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The effects of cyclopiazonic acid on intracellular Ca^{2+} in aortic smooth muscle cells from DOCA-hypertensive rats

R.C.A. Tostes[1,3], D.W. Wilde[2], L.M. Bendhack[4] and R.C. Webb[1]

[1]Departments of Physiology and [2]Anesthesiology, University of Michigan, Ann Arbor, MI 48109-0622, USA
[3]Departamento de Farmacologia, Faculdade de Medicina de Ribeirão Preto and [4]Faculdade de Ciências Farmacêuticas de Ribeirão Preto, Universidade de São Paulo, 14040-900 Ribeirão Preto, SP, Brasil

Abstract

We tested the hypothesis that cyclopiazonic acid (CPA), an inhibitor of the sarcoplasmic reticulum (SR) Ca^{2+}-ATPase, increases intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) in aortic myocytes and that the increase in $[Ca^{2+}]_i$ is higher in aortic cells from deoxycorticosterone acetate (DOCA)-hypertensive rats. Male Sprague-Dawley rats, 250-300 g, underwent uninephrectomy, received a silastic implant containing DOCA (200 mg/kg) and had free access to water supplemented with 1.0% NaCl and 0.2% KCl. Control rats were also uninephrectomized, received normal tap water, but no implant. Intracellular Ca^{2+} measurements were performed in aortic myocytes isolated from normotensive (Systolic blood pressure = 120 ± 3 mmHg; body weight = 478 ± 7 g, N = 7) and DOCA-hypertensive rats (195 ± 10 mmHg; 358 ± 16 g, N = 7). The effects of CPA on resting $[Ca^{2+}]_i$ and on caffeine-induced increase in $[Ca^{2+}]_i$ after $[Ca^{2+}]_i$ depletion and reloading were compared in aortic cells from DOCA and normotensive rats. The phasic increase in $[Ca^{2+}]_i$ induced by 20 mM caffeine in Ca^{2+}-free buffer was significantly higher in DOCA aortic cells (329 ± 36 nM, N = 5) compared to that in normotensive cells (249 ± 16 nM, N = 7, $P<0.05$). CPA (3 µM) inhibited caffeine-induced increases in $[Ca^{2+}]_i$ in both groups. When the cells were placed in normal buffer (1.6 mM Ca^{2+}, loading period), after treatment with Ca^{2+}-free buffer (depletion period), an increase in $[Ca^{2+}]_i$ was observed in DOCA aortic cells (45 ± 11 nM, N = 5) while no changes were observed in normotensive cells. CPA (3 µM) potentiated the increase in $[Ca^{2+}]_i$ (122 ± 30 nM, N = 5) observed in DOCA cells during the loading period while only a modest increase in $[Ca^{2+}]_i$ (23 ± 10 nM, N = 5) was observed in normotensive cells. CPA-induced increase in $[Ca^{2+}]_i$ did not occur in the absence of extracellular Ca^{2+} or in the presence of nifedipine. These data show that CPA induces Ca^{2+} influx in aorta from both normotensive and DOCA-hypertensive rats. However, the increase in $[Ca^{2+}]_i$ is higher in DOCA aortic cells possibly due to an impairment in the mechanisms that control $[Ca^{2+}]_i$. The large increase in $[Ca^{2+}]_i$ in response to caffeine in DOCA cells probably reflects a greater storage of Ca^{2+} in the SR.

Correspondence
R.C.A. Tostes
Laboratório de Hipertensão
Departamento de Farmacologia
Instituto de Ciências Biomédicas I
Universidade de São Paulo
Av. Prof. Lineu Prestes, 1524
05508-900 São Paulo, SP
Brasil
Fax: 55 (011) 818-7433
E-mail: rtostes@usp.br

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- Sarcoplasmic reticulum
- Deoxycorticosterone (DOCA) hypertension

Introduction

Cyclopiazonic acid (CPA), a mycotoxin from *Aspergillus* and *Penicillium*, is a selective inhibitor of the sarcoplasmic reticulum (SR) Ca^{2+}-ATPase and is widely used as a pharmacological tool for investigating the role and turnover of intracellular Ca^{2+}. CPA has been shown to inhibit the SR Ca^{2+}-ATPase in skeletal (1), smooth (2,3) and cardiac muscle (4). The specific inhibition of the SR Ca^{2+}-ATPase by CPA may be related to the inhibition of conformational changes associated with ATP hydrolysis and Ca^{2+} transport (1). By inhibiting the ATP-driven Ca^{2+} sequestration, CPA reduces the storage capacity of the SR for Ca^{2+} and inhibits contractions that depend on intracellular Ca^{2+} release (5-7).

CPA has been reported to increase intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) in endothelial cells from bovine pulmonary arteries (8), but has no effect on membrane currents of single myocardial cells (4). In aorta from deoxycorticosterone acetate (DOCA)-hypertensive rats, CPA induced contractions in the presence of extracellular Ca^{2+}, but not in Ca^{2+}-free buffer, suggesting that CPA activates Ca^{2+} influx (7). CPA-induced contractions were not observed in aorta from normotensive rats.

