

# Estrogen and the $\text{Ca}^{2+}$ -mobilizing agonist ATP evoke acute NO synthesis via distinct pathways in an individual human vascular endothelium-derived cell

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**Sheng J-Z, Arshad F, Braun JE, Braun AP.** Estrogen and the  $\text{Ca}^{2+}$ -mobilizing agonist ATP evoke acute NO synthesis via distinct pathways in an individual human vascular endothelium-derived cell. *Am J Physiol Cell Physiol* 294: C1531–C1541, 2008. First published March 26, 2008; doi:10.1152/ajpcell.00561.2007.—In this study, we have systematically evaluated the signaling mechanisms underlying stimulated nitric oxide (NO) synthesis by estrogen ( $\text{E}_2$ ) and other vasoactive agents at the level of a single endothelium-derived cell. To do so, we have characterized and contrasted rapid  $\text{E}_2$ -evoked NO synthesis with that of ATP using single-cell microfluorimetry and patch-clamp recordings to monitor stimulated changes in cellular NO synthesis (via 4-amino-5-methylamino-2',7'-difluorofluorescein),  $\text{Ca}^{2+}$  transients (via Fluo-3), and membrane hyperpolarization in cultured human EA.hy926 cells.  $\text{E}_2$ -evoked NO synthesis in single cells ( $\text{EC}_{50} \sim 0.3 \text{ nM}$ ) was blocked by the  $\text{E}_2$  receptor antagonist ICI 162,780 and the NO synthase inhibitor  $N^{\omega}$ -nitro-L-arginine methyl ester. Although both  $\text{E}_2$  and ATP stimulated comparable  $\text{Ca}^{2+}$  transients,  $\text{E}_2$ -induced NO synthesis was insensitive to intracellular BAPTA-AM or removal of external  $\text{Ca}^{2+}$ . In contrast, ATP-evoked NO production was abolished by either one of these treatments. ATP-evoked hyperpolarizations ( $\sim 20 \text{ mV}$ ) and NO production were both inhibited by the respective small-conductance and intermediate-conductance calcium-activated  $\text{K}^+$  channel blockers apamin and charybdotoxin.  $\text{E}_2$  minimally affected membrane potential, and stimulated NO synthesis was insensitive to calcium-activated  $\text{K}^+$  channel blockers. Exposure to either the phosphatidylinositol 3-kinase inhibitor LY-294001 or the MAP kinase inhibitor PD-98059 abolished the NO response to  $\text{E}_2$ , but not that to ATP. Finally, the NO response evoked by a combined stimulus of  $\text{E}_2$  plus ATP was similar to that of ATP alone. In conclusion, our data directly demonstrate that an individual human EA.hy926 cell contains at least two distinct mechanisms for stimulated NO synthesis that depend on either calcium or protein kinase signaling events.

nitric oxide; endothelial function; calcium; signal transduction

ESTROGEN PROMOTES A NUMBER of beneficial effects within the cardiovascular system that include vasodilatation, prevention of leukocyte adhesion, angiogenesis, and antiproliferation of vascular smooth muscle (34, 35). Clinically, the loss of estrogen-mediated vasorelaxation in postmenopausal women correlates closely with increased incidence of cardiovascular events such as coronary heart disease and stroke (24). A key action of estrogen is to enhance nitric oxide (NO) production in vascular endothelium, which may occur via transcriptional and nontranscriptional mechanisms (6). Along this line, several recent studies have highlighted the importance of the phosphatidylinositol 3-kinase (PI3-kinase)/Akt signaling cascade to acute NO synthesis evoked by endothelial stimuli such as estrogen

and shear stress (9, 15, 20, 22, 43). Taken together with earlier work demonstrating that endothelial NO production can be readily stimulated by calcium-mobilizing agonists such as ATP, histamine, and acetylcholine (3), these studies strongly suggest the existence of multiple cellular pathways for stimulated NO synthesis in vascular endothelium. Biochemical studies demonstrating that endothelial NO synthase (eNOS) itself may be regulated via  $\text{Ca}^{2+}$ /calmodulin or by direct phosphorylation at Ser1177 (16) have further implied that both these pathways may operate within the same cell. This assumption, however, has not been rigorously examined using direct experimental measurements.

Given the physiological importance of both estrogen and  $\text{Ca}^{2+}$ -mobilizing agonists to endothelial NO production, the goal of our study was to compare and contrast the cellular mechanism(s) underlying the NO response to each type of stimulus in the same endothelial cell. To do so, we have developed experimental approaches to monitor in real time evoked changes in NO synthesis, cytosolic free  $\text{Ca}^{2+}$ , and membrane potential in single human EA.hy926 cells. Our findings provide direct evidence demonstrating for the first time that a single identified endothelial cell is capable of producing NO in response to both  $\text{Ca}^{2+}$ -mobilizing agonists and stimuli that activate eNOS via the PI3-kinase/Akt and MAP kinase signaling cascades. Our data further suggest that these two distinct cellular mechanisms operate in parallel and converge at the level of eNOS itself. Vascular endothelial cells thus appear to have evolved at least two separate stimulatory pathways leading to de novo NO synthesis, which may reflect the critical importance of this multipotent mediator in the long-term regulation of vascular tone and overall function of the circulatory system.

## MATERIALS AND METHODS

**Cell culture and fluorescence measurements.** The EA.hy926 cell line (10), originally derived from human umbilical vein endothelium, was cultured and loaded with the membrane-permeable forms of the fluorescent dyes 4-amino-5-methylamino-2',7'-difluorofluorescein (DAF-FM) or Fluo-3, as recently described (41). Fluorescence measurements were performed in a  $\sim 0.3\text{-ml}$  bath chamber mounted on the stage of a Nikon TE300 inverted microscope equipped with a 75 W xenon arc lamp and SFX-1 microfluorimeter (Solamere Technology Group). Both DAF-FM and Fluo-3 fluorescence signals were measured using excitation and emission band-pass filters centered on 488 and 515 nm, respectively; data were acquired using AxoScope software and analyzed with pClamp 7 and SigmaPlot 2000 software suites. Because the fluorescent intensity of the triazole or NO-bound

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form of DAF-FM originating from a single cell was typically quite modest, the strong excitation light needed to observe reliable fluorescent signals often resulted in some photobleaching of the NO-modified form of DAF-FM during continuous cell illumination. Exposure of the cell to intermittent illumination through the use of a timer-driven, optical shutter reduced, but did not completely eliminate, photobleaching of NO-modified DAF-FM. A manually controlled diaphragm was used to restrict the region of light collection to the single cell of interest.

**Electrophysiology.** Current-clamp recordings of membrane voltage were performed using perforated patch-clamp methodology in combination with an Axopatch 200B amplifier, Digidata 1200B analog/digital interface, and Clampex 8 software; electrical signals were typically sampled at 1 Hz. Borosilicate glass micropipettes (2–4 M $\Omega$  tip resistance) were first briefly dipped into standard filling solution (final concentrations in mM: 100 K-aspartate, 30 KCl, 1 MgCl<sub>2</sub>, 2 Na<sub>2</sub>-ATP, and 10 HEPES, pH 7.2 with 1 M KOH) and then back-filled with the same filling solution containing nystatin (50  $\mu$ g/ml final). Following nystatin-mediated perforation, measured cell membrane capacitance and intracellular access resistance ranged from 14 to 20 pF and 17 to 25 M $\Omega$ , respectively. The bath solution for both fluorescence and electrophysiological recordings contained (in mM) 135 NaCl, 5 KCl, 1 MgCl<sub>2</sub>, 1.5 CaCl<sub>2</sub>, and 10 HEPES, pH 7.4 with 1 M NaOH. For the Ca-free solution, CaCl<sub>2</sub> was omitted and replaced by 2 mM EGTA. Cells in the bath chamber were constantly superfused at  $\sim$ 1 ml/min, and solution changes were performed by gravity flow from a series of elevated solution reservoirs using manually controlled solenoid valves. All fluorescence and electrophysiological recordings were performed at 35°C.

