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Role of Sodium-Calcium Exchange and Effects of Calcium Entry Blockers on Endothelial-Mediated Responses in Rat Isolated Aorta

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SUMMARY

Acetylcholine relaxes rat aorta and increases aortic cyclic GMP levels by a mechanism (or mechanisms) dependent on the endothelium and on extracellular calcium. Therefore, the effects of representatives of different subclasses of calcium entry blockers, verapamil, nifedipine, diltiazem, and bepridil, on maximal acetylcholine (1 μM)-induced increases in cyclic GMP levels were investigated in rat isolated aorta. None of these compounds, at a concentration (3 μM) sufficient to maximally inhibit agonist-stimulated Ca^{2+} influx into vascular smooth muscle cells, significantly affected either the basal or the acetylcholine-stimulated tissue cyclic GMP levels. On replacing all but 20 mM Na^+ by choline, a condition that might be expected to limit or even abolish Na^+-Ca^{2+} exchange, or in the presence of amiloride (1 mM), an inhibitor of Na^+-Ca^{2+} exchange, acetylcholine-stimulated increases in tissue cyclic GMP levels were abolished or inhibited by about 80%, respectively. In choline-containing solution acetylcholine relaxant responses were abolished. The presence of amiloride, or the replacement of Na^+ by choline, had no effect on increases in cyclic GMP levels evoked by sodium nitroprusside (0.3 μM), an agent that stimulates cyclic GMP formation in smooth muscle without intervention of the endothelium. Replacement of Na^+ by Li^+ but not the other treatments depressed basal tissue cyclic GMP levels by about 45% but did not abolish either acetylcholine- or sodium nitroprusside-induced relaxant responses. However, the time course of relaxant responses elicited by both these relaxant agonists in precontracted rat aortic rings with endothelium was altered by Li^+ replacement; the half-time to relaxation to acetylcholine was increased by about 70-fold. It is concluded that calcium channels, as characterized in smooth muscle and cardiac tissue, are not involved in the stimulated liberation of an endothelial-derived relaxant factor by acetylcholine, but that an Na^+-Ca^{2+} exchange process may be of importance.

Compounds such as acetylcholine and histamine relax precontracted vascular smooth muscle only if the endothelium is present (1, 2). Other compounds, such as ATP (3), that also relax precontracted vascular preparations have been shown to be partially dependent on the presence of endothelium. It has been convincingly demonstrated that this endothelium-dependent relaxant effect is mediated by a factor liberated by the endothelium (EDRF) in response to stimulation of agonist-specific receptors (1, 4, 5). The relaxant effect dependent on the endothelium is associated with an increase in tissue levels of cyclic GMP (6–8).

The dependence of this secretory response of the endothelium on extracellular calcium has been shown by both relaxation experiments in rabbit and rat aorta (9, 10) and cyclic GMP measurements in rat aorta and bovine coronary artery (11, 12). The calcium entry blockers tested (variously, nifedipine, verapamil, and flunarizine) were not notably effective as inhibitors of the endothelium-mediated responses although, in the rabbit aorta, nifedipine and verapamil in high concentrations partially inhibited acetylcholine-induced relaxations (9). It has been suggested that an Na^+-Ca^{2+} exchange mechanism may, however, be involved (10), and there is some data in support of this idea (10, 13).

The modulatory effects of endothelium on contractile responses in rat aorta have been extensively studied (8, 11, 14–17), and it has been suggested that endothelium-derived factor(s) may have effects on the mobilization of both extracellular (18, 19) and intracellular (19) calcium for contraction in vascular smooth muscle. Therefore, it is not only important to understand the differences between endothelial and smooth muscle cells in their membranal handling of calcium, but also it is useful to develop pharmacological tools capable of modulating calcium metabolism in each cell type separately.

The purpose of the present work was to investigate the effects of changes in extracellular sodium concentration and of calcium entry blockers of different types (20, 21) on endothelium-mediated responses in rat isolated aorta, subsequent to stimu-

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ABBREVIATION: EDRF, endothelium-derived relaxing factor.

lation by acetylcholine. The results show that none of the calcium entry blockers used affected the release of EDRF, as measured by the acetylcholine-induced increase in cyclic GMP levels, in this tissue. Amiloride and replacement of external sodium by choline inhibited this response. It is concluded that functional calcium channels are probably not involved in activation of endothelial cells by acetylcholine but that an Na^+-Ca^{2+} exchange process may be of importance.