In the present study we tested the hypothesis that CPA increases $[Ca^{2+}]_i$ in rat aortic smooth muscle cells and that the CPA-induced increase in $[Ca^{2+}]_i$ is higher in aortic cells from DOCA-hypertensive rats. Intracellular Ca^{2+} measurements were performed in aortic smooth muscle cells isolated from normotensive and DOCA-hypertensive rats and the effects of CPA on resting $[Ca^{2+}]_i$ and on the increase in $[Ca^{2+}]_i$ stimulated by 20 mM caffeine were evaluated. The effects of CPA and caffeine on $[Ca^{2+}]_i$ were compared to the effects of CPA and caffeine on vessel tone according to a protocol obtained under similar experimental conditions.

Material and Methods

DOCA-salt induced hypertension

Adult male Sprague-Dawley rats weighing 250-300 g (Charles River Laboratories, Inc., Portage, MI) underwent uninephrectomy (small flank incision, left side) under sodium pentobarbital anesthesia (50 mg/kg, *ip*). In the same surgery, a silastic implant containing DOCA (200 mg/kg) was implanted subcutaneously behind the skull. Control (normotensive) rats were also uninephrectomized, but received no implant. After surgery, DOCA-treated rats received water supplemented with 1.0% NaCl and 0.2% KCl and normotensive rats received normal tap water. All animals were fed standard laboratory rat chow and had free access to both food and water. The rats were housed individually in a room kept at constant temperature (24ºC) with a day-night cycle interval of 12 h each. Systolic blood pressure (SBP) was determined in conscious restrained rats by the standard tail cuff method (pneumatic transducer), before and after surgery. The experimental protocols performed in this study were in accordance with the State of Michigan guidelines on animal care as well as the standards and policies of the Unit for Laboratory Animal Medicine, University of Michigan.

Intracellular Ca^{2+} measurements

Four to six weeks after surgery, rats were anesthetized with sodium pentobarbital (60-80 mg/kg, *ip*) and killed by pneumothorax. The thoracic aorta was removed, placed in physiological salt solution (PSS) and cleaned of fat and connective tissue. The PSS contained 130 mM NaCl, 4.7 mM KCl, 1.18 mM KH_2PO_4, 1.17 mM $MgSO_4.7H_2O$, 14.9 mM $NaHCO_3$, 26 µM $CaNa_2$-EDTA, 1.6 mM $CaCl_2.H_2O$ and 5.5 mM dextrose. Ca^{2+}-free buffer contained 1 mM ethylene glycol-bis-

(ß-aminoethyl ether)-N,N,N',N'-tetraacetic acid (EGTA) and no Ca^{2+} was added.

Vascular smooth muscle cells (VSMC) were isolated from aortas of normotensive and DOCA-hypertensive rats according to the method of Furspan and Webb (9). Briefly, the thoracic aorta was rapidly removed, placed in 0.1 mM Ca^{2+} Hanks' solution at 22°C and cleaned of fat and connective tissue. The composition of Hanks' solution was 140 mM NaCl, 5.4 mM KCl, 0.44 mM KH_2PO_4, 0.42 mM NaH_2PO_4, 4.17 mM $NaHCO_3$, 26 µM $CaNa_2$-EDTA, 0.10 mM $CaCl_2.H_2O$, 5.0 mM HEPES and 5.5 mM dextrose, pH 7.35. Vessels were cut into 1-mm segments, and placed into 5 ml of 0.1 mM Ca^{2+} Hanks' solution containing 10 mg/ml bovine serum albumin (type I), 1 mg/ml soybean trypsin inhibitor, 5 mM taurine, 0.4 mg/ml dithiothreitol, 0.6 mg/ml type I collagenase, and 0.6 mg/ml papain (all from Sigma, St. Louis, MO). Vessels were incubated at 37°C with gentle shaking for 40 min. After incubation, vessel fragments were removed with a pipette and placed in 15-ml plastic capped tubes and resuspended in 0.1 mM Ca^{2+} Hanks' solution containing albumin, trypsin inhibitor and taurine as described above. Fragments were washed three times in this solution to remove enzymes and then incubated with 5 µM Fura-2 acetoxy methylester (Fura-2/AM) for 45 min at room temperature. After loading with Fura-2/AM, single cells were released from vessel fragments by gentle pipette agitation. Isolated VSMC in suspension were transferred to a recording chamber on the stage of a Leitz Diavert inverted fluorescence microscope equipped with quartz optics. The recording chamber was maintained at 37°C while being superfused at 3 ml/min with PSS equilibrated with 95% O_2 and 5% CO_2. All VSMC were allowed to rest for 30 min for complete de-esterification of Fura-2 from its acetoxy methylester form to the Ca^{2+}-sensitive Fura-2 pentapotassium salt. Single VSMC with relaxed fusiform morphology were isolated in the window of the photomultiplier (PMT). Fluorescence Fura-2 emission was excited alternately with 340-nm and 380-nm bandpass (± 5 nm) filters mounted in a computer-controlled filter wheel (Lambda-10, Sutter Instruments, Novato, CA). Emitted light was filtered with a 500-530 bandpass filter. Fluorescence emissions were captured with the PMT, recorded and processed on a Mac II fx computer using data acquisition/analysis software (LabView 2, Version 2.2.1). The computer also controlled the filter wheel alternation up to a maximum rate of 90 msec/cycle. Isolated VSMC exhibited no detectable autofluorescence. Maximum and minimum $[Ca^{2+}]_i$ were determined at the end of each experimental protocol by treating the cells with PSS containing 1.6 mM Ca^{2+} plus 1 µM ionomycin and 15 mM EGTA (0 mM Ca^{2+}), respectively. The ratios of 340/380 nm were then used to calculate $[Ca^{2+}]_i$ according to the equation of Grynkiewicz et al. (10):