**Reagents.** Apamin, charybdotoxin, estrogen (17 $\beta$ -estradiol), ICI 182,780, LY-294001, LY-303511, PD-98059, histamine dihydrochloride, Na<sub>2</sub>-ATP, along with the chemicals used to prepare physiological saline solutions (American Chemical Society grade or higher) were purchased from Sigma-Aldrich (St. Louis, MO). DAF-FM diacetate and Fluo-3 AM were obtained from Molecular Probes/Invitrogen (Eugene, OR).

**Statistical analyses.** Data are presented as means  $\pm$  SE. Mean values calculated from independent experiments were statistically analyzed using Student's *t*-test, and differences among means were considered to be significant when *P* < 0.05.

## RESULTS

**Estrogen induces acute NO production in single EA.hy926 cells.** In the present study, we have used the cultured human EA.hy926 cell line as a model vascular endothelial cell to examine stimulus-driven NO production. Earlier studies have shown that these cells display Ca<sup>2+</sup>-dependent signaling events and membrane currents similar to those observed in other types of isolated vascular endothelial cells. Upon loading these cells with the NO-sensitive fluorescent dye DAF-FM diacetate (26), acute exposure to estrogen (17 $\beta$ -estradiol) evoked concentration-dependent increases in cellular fluorescence that were detectable in single cells within seconds of estradiol application (Fig. 1A). The rapidity of this response is consistent with a nongenomic action of estrogen and agrees with earlier studies using alternate NO detection methods that report acute estrogen-evoked NO production within minutes of exposure (5, 8, 27, 46). As depicted by the concentration-response curve in Fig. 1B, estrogen-induced increases in cellular fluorescence occurred over the range of 0.01–10 nM, with a half-maximal fluorescence rise observed at an estrogen concentration of  $\sim$ 0.3 nM (EC<sub>50</sub> value). Importantly, this observed sensitivity to estrogen in our human endothelial cell model agrees closely with the range of circulating plasma levels of estrogen ob-

served during the menstrual cycle in premenopausal women ( $\sim$ 30 to 200 pg/ml or  $\sim$ 0.1 to 0.8 nM), suggesting that these cells have a physiologically appropriate sensitivity to this hormone. In the presence of ICI 182,780, a competitive antagonist of the steroidal estrogen receptor  $\alpha$ - and  $\beta$ -isoforms (48, 50), this observed estrogen-evoked NO synthesis was largely abolished (Fig. 1, C and D). As shown in the same experiment, however, ATP-induced NO synthesis was unaffected by ICI 182,780, demonstrating the pharmacologic selectivity of this inhibitor. Importantly, estrogen-induced increases in DAF-FM fluorescence could be blocked by brief treatment of DAF-FM-loaded cells with the NOS inhibitor N<sup>ω</sup>-nitro-L-arginine methyl ester [L-NAME (25)], which effectively interfered with the stimulated rises in NO production in response to either estrogen or the calcium-mobilizing agonist ATP (Fig. 2, A and B). Moreover, this L-NAME-mediated inhibition was readily reversible, because ATP-evoked NO synthesis could be restored following a 2- to 3-min washout of the NOS inhibitor from the bath. ATP is known to act via endothelial purinergic P2Y receptors to induce L-NAME-sensitive vasodilation in intact blood vessels (2), and we have recently described acute ATP-stimulated NO synthesis in these same human cells (41). Taken together, these observations demonstrate that estrogen acts via ICI-182,780-sensitive receptors to elicit rapid de novo synthesis of NO in single EA.hy926 cells that also display ATP-evoked NO synthesis.

**Estrogen-induced NO production occurs in a Ca<sup>2+</sup>-independent manner.** It is now well recognized that elevations in cytosolic free Ca<sup>2+</sup> concentration play a critical signaling role in the activation of eNOS by calcium-mobilizing agonists (Ref. 40 and references therein). To examine the nature of free Ca<sup>2+</sup> transients evoked by estrogen and ATP in the same cell, we monitored changes in single-cell fluorescence in Fluo-3-loaded EA.hy926 cells in response to both agonists. As shown in Fig. 3A, estrogen evoked dose-dependent elevations in Fluo-3 fluorescence that were kinetically similar to those induced by a maximally effective concentration of ATP but did not reach the same magnitude (Fig. 3B).

A number of studies, including recent data from our own lab (40), have demonstrated that the endothelial production of NO/endothelium-derived relaxing factor (EDRF) evoked by Ca<sup>2+</sup>-mobilizing agonists, such as acetylcholine, ATP, and histamine, is strictly dependent on the presence of extracellular Ca<sup>2+</sup> (23, 28, 29, 32). Because both estrogen and ATP were observed to produce clear Ca<sup>2+</sup> transients in single EA.hy926 cells, we examined further the potential involvement of internal and external Ca<sup>2+</sup> pools in estrogen-mediated NO synthesis. To elucidate a role for extracellular Ca<sup>2+</sup>, we first monitored estrogen- and ATP-evoked NO responses under control conditions and then repeated the agonist exposures in the presence of a physiological saline solution with no added Ca<sup>2+</sup> that also contained the Ca<sup>2+</sup> chelator EGTA (2 mM). As shown in Fig. 4A, estrogen-mediated NO production was unaffected by the rapid removal of external Ca<sup>2+</sup>, whereas the NO response to ATP was virtually abolished, in agreement with our previous observations (40). These data thus highlight a major difference in the signaling mechanisms underlying the NO response evoked by each agonist. To determine whether estrogen-stimulated changes in intracellular free Ca<sup>2+</sup> levels per se may be more physiologically relevant to the estrogen-

