Low Doses of Bisphenol A and Diethylstilbestrol Impair Ca^{2+} Signals in Pancreatic α-Cells through a Nonclassical Membrane Estrogen Receptor within Intact Islets of Langerhans

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Glucagon, secreted from pancreatic α-cells integrated within the islets of Langerhans, is involved in the regulation of glucose metabolism by enhancing the synthesis and mobilization of glucose in the liver. In addition, it has other extrahepatic effects ranging from lipolysis in adipose tissue to the control of satiety in the central nervous system. In this article, we show that the endocrine disruptors bisphenol A (BPA) and diethylstilbestrol (DES), at a concentration of 10^{-9} M, suppressed low-glucose–induced intracellular calcium ion ($[Ca^{2+}]_i$) oscillations in α-cells, the signal that triggers glucagon secretion. This action has a rapid onset, and it is reproduced by the impermeable molecule estradiol (E_2) conjugated to horseradish peroxidase (E-HRP). Competition studies using E-HRP binding in immunocytochemically identified α-cells indicate that 17β-E_2, BPA, and DES share a common membrane-binding site whose pharmacologic profile differs from the classical ER. The effects triggered by BPA, DES, and E_2 are blocked by the Gα*i*- and Gα$_o$-protein inhibitor pertussis toxin, by the guanylate cyclase–specific inhibitor 1H-[1,2,4]oxadiazolo[4,3-a]quinoxalin-1-one, and by the nitric oxide synthase inhibitor *N*-nitro-L-arginine methyl ester. The effects are reproduced by 8-bromo-guanosine 3',5'-cyclic monophosphate and suppressed in the presence of the cGMP-dependent protein kinase inhibitor KT-5823. The action of E_2, BPA, and DES in pancreatic α-cells may explain some of the effects elicited by endocrine disruptors in the metabolism of glucose and lipid. *Key words:* cGMP, endocrine disruptors, environmental estrogens, estrogen receptors, glucagon, islets of Langerhans, nongenomic, second messengers. *Environ Health Perspect* 113:969–977 (2005). doi:10.1289/ehp.8002 available via *http://dx.doi.org/* [Online 18 May 2005]

Glucagon is a 29–amino acid pancreatic hormone that is secreted from the pancreatic α-cells into the portal blood supply in response to hypoglycemia, acting as the counter-regulatory hormone to insulin. Its main biologic effect is the regulation of glucose metabolism by enhancing the synthesis and mobilization of glucose in the liver. There is solid evidence demonstrating that the inhibition of glucagon signaling *in vivo* leads to a reduction of plasma glucose (Jiang and Zhang 2003). In addition, glucagon has many extrahepatic effects, such as the increase of lipolysis in adipose tissue, a positive inotropic effect in the heart, a role in the satiety control in the central nervous system, and the regulation of the glomerular filtration rate (Berne and Levy 1993). In the islets of Langerhans, it participates in the regulation of intraislet hormone, insulin, somatostatin, and glucagon secretion (Gromada et al. 1997a; Ma et al. 2005). When insulin secretion from β-cells is impaired, diabetes mellitus develops. In this pathology, the normal physiologic suppression of glucagon secretion from pancreatic α-cells in response to elevated plasma glucose is lost (Unger and Orci 1981a, 1981b). A concomitant decrease in early insulin secretion in response to oral glucose is also observed. These combined defects alter the insulin-to-glucagon ratio, leading to a failure of the normal suppression of endogenous glucose production that occurs after ingestion of oral glucose

(Gerich 1997). This contributes to the elevation of glucose levels in plasma in individuals with impaired glucose tolerance or diabetes.

Despite the great importance of α-cells, little is known about the stimulus secretion coupling and its regulation by other hormones and neurotransmitters. This is partly due to the scarcity of islet tissue and small proportion of α-cells compared with insulin-releasing β-cells. α-Cells contain a specific set of ion channels, including a voltage-dependent Na$^+$ channel, responsible for their electrical activity (Göpel et al. 2000; Rorsman and Hellman 1988; Salehi et al. 1996). Consequently, the intracellular calcium ion $[Ca^{2+}]_i$ oscillates at low glucose concentrations (Berts et al. 1996; Nadal et al. 1999). Because of the calcium influx, the exocytotic machinery is initiated and glucagon is released (Göpel et al. 2004; Gromada et al. 1997b). When the extracellular glucose concentration increases to the level needed for insulin to be released, the frequency of $[Ca^{2+}]_i$ oscillations diminishes and, as a consequence, glucagon release decreases (Nadal et al. 1999; Opara et al. 1988).

