Cellular mechanisms and role of endothelin-1-induced calcium oscillations in pulmonary arterial myocytes

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Hyvelin, Jean-Marc, Christelle Guibert, Roger Marthan, and Jean-Pierre Savineau. Cellular mechanisms and role of endothelin-1-induced calcium oscillations in pulmonary arterial myocytes. Am. J. Physiol. 275 (Lung Cell. Mol. Physiol. 19): L269–L282, 1998.—The effect of endothelin (ET)-1 on both cytosolic Ca2+ concentration ([Ca2+]i) and membrane current in freshly isolated myocytes, as well as on the contraction of arterial rings, was investigated in rat main pulmonary artery (RMPA) and intrapulmonary arteries (RIPA). ET-1 (5–100 nM, 30 s) induced a first [Ca2+]i peak followed by 3–5 oscillations of decreasing amplitude. In RMPA, the ET-1-induced [Ca2+]i response was fully abolished by BQ-123 (0.1 µM). In RIPA, the response was inhibited by BQ-123 in only 21% of the cells, whereas it was abolished by BQ-788 (1 µM) in 70% of the cells. In both types of arteries, the response was not modified in the presence of 100 µM La3+ or in the absence of external Ca2+ but disappeared after pretreatment of the cells with thapsigargin (1 µM) or neomycin (0.1 µM). In RPMA myocytes clamped at −60 mV, ET-1 induced an oscillatory inward current, the reversal potential of which was close to the equilibrium potential for Cl−. This current was unaltered by the removal of external Ca2+ but was abolished by niflumic acid (50 µM). In arterial rings, the ET-1 (100 nM)-induced contraction was decreased by 35% in the presence of either niflumic acid (50 µM) or nifedipine (1 µM). These results demonstrate that ET-1 via the ETA receptor only in RMPA and both ETA and ETB receptors in RIPA induce [Ca2+]i oscillations due to a release of Ca2+ from an inositol trisphosphate-sensitive Ca2+ store. Ca2+ release secondarily activates an oscillatory membrane Ca2+ current that can depolarize the cell membrane, leading to an influx of Ca2+, this latter contributing to the ET-1-induced vasoconstrictor effect.

PULMONARY VASCULAR TONE is controlled by a variety of circulating and locally released mediators, including the peptides endothelin (ET)-1 and angiotensin II (ANG II), which are the most potent vasoconstrictors (3). These two peptides are also implicated in a variety of pulmonary pathophysiological processes such as cell proliferation (7, 45) or pulmonary hypertension (11, 25). A better knowledge of cellular and molecular mechanisms mediating the effect of these peptides would be of interest to further understand pulmonary vascular diseases.

At the vascular smooth muscle cell site, ET-1 and ANG II bind to specific membrane receptors, inducing an increase in the cytosolic Ca2+ concentration ([Ca2+]i) that triggers the contractile response (28, 33). Unlike for ANG II (15), the detailed mechanisms linking the binding of ET-1 to its receptor to the activation process of the contractile apparatus in pulmonary vascular smooth muscle are not fully elucidated. In all of the different vascular beds, ET-1 produces slowly developing and sustained vasoconstrictor responses. However, the sources of activator Ca2+ and the cellular mechanisms underlying these responses vary. ET-1 increases [Ca2+]i by either stimulating a Ca2+ influx through the plasma membrane (13) or releasing Ca2+ from internal Ca2+ stores (29, 41). Ca2+ influx can result from the opening of dihydropyridine-sensitive Ca2+ channels via a G protein (24) or as a consequence of membrane depolarization due to the activation of Cl− channels (26, 40), nonselective cation channels (4), or inhibition of ATP-sensitive K+ channels (31). More recently, a receptor-mediated Ca2+-permeable nonselective cation channel has been implicated in membrane depolarization and Ca2+ entry activated by ET-1 in aortic smooth muscle (32). Whatever the pathways responsible for this [Ca2+]i increase, the pattern of ET-1-induced [Ca2+]i response generally corresponds to an initial peak followed by a sustained plateau of a smaller amplitude (10). However, in myocytes from small pulmonary arteries, oscillations in [Ca2+]i have been observed in response to ET-1 (2). This pattern of [Ca2+]i response mimics what we have previously described in the main pulmonary artery upon ANG II stimulation (15). Whether ET-1-induced [Ca2+]i oscillations are due to similar cellular mechanisms to those evoked by ANG II is not known. Moreover, in the pulmonary circulation, ET-1-induced vasoconstriction results from the activation of both ETA and ETB receptors, the ratio and the efficacy of coupling of these receptors to the contractile apparatus being species dependent (9) and varying throughout the pulmonary arterial tree (18).

The present work was thus designed to investigate, in both rat main pulmonary artery (RMPA) and intrapulmonary arteries (RIPA) 1) the subtype of ET-1 receptor implicated in the [Ca2+]i response; 2) the sources of Ca2+ and the cellular mechanisms underlying this response; and 3) the contribution of these mechanisms to the ET-1-induced contractile response. Indo 1 microspectrofluorimetry and the whole cell patch-clamp technique were used in freshly isolated myocytes to measure [Ca2+]i and membrane current, respectively. Isometric contraction was measured in arterial rings from the same preparation.

