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Presynaptic α_2-adrenoceptor antagonism by verapamil but not by diltiazem in rabbit hypothalamic slices

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1 Rabbit hypothalamic slices prelabelled with [^{3}H]-noradrenaline and superfused with Krebs solution were stimulated electrically at a frequency of 5 Hz. Exposure to verapamil (0.1 to 10 μM) significantly increased, in a concentration-dependent manner, the electrically-evoked overflow of tritium, without affecting the spontaneous outflow of radioactivity.

2 Exposure to diltiazem in concentrations up to 100 μM had no effect on the electrically evoked release of [^{3}H]-noradrenaline, but increased the basal outflow of radioactivity at 10 and 100 μM.

3 The preferential α_2-adrenoceptor antagonist, yohimbine (0.1 μM) significantly antagonized the inhibitory effect of clonidine or adrenaline on [^{3}H]-noradrenaline overflow elicited by electrical stimulation. Verapamil (3 μM) also antagonized this inhibitory effect of the α_2-adrenoceptor agonists on [^{3}H]-noradrenaline release. In contrast to these results, exposure to diltiazem (10 μM) was ineffective in blocking the action of the α_2-adrenoceptor agonist.

4 These results suggest that the two Ca^{2+}-antagonists verapamil and diltiazem differ in their ability to affect central noradrenergic neurotransmission. While verapamil is a relatively potent α_2-adrenoceptor antagonist, diltiazem is devoid of presynaptic α_2-adrenoceptor antagonist properties.

Introduction

Verapamil and diltiazem are compounds classed as calcium antagonists because of their ability to block the slow calcium channels found in myocardial fibres and smooth muscle cells (Fleckenstein, 1971; 1977; Nayler, 1980). It is also known that these compounds inhibit the excitation-contraction coupling in vascular smooth muscle, which is dependent on the translocation of calcium into cells via potential-dependent calcium channels (Nayler, 1980).

It has recently been demonstrated that in vascular smooth muscle, both under *in vivo* and *in vitro* experimental conditions, the contractile responses to α_2-adrenoceptor agonists are particularly sensitive to blockade by diltiazem and verapamil, while the responses to α_1-adrenoceptor agonists are virtually unaffected by these calcium antagonists (Cavero, Shepperson, Lefèvre-Borg & Langer, 1982). In the rat portal vein, diltiazem is a potent antagonist of the α_2-adrenoceptor mediated phasic activity of the smooth muscle (Hicks, 1982). In addition, in membranes of the rat brain, both verapamil and diltiazem inhibit [^{3}H]-clonidine binding in the low micromolar range (Nayler, Thompson & Jarrot, 1982).

The pharmacological profile of the presynaptic, release-modulating α_2-adrenoceptors is identical to the postsynaptic α_2-adrenoceptors mediating vasoconstriction in vascular smooth muscle (Langer 1980; Langer & Shepperson, 1982). It was therefore considered of interest to investigate the effects of verapamil and diltiazem on the electrically- evoked release of [^{3}H]-noradrenaline from the rabbit hypothalamus and their interactions with the α_2-adrenoceptor-mediated presynaptic modulation of noradrenergic neurotransmission.

Methods

Male rabbits weighing 1.5–3.0 kg were killed by decapitation. The brains were quickly removed at 4°C and the hypothalamus was dissected and chopped to a 0.4 mm thickness by a McIlwain tissue chopper. The slices were immersed in cold Krebs solution of the following composition (mM): NaCl 118, KCl 4.7, glucose 11.1, $NaHCO_3$ 25.0, $MgCl_2$ 1.2, NaH_2PO_4 1.0, $CaCl_2$ 1.3, ascorbic acid 0.11, and sodium ethylenediamine tetraacetic acid (EDTA) 0.04.

