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Na/Ca exchange and tension development in vascular smooth muscle: effect of amiloride

Sergio Bova, Gabriella Cargnelli & [1]Sisto Luciani

Department of Pharmacology, University of Padova, Largo E. Meneghetti 2, I35131 – Padova, Italy

1 The potassium-sparing diuretic, amiloride, has been shown to inhibit the Na/Ca exchange system in various preparations. The effects of this drug have been investigated on the contractions of guinea-pig aortic strips elicited by reduction of external K, by addition of ouabain and by removal of external Na.

2 Amiloride (5×10^{-6} M–5×10^{-4} M) inhibited the mechanical responses when it was added before giving the stimulus for contractions, but was not effective in relaxing the contracted strips. The drug shifted to the right the dose-response curve for Ca in low K solution.

3 The calcium antagonist diltiazem had no effect on the ouabain-, low K- and Na-free-induced contractions.

4 Amiloride decreased the rate of relaxation of aortic strips induced by removal of the low K solution.

5 The pattern of amiloride action on ouabain-, low K- and Na-free-induced contractions suggests that the drug interferes with Ca influx. The effect of amiloride on the relaxation rate of low K-contracted aortic strips is consistent with an interference with Ca efflux.

6 It is suggested that amiloride prevents Ca fluxes through the Na/Ca exchange system of guinea-pig aortic strips.

Introduction

The potassium-sparing diuretic amiloride, a well known inhibitor of two Na transport mechanisms in various tissues, namely a conductive Na entry pathway and a Na/H exchange system (Benos, 1982), has recently been found to inhibit the Na/Ca exchange in plasma membrane vesicles from various types of preparations (Smith *et al.*, 1982; Schellenberg *et al.*, 1983; Floreani & Luciani, 1984; Luciani & Floreani, 1985; Debetto *et al.*, 1987). In cardiac muscle this carrier system, which countertransports Ca for Na across the cell membrane, is thought to play an important role in cellular calcium homeostasis and hence in the regulation of contractility (Glitsch *et al.*, 1970; Mullins, 1977; Reuter, 1982; Langer, 1983; Lee, 1985). It has been shown, in cardiac muscle and squid axon, that the Na/Ca exchange system can move calcium in either direction across the cell membrane in exchange for sodium. The direction is dependent on the prevailing electrochemical gradient for Na and on membrane potential (Carafoli, 1984; Schatzman, 1985; Sheu & Blaustein, 1986; Mechmann & Pott, 1986; Kimura *et al.*, 1986).

Although the occurrence of a Na/Ca exchange system in vascular smooth-muscle has been suggested by several authors (Sitrin & Bohr, 1971; Reuter *et al.*, 1973; Burton & Goodfraind, 1974; Daniel *et al.*, 1982; Grover *et al.*, 1983; Morel & Goodfraind, 1984; Matlib *et al.*, 1985), considerable controversy exists over the contribution of this system to the regulation of the intracellular calcium level in this tissue (Van Breemen *et al.*, 1979; Droogmans & Casteels, 1979; Van Breemen *et al.*, 1982; Brading & Latergan, 1985; Somlyo *et al.*, 1986). Nevertheless, the results of several studies indicate that Ca entry through Na/Ca exchange can play a determining role in inducing smooth muscle contraction (Blaustein, 1974; 1977; Aickin *et al.*, 1984; Bolton, 1985; Ashida & Blaustein, 1987). Vascular smooth muscle preparations respond with a sustained contraction to modification of $[Na]_o/[Na]_i$ induced by reduction of external Na (Reuter *et al.*, 1973; Ashida & Blaustein, 1987; Bova *et al.*, 1986) or by an increase in the intracellular Na through inhibition of the Na pump. The latter effect can be achieved experimentally by lowering the K concentration in the medium or by adding cardiac glycosides (Karaki *et al.*, 1978; Ozaki *et al.*, 1978; Toda, 1980). In all the above mentioned conditions the smooth muscle contractions have been correlated with Ca influx

[1]Author for correspondence.

through the Na/Ca exchange as a result of a decrease of $[Na]_o/[Na]_i$ (Ozaki & Urakawa, 1979; Ashida & Blaustein, 1987).

