Estrogen increases Ca\textsuperscript{2+} efflux from female porcine coronary arterial smooth muscle


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Praakash, Y. S., A. A. Togaibayeva, M. S. Kannan, V. M. Miller, L. A. Fitzpatrick, and G. C. Sieck. Estrogen increases Ca\textsuperscript{2+} efflux from female porcine coronary arterial smooth muscle. Am. J. Physiol. 276 (Heart Circ. Physiol. 45): H926–H934, 1999.—Acute estrogen administration relaxes vascular smooth muscle by decreasing intracellular Ca\textsuperscript{2+} concentration ([Ca\textsuperscript{2+}]i). In the present study, we examined the hypothesis that this reduction in [Ca\textsuperscript{2+}]i is mediated in part by enhanced Ca\textsuperscript{2+} efflux. Coronary artery smooth muscle cells were isolated from gonad-intact, sexually mature female pigs. The [Ca\textsuperscript{2+}]i response to endothelin-1 was measured using fluo 3 and confocal microscopy. 17\beta-Estradiol (E\textsubscript{2}) , but not 17\alpha-estradiol or triamcinolone acetonide, caused a concentration-dependent ([C\textsubscript{50} = 10 nM] decrease in the [Ca\textsuperscript{2+}]i ) response to endothelin-1. This decrease was blocked by the specific estrogen receptor antagonist ICI-182780. Under conditions in which Ca\textsuperscript{2+} influx and sarcoplasmic reticulum Ca\textsuperscript{2+} reuptake were blocked, E\textsubscript{2} still decreased [Ca\textsuperscript{2+}]i . The response was blocked by extracellular lanthanum. These data indicate that E\textsubscript{2} decreases [Ca\textsuperscript{2+}]i in coronary artery smooth muscle by affecting Ca\textsuperscript{2+} efflux via a receptor-mediated mechanism.

vasodilation; endothelin; receptor; calcium adenosine 5’- triphosphatase.

ACUTE ADMINISTRATION of estrogens relaxes vascular smooth muscle, causing a reduction in vascular resistance and an increase in blood flow (3, 5, 8, 9, 16, 19, 21, 25, 30). The rapid relaxation of vasomotor tone induced by estrogens is likely mediated through nongenomic effects (for review, see Ref. 2) and probably involves estrogen receptors (2, 11, 20). However, despite considerable evidence for acute, nongenomic effects of estrogen on vascular tissue, the mechanisms underlying these effects are not completely understood.

Estrogen-induced relaxation of vascular smooth muscle involves a reduction in intracellular Ca\textsuperscript{2+} concentration ([Ca\textsuperscript{2+}]i ) (15, 19). One demonstrated mechanism for this effect is a decrease in Ca\textsuperscript{2+} influx (8, 9, 14). However, a reduction in [Ca\textsuperscript{2+}]i induced by estrogens may also result from an increase in Ca\textsuperscript{2+} efflux, an inhibition of Ca\textsuperscript{2+} release from the sarcoplasmic reticulum (SR), or an increase in SR Ca\textsuperscript{2+} reuptake. Therefore, the purpose of the present study was to examine whether estrogens [17\beta-estradiol (E\textsubscript{2})] affect Ca\textsuperscript{2+} efflux and SR Ca\textsuperscript{2+} release in coronary artery smooth muscle.

METHODS

Animals. Ten sexually mature female Yorkshire pigs (at least 6 mo of age) were obtained from a local supplier. Animals were anesthetized with ketamine (8 mg/kg) and xylazine (12 mg/kg), and their hearts were removed. The three main coronary arteries (right and left circumflex and left anterior descending arteries) were removed and placed in oxygenated modified Krebs-Ringer bicarbonate solution at 4°C. Blood samples were also drawn to verify hormonal status using plasma concentrations of estrogen and progesterone.

All animal surgical and care procedures were approved by the Institutional Animal Care and Use Committee of the Mayo Clinic and were in strict accordance with the American Physiological Society animal care guidelines.