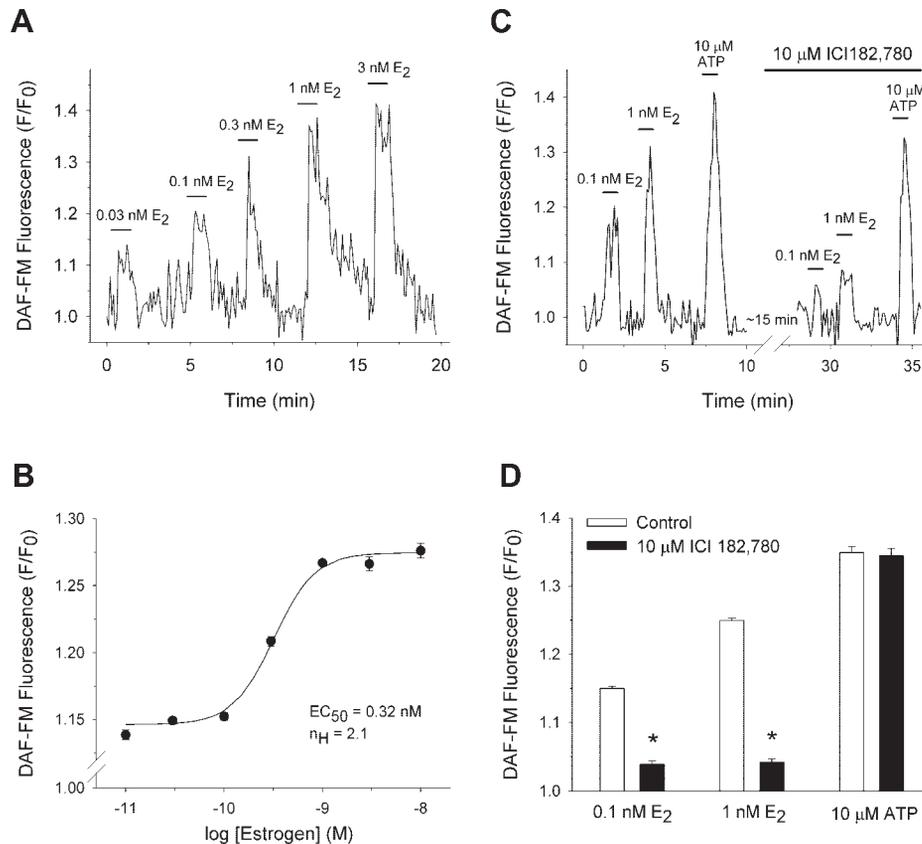


Fig. 1. Estrogen produces concentration-dependent increases in 4-amino-5-methylamino-2',7'-difluorofluorescein (DAF-FM) fluorescence that are sensitive to the estrogen receptor antagonist ICI 182,780. **A**: continuous fluorescence tracing recorded from a single DAF-FM-loaded EA.hy926 cell that was exposed sequentially to increasing concentrations of estrogen ( $E_2$ ) or  $10 \mu\text{M}$  ATP, as indicated by the horizontal bars above the tracing. **B**: line graph of the estrogen-evoked increase in cellular fluorescence at each hormone concentration. The smooth line through the data points was fit using the following equation:  $F = F_{\text{max}}/[1 + (EC_{50}/[E_2])^n] + b$ , where  $F$  is the measured fluorescence,  $F_{\text{max}}$  is the calculated maximal fluorescence,  $[E_2]$  represents the different estrogen concentrations,  $EC_{50}$  is the estrogen concentration producing half-maximal effect,  $n$  is the Hill coefficient, and  $b$  is the baseline fluorescence ratio. **C**, left: increases in cellular fluorescence recorded from a single DAF-FM-loaded EA.hy926 cell in response to either estrogen or  $10 \mu\text{M}$  ATP. Following exposure to the estrogen receptor antagonist ICI 182,780 ( $10 \mu\text{M}$ ) for  $\sim 15$  min, the same cell was restimulated by either estrogen or ATP in the continued presence of ICI 182,780, as indicated by the horizontal bar above the fluorescence tracing. **D**: hormone-evoked increases in cellular DAF-FM fluorescence by estrogen and ATP in both the absence and presence of ICI 182,780 are quantified. Data are means  $\pm$  SE of recordings taken from 5–7 individual cells under each agonist condition. \*Statistically different from the associated control ( $P < 0.05$ ).

mediated NO production, EA.hy926 cells were simultaneously loaded with both the  $\text{Ca}^{2+}$  chelator BAPTA-AM ( $20 \mu\text{M}$ ) and the NO-sensitive dye DAF-FM. Under such conditions, we observed that estrogen-evoked increases in NO production were unaffected by intracellular BAPTA loading, whereas the ATP-stimulated NO response monitored in the same cell was largely inhibited by BAPTA pretreatment (Fig. 4B). The effects of external  $\text{Ca}^{2+}$  removal and intracellular BAPTA loading on estrogen- and ATP-stimulated NO synthesis are summarized in Fig. 4C.

*Estrogen- and ATP-induced NO synthesis display differential sensitivity to inhibition of PI3-kinase, MAP kinase, and small- and intermediate-conductance calcium-activated potassium channels.* Recently, we and others have directly demonstrated that membrane hyperpolarization mediated by endothelial small- and intermediate-conductance Ca-activated  $\text{K}^+$  channels ( $\text{SK}_{\text{Ca}}$  and  $\text{IK}_{\text{Ca}}$ , respectively) plays an essential role in the cellular mechanism underlying stimulated NO synthesis by  $\text{Ca}^{2+}$ -mobilizing agonists, such as ATP, histamine, and acetylcholine (40, 45). Given the estrogen-induced  $\text{Ca}^{2+}$  transients observed in our isolated EA.hy926 cells (Fig. 3),

we examined whether these same  $\text{K}_{\text{Ca}}$  channels also contribute to estrogen-mediated NO synthesis. To do so, EA.hy926 cells were exposed to apamin and charybdotoxin, selective inhibitors of  $\text{SK}_{\text{Ca}}$  and  $\text{IK}_{\text{Ca}}$  channels, respectively, following control NO responses to estrogen and ATP. In agreement with our earlier study (40), the combination of apamin and charybdotoxin abolished the ATP-evoked increase in NO production; however, the estrogen-stimulated NO response was unaffected by the presence of these two channel blockers (Fig. 5). To determine whether estrogen was able to evoke membrane potential changes via other types of ion channels, we performed current-clamp recordings of membrane voltage in single EA.hy926 cells using perforated patch methodology under conditions of estrogen and ATP application. As shown in Fig. 6A, increasing concentrations of estrogen (0.01–1 nM) produced minimal changes in membrane potential, whereas ATP and histamine evoked robust membrane hyperpolarizations in the same cell. The magnitudes of membrane potential changes evoked by estrogen, ATP, and histamine are quantified in the histogram displayed in Fig. 6B.

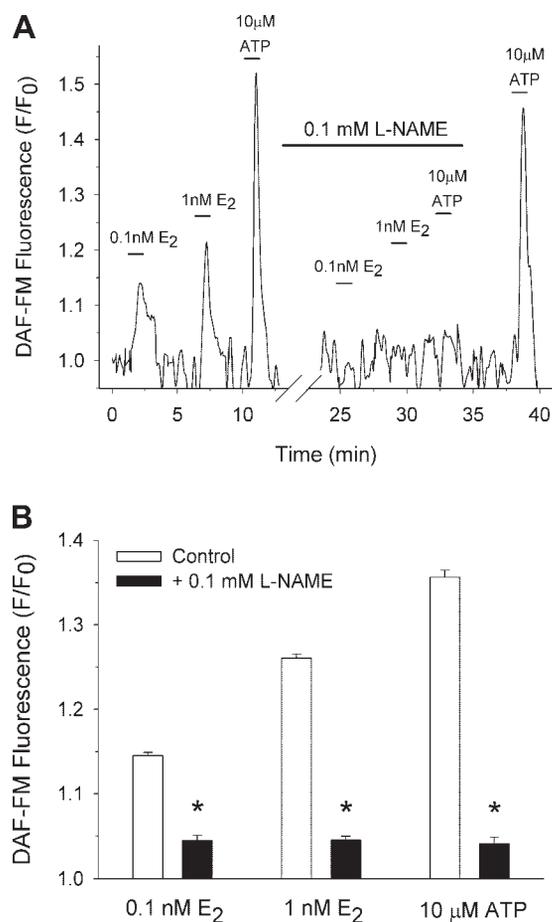


Fig. 2. *N*<sup>ω</sup>-nitro-L-arginine methyl ester (L-NAME), an inhibitor of nitric oxide (NO) synthase (NOS), blocks agonist-evoked increases in DAF-FM fluorescence. *A, left*: increases in cellular fluorescence recorded from a single DAF-FM-loaded EA.hy926 cell in response to either estrogen or 10  $\mu$ M ATP, as indicated by the horizontal bars above the tracing. Following exposure to the endothelial NOS (eNOS) inhibitor L-NAME (0.1 mM) for  $\sim$ 15 min, the same cell was restimulated by either estrogen or ATP in the continued presence of L-NAME. Near the end of the recording, L-NAME was removed from the bath, as indicated by the horizontal bar above the fluorescence tracing, and after 2–3 min, the cell was reexposed to 10  $\mu$ M ATP. *B*: hormone-evoked changes in cellular DAF-FM fluorescence by either estrogen or ATP in both the absence and presence of L-NAME are quantified. Data are means  $\pm$  SE of recordings taken from 5–6 individual cells for each agonist. \*Statistically different from the associated control ( $P < 0.05$ ).