MATERIALS AND METHODS

Tissue preparation. Wistar male rats aged from 8 to 10 wk and weighing 280–340 g were anesthetized by intraperitoneal injection of 40 mg of ethyl carbamate. Heart and lungs
were removed en bloc. The main and small intrapulmonary (277 ± 26.1 µm, n = 22) arteries were then dissected under binocular control, and the adventitial and intimal layers were removed. For contraction experiments, rings (3 mm in length) were dissected from the main artery. For cell dissociation, arteries were cut into several pieces (1 × 1 mm), incubated for 10 min in low-Ca²⁺ (200 µM) physiological saline solution (PSS, composition given below) and then incubated in low-Ca²⁺ PSS containing 0.5 mg/ml collagenase, 0.4 mg/ml pro-

nase, 0.06 mg/ml elastase, and 3 mg/ml bovine serum albumi-

n at 37°C for two successive periods of 20 min, using fresh enzymes each time. After this sequence, the solution was removed and the arterial pieces were incubated again in a fresh enzyme-free solution and triturated with a fire-polished Pasteur pipette to release cells. Cells were stored on glass coverslips at 4°C in PSS containing 0.8 mM Ca²⁺ and used on the same day.

[Ca²⁺]i measurements. To assess the dynamic changes in [Ca²⁺]i of individual arterial myocytes, we used the [Ca²⁺]-
sensitive fluorophore indo 1. In most of the experiments, cells were loaded with indo 1 by incubation in PSS containing 1 mM indo 1-pentaacetoxymethyl ester (indo 1-AM) for 25 min at room temperature and then washed in PSS for 25 min. The coverslip with attached cells was then mounted in a perfusion chamber and continuously perfused. In combined experiments of [Ca²⁺]-i and membrane current measurements, indo 1 (50 µM) was added to the pipette solution and then entered the cells after establishment of the whole cell recording mode (see below). The recording system included a Nikon Diaphot inverted microscope fitted with epifluorescence (Nikon, To-

kyo, Japan). A single cell among those on the coverslip was selected for analysis. The fluorescence ratio (405/480) was measured in the conventional whole cell current recording mode (17) using a Biologic RK 400 patch-clamp amplifier. Membrane currents were recorded with borosili-

cate glass pipettes (170–190 MΩ) from a glass pipette located close to the cell for the period indicated on the records. It was verified, in control experiments, that no change in [Ca²⁺]i was observed during test ejections of PSS. Generally, each record of membrane current and [Ca²⁺]i response to ET-1 alone or in the presence of an additional substance was obtained from a different cell. Each type of experiment was repeated for the number of cells indicated in the text. Experiments were done at room temperature (20–22°C).

Solutions and application of ET-1. The external PSS con-

tained (in mM) 130 NaCl, 5.6 KCl, 1 MgCl₂, 2 CaCl₂, 1.1 d-glucose, and 10 HEPES, pH 7.4 with NaOH. Ca²⁺-free PSS was prepared by replacing CaCl₂ with 0.4 mM EGTA. The internal solution (the solution in the patch pipette and inside the cell) contained (in mM) 120 CsCl, 10 NaCl, and 20 HEPES, pH 7.3 with NaOH. ET-1 was applied to the recorded cell by pressure ejection from a glass pipette located close to the cell for the period indicated on the records. It was verified, in control experiments, that no change in [Ca²⁺]i was observed during test ejections of PSS. Generally, each record of membrane current and [Ca²⁺]i response to ET-1 alone or in the presence of an additional substance was obtained from a different cell. Each type of experiment was repeated for the number of cells indicated in the text. Experiments were done at room temperature (20–22°C).

Isometric contraction measurements. Isometric contraction was measured in rings from RMPA that were mounted between two stainless steel clips in vertical 20-ml organ baths of a computerized isolated organ bath system (IOM1; EMKA Technologies, Paris, France). Baths were filled with Krebs-

Henseleit (KH) solution (composition in mM: 118.4 NaCl, 4.7 KCl, 2.5 CaCl₂, 1.2 MgSO₄, 1.2 KH₂PO₄, 25 NaHCO₃, and 11.1 d-glucose, pH 7.4) maintained at 37°C and bubbled with a 95% O₂-5% CO₂ gas mixture. The upper stainless clip was connected to an isometric force transducer (EMKA Technolo-

gies). Tissues were set at optimal length by equilibration against a passive load of 10 mN as determined in preliminary experiments. At the beginning of each experiment, K⁺-rich (80 mM) solution, obtained by substituting an equimolar amount of KCl for NaCl from KH solution, was repeatedly applied to obtain at least two contractions similar in both amplitude and kinetics. This contraction served as a reference response that was used to normalize subsequent contractile responses. A cumulative concentration-response curve (CCRC) to ET-1 (0.1–100 nM) was then constructed. A concentration increment was made once the maximal contractile effect of the preceding concentration had been recorded (generally 18–20 min). Fifteen minutes before the beginning of the CCRC, the desired channel antagonist (nifedipine or niflumic acid) was administered to one-half of the rings. The unexposed rings served as temporal control.

Chemicals and drugs. Collagenase (type CLS1) was from Worthington Biochemical (Freehold, NJ). Pronase (type E), elastase (type 3), bovine serum albumin, ET-1, neomycin, nifedipine, niflumic acid, phorbol 12,13-dibutyrate (PDBu), ruthenium red, thapsigargin, and tetrodamine were purchased from Sigma (Saint Quentin Fallavier, France). BQ-123, BQ-788, and sarafotoxin (SRTX) S6c were from RBI (Natick, MA). Ca(²⁺) was from Merck (Darmstadt, Germany). Indo 1 was from Calbiochem (France Biochem, Meudon, France). ET-1 was dissolved in distilled water to make aliquots of stock solution (10–¹⁴ M) that were kept frozen (–20°C) until the day of the experiment. Indo 1, niflumic acid, PDBu, and thapsigarin were dissolved in dimethyl sulfoxide (DMSO). The maximal concentration of DMSO used in our experiments was <0.1% and had no effect on the mechanical activity of rings or on the resting value or the variation of the membrane current and [Ca²⁺]i induced by agonists in cells.

