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The effect of diltiazem on noradrenaline release

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1 The effects of diltiazem in rat tail arteries and guinea-pig vasa deferentia have been investigated.

2 Superfusion of the rat tail artery with diltiazem (10^{-6}–10^{-4} M) resulted in a dose-related increase in ^{3}H-overflow ($P < 0.001$) both in Wistar Kyoto (WKY) and in spontaneously hypertensive (SHR) rats. Release of ^{3}H by transmural stimulation (1 Hz, 2 ms, 10 V) was also much greater in vessels perfused with diltiazem; this effect was dose-dependent.

3 Diltiazem did not significantly alter the proportion of noradrenaline and its metabolites in ^{3}H-overflow, as analysed by column chromatography.

4 In the vasa deferentia of guinea-pigs, diltiazem (10^{-9}–10^{-5} M) increased spontaneous ^{3}H-release.

5 The results indicate that diltiazem acts on sympathetic nerves and causes the release of noradrenaline.

Introduction

The effect of calcium entry blockers on sympathetic nerves is not yet clarified. While large doses of nifedipine (Starke & Schnemann, 1973) and verapamil (Göthert *et al.*, 1979) in rabbit hearts, and diltiazem (Zelis *et al.*, 1982) in rabbit pulmonary artery, have been shown by some workers, to inhibit electrical stimulation-induced noradrenaline (NA) release, other investigators have found verapamil (up to 2×10^{-6} M) to be ineffective on NA release (Haeusler, 1972) or on pharmacological responses (Nayler & Szeto, 1972) induced by stimulation of the stellate ganglia in the cat and dog respectively. Our recent experiments (Zsotér *et al.*, 1984) demonstrated that verapamil not only failed to inhibit ^{3}H-release from [^{3}H]-NA labelled tissues, but on the contrary, caused a pronounced dose-related increase in ^{3}H-overflow.

It is important to clarify the effect of calcium antagonists on sympathetic nerves not only for theoretical but also for practical reasons. An action on transmitter release can profoundly alter the function of baroreceptor reflexes and thus, the effect of these drugs on the heart and blood vessels. In order to see whether or not the effect of verapamil on ^{3}H-release is also characteristic for other calcium entry blockers, we decided to study the effect of diltiazem on ^{3}H-release from [^{3}H]-NA labelled tissues with a rich sympathetic innervation. The tail artery of the rat and the guinea-pig vas deferens were chosen. In addition, the composition of ^{3}H-overflow was analysed by separating NA from its metabolites by means of column chromatography.

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Methods

Rats

Seven to nine week old male, spontaneously-hypertensive rats (SHR) of the strain originally bred by Okamoto & Aoki (1963) and normotensive Wistar Kyoto rats (WKY) were used. Systolic blood pressure measured on the tail of unanaesthetized animals (Zsotér *et al.*, 1981) was 119.0 ± 0.9 mmHg in WKY and 171.6 ± 3.9 mmHg in SHR.

The proximal part of the tail artery was removed from rats anaesthetized with ether, carefully cleaned, weighed and suspended in Krebs solution bubbled with 95% O_2 and 5% CO_2 at 37°C for 30 min. The Krebs solution had the following composition (mM): NaCl 118, $NaHCO_3$ 25, KCl 4.7, $CaCl_2$ 2.6, $MgCl_2$ 1.2, NaH_2PO_4 1, glucose 11. Subsequently, the arteries were placed, for 60 min, in a tissue bath containing 10 μCi [^{3}H]-NA (−)-[7^{3}H]-noradrenaline, New England Nuclear; specific activity 17.2 Ci mmol^{-1}) in 5 ml Krebs solution; the concentration of NA was 1.16×10^{-7} M. During the incubation and superfusion, ascrobic acid (0.11 mmol) and Na EDTA (0.004 mmol) were added to the solution to delay the breakdown of NA. After labelling with [^{3}H]-NA the tail arteries were transferred to another tissue bath and

superfused with the modified Krebs solution at 37°C for 180 min at the rate of 1 ml min^{-1}. When the effect of Ca^{2+} withdrawal was studied, after 75 min of superfusion, the Krebs solution was replaced by one in which $CaCl_2$ was replaced by NaCl. The effluent from the artery was collected at 5 min intervals. The dead space between the tissue bath and the tube collecting the ^{3}H-overflow was about 2 ml.

