Ca\textsuperscript{2+} signaling in human fetoplacental vasculature: effect of CGRP on umbilical vein smooth muscle cytosolic Ca\textsuperscript{2+} concentration

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Dong, Yuan-Lin, Sujatha Vegiraju, and Chandrasekhar Yallampalli. Ca\textsuperscript{2+} signaling in human fetoplacental vasculature: effect of CGRP on umbilical vein smooth muscle cytosolic Ca\textsuperscript{2+} concentrations. *Am J Physiol Heart Circ Physiol* 289: H960–H967, 2005; doi:10.1152/ajpheart.00059.2005.—CGRP is a potent vasodilator with increased levels in fetoplacental circulation during late pregnancy. We have recently demonstrated that acute CGRP exposure to fetoplacental vessels in vitro induced vascular relaxation, but the signaling pathway of CGRP in fetoplacental vasculature remains unclear. We hypothesized that CGRP relaxes fetoplacental vascular via regulating smooth muscle cytosolic Ca\textsuperscript{2+} concentrations. In the present study, by using human umbilical vein smooth muscle (HUVE) cells (HUVE-112D), we examined CGRP receptors, cAMP generation, and changes in cellular Ca\textsuperscript{2+} concentrations on CGRP treatment. These cells express mRNA for CGRP receptor components, calcitonin receptor-like receptor, and receptor activity-modifying protein-1. Direct saturation binding for \textsuperscript{125}I-labeled CGRP to HUVE cells and Scatchard analysis indicate specificity of the receptors for CGRP (dissociation constant \(K_D\) = 67 nM, maximum binding capacity \(B_{max}\) = 2.7 pmol/million cells). Exposure of HUVE cells to CGRP leads to a dose-dependent increase in intracellular cAMP accumulation, and this increase is prevented by CGRP antagonist CGRPs. Using fura-2-loaded HUVE cells, we monitored the effects of CGRP on intracellular Ca\textsuperscript{2+} concentration ([Ca\textsuperscript{2+}\textsubscript{i}]). In the presence of extracellular Ca\textsuperscript{2+}, bradykinin (10\^-6 M), a fetoplacental vasoconstrictor, increases HUVE cells [Ca\textsuperscript{2+}\textsubscript{i}], concentration. CGRP (10\^-8 M) abolishes bradykinin-induced [Ca\textsuperscript{2+}\textsubscript{i}] elevation. When the cells were pretreated with glibenclamide, an ATP-sensitive potassium channel blocker, the CGRP actions on bradykinin-induced Ca\textsuperscript{2+} influx were profoundly inhibited. In the absence of extracellular Ca\textsuperscript{2+}, CGRP (10\^-8 M) attenuated the increase of [Ca\textsuperscript{2+}\textsubscript{i}], induced by a sarcoplasmic reticulum Ca\textsuperscript{2+} pump ATPase inhibitor thapsigargin (10\^-2 M). Furthermore, Rp-cAMPS, a cAMP-dependent protein kinase A inhibitor, blocks CGRP actions on thapsigargin-induced Ca\textsuperscript{2+} release from sarcoplasmic reticulum. Our results suggested that CGRP relaxes human fetoplacental vessels by not only inhibiting the influx of extracellular Ca\textsuperscript{2+} but also attenuating the release of intracellular Ca\textsuperscript{2+} from the sarcoplasmic reticulum, and these actions might be attributed to CGRP-induced intracellular cAMP accumulation.

vascular smooth muscle cells; fetoplacental circulation; calcitonin gene-related peptide; cytosolic calcium mobilization

CALCITONIN GENE-RELATED PEPTIDE (CGRP) is one of the most potent endogenous vasodilators known (33). CGRP is primarily synthesized in the sensory neurons of dorsal root ganglia, which extend axons centrally to the spinal cord and peripherally to various organs, including blood vessels (5), and is present in the bloodstream (2). In the pregnant woman, the serum levels of CGRP are significantly increased in both maternal and fetal circulation (31). The magnitude of increases in fetal serum CGRP is related to the fetal weight and gestational age (25), suggesting that CGRP may be involved in fetal growth and development. Recently, we have demonstrated that CGRP-receptor components calcitonin receptor-like receptor (CRLR) and receptor activity-modifying protein-1 (RAMP-1) are abundantly expressed by the vascular endothelium and underlying smooth muscle cells in the umbilical artery and vein, chorionic artery and vein, and stem villous vessels of the human placental villi (10). Furthermore, in an in vitro isometric force measurement showed that CGRP dose dependently relaxes umbilical artery and vein, chorionic artery and vein, and stem villous vessels (10), implying that CGRP present in fetoplacental circulation may play a role in the control of local vascular tone. However, the mechanisms of CGRP actions on human fetoplacental vessels remain under investigation.

