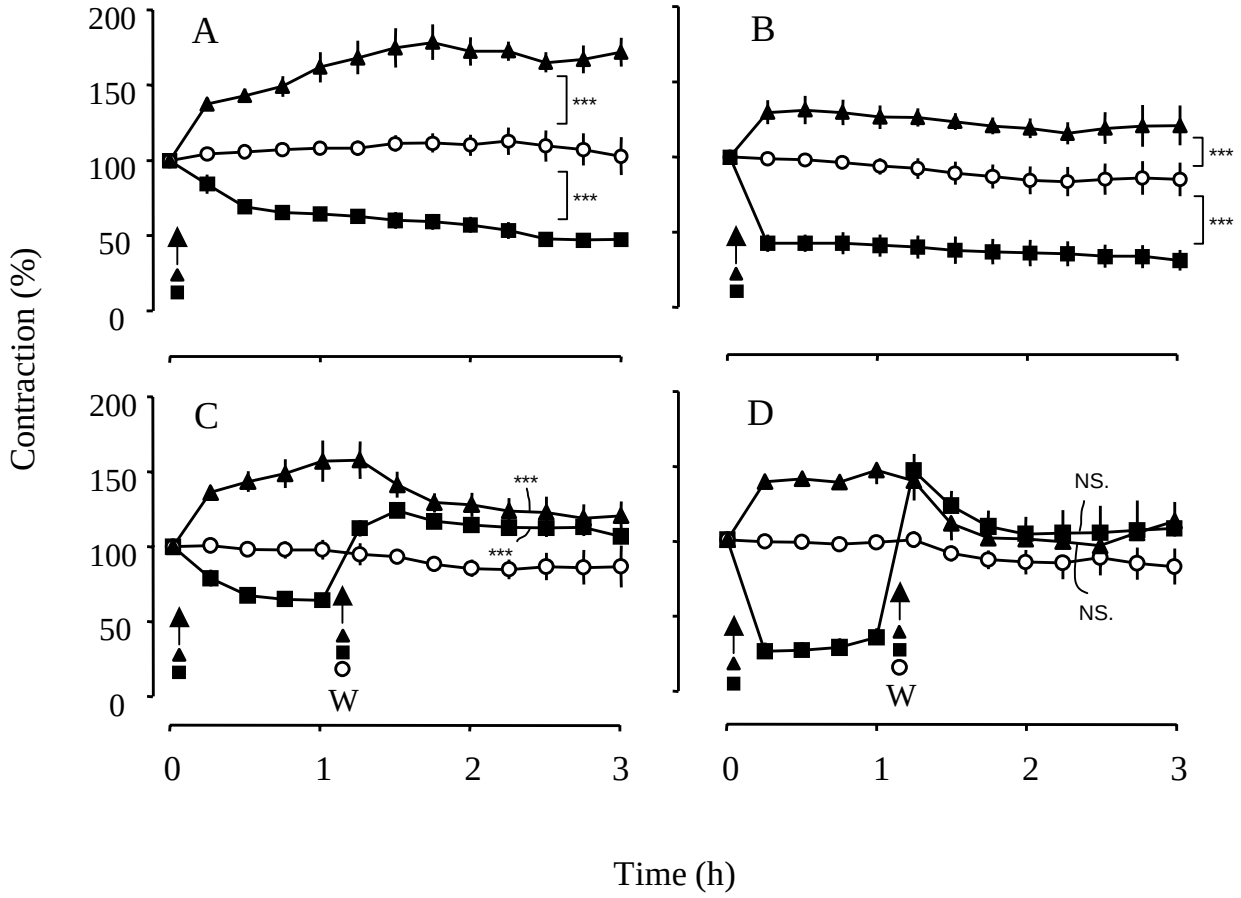
Figure 2.



