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The role of the endocardium in the facilitatory effect of bradykinin on electrically-induced release of noradrenaline in rat cardiac ventricle

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1 The present investigation was undertaken to study the role of bradykinin in noradrenaline release from the ventricle of the rat induced by electrical stimulation. Slices of the left ventricle of adult Wistar rats with or without endocardium were previously loaded with 0.2 μM [^{3}H]-noradrenaline and washed out before electrical stimulation was applied.

2 Bradykinin (0.1-100 nM) concentration-dependently increased tritium release evoked by electrical stimulation (EC_{50} = 3.5 (1.2-10.2) nM; n = 12). The angiotensin converting enzyme inhibitor, captopril (1 μM), which *per se* had no effect on tritium release, caused a marked enhancement of the bradykinin facilitatory effect, shifting the concentration-response curve of bradykinin to the left by about one log unit. The compound Hoe 140, a selective inhibitor of B_2-bradykinin receptors, competitively antagonized the effect of bradykinin, indicating the involvement of these receptors in the action of bradykinin.

3 In endocardium-free ventricle, bradykinin had no effect either in the absence or in the presence of captopril.

4 These results show that: (1) bradykinin is able to facilitate noradrenaline release evoked by electrical stimulation of the rat ventricle through activation of B_2-bradykinin receptors located on endocardial cells; (2) this action of bradykinin which is markedly potentiated by the inhibition of the angiotensin-converting enzyme seems to be exerted through the release of some factor which is formed in the endocardium and diffuses into the myocardium where it acts.

Keywords: Endocardium; bradykinin; noradrenaline release; rat ventricle; angiotensin-converting enzyme inhibitors; B_2-receptors

Introduction

In experiments carried out on strips of rabbit pulmonary artery and on the isolated perfused heart of the rabbit, bradykinin was shown to reduce the overflow of noradrenaline evoked by electrical stimulation (Starke *et al.*, 1977). More recently, indirect evidence was obtained supporting the view that the positive inotropic effect of bradykinin in rat atria and ventricle may be due to prejunctional facilitation of noradrenaline release; this effect was enhanced by ramiprilat, an angiotensin-converting enzyme inhibitor (Minshall *et al.*, 1994). In human kidney cortex slices, Rump *et al.* (1995) showed that bradykinin activates prejunctional B_2-subtype receptors to enhance noradrenaline release. However, this effect was demonstrable only in the presence of captopril, an inhibitor of bradykinin degradation.

On the other hand, after Furchgott & Zawadzki (1980) had reported that the presence of the endothelium was required for acetylcholine to evoke relaxation in isolated rings of aorta, many biologically active substances were described which are produced by the endothelium and play important roles in the regulation of vascular smooth muscle tone (Brutsaert & Andries, 1992; Henderson *et al.*, 1992). Since it is obvious that endocardial endothelium may influence myocardial function in a manner analogous to endothelial regulation of vascular function, the present study was undertaken to look at: (1) the role of bradykinin in noradrenaline release evoked by electrical stimulation from the ventricle of the rat, and (2) the influence of the endocardium on the role of bradykinin.

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Methods

Male, normotensive Wistar rats weighing 200-250 g (Biotério do Instituto Gulbenkian de Ciências, Oeiras, Portugal) were kept on 12 h light/dark cycle and given a standard laboratory chow and water *ad libitum*. After overnight fasting the animals were anaesthetized with pentobarbitone sodium (50 mg kg^{-1}, i.p.) and the hearts were removed and immediately placed in warmed, aerated (95% O_2 + 5% CO_2) modified Krebs-Henseleit solution (Guimarães & Osswald, 1969) of the following composition (mM): NaCl 118.6, KCl 4.70, $CaCl_2$ 2.52, KH_2PO_4 1.18, $MgSO_4$ 1.23, $NaHCO_3$ 25.0; glucose 10.0. To avoid autoxidation of [^{3}H]-noradrenaline, EDTA (0.027 mM) and ascorbic acid (0.57 mM) were added to the medium.