To examine whether the weak estrogen-evoked membrane hyperpolarizations described in Fig. 6 were related to differences in the amplitudes of estrogen- versus ATP-evoked  $\text{Ca}^{2+}$  transients (see Fig. 3), we simultaneously recorded evoked changes in membrane potential and Fluo-3 fluorescence in single EA.hy926 cells in response to equipotent concentrations of estrogen and ATP. As shown in Fig. 7, 1 nM estrogen and 1  $\mu$ M ATP produced similar elevations in cytosolic free  $\text{Ca}^{2+}$ , as judged by Fluo-3 fluorescence. Despite this similarity, ATP produced a considerably larger membrane-hyperpolarizing response compared with estrogen. These data thus rule out the possibility that the estrogen-induced  $\text{Ca}^{2+}$  elevation was below threshold for activation of membrane ion channels (i.e.,  $\text{SK}_{\text{Ca}}$  and  $\text{IK}_{\text{Ca}}$ ) and point to a fundamental difference in the calcium-signaling pathways stimulated by estrogen and ATP in these cells.

In vascular endothelium, protein kinase signaling cascades are known to participate in the regulation of NO synthesis evoked by a variety of stimuli, including shear stress (9, 17), growth factors (38, 51), insulin (37), and estrogen (43, 44). Moreover, phosphorylation of eNOS at Ser1177 appears to be an important activation mechanism used by several agonists to stimulate de novo NO production (7, 15, 21, 33), and modification of this site has been documented in both isolated endothelial cells and intact arterial tissue (9, 15, 17, 47). To examine the potential contributions of two major protein kinase signaling cascades, the PI3-kinase/Akt pathway and MAP kinase pathway, to both estrogen- and ATP-evoked NO production, EA.hy926 cells were exposed to either the selective PI3-kinase inhibitor LY-294002 (49) or PD-98059, a blocker of MAP kinase signaling (1). As shown in Fig. 8A, brief exposure to LY-294002 (10  $\mu$ M) abolished estrogen-induced NO synthesis in a single EA.hy926 cell but did not significantly alter the NO response to ATP in the same cell. Importantly, exposure of cells to the inactive control compound LY-303511 (10  $\mu$ M) had no effect on either the estrogen- or ATP-evoked increases in NO production in the same single-cell preparation. The effects of both LY-294002 and LY-

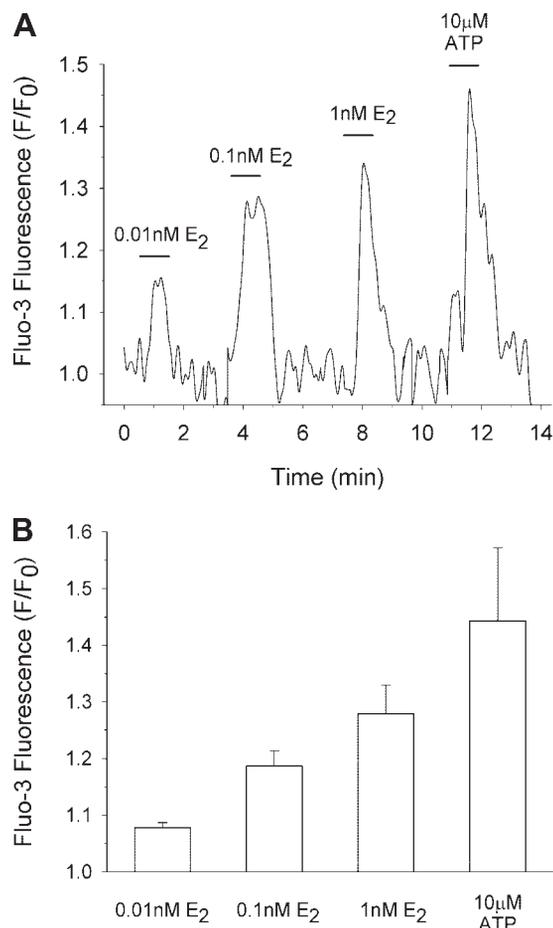


Fig. 3. Estrogen and ATP induce elevations in cytosolic free  $\text{Ca}^{2+}$ . *A*: continuous fluorescence tracing recorded from a single Fluo-3-loaded EA.hy926 cell that was exposed sequentially to increasing concentrations of estrogen or 10  $\mu$ M ATP, as indicated by the horizontal bars above the tracing. *B*: histogram quantifies the hormone-induced increases in cellular fluorescence. Data are means  $\pm$  SE of recordings taken from 6 individual cells for each agonist.

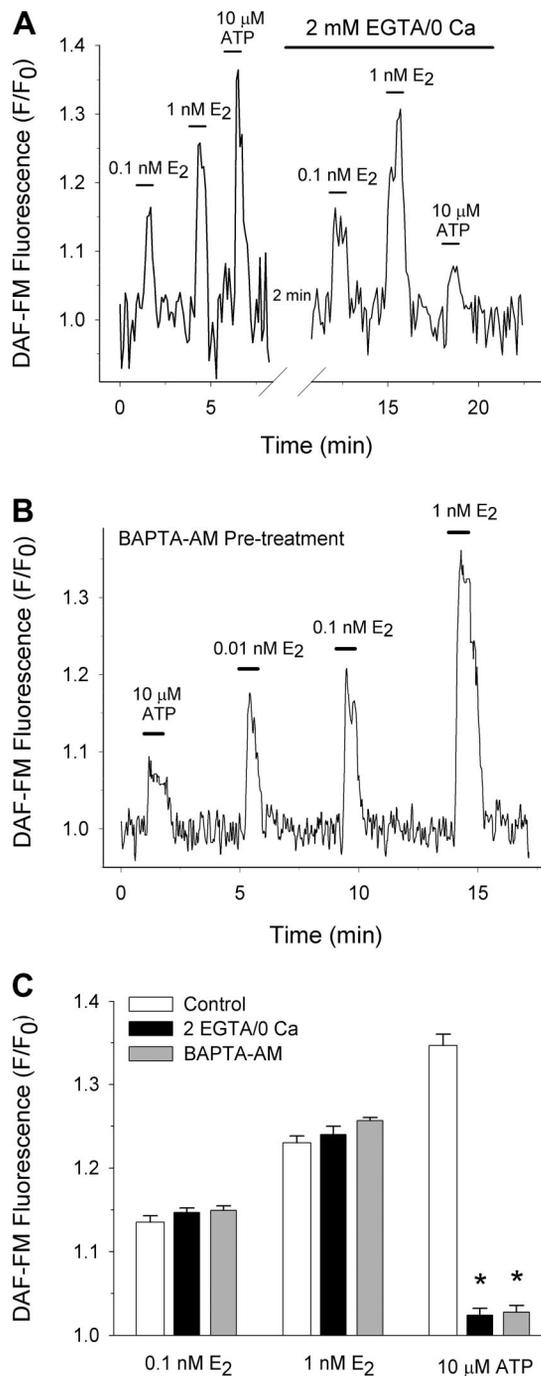


Fig. 4. Removal of external  $\text{Ca}^{2+}$  or buffering of cytosolic free  $\text{Ca}^{2+}$  does not prevent estrogen-induced increases in DAF-FM fluorescence. *A, left*: increases in cellular fluorescence recorded from a single DAF-FM-loaded EA.hy926 cell in response to brief exposures to either estrogen or ATP. Following replacement of the extracellular bath solution by a solution containing 2 mM EGTA and no added  $\text{CaCl}_2$ , the cell was reexposed to either estrogen or ATP, as indicated by the horizontal bars above the tracing. *B*: stimulated increases in cellular fluorescence recorded from a single EA.hy926 cell loaded with both DAF-FM and 20  $\mu\text{M}$  BAPTA-AM. Fluorescence changes were evoked by either ATP (10  $\mu\text{M}$ ) or increasing concentrations of estrogen, as indicated by the horizontal bars above the continuous tracing. *C*: histogram quantifies the effects of external  $\text{Ca}^{2+}$  removal and cytosolic buffering of free  $\text{Ca}^{2+}$  on stimulated increases in DAF-FM fluorescence under each condition. Data are means  $\pm$  SE of recordings taken from 6–7 individual cells for each agonist. \*Statistically different from the associated control ( $P < 0.05$ ).

