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The aziridinium derivative of DSP4 (N-(2-chloroethyl)-N-ethyl-2-bromobenzylamine) accelerates the beating rate of isolated rat atria by enhancing the spontaneous release of noradrenaline

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Summary. The aziridinium derivative of the compound N-(2-chloroethyl)-N-ethyl-2-bromobenzylamine hydrochloride (az-DSP4) depletes endogenous noradrenaline stores and exerts neurotoxic actions on noradrenergic neurons. These effects are persistent in the central nervous system and transient in the periphery. To determine if transmitter release plays a role in the noradrenaline depletion caused by az-DSP4, the action of the compound was studied in isolated and spontaneously beating rat atria. 1. az-DSP4 enhanced atrial beating rate when present in the incubation medium at concentrations ranging from 10^{-7} M to 10^{-4} M but at 10^{-3} M decreased that rate below basal levels. 2. Preincubation of atria for 30 min with the noradrenaline uptake blocker desimipramine (DMI, 10^{-6} M) or with the beta-blocker propranolol (10^{-7} M), abolished the positive chronotropic action of az-DSP4. 3. The rate-accelerating effect of az-DSP4 could be prevented by pretreating the rats with reserpine (5 mg/kg i.p. 24 h) or enhanced by pargyline pretreatment (100 mg/kg i.p. 18 h). 4. az-DSP4 stimulated the spontaneous efflux of tritium from the isolated atria previously labeled with ^{3}H-noradrenaline (4×10^{-7} M), an increase that was mainly accounted for by DOPEG. 5. COMT and MAO activities in atria homogenates were inhibited by az-DSP4 in a concentration-dependent manner. However, MAO inhibition did not result in a change of the metabolic pattern as could be expected. 6. The results obtained indicate that az-DSP4 enhances the rate of spontaneous beating of isolated rat atria. The positive chronotropic effect of az-DSP4 requires the interaction of the compound with the noradrenaline uptake system. The mechanism of the accelerating effect of az-DSP4 most probably involves the release of noradrenaline from adrenergic nerve terminals in the atria and its subsequent interaction with adrenergic beta-receptors.

Key words: Adrenergic nerve terminals – DSP4 – Noradrenaline transport – Release and metabolism – Rat atria

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Abbreviations: DSP4 N-(2-chloroethyl)-N-ethyl-2-bromobenzylamine hydrochloride; az-DSP4, aziridinium derivative of DSP4; NA; noradrenaline; DOMA, 3,4-dihydroxy mandelic acid; DOPEG, 3,4-dihydroxyphenylethyleneglycol; NMN, normetanephrine; OMDA, O-methyl deaminated metabolite fraction, comprising vanillyl-mandelic acid (VMA) plus the 3-methoxy derivative of DOPEG (MOPEG); COMT, catechol-O-methyltransferase; MAO, monoamineoxidase

Introduction

DSP4 (N-(2-chloroethyl)-N-ethyl-2-bromobenzylamine hydrochloride) is a compound with selective neurotoxic effects acting on central and peripheral noradrenergic neurons. It inhibits noradrenaline uptake mechanisms and reduces endogenous noradrenaline levels. Both effects are prevented by pretreatment with the noradrenaline uptake blocker desimipramine (DMI) (Ross et al. 1973; Ross 1976; Jaim-Etcheverry and Zieher 1980; Jonsson et al. 1981).

DSP4 does not seem to have any apparent neurotoxic action on dopamine or adrenaline neurons while transient effects have been described on serotonin neurons (Ross 1976; Jonsson et al. 1981).

The compound, a tertiary haloalkylamine can cross the blood brain barrier when injected systemically. After intramolecular cyclization it develops its neurotoxic action through a reactive aziridinium derivative (az-DSP4), probably, by an alkylating mechanism (Zieher and Jaim-Etcheverry 1980).

In the periphery, noradrenaline uptake and endogenous levels return to normal values one week after treatment with DSP4 while in central nerve terminals, noradrenaline content remains low even at six months after injection (Jonsson et al. 1981).

In previous studies, it was described that the incubation of slices of rat cerebral cortex in the presence of az-DSP4 reduced their noradrenaline content. Although the impairment of noradrenaline uptake was not sufficient to result in a depletion of noradrenaline levels, the compound was shown to increase the spontaneous outflow of neurotransmitter. Such a possible release mechanism could be correlated with the decrease of noradrenaline content observed in vitro (Landa et al. 1984).

In order to establish if az-DSP4 can also enhance spontaneous release of the neurotransmitter in peripheral nerve terminals as it does in central neurons, we studied the spontaneous beating rate of the isolated rat atria in the presence of the compound, expecting to find an accelerating effect as the response to the possible increase of noradrenaline outflow.