The increase in the intracellular Ca\textsuperscript{2+} concentration ([Ca\textsuperscript{2+}\textsubscript{i}]) in vascular smooth muscle cells plays an important role in tension development in smooth muscle (4). Vasorelaxants mobilize Ca\textsuperscript{2+} at both the plasma membrane level and the intracellular level through different mechanisms and finally increase the [Ca\textsuperscript{2+}\textsubscript{i}], and contract the muscle (7). Vasodilators could affect these mechanisms to decrease the [Ca\textsuperscript{2+}\textsubscript{i}], and then relax the vessels (7). Recently, several mechanisms have been suggested for the muscle-relaxing effects of CGRP, including the stimulation of the adenyl cyclase pathway in the human osteosarcoma cells (11), the activation of guanylate cyclase in the neonatal rat spinal cord (26), the modulation of phospholipase expression in skeletal muscle cells (20), and the regulation of the [Ca\textsuperscript{2+}\textsubscript{i}], in osteoblastic UMR-106 cells (16). However, the effects of CGRP on [Ca\textsuperscript{2+}\textsubscript{i}] in fetoplacental vasculature have not yet been clarified. On the other hand, CGRP causes hyperpolarization of the smooth muscle cells in mesenteric arteries by activating an outward potassium channel current (22). Membrane hyperpolarization in response to CGRP was inhibited by glibenclamide, a blocker of ATP-sensitive potassium channels, in mesenteric arteries of rats. However, whether the activation of ATP-sensitive potassium channels was involved in CGRP action on [Ca\textsuperscript{2+}\textsubscript{i}], in human umbilical vein smooth muscle (HUVE) cells has not yet been established. We hypothesized that cytosolic Ca\textsuperscript{2+} mobilization is involved in CGRP-induced fetoplacental vascular relaxation. Thus the aims of the present study are to investigate the effect of CGRP on [Ca\textsuperscript{2+}\textsubscript{i}], and the role of cAMP in [Ca\textsuperscript{2+}\textsubscript{i}], regulation by using fura-2, a fluorescent Ca\textsuperscript{2+} indicator, in HUVE cell line, HUVE-112D.
MATERIALS AND METHODS

Cell culture. HUVS-112D cell line (American Type Culture Collection, Manassas, VA) was grown in Kaighn’s F12K medium containing 2 mM L-glutamine, 1.5 g/l sodium bicarbonate, and supplemented with 0.1 mg/ml heparin, 0.03 mg/ml endothelial cell growth supplement, and 10% fetal bovine serum, and maintained according to the manufacturer’s instructions. The cells were serum starved for at least 12 h before stimulation.

Immunofluorescent staining. HUVS cells were cultured in Lab-Tek two-well chamber slides (Nunc; Naperville, IL) at 1 × 10^5 cells/ml for 48 h. The cells fixed by 70% ice-cold acetone on chamber slides were exposed to anti-α-smooth muscle actin-FITC (mouse monoclonal, 1:200, Santa Cruz Biotechnology, Santa Cruz, CA) and incubated at room temperature for 4 h. The slides were rinsed with PBS (0.1 M, pH 7.4) for 30 min and then mounted with coverslips using 4’6-diamidino-2-phenylindole (Vector Laboratories; Burlingame, CA). The slides were viewed with an Olympus microscope with Image-ProPlus Software (Olympus Optical; Tokyo, Japan).