Immunocytochemical detection of estrogen receptors. Freshly dissociated coronary artery smooth muscle cells were fixed in 4% paraformaldehyde in 0.1 M phosphate buffer and stained for 2–3 h with a polyclonal antibody to the estrogen receptor (Research Biochemicals; 1:2,000 dilution in phosphate buffer). After staining with the primary antibody was completed, the sections were washed in phosphate buffer and reacted with a 1:1,000 Cy3-conjugated donkey anti-rabbit IgG secondary antibody. The fluorescently labeled coronary artery smooth muscle cells were visualized using a Bio-Rad MRC500 confocal microscope equipped with an argon-krypton laser. The 568-nm laser line was used to excite the Cy3, and the emissions were collected using a 590-nm long-pass filter.

Cell preparation for Ca\textsuperscript{2+} imaging. The procedures for dissociation of single porcine coronary artery smooth muscle cells have been described previously (10). Briefly, the endothelium was removed and the tissue was minced thoroughly in Hanks’ balanced salt solution (HBSS) buffered with 10 mM HEPES (pH 7.4; Life Technologies, Gaithersburg, MD). The tissue was incubated first in 20 U/ml papain and 2,000 U/ml DNase (Worthington Biochemical, Freehold, NJ) and subsequently in 1 mg/ml type IV collagenase and 0.1 mg/ml elastase (Worthington). Single cells were released by trituration, centrifugation, and resuspension in minimum essential medium with 10% fetal calf serum. The cells were plated on glass coverslips coated with Cell-Tak (Collaborative Biomedical Products, Bedford, MA) and incubated for 1–2 h in a 5% CO\textsubscript{2} incubation chamber at 37°C. Cells were used within 4 h after dissociation. No time-dependent changes were observed in the gross morphology of the cells that were used for the experimental protocols. Exclusion of the dye trypan blue was used to assess cell viability just after dissociation (>90% of all cells). An anti-smooth muscle myosin antibody (Sigma Immunochemicals, St. Louis, MO) was used to estimate the relative proportion of smooth muscle myocytes (immunoreactive) and fibroblasts (50:1).

Each coverslip was washed with HBSS and incubated for 30–45 min at 37°C in 5 μM fluo 3-AM (Molecular Probes, The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.
Eugene, OR). The coverslip was then washed briefly in HBSS and mounted on an open slide chamber (RC-25F, Warner Instruments, Hamden, CT) mounted on the stage of a Nikon Diaphot and equipped with an argon-krypton laser was used to visualize fluo 3-loaded coronary artery smooth muscle cells (18). An Olympus ×40/1.3 air-immersion objective lens was used to visualize the cells. Image size was set to 640 × 480 pixels, and pixel area was calibrated using a stage micrometer (0.063 mm²/pixel). A fixed combination of laser intensity (20% of maximum) and photomultiplier gain (1,700 from a maximum of 4,096) was set a priori to ensure that pixel intensities within regions of interest ranged between 25 and 255 gray levels across different experimental protocols. Dye bleaching was kept to a minimum by maintaining laser intensity below 3 mW. Continued exposure to the laser did not exceed 3 min. With the use of these precautions, dye bleaching was estimated to be <5% over a 3 min period.

The Odyssey confocal system is controlled by a Silicon Graphics workstation and is capable of acquiring 480 frames/s. In preliminary studies on fluo 3-loaded coronary artery smooth muscle cells, it was determined that an acquisition rate of 30 frames/s was sufficient to measure the changes in [Ca²⁺] during various protocols without frequency aliasing. Therefore, image acquisition was limited to 30 frames/s. When necessary, image noise was reduced by acquiring at 60 or 120 frames/s with frame averaging. The sampling time for any pixel was 100 ns.

A region of the coverslip containing at least 15–20 coronary artery smooth muscle cells was selected, and a region-of-interest software tool was used to define regions within the boundaries of individual cells. Each region of interest had a fixed dimension of 10 × 10 pixels (6 µm²). The optical section thickness for the ×40 lens was set to 1 µm by controlling the confocal slit size. Therefore, Ca²⁺ measurements were obtained from a volume of 6 µm³.

