Mechanism of Ca\textsuperscript{2+} release and entry during contraction elicited by norepinephrine in rat resistance arteries

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Lagaud, G. J. L., V. Randriamboavonjy, G. Roul, J. C. Stoclet, and R. Andriantsitohaina. Mechanism of Ca\textsuperscript{2+} release and entry during contraction elicited by norepinephrine in rat resistance arteries. Am. J. Physiol. 276 (Heart Circ. Physiol. 45): H300–H308, 1999.—The intracellular Ca\textsuperscript{2+} stores and the mechanisms of Ca\textsuperscript{2+} entry produced by norepinephrine (NE) were investigated in small mesenteric resistance arteries of the rat. In Ca\textsuperscript{2+}-free medium, NE (10 µM) elicited a transient increase in both intracellular free Ca\textsuperscript{2+} concentration ([Ca\textsuperscript{2+}]i) and tension that were both drastically reduced by caffeine and only partially reduced by the two sarco(endo)plasmic reticulum Ca\textsuperscript{2+}-ATPase (SERCA) blockers thapsigargin and cyclopiazonic acid, despite the presence of SERCA2a and SERCA2b isoforms in the medial smooth muscle layer of the artery. After depletion of intracellular Ca\textsuperscript{2+} stores with 10 µM NE, addition of exogenous CaCl\textsubscript{2} (2.5 mM) produced large and sustained increases in both [Ca\textsuperscript{2+}]i and contraction of the arteries provided that the agonist was continuously present. In these conditions, the responses to CaCl\textsubscript{2} were inhibited by the voltage-dependent Ca\textsuperscript{2+} entry blocker nitrendipine (1 µM), the putative inhibitor of receptor-operated Ca\textsuperscript{2+} entry SKF-96365 (30 µM), and NiCl\textsubscript{2} (1 mM). The inhibition produced by SKF-96365 and NiCl\textsubscript{2} was greater than that of nitrendipine. Also, the responses to CaCl\textsubscript{2} were greatly reduced or abolished in the presence of the Na\textsuperscript{+}/Ca\textsuperscript{2+} exchanger inhibitors 1,3-dimethyl-2-thiourea, 3',4'-dichlorobenzamil, MgCl\textsubscript{2}, and amiloride or after omission of NaCl in the medium. Also, protein kinase C inhibitors, calphostin C and staurosporine, and tyrosine kinase inhibitors, genistatin and tyrphostin 23, both reduced the responses to CaCl\textsubscript{2}. The inhibitory effect of protein kinase C inhibitor and tyrosine kinase were additive. These results suggest that NE releases Ca\textsuperscript{2+} from intracellular stores that are caffeine sensitive and partially sensitive to SERCA inhibitors. They indicate that in addition to Ca\textsuperscript{2+} influx via nitrendipine-sensitive and SKF-96365-sensitive channels, Na\textsuperscript{+}/Ca\textsuperscript{2+} exchanger participates in the CaCl\textsubscript{2}-induced contraction produced in NE-exposed vessels. The pathway leading to Ca\textsuperscript{2+} entry probably involves tyrosine kinase and protein kinase C. All the above mechanisms require ongoing receptor stimulation.

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IT IS WELL ESTABLISHED that the contractile response to a number of agonists including norepinephrine (NE) comprises two distinct components in Ca\textsuperscript{2+}-containing medium: an initial phasic component that results from

the inositol 1,4,5-trisphosphate [Ins(1,4,5)P\textsubscript{3}]-mediated release of Ca\textsuperscript{2+} from intracellular Ca\textsuperscript{2+} stores followed by a tonic component that requires Ca\textsuperscript{2+} entry in the continuous presence of the agonist, due to Ca\textsuperscript{2+} influx (4).

Despite the existence of a large body of literature exploring the roles of Ca\textsuperscript{2+} influx and release from intracellular stores in the vasoconstriction induced by agonists, the relative contribution of each of these mechanisms is still under debate. In vascular smooth muscle, it seems that the mechanisms of Ca\textsuperscript{2+} release triggering contraction may vary between vascular beds and with the stimulus. For example, in the rat portal vein, one of the two characterized Ca\textsuperscript{2+} stores is sensitive to caffeine [an opener of “Ca\textsuperscript{2+}-induced Ca\textsuperscript{2+} release” ryanodine-sensitive channels (CICR)], whereas both of them are sensitive to Ins(1,4,5)P\textsubscript{3} (33). Conversely, only one of the two Ca\textsuperscript{2+} stores characterized in the dog mesenteric artery is released by Ins(1,4,5)P\textsubscript{3}, whereas the other is mobilized by Ca\textsuperscript{2+} (22). These observations suggest differential involvement of CICR and Ins(1,4,5)P\textsubscript{3}-sensitive Ca\textsuperscript{2+} release channels and associated stores.