Isolation of RNA and RT-PCR analysis. Total RNA was isolated from the cells using TRIzol reagent (GIBCO-BRL; Grand Island, NY), and first-strain cDNA was synthesized by RT as previously described (8). Briefly, 2 μg of RNA were added to the reaction mixture containing 3.5 mM MgCl2, 50 mM KCl, 10 mM Tris-HCl, 0.5 mM dGTP, dATP, dTTP, and dCTP; 5 μM RNase inhibitor; 10 units murine leukemia virus RT; and 0.05 μg random primer. For RT, samples were placed into a Progene thermal cycler (Techne; Princeton, NJ) for one cycle at 25°C for 10 min, 42°C for 40 min, and 94°C for 2 min. PCR was initiated by the specific primer sets for CRLR and RAMP-1 (CRLR: 5’-CAGAATTGCTTGAACCTCTC-3’ and 5’-CAGAATTGCTTGAACCTCTC-3’; RAMP-1: 5’-GAGACGCTGTGTTGACTG-3’ and 5’-TCGCCATCTTGAGCTCTCT-3’). Primer sequences for CRLR and RAMP-1 were derived from published sequences (23). PCR was carried out according to the following conditions: an initial denaturation step at 95°C for 7 min, followed by 35 cycles of 30 s at 95°C, 1 min at 60°C, and 30 s at 72°C. Reactions were terminated by a 7-min elongation step at 72°C. PCR products were loaded onto a 1.8% agarose gel containing 0.5 μg/ml ethidium bromide and run in 0.5% Tris-borate-EDTA buffer at 100 V for 2 h. Gels were placed on a UV light box and imaged. The identity of the amplified sequences has been verified by sequencing the gel-extracted PCR product.

CGRP binding assay. Membranes were prepared from HUVS cells, and radiolabeled CGRP binding assay was performed as previously described (9). Cell membrane preparations were incubated with 1 × 10^-11 M of 125I-labeled CGRP with or without varying concentrations of unlabeled CGRP (10^-14 to 10^-7 M) in a total volume of 300 μl assay buffer with 0.5% heat-inactivated BSA for 150 min at 4°C. After incubation, 600 μl of assay buffer were added to each tube and centrifuged at 12,000 g for 5 min at 4°C. The bound radioactivity remaining in the pellets was counted with a gamma counter. Specific binding was calculated by subtracting the labeled CGRP bound in the presence of 0.5 μM unlabeled CGRP from the total amount of labeled CGRP bound, and the receptor density in the cells was expressed as CGRP femtomole bound per milligram of protein. The data were analyzed with the Scatchard method.

RIA measurement of intracellular cAMP. Cells cultured in 35-mm well plates were initially treated with four doses of CGRP (10^-10 to 10^-7 M) in the presence of 100 μM phosphodiesterase inhibitor IBMX (Sigma; St. Louis, MO) for 5 min. Cells treated with IBMX alone served as the control. Reactions were terminated by replacing the medium with ice-cold ethanol and freezing the cells at -80°C. Supernatant obtained after brief sonication and centrifugation of cells was concentrated in a speed vacuum pump and reconstituted in a 500-μl assay buffer. cAMP was quantified using cAMP 125I assay kit (Amersham Biosciences UK) as described by the manufacturer. The cAMP standards (2–128 fmol/tube) and samples were acetylated by adding triethylamine/acetic anhydride (2:1 vol/vol, 5 μl/tube). Labeled cAMP bound to its antibodies was recovered by using magnetic beads coated with goat anti-rabbit IgG. The radioactivity was quantified with a gamma counter, and the results were expressed as femtomole per million cells.

Measurement of [Ca2+]i. Real-time recordings of [Ca2+]i were performed on single cells as previously described (15). Cells were plated onto glass coverslips at a density of 1 × 10^5/ml in Kaighn’s F12K medium and kept in culture until the cells became confluent. Cells were washed with a physiological salt solution (PSS) containing (in mM) 125 NaCl, 5 KCl, 1.2 KH2PO4, 1.2 MgSO4, 1.25 CaCl2, 6 glucose, and 25 HEPES (pH 7.4). Cells were then incubated with 2 μM fura-2 AM (Molecular Probes; Eugene, OR) at 25°C for 60 min in a dark compartment. After being washed with the PSS buffer, the coverslip with attached cells was placed in a Sykes-Moore chamber of 1 ml volume on the stage of a EPI200-TE200-IUC Quantitative Fluorescence Live-Cell and Multidimensional Imaging System (Nichon). Fluorescence was excited at 340 nm (F340) and 380 nm (F380), and the emission was measured at 510 nm. The ratio of F340/F380 (R340/380) was considered to be an index of [Ca2+]i, in the smooth muscle cells (24). Background fluorescence, obtained by quenching the fura-2 fluorescence with MnCl2 (1 mM), was subtracted. Cells showing a lack of basal responsiveness to KCl (30 mM, defined as an increase of [Ca2+]i ≥ 50% of basal) were excluded from further study. No differences in KCl responsiveness were noted in cells used from passages 4 to 5 (the majority used) compared with those of later passages.