303511 on stimulated NO synthesis in single EA.hy926 cells are quantified in Fig. 8B.

Because acute activation of the Raf-1/ERK1/2 signaling pathway by estrogen has also been reported to increase NO synthesis (7, 8, 21), we used the selective MEK inhibitor PD-98059 (1) to examine the potential contribution of MAP kinase signaling to estrogen-induced NO production. As shown in Fig. 9, pretreatment of EA.hy926 cells with 20  $\mu\text{M}$  PD-98059 abolished estrogen-stimulated NO synthesis but did not significantly affect the ATP-induced response under the same exposure conditions (Fig. 9C).

Finally, the clear differences noted above in estrogen- and ATP-evoked cellular actions begged the question of whether these agonists induced NO production in an additive or synergistic manner. Figure 10A shows simultaneous recordings of membrane potential and DAF-FM fluorescence in a single EA.hy926 cell exposed to pharmacologically maximal concentrations of estrogen and ATP first separately and then together. As can be seen, the combination of 1 nM estrogen and 10  $\mu\text{M}$  ATP did not evoke a larger increase in NO production com-

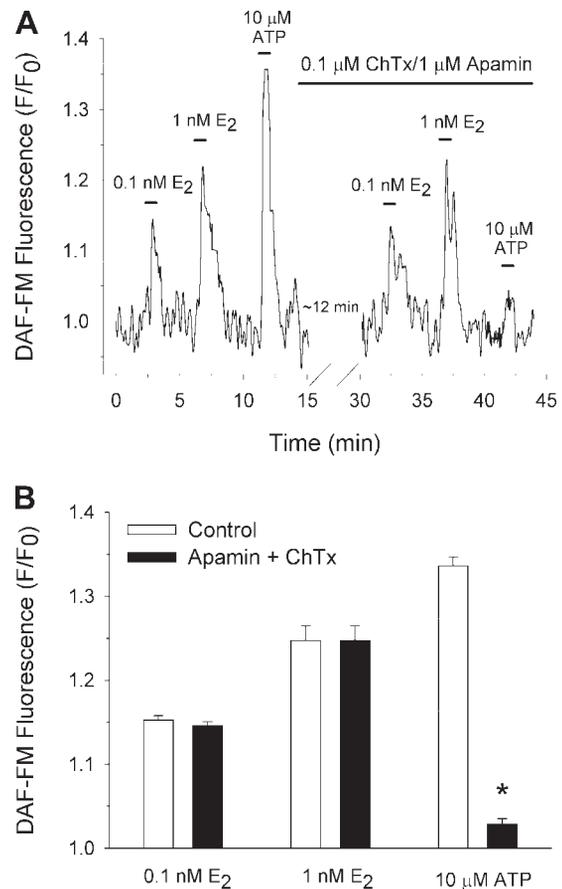


Fig. 5. The estrogen-evoked increase in DAF-FM fluorescence is unaffected in the presence of apamin and charybdotoxin. In response to either estrogen or ATP, increases in cellular fluorescence were observed in single DAF-FM-loaded EA.hy926 cells (*A, left*). Following exposure to 1  $\mu\text{M}$  apamin and 0.1  $\mu\text{M}$  charybdotoxin (ChTx) for ~15 min, the same cell was restimulated by either estrogen or ATP in the continued presence of apamin and ChTx, as indicated by the horizontal bars above the fluorescence tracing (*A, right*). Increases in cellular DAF-FM fluorescence evoked by either estrogen or ATP in both the absence and presence of apamin and ChTx are quantified (*B*). Data are means  $\pm$  SE of recordings taken from 5 individual cells for each agonist. \*Statistically different from the associated control ( $P < 0.05$ ).

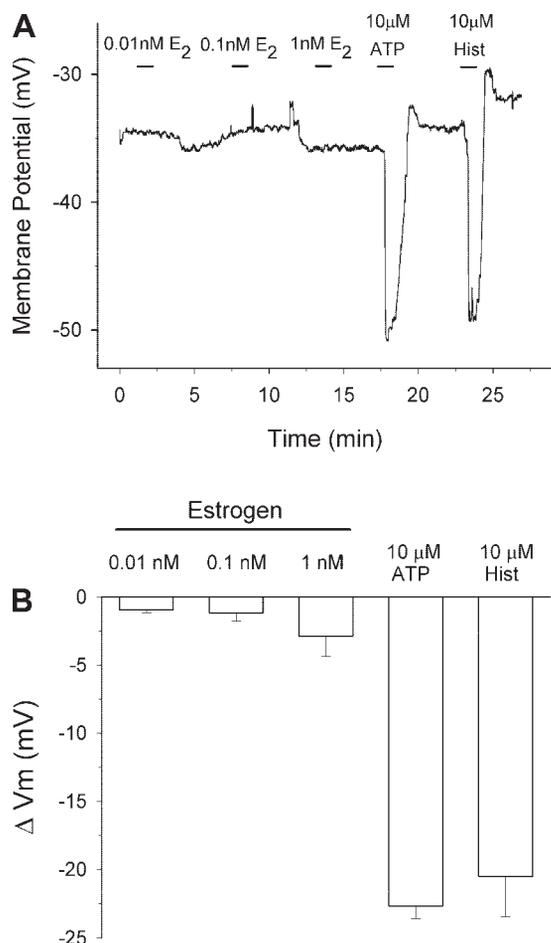


Fig. 6. ATP and histamine (Hist), but not estrogen, induce significant membrane hyperpolarization. *A*: continuous recording of membrane potential from a single EA.hy926 cell held under current clamp using a nystatin-permeabilized patch-clamp method. Brief exposures of the cell to increasing concentrations of estrogen, 10 μM ATP, or 10 μM histamine are indicated by the horizontal bars above the tracing. *B*: hormone-stimulated changes in membrane potential ( $\Delta V_m$ ) are quantified in the histogram. Data are means  $\pm$  SE of recordings taken from 4–5 individual cells under each agonist condition.

pared with 10 μM ATP alone. We did, however, note a modest but statistically insignificant increase in the amplitude of membrane hyperpolarization evoked by estrogen plus ATP compared with ATP alone (Fig. 10B).