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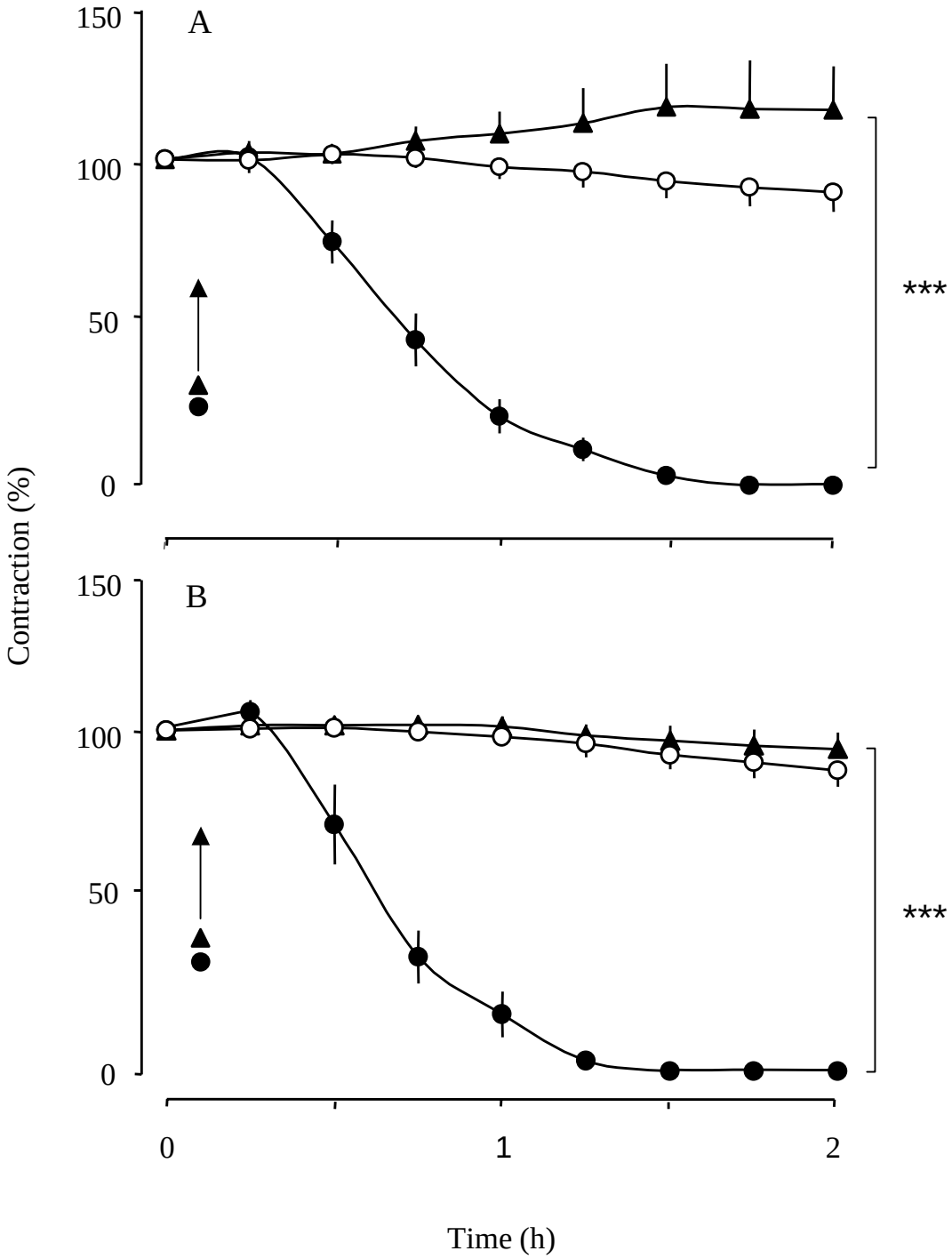

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