To investigate whether the effects of CGRP on [Ca2+]i were due to a block of an influx of Ca2+ from the extracellular milieu, we prepared the cells in the medium containing Ca2+ (1.25 mM) and stimulated the cells with bradykinin, a vasoconstrictor in human umbilical arteries and veins. To determine whether the effects of CGRP on [Ca2+]i were due to an inhibition of Ca2+ mobilization from intracellular stores, we prepared the cells in a Ca2+-free medium and induced the Ca2+ release from intracellular stores by thapsigargin, a selective antagonist of the sarcoplasmic reticulum Ca2+ pump. The changes in R340/380 induced by bradykinin (10^-6 M) and thapsigargin (10^-5 M) in the cell preparations served as the control.

Statistics. Results are expressed as means ± SE. Data were analyzed for statistical differences with one-way ANOVA, followed by post hoc corrections to verify differences between individual groups. A P value of <0.05 was considered significant.
RESULTS

Demonstration of vascular smooth muscle cell property by HUVS cells. By using monoclonal anti-α-smooth muscle actin-FITC, we demonstrated the abundant expression of the smooth muscle cell marker α-actin in the HUVS cells, confirming the smooth muscle cell nature of this cell line (Fig. 1).

Existence of CGRP receptors in HUVS cells. RT-PCR analysis with specific primers showed that both CRLR and RAMP-1 mRNA are abundantly expressed by the HUVS cells, implying the existence of CGRP receptors in these cells (passages 4 and 5) (Fig. 2). Specific binding of CGRP on HUVS cells was examined to determine the binding function of CGRP receptors on these cells and their specificity. The direct saturation-binding curve for 125I-labeled CGRP to HUVS cells was obtained. As shown in Fig. 3, the Scatchard analysis for this binding indicated specificity of the receptors for CGRP in this cell line. CGRP binding in these cells revealed a dissociation constant ($K_D$) of 67 nM, with a maximum binding capacity ($B_{max}$) value of 2.7 pmol/million cells.

Effects of CGRP on cAMP production. To determine whether CGRP is coupled to adenylate cyclase in these muscle cells, we treated the cells with increasing concentrations of CGRP. As shown in Fig. 4, CGRP induced a dose-dependent increase in cAMP production. The direct saturation-binding curve for 125I-labeled CGRP to HUVS cells was obtained. As shown in Fig. 5, the Scatchard analysis for this binding indicated specificity of the receptors for CGRP in this cell line. CGRP binding in these cells revealed a dissociation constant ($K_D$) of 67 nM, with a maximum binding capacity ($B_{max}$) value of 2.7 pmol/million cells.

Fig. 2. RT-PCR analysis of mRNA expression for the CGRP receptor components calcitonin receptor-like receptor (CRLR, 497 bp), receptor activity modifying protein-1 (RAMP-1, 220 bp), and 18S (320 bp) mRNA in HUVS cells (passages 4 and 5) is shown.

Fig. 3. Scatchard analysis of 125I-labeled CGRP binding to HUVS cells. Cell membranes (1 mg/ml membrane protein) prepared from cells were incubated with 2.5 fmol of 125I-labeled human CGRP per tube as described in MATERIALS AND METHODS. Each point represents mean of triplicate incubations of the cells; dissociation constant ($K_D$) = 67 nM, maximum binding capacity ($B_{max}$) = 2.7 pmol/million cells.

Fig. 4. Effect of CGRP on the cAMP production in the HUVS cells. cAMP content was measured in the cells incubated with CGRP at $1 \times 10^{-10}$ M, $1 \times 10^{-9}$ M, $1 \times 10^{-8}$ M, and $1 \times 10^{-7}$ M in the presence of IBMX ($1 \times 10^{-4}$ M). Values are means ± SE ($n = 6$ experiments). Bars with different letters (a, b, c, d, e) at the top vary significantly ($P < 0.05$).

