A Functional Ryanodine-Sensitive Intracellular Ca\(^{2+}\) Store Is Present in Vascular Endothelial Cells


The presence of the ryanodine receptor was recently demonstrated in vascular and endocardial endothelium, but its function has not been established. We investigated whether functional ryanodine-sensitive Ca\(^{2+}\) stores are present in cultured endothelial cells from rat aorta (RAECs), human aorta (HAECs), human umbilical vein (HUVECs), and bovine pulmonary artery (BPAECs) and what role these may play in intracellular Ca\(^{2+}\) regulation. Under resting conditions, HAECs, BPAECs, and HUVECs demonstrated a slow increase in intracellular Ca\(^{2+}\) (indexed by indo 1 fluorescence) on exposure to 5 μmol/L ryanodine, whereas RAECs did not. However, after an initial bradykinin exposure in RAECs, ryanodine markedly blunted the rapid increase in Ca\(^{2+}\) on a second exposure to bradykinin. In HUVECs, ryanodine in buffer with 1.5 mmol/L Ca\(^{2+}\) did not inhibit the agonist-sensitive Ca\(^{2+}\) increase, whereas it blunted the rapid increase in Ca\(^{2+}\) on histamine exposure in buffer with 5 mmol/L Ca\(^{2+}\), suggesting that increasing [Ca\(^{2+}\)] in the extracellular space enhances the binding of ryanodine to its receptor. Thus, functional ryanodine-sensitive Ca\(^{2+}\) stores are present in vascular endothelial cells. These appear to be involved in regulation of Ca\(^{2+}\) storage and release from agonist-sensitive intracellular compartments. (Circ Res. 1994;74:151-156.)

Key Words • endothelium • Ca\(^{2+}\) • ryanodine • indo 1

Received February 16, 1993; accepted September 7, 1993.

From the Laboratory of Cardiovascular Science (R.C.Z., H.A.S., L.C., S.C., E.G.L., M.C.C.) and the Laboratory of Biological Chemistry (R.P., A.P.), Gerontology Research Center, National Institute on Aging, National Institutes of Health; and Johns Hopkins University (R.C.Z.), Baltimore, Md.

Previously presented as a preliminary report in abstract form (Circulation. 1992;86[suppl 1]:I-750).

Correspondence to Maurizio C. Capogrossi, MD, Laboratory of Cardiovascular Science, Gerontology Research Center, NIA, NIH, 4940 Eastern Ave, Baltimore, MD 21224.

An increase in [Ca\(^{2+}\)] is important in mediating many of the functional properties of vascular endothelial cells, particularly release of vasodilatory prostaglandins\(^{12}\) and endothelium-derived relaxation factor.\(^{3}\) Inositol 1,4,5-trisphosphate (InsP\(_3\))–mediated release of Ca\(^{2+}\) from intracellular stores appears largely responsible for the rapid transient increase in cytosolic Ca\(^{2+}\) that occurs on agonist stimulation.\(^{4-12}\) Although considerable information has been learned about endothelial Ca\(^{2+}\) regulation, the identity and sensitivity of the intracellular compartments are not completely known. Further identification of these intracellular stores is facilitated by the use of appropriate pharmacologic probes.

The plant alkaloid ryanodine has been used to study [Ca\(^{2+}\)]\(_{i}\) regulation in striated muscle.\(^{13-15}\) Ryanodine is known to bind to the Ca\(^{2+}\) release channels of the sarcoplasmic reticulum\(^{16,17}\) and to thereby alter its Ca\(^{2+}\) release properties. At low concentrations, ryanodine keeps the Ca\(^{2+}\) release channels in an open state,\(^{17-20}\) causing Ca\(^{2+}\) release.\(^{21}\) Recently, the presence of the ryanodine receptor was demonstrated in vascular and endocardial endothelium.\(^{22}\) Whereas anti–ryanodine receptor antibody binding sites were predominantly dem-
minimum essential medium with d-valine supplemented with 10% fetal calf serum, 100 µg/mL endothelial mitogen, and 16 U/mL heparin. HUVECs and RAECs were identified by demonstrating specific immunofluorescent staining for factor VIII–related antigen (DAKO Corp, Carpenteria, Calif). After treatment with 0.25% trypsin (NIH Medium Center) and 0.5 mmol/L EDTA (Sigma Chemical Co, St Louis, Mo), cells were plated on glass coverslips (BPAECs and HAECs) or in 1-mm² glass capillary tubes (HAECs, HUVECs, and RAECs; Vitro Dynamics, Rockaway, NJ) precoated with 1% gelatin (Sigma) 1 to 2 days before experimental use.

