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

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Prakash, Y. S., A. A. TogaiBayeva, M. S. Kannan, V. M. Miller, L. A. Fitzpatrick, and G. C. Sieck. Estrogen increases Ca\(^{2+}\) 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\(^{2+}\) concentration ([Ca\(^{2+}\)]) 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, Inc., Eugene, OR). The costs of publication of this article were defrayed in part by the payment of page charges. The article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.
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 inverted microscope. The chamber was perfused at 2–3 ml/min at room temperature.

**Real-time Ca**\textsuperscript{2+} **imaging.** An Odyssey XL real-time confocal system (Noran Instruments, Middleton, WI) attached to the 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 oil-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 µm\(^2\)/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 [Ca\textsuperscript{2+}] within regions of interest 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 µm\(^2\)). The optical section thickness for the ×40 lens was set to 1 µm by controlling the confocal slit size. Therefore, Ca\textsuperscript{2+} measurements were obtained from a volume of 6 µm\(^3\).

The fluorescence intensity of fluo 3 was calibrated for Ca\textsuperscript{2+} concentrations as described previously (18). At fixed settings of laser power and photomultiplier gain, fluo 3-loaded coronary artery smooth muscle cells were exposed to A-23187 (Ca\textsuperscript{2+} ionophore) at varying levels of extracellular Ca\textsuperscript{2+}, ranging from 0 (HBSS with EGTA) to 10 µM. Exposure to the ionophore at each extracellular Ca\textsuperscript{2+} concentration was limited to ~1 min to ensure that other Ca\textsuperscript{2+} handling mechanisms such as mitochondria and SR did not compensate for the ionophore-induced Ca\textsuperscript{2+} influx and thus confound the calibration. Furthermore, fluo 3 is also capable of being incorporated into the mitochondria and nucleus with continued exposure. Accordingly, to ensure that the fluo 3 signal represented cytosolic Ca\textsuperscript{2+} only, the total duration of the calibration protocol was minimized. Based on a calibration curve constructed from the gray level values of fluorescence intensities at different Ca\textsuperscript{2+} concentrations, the average gray level within a region of interest was converted to nanomoles per liter of Ca\textsuperscript{2+}.

**Assessment of cell viability.** After each experiment, coronary artery smooth muscle cells on at least one coverslip per animal (~80 cells) were evaluated for the exclusion of trypan blue. These cells were exposed to the confocal laser for varying periods of time ranging from <3 min to repeated exposures across a 30-min period. The exclusion of trypan blue in these cells confirmed that laser exposure did not injure the cells. In a subset of these cells (20 cells), the effect of prolonged laser exposure (three 3-min exposure periods separated by 5 min) on baseline [Ca\textsuperscript{2+}] level was evaluated. Baseline [Ca\textsuperscript{2+}] was found to vary <5% across this entire period. In another subset of cells (15 cells), the reproducibility of the [Ca\textsuperscript{2+}] response to 5 mM caffeine was evaluated over a 20-min period. The average coefficient of variation for the [Ca\textsuperscript{2+}] response to caffeine in these cells was 6.5%.

The [Ca\textsuperscript{2+}] response to endothelin-1. The [Ca\textsuperscript{2+}] response of coronary artery smooth muscle cells was measured in increasing concentrations of endothelin-1 (0.1, 1, 10, and 100 nM and 1 µM). Because tachyphylaxis is a well-known effect with endothelin-1, multiple-agonist exposures for the same cell were not possible. Instead, sets of coronary artery smooth muscle cells were exposed to only one endothelin-1 concentration, and the average response was determined.

**Effect of estrogens on endothelin-1-induced elevation of [Ca\textsuperscript{2+}].** Cells were exposed to 100 nM endothelin-1, and an elevation in [Ca\textsuperscript{2+}] was confirmed. When [Ca\textsuperscript{2+}] had reached a steady-state level (2–3 min), the cells were exposed to solvent (ethanol 10 nM), E\textsubscript{2}β (0.1 nM to 10 µM), 17α-estradiol (E\textsubscript{2}a; 10 nM, the biologically inactive form of estradiol), or triamcinolone acetonide (10 nM; a synthetic steroid unrelated to estrogens).