Six slices (across the entire wall) of about 50 mg were taken from the left ventricle wall and preincubated for 30 min in 5 ml of medium containing 1 mM pargyline (to inhibit monoamine oxidase) and 40 μM U-0521 (3,4-dihydroxy-2-methylpropiophenone) to inhibit extraneuronal removal (Guimarães *et al.*, 1978). Since pargyline irreversibly blocks MAO, only U-0521 was kept in the medium for the remainder of the experiment. After preincubation, the slices were exposed for 60 min to [^{3}H]-noradrenaline (0.2 μM). Thereafter they were mounted in a perifusion chamber and perifused with amine-free medium (aerated and at 37°C) for 110 min, at a flow rate of 0.8 ml min^{-1}. From t = 110 min (t = 0 min being the start of the perifusion) the perifusion fluid was collected continuously in samples of 5 min. Transmural electrical stimulation (1 Hz, 2 ms, 100 V, for 5 min; Stimulator II X, Hugo Sachs Elektronik, March-Hugstetten, Germany) was applied at min 120 (S_1), 160 (S_2) and 200 (S_3). In addition to U-0521, which was present throughout the perifusion, cocaine (12 μM) was also present in the perifusion fluid from min 90 onwards.

The prejunctional effect of bradykinin was determined by the change in tritium overflow evoked by electrical stimulation.

The first period of stimulation was rejected, the second stimulation was taken as control. Drugs under study were added to the perifusion fluid 20 min before S_3. $EC_{50\%}$ represents the concentration of the drug increasing the evoked overflow by 50%.

The outflow of tritium was calculated as a fraction of the amount of tritium in the tissue at the start of the respective collection period (fractional rate of loss min^{-1}).

For the calculation of the overflow induced by the electrical stimulation those 5 min samples were taken into account in which the overflow of tritium exceeded that in the last pre-stimulation control sample (usually this applied to the 3 or 4 samples collected during and after stimulation). The spontaneous outflow measured in the last pre-stimulation sample was assumed to represent the spontaneous outflow in subsequent samples; it was subtracted from the overflow determined in stimulation and post-stimulation samples. The 'total overflow of transmitter' was the sum of all increases (induced by a period of stimulation) above the spontaneous level of outflow of tritium.

Drug effects are expressed as the ratio of tritium overflow evoked by S_3 over that evoked by S_2. In order to detect whether the release of tritium by electrical stimulation remained constant from S_2 to S_3, control experiments were performed in the absence of drugs. As the ratio S_3/S_2 in control experiments remained constantly close to unity, no corrections were introduced.

Removal of the endocardium

The technique described by Brutsaert *et al.* (1988) was used in one group of hearts in which the endocardium was damaged by a quick contact (1 s) with a 1% Triton X-solution injected into the ventricular chamber immediately followed by abundant wash with Krebs-Henseleit solution. In a second group of hearts the endocardium was removed surgically. This procedure was easily carried out because, in this species, there is a clear-cut separation between the endocardium and the myocardium.

Determination of tritium in the ventricle wall and in the overflow

After the experiment, the tissues were kept overnight in 3 ml of 0.2 M perchloric acid at 4°C.

Radioactivity was measured by liquid scintillation counting (liquid scintillation counter 1209 Rackbeta, LKB Wallac, Turku, Finland) from 2 ml aliquots (or 0.5 ml of the acid extract of the tissue + 1.5 ml of Krebs-Henseleit solution), after addition of 8 ml of scintillation mixture (OptiPhase 'HiSafe' 3, LKB, Loughborough, Leics, England).

Assay of total noradrenaline in the ventricle wall

Total noradrenaline content of the ventricle wall was measured by h.p.l.c. with electrochemical detection. Aliquots of the acid extract (see above) were directly injected in an h.p.l.c. system with 5 μm C18 reverse-phase column. The mobile phase consisted of 0.1 M sodium acetate, 0.1 M citric acid, 0.5 mM sodium octyl sulphate, 0.15 mM EDTA, 1 mM dibutylamine and 10% methanol (v/v), pH 3.7 and was pumped at a flow-rate of 1 ml min^{-1}. Endogenous noradrenaline represented the difference between total noradrenaline, determined by h.p.l.c. and [^{3}H]-noradrenaline, determined by scintillation counting.

Antagonism of bradykinin effects

To study the antagonism by the compounds Hoe 140 and (Des-Arg^{10}) Hoe 140, pA_2 values were calculated according to the method of Van Rossum (1963) from the equation: $pA_2 = pA_x + \log(x-1)$ in which x represents the factor of the shift of the concentration-response curve to the right and pA_x the negative logarithm of the molar concentration of the antagonist which caused this shift.

Statistics

The results are presented as arithmetic means ± s.e.mean or as geometric means with 95% confidence limits. One-way analysis of variance was used to test differences between unpaired results. Paired experiments were analysed by ANOVA for repeated measures. Multiple comparisons were corrected by Newman Keuls procedure. P values of 0.05 or less were considered significant.