$$[Ca^{2+}]_i = K_d \left(\frac{F - F_{mn}}{F_{mx} - F} \right) \left(\frac{Sf_2}{Sb_2} \right)$$

where: F = fluorescence ratio at 340/380 nm; F_{mx} = the fluorescence ratio in saturating Ca^{2+} (ionomycin) solution; F_{mn} = the fluorescence ratio in Ca^{2+}-free (EGTA) solution, and Sf_2/Sb_2 = the ratio of 380-nm signals in ionomycin and EGTA solutions. It is important to note that external calibration methods using an assumed dissociation constant (K_d) for Fura-2/AM provide only a reasonable estimate of actual $[Ca^{2+}]_i$. Therefore, we have calculated the K_d (297 nM) for Fura-2 in our system. Calibration curves were obtained for most of the cells (basal ratio values as well as agonist-induced changes in the ratio were constant in all cells) and average values were calculated. $[Ca^{2+}]_i$ was determined for all cells using the derived calibration curve. The experimental protocol was performed in various aortic cells from the same rat and the average values were then determined. "N" represents the number of rats used in each protocol.

Experimental protocol

Caffeine-induced increases in $[Ca^{2+}]_i$ and changes in resting $[Ca^{2+}]_i$ were evaluated in single aortic smooth muscle cells as described in Figure 1. Briefly, aortic cells were stimulated with 3 µM 5-hydroxytryptamine (5-HT) (1 min), and rinsed in Ca^{2+}-free buffer for 10 min to deplete intracellular Ca^{2+} stores (depletion of intracellular Ca^{2+} stores was demonstrated by the lack of changes in $[Ca^{2+}]_i$ upon subsequent exposure to 5-HT or caffeine). After Ca^{2+} depletion, intracellular Ca^{2+} stores were loaded by placing the cells in PSS containing 1.6 mM Ca^{2+} for 10 min (loading period). The bathing medium was then replaced with Ca^{2+}-free buffer and the increase in $[Ca^{2+}]_i$ in response to 20 mM caffeine was induced after a 2-min exposure to Ca^{2+}-free PSS. The magnitude of this last response was taken as a measure of the SR Ca^{2+} content (for additional details, see Refs. 7,11,12). The experiment was performed in the presence of 3 µM CPA or vehicle (DMSO). Experiments previously performed in our laboratory (7) have shown that 3 µM CPA inhibits Ca^{2+} uptake into the SR in the aorta from both DOCA and normotensive rats [IC_{50} (-log M) = 5.52 ± 0.23 normotensive *vs* 5.30 ± 0.05 DOCA, N = 6].

The same protocol was used in aortas from normotensive and DOCA rats for contraction measurements to correlate force and $[Ca^{2+}]_i$. Aortic strips were stimulated with 3 µM 5-HT and, when maximal contraction was achieved, vessels were rinsed in Ca^{2+}-free buffer for 10 min and subsequently in PSS containing 1.6 mM Ca^{2+} for an additional 10 min. Arteries were stimulated with 20 mM caffeine after a 1-min exposure to Ca^{2+}-free buffer. The protocol was performed in the presence of 3 µM CPA or vehicle (DMSO). The magnitude of the last response to caffeine was taken as a measure of the functional capacity of the SR to release Ca^{2+}.

Statistical analysis

Data are reported as means ± SEM. The Student *t*-test was used to compare observations between DOCA-hypertensive and normotensive groups and differences related to treatment. The Bonferroni correction was used during multiple testing procedures. Values of $P<0.05$ were considered to be statistically significant.

Drugs

All drugs were prepared daily and kept on ice during the course of the experiments. 5-Hydroxytryptamine (5-HT) hydrochloride, caffeine, cyclopiazonic acid (CPA) and deoxycorticosterone acetate (DOCA) were purchased from Sigma Chemical Co., St. Louis, MO. Ionomycin (Calbiochem, La Jolla, CA), CPA and Fura-2/AM (Molecular Probes, Eugene, OR) were prepared as stock solutions and dissolved in dimethyl sulfoxide (DMSO, Fisher Chemical, Fair Lawn, NJ). The final concentration of DMSO in the bath chamber did not exceed 0.1% v/v. The DOCA implant was prepared as 1:2 parts DOCA/Silastic (Dow Chemical Co., Midland, MI) .

Results

Systolic blood pressure values were significantly higher ($P<0.05$) in the DOCA group (195 ± 10 mmHg; N = 7) than in the normotensive group (120 ± 3 mmHg; N = 7), 4-6 weeks after surgery. Mean body weight was 358 ± 16 g for DOCA rats (N = 7) and 478 ± 7 g for normotensive rats (N = 7; $P<0.05$).