**Role of estrogen receptors in [Ca\textsuperscript{2+}] response to estrogens.** To determine whether estrogen receptors are necessary for mediation of the effect of E\textsubscript{2}β on [Ca\textsuperscript{2+}] coronary artery smooth muscle cells were exposed for 3 min to the estrogen-receptor antagonist ICI-182780 (1 µM; generously provided by Zeneca Pharmaceuticals, Cheshire, UK). Subsequently, the cells were exposed to endothelin-1, and when steady-state [Ca\textsuperscript{2+}] was reached, the cells were exposed to either E\textsubscript{2}β or E\textsubscript{2}a (10 nM).

**Effect of E\textsubscript{2}β on Ca\textsuperscript{2+} efflux.** To block Ca\textsuperscript{2+} influx, cells were exposed to zero extracellular Ca\textsuperscript{2+} or nifedipine (100 nM). Cells were then exposed to thapsigargin (1 µM) to block SR Ca\textsuperscript{2+} reuptake (23), which resulted in a gradual increase in [Ca\textsuperscript{2+}], plateauing after ~2 min. The cells were then exposed to E\textsubscript{2}β in the presence or absence of endothelin-1. To determine whether estrogen receptors mediate the effect of E\textsubscript{2}β on Ca\textsuperscript{2+} efflux under these conditions, cells were preexposed to ICI-182780 (1 µM) before endothelin-1 exposure. To confirm that Ca\textsuperscript{2+} efflux was actually involved, experiments were repeated in the presence of lanthanum (1 mM), which nonselectively blocks both Ca\textsuperscript{2+} influx and efflux (28).

**Effect of E\textsubscript{2}β on SR Ca\textsuperscript{2+} release and reuptake.** The response to caffeine (5 mM) was evaluated in the presence or absence of preexposure to E\textsubscript{2}β in cells in which Ca\textsuperscript{2+} influx and SR Ca\textsuperscript{2+} release were blocked by nifedipine (or zero extracellular Ca\textsuperscript{2+}) and thapsigargin, respectively. Under these conditions, SR Ca\textsuperscript{2+} release was estimated from the peak [Ca\textsuperscript{2+}] response.

To examine SR Ca\textsuperscript{2+} reuptake, cells were first exposed to nifedipine and lanthanum to block both Ca\textsuperscript{2+} influx and efflux. The [Ca\textsuperscript{2+}] response to endothelin-1 was then evaluated in the presence or absence of E\textsubscript{2}β. SR Ca\textsuperscript{2+} reuptake was estimated from the initial rate of decline in [Ca\textsuperscript{2+}].

**Effect of E\textsubscript{2}β on Ca\textsuperscript{2+} influx.** Cells were exposed to BAY K 8644 (100 nM, 1 and 10 µM) to induce Ca\textsuperscript{2+} influx. Nifedipine (100 nM) completely blocked the elevation of [Ca\textsuperscript{2+}] induced by 100 nM and 1 µM BAY K 8644, but it only partially blocked the [Ca\textsuperscript{2+}] response to 10 µM BAY K 8644. Accordingly, Ca\textsuperscript{2+} influx was induced by 1 µM BAY K 8644 in the presence of E\textsubscript{2}β.

To explore whether the effects of estrogens on Ca\textsuperscript{2+} influx are mediated via Ca\textsuperscript{2+}-activated K\textsuperscript{+} channels (BK,\textsubscript{Ca} (5, 13, 29), cells were exposed to iberiotoxin (100 nM), a specific inhibitor of these channels, and then to endothelin-1. When
[Ca\(^{2+}\)]_i had reached a steady state, the cells were exposed to E2β.

Statistical analysis. In determining the statistical design for various experiments, the influence of interanimal variability in the [Ca\(^{2+}\)]_i 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\(^{2+}\)]_i 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\(^{2+}\)]_i are reported as percent changes from the maximum [Ca\(^{2+}\)]_i 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\(^{2+}\)]_i response to endothelin-1. Basal [Ca\(^{2+}\)]_i 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\(^{2+}\)]_i response of 16 cells was determined. Endothelin-1 at 1 and 10 nM caused a slow and sustained increase in [Ca\(^{2+}\)]_i. In comparison, 100 nM and 1 µM endothelin-1 caused a more rapid increase in [Ca\(^{2+}\)]_i, 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\(^{2+}\)]_i 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\(^{2+}\)]_i. The effect of E2β on the [Ca\(^{2+}\)]_i response to endothelin-1 was concentration dependent with an ED\(_{50}\) of ~10 nM (Fig. 2A). The effect of 10 nM E2β on the endothelin-1 dose-response (1, 10, and 100 nM) was determined (Fig. 2B). Although the [Ca\(^{2+}\)]_i response varied with endothelin-1 concentration, the inhibitory effect of E2β was proportionately similar across all endothelin-1 concentrations. Because the relative effect of 10 nM E2β was comparable across endothelin-1 concentrations, fixed concentrations of E2β (10 nM) and endothelin-1 (100 nM) were used in all subsequent protocols.