The experiments were performed in vitro using the aziridinium derivative of DSP4 because this molecular species is the pharmacologically active form (Zieher and Jaim-Etcheverry 1980). Previously published data show that DSP4, a tertiary amine, cyclizes spontaneously to generate a quaternary ammonium derivative (az-DSP4) in aqueous medium at physiological pH (Ross et al. 1973).

Methods

1. Isolated rat atria. Wistar rats of 250–300 g weight and of both sexes were killed by decapitation. Their hearts were quickly removed and placed in a Petri dish with Krebs solution at room temperature. The Krebs solution had the following composition (mmol/l): NaCl 118.0; KCl 4.7; $CaCl_2$ 2.6; $MgCl_2$ 1.2; NaH_2PO_4 1.0; $NaHCO_3$ 25.0, glucose 11.1; ethylendiamine tetraacetic acid (EDTA) 0.004 and ascorbic acid 0.11.

Once dissected, the atria were set up in a 10 ml organ bath at 37° C and the incubation medium was continuously bubbled with 95% O_2 and 5% CO_2. The uper thread from the atria was connected to a force displacement transducer (Grass FT03) and the spontaneous beating was recorded on a Grass polygraph. The atria were kept in the bath until the rate of their spontaneous beating did not change more than 5 beats/min during a 10 min period; this usually occurred after 60–90 min.

Cumulative dose-response curves to az-DSP4 were performed by increasing drug concentration stepwise (in steps of 10-times higher concentration), maintaining each concentration for 30 min before adding the following one. The concentrations investigated ranged from 10^{-7} M to 10^{-3} M. DSP4 was dissolved in Krebs solution and incubated for 30 min at 37° C before addition to the organ bath to obtain the cyclized derivative of DSP4, az-DSP4 (Ross et al. 1973; Zieher and Jaim-Etcheverry 1981).

Some rats were pretreated with reserpine (5 mg/kg i.p.) 24 h before killing and others with pargyline (100 mg/kg i.p.) 18 h before the experiment.

When used, propranolol (10^{-7} M) or desimipramine (DMI, 10^{-6} M) were added to the organ bath 30 min before beginning the cumulative dose response curves and were present in the bath at the same concentration throughout the experiment.

2. Radioactive labelling of endogenous noradrenaline stores. In some experiments, noradrenaline stores were radioactively labelled by preincubating the atria in 5 ml of Krebs solution at 37° C for 10 min and then for 30 min in 5 ml Krebs containing 4×10^{-7} M (±) ^{3}H-noradrenaline and bubbled with the gas mixture described above. After 30 min incubation with radioactive noradrenaline, the tissue was washed for 1 min 8 times with fresh Krebs solution. Washing of the atria continued until the spontaneous efflux of radioactivity leveled off. This occurred about 60–90 min after the incubation with ^{3}H-noradrenaline was concluded. A volume of 5 ml was used in the bath throughout the experiment.

The total tritium spontaneously released from the atria to the bathing fluid was monitored by counting 0.5 ml aliquots of the Krebs solution added to 5 ml of a liquid scintillator solution (POPOP 100 g; PPO 5 g; ethanol 100 ml; HCl 1 N 20 ml; Triton X 100 300 ml and toluene 600 ml).

When a stable level of tritium efflux was reached, the atria were successively exposed to az-DSP4 10^{-7} M (30 min), 10^{-6} M (30 min) and 10^{-5} M (30 min). From each period, 0.5 ml samples were taken to determine total radioactivity. Controls were carried out by incubating atria in Krebs solution without added drug.

3. Metabolism of ^{3}H-noradrenaline. After incubation the atria in 10^{-5} M az-DSP4 for 30 min, the metabolism of ^{3}H-noradrenaline was determined by the chromatographic method described by Graefe et al. (1973) using purification of ^{3}H-noradrenaline and its metabolites in alumina and Dowex. The results obtained were compared with those from a similar analysis performed in fluid corresponding to 30 min of basal tritium efflux.

In other experiments, the pattern of metabolism was determined in 5 min samples after exposure of the atria to az-DSP4 (5×10^{-4} M) for 60 min, followed by 3 washings, each of 5 min. Total tritium efflux and the percentage of noradrenaline and of each metabolite in that efflux were determined as described above.

4. Determination of enzyme activities. COMT activity was assayed in atria homogenates according to Axelrod et al. (1958) using 3,4-dihidroxybenzoic acid as substrate and 10^{-4} M S-adenosylmethionine as cofactor. When COMT activity was determined in homogenates of atria preincubated with az-DSP4 (10^{-5} M) for 60 min, the drug was also present at the same concentration in the incubation medium used for enzyme assay.

