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THE RELATIONSHIP BETWEEN CALCIUM AND INCREASED SENSITIVITY OF RABBIT AORTAE FOUR HOURS AFTER RESERPINE

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1 Four hours after reserpine, rabbit aortic strips were supersensitive to acetylcholine, isoprenaline and noradrenaline. The threshold concentration of the drugs necessary to induce a response was less and the maximum tension developed by the tissues was greater than in control strips.

2 Reserpine-treatment potentiated the contractile responses to $CaCl_2$.

3 Reserpine-treatment resulted in an increase in calcium uptake and an increase in the slow component of $^{45}Ca^{2+}$ efflux.

4 After resperine-treatment, the rate of relaxation from a potassium-induced contraction was decreased.

5 It is concluded that reserpine-induced supersensitivity is related to an enhanced ability of the tissue to retain and utilize calcium.

Introduction

Non-specific supersensitivity of vascular smooth muscle observed 24 h after administration of reserpine may be related to changes in calcium mobility (Carrier & Jurevics, 1973). We have previously observed that rabbit isolated atria become more sensitive to calcium as little as 4 h after reserpine administration (Jurevics & Carrier, 1973), and comparable changes in vascular calcium homeostasis have been detected (Carrier, Whittington-Coleman, Matheny & Shibata, 1970). This raised the question whether there might be an acute change in vascular sensitivity 4 h after reserpine. The present study was designed to test this possibility and to further our knowledge of reserpine-induced postsynaptic supersensitivity.

The present paper includes characterization of the sensitivity, the tissue calcium changes, and the alterations in radioactive calcium movements, occurring in aortic tissue 4 h after reserpine-treatment of rabbits.

Methods

Tissue preparation and procedure

New Zealand white rabbits of either sex weighing approximately 1.5 kg were given 3 mg/kg reserpine (Serpasil, CIBA) intramuscularly 4 h before they were killed (treated) or were used as untreated controls. They were killed by a blow to the head and exsanguinated. A section of the thoracic aorta between the aortic arch and the diaphragm was removed and placed in oxygenated Ringer solution. Helical aortic strips were then prepared according to the method of Furchgott (1960). For contractile tension studies, aortic strips were divided into two equal segments which were approximately 3 mm in width and 33–35 mm in length. Ligatures were placed on both ends of the muscle segments, and subsequently mounted vertically on a glass rod and placed in a tissue-organ bath containing Ringer solution with a final volume of 20 ml. One end of the muscle segment was attached to a Grass FT-03 force-displacement transducer and isometric contractions were recorded on a Grass Model 7 Polygraph.

The muscle preparations were equilibrated for 90 min and maintained under a baseline tension of 2 g before exposing them to an agonist. During the equilibration period, the tissues were washed with fresh Ringer solution every 15 min to prevent toxic accumulation of waste products (Altura & Altura, 1970). The solutions, both in the chamber and those used for wash, were continuously oxygenated with a 95% O_2 and 5% CO_2 and maintained at a constant temperature of 37°C. In experiments involving La^{3+}, 100% O_2 was used as indicated.

At the end of the initial equilibration period, dose-response relationships were obtained for different agonists as well as for $CaCl_2$. Dose-response relationships were obtained by exposing aortic strips

to cumulatively increasing concentrations of each respective agonist, without any subsequent washout of the bath chambers until the maximal contractile response was reached. Cumulative responses to $CaCl_2$ could only be obtained in a calcium-free depolarizing Ringer solution (Godfraind & Kaba, 1969; Jurevics & Carrier, 1973). Before each successive concentration of agonist was added, the aortic strips were allowed to reach a new steady-state tension. No more than one agonist was utilized in any one preparation. No more than one dose-response curve was obtained with any one preparation.

Several experiments were carried out to check the equality of tone between control and reserpine-treated aortic strips. After the initial equilibration period in normal Ringer solution, sodium nitrite (1 mM), a non-specific relaxant, was added. Both control and reserpine-treated aortic strips showed the same amount of relaxation, indicating similar levels of basal tone.

ED_{50} values were obtained by graphically calculating the concentration of an agonist producing 50% of its maximum response. Mean ED_{50} values were expressed as geometric means (Fleming, Westfall, De La Lande & Jehlett, 1972).