Fig. 5. Effect of CGRP8 –37 on CGRP-induced cAMP production in the HUVS cells. cAMP content was measured in the cells incubated with CGRP at $1 \times 10^{-10}$ M and CGRP8 –37 ($1 \times 10^{-7}$ M) plus CGRP ($1 \times 10^{-8}$ M) in the presence of IBMX ($1 \times 10^{-4}$ M). CTL, control. Values are means ± SE ($n = 6$ experiments). Bars with different letters (a, b) at the top vary significantly ($P < 0.01$).
CGRP. As shown in Fig. 4, in the presence of IBMX, a cAMP phosphodiesterase inhibitor, CGRP (10^{-11} to 10^{-7} M) caused a dose-dependent increase in cAMP production in the HUVS cells, suggesting that adenylate cyclase activation mediated CGRP actions in HUVS cells. Furthermore, CGRP_{8-37}, a CGRP receptor antagonist, completely blocks the elevation in cAMP in cultured cells (Fig. 5), implying that the actions of CGRP on HUVS cell cAMP production were mediated through CGRP receptors.

Effects of CGRP on [Ca^{2+}]_{i} induced by bradykinin in the presence of extracellular Ca^{2+}. In fura-2-loaded HUVS cell monolayers, the application of bradykinin, a vasoconstrictor in the fetoplacental vasculature, induced a transient increase in [Ca^{2+}]_{i} in normal PSS containing 1.25 mM Ca^{2+}. This increase in [Ca^{2+}]_{i} formed a sharp peak, which fell rapidly after ~10 s but remained above the basal levels (Fig. 6A). However, when bradykinin was added at 200 s after incubation with CGRP, the [Ca^{2+}]_{i} increase was completely abolished (Fig. 6B). When the cells were pretreated with glibenclamide, an ATP-sensitive potassium channel blocker, the CGRP actions on bradykinin-induced [Ca^{2+}]_{i} influx were profoundly inhibited (Fig. 6C). Summarized data in Fig. 7 demonstrated that bradykinin stimulates Ca^{2+} influx, which is blocked by CGRP, suggesting that CGRP relaxes fetoplacental vasculature via, at least in part, inhibiting the influx of extracellular Ca^{2+}. Furthermore, glibenclamide abolished CGRP action on bradykinin-induced Ca^{2+} influx, indicating that CGRP inhibits extracellular Ca^{2+} influx via activation of ATP-sensitive potassium channels.

To determine whether bradykinin-induced elevation of [Ca^{2+}]_{i} is an influx of extracellular Ca^{2+} or a release from its intracellular stores, we examined the effect of CGRP on [Ca^{2+}]_{i} in the HUVS cells in the Ca^{2+}-free medium containing 2 mM EGTA. As shown in Fig. 8A, bradykinin (10^{-6} M) failed
to change $[Ca^{2+}]_i$, in the HUVS cells. Taken together with the findings in Fig. 6A, in which bradykinin increases $[Ca^{2+}]_i$, in the presence of extracellular $Ca^{2+}$, these results suggested that in the HUVS cells, bradykinin increases $[Ca^{2+}]_i$, primarily through an influx of extracellular $Ca^{2+}$ rather than the release of $Ca^{2+}$ from intracellular stores.

**Effects of CGRP on the $[Ca^{2+}]_i$, induced by thapsigargin in the absence of extracellular $Ca^{2+}$**. To observe the effect of CGRP on $[Ca^{2+}]_i$, release from intracellular stores, we cultivated the cells in $Ca^{2+}$-free medium. Figure 8A shows the representative recordings of changes in $[Ca^{2+}]_i$, induced by thapsigargin in $Ca^{2+}$-free medium containing 2 mM EGTA. Thapsigargin, a sarcoplasmic reticulum $Ca^{2+}$ pump ATPase inhibitor, caused transient elevation of $[Ca^{2+}]_i$ in the HUVS cells. This increase in $[Ca^{2+}]_i$ formed a sharp peak, which fell rapidly after $\sim 10$ s but remained above the basal levels. However, the application of CGRP in $Ca^{2+}$-free medium substantially attenuated $[Ca^{2+}]_i$, release from sarcoplasmic reticulum (Fig. 8B). Furthermore, as shown in Fig. 8C, Rp-cAMPS, a cAMP-dependent protein kinase A inhibitor, blocks CGRP actions, suggesting that CGRP attenuates sarcoplasmic reticulum $Ca^{2+}$ release via activation of adenylyl cyclase. Summarized data shown in Fig. 9 confirmed the findings that CGRP relaxes fetoplacental vascular smooth muscle cells by not only inhibiting the influx of extracellular $Ca^{2+}$ but also attenuating the release of intracellular $Ca^{2+}$, and these actions might be attributed to cAMP accumulation induced by CGRP.