The fluorescence intensity of fluo 3 was calibrated for Ca²⁺ concentrations as described previously (18). At fixed settings of laser intensity and photomultiplier gain, fluo 3-loaded coronary artery smooth muscle cells were exposed to A-23187 (Ca²⁺ ionophore) at varying levels of extracellular Ca²⁺ ranging from 0 (HBSS with EGTA) to 10 µM. Exposure to the ionophore at each extracellular Ca²⁺ concentration was limited to ~1 min to ensure that other Ca²⁺ handling mechanisms such as mitochondria and SR did not compensate for influx and thus confound the measurements. Therefore, image acquisition was limited to 30 frames/s. When necessary, image noise was reduced by acquiring at 60 or 120 frames/s with frame averaging. The sampling time for any pixel was 100 ns.

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[Ca\textsuperscript{2+}] had reached a steady state, the cells were exposed to E\textsubscript{2\beta}.

Statistical analysis. In determining the statistical design for various experiments, the influence of interanimal variability in the [Ca\textsuperscript{2+}] response to 100 nM endothelin-1 was evaluated for 15 coronary artery smooth muscle cells from each of 10 animals using a one-way ANOVA. Interanimal variability was found to be nonsignificant at P > 0.25. Based on this result, the contribution of interanimal variability was determined to be insignificant, and the pooling of results from coronary artery smooth muscle cells obtained from different animals was justified. However, for each of the experimental protocols assessing the impact of estrogen on the [Ca\textsuperscript{2+}] response to 100 nM endothelin-1, cells from at least five animals were studied, and cells from any one animal did not represent >30% of the total. In addition, individual coronary artery smooth muscle cells were exposed to only one experimental protocol. The numbers of cells used for each protocol are reported in the results, and statistical analysis (one-way ANOVA) was based on this number. Statistical significance was tested at an 0.05 level. Data are reported as means ± SE. Reductions in [Ca\textsuperscript{2+}] are reported as percent changes from the maximum [Ca\textsuperscript{2+}] response.

RESULTS

Estrogen levels and estrogen-receptor status. Serum estrogen ranged from 37 to 103 pM and averaged 56 ± 10 pM in all 10 animals. Immunocytochemical staining confirmed the presence of estrogen receptors in coronary artery smooth muscle cells (Fig. 1). Although the immunocytochemical procedure could not distinguish between nuclear and cytosolic estrogen receptors, the higher intensity of fluorescence staining in the center of the cell suggested the presence of nuclear receptors. However, immunoreactivity for cytosolic estrogen receptors distributed throughout the cell was also clearly present.

[Ca\textsuperscript{2+}] response to endothelin-1. Basal [Ca\textsuperscript{2+}] levels were not significantly different among cells and ranged from 120 to 160 nM (131 ± 3 nM; n = 426). At each concentration of endothelin-1, the [Ca\textsuperscript{2+}] response of 16 cells was determined. Endothelin-1 at 1 and 10 nM caused a slow and sustained increase in [Ca\textsuperscript{2+}]. In comparison, 100 nM and 1 μM endothelin-1 caused a more rapid increase in [Ca\textsuperscript{2+}], reaching an initial peak after 4–30 s but then decreasing slowly over the next 2–3 min to ∼70% of the peak value. The [Ca\textsuperscript{2+}] responses to varying endothelin-1 concentrations at 30 s and 2 min are reported in Table 1.

Effect of estrogens on endothelin-1-induced elevation of [Ca\textsuperscript{2+}]. The effect of E\textsubscript{2\beta} on the [Ca\textsuperscript{2+}] response to endothelin-1 was concentration dependent with an ED\textsubscript{50} of ∼10 nM (Fig. 2A). The effect of 10 nM E\textsubscript{2\beta} on the endothelin-1 dose-response (1, 10, and 100 nM) was determined (Fig. 2B). Although the [Ca\textsuperscript{2+}] response varied with endothelin-1 concentration, the inhibitory effect of E\textsubscript{2\beta} was proportionately similar across all endothelin-1 concentrations. Because the relative effect of 10 nM E\textsubscript{2\beta} was comparable across endothelin-1 concentrations, fixed concentrations of E\textsubscript{2\beta} (10 nM) and endothelin-1 (100 nM) were used in all subsequent protocols.