Tissue incubation solutions

Normal Ringer solution contained (mM): NaCl 154.0, KCl 5.4, $CaCl_2$ 2.4, $NaHCO_3$ 6.0 and dextrose, 11.0. Calcium-free Ringer solution was identical except that $CaCl_2$ was omitted (calcium contamination was below the detection limits of the Perkin-Elmer Model 303 Atomic Absorption Spectrophotometer and therefore < 1 μM).

Zero-calcium, depolarizing Ringer solution contained (mM): NaCl 94.0, KCl 60.0, $NaHCO_3$ 6.0, dextrose 11.0 and ethylenebis (oxyethylenenitrilo) tetraacetic acid (EGTA), 0.01 was added.

Calcium-free depolarizing Ringer solution was identical except that EGTA was omitted.

In $^{45}Ca^{2+}$ experiments, Tris(hydroxymethyl) aminomethane (5 mM) was added instead of $NaHCO_3$ to normal Ringer solution, together with $LaCl_3$ (10 mM) (van Breemen, Farinas, Casteels, Gerba, Wuytack & Deth, 1973).

In the depolarizing solutions, KCl was substituted for an equivalent amount of NaCl to maintain isotonicity of the solution. All solutions were made up in double-distilled deionized water with the final pH adjusted and maintained throughout the experiment at 7.4 by the addition of NaOH as necessary.

Stimulating agents

The stimulating agents used in these studies were calcium chloride, acetylcholine bromide (Nutritional Biochemicals Corp.), (–)-noradrenaline bitartrate (Sigma Chemical Co.) and (±)-isoprenaline hydrochloride (Winthrop Labs). Doses of agonists are expressed in terms of molarity. Calcium chloride was dissolved in double-distilled deionized water in a concentrated stock form. Acetylcholine, noradrenaline, and isoprenaline were prepared immediately before starting an experiment.

Calcium uptake studies

The method of van Breemen *et al.* (1973) was used in these studies. Aortic strips were incubated in Tris-buffered normal Ringer solution for 90 minutes. After this initial equilibration, the bathing solution was changed to a similar Ringer solution containing trace amounts of $^{45}Ca^{2+}$ (New England Nuclear Corp.). Following incubation in the radioactive solution for specific time periods (5, 10, 15 and 30 min) 10 mM $LaCl_3$ was added to the same solution. Exactly 3 min later, the solution was changed to a calcium-free Ringer solution containing Tris buffer (5 m) and $LaCl_3$ (10 mM) and subsequently maintained in this solution for 45 minutes. Tissues were then removed from the bath, blotted, weighed and digested in 1 ml of NCS (Nuclear Chicago Corp. solubilizing solution; Amersham/Searle Corp.) and kept overnight in an oven maintained at 50°C. On the following day, 14 ml of toluene scintillation fluid (100 ml of toluene, 400 mg 2,5-diphenyloxazole (PPO) and 5 mg of 1,4-bis 2-(5-phenyloxazolyl) benzene (POPOP)) was added to the digesting solution. Tissue $^{45}Ca^{2+}$ was counted in a Nuclear Chicago Corp. Unilux Model 3 Liquid Scintillation counter.

Calcium content was calculated using the following equation:

$$\text{Calcium content (mmol/kg wet wt.)} = \frac{\text{ct/min in muscle}}{\text{kg wet wt}} \cdot \frac{\text{mmol } Ca^{2+} \text{ in labelled medium}}{\text{ct/min in labelled medium}}$$

$^{45}Ca^{2+}$ efflux studies

Aortic strips were incubated in normal Ringer solution for 90 min and then incubated for 30 min in a normal Ringer solution containing $^{45}Ca^{2+}$ (5×10^{-5} ct min^{-1} ml^{-1}). After this period of incubation and uptake, the strips were rinsed in 4 tubes containing 5 ml of normal Ringer solution (unlabelled) within a total time period of 20 seconds. These strips were then transferred to successive tubes containing 5 ml of calcium-free Ringer solution containing Tris-buffer (5 mM) at designated time intervals for the next 60 minutes. At the end of the final washout period, the tissue was blotted with mineral-free filter paper for not more than 90 s, following which its wet weight was determined. The tissue was then digested and counted using the same procedure as in the previous uptake studies. One ml of each washout solution was dissolved in 15 ml of liquid scintillation fluid containing 40% ethanol and 60% toluene scintillation solution and counted.

