Endothelial Role in Ouabain-Induced Contractions in Guinea Pig Carotid Arteries

Leocadio Rodríguez-Manas, Ana Pareja, Carlos F. Sánchez-Ferrer, Miguel A. Casado, Mercedes Salaices, and Jesús Marín

The inhibition of sodium-potassium adenosine triphosphatase (Na\(^+\),K\(^-\)-ATPase) by cardiac glycosides or potassium-free solutions induces contractile responses in a wide range of vascular beds from different animal species. These contractions can be produced by a direct effect on vascular smooth muscle (myogenic component) or are mediated by the release of norepinephrine (NE) from perivascular adrenergic nerve endings (neurogenic component); the predominant component depends on the animal and the vessel studied.\(^5\)\(^-\)\(^9\)

On the other hand, ouabain or potassium-free solutions inhibit endothelium-dependent relaxations.\(^1\)\(^-\)\(^2\) This action can be mediated by the blockade of endothelium-derived relaxing factor (EDRF) or nitric oxide (NO) released from endothelial cells or by the impairment of endothelium-dependent hyperpolarization of smooth muscle cells.\(^1\)\(^2\)\(^-\)\(^3\)\(^5\)\(^7\)\(^-\)\(^9\)

Both endothelium and the sodium pump have been involved in the pathogenesis of hypertension.\(^1\)\(^6\)\(^-\)\(^2\)\(^0\) However, in spite of the above discussed results, little is known concerning effects of the endothelium on the vascular actions of cardiac glycosides. Therefore, the present work was undertaken to analyze the possible endothelial influence on contractile responses to ouabain in perfused guinea pig carotid arteries. To simplify the experimental model, only the direct effects of the glycoside on vascular smooth muscle (the myogenic component) were analyzed. For this purpose, the possible ouabain-induced vasoconstrictions due to NE release were avoided by using \(\alpha\)-adrenergic receptor antagonists or by pretreating the animals with reserpine.

Methods

Perfusion of Arterial Segments

Guinea pigs of either sex weighing about 500 g (Dunkin-Hartley, IFFA-CREDO, Domaine des Oncins, France) housed at the facilities of Facultad de Medicina de la Universidad Autónoma, Madrid, Spain, were anesthetized with 35 mg/kg i.p. sodium pentobarbital and killed by bleeding. The carotid arteries were dissected into segments 1.5–2 cm long. The vascular segments were perfused according to the procedure described by De la Lande and Rand, with minor modifications. Both ends of the segments were cannulated with stainless steel tubes (0.4 mm o.d.), which were fixed to allow the vessel to stay at the previously measured in situ length. The cannulated segments were introduced into an organ bath containing 25 ml Krebs-Henseleit solution (KHS) at 37°C and continuously bubbled with a 95% O\(_2\)–5% CO\(_2\) gas mixture that gave a pH of 7.4. The segment was perfused with KHS by means of a peristaltic pump (HP-8, Gilson, Villiers le Bel, France) connected to a silicone circuit, both ends of which were immersed in a reservoir containing 22 ml oxygenated KHS at 37°C. Drugs were added to this reservoir in amounts adequate to obtain the desired final concentrations. The circulating volume of KHS was 7 ml. We tested that the only point in the circuit with significant resistance to flow was the arterial segment; thus, the basal
pressure of the system was related to the vascular tone, and contractions or dilations of the arterial segment produced increases or reductions of the perfusion pressure, respectively.

The perfusion pressure was measured by means of a polyethylene catheter (0.5 mm o.d.) placed in the circuit next to the proximal end of the vessel, which allows for the retrograde transmission of pressure. This catheter was connected to a transducer (P23XL, Gould, Oxnard, Calif.), and the pressure was recorded on a polygraph (4000, Letica, Barcelona, Spain). The recording system was calibrated daily with a mercury manometer.