**Indo 1 Fluorescence Measurements**

Endothelial monolayers were loaded with growth medium containing 12 µmol/L of the ester derivative (AM form) of the fluorescent Ca²⁺ probe indo 1 (Molecular Probes, Eugene, Ore) and kept in a 95% air–5% CO₂ incubator at room temperature for 60 minutes. Monolayers were then gently washed with HEPES-buffered saline solution and maintained an additional 60 minutes to allow for deesterification of the indicator before the beginning of the experiment. Cells were then placed on the stage of a modified inverted fluorescence microscope (Zeiss IM-35) as previously described. Briefly, indo 1 fluorescence was excited at 350±5 nm using a xenon strobe lamp (model 236 stroboscope and model 35S lamp, Chadwick-Helmuth Electronics, El Monte, Calif), and band-pass interference filters (Andover, Lawrence, Mass) selected wavelength bands of emitted fluorescence at 391 to 434 nm (“410 channel”) and 457 to 507 nm (“490 channel”) corresponding to the Ca²⁺-bound and Ca²⁺-free forms of the indicator, respectively. The 410–490 nm ratio was used as an index of [Ca²⁺]. Emitted indo 1 fluorescence was collected from a field of approximately 15 confluent cells. Autofluorescence of a comparable field of unloaded cells was generally <5% of indo 1–loaded cells. Data are reported as emitted indo 1 fluorescence ratio and not as [Ca²⁺], since a reliable multipoint intracellular Ca²⁺ calibration in intact monolayers of endothelial cells was not possible to obtain. This type of calibration is dependent on making repeated measurements over a range of [Ca²⁺] values using ionophores to equilibrate Ca²⁺ between the intracellular and extracellular spaces. It also assumes complete hydrolysis of the indicator to a single Ca²⁺-sensitive fluorescent species. An intracellular Ca²⁺ calibration is superior to the calibration of the free acid form of the probe in solution or to the calibration performed on cells loaded with the ester form of the probe, which are then lyzed since the spectral properties of the indicator may be influenced by the intracellular environment. A variable Mn²⁺-insensitive fluorescent component and the apparent low efficacy of Ca²⁺ ionophores in the endothelial cell plasma membrane prevented accurate intracellular calibration.

**Experimental Protocol**

Cells were exposed to ryanodine (Penick Corp, Lyndhurst, NJ), bradykinin (Sigma), or histamine (Sigma) in a buffer of the following composition (mmol/L): NaCl, 137; KCl, 4.9; MgSO₄, 1.2; NaH₂PO₄, 1.2; d-glucose, 15; HEPES, 20; and CaCl₂, 1.5; pH 7.40 (23°C). When experiments were performed on cells grown in capillary tubes, the tubes were internally perfused using a syringe pump (Harvard Apparatus, South Natick, Mass) at a flow rate (0.5 to 1.0 mL/min) that produced negligible shear stress calculated from flow rate and tube geometry (2<dyne·cm⁻²) and did not affect [Ca²⁺]. Since more significant and rapid intracellular loss of indo 1 occurs at 37°C than at 23°C, all experiments were performed at 23°C.

**Statistics**

Data are reported as mean±SEM. The ryanodine concentration-response relation for RAECs was compared by a one-way ANOVA, followed by a Bonferroni test. A difference was considered significant at P<.05.