Although the endocrine pancreas is not considered a classic estrogen target, estrogen effects on insulin and glucagon secretion and receptors of a classical and nonclassical nature are present in islet cells (Nadal et al. 2004; Sutter-Dub 2002). $[Ca^{2+}]_i$ oscillations have recently been described as finely regulated by

17β-estradiol (17β-E_2) in both α- and β-cells. In the glucagon-containing α-cells, 17β-E_2 provokes the suppression of $[Ca^{2+}]_i$ oscillations generated by low glucose, whereas in β-cells the gonadal hormone potentiates Ca^{2+} signals (Ropero et al. 2002). In both types of cells the 17β-E_2 effect is initiated after binding to a nonclassical membrane estrogen receptor (ncmER) (Nadal et al. 2000; Ropero et al. 2002).

Environmental estrogens are endocrine-disrupting chemicals (EDCs) that in many cases imitate the actions of the natural hormone 17β-E_2, eliciting deleterious effects on humans and wildlife (Colborn et al. 1996; Guillette et al. 1996; Hayes et al. 2002; Hunt et al. 2003). It is well accepted that EDCs exert their effects after binding at the classical ER-α and ER-β, inducing classic nuclear actions (Grünfeld and Bonefeld-Jorgensen 2004; McLachlan 2001). Most of the effects described through the classical pathway occur at micromolar concentrations of EDCs. Evidence accumulated in the last few years indicates that EDCs imitate 17β-E_2 actions through alternative pathways, which in some cases are unrelated to classical ERs (McLachlan 2001; Nadal et al. 2005; Witorsch 2002). Some of these pathways are activated at picomolar and nanomolar concentrations of EDCs (Bulayeva and Watson 2004; Nadal et al.

2000; Quesada et al. 2002; Wozniak et al. 2005).

We have described in pancreatic β-cells the effect of two EDCs: bisphenol A (BPA), found in polycarbonate plastic, in the content of food in coated cans, dental sealant, and composites (Brotons et al. 1995; Sonnenschein and Soto 1998), and diethylstilbestrol (DES), a synthetic estrogen used between the 1940s and the 1970s to prevent miscarriages (Newbold 2004). Both bind to an ncmER to induce $[Ca^{2+}]_i$ signals at concentrations as low as 10^{-9} M (Nadal et al. 2000). In addition, 10^{-9} M BPA via the ncmER activates the transcription factor CREB (Quesada et al. 2002).

In pancreatic β-cells, we used laser scanning confocal microscopy to study $[Ca^{2+}]_i$ movements as an index of changes in intracellular signaling because $[Ca^{2+}]_i$ signals are involved in a large number of signaling processes, from secretion to gene expression (Quesada and Soria 2004; Soria et al. 2004). We used pancreatic α-cells within freshly isolated islets of Langerhans, the physiologic unit of the endocrine pancreas. This preparation is similar to the actual physiologic situation as demonstrated by $[Ca^{2+}]_i$ and electrical activity recordings *in vivo* (Fernandez and Valdeolmillos 2000; Sanchez-Andres et al. 1995). Moreover, this methodology excels when applied to this particular type of cell, which is very difficult to work with because of its scarcity (α-cells represent only about 10–20% of the total number of islet cells, i.e., ~ 150–300 cells per islet). Using this approach, we demonstrated that low doses of BPA and DES imitate the E_2-induced blockade of Ca^{2+} signals in glucagon-releasing α-cells within intact islets of Langerhans. This effect is mediated by the interaction with an ncmER and involves G-proteins, nitric oxide synthase (NOS), and cGMP-dependent protein kinase (PKG).

Materials and Methods

Materials. We obtained Fluo-3 AM from Molecular Probes Inc. (Leiden, the Netherlands); ICI182,780 and 1H-[1,2,4]oxadiazolo[4,3-a]quinoxalin-1-one (ODQ) from Tocris Cookson Ltd. (Avonmouth, UK); and KT-5823 from Alomone Labs (Jerusalem, Israel). Other chemicals were obtained from Sigma (Madrid, Spain). We also obtained estradiol–horseradish peroxidase (E-HRP) from Sigma; however, it is not available from this company at the present time.