Preliminary experiments were performed to establish the optimal perfusion pressure for the vasoactive agents to elicit maximal responses. For this, the segments were subjected to different flow rates ranging from 5 to 15 ml/min, which caused basal perfusion pressures from 5 to 20 mm Hg. When a concentration-response curve from $10^{-7}$ to $3\times10^{-5}$ M NE in the perfusate was plotted, the higher responses were observed at a basal pressure of around 15 mm Hg, which corresponds to a flow rate of approximately 12 ml/min. Therefore, in the rest of the experiments the vessels were submitted to this perfusion pressure for a 60-minute equilibration period, and viability of the segments was systematically checked by adding $3\times10^{-5}$ M NE to the perfusate. When contraction of the vascular segments reached a stable plateau, $3\times10^{-4}$ M acetylcholine (ACh) was administered. The presence of functional endothelium was verified when ACh reduced NE-induced tone by at least 30%. After a washout period, the drugs used in the present study were added to the perfusate. The vascular endothelium was removed from some segments by gently rubbing or by infusing saponin (see below) to analyze the influence of the endothelium in drug-induced responses.

Bioassay Technique

These experiments were performed according to the procedure described by Rubanyi et al. Segments (1.5-2 cm) of guinea pig carotid arteries were cannulated as described above, and placed in an organ bath containing 10 ml oxygenated KHS at 37°C. These segments were considered the donors and were perfused with KHS by means of a peristaltic pump (Eyela, Tokyo Rikakikai Co., Tokyo) at a constant rate of 2 ml/min. A stainless steel tube through which KHS was pumped at the same rate was also placed in the organ bath. A ring of guinea pig carotid artery from which the endothelium had been removed (bioassay ring) was suspended immediately below the organ chamber by means of two steel stirrups passed through its lumen. One stirrup was bound to an isometric force transducer (Panlab, Barcelona, Spain) connected to a polygraph (Houston Instruments, Gistel, Belgium). The assembly of bioassay rings, stirrups, and force transducer could be moved freely below the organ chamber, allowing the preparation to be superfused with the perfusate from either the vascular segment or the stainless steel tube.

A resting tension of 1 g (optimal resting tone determined by previous assays with 75 mM K+) was applied to the bioassay ring, which was superfused with KHS passed through the stainless steel tube (direct superfusion) for a 90-minute equilibration period. During this time the resting tension was readjusted every 15 minutes until the tone became stable. After this period, the bioassay ring was moved to below the outlet from the donor segment. To check the viability of the preparation, $10^{-5}$ M NE was added to the perfusate, and when the contraction reached a stable plateau $10^{-5}$ M ACh was administered through the donor segment or directly to the ring to verify the presence and absence of endothelium in the donor segment and bioassay ring, respectively. During the experiments, drugs could be added through the donor segment or directly to the bioassay ring.

Rubidium-86 Uptake

Uptake studies were done according to the method described by Bukoski et al., with minor modifications. Guinea pig carotid arteries were cleaned of blood traces and divided into segments of similar weights. The endothelium of some vessels was then removed. Each group was tied to a rigid nylon fiber and immersed for 60 minutes in 2 ml oxygenated KHS at 37°C for equilibration. The arteries were exposed to a potassium-free medium for 15 minutes, and then they were incubated for different periods (1-50 minutes) in vials containing 2 ml potassium-free KHS plus $10^{-4}$ M $^8$RbCl (specific activity, 4.68 mCi mg) (Rb-KHS). Subsequently, the tissues were washed by successive immersion in vials with 2 ml potassium-free Rb-KHS for three 30-second periods. Then the arteries were blotted, weighed, digested in 1 ml H$_2$O$_2$ (30% w/vol) by heating for 5 hours at 100°C, and placed in vials with 2 ml Ready Solv (Beckman Instruments, Inc., Fullerton, Calif.); radioactivity was measured in a liquid scintillation counter (LS-2800, Beckman). Total $^8$Rb uptake was expressed as millimoles per kilogram wet weight. In simultaneous experiments, ouabain-insensitive $^8$Rb uptake was studied. The procedure was similar, but $10^{-4}$ M ouabain was added to the bath 10 minutes before the incubation period with Rb-KHS. Therefore, ouabain-sensitive $^8$Rb uptake was estimated by the difference between the total and ouabain-insensitive uptakes. Effects on the total and ouabain-insensitive $^8$Rb uptakes were analyzed by the addition of drugs 15 minutes before the incubation period. $^8$Rb uptake was calculated as $(cpm$ in muscle/wet wt$)\times[(mmol$ Rb/l medium$)+(cpm/l$ medium$)]$, with wet weight expressed in kilograms.