**Results**

**Effect of Ryanodine on [Ca²⁺], Under Baseline Conditions**

To determine the effect of ryanodine on endothelial [Ca²⁺], under “resting” (ie, unstimulated) conditions, indo 1–loaded endothelial monolayers were exposed to 5 µmol/L ryanodine for 10 to 15 minutes. These experimental conditions were chosen on the basis of previous work in suspensions of intact cardiac myocytes, which showed that ryanodine evoked a slow release of Ca²⁺ into the myoplasmic space in a concentration-dependent (up to micromolar) and time-dependent (up to 10 minutes) manner.21 HAECs (Fig 1B), BPAECs, and HUVECs (not shown) showed a slow sustained increase in [Ca²⁺], without significant reversibility on ryodine washout. The rate of increase and the amplitude of the change were reduced when compared with the response to histamine stimulation (Fig 1A). Figure 1 also shows that the ryanodine effect in HAECs was more prolonged than that of histamine, which produced a typical rapid transient increase in [Ca²⁺], followed by a sustained elevation of [Ca²⁺], which eventually returned to control levels after several minutes. In contrast to the sustained increase in [Ca²⁺], produced by a 10-minute ryanodine exposure in most HAECs, BPAECs, and HUVEC monolayers, RAECs did not demonstrate this response under these resting conditions (Fig 2).

**Effect of Ryanodine After Agonist Stimulation in RAECs and HUVECs**

Since ryanodine had no effect on RAEC [Ca²⁺], and only a small effect on HUVEC [Ca²⁺], the ability of ryanodine to influence refilling of the agonist-sensitive intracellular Ca²⁺ pool was examined in these two cell types. In these experiments, RAECs were exposed to
bradykinin, and HUVECs were exposed to histamine, since these agonists reproducibly produce a rapid increase in [Ca\(^{2+}\)]\(_i\), in these cell types. The response of RAECs to histamine and of HUVECs to bradykinin is somewhat less consistent, which may reflect cell type-specific effects of tissue culture conditions on receptor number or function. Although a different agonist was used for each cell type, both bradykinin and histamine are thought to cause an initial rapid rise in [Ca\(^{2+}\)]\(_i\), due to receptor-mediated activation of phospholipase C and to generation of InsP\(_3\).\(^{5,9,10}\)

When RAECs were exposed to bradykinin (Fig 3A), an initial rapid increase in [Ca\(^{2+}\)]\(_i\), was followed by a more prolonged second phase; in contrast to the initial rapid increase, this second phase is thought to be due to the influx of Ca\(^{2+}\) from the extracellular space.\(^8\) A period in the absence of bradykinin appears to be required for restoration of the sensitivity of the bradykinin receptor.\(^8\) Removal of extracellular Ca\(^{2+}\) 5 minutes before a second bradykinin exposure in RAECs abolishes the sustained increase in [Ca\(^{2+}\)]\(_i\), (Fig 3A). When RAECs were instead exposed for 15 minutes to 5 \(\mu\)mol/L ryanodine after an initial bradykinin exposure (Fig 3B), the rapid increase in [Ca\(^{2+}\)]\(_i\), on a second bradykinin exposure was markedly blunted. There was no difference in the inhibition of the bradykinin-induced increase in [Ca\(^{2+}\)]\(_i\), whether or not Ca\(^{2+}\) was removed from the buffer 5 minutes before the second bradykinin exposure (n=8 with and n=7 without buffer Ca\(^{2+}\)). These data suggest that under these experimental conditions the effect of ryanodine on the bradykinin-sensitive intracellular Ca\(^{2+}\) pool is incomplete. Furthermore, the increase in [Ca\(^{2+}\)]\(_i\), on the second bradykinin exposure occurred despite the absence of an increase in [Ca\(^{2+}\)]\(_i\), during ryanodine exposure and may reflect slow Ca\(^{2+}\) release into the cytosol, which is balanced by Ca\(^{2+}\) extrusion from the cell.

In the absence of a prior bradykinin exposure, ryanodine had no effect on the increase in [Ca\(^{2+}\)]\(_i\), due to bradykinin (n=8, not shown). Ryanodine may thus prevent refilling of the bradykinin-sensitive Ca\(^{2+}\) store. Alternatively, a prior agonist-induced increase in [Ca\(^{2+}\)]\(_i\), may be required to render the cells sensitive to ryanodine. Since caffeine also has been shown to release Ca\(^{2+}\) from a ryanodine-sensitive intracellular Ca\(^{2+}\) store in other cell types,\(^{21}\) we exposed RAECs for 5 minutes to 5 \(\mu\)mol/L ryanodine alone and then simultaneously to ryanodine and 10 mmol/L caffeine for 10 minutes before bradykinin stimulation. The inability of ryanodine to inhibit the bradykinin-induced increase in [Ca\(^{2+}\)]\(_i\), in the absence of a prior bradykinin stimulation was not affected by simultaneous exposure to 10 mmol/L caffeine and 5 \(\mu\)mol/L ryanodine (n=8, not shown).