Effects of CPA on increases in $[Ca^{2+}]_i$ stimulated by caffeine

Changes in $[Ca^{2+}]_i$ are indicated by alterations in the fluorescence ratio at 340 and

380 nm (340/380 ratio). Basal $[Ca^{2+}]_i$ values were significantly higher in DOCA aortic cells compared to normotensive cells (86.4 ± 14.4 nM, N = 5 *vs* 43.0 ± 6.7 nM, N = 5, respectively; P<0.05). Since the resting $[Ca^{2+}]_i$ in aortic cells differed between normotensive and DOCA rats, 5-HT- and caffeine-induced increases in $[Ca^{2+}]_i$ as well as changes in resting $[Ca^{2+}]_i$ in aortic smooth muscle cells from normotensive and DOCA rats were reported as the differential increase in $[Ca^{2+}]_i$, i.e., peak $[Ca^{2+}]_i$ minus resting $[Ca^{2+}]_i$. Figure 1 illustrates the protocol employed in this study. The cells were stimulated with 3 µM 5-HT and a phasic increase in $[Ca^{2+}]_i$ was observed. Ca^{2+}-free buffer containing 3 µM CPA or vehicle was then introduced into the recording chamber and the cells were allowed to equilibrate for 10 min. After this period, 1.6 mM Ca^{2+} buffer containing CPA or vehicle was placed in the chamber and the cells were allowed to equilibrate for an additional 10 min. Ca^{2+}-free buffer was then reintroduced into the bath chamber and allowed to equilibrate for 2 min before 20 mM caffeine was introduced. The differential peak increase in $[Ca^{2+}]_i$ induced by caffeine in Ca^{2+}-free buffer was taken as a measure of Ca^{2+} released from intracellular stores. No differences were observed in 3 µM 5-HT-induced increase in $[Ca^{2+}]_i$ between normotensive and DOCA aortic cells. The increase in $[Ca^{2+}]_i$ observed with 5-HT was 445 ± 49 nM for cells from DOCA rats, N = 5, and 350 ± 89 nM for cells from normotensive rats, N = 7. As can be observed in the representative traces in Figure 1, the increase in $[Ca^{2+}]_i$ observed in response to 20 mM caffeine was higher in aortic cells from DOCA rats (Figure 1B, 329 ± 36 nM, N = 5, P<0.05) compared to normotensive aortic cells (Figure 1A, 249 ± 16 nM, N = 7). Incubation with 3 µM CPA significantly reduced (P<0.05) the subsequent increase in $[Ca^{2+}]_i$ stimulated by 20 mM caffeine both in normotensive (Figure 1C) and DOCA aortic cells (Figure 1D). This concentration of CPA was utilized because the effects of CPA, indicated by IC_{50} (-log M) values, on caffeine-induced phasic contractions did not differ between DOCA (5.95 ± 0.10, N = 6) and normotensive (5.76 ± 0.25, N = 6) aorta and this concentration produces significant inhibition of caffeine-induced contractions (7). The increase in $[Ca^{2+}]_i$ in response to caffeine in the presence of 3 µM CPA was DOCA = 141 ± 29 nM (N = 5) *vs* normotensive = 102 ± 14 nM (N = 5).

Effects of CPA on resting $[Ca^{2+}]_i$

During the loading period, no changes in $[Ca^{2+}]_i$ were observed in normotensive aortic cells (Figure 1A). However, in DOCA cells, a small increase in $[Ca^{2+}]_i$ (45 ± 11 nM, N = 5; P<0.05; Figure 1B) was observed when Ca^{2+} was reintroduced into the chamber bath. When the same protocol was used in the presence of 3 µM CPA, both normotensive (Figure 1C) and DOCA-hypertensive rat aortic cells (Figure 1D) exhibited increases in $[Ca^{2+}]_i$ during the loading period. However, the increase was higher in DOCA aortic cells compared to normotensive cells (122 ± 30 nM, N = 5 *vs* 23 ± 10 nM, N = 5, respectively; P<0.05). No changes in $[Ca^{2+}]_i$ during the loading period were observed in aortic cells from DOCA rats when 3 µM CPA and 1 µM of the voltage-activated L-type Ca^{2+} channel antagonist nifedipine were introduced simultaneously into the chamber bath (Figure 2D). These results suggest that an increased extracellular Ca^{2+} influx mediates this response in aortas from hypertensive rats.

Figure 3 summarizes the alterations in force and $[Ca^{2+}]_i$ exhibited by aortic strips and isolated smooth muscle cells, respectively, from DOCA and normotensive rats for the protocol employed in this study. Aortic strips and isolated cells were stimulated with 3 µM 5-HT. Ca^{2+}-free buffer containing 3 µM CPA or vehicle was then introduced