Drugs

The following were used: bradykinin (Sigma, St.Louis, MO, U.S.A.); captopril (Sigma); cocaine hydrochloride (Uquipa, Lisboa, Portugal); (Des-Arg^{10}) Hoe 140 trifluoroacetate (RBI, Natick, MA, U.S.A.); Hoe 140 (D-Arg-(Hyp^3, Thi^5, D-Tic^7, Oic^8) bradykinin trifluoroacetate) (RBI); indomethacin (Sigma); N^G-nitro-L-arginine methyl ester hydrochloride (L-NAME) (Sigma); [^{3}H-7]-(−)-noradrenaline (11.4 Ci $mmol^{-1}$, New England Nuclear, Dreieich, Germany); pargyline hydrochloride (Sigma); U-0521 (3′-4′-dihydroxy-2-methylpropiophenone) (Upjohn, Kalamazoo, U.S.A.). The stock solution for bradykinin was prepared in dimethylsulphoxide (DMSO) while that for indomethacin was prepared in equimolar sodium carbonate. Final concentrations of the solvents (DMSO or sodium carbonate) had no effect of their own. All the other stock solutions were prepared in distilled water. Final dilutions of drugs were made with Krebs-Henseleit solution immediately before use.

Results

Intact tissue

After preloading of the tissue with [^{3}H]-noradrenaline and 120 min of washout, the total noradrenaline content of the tissue was 4.21 (3.53;5.01) nmol g^{-1} ($n=8$) and [^{3}H]-noradrenaline amounted to 580 (529; 636) pmol g^{-1} ($n=64$) (Table 1). Thus, before S_1, [^{3}H]-noradrenaline represented about 13% of the total noradrenaline content of the tissue. In the first series of experiments, the tissues were stimulated electrically three times, each stimulation period (S_1 to S_3) consisting of a train of 300 pulses (1 Hz). In the absence of drugs, the overflow evoked by S_1 amounted to 0.74 (0.66;0.82)% of the tritium content of the tissue (Table 1). The evoked overflow remained approximately constant from S_1 to S_3 in control experiments without any drug.

Bradykinin (0.1 − 100 nM), which was present in the bath from 20 min before S_3 without changing the basal outflow of tritium, caused a concentration-dependent enhancement of tritium release evoked by electrical stimulation. Captopril (1 μM) an inhibitor of the angiotensin-converting enzyme, which *per se* did not change either the spontaneous outflow or the overflow evoked by electrical stimulation, caused a very pronounced potentiation of the bradykinin enhancing effect, shifting the concentration response curve to bradykinin to the left by about one log unit (Figure 1a).

N^G-nitro-L-arginine methyl ester (L-NAME; 300 μM) or indomethacin (10 μM) did not change the effect of bradykinin (results not shown).

Endocardium-free tissue

Two different methods were used to eliminate the endocardium: surgical removal and treatment with Triton X-100 (see Methods).

In ventricle wall cleared of endocardium by Triton X-100 treatment, the noradrenaline content of the tissue after preloading with [^{3}H]-noradrenaline and 120 min of washout [4.04 (3.28;4.98) nmol g^{-1}] ($n=4$) was not significantly different

Table 1 Rat left ventricle slices; influence of endocardium on [^{3}H]-noradrenaline tissue content and overflow elicited by electrical stimulation (1 Hz, 300 pulses) of tissues preloaded with 0.2 μM of the tritiated amine

	n	*Tissue accumulation* (*pmol g^{-1}*)	*Basal efflux* (*fmol g^{-1} min^{-1}*)	(*fractional rate of loss, min^{-1}*)	*Overflow induced by electrical stimulation* (*fmol g^{-1} per pulse*)	(*fractonal release per pulse*)
Endocardium-intact	64	580 (529;636)	457 (383;546)	7.88×10^{-4} (6.78×10^{-4};9.17×10^{-4})	14.2 (12.4;16.2)	2.45×10^{-5} (2.20×10^{-5};2.73×10^{-5})
Endocardium-free (by surgery)	19	675 (583;783)	665 (525;843)	9.85×10^{-4} (8.36×10^{-4};11.6×10^{-4})	15.0 (10.2;22.1)	2.22×10^{-5} (1.63×10^{-5};3.04×10^{-5})
Endocardium-free (by Triton X-100)	23	487 (421;550)	380 (322;448)	7.80×10^{-4} (6.73×10^{-4};9.03×10^{-4})	11.4 (9.9;13.0)	2.95×10^{-5} (2.52×10^{-5};3.42×10^{-5})

Values are geometric means and 95% confidence limits of n experiments.