Statistical analysis. Results are expressed as means ± SE with n the sample size. Significance was tested by means of Student's t-test at a P value < 0.05. In contraction experiments, EC₅₀, the concentration of ET-1 inducing 50% of the maximal response, was graphically determined from the mean CCRC.

RESULTS

Characteristics of the ET-1-induced [Ca²⁺]i response. Short application (30 s) of ET-1 (5–100 nM) induced cyclic variations (oscillations) of [Ca²⁺]i in single myo-

cytes from both RMPA and RIPA. The ET-1-induced [Ca²⁺]i response was typically composed of four to six oscillations of constant duration (6.48 ± 0.53 s, n = 25,
and 6.73 ± 0.2 s, n = 40, in RMPA and RIPA, respectively) but of decreasing amplitude (Fig. 1A; see also Fig. 3A). The values of the resting [Ca²⁺], the first peak in [Ca²⁺], the delay between the beginning of ET-1 ejection and this first peak, and the frequency of oscillations are indicated in Table 1. Generally, the two last oscillations occurred after the cessation of ET-1 microejection. The pattern of the [Ca²⁺] response was relatively independent of the ejected ET-1 concentration (Fig. 1A). Nevertheless, the percentage of cells that responded to ET-1 did depend on that concentration (20, 84, and 100% of responding cells in RMPA and 32, 80, and 100% of responding cells in RIPA for 5, 50, and 100 nM ET-1, respectively; Fig. 1B). Combination of the number of responding cells with the amplitude of the first [Ca²⁺] peak reveals a relationship between ET-1 concentration and the [Ca²⁺] response (Fig. 1C). Although ET-1 induced a response for concentrations in the nanomolar range, in order to assess the effect of ET-1 in conditions where all of the cells respond, we thus used 100 nM ET-1 in subsequent experiments. This ET-1 concentration is also that inducing the maximal contractile response in pulmonary arterial rings (see below).

Fig. 1. Effect of endothelin (ET)-1 on cytosolic Ca²⁺ concentration ([Ca²⁺]) in pulmonary arterial myocytes. A: short (30-s) ejection of ET-1 (0.01–0.1 µM) near the cell induced oscillations in [Ca²⁺] in the rat main pulmonary artery (RMPA). B: relationship between ET-1 concentration ([ET-1]; abscissa) and the percentage of cells generating oscillations in [Ca²⁺] (responding cells, ordinate) in RMPA and rat intrapulmonary arteries (RIPA). C: relationship between [ET-1] (abscissa) and the amplitude of the first [Ca²⁺] peak in RMPA and RIPA. Open bars represent data obtained solely from responding cells; solid bars include data obtained from nonresponding cells. For each ET-1 concentration, 25 cells were tested in A and B. Nos. in parentheses are sample size.
Effect of ET-1 receptor modulators on the ET-1-induced $[Ca^{2+}]_i$ response. We investigated the effect of BQ-123 and BQ-788, two specific inhibitors of type A and type B ET-1 receptors (ET$_A$, ET$_B$), respectively (19, 21), and that of SRTX S6c, a specific agonist of ET$_B$ receptors (42). BQ-123 and BQ-788 alone did not modify the resting value of $[Ca^{2+}]_i$ in myocytes from both RMPA and RIPA (Table 1).

### Table 1. Effect of ET-1 receptor antagonists BQ-123 and BQ-788 on ET-1- and SRTX S6c-induced $[Ca^{2+}]_i$ response in myocytes isolated from RMPA and RIPA

<table>
<thead>
<tr>
<th></th>
<th>Resting $[Ca^{2+}]_i$, nM</th>
<th>First Peak, nM</th>
<th>Delay, s</th>
<th>Oscillation Frequency, min$^{-1}$</th>
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<tr>
<td><strong>RMPA</strong></td>
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<tr>
<td>Control response (0.1 µM ET-1)</td>
<td>103.8 ± 4.9</td>
<td>685 ± 76.2</td>
<td>6.45 ± 0.96</td>
<td>6.48 ± 0.53</td>
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<tr>
<td>ET-1 + BQ-123 (0.1 µM)</td>
<td>101.2 ± 3.1</td>
<td>536.5 ± 61.5</td>
<td>7.8 ± 1.6</td>
<td>7.5 ± 1.2</td>
<td>25</td>
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<tr>
<td>ET-1 + BQ-788 (1 µM)</td>
<td>100.4 ± 3.17</td>
<td>523.6 ± 53.4</td>
<td>6.5 ± 1.2</td>
<td>7.2 ± 0.63</td>
<td>20</td>
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<td><strong>RIPA</strong></td>
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<tr>
<td>Control response (0.1 µM ET-1)</td>
<td>101.6 ± 2.8</td>
<td>506 ± 23.9</td>
<td>7.5 ± 0.5</td>
<td>7.15 ± 0.42</td>
<td>40</td>
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<td>ET-1 + BQ-123 (1 µM)</td>
<td>103 ± 5.6</td>
<td>494.8 ± 39.9</td>
<td>7.15 ± 0.6</td>
<td>7 ± 0.9</td>
<td>26</td>
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<tr>
<td>ET-1 + BQ-788 (1 µM)</td>
<td>104.2 ± 4.3</td>
<td>516.1 ± 38</td>
<td>7 ± 1.2</td>
<td>7.1 ± 0.73</td>
<td>15</td>
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<tr>
<td>SRTX S6c (0.1 µM)</td>
<td>99.7 ± 5.8</td>
<td>523.6 ± 53.4</td>
<td>6.5 ± 1.2</td>
<td>7.2 ± 0.63</td>
<td>20</td>
</tr>
<tr>
<td>SRTX S6c + BQ-123 (1 µM)</td>
<td>100.2 ± 1.2</td>
<td>516.1 ± 38</td>
<td>7 ± 1.2</td>
<td>7.1 ± 0.73</td>
<td>15</td>
</tr>
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Values are means ± SE; n, no. of cells. $[Ca^{2+}]_i$, cytosolic $Ca^{2+}$ concentration; ET, endothelin; SRTX, sarafotoxin; RMPA, rat main pulmonary artery; RIPA, rat intrapulmonary arteries.

