Cytosolic Calcium Transients Differ Between Porcine Coronary Arterial and Aortic Smooth Muscle Cells in Primary Culture

Yoshito Shogakiuchi, Hideo Kanaide, and Motoomi Nakamura

Using quin 2 microfluorometry of porcine vascular smooth muscle cells in primary culture at 25°C, we investigated differences in cytosolic calcium transients between epicardial coronary artery and aorta. Both in coronary arterial and aortic smooth muscle cells, histamine induced transient and dose-dependent elevations of cytosolic calcium concentrations, with a similar time course and EC50 (coronary artery, 1.4×10^{-7} M; aorta, 1.8×10^{-7} M). However, a transient and dose-dependent elevation of cytosolic calcium concentrations was induced by norepinephrine in aortic smooth muscle cells (EC50=2.5×10^{-7} M) but not in coronary arterial smooth muscle cells. Isoproterenol, which produced no change in cytosolic calcium concentrations in aortic vascular smooth muscle cells, significantly and dose dependently decreased concentrations of calcium in coronary arterial smooth muscle cells (EC50=1.5×10^{-7} M). Dibutyl cAMP decreased the concentration of cytosolic calcium both in the coronary arterial and aortic vascular smooth muscle cells with a similar time course and EC50 (coronary artery, 9.8×10^{-8} M; aorta, 1.1×10^{-5} M). Intracellular concentration of cAMP was increased in response to isoproterenol, as determined with radioimmunoassay of the coronary arterial smooth muscle cells but not in the aortic cells. Thus, the characteristics of receptors on the sarcolemma may play a key role in the regulation of responsiveness of vascular smooth muscle cells to various vasoactive substances. Aortic smooth muscle cells are α-receptor dominant, and activation results in a transient elevation of cytosolic calcium concentrations. The epicardial coronary arterial smooth muscle cells are β-receptor dominant, and activation results in an increase in cAMP and a reduction of cytosolic calcium concentrations. These results may account for the poor contraction, or relaxation, of epicardial coronary artery induced by sympathetic stimulation and exogenously applied catecholamines. (Circulation Research 1991;68:818–826)

Responses of the coronary artery to various vasoactive substances, in particular, adrenergic stimulants, often differ from those of other blood vessels. Although there are species and regional differences, poor contraction, or relaxation, induced by exogenously applied norepinephrine or epinephrine has been observed in experiments dealing with responses of isolated coronary rings or strips.1–4 Using a radioligand binding technique and porcine coronary arterial and aortic membranes, we have found that in the relative population of adrenoceptors, the epicardial coronary artery is β-dominant, while the aorta is α-dominant.5

Irrespective of the presence or absence of extracellular Ca^{2+}, norepinephrine was found to mainly activate the α1-adrenoceptor and induce a release of Ca^{2+} from intracellular stores. These events led to a transient and dose-dependent elevation of cytosolic Ca^{2+} concentration, [Ca^{2+}], in rat aortic vascular smooth muscle cells (VSMCs) in primary culture.6 In the present study we used quin 2 microfluorometry7,8 to examine the characteristics of [Ca^{2+}], responses to various adrenergic stimulations in epicardial coronary arterial VSMCs and compared our findings with those seen in aortic VSMCs. To investigate the direct actions of vasoactive substances confined to postjunctional receptors located in the smooth muscle cell...

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membrane, without modification by nonvascular cells, we cultured, in tandem and separately, VSMCs of the epicardial coronary artery and aorta from the pig. The observed effects of norepinephrine and isoproterenol on [Ca\(^{2+}\)], in the coronary artery and aortic VSMCs in primary culture correlated with those expected from the relative populations of adrenoceptors in porcine coronary arterial and aortic membranes.\(^5\)

**Materials and Methods**

**Materials**

Quin 2-AM was purchased from DOTTIE, Kuma-moto, Japan; histamine dihydrochloride from Wako, Osaka, Japan; and ionomycin from Calbiochem Corp., La Jolla, Calif. The following drugs were purchased from Sigma Chemical Co., St. Louis: pyrilamine maleate, cimetidine, \(l\)-norepinephrine hydrochloride, \(dl\)-isoproterenol, yohimbine, propranolol, and dibu-tyrly cAMP (\(db\)-cAMP). Prazosin was kindly donated from the manufacturer, Pfizer Inc., New York. The radioimmunoassay kit for cAMP was purchased from Yamasa Shoyu, Chiba, Japan. Drugs were dissolved in 1 mM Ca\(^{2+}\) physiological saline solution (PSS).