Table 1. [Ca\textsuperscript{2+}] response of coronary artery smooth muscle cells to endothelin-1

<table>
<thead>
<tr>
<th>Endothelin-1 Concentration</th>
<th>Δ[Ca\textsuperscript{2+}] at 30 s, nM</th>
<th>Δ[Ca\textsuperscript{2+}] at 2 min, nM</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 nM</td>
<td>65 ± 8</td>
<td>98 ± 10</td>
</tr>
<tr>
<td>10 nM</td>
<td>121 ± 11</td>
<td>161 ± 14</td>
</tr>
<tr>
<td>100 nM</td>
<td>396 ± 15</td>
<td>291 ± 19</td>
</tr>
<tr>
<td>1 μM</td>
<td>354 ± 19</td>
<td>277 ± 15</td>
</tr>
</tbody>
</table>

Values are means ± SE. Timepoints for measurement of intracellular Ca\textsuperscript{2+} concentration ([Ca\textsuperscript{2+}]) are based on average times to peak and steady-state responses at 100 nM and 1 μM endothelin-1.
Role of estrogen receptors in [Ca\(^{2+}\)]\(_i\) response to estrogens. In the presence of ICI-182780, E\(_{2\beta}\) had no effect on the [Ca\(^{2+}\)]\(_i\) response to endothelin-1 (Fig. 4; n = 51). In contrast, ICI-182780 did not abolish the effect of E\(_{2\alpha}\) on the [Ca\(^{2+}\)]\(_i\) response to endothelin-1 (Fig. 4; n = 32).

Effect of E\(_{2\beta}\) on Ca\(^{2+}\) efflux. When Ca\(^{2+}\) influx was blocked by nifedipine (n = 22) or zero extracellular Ca\(^{2+}\) (n = 13; data not shown), inhibition of SR Ca\(^{2+}\) reuptake by thapsigargin caused a gradual increase in [Ca\(^{2+}\)]\(_i\) (Fig. 5). Subsequent exposure to E\(_{2\beta}\) reduced [Ca\(^{2+}\)]\(_i\) by 95.1 ± 3.4% (Fig. 5; P < 0.05 compared with vehicle control). Under conditions of blocked Ca\(^{2+}\) influx (nifedipine: n = 32; zero extracellular Ca\(^{2+}\): n = 21; data not shown) and SR Ca\(^{2+}\) reuptake (thapsigargin), endothelin-1 induced a large increase in [Ca\(^{2+}\)]\(_i\) (Fig. 6). Subsequent exposure to E\(_{2\beta}\) reduced [Ca\(^{2+}\)]\(_i\) (Fig. 6). This reduction in [Ca\(^{2+}\)]\(_i\) was blocked by ICI-182780 (n = 22) and lanthanum (n = 31) (Fig. 6).

Effect of E\(_{2\beta}\) on SR Ca\(^{2+}\) release and reuptake. Preexposure to E\(_{2\beta}\) had no effect on the amplitude of the initial response to 100 nM and 1 μM endothelin-1. When Ca\(^{2+}\) influx and SR Ca\(^{2+}\) reuptake were blocked, the peak [Ca\(^{2+}\)]\(_i\) response to caffeine was comparable in the presence (n = 24) and absence (n = 25) of E\(_{2\beta}\) (Fig. 7). These results suggest that E\(_{2\beta}\) had no effect on SR Ca\(^{2+}\) release.

In the presence of nifedipine and lanthanum, where Ca\(^{2+}\) influx and efflux were blocked, the rate of decline of the [Ca\(^{2+}\)]\(_i\) response to endothelin-1 was comparable in the presence (n = 15) and absence (n = 14) of E\(_{2\beta}\) (Fig. 7). These results suggest that E\(_{2\beta}\) had no effect on SR Ca\(^{2+}\) reuptake.