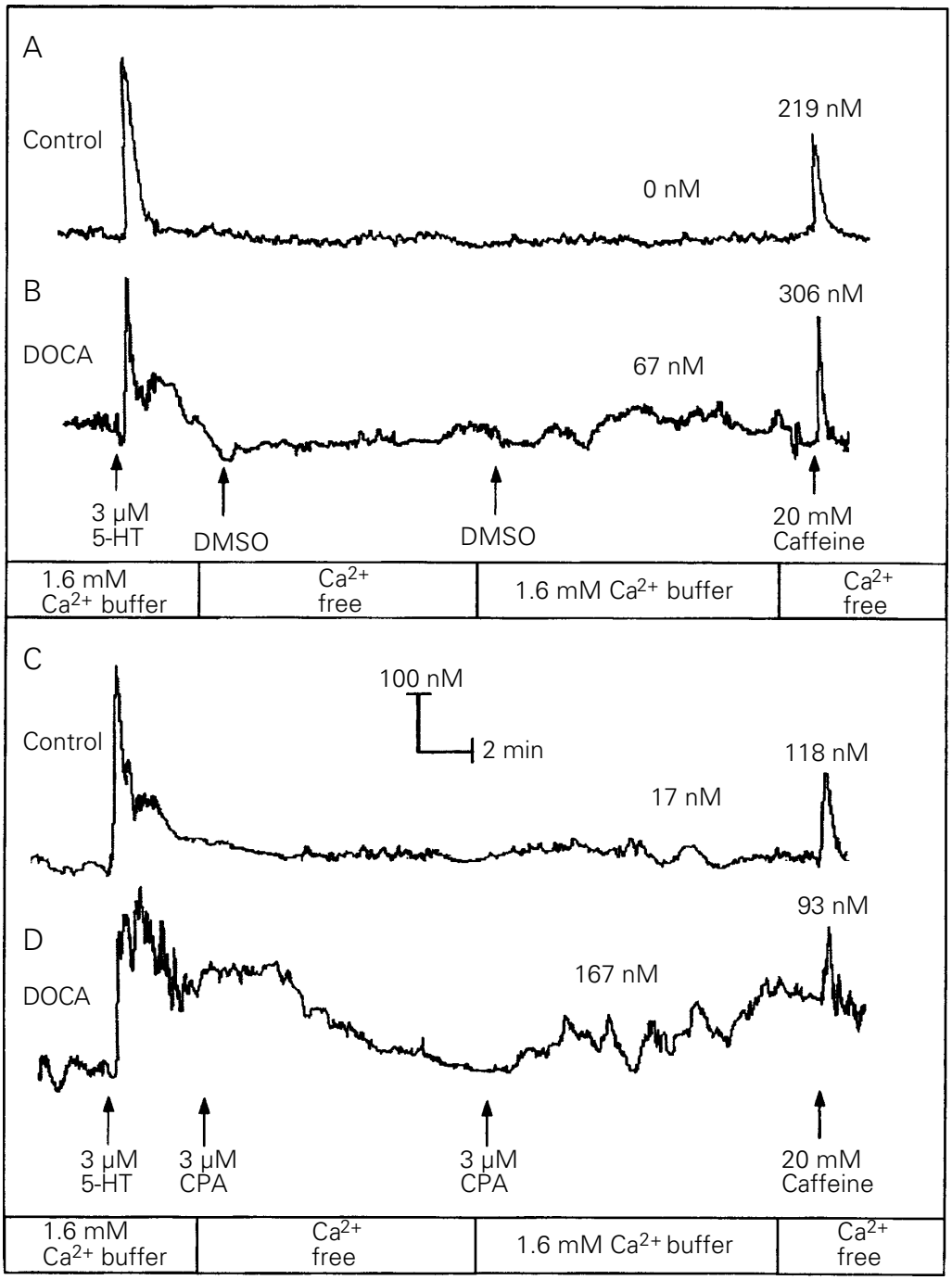

Figure 1 - $[Ca^{2+}]_i$ of rat aortic smooth muscle cells. Aortic myocytes from DOCA-hypertensive and normotensive rats were stimulated with 3 µM 5-HT for 1 min and the protocol described in Methods was performed. Briefly, following stimulation with 5-HT, aortic cells were rinsed in Ca^{2+}-free buffer for 10 min to deplete intracellular Ca^{2+} stores. After Ca^{2+} depletion, intracellular Ca^{2+} stores were loaded by placing the cells in PSS containing 1.6 mM Ca^{2+} for 10 min. After a 2-min exposure to Ca^{2+}-free PSS the cells were stimulated with 20 mM caffeine. Tracings A (normotensive) and B (DOCA) illustrate control (no CPA) responses in aortic cells using DMSO as vehicle. Changes in $[Ca^{2+}]_i$ during the Ca^{2+}-loading period and in response to 20 mM caffeine were higher in aortic cells from DOCA-hypertensive rats compared to normotensive cells. Tracings C (normotensive) and D (DOCA) illustrate the effects of CPA. CPA (3 µM) potentiated the increase in $[Ca^{2+}]_i$ during the loading period both in DOCA and normotensive aortic cells. However, the increase in $[Ca^{2+}]_i$ during the Ca^{2+}-loading period was higher in aortic cells from DOCA-hypertensive rats compared to normotensive cells. CPA also attenuated phasic responses to 20 mM caffeine in DOCA-hypertensive and in normotensive aortic cells. Tracings are representative of 5-7 experiments and values indicated in the figure were obtained from one of these experiments.