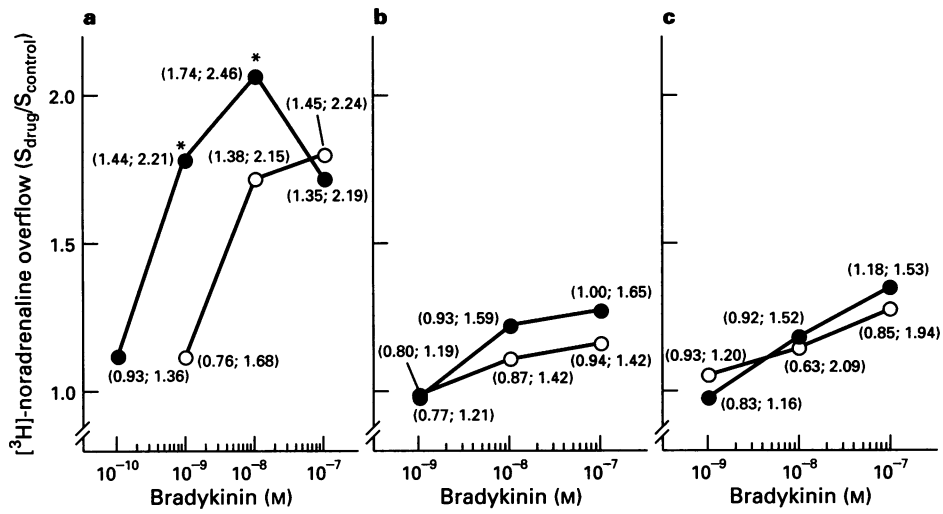

Figure 1 Effect of bradykinin on the overflow of [^{3}H]-noradrenaline induced by electrical stimulation (1 Hz, 300 pulses) of rat left ventricle slices preloaded with 0.2 μM of the tritiated amine. (a) Endocardium-intact ventricle slices; (b) ventricle slices in which endocardium was removed by Triton X-100; (c) ventricle slices in which endocardium was removed by surgery. Experiments in which bradykinin was given alone (○); experiments in which medium contained captopril (1 μM) (●). Results are expressed as the ratios of [^{3}H]-noradrenaline overflow in the presence of bradykinin (S_{drug}) to that in its absence ($S_{control}$). Values are geometric means and 95% confidence limits for 4–6 experiments. *Significantly different from the corresponding values obtained in experiments in which bradykinin was given alone.

from that of intact ventricle wall under the same conditions. In the absence of drugs, the fractional rate of tritium outflow as well as the overflow evoked by electrical stimulation were not significantly different from those obtained in intact tissue (Table 1) and remained generally unchanged from S_1 to S_3. The effect of bradykinin on the electrically-evoked overflow of tritium, in the absence and in the presence of captopril, was markedly reduced or even abolished (Figure 1).

In ventricles surgically cleared of endocardium, noradrenaline content was 4.25 (3.49;5.16) nmol g^{-1} (n=4) after preloading with [^{3}H]-noradrenaline and 120 min washout, a value which was also not significantly different from that of the intact ventricle. As for the ventricle cleared of endocardium by treatment with Triton X-100, in surgically denuded ventricle both the spontaneous outflow and the overflow of tritium evoked by electrical stimulation were not significantly different from the controls (Table 1).

Bradykinin, captopril or the combination of bradykinin and captopril did not change the spontaneous release of tritium. Furthermore, the effect of bradykinin alone and, of the combination of bradykinin with captopril on the electrically-evoked overflow of tritium was markedly reduced or abolished in endocardium-free tissue (Figure 1).

Influence of the compounds Hoe 140 and (Des-Arg^{10}) Hoe 140

The compound Hoe 140 (10–100 nM), a selective B_2-bradykinin receptor antagonist (Dendorfer & Dominiak, 1995), caused a marked displacement to the right of the concentration-response curve to bradykinin ($pA_2=9.56\pm0.40$; $n=8$), while the compound (Des-Arg^{10}) Hoe 140, a B_1-bradykinin receptor antagonist, at a concentration up to 100 nM had no effect (Figure 2).