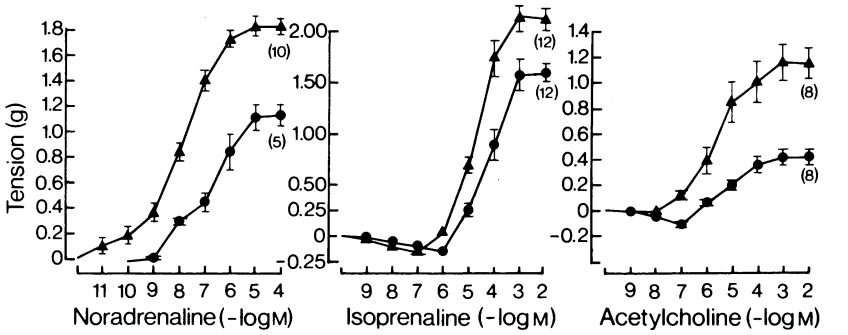

Figure 1 The responses of rabbit aortic strips from control animals (●) and from animals treated 4 h earlier with reserpine 3 mg/kg (▲) to cumulative concentrations of acetylcholine, isoprenaline and noradrenaline. Vertical bars represent s.e. mean. Numbers in parentheses are number of aortae used.

$^{45}Ca^{2+}$ washout data were obtained by determining the $^{45}Ca^{2+}$ present in each washout solution and the $^{45}Ca^{2+}$ remaining in the tissue after the 60 min washout. Curves were then plotted which express the decline in tissue $^{45}Ca^{2+}$ with time, indicating the amount of $^{45}Ca^{2+}$ remaining in the tissue after each washout interval as a percentage of the initial tissue $^{45}Ca^{2+}$ content (desaturation curves).

Statistical significance was determined by the use of Student's *t*-test. Differences were considered significant when $P < 0.05$.

Results

The influence of reserpine pretreatment on the contractile responses of rabbit aortic strips to noradrenaline, isoprenaline, and acetylcholine

The contractile responses to noradrenaline, isoprenaline and acetylcholine of aortic strips from reserpine-treated and untreated rabbits are illustrated in Figure 1. The maximum contractile responses for noradrenaline, isoprenaline, and acetylcholine were obtained at concentrations $\geqslant$, 0.1 μM (10^{-5} M), 1 mM and 1 mM (10^{-3} M), respectively. The aortic strips from the reserpine-treated rabbits were supersensitive to all three agonists. The ED_{50} for acetylcholine was 2.3 times, for isoprenaline 3.9 times, and for noradrenaline 18.1 times less for the aortic strips from reserpine-treated rabbits than for the strips from control rabbits. These differences were significant ($P < 0.02$). The threshold concentration for each agonist was significantly lower with the aortic strips from the reserpine-treated rabbits than with those from the untreated animals. For noradrenaline, all 5 control aortic strips had a threshold of 1 nM (10^{-9} M), while in the reserpine-treated aortic strips, 5 responded at 0.1 nM (10^{-10} M) and 5 at 0.01 nM (10^{-11} M). For isoprenaline, 10 control aortic strips had a threshold response at 10 μM (10^{-5} M) and 2 at 1 μM (10^{-6} M); while in reserpine-treated aortic strips 9 had a threshold at 1 μM (10^{-6} M) and 3 at 0.1 μM (10^{-7} M). For acetylcholine, the control threshold for all 8 of the aortic strips was 1 μM (10^{-6} M), while in the reserpine-treated group, 5 had a threshold response at 0.1 μM (10^{-7} M), and 3 at 0.01 μM (10^{-8} M). Also, with all three agonists, the maximum tensions developed by the aortic strips from the reserpine-treated rabbits were significantly greater than those of strips from untreated rabbits.

Reserpine had an interesting effect on the relaxant response of aortic strips to low doses of isoprenaline and acetylcholine. Strips from reserpine-treated rabbits consistently reached maximum relaxation at a lower concentration of isoprenaline than did aortic strips from untreated rabbits, while reserpine treatment abolished the relaxant phase of acetylcholine responses.