**Primary Culture of Vascular Smooth Muscle Cells**

VSMCs of the coronary artery and aorta from the pig were cultured in tandem and separately by the enzyme digestion method, with minor modifications.\(^9\) Briefly, the heart with aorta was obtained from a local slaughterhouse immediately after the animal had been killed and was transported to our laboratory in ice-cold Hanks’ balanced salt solution containing 5.5 mM glucose and 100 \(\mu\)g/ml streptomycin. The left and right epicardial conduit coronary arteries (about 3–5 mm in diameter) were dissected and opened longitudinally. The intimal layer was scraped off, and the medial layer was mechanically stripped from the adventitia. The aorta was opened longitudinally, and the medial layer was also stripped away. The medial layers of the coronary artery and aorta were then separately dispersed into single cells by incubation with 1.0 mg/ml collagenase (Sigma) and 20 units/ml elastase (Sigma) for 40 minutes. Cells were seeded on Lux chamber slides (No. 4802, Lux Scientific Instrument Corp., New York) containing Dulbecco’s modified Eagle’s medium (GIBCO, Grand Island, N.Y.) supplemented with 10% heat-inactivated fetal bovine serum (GIBCO), 100 \(\mu\)g/ml streptomycin, and 100 units/ml penicillin. The medium was changed every 3 days.

Morphological identification of smooth muscle cells in primary culture was carried out by direct immunofluorescent staining of smooth muscle actin and myosin as described previously.\(^5,10\) Smooth muscle actin was prepared from chicken gizzard, and smooth muscle myosin (chicken gizzard) was purchased from Sigma. The antitactin antiserum and antitrysin antiserum were prepared in rabbits, and the immunoglobulin G fractions of antisera were isolated and conjugated with fluorescein isothiocya-nate (FITC).\(^11\) The antibody solution had an appropriate FITC/protein ratio of 1:2. The cells were fixed with cold 95% ethanol for 30 minutes and then were stained with FITC-conjugated antibody solution for 30 minutes at 25°C.

**Loading Cells With Quin 2**

On days 10–14, just before reaching confluency, VSMCs of the coronary artery and aorta on the culture chamber slides were incubated with growth medium containing 50 \(\mu\)M quin 2-AM for 60 minutes at 37°C. The cells were then washed three times with PSS at 25°C to remove the dye in the extracellular space and then were incubated with PSS at 25°C for 30 minutes before the optical measurements were done, the objective being to compensate for a possible transient chelating effect of quin 2 on cytosolic free Ca\(^{2+}\).\(^12\) The experiments were carried out at 25°C instead of 37°C in a dark shield room with a constant temperature (25°C) and humidity (50%) to prevent leakage of quin 2 from the cells at the higher temperature, as described by Berthon et al.\(^13\) The fluorescence intensity was stable for 60 minutes in normal PSS.\(^7\) The millimolar composition of normal PSS (pH 7.4 at 25°C) was NaCl 135, KCl 5, CaCl\(_2\) 1, MgCl\(_2\) 1, glucose 5.5, and HEPES 10.

There was no apparent morphological change in the cells with and without quin 2 loading throughout all of the experiments, as confirmed by phase-contrast microscopy at \(\times\)400. Therefore, loading the VSMCs with quin 2 probably did not produce cell damage by release of formaldehyde on ester hydrolysis.\(^14\) In addition, a high cell viability (>95%) of the primary cultures was maintained throughout the experiments, as assessed by the trypan blue exclusion test. Since the intracellular quin 2 concentration was 2–3 mM,\(^8\) it should be noted that extremely rapid cytosolic Ca\(^{2+}\) transients may be somewhat slowed or blunted because of the buffering properties of this loading of quin 2.