The hypothalamic slices were incubated for 15 min at 37°C with Krebs solution bubbled with 5% CO_2 in

O_2 containing 0.33 μM [^{3}H]-noradrenaline (specific activity 5.8 Ci/mmol, NEN, Boston, Massachusetts). After the 15 min incubation with [^{3}H]-noradrenaline, the slices were transferred to glass superfusion chambers and superfused at the rate of 0.5 ml/min. Two periods of electrical stimulation (5 Hz, 2 ms, 26 mA) were applied for 2 min at 60 min (S_1) and 104 min (S_2) after the end of the incubation with the radiolabelled neurotransmitter. The effects of adrenaline on the electrically-evoked release of [^{3}H]-noradrenaline were studied in the presence of cocaine (10 μM) at 3 Hz instead of 5 Hz, in order to obtain similar values of fractional release evoked by electrical stimulation under both experimental conditions. The superfusate was collected in 4 min samples. At the end of the experiment, the slices were solubilized in Soluene (0.4 ml) and the tritium content of the slices and superfusate samples was determined by liquid scintillation counting. The overflow of radioactivity elicited by electrical stimulation was expressed as fractional release of the total tritium present in the tissue at the onset of stimulation (Pelayo, Dubocovich & Langer, 1980). Results are expressed as mean ± s.e.mean. Unpaired Student's two-tail t test, and the Mann-Whitney two tail test were used for statistical comparisons.

The following drugs were used: verapamil HCl, diltiazem HCl (Tanabe Seiyaku), clonidine HCl (Boehringer), (−)-adrenaline bitartrate (Sigma), yohimbine HCl (Sigma), and cocaine HCl (La Coopération Pharmaceutique Française).

Results

Effects of verapamil and diltiazem on the stimulation-evoked release of [^{3}H]-noradrenaline in rabbit hypothalamic slices

In the controls, the fraction of the total tissue radioactivity released by electrical stimulation, from slices of the rabbit hypothalamus, was close to 1% (Table 1). We have previously shown that this release is entirely calcium-dependent (Galzin, Dubocovich & Langer, 1982). In the absence of drugs the ratio between two consecutive periods of electrical stimulation (S_2/S_1) was close to unity (Table 1).

When added 20 min before S_2, verapamil (0.1 to 10 μM) significantly increased, in a concentration-dependent manner, the overflow of tritium elicited by electrical stimulation (Table 1). At 10 μM, verapamil also produced a slight but significant increase in the spontaneous outflow of radioactivity (Table 1).

In contrast to verapamil, diltiazem when added 20 min before S_2, had no effect on the electrically-evoked release of [^{3}H]-noradrenaline from hypothalamic slices in concentrations up to 100 μM (Table 1). However, diltiazem at 10 μM produced a small but significant increase in the spontaneous outflow of radioactivity, this effect being more pronounced at 100 μM (Table 1).

Effects of verapamil and diltiazem on the clonidine- or adrenaline- induced inhibition of the stimulation-evoked release of [^{3}H]-noradrenaline in rabbit hypothalamic slices

The α_2-adrenoceptor agonists clonidine (0.1–1 μM) and adrenaline (0.01–0.1 μM) significantly inhibited the stimulation-evoked release of tritium from rabbit hypothalamic slices in a concentration-dependent manner (Table 2, Figure 1). On the other hand the α_2-adrenoceptor antagonist yohimbine (0.1–1 μM) when added to the superfusion medium before S_2 significantly increased the overflow of radioactivity during electrical stimulation without affecting the spontaneous outflow of radioactivity (Table 2). When yohimbine (1 μM) was added 40 min before S_1 and maintained in the perfusion medium for the duration of the experiment, the ratio S_2/S_1 was: 0.91 ± 0.05 ($n = 6$). Under these experimental conditions, clonidine (1 μM) added 20 min before S_2 was significantly less effective in reducing the overflow of [^{3}H]-noradrenaline elicited by electrical stimulation ($S_2/S_1 = 0.43 \pm 0.04$, $n = 4$ in the absence of yohimbine, $P < 0.01$ when compared with the ratio $S_2/S_1 = 0.64 \pm 0.04$, $n = 4$ obtained in the presence of yohimbine).