The present study was carried out to investigate the effect of amiloride on the mechanical response of guinea-pig aortic strips to reduction in external K, to the addition of ouabain and to the removal of external Na. The aim was to find a possible link between the action of amiloride on Na/Ca exchange observed in an isolated system as the plasma membrane vesicles and the effect of the drug on the mechanical function of an isolated vascular tissue. A preliminary account of this work has appeared in abstract form (Bova *et al.*, 1985).

Methods

Aortae excised from female guinea-pigs (350–450 g) were carefully cleaned of connective tissue and cut spirally into 10 mm × 1.5 mm strips. Each strip was placed vertically in a water-jacketed 10 ml organ bath filled with a physiological salt solution (PSS) of the following composition (in mM): NaCl 118, KCl 4.75, $CaCl_2 \cdot 2H_2O$ 2.5, $MgSO_4 \cdot 7H_2O$ 1.2, KH_2PO_4 1.2, $NaHCO_3$ 25, glucose 11. The solution was maintained at 35°C and continuously bubbled with a mixture of 95% O_2: 5% CO_2 to give a pH of 7.35–7.40. The strips were attached by means of a silk thread to an isometric force transducer (Battaglia-Rangoni TRB/200/2) coupled to a pen recorder to monitor force generation. The preparations were preloaded with 0.8 g and equilibrated for at least 90 min before starting the experiments.

The low-K solution was made by omitting KCl. Amiloride was added to the bath approximately 30 min before the substitution of KCl-free solution (which also contained amiloride) for the PSS. An interval of 90 min was allowed between the control contraction (without amiloride) and the test contraction (in the presence of amiloride).

When sodium was omitted, sucrose was added to maintain osmolarity and $KHCO_3$ was substituted for $NaHCO_3$. All the experiments in Na-free solution were carried out in the presence of diltiazem 10^{-5} M in order to block the voltage-dependent calcium channels. The strips were exposed to amiloride about 30 min before changing the PSS to the Na-free solution to which amiloride has been added. Between the control contraction and the contraction in the presence of amiloride an interval of 90 min was allowed.

The dose-response relationship for Ca in low K solution was obtained by first incubating the aortic strips in Ca-free, KCl-free solution containing EGTA 0.1 mM for 30 min, then exposing the preparations to Ca-free, KCl-free solution without EGTA. After 30 min, cumulative concentrations of $CaCl_2$ were added to the bath.

The drugs used in the experiments were as follows: amiloride (Merck & Sharp & Dohme S.p.A., Roma), diltiazem (Schiapparelli S.p.A., Torino) and ouabain (Simes S.p.A., Milano). All drugs were dissolved in distilled water.

Results

Effect of amiloride on contraction induced by Na-free solution

The aortic strips responded to the Na-free solution with a sustained contraction which reached the peak within approximately 30 min and was completely reversible on washing. Amiloride in the concentration range 10^{-5} M to 5×10^{-4} M reduced the contractile responses of the strips when added prior to changing the PSS to Na-free solution (Figure 1), but had negligible effect if added when the contractions had fully developed.

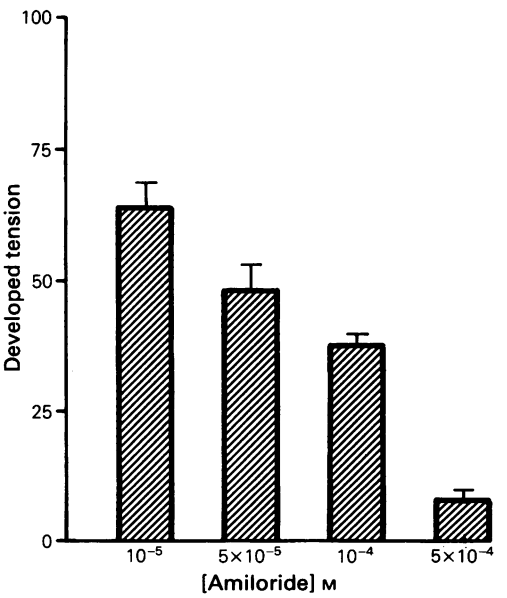

Figure 1 Inhibitory effect of amiloride on Na-free-induced responses of guinea-pig aortic strips. In each strip two contractions were elicited, the first in the absence and the second in the presence of amiloride. Diltiazem (10^{-5} M) was added to the bath about 30 min before changing the PSS to the Na-free solution, which also contained diltiazem. The results are expressed as a percentage of the contraction obtained without the drug. Six strips were used for each amiloride concentration (vertical lines show s.e.mean).