Exposure to 10 nM E2β reduced the elevation of [Ca\(^{2+}\)]_i induced by 100 nM endothelin-1 to near basal levels within a 15– to 45-s period (Fig. 3; n = 56). The endothelin-1-induced elevation in [Ca\(^{2+}\)]_i was also reduced by 10 nM E2α (Fig. 3; n = 53), but to a lesser extent compared with E2β. In contrast, the [Ca\(^{2+}\)]_i response to endothelin-1 was unaffected by triamcinolone acetonide (Fig. 3; n = 14) or ethanol (n = 15).

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

<table>
<thead>
<tr>
<th>Endothelin-1 Concentration</th>
<th>Δ[Ca(^{2+})]_i at 30 s, nM</th>
<th>Δ[Ca(^{2+})]_i 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 ± 7</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. Time points for measurement of intracellular Ca\(^{2+}\) concentration ([Ca\(^{2+}\)]_i) 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 abrogate 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 [Ca\(^{2+}\)]\(_i\) was reduced by both E\(_2\beta\) and an unrelated steroid [triamcinolone acetonide (TAA); n = 15] compared with control, solvent control (ethanol; n = 15) (Fig. 6). SR Ca\(^{2+}\) release and reuptake were blocked, the rate of decline of [Ca\(^{2+}\)]\(_i\) (Fig. 6). These results suggest that E\(_2\beta\) had no effect on SR Ca\(^{2+}\) reuptake.

Effect of E\(_2\beta\) on Ca\(^{2+}\) influx. Exposure to E\(_2\beta\) significantly reduced the Ca\(^{2+}\) influx induced by 1 µM BAY K8644 (Fig. 8). When BK\(_{Ca}\) channels were blocked byiberiotoxin, 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.

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**Fig. 2.** Cumulative dose-response curve of 17\(\beta\)-estradiol (E\(_2\beta\))-induced reduction of endothelin-1-induced intracellular Ca\(^{2+}\) concentration ([Ca\(^{2+}\)]\(_i\)) in coronary artery smooth muscle cells from female pigs. In presence of a fixed concentration of 100 nM endothelin-1, cells were exposed to progressively larger concentrations of E\(_2\beta\) (A; n = 15). Effect of IC\(_{50}\) E\(_2\beta\) (10 nM) on [Ca\(^{2+}\)]\(_i\), response to different endothelin-1 concentrations was determined in separate sets of cells (B; n = 32 per endothelin-1 concentration). [Ca\(^{2+}\)]\(_i\) response is reported as percentage of maximum response at a given endothelin-1 concentration.

**Fig. 3.** Effect of estrogens on endothelin-1-induced elevation of [Ca\(^{2+}\)]\(_i\) in porcine coronary artery smooth muscle cells. Exposure to 100 nM endothelin-1 (A) induced a large elevation of [Ca\(^{2+}\)]\(_i\), which was reduced by both E\(_2\beta\) (B; n = 56) and 17\(\alpha\)-estradiol (E\(_2\alpha\); C; n = 53), albeit to different extents. Compared with control, solvent control (ethanol; n = 15) and an unrelated steroid [triamcinolone acetonide (TAA); n = 14) did not decrease [Ca\(^{2+}\)]\(_i\). (D). *Significant difference (P < 0.05) from control.
The conclusion that E2β enhances Ca2+ efflux was supported by three observations: 1) the reduction in [Ca2+]i by E2β was blocked by the estrogen-receptor antagonist ICI-182780; 2) the E2β-induced reduction in [Ca2+]i was observed even when Ca2+ influx and reuptake were blocked; and 3) the E2β-induced reduction in [Ca2+]i was absent when both Ca2+ influx and efflux were blocked by nifedipine (and/or zero extracellular Ca2+) and lanthanum.