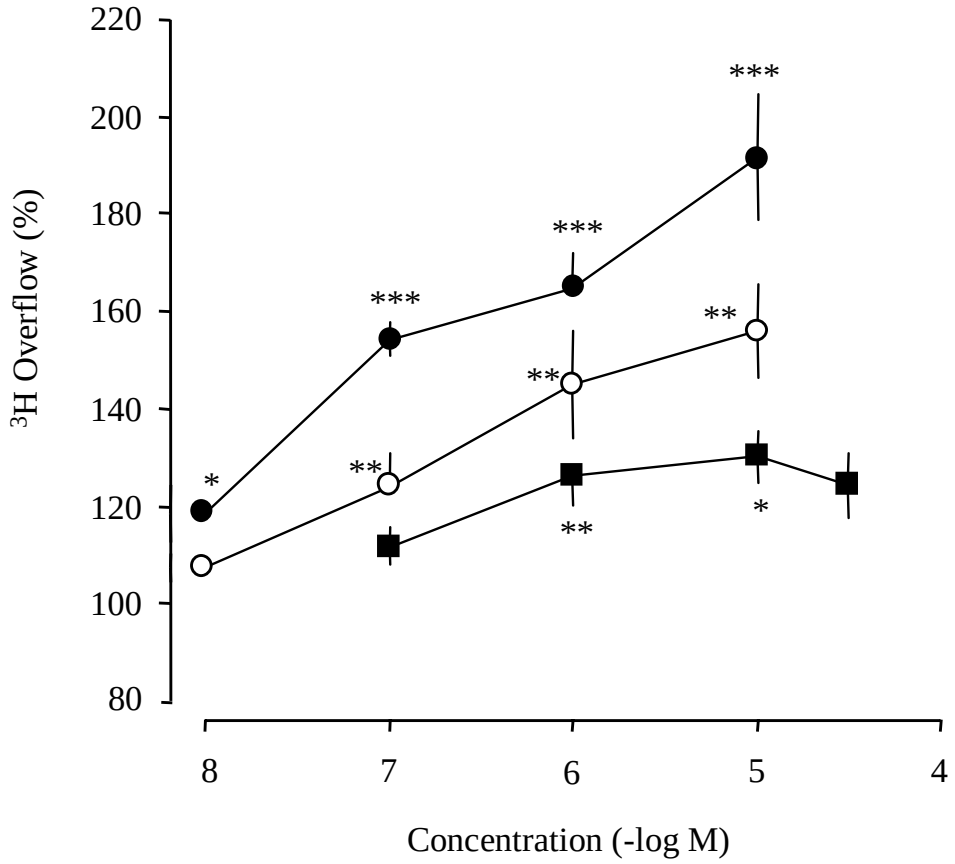

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