Discussion

In this study it was shown that bradykinin facilitated the release of noradrenaline evoked by electrical stimulation from the rat ventricle wall and that this facilitation was very markedly enhanced by captopril. The increase in tritium overflow caused by bradykinin which was by about 100% was much more pronounced than that caused by β-adrenoceptor-mediated stimulation (Osswald & Guimarães, 1983; Guimarães *et al.*, 1995) or than that resulting from the stimulation of any other kind of prejunctional receptors (Starke, 1977; Dohi *et al.*, 1991). Most interestingly, it was shown that both facilitation of noradrenaline release and its enhancement by captopril were markedly reduced or abolished by removal of the endocardium.

In the first study dealing with the influence of bradykinin on noradrenaline release elicited by electrical stimulation of rabbit pulmonary arteries and rabbit hearts it was shown that bradykinin reduced noradrenaline release (Starke *et al.*, 1977). In this first report evidence was presented showing that bradykinin promoted the biosynthesis of prostaglandin E_1 (PGE_1) or PGE_2 and that these prostaglandins mediated bradykinin-

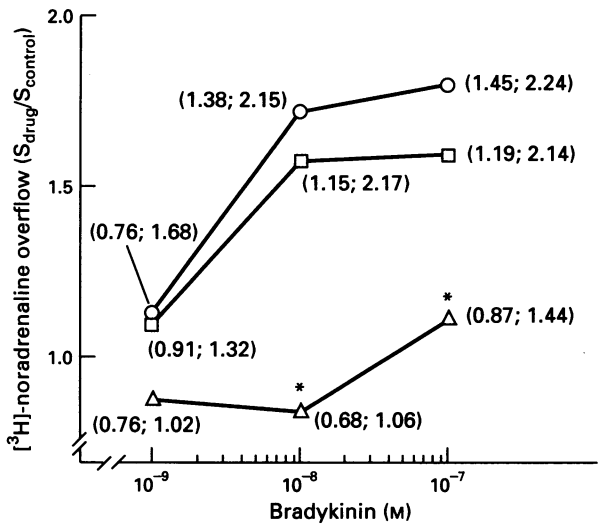

Figure 2 Effect of bradykinin on the overflow of [^{3}H]-noradrenaline induced by electrical stimulation of endothelium-intact rat left ventricle slices preloaded with the tritiated amine (details as in legend to Figure 1). Experiments in which bradykinin was used alone (○); experiments in which bradykinin was used in the presence of Hoe 140 (100 nM) (△); experiments in which bradykinin was used in the presence of (Des-Arg^{10}) Hoe 140 (100 nM) (□). Results are expressed as the ratios of [^{3}H]-noradrenaline overflow in the presence of bradykinin (S_{drug}) to that in its absence ($S_{control}$). Values are geometric means and 95% confidence limits for 4–6 experiments. *Significantly different from the corresponding values obtained in experiments in which bradykinin was given alone.

evoked reduction of noradrenaline release. However, in other tissues, it has been shown that bradykinin enhances noradrenaline release evoked by electrical stimulation. In rat atria and ventricle Minshall *et al.* (1994) presented indirect evidence that the positive inotropic effect of bradykinin, which was more pronounced in the presence of the angiotensin-converting enzyme inhibitor, ramiprilat, might be due to prejunctional facilitation of sympathetic neurotransmitter release. Furthermore, in human kidney cortex slices, Rump *et al.* (1995) showed that bradykinin facilitates noradrenaline release but only after inhibition of the angiontensin-converting enzyme and that this effect was abolished by the compound Hoe 140, a selective bradykinin B_2-receptor antagonist.

Bradykinin was first shown to affect ganglionic sympathetic transmission via receptors distinct from those for nicotine, histamine, muscarine and 5-hydroxytryptamine by Lewis & Reit (1965) and Trendelenburg (1966). Later on it was observed that the majority of the tissues respond to bradykinin through the activation of at least two types of specific receptors denoted B_1 and B_2 (review by Regoli & Barabé, 1980). The present data show that bradykinin-induced facilitation of noradrenaline release from the noradrenergic varicosities is apparently mediated by B_2-bradykinin receptors since it was antagonized by the selective B_2-bradykinin receptor antagonist, Hoe 140 (Dendorfer & Dominiak, 1995). However, this effect does not appear to depend on a direct action of bradykinin on those varicosities, since it disappeared or was markedly reduced when the endocardium was eliminated. This elimination of the effect of bradykinin by removal of the endocardium indicates that it is mediated by some factor which is released from the endocardium by bradykinin and then diffuses into the myocardium where it acts on noradrenergic terminals.