Materials and Methods

Determination of cyclic GMP levels. For each experiment, thoracic aorta were removed from four female Wistar rats aged 12–14 weeks (280–300 g) as previously described (14), care being taken to avoid damage to the endothelium. Each aorta was divided into four segments of approximately equal length, and experimental groups comprised an aortic segment taken from a different level of each rat. The aortic segments, not subject to tension, were incubated for a period of 120 min (during which time the incubation medium was periodically changed) in a physiological solution (composition in mM; NaCl, 112; KCl, 5; $NaHCO_3$, 25; KH_2PO_4, 1; $MgSO_4$, 1.2; $CaCl_2$, 1.25; and glucose 11.5), equilibrated to pH 7.4 by aeration with a mixture of 95% O_2 and 5% CO_2. Calcium entry blockers were left in contact for the last 90 min at a final concentration of 3 μM. In the case of nifedipine, nicergoline, and reserpine, care was taken to shield the incubating solutions from light. Amiloride was added for the last 30 min. When LiCl or choline chloride was substituted for NaCl, the final composition of the physiological solution was (in mM): LiCl (or choline chloride), 117; $NaHCO_3$, 20; $KHCO_3$, 5; KH_2PO_4, 1; $MgSO_4$, 1.2; $CaCl_2$, 1.25; and glucose, 11.5, so that the Na^+ concentration of the bath solution was 20 mM for the last 90 min.

Acetylcholine (1 μM) was then added 1 min before the aortic rings were quickly frozen using aluminum tongs precooled in liquid nitrogen. The peak tissue levels of cyclic GMP stimulated by acetylcholine are achieved after 1 min in these experimental conditons. In some experiments a 2-min stimulation with sodium nitroprusside (0.3 μM) was substituted for the acetylcholine stimulation. Tissues were thawed by adding 1 N $HClO_4$ and were homogenized with a Potter glass/glass homogenizer. Cyclic GMP levels were determined using the radioimmunological methods of Cailla *et al.* (22), including a succinylation step. The DNA content was assayed for reference by an adapted fluorometric method (23).

Contractile experiments. Segments of aorta 2 mm wide with or without endothelium were suspended under 2 g tension in 10-ml organ baths as previously described (14). After a 90-min equilibration period an initial contraction was elicited with 1 μM norepinephrine. Thirty min later, when contractions had stabilized, acetylcholine or sodium nitroprusside was added to the bath to reach a final concentration of 1 μM or 0.3 μM, respectively. The relaxation responses were followed until equilibrium was reached. After washing several times over 30 min, tissues were incubated for 90 min in either normal physiological solution or in Li^+-containing solution before the norepinephrine contraction and acetylcholine relaxation were repeated. In some experiments a 60-min incubation in normal physiological solution followed by a 30-min exposure to a choline-containing solution was used in place of the preincubation with Li^+.

Materials. Diltiazem HCl, verapamil HCl, nifedipine, bepridil HCl, nicergoline, and amiloride HCl were generously donated by LERS-Synthelabo (France), Knoll Biosedra (FRG), Bayer (FRG), CERM (France), Specia (France), and Merck, Sharp and Dohme (France), respectively. Standard cyclic GMP, DNA, acetylcholine chloride, and choline chloride were obtained from Sigma (USA), reserpine was from EGA-Chemie (France), and sodium nitroprusside was from Merck (France). Specific anti-cyclic GMP antibodies were obtained from Immunotech, Marseille-Luminy (France). The labeled antigen was prepared by iodination of the monosuccinyl-cyclic GMP-tyrosyl-methylester (Sigma). Acetylcholine and hydrochloride salts were dissolved in water or physiological solution. Stock solutions of nifedipine, nicergoline, and reserpine were prepared, respectively, in ethanol, 1 N HCl, and glacial acetic acid. Approximately, 3000-fold dilutions of these solutions were made in physiological solution to arrive at the final bath concentration. In all experiments appropriate concentrations of solvents were incorporated in the physiological solution used to incubate control tissues.

Statistical analysis. Data are expressed as means ± standard error. Tests of significance have been made using Student's t test or the paired t test. The p values less than 0.05 were considered to be significant.