**DISCUSSION**

It is now well recognized that estrogen exerts important functional and protective effects on the human cardiovascular system (34, 35), and one of the major mechanisms underlying

this protection involves enhanced production of NO by the vascular endothelium. NO is a major physiological regulator of circulatory function and health as a result of its ability to modulate blood vessel tone, vascular permeability, leukocyte and platelet adhesion, smooth muscle proliferation/migration,

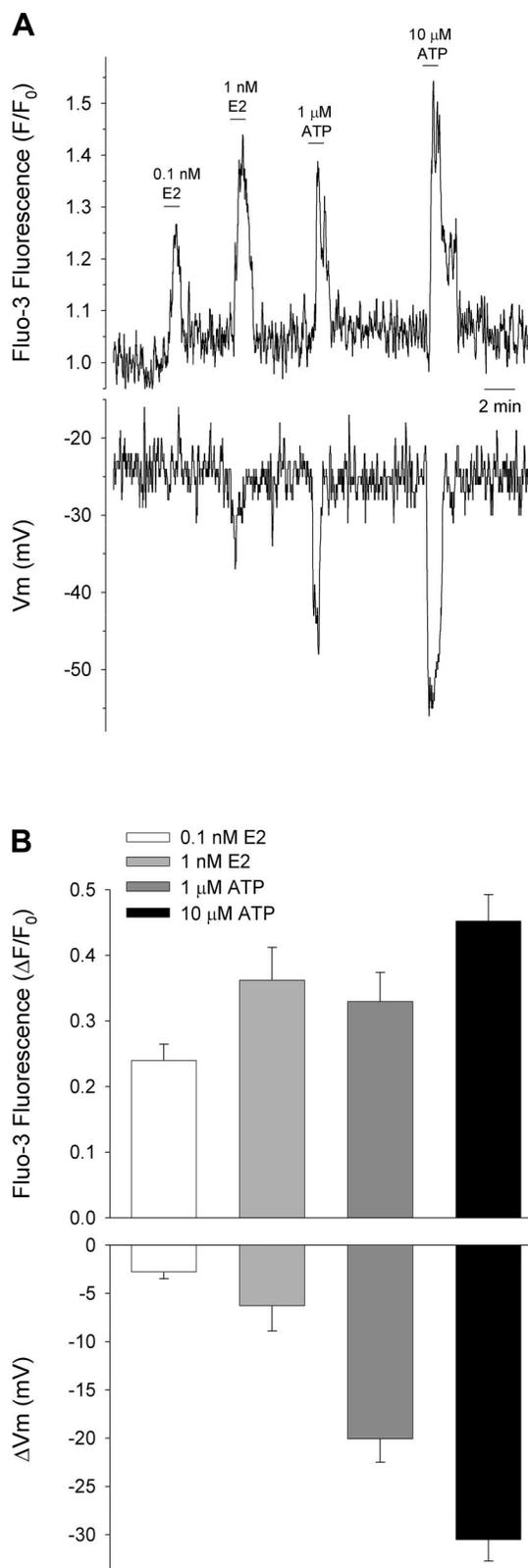


Fig. 7. ATP-evoked Ca<sup>2+</sup> transients are associated with larger membrane-hyperpolarizing responses compared with estrogen. Single EA.hy926 cells loaded with the calcium-sensitive dye Fluo-3 dye were held under current clamp using a nystatin-permeabilized patch-clamp method. *A*: simultaneous and continuous recordings of Fluo-3 fluorescence (*top* trace) and membrane potential (*bottom* trace) from a single cell. As indicated by the bars above the fluorescence tracing, the cell was briefly exposed to two concentrations of either estrogen (0.1 and 1 nM) or ATP (1 and 10 μM). *B*: agonist-stimulated changes in both Fluo-3 fluorescence ( $\Delta F/F_0$ ) and membrane potential are quantified by the upward and downward directed bars, respectively, of the histogram. Data are means  $\pm$  SE of simultaneous recordings from 4 individual cells.

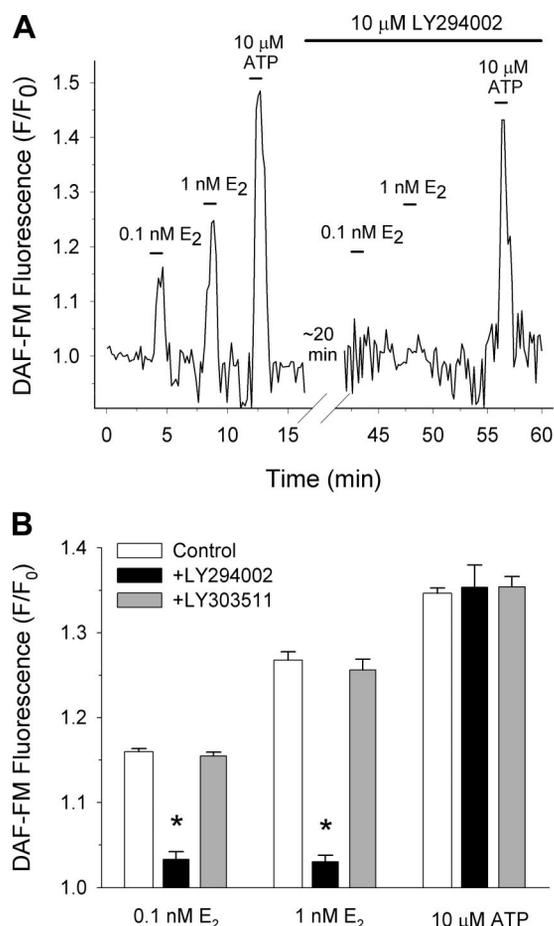


Fig. 8. The phosphatidylinositol 3-kinase (PI3-kinase) inhibitor LY-294002 blocks estrogen-evoked, but not ATP-evoked, increases in DAF-FM fluorescence. *A, left:* increases in cellular fluorescence recorded from a single DAF-FM-loaded EA.hy926 cell in response to either estrogen or 10  $\mu\text{M}$  ATP. Following exposure to the PI3-kinase inhibitor LY-294002 (10  $\mu\text{M}$ ) for ~15 min, the same cell was restimulated by either estrogen or ATP in the continued presence of LY-294002, as indicated by the horizontal bar above the fluorescence tracing. *B:* histogram quantifies the hormone-evoked increases in DAF-FM fluorescence observed under control conditions or in the presence of either 10  $\mu\text{M}$  LY-294002 or its inactive analog LY-303511 (10  $\mu\text{M}$ ). Data are means  $\pm$  SE of recordings taken from 5–6 individual cells for each agonist. \*Statistically different from the associated control ( $P < 0.05$ ).

and atherogenesis. As part of our efforts to establish a more comprehensive picture of vascular NO synthesis and actions, we have used direct, real-time fluorescence- and electrophysiology-based measurements to compare and contrast fundamental aspects of stimulated NO production in single human EA.hy926 cells in response to estrogen and ATP, a Ca<sup>2+</sup>-mobilizing purinergic receptor agonist. EA.hy926 is a permanent cell line derived from human umbilical vein endothelium (10) that has been used in a variety of studies as a model endothelial cell. Using this novel strategy, we report for the first time direct evidence showing that at least two distinct and seemingly parallel signaling mechanisms exist for stimulated NO production within the same single EA.hy926 cell. As we have recently described (40), one such mechanism involves the mobilization of intracellular Ca<sup>2+</sup>, membrane hyperpolarization, and the Ca<sup>2+</sup>-dependent activation of eNOS via external Ca<sup>2+</sup> entry. A second pathway, triggered by stimuli such as estrogen, shear stress, and growth factors, involves the PI3-