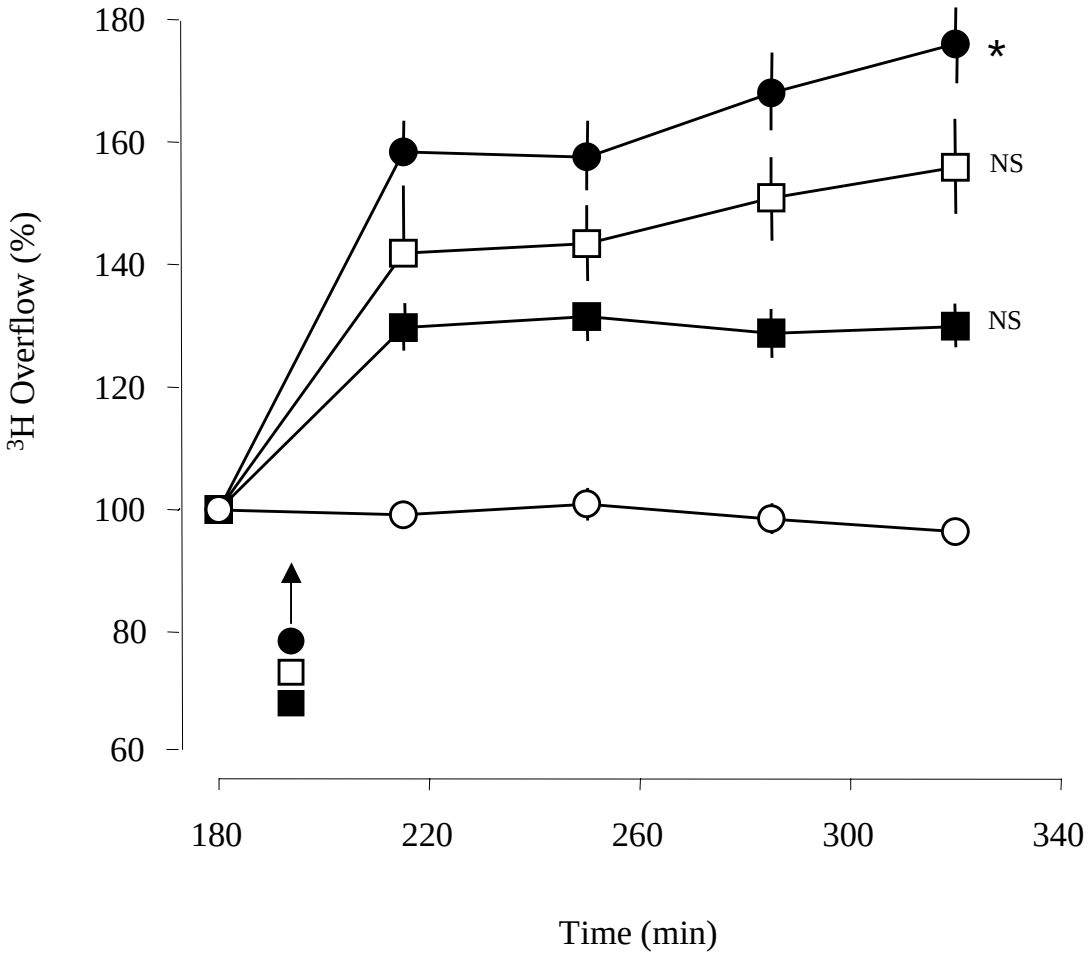

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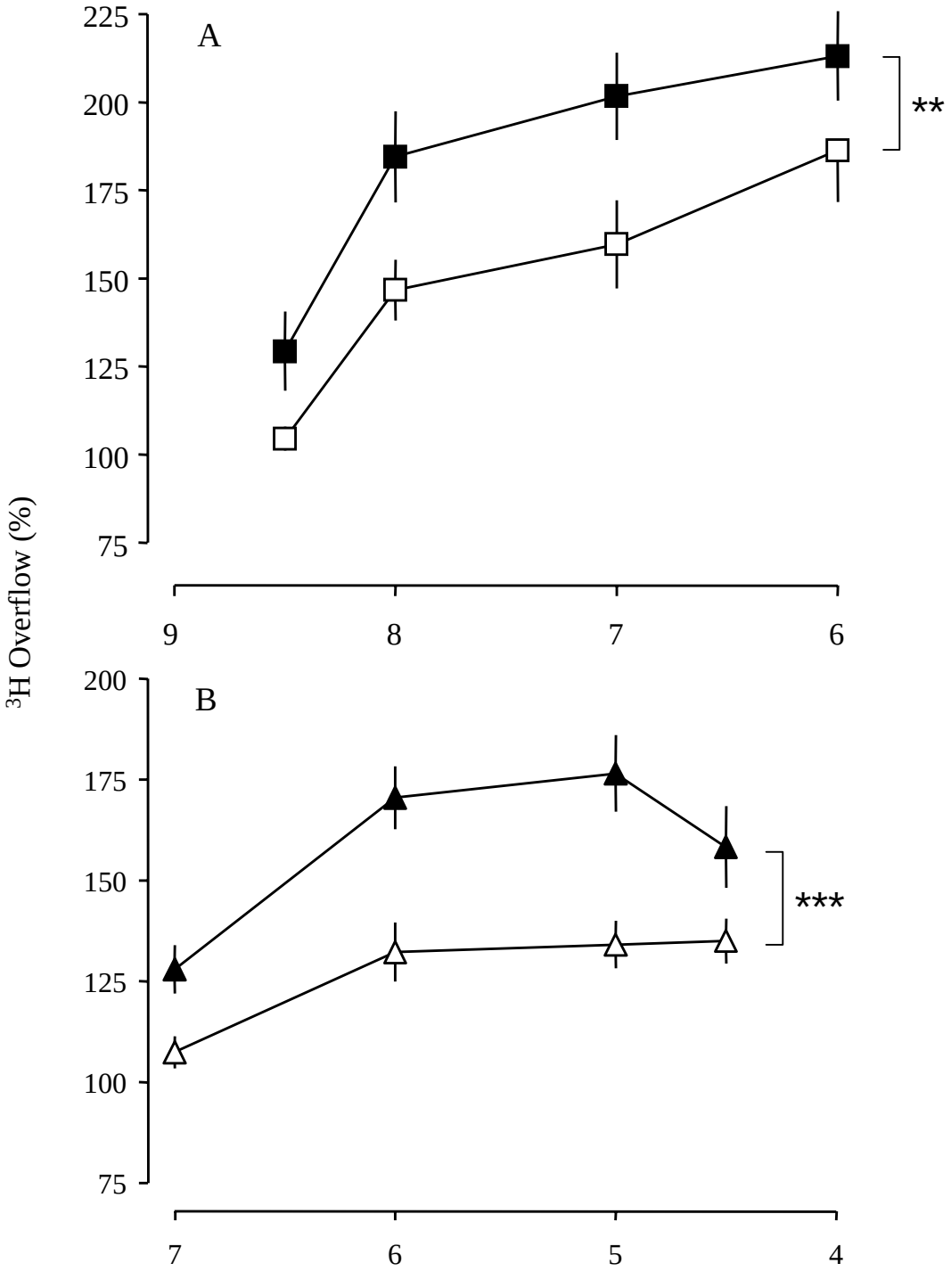