Removal of Vascular Endothelium

The endothelium was removed by gently rubbing or by intraluminal perfusing 0.3 mg/ml saponin in KHS for 1 minute. The latter procedure caused complete endothelial denudation without damaging vascular contractility, as documented by the maintenance of vasoactive responses to 75 mM K+ or $10^{-7}$ to $3\times10^{-5}$ M NE. Deendothelialization was also tested in some cases by histological methods. No differences were observed between vessels denuded by mechanical and chemical procedures.

Solutions, Drugs, and Statistical Evaluations

The millimolar composition of KHS was 119 NaCl, 4.6 KCl, 2.5 CaCl$_2$, 1.2 KH$_2$PO$_4$, 1.2 MgSO$_4$, 7H$_2$O, 25 NaHCO$_3$, 11.1 glucose, and 0.03 disodium salt of ethylenediaminetetraacetic acid. Potassium-free KHS was prepared by omitting KCl and KH$_2$PO$_4$. Potassium-free

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Ouabaln (log M)

FIGURE 1. Line graph shows increases in perfusion pressure induced by ouabain in isolated guinea pig carotid artery segments with (○, n=6) or without (■, n=5) endothelium. Results are mean ± SEM increases from basal perfusion pressure of 15.4 ± 0.5 mm Hg. Three to six animals were used for each curve.

Rb-KHS was obtained by adding 4.6 mM ^8^RbCl to potassium-free KHS.

Distilled water was used to dissolve drugs, except indomethacin and NE, which were dissolved in distilled water with 1.5 mM Na_2_ CO_3 and a saline-ascorbic solution, (0.9% NaCl and 0.1% ascorbic acid wt/vol), respectively. Stock solutions were kept at -20°C. Ouabain solutions were protected from light. Reserpine (3 mg/kg total dose) was administered intraperitoneally to the guinea pigs in two doses 48 and 24 hours before the experiment. Control and experimental responses were obtained from separate vascular preparations because ouabain was not removed from the vessels after repeated washout periods.

The drugs used were ACh chloride, NE hydrochloride, reserpine, saponin, and ouabain octahydrate from Sigma Chemical Co., St. Louis, Mo.; indomethacin from Merck, Darmstadt, FRG; phenolamine mesylate from Ciba-Geigy, Basel, Switzerland; prazosin hydrochloride from Pfizer Inc., New York; N^6^-monomethyl L-arginine (L-NMMA) from Wellcome, Beckenham, UK; and

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Results

Reactivity of Perfused Segments

The addition of 10^{-7} to 10^{-4} M ouabain to the perfusate at 15-minute intervals elicited concentration-dependent increases in the perfusion pressure of guinea pig carotid arteries; under these experimental conditions, removal of the vascular endothelium did not modify the contractile response to the glycoside (Figure 1). To eliminate the neurogenic component of ouabain-induced contraction, the vessels were preincubated for 10 minutes with the α-adrenergic receptor antagonists phenolamine (unspecific) or prazosin (α_1) (both at 3×10^{-6} M) or the animals were pretreated with reserpine. In the presence of the α-adrenergic receptor antagonists, pressure increases evoked by ouabain were markedly reduced in intact segments but were similar to those in control segments after removal of the endothelium (Figure 2). Similarly, in carotid arteries from guinea pigs pretreated with reserpine, in the presence of endothelium, ouabain-induced contractions were less than those observed in control vessels, but after endothelialization these responses were similar to those in arteries from untreated animals (Figure 3). In these experiments, depletion of NE stores by reserpine was documented by the lack of contractile responses to electrical field stimulation (200 mA, 0.3 msec, 4 Hz for 1 minute; results not shown).