Ca\textsuperscript{2+} influx across the plasma membrane generally involves voltage-operated channels and the so-called “capacitative entry pathway” activated by store depletion (10). However, the signaling mechanism for Ca\textsuperscript{2+} entry via the latter pathway is complex and may also be different depending on the cell type. A number of mediators have been proposed to be implicated for this phenomenon, although no such mediator has been unequivocally demonstrated as yet. These mediators included inositol 1,3,4,5-tetrakisphosphate (17), small G proteins (5), a “Ca\textsuperscript{2+}-influx factor,” a small phosphate containing nonprotein compound (30), cytochrome P-450, or one of their products (2). Finally, a role for tyrosine kinase has been proposed in activation of the capacitative Ca\textsuperscript{2+} entry (16).

All of the above findings suggest that agonist stimulation may activate more than one Ca\textsuperscript{2+} entry pathway that may be different depending on the cell type. In resistance vessels, the presence of both ryanodine- and Ins(1,4,5)P\textsubscript{3}-sensitive Ca\textsuperscript{2+} release mechanisms has been characterized in the rat small mesenteric artery (32). However, the involvement of these two mechanisms in NE-induced contraction and the relationship between Ca\textsuperscript{2+} store depletion and Ca\textsuperscript{2+} entry is unknown.

The aim of the present study is to further characterize the intracellular Ca\textsuperscript{2+} stores released by NE and to investigate the mechanism of Ca\textsuperscript{2+} entry during the
tonic contraction produced by this agonist in the small mesenteric resistance artery of the rat, using different pharmacological agents active on the different pathways possibly involved.

**METHODS**

Arterial preparation and mounting. Male Wistar rats (250-350 g) bred in our institute were killed by cervical dislocation and exsanguinated by carotid artery transection. The viscera were exposed, and a proximal segment of the small bowel was removed and pinned in a dissecting dish containing physiological salt solution (PSS) of the following composition (in mM): 119 NaCl, 4.7 KCl, 0.4 KH2PO4, 14.9 NaHCO3, 1.17 MgSO4, 2.5 CaCl2, and 5.5 glucose. In Ca2+-free PSS, Ca2+ was omitted and 1 mM EGTA was added. Branch II or III resistance arteries were cleaned of fat and connective tissue, and a segment 2 mm long was removed. The segment was then mounted in a myograph (as previously described in Ref. 3) filled with PSS continuously kept at 37°C and gassed with a mixture of 95% O2-5% CO2 (pH 7.4). Briefly, two tungsten wires (30 µm diam) were inserted through the lumen of the vessel. Mechanical activity was recorded isometrically by a force transducer (Kistler-Morse, DSG BE4) connected to one of the two tungsten wires, the other being attached to a support carried by a micromanipulator.

After being set up, the vessel was equilibrated for 30 min before being passively stretched to an internal diameter that yields a circumference equivalent to 90% of that given by an internal pressure of 100 mmHg, which requires a load of ~200 mg. The internal diameter of the vessels used in this study ranged between 150 and 200 µm. After the vessel was set to its working length, it was challenged twice with 10 µM NE to elicit reproducible contractile responses. All the experiments were carried out on vessels without endothelium. The endothelium was removed by intraluminal perfusion with 0.5% 3-(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate (CHAPS) for 30 s. The presence of functional endothelium was assessed in all preparations by the ability of acetylcholine (1 µM) to induce relaxation of vessels precontracted with NE (10 µM).

Contraction experiments. In the present study, NE was used throughout the experiments at a concentration (10 µM) giving the maximal contractile response in small mesenteric artery of the rat.

To study the component of NE-induced contraction due to internal Ca2+ release, three intracellular Ca2+ mobilizing agents were used at their maximally active concentrations on rat mesenteric resistance arteries as described previously by Lagaud et al. (19). These agents were thapsigargin (1 µM) and cyclopiazonic acid (20 µM), two inhibitors of the sarcendoplasmic reticulum Ca2+-ATPases (SERCA), and caffeine (10 mM), an activator of the CICR. These experiments were performed in Ca2+-free PSS. The tissue was left 20 min in Ca2+-free PSS before the start of the experiment. The intracellular Ca2+ stores were loaded with Ca2+ by stimulating the vessels with a depolarizing solution, i.e., 100 mM KCl-PSS containing 2.5 mM CaCl2 in which KCl was substituted for an equimolar amount of NaCl. Thapsigargin and cyclopiazonic acid were preincubated for 20 min before the addition of NE.