**Microfluorometry of Quin 2**

The fluorescence intensity of VSMCs was recorded using microfluorometry, as described previously.\(^7,8,10\) Briefly, we used a fluorescence microscope (model Standard 18, Zeiss, Oberkochen, FRG) equipped with a photon-counting system, a water-immersion objective system (Plan-Neofluor 63, Zeiss), and an appropriate combination of filters (Zeiss and Toshiba, Tokyo), in which the cells were excited at wavelengths between 350 and 360 nm and analyzed at fluorescent wavelengths between 470 and 560 nm. By using a pinhole diaphragm (Zeiss) in the light axis, the fluorescence was measured in a spot (<1 \(\mu\)m\(^2\)) of the cytosol 6 \(\mu\)m from the nucleus in the longitudinal axis of the cell to avoid possible fluctuations caused by the uneven thickness of the cell. Each cell was exposed to the excitation light only once, for not longer than 2 seconds, to avoid photobleaching of the dye, which differed in individual cells. To read the fluorescence intensity, an input-output calculator (model 97S, Hew-
lett-Packard Co., Palo Alto, Calif.) was used. Because the VSMCs were multilayered in the “hills,” areas that were one cell thick in the “valleys” were selected for the microfluorometry to avoid possible fluctuations in light signals produced by the presence of underlying cells. Measurement of the fluorescence intensities in a small spot (<1 \( \mu \)m\(^2\)) 6 \( \mu \)m apart from the nucleus of eight cells in the valleys in a culture slide yielded values with a standard deviation of less than 5%. Thus, at each data point in each time of an experimental protocol, we measured the fluorescence intensity of eight cells and obtained mean values. We carried out such measurements in at least five different primary cultures from different animals in each experimental protocol.7,8

The changes in \([Ca^{2+}]\) were expressed in arbitrary units of fluorescence intensity. An estimate of \([Ca^{2+}]\) in relation to fluorescence signal was made, using the method of Tsien et al17 with minor modifications. Cells were permeated with ionomycin (10\(^{-7}\) M) in a calcium-free medium containing 10 mM EGTA to obtain \(F_{\text{min}}\) (approximately 10\(^{-9}\) M \(Ca^{2+}\)). In the presence of excess calcium, \(F_{\text{max}}\) (10\(^{-5}\) M \(Ca^{2+}\)) was determined. Intermediate values for \([Ca^{2+}]\) were calculated using the formula \([Ca^{2+}] = K_d(F - F_{\text{min}})/(F_{\text{max}} - F)\), with the dissociation constant \((K_d = 1.06 \times 10^{-7}\) M\) for \(Ca^{2+}\) binding to quin 2 at 25°C. During the course of all experimental procedures, neither contraction nor swelling of cells was noted, as determined by phase-contrast microscopy at \(\times 400\). However, the cells were contractile, as evidenced by the fact that, when they were dispersed from the culture slide by trypsinization,9 they contracted when histamine, norepinephrine, or high extracellular K\(^+\) in 1 mM \(Ca^{2+}\) PSS was applied.8

Assay of cAMP

VSMCs on the culture dish were exposed to normal PSS containing 10\(^{-5}\) M isoproterenol for 5 minutes at 25°C. The reaction was halted by replacing the medium with ice-cold normal PSS, and the cells were homogenized in 6% trichloroacetic acid. After centrifugation at 1,500g for 10 minutes, the supernatant was adjusted at pH 3.0 by washing three times with diethyl ether. After centrifugation at 1,000g for 10 minutes, the supernatant was used to measure cAMP content with a radioimmunonassay kit. The content of the cAMP was expressed as pmol/10\(^6\) cells.