Effect of ET-1 receptor modulators on the ET-1-induced $[Ca^{2+}]_i$ response. We investigated the effect of BQ-123 and BQ-788, two specific inhibitors of type A and type B ET-1 receptors (ET$_A$, ET$_B$), respectively (19, 21), and that of SRTX S6c, a specific agonist of ET$_B$ receptors (42). BQ-123 and BQ-788 alone did not modify the resting value of $[Ca^{2+}]_i$ in myocytes from both RMPA and RIPA (Table 1).

In RMPA, BQ-123 (0.1–1 µM) fully abolished the ET-1-induced $[Ca^{2+}]_i$ response ($n = 15$; Fig. 2A). This effect was rapid since superfusion of cells for only 1 min with the compound was enough to block the response.

**Fig. 2.** Effect of ET-1 receptor antagonists on ET-1 (0.1 µM)-induced $[Ca^{2+}]_i$ response in myocytes from RMPA. A: superfusion of the cell with the ET$_A$ receptor antagonist BQ-123 rapidly abolished the $[Ca^{2+}]_i$ response. B: the ET$_B$ receptor antagonist BQ-788 had no effect on the ET-1-induced $[Ca^{2+}]_i$ response. In A and B, the first record (a) is the control response obtained in the absence of receptor antagonist. Times in parentheses indicate duration of exposure to ET-1 receptor antagonist before ET-1 ejection. Each trace was recorded from a different cell and is typical of 15 cells.
In contrast, superfusion of the cells for 10 min with BQ-788 (1 µM) did not alter the pattern of ET-1-induced [Ca\textsuperscript{2+}] response. The amplitude of the first rise as well as the duration and the frequency of oscillations were not altered (Fig. 2B; Table 1). SRTX S6c (100 nM) did not evoke a [Ca\textsuperscript{2+}] response (n = 15).

In contrast, in RIPA, BQ-123 abolished the ET-1-induced [Ca\textsuperscript{2+}] response in only 21% of tested cells, and BQ-788 abolished the response in 70% of tested cells (Fig. 3B). In the presence of the combination of the two antagonists, the ET-1-induced response was inhibited in all cells (Fig. 3 and Table 1). In responding cells, the presence of only one or the two antagonists did not modify the ET-1-induced [Ca\textsuperscript{2+}] response when compared with the control response (Table 1). SRTX S6c (100 nM) elicited in 71% of tested cells a [Ca\textsuperscript{2+}] response that was similar in amplitude and kinetics to the ET-1-induced [Ca\textsuperscript{2+}] response. The SRTX S6c-induced response was fully abolished by BQ-788 (1 µM; Fig. 4 and Table 1).

Source of Ca\textsuperscript{2+} involved in the ET-1-induced [Ca\textsuperscript{2+}] response. In myocytes from both RMPA and RIPA, application of La\textsuperscript{3+} (100 µM), a potent inhibitor of Ca\textsuperscript{2+} entry pathways (39), or removal of external Ca\textsuperscript{2+} (Ca\textsuperscript{2+}-free PSS) did not significantly modify the resting [Ca\textsuperscript{2+}] value (Table 2). Ten to twelve minutes after the beginning of each of these pretreatments, ET-1 (100 nM) induced an oscillatory [Ca\textsuperscript{2+}] response, the pattern and the amplitude of which were not significantly different from the control response (Fig. 5, A and B, and Table 2).

Effect of modulators of signal transduction pathways on the ET-1-induced [Ca\textsuperscript{2+}] response. To investigate the cellular mechanisms of the ET-1-induced [Ca\textsuperscript{2+}] response, we used different compounds acting on signal transduction pathways in myocytes from RMPA. Neomycin (0.1–1 µM), an inhibitor of phosphoinositide-phospholipase C (PI-PLC; see Ref. 6), progressively abolished the ET-1-induced [Ca\textsuperscript{2+}] response (Fig. 6A and Table 3). In Ca\textsuperscript{2+}-free PSS, PDBu (1 µM), a potent protein kinase C (PKC) activator (35), time dependently inhibited the ET-1-induced [Ca\textsuperscript{2+}] response (Fig. 6B and Table 3).