Likewise, when verapamil 3 μM was added to the medium 40 min before S_1 and maintained throughout the rest of the experiment it significantly antagonized the inhibitory effect of 0.1 μM clonidine (added before S_2) on the stimulation-evoked overflow of tritium (Table 3). In contrast to the results obtained with verapamil, exposure to 10 μM diltiazem, added 40 min before S_1, did not antagonize the clonidine-induced inhibition of the electrically-evoked overflow of [^{3}H]-noradrenaline (Table 3).

When verapamil 3 μM was added before S_1 together with cocaine 10 μM, it also antagonized significantly the inhibitory effect of adrenaline (0.01–0.1 μM) on the overflow of tritium elicited by electrical stimulation (Figure 1). Like yohimbine, exposure to verapamil antagonized completely the inhibition produced by the low concentration of adrenaline (0.01 μM) and only partially the inhibition caused by the higher concentration (0.1 μM) of adrenaline (Figure 1).

Verapamil increases [^{3}H]-noradrenaline overflow when neuronal uptake is inhibited with cocaine

Exposure to 10 μM cocaine increased significantly the overflow of [^{3}H]-noradrenaline evoked by electrical

Table 1 Effects of diltiazem and verapamil on the electrically evoked [^{3}H]-noradrenaline overflow from rabbit hypothalamic slices

Experimental Group		*^{3}H-transmitter overflow* (a) *fractional release* $\times 10^{-2}$			*Spontaneous outflow fractional release* $\times 10^{-2}$			*Tissue*
	n	S_1	S_2	Ratio[b] S_2/S_1	Sp_1	Sp_2	Ratio[c] Sp_2/Sp_1	nCi/slice[d]
Control	7	1.03 ± 0.11	1.01 ± 0.10	0.99 ± 0.06	1.05 ± 0.03	0.79 ± 0.04	0.75 ± 0.02	58.4 ± 5.0
Diltiazem 10 μM	5	1.04 ± 0.24	0.84 ± 0.20	0.84 ± 0.18	1.00 ± 0.04	0.85 ± 0.03	0.85 ± 0.02*	51.0 ± 6.6
Diltiazem 100 μM	3	1.24 ± 0.25	1.39 ± 0.25	1.04 ± 0.28	0.89 ± 0.11	1.63 ± 0.38	1.74 ± 0.16**	50.8 ± 6.9
Verapamil 0.1 μM	4	1.19 ± 0.08	1.27 ± 0.16	1.05 ± 0.07	0.91 ± 0.08	0.72 ± 0.07	0.79 ± 0.02	56.1 ± 9.3
Verapamil 1 μM	5	1.34 ± 0.14	1.91 ± 0.19	1.43 ± 0.05*	0.90 ± 0.05	0.76 ± 0.04	0.84 ± 0.02	55.4 ± 3.5
Verapamil 3 μM	4	1.06 ± 0.10	1.84 ± 0.36	1.77 ± 0.34**	0.80 ± 0.07	0.62 ± 0.06	0.77 ± 0.04	62.1 ± 7.2
Verapamil 10 μM	6	1.18 ± 0.08	3.18 ± 0.25	2.69 ± 0.11**	1.05 ± 0.11	1.03 ± 0.11	0.97 ± 0.02**	59.1 ± 8.4

[a] Fraction of the total tissue radioactivity released by a 2 min period of electrical stimulation (5 Hz, 2 ms, 26 mA), at 60 min (S_1) and 104 min (S_2) after the end of the incubation with [^{3}H]-noradrenaline.
[b] Ratio of fractional release obtained between the second period of stimulation (S_2) and the first one (S_1).
[c] Ratio between the spontaneous outflow of radioactivity obtained during the 4 min preceding the second stimulation (Sp_2) and the corresponding fraction of radioactivity released spontaneously before the first stimulation period (Sp_1).
[d] Radioactivity retained by the tissue after 130 min of superfusion expressed in nCi/slice.
Diltiazem or verapamil in the concentrations indicated was added to the medium 20 min before S_2.
Shown are mean values ± s.e. mean. n = number of experiments.
* $P<0.05$; ** $P<0.01$ when compared to the control value.