To determine the concentration dependence of the ryanodine effect in RAECs, experiments using sequential bradykinin stimulation were performed in which the concentration of ryanodine was varied without altering the duration of exposure. Fig 4 shows that the magnitude of the increase in [Ca\(^{2+}\)]\(_i\), due to the second bradykinin exposure after a 15-minute period under control conditions without ryanodine was just less than one half (0.49±0.08, n=7) that of the first exposure. This is similar to the findings of others.\(^8\) Although 0.05 \(\mu\)mol/L ryanodine had no effect on the bradykinin-induced rapid [Ca\(^{2+}\)]\(_i\), increase, higher concentrations
Fig 4. Concentration dependence of the effect of ryanodine on the bradykinin (BK)-induced rapid \([Ca^{2+}]\) increase in rat aortic endothelial cells (RAECs). The change in the indo 1 ratio from baseline conditions to the peak of the BK response of the second exposure (\(\Delta BK_2\)) is compared with that of the first exposure (\(\Delta BK_1\)) and is plotted against ryanodine concentration. In each case, the second 0.5 \(\mu\)mol/L BK exposure was separated from the first by a 15-minute period either in control buffer or in buffer with ryanodine. Ryanodine inhibited the BK-induced rapid increase in RAEC \([Ca^{2+}]\), in a concentration-dependent manner. Data represent mean±SEM of six to nine experiments at each ryanodine concentration. *P<.05 vs control.

resulted in increasing inhibition, with significant inhibition noted with 5 \(\mu\)mol/L ryanodine. Thus, ryanodine receptors appear to control, at least in part, bradykinin-sensitive \(Ca^{2+}\) pools in RAECs.

In HUVECs, histamine stimulation resulted in a typical biphasic increase in \([Ca^{2+}]\), that was not different in magnitude on a second histamine exposure after a 15-minute superfusion with control buffer. In contrast to the effect of ryanodine on agonist stimulation in RAECs, a 15-minute exposure of HUVECs to 5 \(\mu\)mol/L ryanodine after histamine stimulation did not inhibit the rapid increase in \([Ca^{2+}]\) on a second histamine exposure (Fig 5). The absence of an effect of 5 \(\mu\)mol/L ryanodine was noted whether or not ryanodine exposure was continued during exposure to histamine. Such an apparent lack of effect of ryanodine may be due to inefficient binding of the drug to its receptor, an interaction known to have an absolute dependence on the presence of \(Ca^{2+}\), which has been suggested to form a complex with the ryanodine receptor as a prerequisite to ryanodine binding.16 That increasing \([Ca^{2+}]\) enhances ryanodine binding and modulates the effect of the drug was inferred in studies demonstrating a “use-dependent” effect of the alkaloid in ferret cardiac muscle27 and was more directly demonstrated in studies with rat cardiac myocytes in which the ryanodine effect was attenuated when extracellular \(Ca^{2+}\) entry was inhibited.21

To determine whether bath \(Ca^{2+}\) ([\(Ca^{2+}\])\textsubscript{b} ) could modulate the effect of ryanodine in HUVECs, the identical protocol using sequential histamine stimulation was performed in 5 mmol/L \([Ca^{2+}]\). When HUVECs in buffer with 1.5 mmol/L \(Ca^{2+}\) were then exposed to buffer with 5 mmol/L \(Ca^{2+}\), there was a slight increase in basal \([Ca^{2+}]\). Although the response of sequential histamine exposures in 5 mmol/L \([Ca^{2+}]\), in the absence of ryanodine was not affected (not shown), a 15-minute exposure to ryanodine substantially inhibited the rapid increase in \([Ca^{2+}]\) on a second histamine exposure in 5 mmol/L \([Ca^{2+}]\), in five of eight monolayers studied (Fig 6). The magnitude of the increase in indo 1 ratio of the second histamine exposure after 15 minutes of ryanodine \((\Delta HIS)\) compared with that of the first histamine exposure before ryanodine \((\Delta HIS)\) was significantly less in 5 mmol/L \([Ca^{2+}]\), than in 1.5 mmol/L \([Ca^{2+}]\). \(\Delta HIS/\Delta HIS\) was 0.54±0.11 in 5 mmol/L \([Ca^{2+}]\), \(n=8\) and 0.83±0.06 in 1.5 mmol/L \([Ca^{2+}]\), \(n=10, P<.05\).