In the subsequent series of experiments, we used tetracaine and ruthenium red to assess the role of the Ca\textsuperscript{2+}-induced Ca\textsuperscript{2+} release (CICR) mechanism in the ET-1-induced [Ca\textsuperscript{2+}] response. We have previously...
demonstrated the ability of tetracaine to block the caffeine-induced \( [Ca^{2+}] \) response in rat pulmonary artery myocytes (16). Pretreatment of the cells with tetracaine (100–300 \( \mu \)M) for 10–12 min did not modify the ET-1-induced \( [Ca^{2+}] \) response (Table 3). Ruthenium red is also known to inhibit the functioning of the ryanodine-sensitive \( Ca^{2+} \) release channel in muscular tissues (46), including smooth muscle (22). Pretreatment of RMPA myocytes with 10–300 \( \mu \)M ruthenium red concentration dependently inhibited the caffeine (5 mM)-induced \( [Ca^{2+}] \) transient response but had no effect on the ET-1-induced response (Fig. 7, A and B, and Table 3).

Finally, we used niflumic acid, a specific blocker of \( Cl^{-} \) channels (27). Pretreatment of cells with 25 or 50 \( \mu \)M niflumic acid for 10–15 min had no significant effect on the ET-1-induced \( [Ca^{2+}] \) response (Fig. 7C and Table 3).

Effect of ET-1 on the membrane current. In myocytes from RMPA clamped at –60 mV, a value close to that of Fig. 4. Effect of the ET\(_{B}\) receptor agonist sarafotoxin (SRTX) S6c on \( [Ca^{2+}] \) in myocytes from RIPA. A: in most of the tested cells (71%), SRTX S6c (0.1 \( \mu \)M) induced a \( [Ca^{2+}] \) response similar to that evoked by ET-1 (a). SRTX S6c-induced response was not altered in the presence of the ET\(_{A}\) receptor antagonist BQ-123 (b) but was inhibited by the presence of the ET\(_{B}\) receptor antagonist BQ-788 (c). Time in parentheses indicates duration of exposure to ET-1 receptor antagonist before SRTX S6c ejection. Each trace was recorded from a different cell and is typical of 20 and 10 cells, respectively. B: percentage of tested cells exhibiting a \( [Ca^{2+}] \) response in the presence of SRTX S6c alone and in the additional presence of BQ-788.

### Table 2. Effect of \( La^{3+} \) or removal of external \( Ca^{2+} \) on the ET-1-induced \( [Ca^{2+}] \) response in myocytes isolated from RMPA and RIPA

<table>
<thead>
<tr>
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<th>([Ca^{2+}] ) Resting Value, nM</th>
<th>First Peak, nM</th>
<th>Delay, s</th>
<th>Oscillation Frequency, min(^{-1})</th>
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<tr>
<td>RMPA</td>
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<tr>
<td>Control response (0.1 ( \mu )M ET-1)</td>
<td>105.4 ± 5.5</td>
<td>656.4 ± 34.5</td>
<td>6.6 ± 0.6</td>
<td>6.2 ± 0.48</td>
<td>17</td>
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<tr>
<td>ET-1 (0 mM ( Ca^{2+} ))</td>
<td>99.3 ± 2.1</td>
<td>607.2 ± 50.1</td>
<td>6 ± 0.25</td>
<td>6.41 ± 0.64</td>
<td>25</td>
</tr>
<tr>
<td>ET-1 ( La^{3+} ) (100 ( \mu )M)</td>
<td>106.5 ± 3.2</td>
<td>642.7 ± 33.1</td>
<td>6.27 ± 0.31</td>
<td>6.3 ± 0.57</td>
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<td>RIPA</td>
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<tr>
<td>Control response (0.1 ( \mu )M ET-1)</td>
<td>100.3 ± 7.7</td>
<td>505 ± 15.2</td>
<td>6.6 ± 0.98</td>
<td>6.77 ± 0.72</td>
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<tr>
<td>ET-1 (0 mM ( Ca^{2+} ))</td>
<td>97.2 ± 3.45</td>
<td>470.6 ± 37</td>
<td>7.1 ± 1.32</td>
<td>6.64 ± 0.64</td>
<td>15</td>
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Values are means ± SE; n, no. of cells.
the resting potential in these cells (5), application (30 s) of ET-1 (100 nM) induced an oscillatory inward current composed of a first large transient peak of 345 ± 44.2 pA (n = 20) followed by four to six peaks of decreasing amplitude (Fig. 8Aa). Generally, the two last current peaks occurred after the cessation of ET-1 microejection. The I-V relation, obtained by applying a ramp pulse from −60 to +40 mV at the peak of the first current oscillation, was mainly linear, and the $E_{rev}$ was −2.3 mV, a value close to that of the theoretical equilibrium potential for Cl$^-$ ($E_{Cl}$), which was −2.1 mV (Fig. 8B). The ET-1-induced current was not significantly altered in Ca$^{2+}$-free PSS (n = 5; Fig. 8Ab) but vanished after pretreatment of the cells with 1 µM thapsigargin (n = 5, data not shown). Superfusion of the cell with niflumic acid (10–50 µM) concentration dependently abolished the ET-1-induced membrane current (Fig. 8C). Combined recordings of membrane current and [Ca$^{2+}$]$_i$ showed that oscillatory inward current evoked by ET-1 appeared simultaneously with oscillations in [Ca$^{2+}$]$_i$ (n = 4; Fig. 9A). The first and large peak of the inward current (Fig. 9Aa) corresponded to the first and large increase in [Ca$^{2+}$]$_i$ (Fig. 9Ab), whereas subsequent peak currents of decreasing amplitude were associated with subsequent [Ca$^{2+}$]$_i$ oscillations also of decreasing amplitude. Combined recordings also confirmed that niflumic acid (50 µM) fully inhibited ET-1-induced membrane current but had no effect on [Ca$^{2+}$]$_i$ (n = 4; Fig. 9B), as mentioned above for independent measurements.
Effect of ET-1 on the mechanical activity of pulmonary artery. In rings isolated from RMPA, ET-1 (0.1–100 nM) concentration dependently induced slowly developing contractions. The maximal ET-1-induced response was 144.7 ± 6.2% (n = 6) of the 80 mM KCl-induced contraction, and the mean EC50 was 5.8 nM (Fig. 10). Pretreatment of the arterial rings with niflumic acid (50 µM) for 15 min decreased the amplitude of ET-1-induced contraction. The maximal force was reduced by 35.7 ± 6.8% (n = 6). A similar inhibitory effect (34 ± 5%, n = 6) was observed in the presence of nifedipine (1 µM), a voltage-activated Ca2+ channel blocker (data not shown). When niflumic acid (50 µM) and nifedipine (1 µM) were used in combination, the inhibitory effect on the contraction was not significantly different (35 ± 6%, n = 6) from that of each of the