The influence of reserpine pretreatment on the contractile responses of rabbit aortic strips to calcium

Contractile responses to calcium can be induced when aortic strips are incubated in a calcium-free, depolarizing Ringer solution (Godfraind & Kaba, 1969; Carrier & Jurevics, 1973). The responses of rabbit aortic strips incubated in this media to increasing doses of calcium are illustrated in Figure 2. Although the ED_{50}'s for the strips from reserpine-treated and untreated rabbits were not different, all responses to calcium above the ED_{50} level of the strips from reserpine-treated rabbits were significantly ($P < 0.01$) greater than those of the strips from the untreated rabbits. This became more pronounced as the concentration of calcium approached normal physiological values. The maximum contractile response of the aortic strips from reserpine-treated

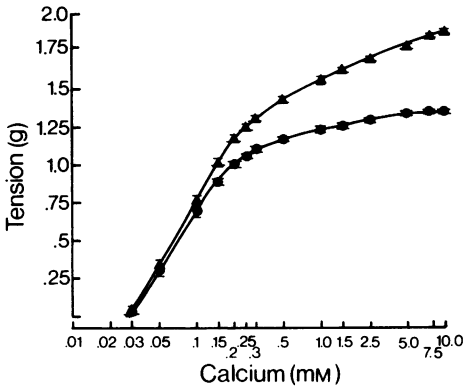

Figure 2 The responses of rabbit aortic strips from control animals (●) and from animals treated 4 h earlier with reserpine 3 mg/kg (▲) to cumulative concentrations of $CaCl_2$ in a calcium-free, depolarizing Ringer solution. Vertical bars represent s.e. mean. Number of aortae used was 12 for both control and reserpine-treated rabbits.

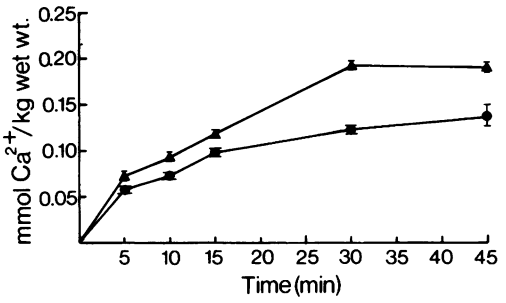

Figure 3 Uptake of calcium by rabbit aortic strips from control animals (●) and from animals treated 4 h earlier with reserpine 3 mg/kg (▲). Vertical bars represent s.e. mean. Number of aortae used was 6 for both control and reserpine-treated rabbits.

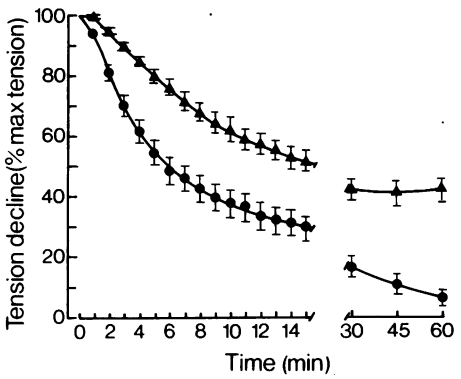

Figure 4 The decline in tension in a zero-calcium depolarizing Ringer solution of rabbit aortic strips from control animals (●) and from animals treated 4 h earlier with reserpine 3 mg/kg (▲). Vertical bars represent s.e. mean. Number of aortae used was 10 for both control and reserpine-treated rabbits.

rabbits (1.85 ± 0.01 g) was significantly ($P < 0.01$) greater than the responses (1.38 ± 0.03 g) of aortic strips from untreated rabbits.

The influence of resperine pretreatment on calcium uptake of rabbit aortic strips

The La^{3+} technique was utilized in order to determine the net transmembrane fluxes without interference from the extensive exchangeability of the calcium in the extracellular space and bound to external membranes. This method presumably defines cellular calcium, not only by eliminating extracellular calcium, but also by completely inhibiting further calcium influx, and almost completely inhibiting calcium efflux (70–90%) in rabbit aortic strips (van Breemen *et al.*, 1973; Freeman & Daniel, 1973). The accumulation of calcium by rabbit aortic strips, illustrated in Figure 3, was significantly greater at 5, 10, 15 and 30 min in the strips from reserpine-treated rabbits than in aortic strips from untreated rabbits. Both groups of aortic strips essentially attained maximum uptake at 30 minutes.