Discussion

The present study is the first to document the existence of a ryanodine-sensitive \(Ca^{2+}\) store that is in-

Fig 5. Tracings showing the effect of ryanodine (RY) on the histamine-induced rapid increase in \([Ca^{2+}]\), in human umbilical vein endothelial cells (HUVECs). A, A representative example of fluorescence measurements was obtained from an indo 1-loaded HUVEC monolayer exposed sequentially to histamine (HIST). A typical biphasic increase in \([Ca^{2+}]\), is noted on both the initial and subsequent exposure to 100 \(\mu\)mol/L HIST. B, A representative example of fluorescence measurements was obtained from an indo 1-loaded HUVEC monolayer during HIST stimulation before and after RY exposure. After a 15-minute period in buffer with 1.5 mmol/L \(Ca^{2+}\) and 5 \(\mu\)mol/L RY, cells were exposed a second time to 100 \(\mu\)mol/L HIST (n=10). RY had no effect on the rapid increase in \([Ca^{2+}]\), due to HIST. Continuing the exposure to RY throughout exposure to HIST (n=8) did not alter the results (not shown).

Fig 6. Tracings showing the effect of ryanodine (RY) on the histamine (HIST)-induced rapid increase in human umbilical vein endothelial cell \([Ca^{2+}]\), in buffer with 5 mmol/L \(Ca^{2+}\). The slight increase in indo 1 ratio that occurred when the buffer with 1.5 mmol/L \(Ca^{2+}\) was replaced with the buffer with 5 mmol/L \(Ca^{2+}\) is not shown. Cells were stimulated with 100 \(\mu\)mol/L HIST before and after a 15-minute exposure to RY (5 \(\mu\)mol/L). Under conditions of elevated \([Ca^{2+}]\), RY inhibited the HIST-induced rapid increase in \([Ca^{2+}]\), in five of eight monolayers.
volved in intracellular Ca\(^{2+}\) regulation in vascular endothelial cells. The study thus extends the recent work of Lesh et al,\(^{22}\) who demonstrated the presence of the ryanodine receptor in vascular and endocardial endothelium. In HAECS, BPAECs, and HUVECs, ryanodine exposure resulted in a slow sustained increase in [Ca\(^{2+}\)]. Whereas ryanodine did not increase [Ca\(^{2+}\)], in unstimulated RAECs, a 15-minute exposure inhibited Ca\(^{2+}\) release from the agonist-sensitive intracellular pool in this cell type. The maximal concentration used in the present study (0.1 \(\mu\)mol/L) is in the range used by others to probe the Ca\(^{2+}\) uptake and release properties of the sarcoplasmic reticulum. Lattanzio et al\(^{29}\) found that similar concentrations (0.01 to 10 \(\mu\)mol/L) increased the Ca\(^{2+}\) permeability of both skeletal and cardiac muscle sarcoplasmic reticulum membranes, and Hansford and Lakatta\(^{29}\) found that 0.01 to 1 \(\mu\)mol/L ryanodine caused a slow and prolonged increase in [Ca\(^{2+}\)], in suspensions of intact cardiac myocytes.

The characteristics of the ryanodine-induced Ca\(^{2+}\) elevation in HAECS, BPAECs, and HUVECs are different from that due to agonist stimulation of endothelial cells. Exposure of indo 1–loaded endothelial cells to either bradykinin (Fig 2) or histamine (Fig 1) results in a rapid increase in [Ca\(^{2+}\)], which then decreases to a level above baseline for several minutes before returning to control values. In contrast, ryanodine exposure in HAECS (Fig 1), BPAECs, and HUVECs results in a slower, more sustained elevation in [Ca\(^{2+}\)] that is smaller in magnitude than that due to agonist exposure. This gradual increase in [Ca\(^{2+}\)], may be the result of Ca\(^{2+}\) release from the endoplasmic reticulum exceeding the rate of extrusion from the cytosol. Alternatively, it may reflect activation of Ca\(^{2+}\) influx from the extracellular space by depletion of an internal Ca\(^{2+}\) store. The activation of Ca\(^{2+}\) influx by depletion of an intracellular Ca\(^{2+}\) store in cultured vascular endothelial cells has recently been reported,\(^{38,29}\) although the precise manner in which the regulation of extracellular Ca\(^{2+}\) influx occurs remains unclear. Although the mechanism of action of ryanodine is likely the same in all endothelial types studied, the rate of Ca\(^{2+}\) release and the Ca\(^{2+}\) dependence of ryanodine binding to its receptor may differ among them.