Data Analysis

All data are presented as mean±SD. Statistical assessment of the data was made by analysis of variance and Student’s t test. Differences were considered significant at \(p<0.05\). EC\(_{50}\) values (the concentrations of the vasoactive substance that produced 50% of the maximum fluorescence response) and IC\(_{50}\) values (the concentration of antagonists that inhibits intracellular \(Ca^{2+}\) elevation by 50%) were determined by dose-response curves fitted according to a four-parameter logistic model.18

### Results

**Cultured Vascular Smooth Muscle Cells and Loading With Quin 2**

Direct immunofluorescent staining of actin and myosin showed positive staining in the cultured VSMCs, both from the coronary artery and aorta. Large numbers of stress fibers of actin and myosin in the cytoplasm were seen parallel to the longitudinal axis of the cells (Figure 1). These findings indicate that these cultured cells from the coronary artery and aorta were all smooth muscle cells. Chamley et al.15 proposed that immunofluorescent staining of smooth muscle actin and myosin allowed for a correct identification of VSMCs. Fluorescence of the quin 2–\(Ca^{2+}\) complex was observed exclusively in the cytosol, both in coronary arterial and aortic VSMCs in primary culture. The extracellular space, nuclei, and stress fibers were not stained with quin 2 (Figure 2). This was consistent with an earlier report that quin 2 was freely available for binding to \(Ca^{2+}\) in the cytosol and did not itself bind to macromolecules.17 In normal PSS containing 5 mM K\(^+\) and 1 mM Ca\(^{2+}\) (the resting condition), the levels of \([Ca^{2+}]\) of the coronary artery and aorta, calculated by using the method of Tsien et al.17 were 109±13 and 114±14 nM (\(n=5\)), respectively. There was no significant difference in \([Ca^{2+}]\) of VSMCs in the resting condition between coronary artery and aorta.

**Effects of Histamine on \([Ca^{2+}]\), of Vascular Smooth Muscle Cells**

To identify whether these cultured VSMCs exhibit elevations in \([Ca^{2+}]\), in response to vasoactive substances, we first examined effects of histamine on \([Ca^{2+}]\) in VSMCs because of the known ability of histamine to elevate \([Ca^{2+}]\) in these cells via H\(_1\)-receptors.19,20 The time course of the responses to extracellularly applied histamine (10\(^{-5}\) M) is shown in Figure 3A. Histamine caused a transient elevation of cytosolic fluorescence signals in VSMCs, both from coronary artery and aorta, with a similar time course and extent. The maximal levels of elevation were observed at 2 minutes and were dose dependent (Figure 3B). Peak levels of \([Ca^{2+}]\), induced by 10\(^{-5}\) M histamine in VSMCs of the coronary artery and aorta were estimated to be 646±236 and 681±271 nM (\(n=5\), respectively. EC\(_{50}\) values for coronary arterial and aortic VSMCs were 1.4×10\(^{-7}\) and 1.8×10\(^{-7}\) M, respectively. After reaching a maximum, the cytosolic fluorescence signal then gradually decreased to reach a level slightly but significantly higher than that before stimulation, both in coronary arterial and aortic VSMCs.

To identify the contributions of subtypes of histamine receptors (H\(_1\) and H\(_2\)) to the \([Ca^{2+}]\), transients in cultured VSMCs from coronary artery and aorta, the effects of pyrilamine (H\(_1\)-antagonist) and cimetidine (H\(_2\)-antagonist) on the \([Ca^{2+}]\), transient induced by 10\(^{-5}\) M histamine were studied. Both in the coronary artery and aorta, pyrilamine reduced these
Effects of Norepinephrine on \([\text{Ca}^{2+}]\), of Vascular Smooth Muscle Cells

There was no change in \([\text{Ca}^{2+}]\), on application of the \(\alpha\)-adrenergic agent norepinephrine to the VSMCs cultured from the coronary artery; yet a marked, transient elevation of \([\text{Ca}^{2+}]\) was observed in the VSMCs cultured from the aorta (Figure 4A). The elevation of cytosolic fluorescence reached a maximum at 2 minutes and then gradually declined, despite the continued application of norepinephrine, but cytosolic fluorescence remained significantly elevated throughout the period of stimulation. The extent of elevation at 2 minutes was dose dependent to give an \(\mathrm{EC}_{50}\) of 2.5\(\times\)10\(^{-7}\) M (Figure 4B) and with 10\(^{-5}\) M norepinephrine was estimated to be 721\(\pm\)256 nM \((n=5)\).