MAO activity was measured in atria homogenates according to McCaman et al. (1965) in the presence of a subsaturating concentration (10^{-4} M) of ^{3}H-tyramine. Atria homogenates, performed in 1.25% (w/v) KCl, were incubated in the presence of the different concentrations of az-DSP4 for 15 min before addition of the radioactive substrate, the drug being present throughout the incubation period.

Agents used in this study: pargyline hydrochloride (Abbott Ltd., USA); reserpine and DMI (Ciba-Geigy Argentina); propranolol hydrochloride (ICI, UK); DSP4 (kind gift of Dr. R. Dahlbom and collaborators, Uppsala, Sweden); DL-(^{3}H)-noradrenaline hydrochloride (sp. activity 13.4 Ci/mmol), adenosyl-L-methionine S- (methyl ^{14}C) (sp. activity 5 Ci/mmol) and (^{3}H)-tyramine hydrochloride (sp. activity 2 Ci/mol) (New England Nuclear, Boston, MA, USA).

Results

1. Chronotropic effects of az-DSP4

The effect of az-DSP4 on the rate of spontaneous beating of isolated rat atria was investigated in a concentration range beween 10^{-7} M to 10^{-3} M. Atrial beating rate was determined after 30 min exposure to each concentration of the compound and, subsequently, az-DSP4 was added to the bath to achieve a 10-times higher concentration. As shown in Fig. 1, at 10^{-7} M to 10^{-4} M, az-DSP4 produced a concentration-dependent increase of the beating rate of the isolated atria when present in the bath. The highest concentration of az-DSP4 tested (10^{-3} M), however reduced the rate of spontaneous beating.

Experiments were carried out to determine if the interaction of az-DSP4 with the noradrenaline uptake system was in some way required for its chronotropic action. Preincubation of atria with the noradrenaline uptake blocker DMI (10^{-6} M, 30 min) increased their basal spontaneous beating rate only about 30 beats/min but completely blocked the rate-increassing effects of 10^{-7} M to 10^{-4} M az-DSP4 (Fig. 1). At 30^{-3} M, az-DSP4 increased by 30 beats/min the rate of atria preincubated with DMI, a change that was not significant.

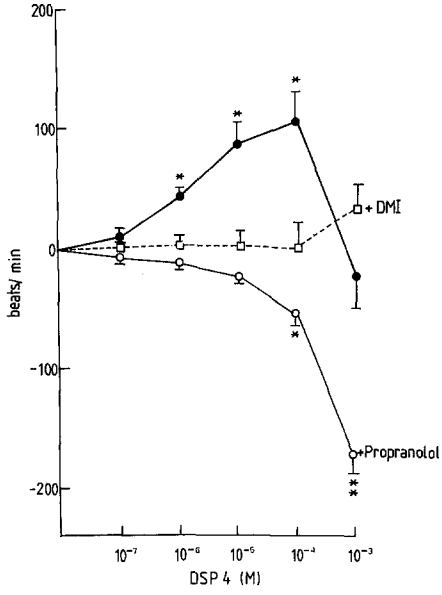

Fig. 1. Effects of increasing concentrations of az-DSP4 on the spontaneous beating rate of isolated rat atria. The cumulative concentration-response curves to az-DSP4 were carried out in Krebs solution at 37° C. *Ordinate:* rate changes from basal values in beats per minute; *abscissa:* concentration of az-DSP4 during a 30 min exposure to each concentration. ●——● az-DSP4, ○——○ az-DSP4 after 30 min preincubation of atria in the presence of 10^{-7} M propranolol or □- - - -□ of 10^{-6} M DMI. In the latter, the basal rate corresponds to the value obtained after a 30 min preincubation with DMI. Each point represents means ± SE of at least 4 determinations per group ($*p < 0.05$; $**p < 0.01$). Baseline atrial rate: 220 ± 15 (*control*), 193 ± 12 (*propranolol*), 228 ± 13 (*DMI*)

To establish if the effect produced by az-DSP4 involved the activation of beta-adrenergic receptors, atria were pre-incubated with the beta-blocker propranolol (10^{-7} M, 30 min) before performing the concentration-response curve to az-DSP4. Propranolol did not modify the basal atria rate observed in controls but completely blocked the positive chronotropic effect of az-DSP4 (Fig. 1). In this condition, az-DSP4 exerted a dose-dependent negative chronotropic effect and, after a 30 min exposure to 10^{-3} M az-DSP4, atrial rate was reduced from 193 ± 12 to 23 ± 29 beats/min.

Figure 2 shows that the positive chronotrophic effect develops rapidly since the maximal action was observed 5 min after addition of 10^{-5} M az-DSP4.