Embryological, anatomical and functional data suggest that the endocardium may influence myocardial contractility in a manner analogous to endothelial regulation of vascular smooth muscle tone (for a review see Brutsaert & Andries, 1992). It is well documented that vascular endothelium modulates vessel tone through the release of several agents namely nitric oxide (NO) (Furchgott & Zawadzki, 1980; Palmer *et al.*, 1987), prostacyclin (Dusting *et al.*, 1977), endothelin (Yanagisawa *et al.*, 1988), ATP and its degradation product adenosine (Sedaa *et al.*, 1990; Shinozuka *et al.*, 1991; Takeuchi *et al.*, 1994; Vaz-da-Silva *et al.*, 1995). An accumulating body of evidence indicates that the endocardium may also play an important role in cardiac regulation, through the release of several diffusible substances (Brutsaert & Andries, 1992; Hendersen *et al.*, 1992). The first report of an endocardial-mediated influence on myocardial contraction was published by Brutsaert *et al.* (1988). These authors suggested that some endocardial released factor would induce premature relaxation by decreasing the sensitivity of the contractile proteins to calcium. Furthermore, they speculated that a second factor released by the endocardium might increase the sensitivity of the contractile proteins to calcium and sugested that this factor might be ATP, because ATP was the only substance that in the absence of a functional endocardium, delayed the onset of isometric tension decline of relaxation, thereby reversing the abbreviation elicited by removal of a functional endocardium. There is now experimental evidence showing that at least two substances are released from the endocardium which modulate myocardium contraction: NO which is short-acting and labile and shortens twitch contraction (Smith *et al.*, 1991; Shah *et al.*, 1991) and an unknown factor, named 'endocardin' (Smith *et al.*, 1991) which is stable and prolongs twitch duration (Ramaciotti *et al.*, 1992). NO does not seem to be the hypothetical candidate responsible for the indirect facilitatory effect of bradykinin observed here. If NO were involved in this facilitatory role of the endocardium, L-NAME, an inhibitor of NO synthesis should have reduced the bradykinin effect and this was not observed. In fact, in concentrations up to 300 μM which were shown to be fully effective in inhibiting NO synthesis (Palmer *et al.*, 1987), L-NAME did not change the transmitter release. Furthermore, a prostanoid seems not to be a very likely candidate since indomethacin did not change the facilitatory role of bradykinin as it should do if a prostanoid were involved in this facilitatory effect. Recently, Chulak *et al.* (1995) reported that diclofenac (1 μM) a cyclo-oxygenase inhibitor also did not change the facilitatory effect of bradykinin.

Endothelin, which is another substance produced by endocardial cells, has been shown to exert direct chrono- and inotropic effects in guinea-pig and rat hearts (Ishikawa *et al.*, 1988; Baydoun *et al.*, 1989). However no evidence has been reported in favour of any facilitatory effect of endothelin on electrically-evoked noradrenaline release.

Furthermore, in certain blood vessels, some derivatives of arachidonic acid, such as hydroxyeicosatetraenoic acid, which are produced by the endothelium are able to cause vasoconstriction by blocking potassium channels (Roman & Harder, 1993). However, again, no facilitatory effect of these products on noradrenaline release evoked by electrical stimulation, has been reported for the cardiac muscle.

In the present study the elimination of the endocardium was brought about by two different techniques which caused virtually identical results indicating that the alterations observed were really due to the absence of the endocardium and not to some kind of myocardial injury caused by the technique used to eliminate the endocardium.

Coronary vascular endothelium, which at the microvascular level is in close proximity to a large amount of myocardial cells, may also influence myocardial contraction by releasing diffusible factors. However, in our experiments, the removal of the endocardium practically eliminated not only the role of bradykinin but also the potentiation of its effect by inhibition of the angiotensin-converting enzyme. Some minor effect of bradykinin remaining after the elimination of the endocardium may be due to a residual influence of the coronary vascular endothelium.

In conclusion, it was shown that bradykinin is able to facilitate noradrenaline release from the sympathetic nerve endings through the activation of B_2 receptors, located in endocardial cells. Additionally, evidence is presented indicating

that the facilitatory effect of bradykinin is an indirect one because it is due to some substance which is formed in the endocardium and diffuses into the myocardium to act on the noradrenergic varicosities.

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