The influence of reserpine pretreatment on the tension decline of rabbit aortic strips in a zero-calcium, depolarizing Ringer solution

Aortic strips placed in a zero-calcium, depolarizing Ringer solution exhibited an initial contractile response which was not sustained. The tension of aortic strips from both reserpine-treated and untreated rabbits declined in 60 min to a new resting tension (Figure 4) after which there was no further significant decline. The control aortic strips developed 1.4 ± 0.1 g in 2.6 ± 0.1 min, while the treated aortic strips developed 1.5 ± 0.1 g in 2.3 ± 0.1 minutes. The tensions developed were not significantly different; however, the time to peak tension of the treated tissue was significantly less than that of the control group. The rate of decline in tension of the aortic strips from reserpine-treated rabbits was significantly less at all times than the rate of decline in tension of the strips from untreated animals. The time to decline to one-half maximum response was 7.3 ± 1.2 min for control aortic strips, and 25.6 ± 6.1 min for reserpine-treated strips ($P < 0.01$). In addition, the aortic strips from the

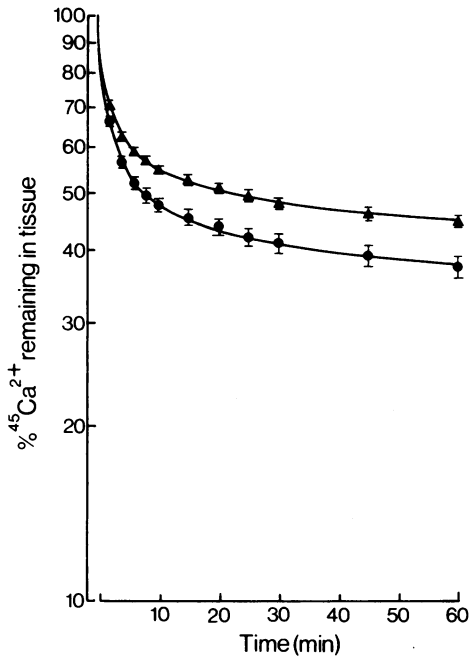

Figure 5 $^{45}Ca^{2+}$ efflux into calcium-free Ringer solution from rabbit aortic strips taken from control animals (●) and from animals treated 4 h earlier with reserpine 3 mg/kg (▲). The curves shown are desaturation curves, i.e. points represent $^{45}Ca^{2+}$ remaining in the tissue at the indicated time. Vertical bars represent s.e. mean. Number of aortae used was 4 for both control and reserpine-treated rabbits.

reserpine-treated rabbits only declined to $42.0 \pm 3.7\%$ of their initial tension in 60 min, whereas the aortic strips from the untreated rabbits declined to $6.3 \pm 2.7\%$ of their initial tension in the same time period.

The influence of reserpine pretreatment on the $^{45}Ca^{2+}$ efflux from aortic strips

Since tension decline studies indicated that reserpine-treated tissues had an increased ability to maintain, or a decreased ability to remove, that ionized calcium pool needed to sustain contractile tension, studies of radioactive calcium efflux might give more insight into that calcium affected by reserpine pretreatment. Figure 5 illustrates the effect of reserpine pretreatment on the loss of $^{45}Ca^{2+}$ from aortic strips into a calcium-free Ringer solution after a 30 min equilibration period with the isotope. At the end of a 60 min washout period, control aortic strips still retained $37.2 \pm 1.9\%$ $^{45}Ca^{2+}$, while reserpine-treated aortic strips retained $44.2 \pm 1.1\%$ $^{45}Ca^{2+}$. The aortic strips from treated animals had a significant delay ($P < 0.01$) in $^{45}Ca^{2+}$ efflux from that of control strips at all points on the desaturation curve.