**DISCUSSION**

Regulation of placental blood flow in both maternal and fetal compartments affects placental transport of oxygen and nutrients, which ultimately determines fetal growth and well being. Unlike other vascular systems, the fetoplacental unit lacks innervation and therefore depends on humoral substances for the control of vascular tone (6). CGRP is a powerful vasodilator circulating in fetal placental units (25). We have recently reported that CGRP dose dependently relaxes the umbilical and chorionic artery and vein in the human placenta, suggesting that CGRP may play a role in human fetoplacental vascular relaxation. However, the mechanisms of CGRP actions on the fetoplacental vasculature have not yet been fully documented. In the present study, we demonstrated the existence of CGRP receptors on HUVS cells by RT-PCR analysis and competitive binding assay. We also found that CGRP acts on HUVS cells to cause intracellular cAMP accumulation. Furthermore, CGRP was found to decrease $[Ca^{2+}]_i$, via two mechanisms. First, it blocked the influx of $Ca^{2+}$ from the extracellular milieu via activation of ATP-sensitive potassium channels as indicated by the experiments with glibenclamide. Second, CGRP inhibits the release of $Ca^{2+}$ from the sarcoplasmic reticulum in HUVS cells as indicated by the experiments with thapsigargin. Therefore, CGRP may act as a circulating hormone in the fetoplacental circulation and may play an important role in the control of fetoplacental vascular tone.

**Involvement of ATP-sensitive potassium channels in CGRP actions**. Intracellular $Ca^{2+}$ homeostasis has been thought to be a primary factor in regulating contractility of all kinds of muscles, including vascular smooth muscles. Previous studies (22, 30) have suggested that CGRP activates, in addition to adenylyl cyclase, ATP-sensitive potassium channels to produce membrane hyperpolarization. The resultant inhibition of $Ca^{2+}$ uptake through voltage-dependent $Ca^{2+}$ channels is thought to be central to the muscle relaxation (22, 30). It has been reported that CGRP decreases $[Ca^{2+}]_i$, and $Ca^{2+}$ sensitivity of contractile elements in intact and $\alpha$-toxin-permeabilized smooth muscle strips in the porcine coronary artery (12), but the potential disadvantage in using a muscle strip for the measurement of $[Ca^{2+}]_i$, could be the heterogeneity of cell types. In that case, the fluorescence signal from cell types other than smooth muscle cells could interfere with the measurement and the interpretation of the results. The present study, by using fura-2-loaded HUVS cells, demonstrates that bradykinin, a powerful vasoconstrictor in the human fetoplacental circulation (1, 13), induces a substantial increase in $[Ca^{2+}]_i$, in normal PSS containing 1.25 mM $Ca^{2+}$. Application of CGRP on the cells completely abolishes $[Ca^{2+}]_i$, increase induced by bradykinin. However, when the cells were pretreated with glibenclamide, an ATP-sensitive potassium channel blocker, the CGRP action on $[Ca^{2+}]_i$, influx was significantly attenuated. Because the ability of CGRP to decrease bradykinin-induced $[Ca^{2+}]_i$, could be blocked by glibenclamide, we suggested that opening of $K^+$ channels and resultant plasma membrane hyperpolarization might mediate such an action. Our present results support the hypothesis that CGRP relaxes fetoplacental vasculature via, at least in part, inhibiting the net uptake of extracellular $Ca^{2+}$ into smooth muscle cells.

**Regulation of intracellular $Ca^{2+}$ by CGRP**. Alternatively, the release of $Ca^{2+}$ from the intracellular stores is another key step in excitation-contraction coupling events in vascular smooth muscle. Thapsigargin, a selective antagonist of the sarcoplasmic reticulum $Ca^{2+}$ pump, increases cytosolic $Ca^{2+}$ by emptying intracellular $Ca^{2+}$ stores (14). The present study showed that CGRP attenuated thapsigargin-induced $[Ca^{2+}]_i$, increase in $Ca^{2+}$-free PSS. These results strongly indicate that CGRP functions as a vasodilator in fetoplacental circulation by inhibiting both the influx of extracellular $Ca^{2+}$ and the release...
of intracellular Ca\(^{2+}\) from sarcoplasmic reticulum of the smooth muscle cells. To the best of our knowledge, this is the first report demonstrating the regulation of CGRP on intracellular Ca\(^{2+}\) mobilization in HUVS cells.