To study the mechanisms involved in the Ca2+ entry after store depletion induced by NE, the vessels were challenged with 10 µM NE in Ca2+-free medium containing 1 mM EGTA. After 20 min, exogenous CaCl2 (2.5 mM) was added in the bath in the continuous presence of the agonist. The same experimental condition was used in the absence and presence of different pharmacological agents. A washout period of 30 min was allowed between each experimental protocol.

Ca2+ entry blockers were used at their maximally active concentrations, being 1 µM for the voltage-operated Ca2+ channel blocker nifedipine, 30 µM for the reported receptor-mediated Ca2+ entry blocker SKF-96365 (1-[β-[3-(methylphenyl)]-propoxy]-1-p-methoxyphenethyl]-1H-imidazole hydrochloride) (21), and 1 mM for the inorganic Ca2+ channel blocker NiCl2.

Four inhibitors of the Na+/Ca2+ exchanger were also used: 1,3-dimethyl-2-thiourea (DMTU) at 25 mM, 3’,4’-dichlorobenzenzimidazole (DCB) at 10 µM, MgCl2 at 10 mM, and amiloride at 1 mM, as described previously (20, 34). In some vessels, experiments were performed in PSS in which NaCl was omitted and replaced with an equimolar amount of choline chloride.

The involvement of different protein kinases in the mechanism of Ca2+ entry was tested using the tyrosine kinase inhibitors genistein (30 µM) and tyrphostin 23 (100 µM) and the protein kinase C inhibitors staurosporine (30 nM) and calphostin C (1 µM). All the inhibitors were used at their maximally active concentrations and were preincubated 30 min before activation by NE (10 µM).

Measurement of intracellular Ca2+. Simultaneous measurements of intracellular Ca2+ concentration ([Ca2+]i) and contraction were performed to study the relationships between Ca2+ stores, Ca2+ entry, and the contraction induced by NE in small mesenteric arteries. Changes in [Ca2+]i were determined by measuring the fluorescence of trapped fura 2 with a dual-excitation wavelength fluorometer (Fluorolog II, SPEX, Edison, NJ) as described by Andriantsitohaina et al. (3). The vessel segments were loaded over a 2-h period with fura 2 by incubation in the dark in PSS containing both 5 µM fura 2-AM and 2% pluronic acid. The tissue was then returned to the chamber and washed three times with PSS (37°C) to remove excess external fura 2-AM. The experiments were performed in PSS (37°C) continuously gassed with 95% O2-5% CO2 (pH 7.4). At the end of each experiment, the Ca2+ signal was calibrated using ionomycin (20 µM), NE (10 µM), and CaCl2 (5 mM) for the maximal fluorescence and 20 mM EGTA in Ca2+-free solution for the minimal fluorescence. The ratio of fluorescence of the emission of fura 2 was obtained at 510 and 505 nm and was calculated after subtraction of the autofluorescence at 340 and 380 nm. Background fluorescence and autofluorescence were measured by looking at the fluorescence of the vessel without the dye at 510 nm after its excitation at 340 and 380 nm.

Immunofluorescence labeling of SERCA2a and SERCA2b. Segments of rat small mesenteric arteries were immersed in freshly prepared 4% paraformaldehyde in 0.1 M sodium phosphate buffer (pH 7.4) for 6 h. The samples were then soaked overnight in buffer containing 20% sucrose. The samples were then frozen in cooled isopentane (−50°C) and sectioned on a cryostat microscope; 12-µm-thick sections were flow-mounted on gelatin-coated slides. The sections were then processed with the conventional indirect immunofluorescence technique. The antibodies used were the SERCA2a and SERCA2b isomeric-specific monoclonal antibodies elicited in rabbits against peptides encompassing the 12 COOH-terminal amino acids (1031–1042) of SERCA2b and 9 COOH-terminal amino acids (989–997) of SERCA2a. The preparation and specificity of these antibodies have both been previously described (35).

The antibodies against SERCA2a and SERCA2b were diluted of these antibodies have both been previously described (35). The preparation and specificity of these antibodies have both been previously described (35). The antibodies against SERCA2a and SERCA2b were diluted 1:50 in 0.05 M phosphate-buffered 0.9% saline, pH 7.3, containing 0.5% Triton X-100. The primary antibodies were applied overnight at 4°C. The secondary antibody was the FITC-labeled anti-rabbit IgG (Biosys, France). FITC-labeled sections were coverslipped in polyvinyl alcohol mounting.
medium containing paraphenylene diamine. Control experiments were similarly processed omitting the primary antibodies.

Expression of results and statistical analysis. Contractions were expressed as a percentage of the maximal contractile response obtained with 10 µM NE. [Ca²⁺]_i was calculated using the equation previously described by Grynikiewicz et al. (15) and expressed in nanomolar. In the resting state, basal [Ca²⁺]_i was 108 ± 14 (n = 5).