To identify the contributions of subtypes of adrenergic receptors to the \([\text{Ca}^{2+}]\), transient in cultured VSMCs from the porcine aorta, the effects of prazosin (\(\alpha_1\)-selective antagonist) and yohimbine (\(\alpha_2\)-selective antagonist) on the \([\text{Ca}^{2+}]\), elevation induced by 10\(^{-5}\) M norepinephrine were studied. Prazosin reduced the \([\text{Ca}^{2+}]\), transient dose dependently (\(\mathrm{IC}_{50}=1.3\times10^{-9}\) M), and at higher concentrations of prazosin, the \([\text{Ca}^{2+}]\), transient was totally blocked. Yohimbine inhibited the \([\text{Ca}^{2+}]\), transient dose dependently (\(\mathrm{IC}_{50}=2.1\times10^{-6}\) M), but the inhibition was never complete even with a high concentration (10\(^{-3}\) M) of yohimbine. Thus, it appeared that the \([\text{Ca}^{2+}]\), transient induced by norepinephrine is mediated by both \(\alpha_1\)- and \(\alpha_2\)-receptors and that the initial transient was largely dependent on \(\alpha_2\)-receptor activation.

Effects of Isoproterenol on \([\text{Ca}^{2+}]\), of Vascular Smooth Muscle Cells

Figure 5A shows the effects of isoproterenol, a \(\beta\)-adrenergic agent, on the cytosolic fluorescence signal in cultured VSMCs of porcine coronary artery and aorta. Isoproterenol did not affect fluorescence in
aortic VSMCs but did significantly decrease the cytosolic fluorescence in the coronary arterial VSMCs over the course of 5 minutes (Figure 5B). This decrease was dose dependent with the EC₅₀ of 1.5×10⁻⁷ M and with 10⁻⁵ M isoproterenol was estimated to decline to 39±8 nM (n=5). After 5 minutes, [Ca²⁺], gradually increased to reach a new steady-state level, which was significantly lower than observed before the application of drug. Because propranolol inhibited this decrease in [Ca²⁺], dose dependently (IC₅₀=2.4×10⁻⁶ M), it appeared that the transient decrease of [Ca²⁺] was mediated by β-adrenergic receptors. Subtypes of β-adrenergic receptors responsible for this inhibition were not determined.

Effects of Dibutyryl cAMP on [Ca²⁺] of Vascular Smooth Muscle Cells

To clarify whether an elevation of intracellular cAMP causes a decrease in [Ca²⁺], both in coronary arterial and aortic VSMCs, we investigated the effects of db-cAMP, a membrane-permeable analogue of cAMP, on [Ca²⁺] of these two cultured VSMCs. The most typical time courses of the effects of extracellularly applied 10⁻⁴ M db-cAMP on the cytosolic fluorescence signals in K⁺-depolarized coronary arterial and aortic VSMCs are shown in Figure 6A. When VSMCs were exposed to 60 mM K⁺ PSS, cytosolic fluorescence signals rapidly increased to an elevated steady state within 6 minutes. The subsequent application of 10⁻⁴ M db-cAMP induced a gradual decrease in the fluorescence signals. When db-cAMP was washed out, the fluorescence signal rapidly reverted within 6 minutes to a level equal to that observed before the application of db-cAMP in both the coronary arterial and aortic VSMCs in the presence of 60 mM K⁺. The increase in levels of [Ca²⁺], in coronary arterial and aortic VSMCs in the presence of 60 mM K⁺ were estimated to be 858±189 and 1,023±251 nM, respectively (n=5). After application of db-cAMP, the decrease in [Ca²⁺] was dose dependent to give an EC₅₀ for coronary arterial and aortic VSMCs of 9.8×10⁻⁶ and 1.1×10⁻⁵ M, respectively (Figure 6B).