To further determine the possible relation of the chronotropic effect of az-DSP4 with endogenous noradrenaline stores, concentration-response curves were done after depleting endogenous noradrenaline and after blocking noradrenaline metabolism. Figure 3 shows that, when added to atria of rats pretreated with reserpine (5 mg/kg i.p., 24 h before the experiment), az-DSP4 not only failed to stimulate atrial beating but produced a dose-dependent negative chronotropic effect. After exposing the atria for 30 min to 10^{-3} M az-DSP4, their beating rate was reduced by 142 beats/min below the basal level.

On the other hand, when the rats were pretreated with the MAO inhibitor pargyline (100 mg/kg i.p., 18 h before the experiment), the positive chronotropic effect of az-DSP4 was markedly enhanced (Fig. 3). The dose response curve

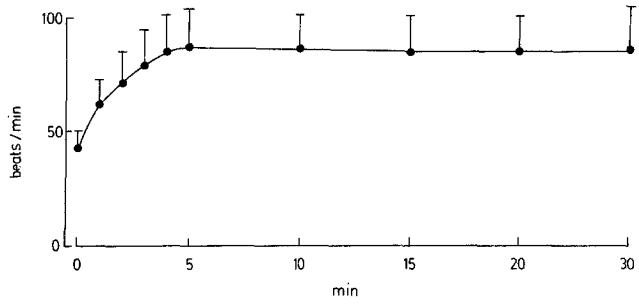

Fig. 2. Time course of the positive chronotropic effect of az-DSP4 (10^{-5} M). Changes in atrial rate from basal values (*ordinate*) is plotted against time in min (*abscissa*). The value at time zero corresponds to the chronotropic effect observed after 30 min incubation with 10^{-6} M az-DSP4. Each point represents means ± SE of at least 4 determinations per group

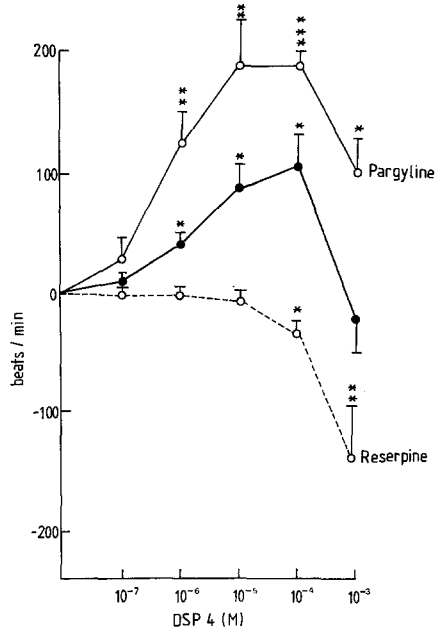

Fig. 3. Actions of az-DSP4 on the spontaneous beating of isolated atria from rats pretreated with reserpine or pargyline. Cumulative-response curves to az-DSP4 were carried out in Krebs solution at 37° C. *Ordinate:* rate changes from basal values in beats per minute; *abscissa:* concentration of az-DSP4 during a 30 min exposure to each concentration. ●——● atria of control rats; ○——○ atria of rats pretreated with pargyline (100 mg/kg ip 18 h before killing) or ○- - - -○ with reserpine (5 mg/kg ip 24 h before killing). Each point represents the means ± SE of at least 4 determinations per group ($*p < 0.05$; $**p < 0.01$; $***p < 0.001$) compared with basal rate; Baseline atrial rate: 220 ± 15 (*control*), 227 ± 6 (*reserpine*), 194 ± 11 (*pargyline*)

was shifted to the left and the maximum increased from 106 ± 27 beats/min in controls to 191 ± 78 in the pargyline pretreated rats.

2. Effects of az-DSP4 on atria labelled with ^{3}H-noradrenaline

To determine if az-DSP4 modified the spontaneous release of noradrenaline in vitro, isolated atria were incubated in the presence of ^{3}H-noradrenaline (4×10^{-7} M, specific activity 13.4 Ci/mmol, 30 min) and washed for 60 – 90 min to reach a

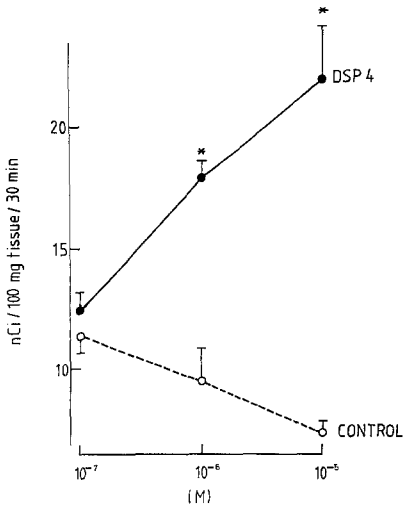

Fig. 4. Spontaneous efflux of tritium from atria labelled with ^{3}H-noradrenaline and then exposed to az-DSP4. Atria were labelled with ^{3}H-noradrenaline as described in *Methods* and after tritium efflux reached equilibrium, the tissue was incubated in drug-free Krebs (*control*) or with increasing concentrations of az-DSP4. Tissue was exposed to each concentration of az-DSP4 for 30 min. Each point represents the mean ± SE of at least 3 experiments per group. $*p < 0.005$)

plateau of tritium efflux. Once this equilibrium was reached, atria were exposed to 10^{-7} M, 10^{-6}M and 10^{-5} M az-DSP4. Atria remained for 30 min with each concentration of az-DSP4 and the radioactivity released during that period was determined in the bathing fluid.