Effect of amiloride on contractions induced by KCl-free solution or by ouabain

Substitution of the KCl-free solution for PSS induced sustained contractions which were slow in onset (about 30 min) and reached the maximum within 150–180 min. The contractions were completely reversible and were reproducible. The contractions induced by ouabain were faster in onset and in reaching the peak (about 50–60 min), but the glycoside was not easily removed on washing. Thus the experiments on ouabain-induced contractions were made on paired strips. Amiloride (5×10^{-6} M to 5×10^{-4} M) induced a dose-dependent inhibition of the contractile responses of the aortic strips to both the KCl-free solution and ouabain (2.5×10^{-5} M) (Figures 2 and 3). Amiloride showed a negligible effect on the fully developed contractions in that the drug was able to inhibit the response to KCl-free solution and ouabain but did not relax the contracted strips. The calcium antagonist diltiazem (5×10^{-6} M) failed to produce any effect in the same experimental conditions.

Effect of amiloride on the dose-response relationship for Ca in KCl-free solution

In the aortic strips exposed to KCl-free, Ca-free solution, contractile responses were obtained with cumulative concentrations of $CaCl_2$ (0.5×10^{-3} M to 2.5×10^{-3} M). In the presence of amiloride (5×10^{-4} M) the dose-response curve for Ca was shifted to the right (Figure 4). The maximal concentration of $CaCl_2$ used was the same as the normal Ca concentration in PSS.

Effect of amiloride on the rate of relaxation induced by the readmission of PSS

The KCl-free induced contractions were completely reversed on re-admission of PSS. The time course of relaxation was calculated on the basis of the time taken for contracted aortic strips to relax by 25%, 50% and 75%. After the first contraction, the tissues were washed with PSS for 90 min and again exposed to KCl-free solution. At the maximum of this second

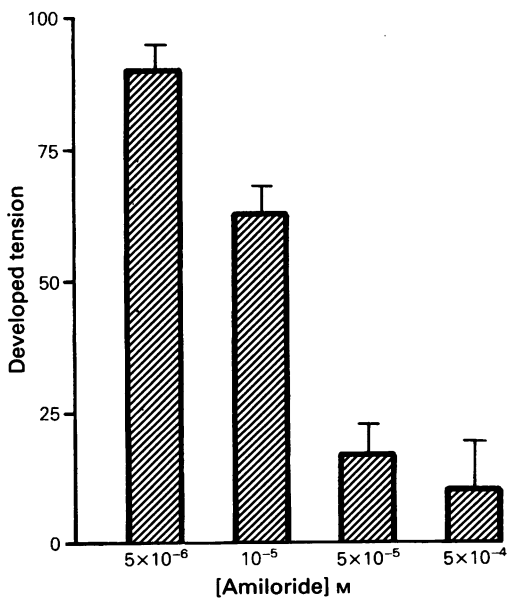

Figure 2 Inhibitory effect of amiloride on the KCl-free-induced contraction of guinea-pig aortic strips. In each strip two contractions were elicited, the first in the absence and the second in the presence of the drug. The results are expressed as a percentage of the contractions obtained without the drug. Six strips were used for each amiloride concentration (vertical lines show s.e.mean).

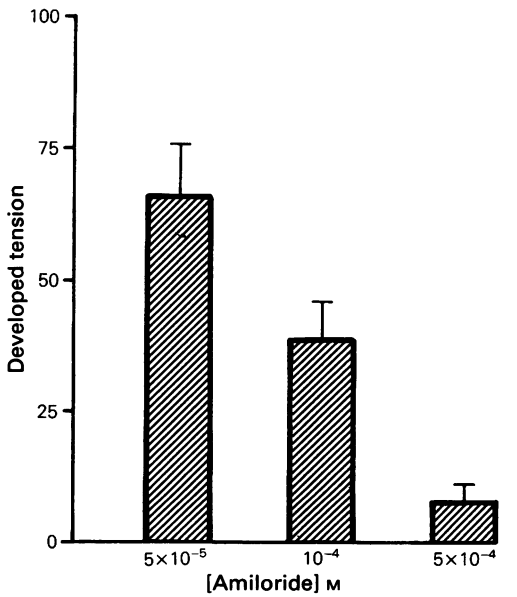

Figure 3 Inhibitory effect of amiloride on the ouabain (2.5×10^{-5} M)-induced contraction of guinea-pig aortic strips. Since the ouabain-contractions were not completely reversible, in this study two groups of six strips were used for each amiloride concentration, one group serving as control. The results are expressed as a percentage of the mean contraction obtained in the absence of amiloride (there were 18 control strips; the magnitude of the control contractions induced by 2.5×10^{-5} M ouabain was 988 ± 50 mg; vertical lines show s.e.mean).