All results are expressed as means ± SE of n experiments. ANOVA was used for statistical analysis. Means were considered significantly different when the P value was < 0.05.

Drugs. Acetylcholine chloride, caffeine, CHAPS, cyclopiazonic acid, DMTU, genistein, MgCl₂, NE bitartrate, NiCl₂, thapsigargin, tyrophostin 23, and staurosporine were purchased from Sigma (Grenoble, France). Calphostin C and ryanodine were purchased from Calbiochem (France). Fura 2-AM was purchased from Molecular Probes (Eugene, OR). DCB was a generous gift from Dr. C. Frelin (Nice, France). Nitrendipine was a generous gift from Bayer (Paris, France). SKF-96365 was a generous gift from SmithKline Beecham Pharmaceuticals (London, UK). SERCA2a and SERCA2b were a generous gift from Dr. F. Wuytack (Katholieke Universiteit Leuven, Belgium). Nitrendipine was dissolved in absolute ethanol. Calphostin C, DCB, and thapsigargin were dissolved in dimethyl sulfoxide (1 mg/ml), and the final concentration of dimethyl sulfoxide in the bath was 0.1%. Genistein, tyrophostin 23, and staurosporine were dissolved in dimethyl sulfoxide, and the maximal concentration of dimethyl sulfoxide in the bath was 1.2% for 100 µM tyrophostin 23. All other drugs were diluted in deionized water (Q10, Millipore).

RESULTS

Acetylcholine was not able to produce relaxation in vessels precontracted with NE after endothelium denudation with CHAPS. Also, the latter procedure resulted in a 10-fold increase in sensitivity to NE, the EC₅₀ values being reduced from 1.01 ± 0.01 µM (n = 90) to 0.101 ± 0.01 µM (n = 62; P < 0.001). Maximal peak response obtained with 10 µM NE was not significantly different in vessels with and without functional endothelium [1.53 ± 0.48 g (n = 90) and 1.14 ± 0.47 g (n = 62), respectively]. These data show that the use of CHAPS to functionally denude the endothelium removed the inhibitory effect of the endothelium without significantly altering the contractile function of the vessel.

Characterization of intracellular stores released by NE. Immunostainings using specific antibodies for SERCA2a and SERCA2b isoforms were performed to assess the presence and distribution of SERCA isoforms in different layers of the vessels (Fig. 1). SERCA2a (Fig. 1A) and SERCA2b (Fig. 1B) stainings were observed in the endothelial and in the medial layers of the vessels. No staining was observed in negative controls performed in vessels in the absence of SERCA2a and SERCA2b antibodies (Fig. 1C).

In Ca²⁺-free medium, NE produced a fast transient increase in both [Ca²⁺]_i and tension (Fig. 2). In the same condition, caffeine also elicited a fast transient increase in [Ca²⁺]_i, and tension and drastically reduced a subsequent response to NE. It should be noted that in the same condition, the inhibitor of CICR, ryanodine (10 µM), did not elicit contraction but prevented a subsequent response to NE (Fig. 2B). Also in Ca²⁺-free medium, thapsigargin alone did not induce any change in [Ca²⁺]_i and tension, but it reduced the increase in [Ca²⁺]_i and the contractile response subsequently induced by NE (Fig. 2). Similar results were obtained with another inhibitor of SERCA, cyclopiazonic acid, used under the same experimental conditions (Fig. 2B). The caffeine response was not affected by prior stimulation with thapsigargin or cyclopiazonic acid, but it was prevented by NE.

Mechanisms of Ca²⁺ entry after depletion of intracellular stores with NE. None of the pharmacological agents used significantly affected the contraction produced by NE in Ca²⁺-free medium except DCB (Table 1). When added after both [Ca²⁺]_i, and tension had returned to the baseline, CaCl₂ elicited a large and sustained rise in both [Ca²⁺]_i and contraction that was reduced by 50% by nitrendipine and significantly more inhibited by SKF-96365 (76%) and NiCl₂ (74%) (Fig. 3). Under the same experimental conditions, no responses were observed after addition of CaCl₂ when caffeine, thapsigargin, or cyclopiazonic acid was used instead of NE (data not shown).