It has been reported that the release of Ca\(^{2+}\) from sarcoplasmic reticulum is mediated by activation of the phospholipase C-inositol 1,4,5-trisphosphate [Ins(1,4,5)P\(_3\)] cascade, which couples to receptors by G proteins (18). Inhibitors of phospholipase C (neomycin) and Ins(1,4,5)P\(_3\) (heparin) block the release of intracellular Ca\(^{2+}\) (34). Further studies (17, 28) have shown that cAMP stimulates Ca\(^{2+}\) uptake into the sarcoplasmic reticulum through activation of Ca\(^{2+}\) pump ATPase. Our study showed that Rp-cAMPS, a cAMP-dependent protein kinase A inhibitor, attenuated the action of CGRP. It is likely that CGRP-induced cAMP accumulation in the HUVS cells activates Ca\(^{2+}\) pump ATPase on the sarcoplasmic reticulum membrane, and the activation of Ca\(^{2+}\) pump stimulates Ca\(^{2+}\) uptake into the sarcoplasmic reticulum and results in decreased cytosolic free Ca\(^{2+}\) concentration.

**Role of cAMP in CGRP signaling pathway.** In many vascular preparations, there is a strong correlation between CGRP-induced relaxation and the elevation in intracellular cAMP. CGRP stimulates the accumulation of cAMP in cultured vascular smooth muscle cells from rat thoracic aortas (19). CGRP-induced dilation of the rat middle cerebral artery was accompanied by an increase in adenylate cyclase activity (32). We (10) have previously demonstrated that CGRP-induced relaxation of human chorionic arteries was inhibited by cAMP-dependent protein kinase A inhibitor Rp-cAMPS, implying that cAMP accumulation is involved in the smooth muscle relaxation in fetoplacental circulation. It has been proposed that cAMP induces vasodilation through three distinct ways: 1) an activation of Ca\(^{2+}\)-activated potassium channels, 2) an inhibition of myosin phosphorylation through phosphorylation
ATPase (Ca\(^{2+}\)) effects on different vascular beds is unknown. Two mammalian mechanisms by which bradykinin induces directionally opposite responses in isolated human umbilical arteries and veins (1, 13). The systemic vasodilator but has been shown to cause vasoconstriction in rodent (21). Bradykinin-induced vasoactive response is associated with changes in intracellular Ca\(^{2+}\) levels. In human and rabbit aortic vascular smooth muscle cells (3), bradykinin activates R-, T-, and L-type Ca\(^{2+}\) channels and induces a sustained increase of nuclear Ca\(^{2+}\). In the rat afferent and efferent glomular arterial microdissected from the juxtaglomerular apparatus, bradykinin increases [Ca\(^{2+}\)], through activation of B2 receptors located on the endothelium, opens voltage-independent channels, and mobilizes intracellular Ca\(^{2+}\) (27). The present study demonstrated that bradykinin increases [Ca\(^{2+}\)]; in HUVS cells in normal PSS containing 1.25 mM Ca\(^{2+}\) but failed to change the [Ca\(^{2+}\)] levels in the cells in the Ca\(^{2+}\)-free medium. These results suggested that in the HUVS cells, bradykinin increases [Ca\(^{2+}\)], mainly through an influx of extracellular Ca\(^{2+}\) rather than the mobilization of Ca\(^{2+}\) in intracellular stores. Further study is apparently warranted to determine the type of bradykinin receptor on the HUVS cells and the Ca\(^{2+}\) channels on the cell membrane that was involved in the extracellular Ca\(^{2+}\) mobilization.

In summary, the present study supports the notion that the direct Ca\(^{2+}\)-dependent, vascular-relaxant effects of CGRP play a central role in the control of the fetoplacental vascular tone. These direct vascular actions of CGRP may explain the extremely low vascular resistance in fetoplacental circulation in normal pregnancy. Further studies are needed to examine the direct effects of CGRP on the L-type Ca\(^{2+}\) channel and on potassium chloride-induced increases in Ca\(^{2+}\) and to explore the possible link between CGRP inhibited [Ca\(^{2+}\)] mobilization in HUVS cells and increased vascular resistance in complicated pregnancies, such as intruteral fetal growth restriction and preeclampsia.

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GRANTS

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