Table 2 Effect of clonidine and yohimbine on the electrically-evoked release of [^{3}H]-noradrenaline from rabbit hypothalamic slices

Experimental Group		*^{3}H-Transmitter overflow* [a] *fractional release* $\times 10^{-2}$			*Spontaneous outflow fractional release* $\times 10^{-2}$			*Tissue*
	n	S_1	S_2	Ratio[b] S_2/S_1	Sp_1	Sp_2	Ratio[c] Sp_2/Sp_1	nCi/slice[d]
Control	7	1.03 ± 0.11	1.01 ± 0.10	0.99 ± 0.06	1.05 ± 0.03	0.79 ± 0.04	0.75 ± 0.02	58.4 ± 5.0
Clonidine								
0.1 μM	4	0.99 ± 0.13	0.70 ± 0.10	0.72 ± 0.08*	0.87 ± 0.06	0.68 ± 0.05	0.78 ± 0.02	59.1 ± 2.1
1 μM	4	0.94 ± 0.11	0.40 ± 0.03	0.43 ± 0.04**	0.91 ± 0.06	0.77 ± 0.05	0.77 ± 0.03	43.4 ± 10.1
Yohimbine								
0.1 μM	3	1.24 ± 0.21	2.47 ± 0.21	2.05 ± 0.17**	0.86 ± 0.08	0.78 ± 0.09	0.91 ± 0.05	61.9 ± 6.3
1 μM	7	1.14 ± 0.13	3.25 ± 0.44	2.81 ± 0.13**	0.87 ± 0.07	0.74 ± 0.05	0.85 ± 0.01	48.2 ± 5.6

[a], [b], [c] and [d] as in Table 1
Clonidine or yohimbine in the concentrations indicated were added to the medium 20 min before S_2.
Shown are mean values ± s.e.mean. n = number of experiments.
* $P<0.05$; ** $P<0.01$ when compared to the corresponding control.