Effect of E\(_{2\beta}\) on Ca\(^{2+}\) efflux. Exposure to E\(_{2\beta}\) significantly reduced the Ca\(^{2+}\) influx induced by 1 μM BAY K 8644 (Fig. 8). When BK\(_{Ca}\) channels were blocked by iberiotoxin, E\(_{2\beta}\) still reduced the [Ca\(^{2+}\)]\(_i\) response to endothelin-1 (Fig. 8). However, this reduction in [Ca\(^{2+}\)]\(_i\) was significantly less than that in the absence of iberiotoxin (57.5 ± 4.1 vs. 95.1 ± 3.1%, respectively).

DISCUSSION

In addition to confirming the inhibitory effect of estrogens on Ca\(^{2+}\) influx in coronary artery smooth muscle cells, the results of the present study also demonstrated that estrogens decrease [Ca\(^{2+}\)]\(_i\) by enhancing Ca\(^{2+}\) efflux via a receptor-mediated mechanism.
The conclusion that $E_2\beta$ enhances $Ca^{2+}$ efflux was supported by three observations: 1) the reduction in $[Ca^{2+}]_i$ by $E_2\beta$ was blocked by the estrogen-receptor antagonist ICI-182780; 2) the $E_2\beta$-induced reduction in $[Ca^{2+}]_i$ was observed even when $Ca^{2+}$ influx and reuptake were blocked; and 3) the $E_2\beta$-induced reduction in $[Ca^{2+}]_i$ was absent when both $Ca^{2+}$ influx and efflux were blocked by nifedipine (and/or zero extracellular $Ca^{2+}$) and lanthanum.

The enhancement of $Ca^{2+}$ efflux by $E_2\beta$ represents a novel mechanism by which estrogens regulate $[Ca^{2+}]_i$ in response to agonist stimulation. As would be expected, the relative enhancement of $Ca^{2+}$ efflux was independent of the level of endothelin-1 stimulation, as indicated by the comparable reduction in $[Ca^{2+}]_i$ at different endothelin-1 concentrations, relative to the peak response at each concentration (e.g., ~50% decrease from the peak response with 10 nM $E_2\beta$). Confirmation of the effect of $E_2\beta$ on $Ca^{2+}$ efflux was derived primarily from the fact that the response was blocked by lanthanum. However, lanthanum also blocks $Ca^{2+}$ influx, which has been previously demonstrated to be inhibited by $E_2\beta$. Therefore, we designed studies to block $Ca^{2+}$ influx independent of any inhibition on $Ca^{2+}$ efflux.

In experimental protocols designed to examine the underlying mechanisms of $E_2\beta$ action, a 100 nM endothelin-1 concentration was selected based on studies by other investigators (24, 28), who found that the initial $[Ca^{2+}]_i$ response at similar endothelin-1 concentrations represents SR $Ca^{2+}$ release and that the subsequent response represents a balance between $Ca^{2+}$ influx and efflux. In this regard, the initial peak $[Ca^{2+}]_i$ response at 100 nM endothelin-1 observed in our study most likely reflects SR $Ca^{2+}$ release, whereas the lower steady state reflects a balance between $Ca^{2+}$ influx and efflux. In contrast, endothelin-1 concentrations of 10 nM or less elicit only slow, monotonic elevations in $[Ca^{2+}]_i$ (also observed in the present study), which most likely reflect $Ca^{2+}$ influx (24). Accordingly, the selection of 100 nM allowed the examination of both SR $Ca^{2+}$ release and $Ca^{2+}$ flux across the cell membrane. In contrast to these measurements, studies using multicellular coronary artery smooth muscle strips as well as single cells have reported a decline in $[Ca^{2+}]_i$ to baseline after the initial peak $[Ca^{2+}]_i$ response to endothelin-1. However, it must be noted that this decline in multicellular preparations occurred over a considerably longer
time period (>10 min) and most likely reflects the ensemble of individual cellular responses. The serum estrogen levels of ~50 pM are consistent with previous reports in swine of 20–200 pM (7, 26). However, because estrous stage was not controlled in the present study, the 50 pM value most likely reflects an average across different stages. Given the picomolar serum estrogen levels, the 10 nM concentration of E₂β used to examine [Ca²⁺]ᵢ regulation is supraphysiological. It is difficult to directly extrapolate the reduction in [Ca²⁺]ᵢ observed with 10 nM E₂β in single coronary artery smooth muscle cells to the extent of reduction in vascular tone in vivo because, if anything, the estrogen concentration at the tissue is likely to be smaller.