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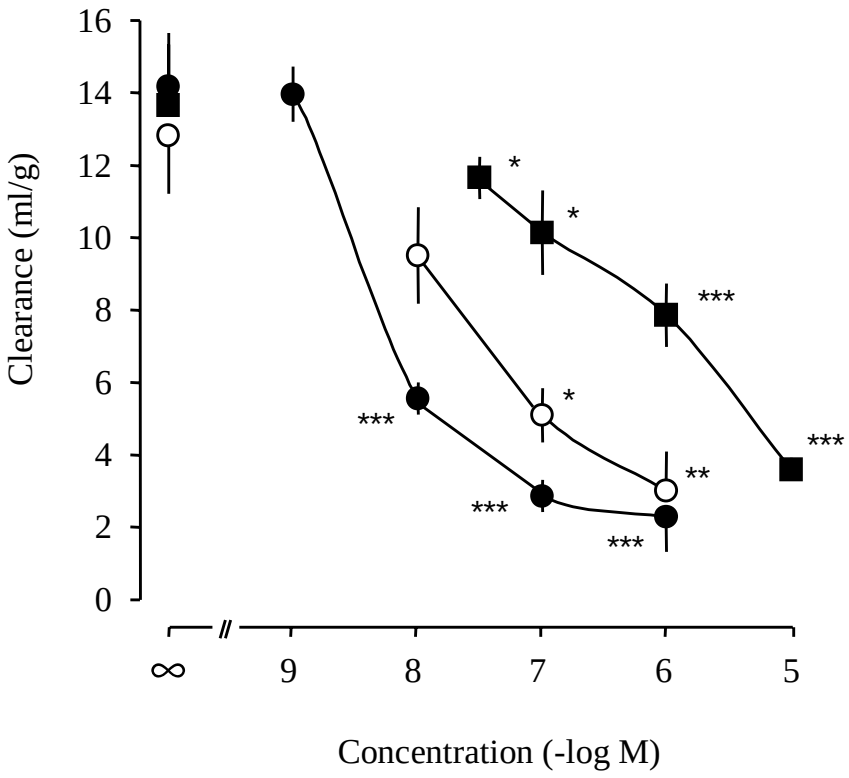

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