Figure 4 shows the spontaneous efflux of radioactivity from atria incubated either without az-DSP4 or with increasing concentrations of the compound. Tritium efflux from the atria diminished with time in controls but was significantly increased above control values after exposure both to 10^{-6} M and 10^{-5} M az-DSP4.

To correlate the release of noradrenaline caused by az-DSP4 with its positive chronotropic action, in another series of experiments both properties of the compound were determined simultaneously in the presence of increasing concentrations of az-DSP4 (10^{-7} M to 10^{-3} M). As shown in Table 1, both the efflux of radioactivity and the atrial beating rate increased in a dose-dependent manner from 10^{-7} M DSP4 to 10^{-4} M. The correlation coefficient of the curve obtained after plotting these values was 0.92 ($p < 0.001$).

3. *Metabolism of the ^{3}H-noradrenaline released by az-DSP4*

To determine if the increased tritium efflux resulted from an inhibition of the noradrenaline uptake system or from an enhanced release of noradrenaline, the metabolic pattern of the ^{3}H-noradrenaline released by az-DSP4 from isolated rat atria was studied. The analysis was carried out by comparing values obtained during 30 min of release under basal conditions or after a 30 min exposure to 10^{-5} M az-DSP4 (following the cumulative exposure to 10^{-7} M and 10^{-6} M az-DSP4 as described above). Figure 5 shows the pattern of efflux of various metabolites, expressed as the percentage distribution of the total tritium efflux. az-DSP4 preferentially enhanced the efflux of DOPEG.

Table 1. Simultaneous effects of varying concentrations of az-DSP4 on the beating rate of isolated rat atria and the efflux of tritium from the tissue

az-DSP4 concentration	nCi/100 mg tissue/30 min	tissue/Rate change (beats/min)
10^{-7} M	11.79 ± 1.36	− 2 ± 8
10^{-6} M	14.61 ± 2.29	+ 63 ± 18
10^{-5} M	19.78 ± 0.79*	+159 ± 28*
10^{-4} M	22.07 ± 0.8*	+179 ± 10*
10^{-3} M	19.86 ± 2.38*	− 70 ± 17

Isolated atria were labelled with ^{3}H-noradrenaline and placed in an organ bath for recording their spontaneous beating rate. The tissue was washed until basal tritium efflux was reached. At that time, basal beating rate was recorded. The cumulative concentration response curve was performed by exposing the tissue to each concentration of az-DSP4 for 30 min. At the end of each 30 min period, the radioactivity was determined in the bath and the rate of atrial beating was recorded (results are expressed as the net change from basal beating rate). The correlation coefficient of the curve obtained between 10^{-7}M to 10^{-4}M was $r = 0.92$ ($p < 0.001$)
Data shown represent mean value ± SE of 3 experiments ($*p < 0.01$)

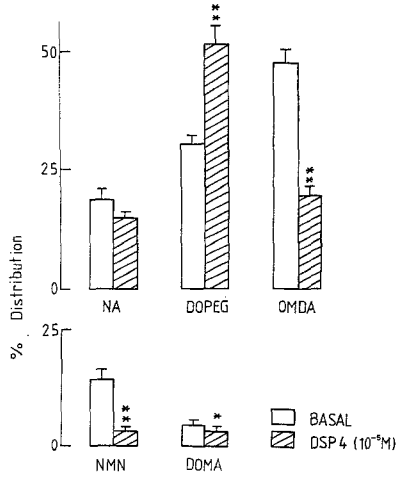

Fig. 5. Metabolic pattern of the ^{3}H outflow from atria previously labelled with ^{3}H-noradrenaline and subsequently incubated for 30 min in 10^{-5} M az-DSP4 or in drug-free Krebs (*controls*). Results are expressed as percentage of noradrenaline and of each metabolite with respect to total tritium efflux. Results correspond to means ± SE of at least 3 determinations per group. ($*p < 0.05$; $**p < 0.01$)

The relative contribution made by OMDA and NMN to the radioactivity appearing in the bathing fluid after exposure to az-DSP4, was significantly lower than in basal conditions. Since this could by explained by the inhibition of COMT by az-DSP4, enzymatic activity was assayed in atrial homogenates preincubated for 60 min with 10^{-5} M az-DSP4. The compound reduced COMT activity to 65% of controls (controls 728 ± 72 and 10^{-5} M az-DSP4 473 ± 33 μmoles/g/h; $p < 0.001$). A reversible inhibition of the enzyme by az-DSP4 cannot be excluded because az-DSP4 (10^{-5} M) was present throughout the incubation performed for assaying enzyme activity.