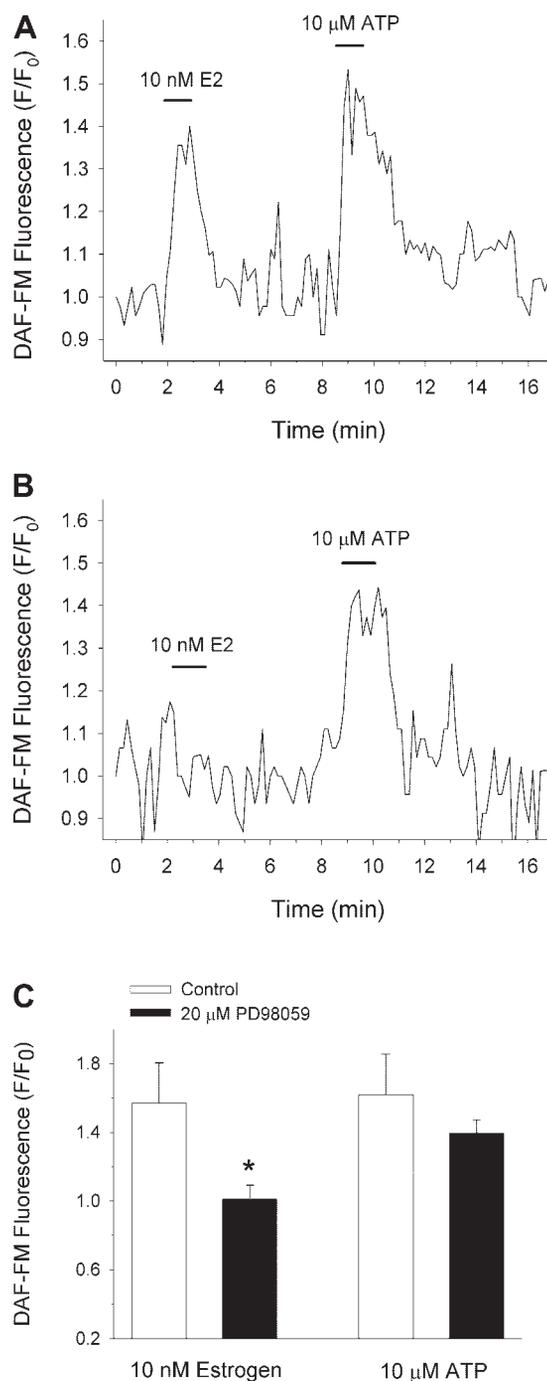


Fig. 9. The MAP kinase inhibitor PD-98059 blocks estrogen-stimulated, but not ATP-stimulated, rises in DAF-FM fluorescence. EA.hy926 cells were pretreated for ~60 min with either solvent (DMSO) alone (control) or 20  $\mu\text{M}$  PD-98059 in DMEM without added serum at 37°C in a 5% CO<sub>2</sub> incubator. Following ~30 min of pretreatment, DAF-FM (0.5  $\mu\text{M}$  final) was added to each dish of cells, and dye loading was carried out for ~30 min, followed by a wash step. Cells receiving either control (*A*) or PD-98059 (*B*) pretreatment were then placed in a bath on the microscope stage and exposed sequentially to 10 nM estrogen and 10  $\mu\text{M}$  ATP using a gravity-fed bath perfusion system. Histogram quantifies agonist-evoked changes in DAF-FM fluorescence (*C*). Data are means  $\pm$  SE of recordings taken from 4 individual cells under each pretreatment condition. \*Statistically significant difference in the agonist-evoked response observed between the control and PD-98059 pretreated cells ( $P < 0.05$ ).

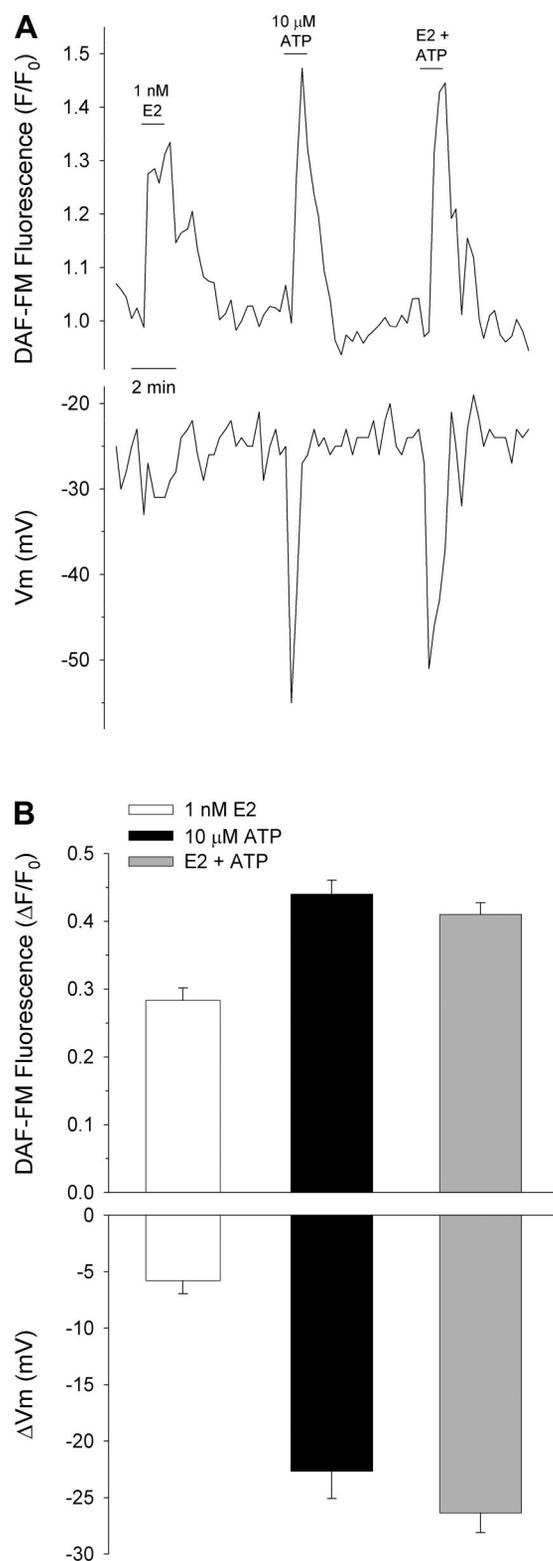


Fig. 10. Estrogen and ATP evoke NO production in a nonadditive manner. Single EA.hy926 cells loaded with DAF-FM dye were held under current clamp using a nystatin-permeabilized patch-clamp method. *A*: simultaneous recordings of DAF-FM fluorescence (*top* trace) and membrane potential (*bottom* trace) from a single cell. Brief exposures of the cell to either 1 nM estrogen or 10  $\mu$ M ATP alone or in combination are indicated by the bars above the *top* tracing. *B*: histogram quantifies agonist-stimulated changes in both cellular DAF-FM fluorescence (*top*) and membrane potential (*bottom*). Data are means  $\pm$  SE of recordings taken from 4 individual cells.

kinase/Akt and MAP kinase signaling cascades and appears to culminate in the phosphorylation-dependent activation of eNOS.