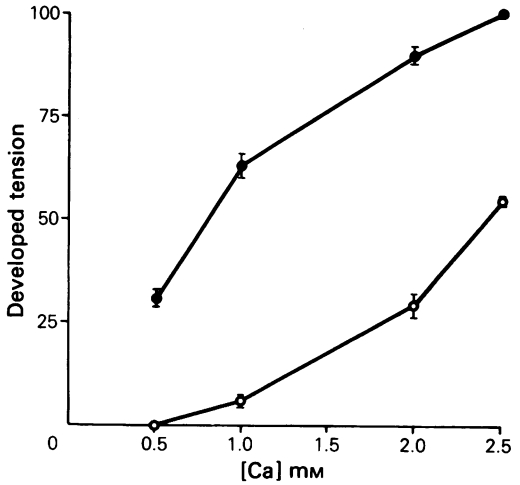

Figure 4 Effect of amiloride on the response to Ca of aortic strips exposed to KCl-free solution. The concentration-response curves for Ca were obtained in each aortic strip in the absence (●) and in the presence (○) of amiloride (5×10^{-4} M). The results are expressed as a percentage of the contraction elicited by $CaCl_2$ 2.5 mM. Each point represents the mean of six experiments (vertical lines show s.e.mean).

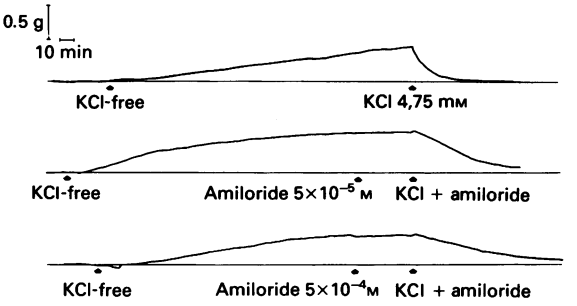

Figure 5 Records showing the effect of two concentrations of amiloride (5×10^{-5} M, 5×10^{-4} M) on the time course of relaxation from KCl-free-induced contraction obtained by the readmission of KCl (4.75 mM). Note the lack of effect of amiloride on the fully developed contraction.

contraction amiloride was added approximately 40 min before substituting PSS, which also contained amiloride. The drug did not affect the contraction but induced a dose-dependent (5×10^{-5} M; 5×10^{-4} M) decrease in the rate of relaxation (Figures 5 and 6).

Discussion

The results of this study show that amiloride prevents the mechanical response of aortic strips induced by reduction of external K, by addition of ouabain and by removal of external Na.

The activation of contractile process in smooth muscle is thought to be due to an increase in free myoplasmic calcium concentration (Bolton, 1979; Borle, 1981). It is generally accepted that the rise in free Ca in smooth muscle cells is dependent on calcium entry from extracellular fluid and calcium mobilization from intracellular binding sites (Bolton, 1979; Johansson & Somlyo, 1980). It is not clear to what extent these calcium pools contribute respectively to trigger the contraction (Bolton, 1985; Deth & Van Breemen, 1974). There are several reports showing that a component of vascular smooth muscle response to stimulant substances persists even after prolonged incubation in Ca-free medium, indicating that it is possible to release sufficient Ca from intracellular bound sites to elicit contraction (Bolton, 1985). However, the mechanical responses observed in aortic strips exposed to Na-free, KCl-free medium or ouabain appear to be dependent on transmembrane Ca supply, as contraction did not occur in the absence of Ca in the medium.