The effect of the Na⁺/Ca²⁺ exchange inhibitors DMTU and amiloride on the increase in both [Ca²⁺]_i and contraction produced by CaCl₂ has been studied in NE-exposed vessels after depletion of intracellular stores (Fig. 4). Both responses were reduced by DMTU and drastically reduced by amiloride. It should be noted that DMTU and amiloride did not produce any rise in [Ca²⁺]_i and contraction when added to unstimulated vessels. Also as shown in Fig. 4, the other inhibitors of

Fig. 1. Immunofluorescence labelings of sarcoplasmic reticulum Ca²⁺-ATPase (SERCA)2a and SERCA2b of small mesenteric arteries representative of 3 different experiments. A: SERCA2a. B: SERCA2b. C: negative control. Calibration bar, 50 µm.
Na+/Ca2+ exchange, DCB and MgCl2, greatly reduced or abolished the contraction produced by CaCl2 in NE-exposed vessels after depletion of intracellular stores. The rank order of efficacy of these inhibitors was DMTU, DCB, MgCl2, and amiloride. Also, omission of NaCl in the PSS and its replacement with choline did not produce any rise in [Ca2+]i and contraction but abolished the contractile response to CaCl2. It should be noted that nitrendipine reduced the DMTU-insensitive response to CaCl2, and SKF-96365 abolished this response. The responses to CaCl2 in NE-exposed vessels were 99 ± 2.2, 47 ± 4.5, 10 ± 0.9, and 2 ± 1.3% (n = 5) in the absence and presence of DMTU, DMTU plus nitrendipine, and DMTU plus SKF-96365, respectively.

The tyrosine kinase inhibitor genistein reduced the increases in both [Ca2+]i and contraction produced by CaCl2 in vessels that had previously been depleted with NE (Fig. 5). The mean of CaCl2-induced contractile force was significantly decreased by genistein and tyrphostin 23. In the presence of either genistein or tyrphostin 23, nitrendipine failed to inhibit further CaCl2-induced contraction elicited by NE.

The effect of the protein kinase C inhibitor staurosporine on the increase in both [Ca2+]i and contraction produced by CaCl2 has been studied in NE-exposed vessels after depletion of intracellular stores (Fig. 6). Staurosporine reduced the increases in both [Ca2+]i and contraction produced by CaCl2 in NE-exposed arteries. The exogenous CaCl2-induced contractile response of NE exposed vessels was also significantly reduced by another protein kinase C inhibitor, calphostin C (Fig. 6). The inhibitory effects of genistein and staurosporine or genistein and calphostin C on the CaCl2-induced contraction elicited by NE were additive (Fig. 6), suggesting that tyrosine kinase inhibition and protein kinase C inhibition decreased the CaCl2-induced contractile response of NE-exposed vessels through different pathways.

**DISCUSSION**

The above results characterize intracellular stores from which Ca2+ are released by NE and the mechanisms of Ca2+ entry triggered by the agonist in rat
small mesenteric artery smooth muscle cells. One major finding is that, in NE exposed arteries, tonic contraction induced by the addition of CaCl$_2$ after depletion of Ca$^{2+}$ stores involved the Na$^+$/Ca$^{2+}$ exchanger in addition to Ca$^{2+}$ entry through both dihydropyridine- and SKF-96365-sensitive mechanisms. In addition, the data obtained with inhibitors support the involvement of both tyrosine kinase and protein kinase

Fig. 3. A: representative traces showing effects of nitrendipine (1 µM) and SKF-96365 (30 µM) on increase in both [Ca$^{2+}$] (top traces) and tension (bottom traces) induced by CaCl$_2$ (2.5 mM) on NE (10 µM)-exposed vessels after depletion of intracellular stores. B: histograms showing contractions induced by addition of CaCl$_2$ (2.5 mM) in small mesenteric resistance arteries exposed to 10 µM NE in absence (1st bar, n = 44) and presence of either 1 µM nitrendipine (2nd bar, n = 9), 30 µM SKF-96365 (3rd bar, n = 5), or 1 mM NiCl$_2$ (4th bar, n = 5). Contractions are percentage of maximal contraction elicited by 10 µM NE in same arteries in normal PSS. Values are means ± SE. ***P < 0.001, significantly different from control. †P < 0.05, significantly different from contraction produced by NE in presence of nitrendipine.

Fig. 4. A: representative traces showing effects of 1,3-dimethyl-2-thiourea (DMTU; 25 mM) and amiloride (1 mM) on increase in both [Ca$^{2+}$] (top traces) and tension (bottom traces) induced by CaCl$_2$ (2.5 mM) on NE (10 µM)-exposed vessels after depletion of intracellular stores. B: histograms showing contractions induced by addition of CaCl$_2$ (2.5 mM) in small mesenteric resistance arteries exposed to 10 µM NE in absence (1st bar, n = 4) and in presence of either 25 mM DMTU (2nd bar, n = 5), 10 µM 3',4'-dichlorobenzamil (3rd bar, n = 5), 1 mM amiloride (4th bar, n = 5), 10 mM MgCl$_2$ (5th bar, n = 5), or substitution of NaCl with choline (6th bar, n = 5). Contractions are percentage of maximal contraction elicited by 10 µM NE in same arteries in normal PSS. Values are means ± SE. ***P < 0.001, significantly different from control.
Fig. 5. A: representative traces showing effects of genistein (30 µM) on increase in both [Ca\(^{2+}\)]\(_i\) (top traces) and tension (bottom traces) induced by CaCl\(_2\) (2.5 mM) on NE (10 µM)-exposed vessels after depletion of intracellular stores. B: histograms showing contractions induced by addition of CaCl\(_2\) (2.5 mM) in small mesenteric resistance arteries exposed to 10 µM NE in absence (1st bar, n = 44) and presence of either 30 µM genistein (2nd bar, n = 6), 100 µM tyrphostin 23 (3rd bar, n = 5), genistein plus nitrendipine (4th bar, n = 5), or tyrphostin 23 plus nitrendipine (5th bar, n = 5). Contractions are percentage of maximal contraction elicited by 10 µM NE in same arteries in normal PSS. Values are means ± SE. ***p < 0.001, significantly different from control.