![Fig. 6. Effect of neomycin (A) and phorbol 12,13-dibutyrate (PDBu; B) on [Ca2+]i response induced by 0.1 µM ET-1 in myocytes from RMPA. Superfusion of arterial myocytes with 0.1 µM neomycin or 1 µM PDBu time dependently inhibited [Ca2+]i oscillations induced by ET-1. In A and B, the first record (a) is the control response obtained in the absence of the tested compound. Times in parentheses indicate duration of exposure to the tested compound before ET-1 ejection. Each trace was recorded from a different cell and is typical of 6–17 cells.](image)

### Table 3. Effect of various substances on the ET-1- and CAF-induced [Ca2+]i responses in myocytes isolated from RMPA

<table>
<thead>
<tr>
<th>Substance</th>
<th>[Ca2+]i Resting Value, nM</th>
<th>First Peak, nM</th>
<th>Delay, s</th>
<th>Oscillation Frequency, min⁻¹</th>
<th>n</th>
</tr>
</thead>
<tbody>
<tr>
<td>ET-1 (0.1 µM)</td>
<td>101.1 ± 1.8</td>
<td>708.3 ± 41.4</td>
<td>6.6 ± 0.6</td>
<td>6.9 ± 0.9</td>
<td>17</td>
</tr>
<tr>
<td>ET-1 + neomycin (0.1 µM, 7 min)</td>
<td>107.6 ± 5.8</td>
<td>621.2 ± 62.8</td>
<td>8.6 ± 0.25</td>
<td>3.84 ± 1.05</td>
<td>6</td>
</tr>
<tr>
<td>ET-1 (0.1 µM)</td>
<td>103.4 ± 2.3</td>
<td>665.2 ± 33.6</td>
<td>6.27 ± 0.31</td>
<td>7.3 ± 0.6</td>
<td>15</td>
</tr>
<tr>
<td>ET-1 + PDBu (1 µM, 12 min)</td>
<td>101.2 ± 2.37</td>
<td>546 ± 71.4</td>
<td>7.8 ± 0.4</td>
<td>3.2 ± 0.8</td>
<td>7</td>
</tr>
<tr>
<td>CAF (5 mM)</td>
<td>99.78 ± 5.6</td>
<td>735.2 ± 56.7</td>
<td>1.51 ± 0.1</td>
<td>15</td>
<td></td>
</tr>
<tr>
<td>CAF + tetracaine (300 µM)</td>
<td>101.1 ± 0.3</td>
<td>696.1 ± 73.4</td>
<td>1.2 ± 0.2</td>
<td>4</td>
<td></td>
</tr>
<tr>
<td>CAF (5 mM)</td>
<td>60.7 ± 0.4</td>
<td>53.2 ± 3.4</td>
<td>4</td>
<td>7</td>
<td></td>
</tr>
<tr>
<td>CAF + ruthenium red (200 µM)</td>
<td>103.7 ± 3.1</td>
<td>688.9 ± 41.4</td>
<td>7.02 ± 0.9</td>
<td>6.85 ± 0.9</td>
<td>13</td>
</tr>
<tr>
<td>ET-1 (0.1 µM)</td>
<td>100.15 ± 3.9</td>
<td>686.1 ± 49.6</td>
<td>6.88 ± 0.71</td>
<td>15</td>
<td></td>
</tr>
<tr>
<td>ET-1 + tetracaine (300 µM)</td>
<td>98.6 ± 2.8</td>
<td>588.8 ± 25.7</td>
<td>6.79 ± 0.4</td>
<td>5</td>
<td></td>
</tr>
<tr>
<td>ET-1 (0.1 µM)</td>
<td>70.37 ± 3.4</td>
<td>602.2 ± 38.4</td>
<td>7.34 ± 0.8</td>
<td>5</td>
<td></td>
</tr>
<tr>
<td>ET-1 + ruthenium red (200 µM)</td>
<td>103.7 ± 3.31</td>
<td>629.1 ± 35.2</td>
<td>6.08 ± 0.48</td>
<td>5</td>
<td></td>
</tr>
<tr>
<td>ET-1 (0.1 µM)</td>
<td>109.54 ± 5.77</td>
<td>625.5 ± 32.8</td>
<td>6.8 ± 0.21</td>
<td>20</td>
<td></td>
</tr>
<tr>
<td>ET-1 + niflumic acid (50 µM)</td>
<td>103.7 ± 3.31</td>
<td>625.5 ± 32.8</td>
<td>6.8 ± 0.21</td>
<td>20</td>
<td></td>
</tr>
</tbody>
</table>

Values are means ± SE; n, no. of cells. CAF, caffeine; PDBu, phorbol 12,13-dibutyrate.
channel antagonists (Fig. 10). In control experiments, we verified that the 80 mM KCl-induced contraction was not altered by 50 µM niflumic acid ($n = 4$), whereas it was fully abolished by 1 µM nifedipine ($n = 4$; data not shown).