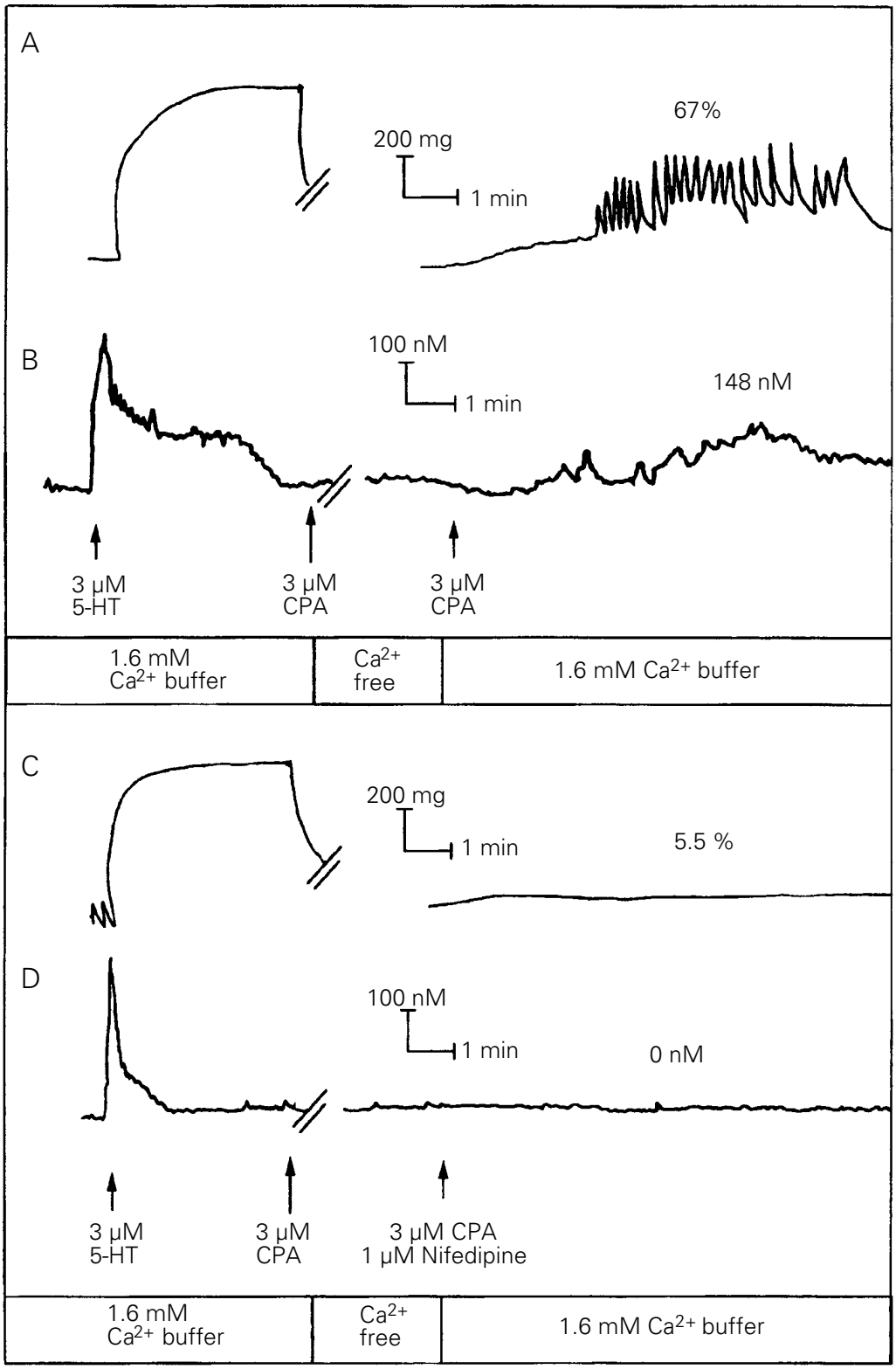

Figure 2 - Effects of nifedipine on CPA-induced contraction and the increase in $[Ca^{2+}]_i$ in DOCA aortic smooth muscle cells. The same protocol as described in the legend to Figure 1 was performed in aortas and isolated aortic myocytes from DOCA-hypertensive rats in the presence of 3 µM CPA (A and B) or CPA plus 1 µM nifedipine (C and D). Nifedipine inhibited both contractile responses and the increase in $[Ca^{2+}]_i$ during the Ca^{2+}-loading period in DOCA aortic cells. Tracings are representative of 6 experiments and values indicated in the figure were obtained from one of these experiments.

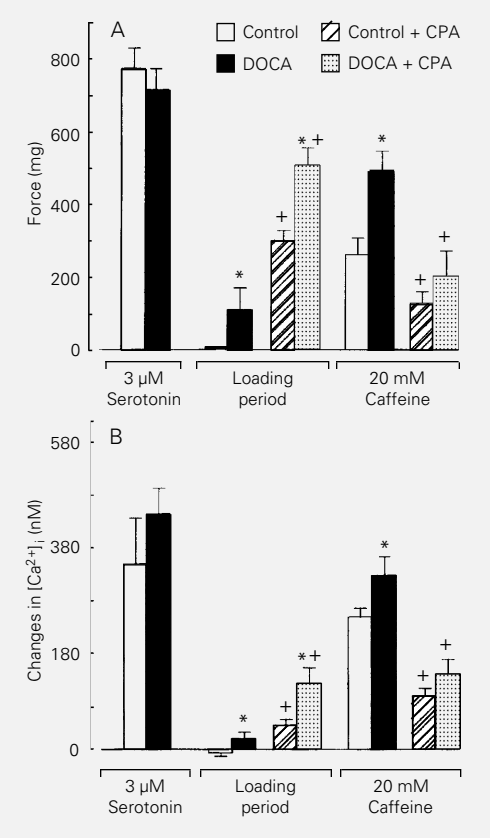

Figure 3 - Changes in tone (A) and $[Ca^{2+}]_i$ (B) in DOCA-hypertensive and normotensive aortic smooth muscle cells. Alterations in force observed in DOCA aortas in the presence of vehicle or CPA, during the protocol previously described (Figures 1 and 2), are comparable to the alterations in $[Ca^{2+}]_i$. The bars indicate mean ± SEM for changes in force (mg, N = 10-12) and $[Ca^{2+}]_i$ (nM, N = 5-7). DOCA, DOCA-hypertensive; control, normotensive; CPA, cyclopiazonic acid (3 µM). *P<0.05 compared to the normotensive group; $^{+}$P<0.05 compared to control conditions (vehicle).