Fig. 6. A: representative traces showing effects of staurosporine (30 µM) on increase in both [Ca\(^{2+}\)]\(_i\) (top traces) and tension (bottom traces) induced by CaCl\(_2\) (2.5 mM) on NE (10 µM)-exposed vessels after depletion of intracellular stores. B: histograms showing contractions induced by addition of CaCl\(_2\) (2.5 mM) in small mesenteric resistance arteries exposed to 10 µM NE in absence (1st bar, n = 24) and presence of either 30 µM staurosporine (2nd bar, n = 7), 1 µM calphostin C (3rd bar, n = 4), genistein plus staurosporine (4th bar, n = 5), or genistein plus calphostin C (5th bar, n = 4). Contractions are percentage of maximal contraction elicited by 10 µM NE in same arteries in normal PSS. Values are means ± SE. ***p < 0.001, significantly different from control.
C in \(Ca^{2+}\) entry elicited by NE. All of the above mechanisms required the continuous presence of the agonist.

The results indicate that the release of \(Ca^{2+}\) induced by NE takes place at the level of an intracellular \(Ca^{2+}\) store that can also be mobilized by caffeine and ryanodine.

Two SERCA inhibitors, thapsigargin and cyclopiazonic acid, with different chemical structures, were used at concentrations that are able to cause a rapid increase in \([Ca^{2+}]_i\), and to empty \(Ca^{2+}\) stores in other vascular smooth muscle cells (9) and vessels (23, 27). Despite the presence in the medial layer of the two SERCA isoforms (SERCA2a and SERCA2b), which are generally found in vascular smooth muscle cells and are inhibited by both thapsigargin and cyclopiazonic acid (21), both drugs failed to increase \([Ca^{2+}]_i\) and tension in rat small mesenteric arteries. It cannot be ruled out that both thapsigargin and cyclopiazonic acid did not reach the SERCA at sufficient concentrations to entirely inhibit them, although this is unlikely because the small mesenteric arteries are much thinner than other vessels like the aorta. It is possible that the fura 2 probe could not detect local increase in \([Ca^{2+}]_i\) produced by SERCA inhibitors in the absence of any signal inducing sufficient \(Ca^{2+}\) release (31). However, the data reported above suggest that NE induced the release of \(Ca^{2+}\) from two storage compartments in small mesenteric arteries (23, 27). The activation of this current relies on store depletion by thapsigargin or by cyclopiazonic acid, and both of them can be depleted by the CICR reagents caffeine and ryanodine. This interpretation is consistent with the recent finding that a thapsigargin-insensitive \(Ca^{2+}\) pool is present in a subcompartment of the endoplasmic reticulum in different mammalian cells (29). In addition, the presence of SERCA2a and SERCA2b in the endothelium layer of small mesenteric arteries found in the present study is consistent with previously reported endothelium-dependent relaxation elicited by SERCA inhibitors in these vessels (11).

In general, depletion of \(Ca^{2+}\) stores is believed to induce the so-called capacitative \(Ca^{2+}\) entry. This phenomenon has been described in a vascular muscle cell line (A7r5) after store depletion by thapsigargin or by an agonist, vasoressin (7). It probably results from \(Ca^{2+}\) release activated current described in various cells (10). The activation of this current relies on store depletion, but it does not require the continuous presence of the agonist (10). The ionic channels involved in the so-called capacitative \(Ca^{2+}\) entry have been studied in rat aorta (26). It was found that \(Ca^{2+}\) entry occurred through a cromakalim- and dihydropyridine-sensitive \(Ca^{2+}\) channel after store depletion by a mechanism independent of the presence of the agonist. In contrast, the continuous presence of NE was necessary, in rat small mesenteric arteries, for the increase in \([Ca^{2+}]_i\), and contraction elicited by the addition of \(CaCl_2\) after store depletion. It has been reported that SKF-96365 is able to inhibit \(Ca^{2+}\) release activated current (10); however, because this drug also inhibits dihydropyridine-sensitive \(Ca^{2+}\) channels, as discussed below, its effect is not specific to \(Ca^{2+}\) entry through the capacitative entry route.