To analyze the possible role for endothelial factors such as NO or prostacyclin, carotid arteries from reserpine-pretreated animals were pretreated with the inhibitor of NO synthesis L-NMMA and/or the cyclooxygenase inhibitor indomethacin (both at 10^{-5} M). These agents did not modify the contractile responses evoked by ouabain (Figure 4, left). For comparative purposes, the effects of such inhibitors were also tested on the contractions induced by 10^{-7} to 3×10^{-5} M NE. Pretreat-

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Figure 2. Line graphs show effects of preincubation for 10 minutes with phenolamine or prazosin (both at 3×10^{-6} M) on increases in perfusion pressure evoked by ouabain in pressurized guinea pig carotid artery segments with (left) or without (right) endothelium. Results are mean ± SEM percentage of previous increase evoked by 10^{-7} M norepinephrine, which was 41.2 ± 2.8% and 39.5 ± 4.4 mm Hg in segments with and without endothelium, respectively. Three to six animals were used for each curve; number of segments used is in parentheses. *p<0.05, **p<0.001 different from control value.
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FIGURE 3. Line graphs show increases in perfusion pressure induced by ouabain in perfused carotid artery segments from control and reserpinized guinea pigs with (left) or without (right) endothelium. Results are mean ± SEM percentage of previous increase evoked by 10⁻³ M norepinephrine, which was 41.2±2.8, 39.5±4.4, 40.1±4.1, and 42.1±4.0 mm Hg in control and reserpinized segments with and without endothelium, respectively. Four to six animals were used for each curve; number of segments used is in parentheses. *p<0.05, **p<0.001 different from control value.

Bioassay Experiments

To confirm the effects of endothelium on the vascular responses to ouabain, bioassay experiments were designed. To eliminate effects mediated by NE release, the bioassay ring was isolated from guinea pigs pretreated with reserpine and the donor segment was taken from either control or reserpinized animals, with similar results. Ouabain (10⁻⁴ M) was continuously administered to the bioassay ring for 60 minutes, but with two different protocols. In one protocol, the drug was passed first through the donor segment for 30 minutes, which caused contraction in the bioassay ring of 662.2±64.8 mg (n=5); then ouabain was added directly to the bioassay ring for another 30 minutes, producing additional increases in vascular tone of 440.0±117.1 mg (Figure 5A). In the second protocol, ouabain was first administered directly to the bioassay ring for 30 minutes, causing a significantly greater contraction than when it was added first through the donor segment (1,062.1±155.1 mg, n=5, p<0.05); this response was slightly decreased by 55±25 mg after drug perfusion through the donor segment for 30 minutes (Figure 5B). Similar experiments were performed using endothelium-denuded donor segments. In this situation, the first addition of ouabain evoked similar contractions in both protocols (957.7±62.2 and 1,146.5±157.3 mg, respectively; n=5), and small similar increases in tone (113.7±61.5 and 135.3±74.1 mg, respectively) were observed after changing the route of glycoside administration (Figures 5C and 5D).

Rubidium-86 Uptake

Guinea pig carotid arteries exhibited time-dependent Rb uptake, which reached a stable level at 30 minutes; preincubation of the vessels with 10⁻⁴ M ouabain for 10 minutes significantly decreased the uptake (Figure 6, left). The difference between total and ouabain-insensitive uptakes was considered a measure of sodium pump activity. Since the highest specific uptake was observed at 30 minutes of incubation, this period was...
chosen for the rest of the experiments. Under these conditions, 10^{-9} to 10^{-4} M ouabain exerted a concentration-dependent inhibition on radioactivity uptake (Figure 6, right). When the vascular endothelium was removed, a significant reduction of total ^{86}Rb uptake was observed; in this case, the glycoside caused an additional concentration-dependent decrease of radioactivity accumulation (Figure 6, right).

Discussion

Ouabain evoked concentration-dependent contractions in perfused guinea pig carotid arteries, as observed in other vascular preparations.\(^1\)\(^-\)\(^7\) In the absence of any other treatment, contractile responses to the glycoside were not affected by removal of the vascular endothelium. In these arteries a major part of ouabain contraction is due to the release of NE from perivascular nerve endings because the \(\alpha\)-adrenergic receptor blockers phentolamine and prazosin markedly inhibited these responses, and similar effects were observed after treating the animals with reserpine, which depletes catecholamine stores. An analogous neurogenic component of ouabain-induced contraction has been described in other vascular preparations.\(^3\)\(^-\)\(^9\)\(^-\)\(^26\)\(^-\)\(^29\) The mechanism by which ouabain produces the secretion of NE involves sodium pump blockade of adrenergic nerve endings, which causes Ca\(^{2+}\) entry through Na\(^{+}\)-Ca\(^{2+}\) exchange.\(^6\)\(^-\)\(^30\)