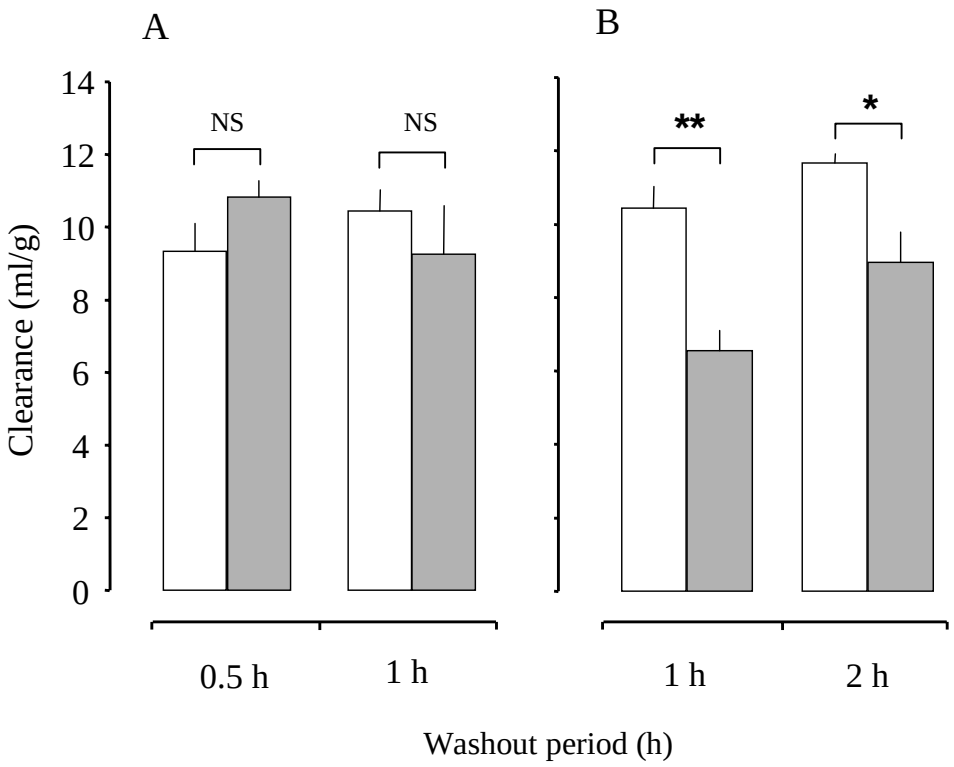

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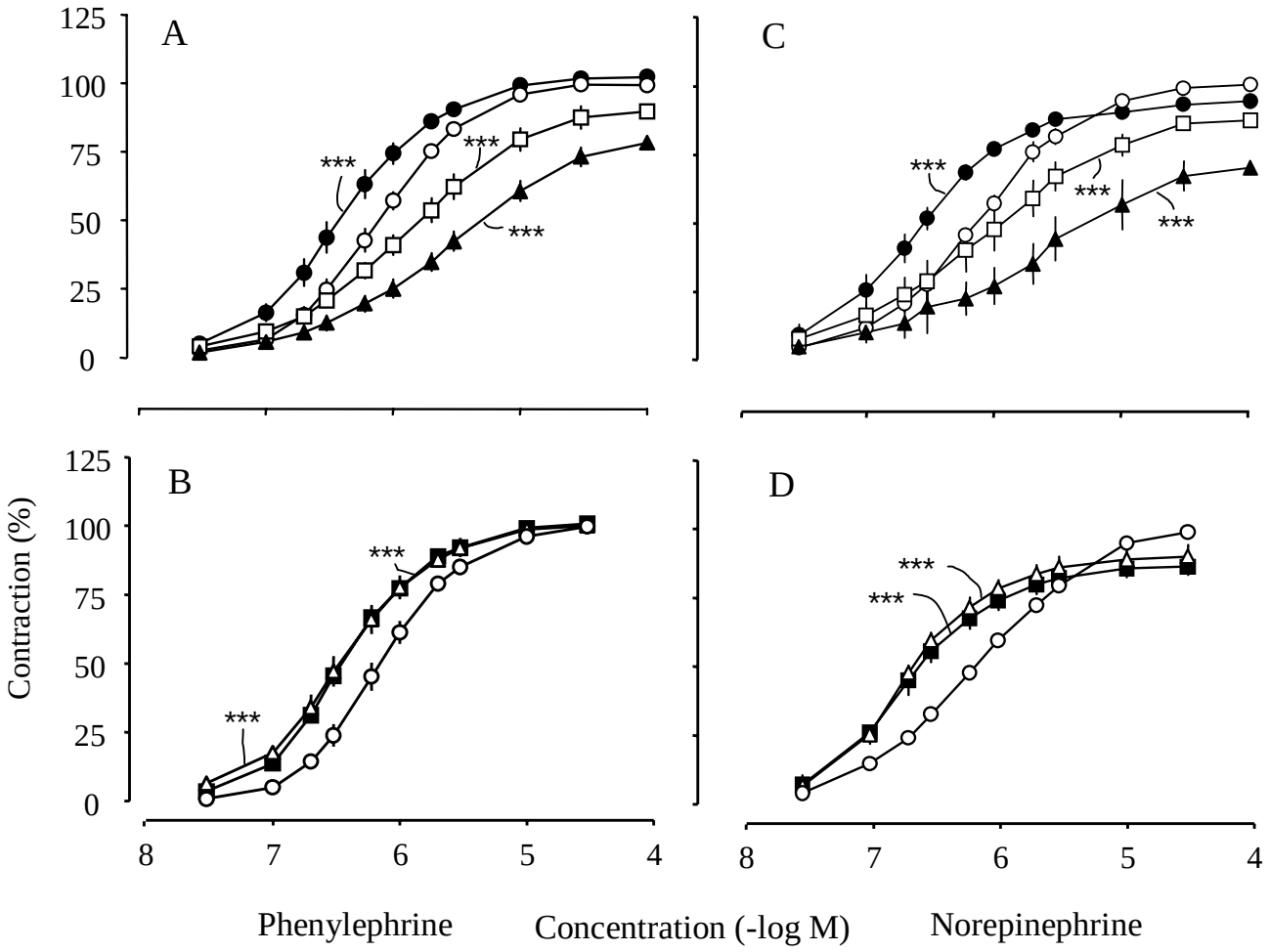