Results

Cyclic GMP measurements. The mean basal level of cyclic GMP in rat isolated aorta, measured in the presence of the vehicles used to solubilize the various antagonists studied in these experiments, was 32.5 ± 2.2 fmol/μg of DNA (n = 54). Acetylcholine (1 μM, 1 min), under the same conditions, stimulated a mean increase in cyclic GMP levels of about 17-fold to 550 ± 33 fmol/μg of DNA (n = 53).

None of the generally recognized calcium entry blockers tested—diltiazem, verapamil, nifedipine, and bepridil (20, 21, 24, 25)—at a concentration of 3 μM, significantly affected the basal or acetylcholine-stimulated increase in tissue cyclic GMP levels (Table 1). Two other compounds, reportedly displaying calcium entry-blocking activity, reserpine (26) and nicergoline (27), were also devoid of any significant effect either on basal or stimulated tissue cyclic GMP levels when tested at the same concentration.

Amiloride, 1 mM, an antagonist of Na-Ca exchange (28, 29), significantly ($p < 0.001$) depressed the acetylcholine-mediated stimulation of tissue cyclic GMP levels by about 75%. A 10-fold lower concentration of amiloride did not significantly inhibit the stimulatory effect of acetylcholine (Table 2). Neither of these concentrations of amiloride affected the basal tissue levels of cyclic GMP. Replacement of all but 20 mM Na^+ by Li^+ reduced the acetylcholine-stimulated increase in tissue cyclic GMP by about 80% when measured after 1 min of contact and reduced the basal cyclic GMP level by about 50% (Table 2). A similar replacement of Na^+ by choline abolished the

TABLE 1

Effect of various calcium entry blockers on basal and acetylcholine-stimulated increases in cyclic GMP levels of rat aorta

Tissues complete with endothelium were stimulated by acetylcholine (1 μM) for 1 min. The effects of various calcium entry blockers (3 μM) were tested after a 90-min preincubation period. All values are the mean ± SE of the mean of four to eight observations.

	cGMP content (fmol/μg of DNA)	
	Basal	Acetylcholine (1 μM)
Control	54.3 ± 10.8	464 ± 66
Verapamil	48.2 ± 8.4	488 ± 53
Control	51.5 ± 10.9	617 ± 140
Nifedipine	31.9 ± 5.6	400 ± 70
Control	26.3 ± 5.7	724 ± 183
Diltiazem	21.7 ± 2.5	524 ± 42
Control	29.9 ± 5.8	582 ± 64
Bepridil	26.7 ± 4.9	442 ± 77
Control	28.9 ± 4.7	394 ± 57
Reserpine	27.8 ± 5.7	374 ± 50
Control	21.5 ± 3.4	302 ± 110
Nicergoline	21.4 ± 2.5	242 ± 65

TABLE 2

Effect of amiloride and of replacement of Na^+ by Li^+ or choline on basal and acetylcholine-stimulated increases in cyclic GMP levels of rat aorta

Tissues complete with endothelium were stimulated by acetylcholine (1 μM) for 1 min. Tissues were preincubated for 30 min with amiloride and for 90 min with Li^+ or choline replacement of all except 20 mM Na^+ in the bathing solution. All values are the means ± SE of four to eight observations.

	cGMP content (fmol/μg of DNA)	
	Basal	Acetylcholine (1 μM)
Control	22.8 ± 2.8	575 ± 63
Amiloride, 1 mM	20.1 ± 1.4	143[a] ± 35
Control	35.7 ± 4.4	547 ± 88
Amiloride, 0.1 mM	37.8 ± 3.7	499 ± 116
Control	20.9 ± 1.3	650 ± 56
Li^+	10.8[a] ± 0.8	48.6[a] ± 7.7
Control	34.4 ± 3.2	702 ± 135
Choline	35.9 ± 5.3	38.8[a] ± 5.6

[a] Significantly different ($p < 0.001$) from respective control values.