For transmural stimulation, platinum electrodes, 35 mm long, placed parallel to the vessels were used. Square wave direct current impulses (1 Hz, 10 V, 2 ms) were triggered by a Grass S44 stimulator at 60–65, 90–95, 120–125 and 150–155 min during the superfusion period. The artery was perfused with 10^{-6} M diltiazem (Nordic Lab., Montreal) at 80–110 min, with 10^{-5} at 110–140 min and with 10^{-4} M at 140–180 min. In one series of experiments, no diltiazem was given during the superfusion. In order to measure the residual ^{3}H content, arteries were digested at 56°C for approximately 5 h in Nuclear Chicago solubilizer (Amersham Corp., Illinois). Radioactivity was counted in each sample of the efflux and in the digested artery by a Beckman 9000 Liquid Scintillation Counter. The results were expressed as d.p.m. mg^{-1} wet weight of vessel and as fractional release, that is ^{3}H-efflux as a percentage of the amount of ^{3}H in the tissue.

Column chromatography (Graefe *et al.*, 1973) was used to separate NA from its metabolites in ^{3}H-overflow. In these experiments, one sample (3.4 ml) collected at 55–60 min (i.e. before transmural stimulation and diltiazem administration) and another one collected at 125–130 min during the superfusion, were analysed for NA, normetanephrine (NMN), O-methylated deaminated products (OMDA) i.e. methoxyhydroxyphenylglycol (MOPEG) and methoxy-hydroxy-mandelic acid (VMA)), dihydroxyphenylglycol (DOPEG) and dihydroxymandelic acid (DOMA) content as described previously (Zsotér *et al.*, 1982).

For statistical analysis, *t* tests for unpaired and paired samples and regression analysis were used as indicated in the text.

Guinea-pigs

Male, albino guinea-pigs (average weight 327 ± 6.7 g) were used. One vas deferens was removed from each animal under ether anaesthesia, cleaned, incubated in 10 μCi [^{3}H]-NA for 60 min and superfused for 180 min as in the rat experiments. The collection of efflux from the tissue, stimulation with platinum electrodes at 60–65, 90–95, 120–125 and 150–155 min during the superfusion period, and the measurement of ^{3}H in the samples and in digested tissues, were performed as for the rats. However, in the guinea-pig diltiazem was given in a wider range of concentrations: 10^{-9} M at 85–115 min, 10^{-7} M at 115–145 min and 10^{-5} M at 145–180 min of the superfusion period.

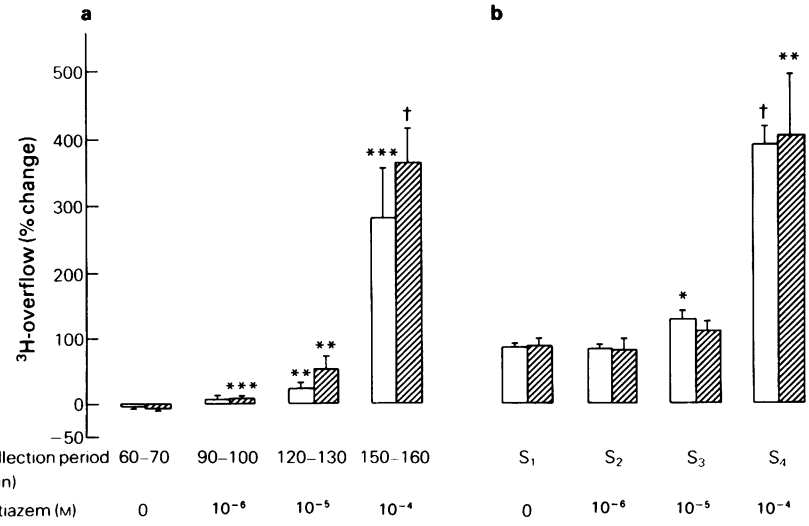

Figure 1 Effect of diltiazem (10^{-6}–10^{-4} M) on ^{3}H-overflow in the tail artery of WKY (open columns) and SHR (hatched columns). Each column represents the mean percentage change in ^{3}H-efflux, expressed as dpm mg^{-1} vessel, compared to the efflux from preceding 5 min to the periods shown; vertical bars show s.e.mean. (a) Non-stimulated vessels. (b) Results over identical periods of superfusion in vessels stimulated transmurally (1 Hz, 10 V, 2 ms) during the 60th–65th (S_1), 90th–95th (S_2), 120th–125th (S_3) and 150th–155th (S_4) min of the superfusion. * $P<0.05$, ** $P<0.02$ *** $P<0.01$ and † $P<0.001$, compared to effect before the addition of diltiazem.