Measuring intracellular calcium in α-cells within intact islets of Langerhans. Swiss albino OF1 male mice (8–10 weeks of age) were killed by cervical dislocation according to national guidelines provided by our animal house. An internal animal care and use committee reviewed and approved the method used.

Pancreatic islets of Langerhans were isolated by collagenase digestion as previously described (Nadal et al. 1998) and loaded with 5 µM Fluo-3 AM for at least 1 hr at room temperature. Loaded islets were kept in a medium containing 115 mM NaCl, 10 mM $NaHCO_3$, 5 mM KCl, 1.1 mM $MgCl_2$, 1.2 mM NaH_2PO_4, 2.5 mM $CaCl_2$, and 25 mM HEPES, plus 1% albumin and 5 mM D-glucose, continuously gassed with a mixture of 95% O_2 and 5% CO_2 (pH 7.35). Islets were perfused at a rate of 1 mL/min with a modified Ringer solution containing 120 mM NaCl, 5 mM KCl, 25 mM $NaHCO_3$, 1.1 mM $MgCl_2$, and 2.5 mM $CaCl_2$ (pH 7.35 when gassed with 95% O_2 and 5% CO_2). It took 30–60 sec to change the bath volume completely; this may explain the different response times for some of the experiments.

Calcium records in individual cells were obtained by imaging intracellular calcium under a Zeiss PASCAL5 confocal microscope using a Zeiss 40× oil-immersion lens, with numerical aperture 1.3 (Zeiss, Jena, Germany). Images were collected at 2-sec intervals, and the time course of fluorescent signals from individual cells were measured using the Zeiss LSM software package (Zeiss, Heidelberg, Germany). Experiments were performed at

34°C. Results were plotted using commercially available software (Sigmaplot; Jandel Scientific, Erkrath, Germany) in which the change in fluorescence (ΔF) is expressed as a percentage of the basal fluorescence (F_0) observed in the absence of stimulus or during the intervals between spikes. α-Cells within the islets were identified by their $[Ca^{2+}]_i$ oscillatory pattern in 0.5 mM glucose (Nadal et al. 1999; Quesada et al. 1999). The frequency of $[Ca^{2+}]_i$ oscillations was calculated during a period of 2–4 min before stimuli application (control), and the frequency was measured during 5 min of stimuli application calculated over the period from minute 5 to minute 10 after stimuli were applied. Because a stable baseline before stimuli application was nonexistent, a spike was defined by a rapid increase in $[Ca^{2+}]_i$ higher than twice the SD of background signals at the intervals between spikes. Spikes were counted manually.

Cell isolation and culture. Isolated islets were dispersed into single cells and cultured as previously described (Nadal et al. 1998). Briefly, islets were disaggregated into single cells with trypsin. Cells were then centrifuged, resuspended in RPMI 1640 culture medium supplemented with 10% fetal calf serum, 200 U/mL penicillin, 0.2 mg/mL streptomycin, 2 mM
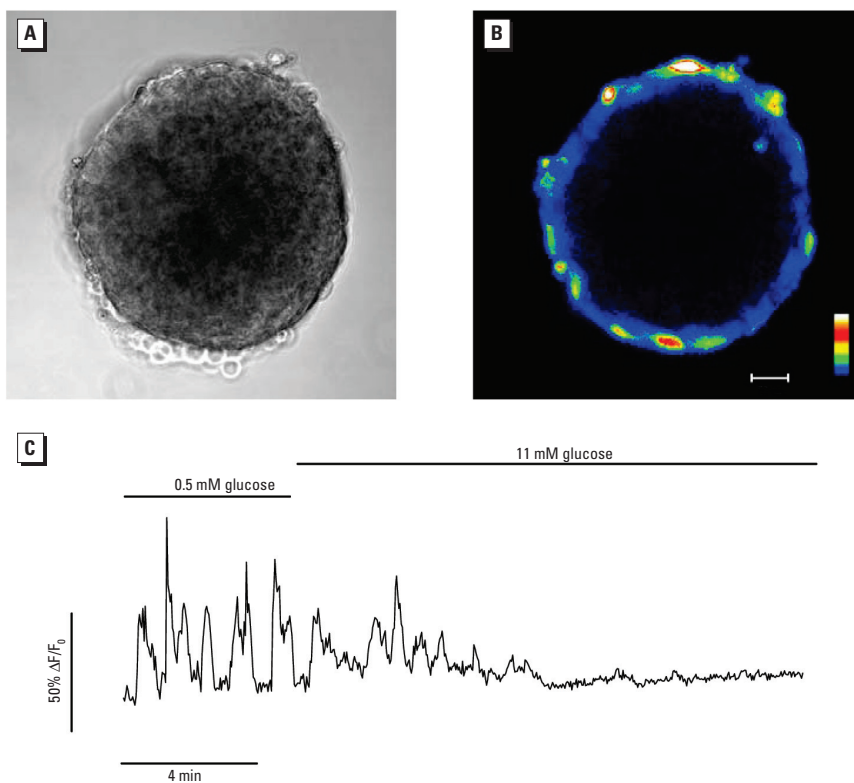