TABLE 3

Effect of amiloride and of replacement of Na^+ by Li^+ or choline on basal and sodium nitroprusside (SNP)-stimulated increases in cyclic GMP levels of rat aorta

Tissues complete with endothelium were stimulated by SNP for 2 min. Tissues were preincubated for 30 min with amiloride (1 mM) and for 90 min with Li^+ or choline replacement of all except 20 mM Na^+ in the bathing solution. All values are the means ± SE of the mean of four observations.

	cGMP content (fmol/μg of DNA)	
	Basal	SNP (0.3 μM)
Control	20.3 ± 3.7	442 ± 120
Li^+	11.7 ± 1.0	58[a] ± 7.4
Control	35.6 ± 5.1	588 ± 120
Choline	38.0 ± 5.2	490 ± 38
Control	33.3 ± 2.5	722 ± 230
Amiloride	30.3 ± 4.8	659 ± 132

[a] Significantly different ($p < 0.005$) from respective control values.

acetylcholine-stimulated increase in cyclic GMP but had no effect on basal levels (Table 2).

Replacement of Na^+ by Li^+ or choline could conceivably inhibit smooth muscle guanylate cyclase activity by another mechanism not related to an effect on endothelial cells, as could amiloride. Therefore, the stimulatory effect of sodium nitroprusside (0.3 μM), an agent known to stimulate guanylate cyclase by a mechanism independent of endothelium (8), on tissue levels of cyclic GMP was tested in the presence of Li^+, choline, and amiloride. This concentration of sodium nitroprusside was chosen because it increased tissue cyclic GMP levels to about the same extent as did 1 μM acetylcholine. That is, from 29.8 ± 2.9 to 584 ± 93 fmol/μg of DNA ($n = 12$), about 20-fold. Neither amiloride nor the replacement of Na^+ by choline had any significant effect on either the basal or sodium nitroprusside-stimulated increases in tissue cyclic GMP levels (Table 3). However, as well as reducing basal tissue levels of cyclic GMP by about 45% (Tables 2 and 3), replacing Na^+ by Li^+ also inhibited the effect of sodium nitroprusside stimulation by about 75% (Table 3).

Contractile experiments. Replacement of Na^+ and Li^+ inhibited norepinephine-induced contractions by about 20% (mean contraction 1.3 ± 0.1 g, $n = 11$), but did not significantly alter the maximal relaxation of these contractions elicited by 1 μM acetylcholine (Table 4). However, the time course of relaxation of these contractions elicited by acetylcholine (1 μM) was

TABLE 4

Effect of replacing all except 20 mM Na^+ by Li^+ or choline on relaxations of rat isolated aorta induced by acetylcholine (Ach) or sodium nitroprusside (SNP)

Contractions of rat aorta complete with endothelium were induced by 1 μM norepinephrine and relaxations by 1 μM acetylcholine or 0.3 μM SNP in the absence (control) and presence of Li^+ or of choline. Tissues were preincubated for 90 min with Li^+ or choline replacement of all except 20 mM Na^+ in the bathing solution. Values are the means ± SE of the mean of at least four observations.

	Agonist	Relaxation	Time to half-relaxation
		%	sec
Control	Ach	57.7 ± 8.3	8.2 ± 0.6
Li^+	Ach	41.4 ± 2.1	607 ± 88[a]
Control	SNP	98.7 ± 0.9	14.3 ± 0.7
Li^+	SNP	86.3 ± 2.4[b]	58.7 ± 9.1[a]
Control	SNP	101.1 ± 1.1	15.3 ± 1.7
Choline	SNP	101.5 ± 7.4	21.3 ± 2.4[b]

[a] Significantly different ($p < 0.001$, paired t test) from control values.
[b] Significantly different ($0.05 < p < 0.025$, paired t test) from control values.