Table 1 Effect of diltiazem on ^{3}H-overflow from tail arteries of Wistar Kyoto (WKY) and spontaneously hypertensive (SHR) rats

			n	*60–70 min*	*90–100 min*	*120–130 min*	*150–160 min*
	Control (no drug)	WKY	6	32.0 ± 10.0	34.8 ± 7.0	36.1 ± 8.3	39.4 ± 8.3
		SHR	6	68.4 ± 13.0	59.3 ± 11.9	65.4 ± 13.7	66.5 ± 12.9
A	Diltiazem	WKY	4	89.1 ± 6.6	85.8 ± 6.0	134.3 ± 15.4*	436.3 ± 39.7****
		SHR	4	90.4 ± 13.1	81.8 ± 17.1	115.8 ± 17.9	454.6 ± 96.9***
B	Diltiazem— no transmural stimulation	WKY	4	− 0.9 ± 2.6	10.7 ± 6.4	29.0 ± 8.4**	288.3 ± 100.7*
		SHR	4	− 5.3 ± 2.8	11.3 ± 3.2***	56.1 ± 20.1**	403.5 ± 55.4****

Results are expressed as % change (mean ± s.e.mean) of fractional release of ^{3}H compared to values during preceding 5 min periods. Diltiazem was given at a concentration of 10^{-6} M at 80–110 min, 10^{-5} M at 110–140 min and 10^{-4} M at 140–180 min of superfusion. * $P<0.05$, ** $P<0.02$, *** $P<0.01$, **** $P<0.001$, compared to results obtained during the 60–70 min control period.

Results

Rats

Diltiazem caused a marked dose-dependent increase in ^{3}H-overflow from [^{3}H]-NA-labelled tail arteries in both WKY and SHR (Figure 1a). Before the administration of diltiazem (at 60–70 min), there was a decline in ^{3}H-efflux, expressed as percentage change. During superfusion with the drug, a dose-related increase in ^{3}H-overflow took place. The percentage change in ^{3}H-efflux expressed as d.p.m. mg^{-1} vessel (Figure 1a) or as fractional release (Table 1b) was similar. ^{3}H-overflow was significantly greater, in both WKY and SHR, during perfusion with 10^{-5} or 10^{-4} M diltiazem than in the control period.

The effect of transmural stimulation on ^{3}H-overflow in diltiazem-treated and in control vessels was also compared. The response to transmural stimulation, repeated at 30 min intervals, between the 60th and 70th, 90th and 100th, 120th and 130th and 150–160 min, remained the same in untreated arteries from both WKY and SHR (Table 1). In contrast, the increase in ^{3}H-overflow following identical periods of stimulation in vessels superfused with 10^{-5} M diltiazem (WKY only) and particularly with 10^{-4} M diltiazem (150–160 min) became significantly greater. Results expressed as fractional release (Table 1) or d.p.m. mg^{-1} values (Figure 1b) are almost identical. Transmural stimulation of vessels perfused with 10^{-4} M diltiazem caused about a 5 times greater release of ^{3}H than occurred in the control stimulation period. The increase in ^{3}H-overflow during superfusion with 10^{-4} M diltiazem was similar in transmurally-stimulated and non-stimulated arteries in both WKY and SHR (Figure 1b), indicating that transmural stimulation did not enhance diltiazem-induced ^{3}H-release. With the lower doses of diltiazem the greatest