Since az-DSP4 has been shown to inhibit MAO activity (Lyles and Callingham 1981), it was surprising to find that

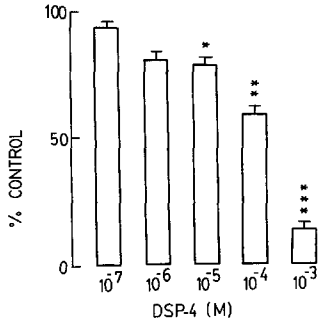

Fig. 6. Effect of varying concentrations of az-DSP4 on MAO activity of rat atria homogenates. Enzyme assays were carried out in the presence of subsaturating concentrations of substrate (10^{-4} M tyramine). Tissue was incubated during 15 min with az-DSP4 and the drug was present in the medium used for enzyme assay. Results correspond to means ± SE of 3 determinations and are expressed as percentage of control value (0.955 ± 0.067 mmol/g/h) (* $p < 0.05$; ** $p < 0.005$; *** $p < 0.001$)

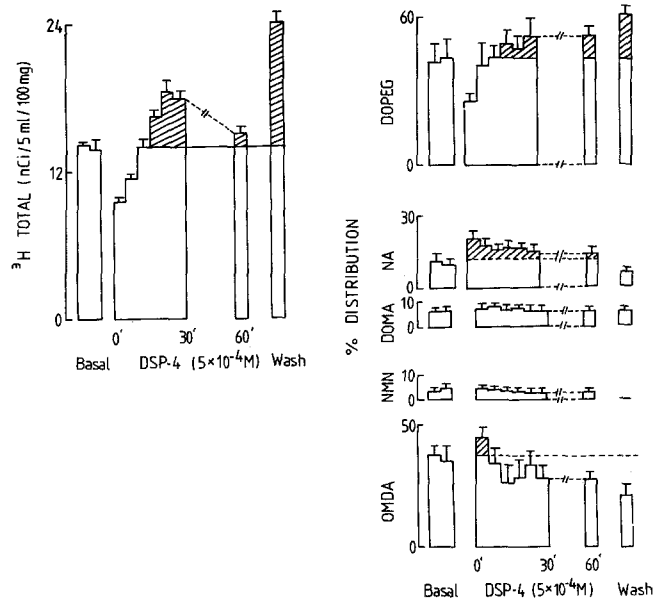

Fig. 7. Spontaneous efflux of total tritium (noradrenaline and metabolites) from rat atria labelled with ^{3}H-noradrenaline and incubated in the presence of 5×10^{-4} M az-DSP4. Tritium efflux is expressed as nCi of tritium released by 100 mg tissue to 5 ml of medium in 5 min. Results are expressed as the percentage distribution of each metabolite from the total tritium efflux. Medium was collected at 5 min intervals twice during the last 10 min of washing before exposure to az-DSP4 (*basal*), during the first 30 min and the last 5 min of exposure to the drug and after the third wash, each of 5 min. *Open bars:* spontaneous tritium efflux from atria incubated in drug-free medium; *shaded bars:* release above spontaneous efflux during exposure to 5×10^{-4} M az-DSP4 and subsequent washings. Results correspond to means ± SE of 3 determinations

in the presence of 10^{-5} M az-DSP4 the glycol DOPEG that results from MAO activity accounted for half of the total radioactivity released to the incubation medium (Fig. 5). Thus, to determine if a poor drug accumulation was responsible for the lack of enzyme inhibition, the metabolic pattern was determined in another set of experiments after exposure to a higher concentration of az-DSP4.

To determine the concentration of az-DSP4 that could block MAO activity when placed in contact with the enzyme, MAO activity was first assayed in atrial homogenates after a 15 min preincubation and in the presence of the drug. Figure 6 shows that az-DSP4 inhibited enzyme activity in a concentration-dependent manner. Since MAO activity was 60% and 15% of controls in the presence of 10^{-4} M and 10^{-3} M az-DSP4 respectively, it was assumed that approximately 50% of MAO activity was blocked by 5×10^{-4} M az-DSP4.