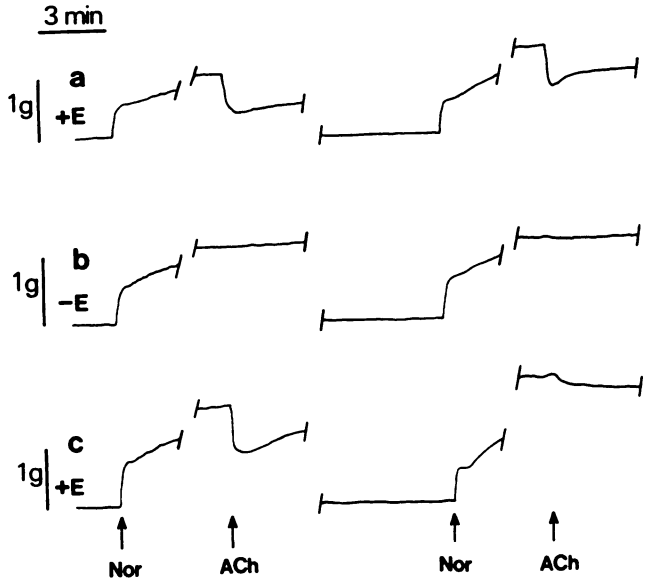

Fig. 1. A typical experiment showing the effect of 1 μM acetylcholine (*ACh*) on rat isolated aorta preparations contracted by 1 μM norepinephrine (*Nor*) in the absence (*−E*) and presence (*+E*) of endothelium. a. Control responses in normal physiological solution. b and c. Responses in normal physiological solution in the absence and presence of endothelium, respectively, followed by responses in the presence of Li^+. Note that in c but not in b, relaxant responses to acetylcholine continued to develop over a prolonged period (see Table 4).

markedly changed (Fig. 1). In the Li^+-containing solution the half-time to maximum relaxation was increased by about 70-fold compared to that in normal Na^+-containing physiological solution (Table 4). In the presence of Li^+, maximal relaxations elicited by 0.3 μM sodium nitroprusside were slightly but significantly ($0.05 < p < 0.025$, paired t test) less than those in normal physiological solution and, as in the case of acetylcholine, the time to half-maximal relaxation was significantly increased, but only by about 4-fold (Table 4). Contractions of aorta could not be elicited by norepinephrine (1 or 10 μM) in the presence of amiloride (1 mM), a concentration known to inhibit K^+-induced contractions of rat aorta (30). This inhibitory effect of amiloride was reversible.

Changing from normal physiological solution to one containing choline (117 mM) as a substitute for sodium evoked a transitory contraction that relaxed to baseline over about 15–

20 min. This contractile effect was inhibited by 50 nM atropine. In this solution norepinephrine-induced contractions were inhibited by about 20% (mean contraction 1.3 ± 02. g, $n = 10$) and acetylcholine relaxant responses in the presence of endothelium were abolished. In normal physiological solution, acetylcholine relaxed these norepinephrine-contracted arteries by about 60%. In the presence of choline, sodium nitroprusside relaxed precontracted arteries and the time to half-relaxation was increased slighly (by about 1.4-fold) in comparison to relaxant responses in normal physiological solution (Table 4). On washing in normal physiological solution for 90 min after exposure to either Li^+- or choline-containing solution, responses to sodium nitroprusside returned to normal (data not shown).

Discussion

It has been demonstrated previously in several vessel types from different species that endothelial-mediated relaxant responses evoked by acetylcholine are associated with increased tissue levels of cyclic GMP (6–8, 11). In the present study this increase in cyclic GMP levels in the rat aorta, induced by acetylcholine, has been used as an index of the stimulation of the release of EDRF from the endothelial cells.

In rat aorta it has been demonstrated previously that, although extracellular calcium is essential for the liberation of EDRF (9–11), the calcium entry blocker flunarizine, at a concentration (3 μM) sufficient to maximally inhibit agonist-stimulated influx of ^{45}Ca in vascular tissue (31, 32), does not affect acetylcholine-induced increases in tissue cyclic GMP levels and does not seem to affect maximal relaxant responses to acetylcholine (11). Similarly, high concentrations of nifedipine and verapamil (in comparison to those active on smooth muscle) have been shown to be relatively inactive as inhibitors of endothelium-dependent responses in rabbit and rat aorta (9, 10), and verapamil has little antagonistic effect against acetylcholine-induced relaxations of rat tail artery (33) and dog femoral artery (13).

The observations reported here, of the lack of inhibitory activity of a number of recognized calcium entry blockers (calcium antagonists), comprising examples from all three calcium antagonist subgroups (20, 21), extend these previous observations. Furthermore, two compounds that have also been shown to exhibit submaximal (about 40% of maximal) calcium entry-blocking activity at the concentrations used in this study, reserpine (26) and nicergoline (27), that have different structures to the other blockers used and have not yet been classified into one or the other subgroup, were also inactive. Altogether, these observations indicate that if calcium entry into endothelial cells in response to stimulation by acetylcholine occurs via specific channels, then such channels have characteristics different from those generally associated with smooth muscle cells.