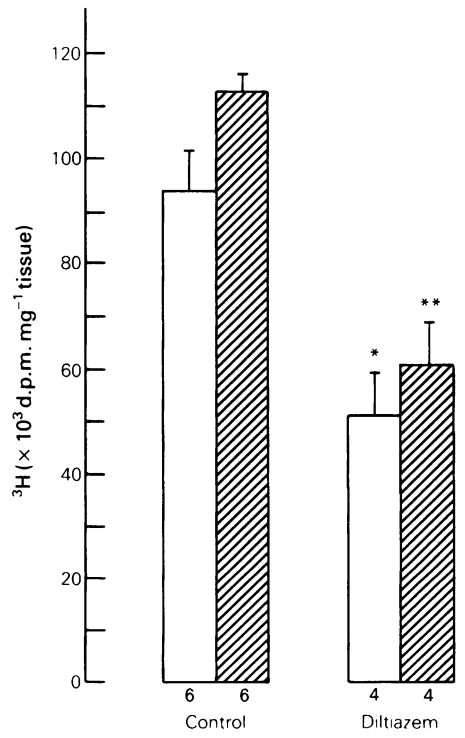

Figure 2 Mean residual ^{3}H measured in digested tail arteries from WKY (open columns) and SHR (hatched columns) after transmural stimulation in the absence (control) or presence of diltiazem. * $P<0.01$, ** $P<0.001$, compared to results from untreated arteries (t test for unpaired samples). Vertical bars show s.e. mean; numbers of arteries shown beneath columns.

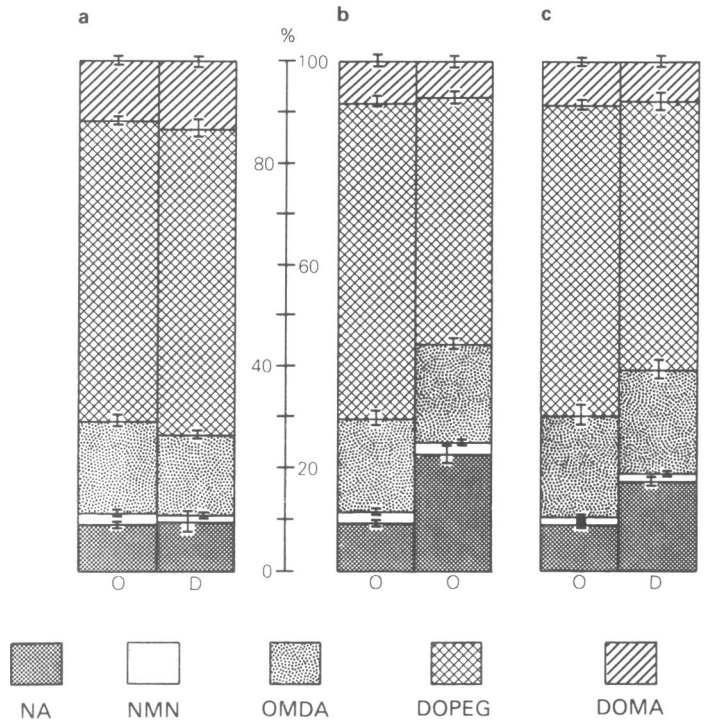

Figure 3 Mean percentage composition of ^{3}H-overflow, with respect to (NA) and its metabolites, from rat tail artery in the presence (D) and absence (0) of diltriazem (10^{-5} M), as analysed by column chromatography. (a) Results from non-stimulated arteries. (b and c) Composition from arteries before and after transmural stimulation. In each pair of columns that on the lefthand side represents results before treatment with diltiazen (a and c) or before transmural stimulation (b and c). Vertical bars show s.e.mean. Abbreviations: NMN, normetanephrine; OMDA, -methylated deaminated products; DOPEG, dihydroxyphenylglycol; DOMA, dihydroxymandelic acid.

increase in ^{3}H-overflow occurred in the first 10 min after stimulation, i.e. in the periods shown in the figures and in the Table, but with the highest concentration of diltiazem (10^{-4} M) peak values were observed in subsequent 5 min periods and ^{3}H-overflow remained higher, usually for at least 20 min, both in stimulated and unstimulated arteries.

The effect of diltiazem on ^{3}H-overflow expressed in d.p.m. mg^{-1} was dose-related. Regression analysis revealed a significant correlation both in WKY and SHR. This was true both for unstimulated arteries ($P < 0.001$) and for vessels where transmural stimulation followed superfusion with diltiazem ($P < 0.001$). Mean ^{3}H-overflow, expressed as d.p.m. mg^{-1} tissue, was similar in SHR and WKY. The slopes for WKY (0.573 ± 0.084) and SHR (0.578 ± 0.079) were parallel indicating that diltiazem was comparably effective in both normotensive and hypertensive rats.