Mechanistically, Busse and colleagues (31, 32) were the first to identify the importance of endothelial  $Ca^{2+}$  signaling in the production of NO or EDRF. While we observed that estrogen was capable of producing rapid intracellular  $Ca^{2+}$  transients in single Fluo-3-loaded EA.hy926 cells (Fig. 3), estrogen-evoked NO synthesis was not significantly altered following the acute removal of external  $Ca^{2+}$  by EGTA or buffering of cytosolic free  $Ca^{2+}$  by BAPTA (Fig. 4, *A* and *B*, respectively). This apparent lack of  $Ca^{2+}$  dependence of estrogen-evoked NO synthesis is thus in agreement with earlier findings (5, 20) and is consistent with an alternative (i.e., phosphorylation-dependent) signaling mechanism for eNOS activation by estrogen. Under some experimental conditions, however, intracellular  $Ca^{2+}$  buffering has been reported to interfere with the estrogen-evoked NO response (27, 46). In contrast, ATP-induced NO production monitored in the same cells following estrogen removal was abolished by either external  $Ca^{2+}$  removal or introduction of an intracellular  $Ca^{2+}$  chelator, in agreement with earlier reports and observations from our own lab (23, 28, 29, 32, 40). Moreover,  $Ca^{2+}$  transients evoked by either ATP or histamine were associated with large membrane hyperpolarizations, whereas estrogen stimulation evoked very modest changes in membrane potential, despite producing clear elevations in cytosolic free  $Ca^{2+}$ . This observation held true even under conditions in which estrogen- and ATP-stimulated  $Ca^{2+}$  transients were of equal magnitude (see Fig. 7), indicating that the estrogen-evoked  $Ca^{2+}$  elevation could not be below the calcium threshold required to activate membrane ion channels. One likely explanation for this somewhat paradoxical set of observations is the fact that second messenger signaling may be "compartmentalized" within a cell, occurring within restricted compartments or microdomains. For example, the compartmentalization of agonist-stimulated cyclic AMP production in cardiac myocytes has been shown to underlie the differential effects of agonists on cellular contractility and metabolism (4, 30). More recently, several calcium-mobilizing hormones have been shown to induce differential activation of  $Ca^{2+}$ -activated  $K^+$  currents and membrane potential responses in isolated vascular endothelial cells (11–13, 19), data that are consistent with our own observations presented in Figs. 6 and 7. Collectively, these findings support the concept of functionally distinct microdomains of intracellular  $Ca^{2+}$  signaling. Such compartmentalization of  $Ca^{2+}$  signals (39) could thus readily explain how estrogen-evoked  $Ca^{2+}$  transients in one cellular compartment may be unable to activate membrane ion channels and alter membrane potential compared with  $Ca^{2+}$  elevations produced by ATP and histamine that likely occur in a distinct compartment (i.e., subplasmalemmal domain). This last point remains quite speculative, because Fluo-3 typically reports global changes in cytosolic free  $Ca^{2+}$ , and we did not have the necessary spatial resolution in our single-cell measurements to detect the absence or presence of  $Ca^{2+}$  microdomains within defined regions of a cell. The lack of association between stimulated  $Ca^{2+}$  transients and membrane hyperpolarization in the case of estrogen, but not ATP, argues nonetheless for differential  $Ca^{2+}$  signaling by each agonist. If estrogen-induced  $Ca^{2+}$  transients do not lead to changes in membrane potential, could they modulate a different physio-

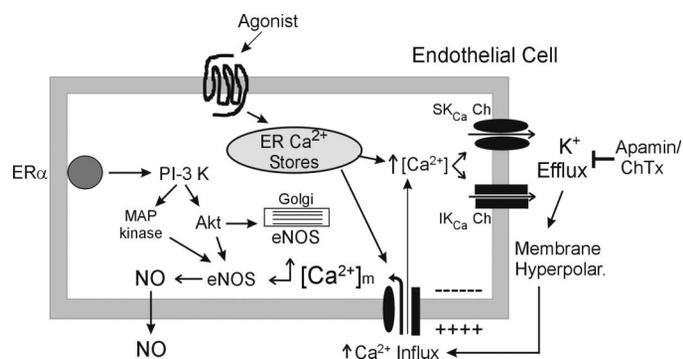


Fig. 11. Cartoon summarizing the primary signaling cascades initiated by estrogen and  $\text{Ca}^{2+}$ -mobilizing hormones to trigger the production of NO in a single vascular endothelial cell. Estrogen initiates activation of the protein kinases PI3-kinase (PI3-K) and Akt, along with the MAP kinase cascade, leading to eNOS phosphorylation and stimulation. Intracellular  $\text{Ca}^{2+}$  appears to play a minimal role in this activation cascade. In contrast, stimulation of NOS (eNOS) by  $\text{Ca}^{2+}$ -mobilizing hormones acting via G protein-coupled receptors involves release of intracellular  $\text{Ca}^{2+}$  stores (ER) and elevation of cytosolic  $[\text{Ca}^{2+}]_i$ , which further triggers store-operated, channel-mediated  $\text{Ca}^{2+}$  influx. Increased cytosolic  $[\text{Ca}^{2+}]_i$  results in activation of small conductance and intermediate conductance calcium-activated potassium channels (SK<sub>Ca</sub> Ch and IK<sub>Ca</sub> Ch, respectively), and the ensuing membrane hyperpolarization (hyperpolar) increases  $\text{Ca}^{2+}$  influx via store-operated channels in the plasma membrane (PM). Enhanced  $\text{Ca}^{2+}$  influx is critical for the stimulation of adequate NO production by PM-associated eNOS; inhibition of SK<sub>Ca</sub> and IK<sub>Ca</sub> channel-mediated membrane hyperpolarization by apamin and ChTx/TRAM-34, respectively, reduces store-operated  $\text{Ca}^{2+}$  influx, thereby preventing calcium-dependent eNOS activation.

logical response? One possibility is that such  $\text{Ca}^{2+}$  elevations underlie rapid estrogen-stimulated prostacyclin synthesis (42) or the  $\text{Ca}^{2+}$ -dependent cellular redistribution of eNOS induced by acute estrogen exposure (18).

Our finding that estrogen-evoked NO production could be prevented by the PI3-kinase inhibitor LY-294002, but not by the inactive control compound LY-303511 (Fig. 8), is consistent with studies demonstrating that the PI3-kinase/Akt signaling cascade is capable of directly activating eNOS via phosphorylation at Ser1177 in both isolated endothelial cells (9, 15, 17) and intact arterial vessels (47). Similarly, the observed inhibition of the estrogen-induced NO response by the selective MEK inhibitor PD-98059 (Fig. 9) is in agreement with earlier reports (7, 8, 21) and further indicates that MAP kinase signaling contributes to stimulated NO production in human EA.hy926 cells. The fact that inhibition of either the PI3-kinase/Akt pathway or MAP kinase cascade prevents acute estrogen-evoked NO production implies that both pathways participate in the estrogen-mediated response and may possibly converge at the level of eNOS. Similar effects of LY-294002 and PD-98059 on estrogen- and high-density lipoprotein-mediated eNOS activation have been recently described in other endothelial cell preparations (21, 36).

Interestingly, ATP-evoked NO synthesis was unaffected by exposure to either LY-294002 or PD-98059, demonstrating that intracellular  $\text{Ca}^{2+}$  mobilization leading to external  $\text{Ca}^{2+}$  entry (40) is the primary mechanism underlying eNOS activation by this and similar agonists (i.e., histamine). Furthermore, our observation that estrogen and ATP stimulated NO production in a nonadditive manner (Fig. 10) suggests that EA.hy926 cells may contain only a single "functional pool" of eNOS that is sensitive to both calcium- and phosphorylation-dependent

signaling pathways. On the basis of recent work from Fulton and colleagues (14, 52), this functional pool of eNOS may be spatially distributed between the plasma membrane and Golgi complex, with plasma membrane-bound eNOS somewhat more sensitive to calcium- versus kinase-dependent signaling and Golgi-bound eNOS displaying similar sensitivities. Whether the existence of spatially distinct pools of eNOS gives rise to physiologically distinct effects of NO remains unclear. A cartoon summarizing the cellular mechanisms underlying endothelial NO production in response to stimulation by estrogen and  $\text{Ca}^{2+}$ -mobilizing agonists is shown in Fig. 11.

In summary, the novel findings of our study provide the first direct experimental evidence for the existence of at least two distinct hormone-driven signaling pathways for NO production within a single identified human EA.hy926 cell. On the basis of biochemical data, it has been largely assumed that eNOS activation by both  $\text{Ca}^{2+}$ /calmodulin and protein phosphorylation events may occur within a single cell. By using direct experimental measurements, the results of our study thus serve as the crucial functional correlate to these earlier reports. Given the widespread sensitivity of the vasculature to the relaxant effects of estrogen and  $\text{Ca}^{2+}$ -mobilizing hormones, we speculate that these two separate mechanisms will function in parallel in individual endothelial cells present in a variety of vascular beds. Moreover, it is possible that such pathways could result in spatial and/or temporal differences in NO synthesis and signaling that differentially affect vascular tone and health both acutely and in the long term. Finally, although human EA.hy926 cells are reported to display a number of features in common with primary vascular endothelial cells, the extent to which data derived from such a model endothelial cell can be extrapolated to native endothelium remains a consideration.

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