After depletion of the stores, the increases in \([Ca^{2+}]_i\) and contraction induced by \(CaCl_2\) in NE-exposed arteries were reduced by the dihydropyridine \(Ca^{2+}\) entry blocker nitrendipine and by a drug which can inhibit both voltage-operated and receptor-operated \(Ca^{2+}\) channels, SKF-96365 (19, 24). It should be noted that the inhibitory effect of SKF-96365 was greater than that of nitrendipine, whereas the latter was not able to produce further inhibition in the presence of SKF-96365. Thus, after \(Ca^{2+}\) store depletion, NE stimulates \(Ca^{2+}\) entry mainly through both voltage-dependent \(Ca^{2+}\) channels sensitive to nitrendipine. In accordance with data reported in the literature obtained in the same type of artery, voltage-dependent \(Ca^{2+}\) channels appear to play a significant role in the regulation of \([Ca^{2+}]_i\) in these vessels (25). Also, the above data showed that NE might stimulate the so-called receptor-operated channels sensitive to SKF-96365. This conclusion is consistent with the dual effect of SKF-96365 on the \(Ca^{2+}\) entry through inhibition of the opening of both receptor-operated and voltage-operated \(Ca^{2+}\) channels in rat small resistance arteries previously described by Lagaud et al. (19) in the case of ATP. It is reinforced by the finding that nonselective blockade of \(Ca^{2+}\) channels by high concentration of NiCl_2 (known to produce nonselective \(Ca^{2+}\) channels blockade) reduced the responses to \(CaCl_2\) of NE-exposed vessels to the same extent as that observed in the presence of SKF-96365.

The hypothesis that another possible route for \(Ca^{2+}\) entry into vascular smooth muscle cells via the Na\(^{+}/Ca^{2+}\) exchanger has also been tested here. The extracellular \(CaCl_2\)-induced responses on NE-exposed mesenteric arteries were abolished when extracellular Na\(^+\) was omitted from PSS. Also, the \(CaCl_2\)-induced increase in \([Ca^{2+}]_i\), and contraction produced by NE were greatly reduced or abolished by the Na\(^{+}/Ca^{2+}\) exchange inhibitors DMTU and DCB or MgCl_2 and amiloride, respectively. Moreover, the DMTU-insensitive component of the \(CaCl_2\)-induced responses was reduced or abolished by nitrendipine or SKF-96365, respectively. The reported specific inhibitor of the Na\(^+)/Ca^{2+}\) exchanger, DCB, at a concentration below the IC_{50} values at which it suppressed the inward Na\(^{+}/Ca^{2+}\) exchange current in cardiac ventricular cell (34), also reduced the extracellular \(CaCl_2\)-induced responses on NE-exposed vessels. Taken together, these data show that extracellular Na\(^+\) is needed for the extracellular \(CaCl_2\)-induced responses, and they indicate that the Na\(^{+}/Ca^{2+}\) exchanger may contribute to the pathway leading to \(Ca^{2+}\) entry produced by NE. Such observations are consistent with previous reports showing that NE or KCl, by depolarizing the cells, produces a rise in intracellular Na\(^+\) that thereby activates a reverse mode of the Na\(^{+}/Ca^{2+}\) exchanger, leading to an increase of \(Ca^{2+}\) influx in vascular smooth muscle cells in addition to the opening of \(Ca^{2+}\) channels (18). Also, the present results are consistent with those reported in the literature showing the presence of the Na\(^{+}/Ca^{2+}\) exchanger as a viable mechanism for \(Ca^{2+}\) transport in other cell types such as the endothelium of
rabbit cardiac valve, in which the Ca\(^{2+}\) entry component is enhanced when intracellular Na\(^+\) concentration is elevated (20). Moreover, the present data are in agreement with those reported by Blaustein (6) showing that NE elicited a rise in intracellular Na\(^+\) concentration (via activation of the Na\(^+\)/H\(^+\) exchanger, following its phosphorylation by protein kinase C) and a subsequent reverse mode of Na\(^+\)/Ca\(^{2+}\) exchange operation. Indeed, we found here that the protein kinase C inhibitors staurosporine and calphostin C reduced the CaCl\(_2\)-induced responses on NE-exposed vessels. However, it is difficult to determine the exact intracellular target of protein kinase C on the CaCl\(_2\)-induced responses on NE-exposed vessels, inasmuch as its role in agonist-induced contraction is not essential in rat mesenteric small arteries (28). Nevertheless, the present results suggest that the Na\(^+\)/Ca\(^{2+}\) exchange may participate in the increase of [Ca\(^{2+}\)]\(_i\), and contraction produced by CaCl\(_2\) in NE-exposed vessels after depletion of intracellular stores. Finally, the fact that nitrendipine reduced the DMTU-insensitive response to CaCl\(_2\) and SKF-96365 abolished this response reinforce the hypothesis that the Ca\(^{2+}\) entry produced by NE is composed of two parts, activation of voltage-dependent Ca\(^{2+}\) channels and receptor-dependent Ca\(^{2+}\) channels sensitive to SKF-96365.