**DISCUSSION**

Characteristics of ET-1-induced Ca$^{2+}$ signaling and ET-1 receptor subtypes involved. The present work demonstrates that the vasoconstrictor effect of ET-1 in pulmonary arteries is underlined by a complex Ca$^{2+}$ signaling at the site of the arterial myocyte. The induction of [Ca$^{2+}$]$_i$ oscillations seems to be a property shared by a variety of pulmonary vascular tone modulators (15, 16). As previously observed for ANG II in RMPA myocytes, the pattern and the amplitude of ET-1-induced [Ca$^{2+}$]$_i$ response are relatively independent of the ET-1 concentration, whereas the percentage of responding cells increases with increasing ET-1 concentration. A similar lack of dependence between the amplitude of agonist-induced Ca$^{2+}$ release and agonist concentration (the so called all-or-none response) has been shown in other cell types. Nevertheless, combining the percentage of responding cells with
the all-or-none character of the ET-1 response (Fig. 1C) reveals a concentration-dependent relationship in the entire population of tested cells and may account for the graduated physiological response in the whole tissue. Unlike for ANG II- and ATP-induced [Ca\(^{2+}\)]\(_i\) oscillations in the same preparation (15, 16), [Ca\(^{2+}\)]\(_i\) did not return to its baseline value between each [Ca\(^{2+}\)]\(_i\) rise induced by ET-1. In this respect, [Ca\(^{2+}\)]\(_i\) oscillations observed in the present work exhibit an intermediary pattern between those generally observed in nonexcitable cells and referred to as baseline spikes and sinusoidal oscillations (38).

Although the ET-1-induced [Ca\(^{2+}\)]\(_i\) response displays a similar pattern in RMPA and in RIPA, the type of membrane receptor involved is different according to the site along the pulmonary arterial tree. In RMPA, the [Ca\(^{2+}\)]\(_i\) response is entirely mediated by the \(\alpha\)-type receptor of ET-1 (ETA), since it is fully blocked by the compound BQ-123 and, conversely, is not altered by the compound BQ-788. This result is in accordance with contractile experiments showing that the ET-1 mechanical response in RMPA is mediated by only ETA receptors (18). This ETA receptor subtype distribution is similar to that observed in human pulmonary arteries (9). In contrast, in RIPA, both ETA and ETB receptors are involved in the ET-1-induced [Ca\(^{2+}\)]\(_i\) response, with a major distribution of the B subtype. This is assessed by the effect of SRTX S6c, which produces oscillations in 71% of the cells, and that of BQ-123, the ETA inhibitor and BQ-788, the ETB inhibitor, which
inhibit oscillations in 21 and 70% of the cells, respectively. These findings suggest that ~70% of myocytes mainly exhibit ET$_B$ receptor and the others the ET$_A$ receptor. It is known that contraction in both the rabbit and the rat small intrapulmonary arteries is mainly dependent on the activation of ET$_B$ receptors (9, 18). Moreover, in the rat small pulmonary arteries, ET$_B$-mediated electrophysiological responses have also been described (34). The physiological meaning of this heterogeneity is unclear. However, a similar heterogeneity of pulmonary myocytes for the expression of other membrane proteins along the arterial tree has been previously reported in the case of potassium and Ca$^{2+}$ voltage-operated channels (1, 8).

Mechanisms of ET-1-induced [Ca$^{2+}$]$_i$ response. Our results clearly show that the ET-1-induced [Ca$^{2+}$]$_i$ response in myocytes from RMPA and RIPA involves the mobilization of an intracellular Ca$^{2+}$ source, presumably the SR, since the response was not altered by La$^{3+}$ or in Ca$^{2+}$-free solution but vanished after pretreatment of the cells with thapsigargin. The ET-1-induced internal Ca$^{2+}$ mobilization operates via an inositol trisphosphate-sensitive signaling pathway, since the ET-1-induced [Ca$^{2+}$]$_i$ response was inhibited by neomycin, a selective inhibitor of PI-PLC (6), and by the addition, in Ca$^{2+}$-free PSS, of PDBu, a potent PKC activator in this preparation (35). In vascular smooth
muscle, it is known that phorbol ester-activated PKC negatively regulates the activity of PI-PLC and inhibits agonist-induced inositol trisphosphate production and the contractile response (30). Moreover, ET-1 increases both \([Ca^{2+}]\) and the production of inositol trisphosphate in a variety of vascular preparations (23, 29). The oscillatory pattern of the ET-1-induced \([Ca^{2+}]\) response could thus be in relation with a cyclic \(Ca^{2+}\) release through the inositol trisphosphate receptor \(Ca^{2+}\) release channel and explained by the well-known biphasic \(Ca^{2+}\) regulation of the smooth muscle inositol trisphosphate receptor activity (20). Evidence for such a model of inositol trisphosphate-mediated \(Ca^{2+}\) oscillations has also been provided recently in permeabilized epithelial cells (36). Although RMPA myocytes contain another \(Ca^{2+}\) release channel in the SR membrane, i.e., the ryanodine receptor (16), it is unlikely that such a channel is involved in the ET-1-induced \(Ca^{2+}\) release, since tetracaine and ruthenium red, two potent inhibitors of the CICR mechanism in smooth muscle (16, 22), did not alter ET-1-induced \([Ca^{2+}]\) oscillations. In this respect, \([Ca^{2+}]\) oscillations in pulmonary vascular smooth muscle are different from those observed in airway smooth muscle where it has been suggested that the ryanodine receptor could contribute to acetylcholine-induced \([Ca^{2+}]\) oscillations (22).