Therefore, in the presence of phentolamine or prazosin or in vessels from reserpinized guinea pigs, it can be assumed that the remaining contraction is mainly due to the direct inhibition by ouabain of the sodium pump in vascular smooth muscle. The reduced contractile responses evoked by the glycoside under these experimental conditions were markedly increased by removal of the vascular endothelium, until contractions were similar to those found in the control situation. The endothelial modulation of ouabain-induced contraction could be explained by the loss of a tonic release of vasorelaxant factors, EDHF/NO or prostacyclin, as suggested for other vasoconstrictors such as NE or serotonin in different vascular beds.\(^3\)\(^-\)\(^4\)\(^-\)\(^8\)\(^-\)\(^9\)\(^-\)\(^26\)\(^-\)\(^29\) In the present work, the vasoconstriction elicited by NE in guinea pig carotid arteries was also increased after removal of the endothelium or with the NO blocker L-NMMA.\(^2\)\(^6\)\(^27\) However, the present results suggest that the mechanisms by which endothelial cells modulate ouabain- or NE-induced contractions are different. Indeed, the inhibition of NO synthesis with L-NMMA did not alter ouabain-

![Figure 5. Representative tracings from experiments (n=5) showing contractile effects of 10^{-4} M ouabain on denuded bioassay rings from reserpinized guinea pig carotid arteries. Tracing A: Ouabain was first added for 30 minutes through donor segment from control guinea pig carotid artery with endothelium; afterward, ouabain was administered for 30 more minutes directly to bioassay ring. Tracing B: Ouabain was first added directly to bioassay ring for 30 minutes and after was added through donor segment with endothelium for 30 more minutes. Three to six animals were employed. Protocols for tracings C and D are given in figure.]

![Figure 6. Uptake of rubidium-86 (^{86}Rb) in guinea pig carotid arteries. Left: Total (○) and ouabain-insensitive (■) time-dependent uptake in presence of 10^{-4} M ouabain. Right: Effects of ouabain on ^{86}Rb uptake in vessels with (○) or without (■) endothelium incubated for 30 minutes with radioisotope; c, control uptake without ouabain. Results are mean±SEM. Seven to ten vascular rings were used for each point; 25 animals were needed. *p<0.05 different from □.]

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evoked vasoconstrictions but markedly increased NE-induced contractions. This suggests that the release of NO modulates NE-evoked contractions but is not involved in endothelial modulation of glycoside effects. On the other hand, the blockade of cyclooxygenase with indomethacin modified neither ouabain- nor NE-induced contractions, disproving a role for prostaglandins in these responses.

From these results, it is possible to conclude the existence of a specific antagonistic effect of the endothelium on myogenic contractions elicited by ouabain in guinea pig arteries. To further analyze the interference of endothelium in the smooth muscle sodium pump, bioassay experiments were performed using only reserpined endothelium-denuded bioassay rings (donor segments from control or reserpined guinea pigs were used and gave similar results). Direct perfusion of the glycoside into the bioassay ring evoked greater contractions than did ouabain's addition through a donor segment with endothelium. Changing the route of drug addition produced different effects; an additional increase in the contraction was observed when ouabain was added directly, whereas slight relaxations occurred when the glycoside was added through the donor segment. These effects were dependent on the presence of endothelium in the donor segment.

Activity of the sodium pump can also be tested by means of the uptake of $^{85}$Rb (a potassium carrier). Differences between total and ouabain-insensitive $^{85}$Rb uptakes represent the activity of Na$^+$,K$^+$-ATPase. In guinea pig carotid arteries maximal at 30 minutes of incubation. When the endothelium had been removed, total $^{85}$Rb uptake significantly decreased. Radioisotope uptake in segments with or without endothelium was reduced in a concentration-dependent manner by ouabain, further indicating its dependence on sodium pump activity. However, the radioactivity accumulated in the presence of low concentrations of the glycoside (10$^{-9}$ to 10$^{-7}$ M) was significantly higher in segments with than without endothelium. A possibility to be considered is that the reduction of $^{85}$Rb uptake is due to the absence of endothelium, but this seems unlikely since the ratio of endothelial versus smooth muscle cells is very low and the removal of endothelial cells would probably not account for the observed decrease in $^{85}$Rb uptake.