Assay of cAMP

Changes in the intracellular concentrations of cAMP in response to isoproterenol are shown in Table 1. The resting levels of the intracellular cAMP concentration both in the porcine coronary arterial and aortic VSMCs in normal PSS were 7.74±0.12 and 5.44±0.66 pmol/10⁶ cells, respectively. The resting level of cAMP in the coronary VSMCs was slightly but significantly higher than that of the aortic VSMCs (p<0.01, Student’s t test). When coronary VSMCs were exposed to 10⁻⁵ M isoproterenol, the intracellular cAMP concentration was significantly increased to reach twice the resting level at 5 minutes. In the aortic VSMCs, the intracellular cAMP level remained unchanged. These findings are interpreted to mean that a reduction of [Ca²⁺], induced by isoproterenol in the coronary VSMCs was associated with an increase in intracellular cAMP.

Discussion

The activity of vascular smooth muscle can be regulated by catecholamines that are released from the autonomic nervous system and the adrenal medulla. There is, however, considerable variability in the re-
response of arterial muscles from different tissues, in particular, responses of the coronary artery.\textsuperscript{1,2} It has been reported that coronary arterial muscle exhibits weak contraction when stimulated through sympathetic fibers as compared with the contraction observed with other segments of the systemic arterial tree.\textsuperscript{1,2} In addition, coronary arteries relax in response to circulating norepinephrine and epinephrine.\textsuperscript{3,4} These variations in responses may be due to corresponding variations in the types and densities of adrenergic receptors on the sarcolemma. But another complexity arises from the contribution of other active substances that may be released from nerve fibers, ganglion cells, and endothelium,\textsuperscript{1,2,21} which may indirectly influence the direct action of catecholamines on the particular VSMCs.

**Figure 3.** Panel A: Typical time courses of the effects of 10\textsuperscript{-5} M histamine on fluorescence signals in cultured vascular smooth muscle cells (VSMCs) of porcine coronary artery (○) and aorta (●) in 1.0 mM Ca\textsuperscript{2+} physiological saline solution (PSS). Data are mean±SD of eight cells. [Ca\textsuperscript{2+}]\textsubscript{i} was calculated using Tsien's equation: [Ca\textsuperscript{2+}]\textsubscript{i}=[K\textsubscript{d}(F-F\textsubscript{mm})/(F\textsubscript{max}-F)]. K\textsubscript{d} is the dissociation constant for Ca\textsuperscript{2+} binding to quin 2 at 25°C (K\textsubscript{d}=1.06×10\textsuperscript{-7} M). Panel B: Dose-dependent effects of histamine on fluorescence signals (at the peak levels) in cultured VSMCs of porcine coronary artery (○) and aorta (●) in 1.0 mM Ca\textsuperscript{2+} PSS. Data are mean±SD of five experiments. ★p<0.05 when compared with control (no drug) values. There were no significant differences between coronary artery and aorta.

Most smooth muscles seem to contain several subtypes of adrenergic receptors, but it is difficult to define whether different types are present on the same muscle cell or whether they occur on different cells within small regions. It is known, for example, that, after blocking α\textsubscript{1} and β-adrenoceptors, norepinephrine exerts a relaxing effect in vascular smooth muscles, mediated through α\textsubscript{2}-adrenoceptors located on the endothelium.\textsuperscript{21} Thus, there is controversy concerning possible actions of catecholamines on coronary arterial smooth muscles because of the complexity of the components in tissue preparations.

The use of VSMCs in primary culture circumvent some of the difficulties in studies of catecholamine on intact vascular smooth muscle. In contrast to experiments with whole tissue preparations, cells are derived entirely from the medial layer of the artery and aorta, a location that consists entirely of VSMCs, and
Dose-dependent effects (PSS).