Physiological implications of ET-1-Induced \([Ca^{2+}]\) oscillations. Using electrophysiological and mechanical measurements, we tentatively investigated the role of ET-1-induced \([Ca^{2+}]\) oscillations in RMPA. In patch-clamped myocytes, ET-1 evoked an oscillatory membrane current that was obviously caused by \(Cl^-\) for the following reasons. First, in our experimental conditions, i.e., \(E_{Cl} = -2.1\) mV and holding potential = \(-60\) mV, the current was inward as expected from the large outward electrochemical gradient for \(Cl^-\). Second, its \(E_{rev}\) was similar to \(E_{Cl}\). Third, it was not altered in \(Ca^{2+}\)-free solution but was fully abolished by 50 \(\mu M\) niflumic acid, a selective inhibitor of voltage- and agonist-activated \(Cl^-\) current in vascular smooth muscle (27, 44). Simultaneous recordings of membrane current and \([Ca^{2+}]\) showed that oscillations of the inward current coincided with oscillations in \([Ca^{2+}]\). Moreover, 1) both phenomena remained unchanged in \(Ca^{2+}\)-free solution but disappeared after treatment of the cells with thapsigargin, and 2) niflumic acid, as mentioned above, abolished only the oscillatory current but not \([Ca^{2+}]\) oscillations. These results strongly suggest that oscillations in membrane current are triggered by oscillations in \([Ca^{2+}]\) and thus that ET-1-induced current in these conditions is a \(Ca^{2+}\)-activated \(Cl^-\) current \((I_{Cl(Ca)})(Ca)) in vivo.

In vascular myocytes, it is generally proposed that the main physiological role of agonist-induced \(I_{Cl(Ca)}\) is to depolarize the membrane to trigger the contraction by activation of a voltage-dependent \(Ca^{2+}\) influx (27, 44). In the present work, niflumic acid reduced by 35% the amplitude of ET-1-induced contraction. Interestingly, the same proportion of the contractile response was inhibited by either nifedipine alone or by the combination of both substances. These results confirm that ET-1-activated \(I_{Cl(Ca)}\) contributes to depolarize the cell to the threshold activation of voltage-dependent \(Ca^{2+}\) channels. Nevertheless, although the resulting \(Ca^{2+}\) influx plays a role in ET-1-induced contraction, the main part of the contractile response seems to be due to a \(Ca^{2+}\) release from the internal store. The fact that the contribution of this \(Ca^{2+}\) influx is somewhat limited in the ET-1-induced response could explain, at least in part, why it is difficult to demonstrate the presence of such an influx in our experiments in isolated cells. Moreover, 1) in patch-clamp experiments, myocytes were set at a membrane potential of \(-60\) mV, a value much more polarized than the threshold value for activating \(L\)-type \(Ca^{2+}\) channels; 2) when myocytes were not clamped, they may be slightly depolarized, the value of the membrane potential then corresponding to activation of \(L\)-type \(Ca^{2+}\) channels; and 3) finally, it is difficult to separate a sustained small increase in \([Ca^{2+}]\) related to \(Ca^{2+}\) influx when oscillations are superimposed unless oscillations are blocked; however, then \(I_{Cl(Ca)}\) leading to \(Ca^{2+}\) influx is also blocked. In the same preparation, we did perform such a separation in a previous study in response to ATP (16); the \([Ca^{2+}]\) response to ATP combines \(Ca^{2+}\) influx and \(Ca^{2+}\) oscillations, but these are mediated by different membrane receptors, \(P_{2X}\) and \(P_{2U}\), respectively (16). In the case of ET-1, the two mechanisms are linked and activated by the same receptor. Finally, it must be kept in mind that additional cellular mechanisms could participate in the ET-1-induced vasoconstrictor response, such as an increase in the sensitivity to \(Ca^{2+}\) of the contractile apparatus as observed in rabbit mesenteric artery (43).

In conclusion, this study has demonstrated that ET-1, via the activation of the receptor subtype A in RMPA and both receptor subtypes A and B receptor subtypes in RIPA induce \([Ca^{2+}]\) oscillations due to an inositol trisphosphate-mediated release from the SR. \(Ca^{2+}\) release directly accounts for the main part of the contractile response on the one hand and on another hand activates an oscillatory \(I_{Cl(Ca)}\) that depolarizes the cell to the threshold activation of voltage-dependent \(Ca^{2+}\) channels. The resulting \(Ca^{2+}\) influx is responsible for an additional component of the ET-1-induced contraction.

This work was supported by grants from Contrat de Recherche Institut National de la Santé et de la Recherche Médicale 9806, the Ministère de l’Environnement [Agence De l’Environnement et de la Maîtrise de l’Energie (ADEME), PRIMEQUAL 9593017], and Conseil Régional d’Aquitaine (96301117). J.-M. Hyvénin is an ADEME studentship recipient.

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Received 16 September 1997; accepted in final form 24 April 1998.

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