The time course of ^{3}H-DOPEG efflux from the atria was followed during 60 min incubation with 5×10^{-4} M az-DSP4 to establish the time required to achieve an intraneuronal concentration of az-DSP4 that could result in a reduction of DOPEG levels due to the inhibition of MAO. Figure 7 shows the results of experiments in which tritium efflux and the metabolic pattern of ^{3}H-noradrenaline were determined twice during the 10 min preceeding the exposure of atria to az-DSP4, during the 60 min incubation with 5×10^{-4} M az-DSP4 and after washing. The left panel shows that tritium efflux increased between 15 and 30 min of exposure to az-DSP4. This effect was not maintained during the 60 min incubation because in the last 5 min (55th–60th min) the radioactivity in the bath was almost similar to that appearing in basal conditions. During the last of the 3 washings, each of 5 min duration, tritium efflux increased again reaching even higher levels than those attained when az-DSP4 was present in the medium. In the figure, only the third washing is shown.

The metabolic distribution of ^{3}H efflux is shown in Fig. 7, right panel. During the first minutes of incubation of the tissue with the compound, the efflux of noradrenaline and OMDA, was increased above basal values, although these changes did not reach significance. However, as the incubation progressed, noradrenaline efflux decreased gradually whilst OMDA levels fell abruptly below controls. On the other hand, the percentage of total radioactivity corresponding to DOPEG, that was reduced at the beginning of the exposure to az-DSP4, was increased after 15 min and remained constantly elevated during the rest of the experiment. When the tissue was washed, the increase in DOPEG and the decrease of noradrenaline and OMDA, became even more marked.

These changes increased the ratio noradrenaline/DOPEG from 0.26 ± 0.01 (basal value) to 0.81 ± 0.001, 5 min after the addition of az-DSP4 ($p < 0.05$). After a 15 min exposure to az-DSP4, this ratio returned to basal values (0.25 ± 0.006).

Discussion

The results obtained in the present study indicate that az-DSP4 modifies the spontaneous beating rate of the isolated rat atria. Depending on the concentration used, the compound produces a positive (10^{-6} M to 10^{-4} M) or a negative (10^{-3} M) chronotropic effect.

The positive chronotropic actions of az-DSP4 required the stores of endogenous noradrenaline since they disappear after reserpine pretreatment. Moreover, beta-adrenergic receptors are involved in these actions since they are abolished when atria are preincubated in the presence of propranolol.

az-DSP4 must interact with the noradrenaline-carrier system to enhance neurotransmitter outflow as shown by the finding that this effect can be prevented by preincubation with DMI.

Another evidence indicating the participation of endogenous noradrenaline in the acceleration of atrial beating rate is the finding that when the endogenous concentration of noradrenaline was increased after pretreatment with the MAO inhibitor pargyline (Luchelli-Fortis and Langer 1973), the chronotropic effect was enhanced.

In the range of concentration of az-DSP4 between 10^{-7} M to 10^{-4} M, there was a close correlation between increase in atrial rate and increase of ^{3}H-noradrenaline outflow.

This rate-accelerating effect may be due to the initial release of noradrenaline (Fig. 7) observed in the metabolic pattern during the first 10 min incubation with az-DSP4 5×10^{-4} M. Since az-DSP4 is actively taken up into the neuron by the noradrenaline carrier (Jonsson et al. 1985), we assume that this compound releases noradrenaline via a counter transport mechanism. There are reports in the literature showing that the extra supply of Na^+ in the axoplasm, cotransported with indirect amines, lowers the K_m of noradrenaline carrier in the inner face of the membrane leading to an accelerative exchange diffusion (Paton 1973; Stute and Trendelenburg 1983). In support of this interpretation, the release pattern (initial increase in noradrenaline and OMDA and decrease in DOPEG) is in accordance with the counter transport hypothesis. The brisk outward pumping of noradrenaline decreased the rate of MAO deamination by lowering the amine axoplasmic concentration (reducing DOPEG efflux) while the extra noradrenaline release is inactivated in OMDA metabolites by the extraneuronal system.

As the incubation proceeds, the difference between the actions of az-DSP4 and of indirectly acting amines became greater because DOPEG levels rose while noradrenaline and OMDA decreased. We assumed that this DOPEG increase corresponds to the in vitro effect of a reserpine-like drug, first described by Adler-Graschinsky et al. (1972), where the noradrenaline mobilized from its vesicular stores increased the axoplasmic concentration of the amine, overcoming the capacity of the noradrenaline efflux carrier system (Paton 1973; Trendelenburg 1980). In consequence, the neurotransmitter is exposed to the mitochondrial MAO for prolonged periods, resulting in the spontaneous efflux enriched in DOPEG. On the other hand, OMDA levels decreased because of the inhibition of COMT activity caused by az-DSP4 as well as the reduction of noradrenaline substrate.

The possibility that az-DSP4 was acting simply as an uptake blocker could be ruled out since, in that case, the metabolic pattern of the released tritium would have only shown an increase in noradrenaline, as is the case with cocaine (Adler-Graschinsky et al. 1972).