Discussion

The results obtained in the present study clearly show the development of a non-specific supersensitivity of rabbit aortic strips following a relatively acute treatment with reserpine. The dose-response curves for the three agonists, noradrenaline, isoprenaline, and acetylcholine, were shifted to the left and the threshold concentration of each was significantly decreased. The maximum tension obtained with each agonist was significantly increased. The significant decrease in the ED_{50} values is indicative of an increased sensitivity (Fleming, McPhillips & Westfall, 1973). It has been reported (Fleming & Trendelenburg, 1961; Hudgins & Fleming, 1966) that non-specific supersensitivity is usually associated with a slow time course of development with variations accompanying different methods, species and tissues. The present results clearly demonstrate that a non-specific supersensitivity can be acutely induced. Short-term development of supersensitivity characterized by similar changes has been also demonstrated in the heart (Jurevics & Carrier, 1973) and in the pineal gland (Romero & Axelrod, 1975). The possibility that this is a phenomenon different from the supersensitivity seen after 24 h or longer cannot be excluded.

There are many available theories explaining the mechanism of reserpine-induced supersensitivity. Hudgins & Fleming (1966) proposed that the change after reserpine-treatment of rabbits which results in supersensitivity occurs at a step beyond the level of receptor activation. Carrier & Shibata (1967) proposed that reserpine-treatment induced changes in essential ions (Na^+, K^+, and Ca^{2+}) leading to an increased cellular excitability and consequential supersensitivity. Many authors (Carrier & Shibata, 1967; Hudgins, 1969; Hudgins & Harris, 1970; Garrett & Carrier, 1971; Carrier & Jurevics, 1973) have been proponents of the involvement of the calcium ion in reserpine-induced supersensitivity. Fleming (1972) has demonstrated a partial depolarization of cell membranes of the vas deferens following treatment of guinea-pigs with reserpine and suggests it plays a role in supersensitivity. A partial depolarization was also reported for isolated hearts from reserpine-treated guinea-pigs (Taylor, Westfall & Fleming, 1974).

The concepts of a membrane depolarization and of electrolyte involvement in supersensitivity correlates well with the data obtained in these studies, for the increased calcium uptake by the reserpine-treated aortic strips essentially demonstrates an increase in the tissue calcium available for exchange. This might

be explained by increased permeability of the membrane to calcium (Carrier & Shibata, 1967; Hudgins & Harris, 1970) as well as a partial membrane depolarization (Fleming, 1974) because a major portion of the measurable inward current which accompanies either depolarization or other increases in membrane permeability in smooth muscle is a result of calcium fluxes (Bohr, 1973). These data, in conjunction with the decrease in the rate of tension decline, and the apparent increase in the size of the slow-clearing component of $^{45}Ca^{2+}$ loss, indicate that the tissue has an increased exchangeable fraction and, in addition, has a greater ability to retain and maintain the calcium stores involved in contraction. Previous studies (Hudgins & Harris, 1970; Garrett & Carrier, 1971; Carrier & Jurevics, 1973) have shown that reserpine-induced supersensitivity is associated with an increased ability of the tissue to bind calcium. This may explain the increase in maximum tension seen in these studies.

The increase in sensitivity to different agonists helps to elucidate the possible mechanism of action of reserpine-induced supersensitivity. Carrier & Jurevics (1973) have demonstrated the dependence of acetylcholine's action upon extracellular calcium in supersensitive tissues. This is in agreement with the possibility of increased membrane excitability or permeability following reserpine treatment. Also, Triggle (1972) has shown that acetylcholine exerts its contractile stimulation by first mobilizing membrane bound calcium which is closely associated with the cholinoceptor. The increased sensitivity to noradrenaline and isoprenaline following reserpine treatment suggests reserpine has an effect on intracellular calcium stores, because these agonists activate contractile proteins by primarily mobilizing intracellular calcium (Seidel & Bohr, 1971; van Breemen *et al.*, 1973).

The data obtained in the present studies appear to indicate an increased ability of the reserpine-treated aortic strips to maintain the calcium pool essential to the contractile process. Essentially, we believe that reserpine acts as a 'pharmacological rectifier' in that it allows more calcium to be taken up due to an apparent increase in permeability and to enhanced calcium mobilization in response to these agonists, while concomitantly limiting or decreasing the amount that is actively sequestered and extruded.

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