into the recording chamber for 10 min to deplete intracellular Ca^{2+} stores. During the loading period, 1.6 mM Ca^{2+} buffer containing CPA or vehicle was placed in the chamber for an additional 10 min. Ca^{2+}-free buffer was then reintroduced into the bath before 20 mM caffeine was added. The bars represent the changes in tone and $[Ca^{2+}]_i$ observed after 3 µM 5-HT stimulation, during the loading period, and in response to 20 mM caffeine. No differences in 5-HT-induced contraction or increase in $[Ca^{2+}]_i$ were observed between the DOCA and normotensive groups. Changes in tone or $[Ca^{2+}]_i$ during the loading period under control conditions (vehicle) were observed only in DOCA preparations. In the presence of 3 µM CPA, aortic strips and isolated cells from both DOCA and normotensive rats presented changes in tone and $[Ca^{2+}]_i$ during the loading period, but the changes were higher in DOCA preparations. The contractile responses and increases in $[Ca^{2+}]_i$ stimulated by caffeine were higher in the DOCA group compared to those in the normotensive group. CPA inhibited caffeine-induced contractions and increases in $[Ca^{2+}]_i$ in both groups.

Discussion

The present study was performed to determine the effects of CPA, a specific and reversible inhibitor of the SR Ca^{2+}-ATPase, on $[Ca^{2+}]_i$ of aortic cells isolated from normotensive and DOCA-hypertensive rats. We have tested the hypothesis that CPA increases $[Ca^{2+}]_i$ in aortic smooth muscle cells and that the increase in $[Ca^{2+}]_i$ is higher in cells from DOCA-hypertensive rats. Changes in $[Ca^{2+}]_i$ stimulated by caffeine as well as the effects of CPA on caffeine-induced responses were also evaluated in both types of vascular smooth muscle cells. Cyclopiazonic acid is an indole tetramic acid metabolite derived from tryptophan which is produced by fungi such as *Aspergillus* and *Penicillium.* The specific inhibition of the SR Ca^{2+}-ATPase by CPA may be related to the inhibition of conformational changes associated with ATP hydrolysis and Ca^{2+} transport (1). However, unlike thapsigargin, a tumor-promoting sesquiterpene lactone which binds irreversibly to the SR Ca^{2+}-ATPase, CPA does not block the enzyme in the E_1 or E_2 state (1,13,14).

These agents promote depletion of intracellular Ca^{2+} stores by inhibiting Ca^{2+} uptake into the SR and, consequently, they inhibit responses to drugs that release Ca^{2+} from intracellular stores (5-7). CPA reduces or abolishes contractions induced by caffeine in guinea pig ileum (3), in rat skeletal muscle and mesenteric arteries (5,6) and in aortas from normotensive and DOCA-hypertensive rats (7). In the present study, CPA also decreased the caffeine-induced phasic increase in $[Ca^{2+}]_i$ (Figure 1C,D) in rat aortic myocytes, consistent with an inhibitory action of CPA on Ca^{2+} uptake into the SR. The CPA-induced inhibition of the caffeine-induced increase in $[Ca^{2+}]_i$

is not related to the inhibition of cyclic adenosine 3',5'-monophosphate (cAMP) phosphodiesterase or the increase in cAMP, since no inhibition of caffeine-induced responses was observed in the presence of vehicle.

The caffeine-induced differential peak increase in $[Ca^{2+}]_i$ in Ca^{2+}-free buffer, a reflection of Ca^{2+} released from the SR, was higher in DOCA aortic cells compared to normotensive cells (Figure 1A,B). We have also observed that phasic contractile responses to caffeine in Ca^{2+}-free buffer were enhanced in aorta from DOCA rats (7). Taken together, these observations support our previous suggestion of an expansion of intracellular Ca^{2+} stores in vessels from DOCA-hypertensive rats (7).

The basal $[Ca^{2+}]_i$ was higher in DOCA aortic smooth muscle cells compared to normotensive cells. Elevated intracellular Ca^{2+} levels have been reported in aortic smooth muscle cells from spontaneously hypertensive rats (SHR) compared to normotensive Wistar Kyoto (WKY) rats (15,16) and have been associated with increased vascular reactivity in hypertension (17-21). Cytochemical studies on aortic smooth muscle cells from DOCA rats have demonstrated increased cytoplasmic Ca^{2+} and elevated Ca^{2+} in the sarcoplasmic area (22,23), a fact that also supports our observations.