The enhancement of Ca2+ efflux by E2β represents a novel mechanism by which estrogens regulate [Ca2+]i in response to agonist stimulation. As would be expected, the relative enhancement of Ca2+ efflux was independent of the level of endothelin-1 stimulation, as indicated by the comparable reduction in [Ca2+]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 E2β). Confirmation of the effect of E2β on Ca2+ efflux was derived primarily from the fact that the response was blocked by lanthanum. However, lanthanum also blocks Ca2+ influx, which has been previously demonstrated to be inhibited by E2β. Therefore, we designed studies to block Ca2+ influx independent of any inhibition on Ca2+ efflux.

In experimental protocols designed to examine the underlying mechanisms of E2β action, a 100 nM endothelin-1 concentration was selected based on studies by other investigators (24, 28), who found that the initial [Ca2+]i response at similar endothelin-1 concentrations represents SR Ca2+ release and that the subsequent response represents a balance between Ca2+ influx and efflux. In this regard, the initial peak [Ca2+]i response at 100 nM endothelin-1 observed in our study most likely reflects SR Ca2+ release, whereas the lower steady state reflects a balance between Ca2+ influx and efflux. In contrast, endothelin-1 concentrations of 10 nM or less elicit only slow, monotonic elevations in [Ca2+]i (also observed in the present study), which most likely reflect Ca2+ influx (24). Accordingly, the selection of 100 nM allowed the examination of both SR Ca2+ release and Ca2+ 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 [Ca2+]i to baseline after the initial peak [Ca2+]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 E$_2$ used to examine [Ca$^{2+}$]$_i$ regulation is supraphysiological. It is difficult to directly extrapolate the reduction in [Ca$^{2+}$]$_i$ observed with 10 nM E$_2$ 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.

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**Fig. 6. Effect of E$_2$ on Ca$^{2+}$ efflux after endothelin-1 (ET) activation.** When Ca$^{2+}$ influx and SR Ca$^{2+}$ reuptake were blocked by nifedipine (Nif; or zero extracellular Ca$^{2+}$; not shown) and thapsigargin (Tg), respectively, endothelin-1 induced a large, sustained elevation of [Ca$^{2+}$]$_i$ (A; n = 32). Subsequent exposure to E$_2$ reduced [Ca$^{2+}$]$_i$ (B; n = 32). This effect of E$_2$ on [Ca$^{2+}$]$_i$ was inhibited by lanthanum (La; C; n = 31), indicating that E$_2$ enhances Ca$^{2+}$ efflux. D: summary of [Ca$^{2+}$]$_i$ responses. *Significant difference (P < 0.05) from response to endothelin-1 alone.

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**Fig. 7. Effect of E$_2$ on SR Ca$^{2+}$ release and reuptake.** In absence of Ca$^{2+}$ influx and SR reuptake, the large, transient [Ca$^{2+}$]$_i$ response to caffeine (A; n = 25) was not affected by preexposure to E$_2$ (B; n = 24). In absence of Ca$^{2+}$ influx and efflux (blocked by nifedipine and lanthanum), endothelin-1 produced a large, sustained elevation of [Ca$^{2+}$]$_i$ (C; n = 14). Rate of decline of [Ca$^{2+}$]$_i$ response to endothelin-1 was not affected by preexposure to E$_2$ (D; n = 15). These data suggest that SR Ca$^{2+}$ 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{2β} 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{2β} 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+} influx versus other mechanisms of estrogen action. It is difficult to compare the results of the present study, in which 10 nM E\textsubscript{2β} 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 circulating estrogen levels and considerably higher than that 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+} influx plays a role in estrogen-induced reduction of [Ca\textsuperscript{2+}] even in vivo.

The specificity of the E\textsubscript{2β} 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{2β}. In addition, compared with E\textsubscript{2β}, the biologically inactive isomer E\textsubscript{2α} 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{2α} by ICI-182780 suggests a nonspecific non-receptor-mediated effect of E\textsubscript{2α}. However, the mechanisms underlying the effects of E\textsubscript{2α} 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{2α} 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{2β}, 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{2β} inhibits Ca\textsuperscript{2+} influx (14, 19, 22, 29, 31). For example, E\textsubscript{2β} 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{2β} 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{2β} had no effect on SR Ca\textsuperscript{2+} release. In the present study, E\textsubscript{2β} 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 Eβ did not affect the initial [Ca2+]i 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 Eβ. 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 E2 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 Eβ effect of the rate of [Ca2+]i 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 Eβ enhances Ca2+ efflux, thereby providing a novel mechanism by which estrogens decrease the [Ca2+]i response to endothelin-1 in coronary artery smooth muscle cells from gonad-intact female pigs. This effect of Eβ requires estrogen receptors.

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