There is evidence that tyrosine kinases participate in the regulation of Ca\(^{2+}\) entry associated with agonist-induced contraction in smooth muscle cells (16). In the present study, we used two groups of tyrosine kinase inhibitors, a compound interacting with the ATP binding site of the enzyme, genistein, and a compound that interacts with the substrate binding site of the enzyme, tyrphostin 23. Both tyrosine kinase inhibitors reduced the CaCl\(_2\)-induced increase in [Ca\(^{2+}\)]\(_i\) and the resultant contraction in NE-exposed vessels. Although the inhibitory effect of genistein and tyrphostin 23 on tyrosine phosphorylation was not directly assessed in the present study, the properties of these agents at the concentrations used have been previously well documented (1, 13). Multiple effects of tyrosine kinase inhibitors on vascular smooth muscle contraction have been reported in the literature. These effects include blockade of a step involved in Ca\(^{2+}\) entry and Ca\(^{2+}\) store refilling by the agonist and blockade of the effect of Ca\(^{2+}\) on the contractile apparatus. In the present study, neither genistein nor tyrphostin 23 affected the increase in [Ca\(^{2+}\)]\(_i\) and contraction produced by NE in Ca\(^{2+}\)-free medium, suggesting that tyrosine kinase did not play a role in mediating the responses linked to the release of intracellular Ca\(^{2+}\) produced by NE. Furthermore, the inhibitory effects of genistein and staurosporine or genistein and calphostin C on the CaCl\(_2\)-induced responses in NE-exposed vessels were additive, suggesting that tyrosine kinase did not affect the protein kinase C-sensitive component of the contraction associated with the entry of Ca\(^{2+}\) produced by NE. Because nitrendipine did not produce an additional inhibitory effect on the Ca\(^{2+}\)-induced responses on NE-exposed vessels in the presence of either genistein or tyrphostin 23, it is most likely that tyrosine kinase activation by NE modulates the entry of Ca\(^{2+}\) linked to the opening of voltage-dependent Ca\(^{2+}\) channels sensitive to nitrendipine. Tyrosine kinase inhibitors might reduce the Ca\(^{2+}\)-induced responses in vessels exposed to NE at the level of the contractile apparatus. However, neither genistein nor tyrphostin 23 modified significantly responses to KCl depolarization (KCl, 100 mM) in the same arteries. Also, genistein did not significantly affect the response to CaCl\(_2\) (3 µM) on vessels permeabilized with β-escin (data not shown). Taken together, these data suggest that tyrosine kinase inhibitors might not mediate their effects through the inhibition of contractile machinery. Also, it should be noted that at the concentrations used, both genistein and tyrphostin 23 have been reported to inhibit tyrosine kinase activity but not other kinases linked to the signal transduction leading to vascular contraction such as myosin light-chain kinase (1, 13). Therefore, the present study provides pharmacological evidence supporting the involvement of tyrosine kinase in the Ca\(^{2+}\) influx activated by NE-mediated store depletion at the level of dihydroxyridine-sensitive Ca\(^{2+}\) entry in rat small resistance arteries.

We cannot exclude that the complexity of the signal transduction involved in the responses to NE might be due to the activation of multiple α\(_1\)-adrenoceptor populations. Indeed, the characterization of α\(_1\)-adrenoceptor population in small resistance arteries shows that NE mainly activates α\(_1\)-adrenoceptor population, which belongs to α\(_1\)A-, α\(_1\)B-, and the pharmacologically defined α\(_1\)II-subtypes (8, 12, 33). Further studies are needed to better understand the nature of the receptor subtypes implicated in the observed different pathways in these arteries.

In summary, the present study shows that NE releases Ca\(^{2+}\) from intracellular stores that are sensitive to caffeine and partially sensitive to SERCA inhibitors. They also suggest that, in addition to the participation of nitrendipine-sensitive and SKF-96365-sensitive channels, the Na\(^+\)/Ca\(^{2+}\) exchange may contribute to the Ca\(^{2+}\) influx produced by NE after depletion of intracellular stores. Finally, this Ca\(^{2+}\) influx requires ongoing receptor activation and probably involves tyrosine kinase and protein kinase C.

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REFERENCES