Calcium entry into activated smooth muscle cells normally occurs through potential- and receptor-gated calcium channels. In our experimental conditions it is unlikely that these routes of Ca influx were operative. In many types of vascular smooth muscle treated with cardiac glycosides or low K solutions the contractions are partially mediated by noradrenaline released from adrenergic nerve endings (Karaki *et al.*, 1978; Katsuragi & Su, 1982; Hayashi & Park, 1984). However, experimental evidence demonstrates that, in guinea-pig aortic strips, the mechanical response to the above stimuli is the result of a purely myogenic mechanism (Ozaki *et al.*, 1978; Karaki *et al.*, 1978). Our results confirm this observation, as we obtained similar contractile responses in aortic strips from reserpinized and non-reserpinized guinea-pigs (data not shown).

The contractions of vascular smooth muscle induced by cardiac glycosides or lowering external K have often been attributed to Ca influx through Ca channels activated by the depolarization resulting from the inhibition of the electrogenic Na pump (Van Breemen *et al.*, 1979; Fleming, 1980). In our experiments, the contractile response of the aortic strips to KCl-free medium or ouabain, although strictly dependent on the presence of external Ca, was not affected by the Ca antagonist diltiazem. This

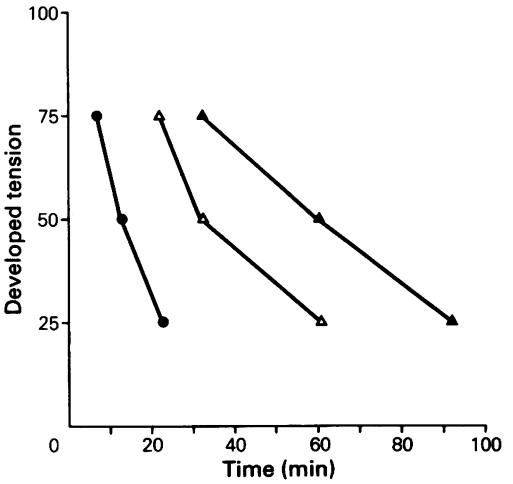

Figure 6 Effect of amiloride on the time course of relaxation from the KCl-free-induced contraction of guinea-pig aortic strips. Each point of each curve represents the mean of the times at which the strips had relaxed by 25%, 50% and 75%. In each strip the relaxation was obtained first in the absence (●) and then in the presence of amiloride 5×10^{-5} M (△) and 5×10^{-4} M (▲). Amiloride was added when the maximum contraction had been reached in KCl-free solution which after 40 min was then substituted with PSS (containing amiloride). Six aortic strips were used for each amiloride concentration.

suggests that Ca entry through voltage-activated Ca channels does not contribute to the contractions. The same conclusion has been drawn by Ozaki *et al.* (1978) and Ozaki & Urakawa (1979) who investigated the mechanism of the contractile response of guinea-pig aortic strips following inhibition of the Na pump. These authors suggested that contractions elicited by manoeuvres which inhibit the Na pump are produced mainly by an influx of Ca through the Na/Ca exchange mechanism. That the Na/Ca exchange may operate in the Ca entry mode, extruding Na in exchange for incoming Ca when the intracellular Na is increased by the inhibition of the Na pump (lowered $[Na]_o/[Na]_i$), has also been demonstrated in other types of smooth muscle (Aickin *et al.*, 1984). Moreover in cardiac myocytes the Na/Ca exchange has recently been demonstrated to generate a membrane current which depends on the transmembrane gradients of Na and Ca and on membrane potential (Mechmann & Pott, 1986; Kimura *et al.*, 1986). Therefore on depolarization Ca entry would occur not only via Ca channels but also via Na/Ca exchange (Mullins, 1979; Eisner & Lederer, 1985). If the Na/Ca exchange of vascular smooth muscle is regulated by the same factors as cardiac muscle, then the inhibition of the Na pump can promote Ca influx through Na/Ca exchange by depolarization as well as by a decrease of the ratio $[Na]_o/[Na]_i$. A decrease of $[Na]_o/[Na]_i$ can also be achieved by lowering the external Na concentration. Ashida & Blaustein (1987) have demonstrated in rat aorta that a progressive reduction of $[Na]_o$ results in tension development and that the relative rate of contractions is inversely related to $[Na]_o$. The same authors have found, using the Ca-sensitive fluorescent dye fura 2, that in single myocytes from bovine tail artery the intracellular Ca increase induced by K-free medium was further augmented by subsequent reduction of extracellular Na. These findings strongly support the idea of Ca entry mediated by Na/Ca exchange when $[Na]_o/[Na]_i$ is reduced.