In an earlier study on the relaxant effect of acetylcholine in dog femoral arteries, before the essential role of endothelium in this response was recognized, De Mey and Vanhoutte (13) presented some evidence consistent with the idea that the acetylcholine response might involve an Na^+-Ca^{2+} exchange process. Also, the Na^+-Ca^{2+} exchange inhibitor, dichlorobenzamil, inhibits acetylcholine- and calcium ionophore A 23187-induced relaxation of precontracted rat aorta (endothelium-dependent responses) but has little effect on maximal endothelium-independent relaxations induced by sodium nitroprusside (10). Here it has been shown that replacement of external Na^+ by Li^+ or choline will inhibit the acetylcholine-stimulated increase in tissue cyclic GMP levels. However, the two treatments did not produce identical effects. Choline replacement abolished the acetylcholine responses without affecting basal tissue levels of cyclic GMP and did not affect responses to sodium nitroprusside. In contractile experiments choline replacement abolished acetylcholine-induced relaxations and also had no effect on the maximal responses to sodium nitroprusside. Li^+ replacement reduced the basal tissue levels of the cyclic nucleotide and inhibited, but did not abolish, acetylcholine- and sodium nitroprusside-stimulated increases in tissue cyclic GMP levels at the time measured. In contractile experiments Li^+ replacement did not abolish acetylcholine- or sodium nitroprusside-induced relaxant responses but changed their kinetic characteristics, decreasing the rate of relaxation (Fig. 1, Table 4).

A reduction in basal levels of cyclic GMP has been associated with the absence of endothelium or with the withdrawal of Ca^{2+} (8, 11, 12, 14). In either case, there is a marked inhibition of endothelium-dependent acetylcholine-induced responses. The results of contractile experiments in the presence of Li^+ demonstrate that, even though basal tissue levels of cyclic GMP were reduced, endothelium-dependent relaxant responses could still be elicited (Table 4). That is, the endothelial cells were still present and could be activated by acetylcholine, and might imply a direct effect of Li^+ on the smooth muscle. Indeed, Li^+ antagonized the stimulatory effect of sodium nitroprusside as measured by increases in tissue cyclic GMP levels (Table 3) and has been shown to antagonize stimulated increases in tissue cyclic GMP levels in other tissues, probably due to an effect on guanylate cyclase (34, 35). This effect, however, may be partly attributed to the decrease in external Na^+ since time to half-maximal relaxation induced by 0.3 μM sodium nitroprusside was also slightly but significantly increased when choline was used to replace Na^+.

Amiloride, an inhibitor of Na^+-Ca^{2+} exchange (28, 29), inhibited acetylcholine-stimulated increases in tissue cyclic GMP levels without affecting increases due to stimulation by sodium nitroprusside and also had no significant effect on basal tissue levels of cyclic GMP. Therefore, like choline, amiloride treatment did not directly inhibit activation of guanylate cyclase or depress the basal release of EDRF. Taken together, these results indicate that Na^+-Ca^{2+} exchange might be an important mechanism for the entry of extracellular calcium into endothelial cells subsequent to acetylcholine stimulation (10). The lack of effect of calcium entry blockers could then be due to a lack of calcium channels with characteristics similar to those of the smooth muscle, in the sarcolemma of the endothelial cells. Such a lack of calcium channels is further supported by the observation that the calcium agonist Bay K 8644, which prolongs the opening time of potential dependent calcium channels (36), does not stimulate EDRF release from endothelial cells (37). The results with Li^+ substitution for Na^+ seem to indicate that Li^+ can, at least in part, substitute for Na^+ in the endothelial cells.

Basal tissue levels of cyclic GMP varied significantly (about 2-fold) from one experimental group to another (Tables 1–3). Such variability (or even to a more marked extent) is often evident in vascular smooth muscle from different species (see,

for example Refs. 8 and 38). Reasons for this variability, which is also seen with tissue cyclic AMP levels, are unknown.

In conclusion, these results demonstrate that, although activation of endothelial cells to release EDRF is dependent on extracellular Ca^{2+}, calcium entry blockers do not inhibit the release or the effects of EDRF. It is therefore unlikely that calcium channels having characteristics similar to those found in smooth or cardiac muscle are involved in the release of EDRF. However, an Na^+-Ca^{2+} exchange process may be involved.

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