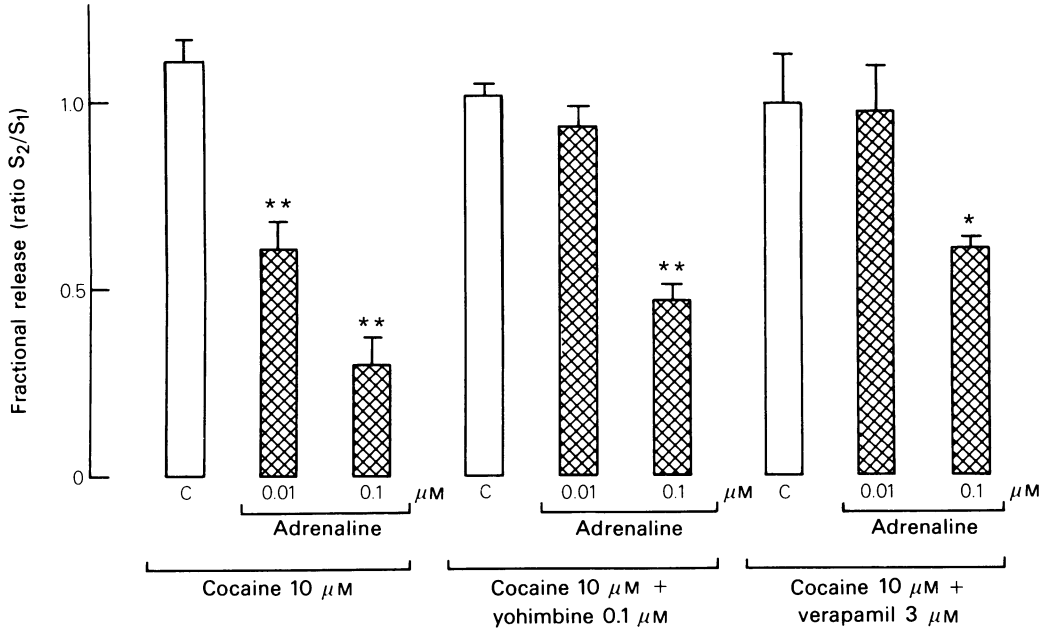

Figure 1 Antagonism by yohimbine and verapamil of the adrenaline-induced inhibition of noradrenergic neurotransmission in slices of the rabbit hypothalamus. Ordinate scale: fraction of the total tissue radioactivity released by a 2 min period of electrical stimulation (3 Hz, 2 ms, 26 mA) expressed as the ratio obtained between the second (S_2) and the first (S_1) periods of stimulation carried out within the same experiment. Cocaine (10 μM) and yohimbine (0.1 μM) or verapamil (3 μM) when used, were added to the superfusion medium 40 min before S_1 and were present throughout the experiment. Adrenaline (0.01 and 0.1 μM) was added to the medium 20 min before S_2. C = Control. The mean values of at least 3 experiments per group are shown; vertical lines indicate s.e.mean $^*P<0.05$; $^{**}P<0.01$ when compared to the corresponding control.

stimulation at 5 Hz in rabbit hypothalamic slices ($S_2/S_1 = 2.05 \pm 0.28$ ($n=4$), $P<0.01$ when compared with the controls in Table 1). In experiments in which cocaine (10 μM) was added 40 min before S_1 and remained present throughout the experiment, the ratio S_2/S_1 was 0.95 ± 0.04, $n=7$. Under these conditions, exposure to verapamil 3 μM added before S_2, significantly increased the overflow of tritium elicited by electrical stimulation ($S_2/S_1 = 2.14 \pm 0.19$, $n=4$, $P<0.001$ when compared to the corresponding control value). This increase of tritium overflow elicited by verapamil in the presence of cocaine was of the same order of magnitude as that observed in the absence of the inhibitor of neuronal uptake (Table 1).

Discussion

It has been demonstrated recently that vascular smooth muscle contains not only α_1- but also α_2-adrenoceptors, both adrenoceptors mediating contractile responses (for review see Langer & Shepperson 1982). An investigation into the effects of verapamil and diltiazem on the responses of vascular smooth muscle to selective α_1- and α_2-adrenoceptor agonists, has shown that responses to the α_2-adrenoceptor agonists are inhibited by both calcium antagonists, verapamil and diltiazem, whilst responses to α_1-adrenoceptor agonists are relatively resistant to these calcium antagonists (Van Meel, De Jonge, Wilffert, Kalkman, Timmermans & Van Zwieten, 1981; Cavero *et al.*, 1982). More recently (Hicks, 1982) it was reported that under *in vitro* conditions diltiazem in low concentrations antagonizes the α_2-adrenoceptor-mediated increase in phasic activity in the rat portal vein. We have therefore considered it of interest to investigate the effects of both calcium antagonists on the presynaptic α_2-adrenoceptors which modulate the electrically-evoked release of noradrenaline in the central nervous system.

Verapamil and diltiazem are known to block specifically the potential dependent translocation of calcium into cardiac and vascular smooth muscle cells via the so called 'slow calcium channels' (Nayler, 1980; Henry, 1980). Although calcium is known to be essential for the excitation-secretion coupling in nerve terminals there are different sources of in-

Table 3 Effect of verapamil and diltiazem on the inhibition by clonidine of the electrically evoked release of [^{3}H]-noradrenaline from rabbit hypothalamic slices