_vascular smooth muscle_ (VSMCs) of porcine coronary artery (○) and aorta (●) in 1.0 mM Ca\(^{2+}\) physiological saline solution (PSS). Data are mean±SD of eight cells. Panel B: Dose-dependent effects of isoproterenol on fluorescence signals in cultured VSMCs of porcine coronary artery (○) and aorta (●) in 1.0 mM Ca\(^{2+}\) PSS. The effects on the lowest level are plotted. Data are mean±SD of five experiments. \*p<0.05 when compared with control (no drug) values. tp<0.05 when compared with aortic VSMCs.

there is no contamination with other vascular or nonvascular cell types.\(^{15,22,23}\) This facilitates direct observation of the action of catecholamines in VSMCs, without modification by nonvascular cells. We cultured VSMCs from the pig coronary artery and aorta, in tandem and separately, and investigated characteristics of the responsiveness of these cells to various vasoactive substances confined to receptors on the sarcolemma.

Pharmacological studies showed that histamine contracts vascular smooth muscles by acting on the H\(_1\)-receptor and relaxes the muscles by the H\(_2\)-receptor.\(^{24,25}\) In the present study, histamine caused a transient elevation of [Ca\(^{2+}\)]\(_i\), with a similar time course both in the coronary arterial and aortic VSMCs. Pyrilamine, an H\(_1\)-antagonist, dose dependently inhibited this increase in [Ca\(^{2+}\)]\(_i\), induced by histamine, but cimetidine, an H\(_2\)-antagonist, had little or no such effect. This suggests that these histamine receptors are of the type H\(_1\) species. We have used a pyrilamine binding assay for the sarcolemmal fractions and noted a single type of histamine receptor (H\(_1\)-receptor) both in the porcine coronary artery and aorta.\(^{19}\) These findings indicate that activation of the histamine H\(_1\)-receptor causes an elevation of [Ca\(^{2+}\)]\(_i\) and that both coronary arterial and aortic VSMCs in primary culture possess mecha-

**TABLE 1. Intracellular cAMP Content**

<table>
<thead>
<tr>
<th>cAMP concentration (pmol/10(^6) cells)</th>
<th>Coronary SMCs</th>
<th>Aortic SMCs</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>7.74±0.12*</td>
<td>5.44±0.66</td>
</tr>
<tr>
<td>10(^{-5}) M isoproterenol</td>
<td>16.17±0.62*(\dagger)</td>
<td>5.42±0.15</td>
</tr>
</tbody>
</table>

*Values are mean±SD of five experiments. SMCs, smooth muscle cells.
\(\dagger\)p<0.01 vs. aortic SMCs.
\*p<0.01 vs. control (Student's t test).
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organisms, such as receptor-mediated calcium release and influx, that will elevate [Ca\(^{2+}\)]. This is also supported by our data on rat aortic cultured VSMCs.\(^{20}\)

Norepinephrine, an \(\alpha\)-adrenergic agent, elevated [Ca\(^{2+}\)] in aortic VSMCs, but there was no change in coronary arterial VSMCs despite the finding that [Ca\(^{2+}\)] elevation was induced by histamine in these VSMCs. An increase in [Ca\(^{2+}\)], of the aortic VSMCs induced by norepinephrine was almost completely inhibited by the \(\alpha\)-adrenergic blocker prazosin and was partially inhibited by the \(\alpha\)-adrenergic blocker yohimbine. These findings indicate that there are \(\alpha\)-adrenergic receptors in the aortic VSMCs, whereas in the coronary arterial VSMCs there are few, if any. In contrast, [Ca\(^{2+}\)], was decreased by application of the \(\beta\)-adrenergic agent isoproterenol to coronary arterial VSMCs but not to aortic VSMCs; nevertheless, [Ca\(^{2+}\)] was decreased in both cell types by db-cAMP. The \(\beta\)-adrenergic blocker propranolol dose dependently inhibited the decrease in [Ca\(^{2+}\)], induced by isoproterenol in coronary arterial VSMCs. These findings indicate that there are \(\beta\)-adrenergic receptors in the coronary arterial VSMCs, while in the aortic VSMCs there are few or none. Thus, it would appear that aortic VSMCs are \(\alpha\)-receptor dominant, whereas coronary arterial VSMCs are \(\beta\)-receptor dominant. We have reported previously that the porcine coronary artery is \(\beta\)-receptor dominant (\(\beta/\alpha=11\)), whereas the aorta is \(\alpha\)-receptor dominant (\(\beta/\alpha=0.02\)).\(^{3}\) Therefore, the characteristic [Ca\(^{2+}\)] transients induced by catecholamines (norepinephrine and isoproterenol) in the VSMCs from porcine coronary artery and aorta were consistent with our previous findings.