![Diagram](image-url)

**Fig. 6.** Effect of E₂β on Ca²⁺ efflux after endothelin-1 (ET) activation. When Ca²⁺ influx and SR Ca²⁺ reuptake were blocked by nifedipine (Nif; or zero extracellular Ca²⁺; not shown) and thapsigargin (Tg), respectively, endothelin-1 induced a large, sustained elevation of [Ca²⁺] (A; n = 32). Subsequent exposure to E₂β reduced [Ca²⁺] (B; n = 32). This effect of E₂β on [Ca²⁺] was inhibited by lanthanum (La; C; n = 31), indicating that E₂β enhances Ca²⁺ efflux. D: summary of [Ca²⁺] responses. *Significant difference (P < 0.05) from response to endothelin-1 alone.

![Diagram](image-url)

**Fig. 7.** Effect of E₂β on SR Ca²⁺ release and reuptake. In absence of Ca²⁺ influx and SR reuptake, the large, transient [Ca²⁺] response to caffeine (A; n = 25) was not affected by preexposure to E₂β (B; n = 24). In absence of Ca²⁺ influx and efflux (blocked by nifedipine and lanthanum), endothelin-1 produced a large, sustained elevation of [Ca²⁺] (C; n = 14). Rate of decline of [Ca²⁺] response to endothelin-1 was not affected by preexposure to E₂β (D; n = 15). These data suggest that SR Ca²⁺ release and reuptake are not substantially affected by estrogens.
However, it must be noted that we observed decreases of 20–30% in [Ca\textsuperscript{2+}] even at E\textsubscript{2\beta} concentrations between 100 pM and 1 nM. These observations would suggest that circulating estrogen levels do decrease [Ca\textsuperscript{2+}] to a somewhat smaller extent, which would be consistent with a role of estrogen as a modulator of vascular tone. We opted for a concentration of 10 nM E\textsubscript{2\beta} because it approximates an IC\textsubscript{50} and thus provided a greater sensitivity for assessing the effects of various inhibitors and activators and distinguishing between the effects of Ca\textsuperscript{2+} efflux versus other mechanisms of estrogen action. It is difficult to compare the results of the present study, in which 10 nM E\textsubscript{2\beta} was used, with those of previous reports because a wide range of estrogen concentrations have been used in these studies (4, 14, 16, 19, 21a, 29) that are often greater than those used in the present study. Regardless, it appears that the mechanisms by which estrogen modulates vascular tone are similar, and it is likely that Ca\textsuperscript{2+} efflux plays a role in estrogen-induced reduction of [Ca\textsuperscript{2+}] even in vivo.

The specificity of the E\textsubscript{2\beta} effect on [Ca\textsuperscript{2+}] regulation is supported by the fact that the estrogen-receptor antagonist ICI-182780 blocked the [Ca\textsuperscript{2+}] response to E\textsubscript{2\beta}. In addition, compared with E\textsubscript{2\beta}, the biologically inactive isomer E\textsubscript{\alpha\beta} at a similar concentration was significantly less potent in reducing agonist-induced elevation of [Ca\textsuperscript{2+}], consistent with previous studies (25, 31). The lack of inhibition of the [Ca\textsuperscript{2+}] response to E\textsubscript{\alpha\beta} by ICI-182780 suggests a nonspecific non-receptor-mediated effect of E\textsubscript{\alpha\beta}. However, the mechanisms underlying the effects of E\textsubscript{\alpha\beta} are not clear. Finally, triamcinolone acetonide, a steroid not related to estrogens, did not reduce the endothelin-1-induced elevation of [Ca\textsuperscript{2+}], in coronary artery smooth muscle cells. This also suggests that the nonspecific effect of E\textsubscript{\alpha\beta} is unlikely to be an experimental artifact.