Experimental Group		*^{3}H-transmitter overflow [(a)] fractional release × 10^{-2}*			*Spontaneous outflow fractional release × 10^{-2}*			*Tissue*
	n	S_1	S_2	Ratio[(b)] S_2/S_1	Sp_1	Sp_2	Ratio[(c)] Sp_2/Sp_1	nCi/slice[(d)]
Verapamil 3 μM								
Control ($S_1 - S_2$)	4	1.66 ± 0.44	1.76 ± 0.49	1.07 ± 0.08	0.95 ± 0.06	0.69 ± 0.07	0.73 ± 0.05	48.4 ± 7.6
Clonidine 0.1 μM	4	1.19 ± 0.15	1.17 ± 0.09	1.01 ± 0.08	1.03 ± 0.07	0.78 ± 0.09	0.75 ± 0.03	50.4 ± 8.0
Diltiazem 10 μM								
Control ($S_1 - S_2$)	4	1.09 ± 0.20	1.06 ± 0.20	1.02 ± 0.15	0.98 ± 0.09	0.80 ± 0.08	0.82 ± 0.03	55.3 ± 11.9
Clonidine 0.1 μM	4	0.78 ± 0.23	0.47 ± 0.13	0.65 ± 0.07*	0.97 ± 0.04	0.76 ± 0.03	0.79 ± 0.02	58.5 ± 12.0

[(a)], [(b)], [(c)] and [(d)] as in Table 1.
Verapamil (3 μM) or diltiazem (10 μM) were added to the medium 40 min before S_1 and remained present throughout the experiment.
Clonidine (0.1 μM) was added to the medium 20 min before S_2.
Shown are mean values ± s.e.mean. n = number of experiments.
*$P < 0.05$ when compared to the corresponding control.

tracellular calcium. In addition the reports of the effect of calcium antagonists on transmitter release tend to differ. High concentrations of verapamil and methoxyverapamil (D600) were reported to inhibit the potassium-stimulated calcium influx into synaptosomes from rat brain (Nachshen & Blaustein, 1979; Ichida, Okada & Terao, 1980). On the other hand Haeusler (1972) reported that verapamil in concentrations up to 2 μM had no effect on the stimulation-evoked noradrenaline release from cat isolated hearts. In the isolated heart of the rabbit, Göthert, Nawroth & Neumeyer (1979) found that high concentrations of verapamil inhibited the stimulated release of noradrenaline with an IC_{50} of 73 μM. In the central nervous system, it was reported that exposure to 10 μM D600 inhibited the potassium-evoked release of noradrenaline from rat brain cortex slices and mouse forebrain synaptosomes (Vargas, de Lorenzo, Saldate & Orrego, 1977; Haycock, White & Cotman, 1978).

Under our experimental conditions, in slices of the rabbit hypothalamus, diltiazem in concentrations up to 100 μM had no significant effect on the electrically-evoked release of [^{3}H]-noradrenaline. The slight decrease in ^{3}H-transmitter overflow observed in the presence of 10 μM diltiazem did not reach statistical significance and at 100 μM, diltiazem produced a marked increase in the spontaneous outflow of tritium, which interfered with the determination of its effects on the stimulation-evoked release of [^{3}H]-noradrenaline. In contrast with the results obtained with diltiazem, exposure to verapamil, in concentrations between 1 and 10 μM increased in a concentration-dependent manner the electrically-evoked overflow of [^{3}H]-noradrenaline without affecting the spontaneous outflow of radioactivity. This result differs from the findings reported in peripheral sympathetic nerves of rabbit isolated hearts, in which verapamil inhibited the noradrenaline output evoked either by addition of $CaCl_2$ to calcium-free potassium-rich solutions, or by electrical stimulation of the postganglionic sympathetic nerves (Göthert *et al.*, 1979). An important difference between our results and those of Göthert *et al.* (1979) is the range of concentrations of verapamil that were studied. In the present study we explored the range of concentrations between 0.1 and 10 μM verapamil while Göthert *et al.* (1979) studied much higher concentrations.