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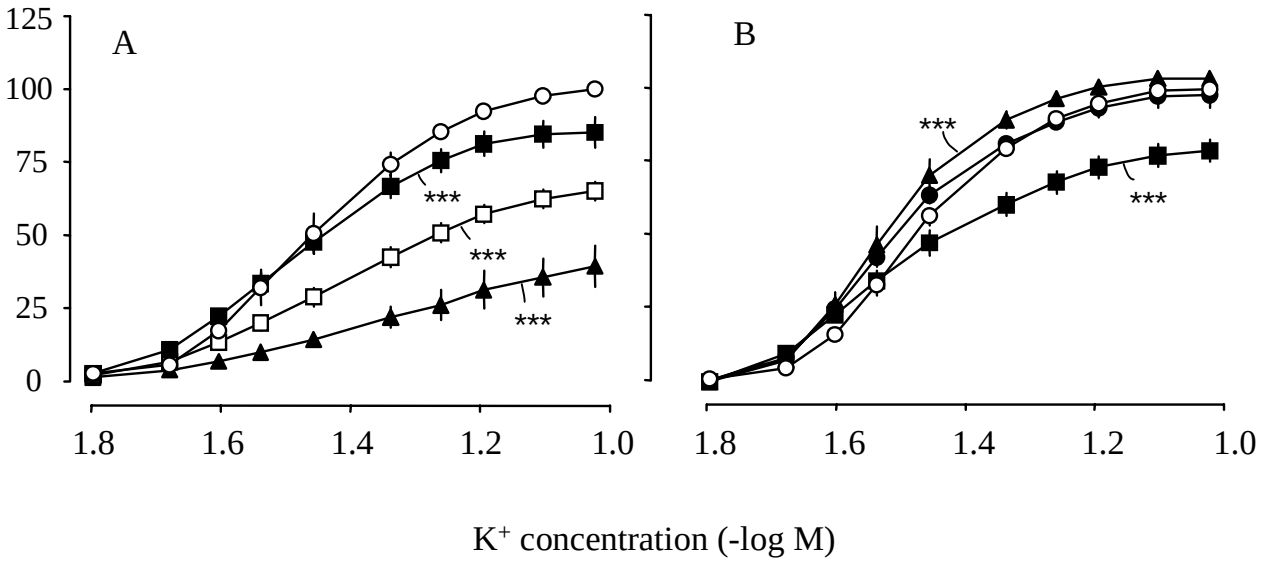

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