The relatively rapid changes in [Ca\textsuperscript{2+}] observed in the present study, occurring over seconds rather than minutes or hours, are clearly not compatible with the classic genomic mechanism for the action of E\textsubscript{2\beta}, which involves translocation of receptors to the nucleus and protein synthesis (1, 12). Therefore, the acute effects observed in the present study are nongenomic in nature and are also consistent with previous studies both in vivo (13, 17, 30) and in vitro (5, 8, 9, 19, 21a, 25) (also see Ref. 2 for a review). The existence of cytosolic estrogen receptors and the recent interest in the role of plasma membrane estrogen receptors are also indicative of nongenomic effects. It is possible that these plasma membrane receptors are also involved in [Ca\textsuperscript{2+}] regulation by estrogens and mediate some of the effects on [Ca\textsuperscript{2+}] observed in the present study. However, with the use of light microscopy, it was not possible to localize the estrogen receptors to the plasma membrane.

In addition to an enhancing effect on Ca\textsuperscript{2+} efflux, the results of the present study also confirmed previous observations that E\textsubscript{2\beta} inhibits Ca\textsuperscript{2+} influx (14, 19, 22, 29, 31). For example, E\textsubscript{2\beta} inhibited the BAY K 8644-induced elevation of [Ca\textsuperscript{2+}]. These results of the present study extend previous reports by establishing that Ca\textsuperscript{2+} influx, at least through L-type Ca\textsuperscript{2+} channels, is blocked even by nanomolar concentrations of estrogen, considerably smaller than those used in previous studies. In porcine coronary artery smooth muscle from castrated males, estrogens were shown to activate BK\textsubscript{Ca} channels through a cGMP-dependent mechanism, thus indirectly reducing Ca\textsuperscript{2+} influx (29). In the present study, the observation that iberiotoxin (a potent inhibitor of BK\textsubscript{Ca} channels) decreased the extent to which E\textsubscript{2\beta} reduced the endothelin-1-induced elevation of [Ca\textsuperscript{2+}] supports this mechanism.

Also in general agreement with previous studies (14, 31) is the observation that E\textsubscript{2\beta} had no effect on SR Ca\textsuperscript{2+} release. In the present study, E\textsubscript{2\beta} did not affect the [Ca\textsuperscript{2+}] response to caffeine, an agonist for ryanodine-
receptor channels. Endothelin-1 is also known to activate inositol 1,4,5-trisphosphate (IP₃) production and release Ca²⁺ through IP₃-receptor channels (10, 27). In the present study, preexposure to E₂β did not affect the initial [Ca²⁺] response to endothelin-1, suggesting that SR Ca²⁺ release through IP₃-receptor channels is also not affected. Indeed, in permeabilized vascular smooth muscle, Kitazawa et al. (14) found that the force response to exogenous IP₃ was unaffected by E₂β. Therefore, it is unlikely that SR Ca²⁺ release through either IP₃- or ryanodine-receptor pathways is affected by E₂β. However, these observations do not rule out the possibility that estrogens inhibit SR Ca²⁺ release via other agonists and/or signal transduction pathways. For example, Han et al. (4) found that E₂β does inhibit SR Ca²⁺ release induced by thromboxane A₂. Further experimentation is required to distinguish between these potentially differential mechanisms of estrogen action on the SR. In the present study, the lack of an E₂β effect of the rate of [Ca²⁺] decline under conditions of blocked Ca²⁺ influx and efflux suggests that SR Ca²⁺ reuptake is also unaffected by estrogens. Thus the predominant site of action for estrogens appears to be the plasma membrane.

In summary, the results of the present study demonstrate that E₂β enhances Ca²⁺ efflux, thereby providing a novel mechanism by which estrogens decrease the [Ca²⁺] response to endothelin-1 in coronary artery smooth muscle cells from gonad-intact female pigs. This effect of E₂β requires estrogen receptors.

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