If diltiazem enhances ^{3}H-efflux from vessels, residual radioactivity in the tissues should be decreased after superfusion with the drug. That this was the case is shown in Figure 2. Residual ^{3}H was almost 50% less in diltiazem-treated than in untreated arteries of WKY and SHR.

To measure whether diltiazem induced the release of mainly NA or of its metabolites from [^{3}H]-NA labelled vessels, samples of ^{3}H-efflux were analysed using column chromatography before and after superfusion with the drug (at 125–130 min). Diltiazem (10^{-5} M) did not significantly alter the composition of ^{3}H-overflow (Figure 3a); i.e. diltiazem released NA and its metabolites from the tail artery in almost the same proportion as that found in the spontaneous efflux. Transmural stimulation increased the percentage of NA (Figure 3b) and decreased that of DOPEG ($P < 0.01$) in both untreated arteries and in those superfused with 10^{-5} M diltiazem (Figure 3c). The composition of the overflow remained similar in untreated and treated vessels both before and after transmural stimulation.

In a few experiments, the effect of superfusion with a Ca^{2+}-free solution was studied. In two WKY and two

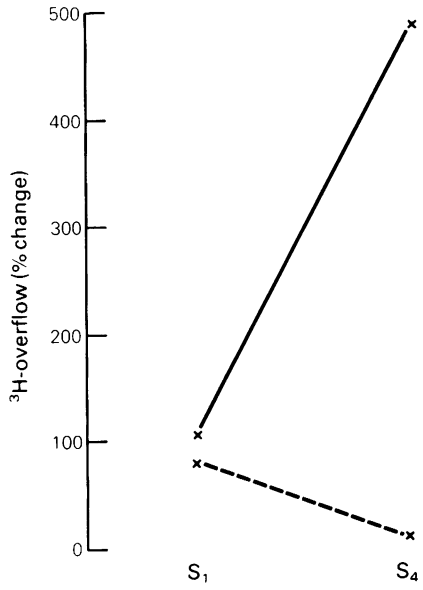

Figure 4 The increase in ^{3}H-overflow in 2 SHR induced by transmural stimulation during perfusion with normal Krebs solution (S_1) and with Ca^{2+}-free Krebs (S_4) in the presence (———) and absence (– – – –) of diltiazem (10^{-4} M). S_1 reflects changes in control period between the 60th–70th min of superfusion (stimulation at 1 Hz, 10 V, 2 ms from 60–65 min) and S_4 during perfusion with Ca^{2+}-free solution between 150th–160th min of superfusion (stimulation at 1 Hz, 10 V, 2 ms from 150–155 min.).

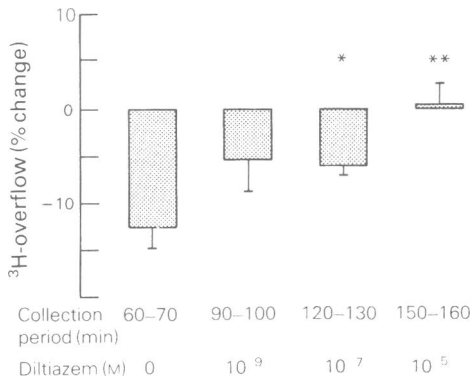

Figure 5 Effect of diltiazem (10^{-9}–10^{-5} M) on ^{3}H-overflow from the guinea-pig vas deferens. Percentage changes in fractional release were compared in the absence (control) and presence of diltiazem at the times and concentrations indicated; $*P<0.05$ and $**P<0.01$, significantly different from control. Vertical bars show s.e.mean; $n = 6$.

SHR, the Krebs solution perfusing the arteries, at 75 min (i.e. after control stimulation) was replaced by one with no $[Ca^{2+}]_o$ (Figure 4). In the untreated artery after withdrawal of $[Ca^{2+}]_o$ the increase in ^{3}H-overflow induced by transmural stimulation was markedly reduced. However, in the diltiazem-treated artery, ^{3}H-overflow increased; the increment caused by stimulation in the presence of 10^{-4} diltiazem was almost 5 times greater than after stimulation in the absence of the drug. Experiments in two WKY gave similar results.