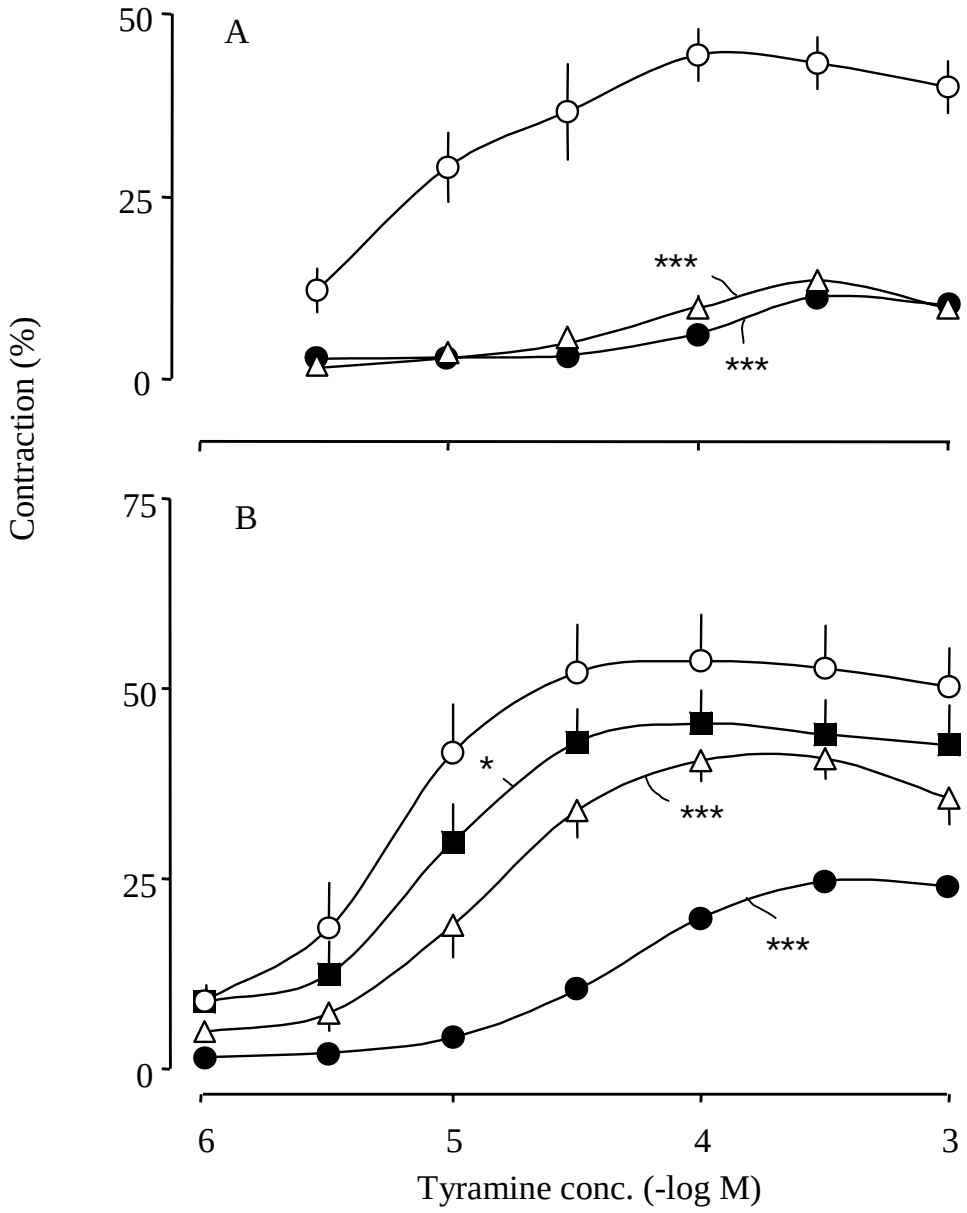
Figure 11.



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