All these results suggest 1) there is a clearly greater myogenic contraction to ouabain in the absence of endothelium, 2) endothelium seems to protect the smooth muscle sodium pump from blockade by ouabain, and 3) this effect appears to be due to a diffusible factor (or factors) that is neither NO nor prostacyclin. These facts allow for speculation that endothelium releases a substance (or substances) that interacts with Na$^+$,K$^+$-ATPase in smooth muscle cells, stimulating sodium pump activity or antagonizing the inhibitory actions of ouabain, or both.

It is noteworthy that in cultured vascular smooth muscle from dog aorta, the uptake of $^{85}$Rb is inhibited by plasma from hypertensive animals, which contains endogenous digitalis. This inhibition does not occur when those cells are cocultured with bovine endothelial cells. Although those authors did not provide any explanation for their finding, it is consistent with our data. Also in agreement with our data, in guinea pig isolated trachea contractions induced by ouabain are potentiated by epithelium removal. Two possibilities have been proposed to explain this effect: first, blockade by the glycoside of a relaxant factor (or factors) released from epithelial cells, and second, secretion by the epithelium of a factor (or factors) able to stimulate activity of the underlying smooth muscle sodium pump. It is possible that this endothelial factor could be potassium, although some facts argue against this hypothesis: first, the involvement of potassium in endothelium-mediated responses to ouabain would indicate a high release of this ion from this thin cell layer, which is perhaps unlikely, and second, this release of potassium by ouabain should be very specific for endothelium, without affecting underlying smooth muscle cells, but the present results demonstrate that the glycoside acts on smooth muscle.

It is important to remark that, in the presence of an intact adrenergic innervation, removal of the endothelium did not increase ouabain-induced contraction, as would be expected if a facilitation of sodium pump inhibition in the vascular smooth muscle had occurred. This fact lacks an appropriate explanation, unless we accept that endothelium also modulates the NE-mediated ouabain-induced contraction. Some preliminary results from our laboratory suggest that this could be the case. Indeed, in guinea pig carotid arteries the presence of endothelium appears to be necessary for ouabain-evoked NE release (unpublished results).

In vascular preparations, sodium pump blockade can interfere with the action of an endothelial factor on smooth muscle cells, as reported in canine coronary arteries and femoral arteries. Because NO-induced relaxations are not blocked by ouabain, it is reasonable to conclude that endothelium is able to release a factor (or factors) distinct from NO. On the other hand, it is known that ACh can cause smooth muscle hyperpolarization by the release of endothelial-derived hyperpolarizing factor (EDHF), a factor that is not NO. Feletou and Vanhoutte proposed that the mechanism of action of EDHF could be the stimulation of a K$^+$ channel. In our case, this potassium channel could be the underlying smooth muscle sodium pump, based on the inhibition of hyperpolarizing responses by sodium pump blockade. This fact permits us to establish a certain analogy with the endothelium protective effect on the smooth muscle sodium pump in guinea pig carotid arteries. Thus, one can speculate that this action can be mediated by an EDHF-like factor. In addition, as occurs with EDHF-mediated responses, endothelial protection of the sodium pump is due to neither cyclooxygenase products nor NO-related compounds. However, some differences exist between the present results and the described EDHF responses. The observed endothelium-dependent hyperpolarizations are transitory, whereas our experiments suggest that activity of the sodium pump is modified by deendothelialization in a long-lasting manner. In addition, some authors suggest that EDHF's actions are not related to sodium pump activity.

Our results can be of physiological relevance by possible implication in pathological events such as hypertensive disease, in which a circulating factor that blocks Na$^+$,K$^+$-ATPase activity, as well as abnormalities in the endothelium-dependent responses, have been detected. Recent data suggest that the en-
dogenous sodium pump inhibitor isolated from human plasma can be identified as a compound identical to ouabain. Inhibition of the vascular sodium pump by these compounds can be blocked by endothelial cells. In conclusion, one can speculate that the loss of a protective effect of the endothelium on sodium pump activity could facilitate vascular Na\(^+\), K\(^-\)-ATPase inhibition by endogenous circulating ouabainlike compounds. It is worth noting that the contractile effect of digitals in human vessels is mainly due to direct actions of this agent on smooth muscle cells, through which the possible antagonistic effects of the endothelium may have a greater physiological role.

Acknowledgments

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