In our study the removal of extracellular Na induced a sustained contraction of guinea-pig aortic strips which was unaffected by diltiazem, but was dose-dependently reduced by amiloride. Pretreatment of the tissue with amiloride effectively prevented the contractile responses but had no effect on established contractions. This pattern strongly suggests that amiloride interacts through a mechanism that does not involve intracellular Ca sequestration because if this were so it should have not only prevented contraction but also relaxed the previously contracted aortic strips. Considering that amiloride has been shown to inhibit Na/Ca exchange in several preparations (Smith *et al.*, 1982; Schellenberg *et al.*, 1983) including cardiac sarcolemma (Floreani & Luciani, 1984; Debetto *et al.*, 1987), it is likely that a similar action on the Na/Ca exchange working in the Ca entry mode might explain the inhibition of the contractions of aortic strips. Amiloride was not equi-active in inhibiting the contractions elicited by ouabain (2.5×10^{-5} M), low K and Na-free solutions. This result can be explained by the fact that the above conditions do not alter to the same extent the ratio $[Na]_o/[Na]_i$ and consequently the extent of the calcium transport mediated by Na/Ca exchange. Thus, differences in the level of operation of the exchange system may be responsible for the differences in the activity of amiloride.

That the inhibition of Na/Ca exchange by amiloride has functional consequences in aortic strips may also be consistent with the observation that the drug significantly decreased the rate of relaxation of the contracted preparations (low-K medium).

The relative importance of Ca extrusion from the cells and of intracellular sequestration in inducing relaxation of smooth muscle is still a controversial issue (Ma & Bose, 1977; Van Breemen *et al.*, 1979; Somlyo *et al.*, 1982; Bolton, 1985). Nevertheless, the maintenance of intracellular Ca concentration far below the external concentration in spite of the Ca influx during rest (Ca leakage) and activation requires

the presence of some mechanism of Ca extrusion. In smooth muscle it has been shown that Ca efflux from the cells involves an external Na-dependent pathway (Ma & Bose, 1977) presumably through the Na/Ca exchange mechanism, and an ATP-driven Ca pump (Blaustein, 1982; Schatzmann, 1985). The inhibition of the Na/Ca exchange mechanism operating in the Ca extrusion mode may result in a decrease of the relaxation rate of the smooth muscle (Ashida & Blaustein, 1987), as we observed in the presence of amiloride.

However, alternative explanations of the effects of amiloride should be considered. Amiloride has been reported to be a good inhibitor of Na/H exchange (Kinsella & Aaronson, 1980; Piwnica-Worms & Lieberman, 1983; Frelin *et al.*, 1984) and of several protein kinases (Holland *et al.*, 1983).

The Na/H exchange system is an important mechanism for the regulation of intracellular pH. Although the role of Na/H exchange in vascular smooth muscle has not been investigated in detail (Roos Boron, 1981), the consequence of its inhibition would be a reduction of intracellular pH that results in a decrease of developed tension (Peiper *et al.*, 1976). That the effect of amiloride on aortric strips contractions was the result of an inhibition of Na/H exchange cannot be excluded (Little *et al.*, 1986) although it is difficult to reconcile this mechanism of action with the drug's lack of effect on established contractions and with the slowing of relaxation.

A protein kinase plays a major role in the activation of smooth muscle contraction. Many observations made with several types of smooth muscle led to the formulation of the phosphorylation theory of the regulation of smooth muscle contraction (Walsh, 1985). According to this theory, the phosphorylation of the two 20K light chains of myosin operated by a myosin light chain kinase is a fundamental stage involved in smooth muscle contraction. Given that amiloride is known to inhibit several protein kinases (Holland *et al.*, 1983), inhibition of myosin light chain kinase could explain the inhibition of contraction caused by amiloride. If this were the case, however, one would expect amiloride to affect the established contractions and to increase, rather than decrease, the relaxation rate.

In conclusion we suggest that the most parsimonious explanation of the pattern of action of amiloride on vascular smooth muscle contractions induced by Na-free, KCl-free solutions and ouabain is the inhibition of Ca movements through Na/Ca exchange. Effects on other mechanisms involved in the regulation of vascular smooth muscle contractility cannot be excluded, but if they occur, do not play a determinant role in our experimental conditions.

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