An increase in $[Ca^{2+}]_i$ during the loading period was only observed in aortic cells from DOCA-hypertensive rats. Chen et al. (24) have proposed that the SR regulates $[Ca^{2+}]_i$ and acts as a superficial buffer barrier by taking up Ca^{2+} that enters the smooth muscle cell. In this model, the SR may determine the threshold $[Ca^{2+}]_i$ that induces contraction and may regulate the steady-state $[Ca^{2+}]_i$. A dysfunction in the SR Ca^{2+} uptake would decrease the ability of the smooth muscle cells to control $[Ca^{2+}]_i$ and would result in increased $[Ca^{2+}]_i$. We have previously suggested that the increase of $[Ca^{2+}]_i$ in DOCA aorta, indicated by spontaneous contractions, is not due to an impairment of Ca^{2+} uptake into the SR, but to increased cell membrane permeability to Ca^{2+} (7). Since Ca^{2+} uptake into the superficial SR is mediated mainly by Ca^{2+}-ATPase, which is a saturable active transporter, it could be bypassed by a high rate of Ca^{2+} entry. Ca^{2+} entering at slower rates could be effectively removed from the cytoplasm before activation of the contractile proteins. Since the spontaneous increase in $[Ca^{2+}]_i$ in DOCA aortic cells is only observed in the presence of external Ca^{2+}, a higher Ca^{2+} influx may occur in these cells.

CPA-induced increase in $[Ca^{2+}]_i$ was observed in both aortic smooth muscle cells from DOCA and normotensive rats. Baró and Eisner (25) have shown that thapsigargin increases $[Ca^{2+}]_i$ in smooth muscle cells isolated from the rat mesenteric artery by inducing Ca^{2+} release from the norepinephrine- and caffeine-sensitive stores. In our studies, the $[Ca^{2+}]_i$ increase was not due to Ca^{2+} release from intracellular stores since it was also observed after depletion and during loading of the intracellular Ca^{2+} stores. The modulatory effects of the SR on Ca^{2+} entry through the plasma membrane have been proposed in the capacitative model (26,27), which suggests that depletion of intracellular Ca^{2+} stores activates extracellular Ca^{2+} entry, possibly in order to refill the intracellular Ca^{2+} stores. In lymphocytes (28) and in *Xenopus* oocytes (29), the emptying of intracellular Ca^{2+} stores activates Ca^{2+} influx through the release of an intracellular messenger. CPA can indirectly promote intracellular Ca^{2+} store depletion since it inhibits Ca^{2+} uptake into the SR. The empty stores may trigger the influx of external Ca^{2+} and, in this sense, may explain the different effects of CPA which have been reported. In endothelial cells, CPA induces external Ca^{2+} influx through non-selective cation channels probably by a continuous leak of Ca^{2+} from the endoplasmic reticulum (8). However, in myocardial cells no changes were observed in membrane currents in response to CPA (4). In the present study, the observation that the CPA-induced increase in $[Ca^{2+}]_i$ as

well as the CPA-induced changes in tone did not occur in Ca^{2+}-free buffer and were blocked by nifedipine supports the idea that CPA activates Ca^{2+} influx. Similarly, in skeletal muscle arterioles, it has been shown that the CPA-induced increase in tone is inhibited by nifedipine and Ca^{2+}-free PSS (6). The mechanisms by which CPA increases $[Ca^{2+}]_i$ are unknown, but there are suggestions that CPA inhibits Ca^{2+}-dependent K^+ channels (30) and increases Ca^{2+} influx through voltage-operating Ca^{2+} channels (31).

An increased transmembrane Ca^{2+} flux as well as altered activity of Ca^{2+} channels have been reported in vessels from hypertensive animals (32-36). Furthermore, Soltis and Field (37) reported an increased reactivity to KCl and to norepinephrine in femoral arteries from DOCA rats, which was related to increased sensitivity to extracellular Ca^{2+}, supporting the suggestion of an augmented membrane permeability to Ca^{2+}. While an increased membrane permeability to Ca^{2+} in DOCA aortic cells can account for the alterations observed, alternative mechanisms must be considered. For example, reduced Ca^{2+} extrusion through plasma membrane Ca^{2+}-ATPase, and increased sensitivity of the contractile proteins to Ca^{2+} have been reported in the literature (19,20). Interactions between Na^+ and $[Ca^{2+}]_i$ should also be taken into consideration. Changes in the transmembrane Na^+ gradient may alter, for example, the activity of the Na^+-Ca^{2+} exchanger, which couples Ca^{2+} efflux to Na^+ influx, an important mechanism for Ca^{2+} extrusion following a transient rise in $[Ca^{2+}]_i$ (38). In fact, an increased intracellular Na^+ content has been shown in vascular smooth muscle from DOCA-hypertensive rats (39,40). The increased intracellular Na^+ content may alter the Na^+-Ca^{2+} exchange system, with a consequent increase in $[Ca^{2+}]_i$.

The data presented here support the hypothesis that CPA increases $[Ca^{2+}]_i$ in vascular smooth muscle from DOCA-hypertensive and normotensive rats and that the increase in $[Ca^{2+}]_i$ is higher in DOCA aortic smooth muscle cells. The alterations in Ca^{2+} handling observed in DOCA aorta do not involve an inadequate SR buffering ability, as previously suggested, but are possibly related to an increased membrane permeability to Ca^{2+}. The augmented intracellular Ca^{2+} levels in DOCA aorta may be the consequence of increased Ca^{2+} influx and may be responsible for the greater contractile responses to caffeine observed in this hypertensive model.

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