Figure 1. Fluorescence changes monitored from individual cells within intact islets of Langerhans. (*A*) Transmission image of an intact islet of Langerhans. (*B*) Color image of the same islet of Langerhans shown in *A* loaded with the calcium-sensitive dye Fluo-3 and exposed to 0.5 mM glucose; bar = 20 µm (color scale: blue, low calcium; red, high calcium). (*C*) Islets exposed to 0.5 mM glucose were switched to 11 mM glucose, as indicated by the bars above the trace. Note that the low-glucose oscillatory pattern is suppressed in the presence of a high glucose concentration (11 mM).

L-glutamine, and 11 mM glucose and plated on poly-L-lysine–coated glass coverslips. Cells were kept at 37°C in a humidified atmosphere of 95% O_2 and 5% CO_2 for 24 hr.

Estradiol-HRP binding assay. The E-HRP binding assay was performed as previously described (Nadal et al. 2000; Ropero et al. 2002). Briefly, after 24 hr in culture, cells were gently fixed to avoid permeabilization and exposed overnight to 4.5 µg/mL E-HRP plus the testing reagent at 4°C. This was the appropriate concentration of E-HRP to obtain a suitable labeling of the cell membrane. E-HRP binding was visualized by the precipitate formed when reacting with 3,3′-diaminobenzidine tetrahydrochloride (DAB) in the presence of urea hydrogen peroxide and $CoCl_2$ for 15 min [SIGMA FAST DAB with metal enhancer tablet set (Co-DAB)]. The light absorbed by the precipitate was measured; the lower the percentage of absorbed light, the higher the competition for an E-HRP binding site. The quantity of E-HRP bound is expressed as the percentage of absorbed light compared with the control condition. In order to obtain an appropriate staining background, incubation and developmental procedures with peroxidase were performed in identical conditions. All of the chemicals tested for competition with E-HRP were used in a concentration 300-fold higher than that of E-HRP.

Immunocytochemistry. α-Cells were identified using antiglucagon antibody labeling. After staining with E-HRP and Co-DAB, the cells were permeabilized with 1% Triton X-100 for 2 min, and immunocytochemistry was performed as previously described (Ropero et al. 2002).

Results

Effect of EDCs and E_2 on low-glucose–induced $[Ca^{2+}]_i$ oscillations.

To investigate the effect of E_2 and EDCs in pancreatic α-cells, $[Ca^{2+}]_i$ recordings were obtained from intact islets of Langerhans (Figure 1A) loaded with the fluorescent calcium-sensitive dye Fluo-3 and imaged using laser scanning confocal microscopy (Figure 1B). Although only the periphery of the islet is loaded, as previously described (Nadal et al. 1999; Ropero et al. 2002), all the different types of cells within the islet are represented and can be easily identified by their $[Ca^{2+}]_i$ response to glucose (Nadal et al. 1999; Quesada et al. 1999). Pancreatic α-cells are distinguished by their particular oscillatory $[Ca^{2+}]_i$ pattern in the absence of glucose, which diminishes as the glucose concentration is increased (Figure 1C).