Evidence in favor of the existence of two sites of action for az-DSP4 were previously provided by the study of the curve corresponding to the DMI-sensitive accumulation of ^{3}H-xylamine (a compound related to DSP4) in which two plateaus could be identified (Fischer et al. 1983).

Lyles and Callingham (1981) have shown that az-DSP4 inhibits MAO activity, a finding that has been confirmed in the present study (Fig. 6). Thus, it is interesting to determine why the percentage of DOPEG increased after exposure of the atria to the compound. The experiments show that this is not due to the failure to achieve a high concentration of the compound nor to the time needed to reach a high intraneuronal concentration of az-DSP4. From these results it can be concluded that the analysis of noradrenaline metabolic pattern is not the best indicator of MAO inhibition. A similar idea has been suggested by Stefano and Trendelenburg (1984) who have pointed out the importance of changes in MAO saturation when the enzyme is partially inhibited. These authors argue that usually the concentration of cytoplasmic noradrenaline is very low due to the combined activities of MAO and the noradrenaline carrier. In this condition the DOPEG measured is the result of an unsaturated enzymatic system. Following inhibitor treatment, these conditions change because some MAO activity is not completely blocked, its degradative capacity is partially lost and consequently, noradrenaline accumulates in the cytoplasm thus increasing substrate availability. The enzyme not inhibited and exposed to a higher substrate offer will produce a relatively high level of product thus masking the degree of real inhibition.

The case of az-DSP4 is not quite the same as that of pargyline because az-DSP4 is a reversible blocker in contrast to pargyline which blocks irreversibly enzyme activity. This could make possible for the noradrenaline released from vesicles into the cytoplasm to displace az-DSP4 from its binding site in MAO. The present data show that during washing the percentage of DOPEG was higher than when the compound was added to the incubation medium, indicating that MAO activity was apparently inhibited by az-DSP4 present in the medium.

Most probably the release of DOPEG is enhanced by az-DSP4 because the compound is an irreversible blocker of the noradrenaline uptake system (Ross 1976) and in this manner prevents the outflow of noradrenaline from the sympathetic terminal by the cocaine sensitive carrier system described by Paton (1973). This in turn may enhance even more the accumulation of noradrenaline in the cytoplasm. Noradrenaline is deaminated to DOPEG by MAO and this lipophylic metabolite then leaves the terminal by a passive diffusion process (Trendelenburg et al. 1980).

On the other hand, az-DSP4 by blocking the noradrenaline reuptake mechanism may potentiate the response to the relatively low proportion of noradrenaline released. A similar mechanism although with a slower time course was described for phenoxybenzamine (Adler-Graschinsky et al. 1972). The lag of 10 min needed to change the ratio noradrenaline/DOPEG in the radioactivity released from the tissue, may represent the time required to install the irreversible blockade of the neurotransmitter uptake and/or to saturate the MAO enzymatic system with the cytoplasmic accumulation of noradrenaline mobilized from vesicles.

Since az-DSP4 can be considered to be an irreversible noradrenaline uptake blocker, the cumulative dose response approach used in this study probably does not reflect the real effect of high concentrations of az-DSP4.

The negative chronotropic action of the highest concentration of az-DSP4 (10^{-3} M) seems to be due to pacemaker damage, because when the spontaneous neurotransmitter released and the beating rate are simultaneously determined, ^{3}H outflow is still elevated at 10^{-3} M az-DSP4 while the rate is diminished below basal levels. A similar effect is produced by phenoxybenzamine (Adler-Graschinsky et al. 1972) and many indirectly acting amines when used in high concentrations (Smith 1966). Bretylium has also been shown to develop a negative chronotropic effect of the isolated rat atria, acting directly on the plasma membrane of cardiac muscle cells (Naumm et al. 1970). The fact that the molecular

structure of these compounds is related to that of az-DSP4 makes possible the existence of a similar direct effect. When the positive chronotropic effect was blocked after reserpine pretreatment or with propranolol, az-DSP4 produced a negative chronotropic response at lower concentrations. This indicates that the response curve to different concentrations of az-DSP4 most probably results from two different chronotropic effects, a positive one mediated by the noradrenaline released from nerve endings and a negative one, observed at higher concentrations, resulting from a direct action of az-DSP4 on the contractile properties of muscle cells or from a damage of the pacemaker.

The reserpine-like effect of az-DSP4 described may explain in some way why the reduction of noradrenaline content observed in the heart after injecting DSP4 is not accompanied by a reduction of dopamine-beta-hydroxylase activity as is the case in central neurons (Ross 1976).

The present results allow us to conclude that neurotransmitter release from noradrenergic neurons produced by az-DSP4 is not restricted to the central nervous system because it can be also demonstrated in the terminals of sympathetic neurons in the periphery.

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