Although ryanodine did not affect [Ca\(^{2+}\)], in unstimulated RAECs, a 15-minute exposure to ryanodine after initial agonist stimulation considerably inhibited the increase in [Ca\(^{2+}\)] induced by a second agonist stimulation. This indicates that at least some of the ryanodine effect in this cell type is on an agonist- and InsP\(_3\)-sensitive intracellular Ca\(^{2+}\) pool. In a similar manner, Giannini et al\(^{29}\) recently reported the existence of a functional ryanodine receptor in mink lung epithelial cells treated with transforming growth factor-\(\beta\). Although exposure of these cells to ryanodine (100 to 200 \(\mu\)mol/L for up to 20 minutes) also did not alter [Ca\(^{2+}\)], 10 \(\mu\)mol/L ryanodine prevented the increase in [Ca\(^{2+}\)], induced by bradykinin, as in the present study.

The effects of ryanodine in vascular endothelial cells appear to vary with species, the vascular bed of origin of the cell type, or both. This is similar to the variation in the effect of ryanodine on inhibition of the twitch in cardiac muscle preparations from different species.\(^{31-34}\) Although the etiology of this species variation for both vascular endothelium and cardiac muscle is unknown, it may relate to a species difference in the extent of endoplasmic or sarcoplasmic reticulum loading. Alternatively, it may relate to differences in membrane permeability to ryanodine. Our results also indicate some variability even within a given cell type.

In summary, we have documented the existence of functional ryanodine-sensitive Ca\(^{2+}\) stores in cultured vascular endothelial cells, which largely deplete the InsP\(_3\)-sensitive intracellular Ca\(^{2+}\) pool. The intracellular Ca\(^{2+}\) pool in endothelial cells plays an important role in the rapid [Ca\(^{2+}\)], rise seen on exposure to several physiological agonists such as adenosine nucleotides,\(^{4}\) bradykinin,\(^{5,8}\) histamine,\(^{9-12}\) and thrombin\(^{2-10}\) through generation of InsP\(_3\). Like InsP\(_3\) receptors, endothelial ryanodine receptors are involved in Ca\(^{2+}\) regulation of agonist-sensitive intracellular compartments. The development of pharmacologic probes of the intracellular Ca\(^{2+}\) pool in vascular endothelial cells will enhance our knowledge of endothelial Ca\(^{2+}\) homeostasis.

Acknowledgments

The statistical expertise of Frances O’Connor and the secretarial assistance of Miriam Glaser are greatly appreciated.

References


29. Schilling WP, Cabello OA, Raja L. Depletion of the inositol 1,4,5-trisphosphate-sensitive intracellular Ca\textsuperscript{2+} store in vascular endothelial cells activates the agonist-sensitive Ca\textsuperscript{2+}-influx pathway. *Biochem J.* 1992;284:521-530.


A functional ryanodine-sensitive intracellular Ca2+ store is present in vascular endothelial cells.
R C Ziegelstein, H A Spurgeon, R Pili, A Passaniti, L Cheng, S Corda, E G Lakatta and M C Capogrossi

Circ Res. 1994;74:151-156
doi: 10.1161/01.RES.74.1.151

The online version of this article, along with updated information and services, is located on the World Wide Web at:
http://circres.ahajournals.org/content/74/1/151

Permissions: Requests for permissions to reproduce figures, tables, or portions of articles originally published in Circulation Research can be obtained via RightsLink, a service of the Copyright Clearance Center, not the Editorial Office. Once the online version of the published article for which permission is being requested is located, click Request Permissions in the middle column of the Web page under Services. Further information about this process is available in the Permissions and Rights Question and Answer document.

Reprints: Information about reprints can be found online at:
http://www.lww.com/reprints

Subscriptions: Information about subscribing to Circulation Research is online at:
http://circres.ahajournals.org//subscriptions/