Typical α-cells presented a $[Ca^{2+}]_i$ oscillatory pattern in the absence of glucose, which was completely suppressed by 1 nM BPA in almost 50% (20 of 41) of the cells (Figure 2A,D) and by 1 nM DES in 31% of the cells (14 of 45 cells; Figure 2B,D). In all the remaining cells, both EDCs largely reduced the frequency of $[Ca^{2+}]_i$ oscillations (Figure 2E). To calculate the average effect on the frequency of $[Ca^{2+}]_i$ (Figure 2E), an average of all cells was performed. The pesticide 1,1,1-trichloro-2-[*o*-chlorophenyl]-2,2[*p*-chlorophenyl]ethane (*o,p*′-DDT), was less active, producing a complete blockade in only 7% (3 of 41) of the cells tested (Figure 2C,D). The onset of the effect of BPA and DES was very rapid, and the decrease of the frequency of $[Ca^{2+}]_i$ oscillations was similar to the one obtained with E_2 and the membrane-impermeable E-HRP (Figure 2E,F). We used a concentration of E-HRP that was equivalent to the molecules of E_2. Because the effect was present at a concentration equivalent to 1 nM E_2, the effect should be produced by the conjugated form; if free 17β-E_2 was present, it would be in a much lower concentration. The effect of *o,p*′-DDT was significantly smaller than that of the other estrogenic compounds (Figure 2E,F). E_2 and EDC effects were irreversible after 20 min in a solution of 0.5 mM glucose, as previously described for E_2 (Ropero et al. 2002). The speediness of the action and the fact that E-HRP mimics the actions of E_2, BPA, and DES indicate that these EDCs act through an E_2 binding site, probably located at the plasma membrane.

A common membrane-binding site is shared by E_2, BPA, and DES.

To further demonstrate the implication of a membrane binding site, we used a binding assay method based on the interaction of E-HRP as a specific probe to detect E_2 binding sites at the plasma membrane of nonpermeabilized cells. E-HRP bound to the plasma membrane is developed using DAB; the DAB-based primary reaction
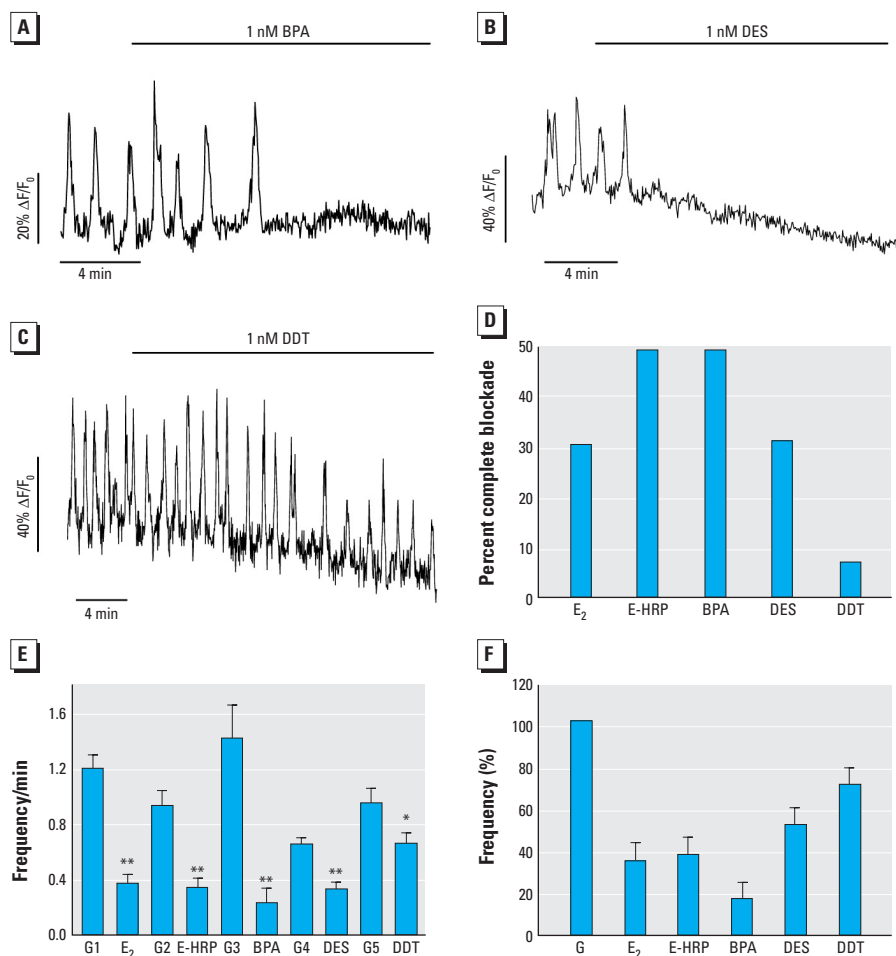