Guinea-pigs

In non-stimulated, [^{3}H]-NA-labelled vasa deferentia, ^{3}H-overflow declined exponentially. However, this decrease was significantly less in tissues perfused with 10^{-7} M diltiazem and was replaced by a slight increase in the presence of 10^{-5} M diltiazem (Figure 5). Results expressed in d.p.m. mg^{-1} revealed a significantly smaller decline in ^{3}H-overflow during superfusion with 10^{-9} ($P<0.05$), 10^{-7} ($P<0.01$) or 10^{-5} M diltiazem ($P<0.001$) than before the drug was given. The response to transmural stimulation, stable in each of the untreated vasa deferentia from 6 guinea-pigs, did not increase significantly after superfusion with 10^{-9}, 10^{-7} or 10^{-5} M diltiazem.

Discussion

Diltiazem like verapamil (Zsotér *et al.*, 1984) caused a consistent increase in ^{3}H-release from [^{3}H]-NA-labelled tissues in both rat and guinea-pig and was equally effective in arteries of WKY and SHR as indicated by the parallel dose-response curves. There was no significant difference between the slopes, obtained with regression analysis, characterizing the dose-response relationship for verapamil and for diltiazem in rat tail artery. Nevertheless, certain other differences were observed between the two drugs. Verapamil (10^{-4} M), in contrast to diltiazem, increased the ^{3}H-overflow from transmurally stimulated arteries less than from non-stimulated vessels. If verapamil induces the release of NA by blocking presynaptic α_2-adrenoceptors, as suggested by Galzin & Langer (1983), the opposite effect would be expected, that is a greater rather than a reduced release of ^{3}H on stimulation of sympathetic nerves.

It was important to know whether or not diltiazem increased ^{3}H-overflow from tissues by releasing NA, its metabolites or both. When NA and its metabolites in the ^{3}H-efflux were separated by column chromatography, no significant difference in the composition from untreated and diliazem-treated vessels was revealed. Transmural stimulation increased the proportion of NA and decreased that of DOPEG in

the ^{3}H-overflow from rat artery (Zsotér *et al.*, 1982). The composition of stimulation-induced ^{3}H-efflux diltiazem was similar to that in untreated vessels. These results differ from those of Chaudhry & Vohra (1984) who showed that verapamil in high concentration released mainly DOPEG from rat atrium, i.e. it had a reserpine-like effect. The present findings suggest that both diltiazem and stimulation of sympathetic nerve terminals release NA and its deaminated metabolites from the same intracellular storage sites. Thus, prolonged exposure of tissues to diltiazem may conceivably deplete NA.

Verapamil and diltiazem appear to have a similar, although not an identical, effect on ^{3}H-release. However, this does not mean that each drug acts by inhibition of Ca^{2+} entry into sympathetic nerves. Calcium is required for exocytosis of NA and therefore, exposure of tissues to calcium entry blockers or to perfusion with a Ca^{2+}-free solution would be expected to decrease the release of NA by transmural stimulation. We found that diltiazem, in contrast to superfusion with Ca^{2+}-free solution, did not abolish ^{3}H-release and its effect was neither enhanced nor abolished by perfusion of the arteries with Ca^{2+}-free solution. The effect of diltiazem and verapamil on NA release is mediated by an action other than the inhibition of Ca^{2+} influx into the cells. An intracellular effect, similar to that of tyramine (Lindmar *et al.*, 1967), which is calcium independent, is proposed. The assumption that calcium entry blockers act not only on the slow channels but also intracellularly seems justified (Zsoter & Church, 1983). The exact mechanism whereby calcium antagonists can release NA and its metabolites is not yet clear.

Although diltiazem was effective in releasing NA in as low a concentration as 10^{-7} M, the most dramatic effect was observed with 10^{-5} and 10^{-4} M diltiazem, that is, at a level higher than that encountered with therapeutic administration of the drug (Kates, 1983). It is questionable, therefore, whether the higher plasma NA levels found in humans after nifedipine (Kiowski *et al.*, 1983) and diltiazem (Petru *et al.*, 1983) administration could be explained by an effect on sympathetic nerves, as described here for diltiazem; rather, they may reflect the activation of baroreceptor reflexes. However, the results of the present study may have important clinical applications for the chronic administration of calcium channel blockers as these drugs could lead to a depletion of NA in sympathetic nerve endings.

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