In order to determine the mechanism by which verapamil increased the overflow of tritium from rabbit hypothalamic slices, the effect of the calcium antagonist on the inhibition of transmitter release by clonidine was investigated. In rabbit hypothalamic slices, clonidine inhibits ^{3}H-transmitter release through the stimulation of α_2-adrenoceptors, an effect which is antagonized by yohimbine (Galzin *et al.*, 1982). Under these experimental conditions, verapamil antagonized the inhibitory effects of clonidine on noradrenergic neurotransmission, while diltiazem failed to do so. Exposure to adrenaline in the presence of cocaine inhibited the stimulation-evoked overflow of tritium through the activation of presynaptic α_2-adrenoceptors as previously reported (Galzin *et al.*, 1982). Verapamil also antagonized the adrenaline-induced inhibition of ^{3}H-transmitter overflow. It should be noted that verapamil was approximately thirty times less potent than yohimbine in blocking the inhibitory effects of adrenaline on noradrenergic neurotransmission.

Since Göthert *et al.* (1979) carried out their experiments on noradrenaline release from the rabbit heart

in the presence of the α-adrenoceptor antagonist, phentolamine, they were unable to study the α_2-blocking properties of verapamil and only reported the inhibitory effects of rather high concentrations of this calcium antagonist.

In rat cerebral cortex slices, the inhibition of neuronal uptake by cocaine or desipramine antagonizes the inhibition of noradrenergic neurotransmission by α_2-adrenoceptor agonists of the imidazoline type (Pelayo *et al.*, 1980). In order to rule out such an interaction between verapamil and clonidine in rabbit hypothalamic slices, we studied the effect of verapamil in the presence of cocaine (10 μM) to inhibit neuronal uptake of noradrenaline. The increase in the stimulation-evoked release of tritium elicited by 3 μM verapamil in the presence of cocaine was of the same magnitude as that obtained in the absence of the uptake inhibitor, indicating that verapamil did not increase transmitter overflow by inhibiting neuronal uptake of noradrenaline.

Our results are consistent with the view that verapamil is an α_2-adrenoceptor antagonist: the increase in the stimulation-evoked release of [^{3}H]-noradrenaline resulted from the blockade by verapamil of the α_2-adrenoceptor mediated negative feedback mechanism that modulates noradrenergic neurotransmission (Langer, 1974; 1980). The antagonism by verapamil of the inhibitory effect of clonidine and adrenaline is consistent with the α_2-adrenoceptor blocking properties of this calcium antagonist. Although both verapamil and diltiazem inhibit the calcium-dependent contractile responses to postsynaptic α_2-adrenoceptor stimulation in the dog isolated saphenous vein and rat portal vein preparations (Langer & Shepperson, 1981; Hicks, 1982), diltiazem unlike verapamil does not block presynaptic, release-modulating α_2-adrenoceptors and does not affect central noradrenergic neurotransmission.

Our results suggest that differences may exist between the properties of potential-dependent slow calcium channels linked to the stimulation of presynaptic and postsynaptic α_2-adrenoceptors. At the level of vascular smooth muscle, stimulation of α_2-adrenoceptors is probably linked to a potential-dependent slow calcium channel and leads preferentially to an increase in intracellular Ca^{2+} which can be blocked by verapamil and diltiazem (Cavero *et al.*, 1982). On the other hand, the stimulation of presynaptic α_2-adrenoceptors modulating transmitter release in sympathetic neurones reduces the release of the neurotransmitter by inhibiting Ca^{2+} influx through a voltage-sensitive Ca^{2+} conductance mechanism (Horn & McAfee 1980). Our results suggest that the latter mechanism may be linked to a potential-dependent calcium channel which is different from the postsynaptic slow Ca^{2+} channel in vascular smooth muscle. Compatible with this view is the observation that diltiazem has no effect on noradrenergic neurotransmission in rabbit hypothalamic slices. On the other hand the α_2-blocking properties of verapamil explain the increase in transmitter release observed in the presence of this drug.

It is concluded that diltiazem is a calcium antagonist devoid of α_2-adrenoceptor blocking properties at the level of presynaptic release-modulating receptors. On the other hand, verapamil enhances noradrenergic neurotransmission by blocking the α_2-adrenoceptors that modulate transmitter release through a negative feed-back mechanism.

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