The question of whether the vasodilation induced by \(\beta\)-agonists is mainly based on the decrease in [Ca\(^{2+}\)], remains unsolved.\(^{2,26,27}\) Adelstein and coworkers\(^{28-29}\) showed that gizzard myosin light chain kinase, in the absence of bound Ca\(^{2+}\)-calmodulin, was phosphorylated by cAMP-dependent protein kinase. This would suggest that relaxation can be achieved by reducing the activity of myosin light chain kinase without a decrease in [Ca\(^{2+}\)]. Although documentation of changes in [Ca\(^{2+}\)] in VSMCs during \(\beta\)-stimulation is scanty, the available data suggest decreases in [Ca\(^{2+}\)], by \(\beta\)-stimulants in isolated intact VSMCs\(^{30,31}\) and ileal smooth muscle.\(^{32}\) The effect of \(\beta\)-stimulation on [Ca\(^{2+}\)] of coronary arterial smooth muscle has heretofore not been reported. We clearly demonstrated that [Ca\(^{2+}\)] is decreased by isoproterenol in the coronary VSMCs, a finding that leads to the hypothesis that the reduced phosphorylation of myosin light chain, as mediated by a decrease in [Ca\(^{2+}\)], is a major mechanism involved in the relaxation induced by isoproterenol-mediated \(\beta\)-stimulation under physiological conditions.

Isoproterenol-induced relaxation of vascular smooth muscle is accompanied by an increase in cAMP levels.\(^{33-35}\) Relaxation of smooth muscle may be directly due to elevations of cAMP, because membrane-permeable db-cAMP directly relaxes smooth muscle.\(^{33,36}\) In the present study, the exposure to db-cAMP led to reductions in [Ca\(^{2+}\)], both in the coronary arterial and aortic VSMCs (Figure 6), although isoproterenol did not reduce [Ca\(^{2+}\)] and did not increase cAMP of aortic VSMCs in primary culture (Table 1), possibly because of the lack of \(\beta\)-receptors.\(^{5}\) Thus, not only coronary arterial VSMCs but also aortic VSMCs may possess mechanisms that will reduce [Ca\(^{2+}\)], in response to an increase in cytosolic cAMP concentrations. In the present study, depolarization of VSMCs with high extracellular K\(^{+}\) led to elevations in [Ca\(^{2+}\)], thereby indicating that both coronary arterial and aortic VSMCs in primary culture possess voltage-dependent Ca\(^{2+}\) channels. We also noted that K\(^{+}\) depolarization directly releases Ca\(^{2+}\) from the caffeine-sensitive intracellular Ca\(^{2+}\) store site in VSMCs in primary culture.\(^{7}\) In the present study, cAMP decreased the levels of [Ca\(^{2+}\)], during K\(^{+}\) depolarization, and this low level was maintained as long as db-cAMP was present. It seems probable that not only the sequestration of Ca\(^{2+}\) into intracellular stores\(^{34,36-38}\) but also Ca\(^{2+}\) extrusion from the cell or the suppression of Ca\(^{2+}\) influx may participate in the reduction of [Ca\(^{2+}\)] induced by cAMP.

Although cultured cells used in the present study had the capacity to contract when dispersed from the culture slide by trypsinization, the contraction did not occur in our experimental condition, possibly because the cells adhered strongly to the culture slide. In addition, it is known that there is a modulation in the structure and, hence, in the function of myosin in the VSMCs during culture.\(^{9}\) Thus, the limitation of the present study regarding contraction of VSMCs in situ has to be kept in mind.

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References


KEY WORDS * calcium * coronary artery * aorta * quin2 * isoproterenol * cAMP
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