Figure 2. Effect of EDCs on $[Ca^{2+}]_i$ in pancreatic α-cells within the islets of Langerhans in response to 1 nM BPA (*A*), 1 nM DES (*B*), and 1 nM *o,p*′-DDT (*C*), in the presence of 0.5 mM glucose. (*D*) Percentage of cells with a complete blockade of $[Ca^{2+}]_i$ oscillations 5 min after application of stimuli. (*E*) Effect on the frequency of $[Ca^{2+}]_i$ oscillations after the stimuli were applied for 5 min and calculated over a 5–10 min period. G1–G5 are the frequencies of $[Ca^{2+}]_i$ in the presence of 0.5 mM glucose measured during a 2–4 min interval before stimuli application, and E_2, HRP, BPA, EDS, and DDT represent the frequency values after 5 min application of the compound (see "Materials and Methods" for details). (*F*) Frequency (%) of $[Ca^{2+}]_i$ after stimuli compared with control conditions. Note that the decrease in the frequency is 80% in the presence of 1 nM BPA. Error bars in *E* and *F* indicate SE.

*p < 0.01, **p < 0.0001, Student *t*-test, comparing each stimulus with its own control.

product of peroxidase was used as an indication of the amount of E-HRP bound to the estrogen-binding sites. This primary reaction product is highly absorptive of laser light at 543 nm and can be easily visualized with high contrast by using transmission laser scanning microscopy (Nadal et al. 2000). This assay has been combined with immunocytochemistry against glucagon performed afterward to study specifically the characteristics of the E_2 binding site in glucagon-containing α-cells (Ropero et al. 2002). Figure 3A shows several E-HRP control cells after being developed with Co-DAB; two of them were identified as glucagon-containing α-cells by immunostaining (Figure 3D). These control cells presented a black staining that absorbed the light of 543 nM from the laser beam (Figure 3G).

When DES was added at the same time as E-HRP, it decreased the binding of E-HRP to its binding site at the plasma membrane, producing a reduced amount of precipitate. This can be easily visualized by a lower light absorption (Figure 3B,E), and it has been quantified in Figure 3G. Figure 3C shows the basal level when α-cells are incubated only with HRP. This background level has been subtracted in all experiments.
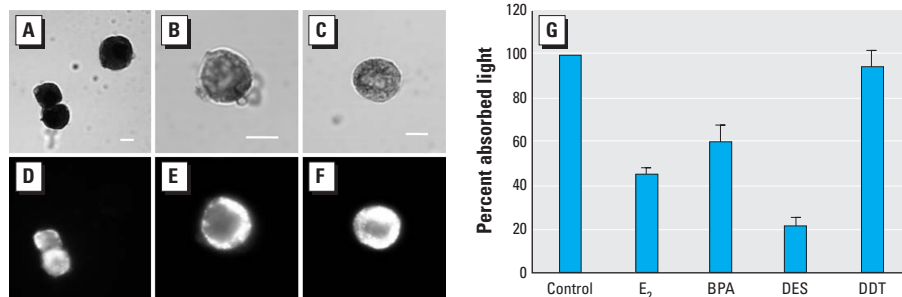
Figure 3G shows that BPA, DES, and E_2 bind to the same membrane-binding site. Remarkably, the pesticide o,p'-DDT, which was weaker in inducing the abolishment of $[Ca^{2+}]_i$ signals, presented a very low competition. This may indicate that the EDCs containing phenol groups, that is, DES and BPA, mimic the effects of 17β-E_2, which also contains a phenolic A-ring in its structure, by binding at the same membrane-binding site. However, o,p'-DDT, which contains no hydroxylated moieties in its structure, was less potent.

To characterize whether the membrane-binding site is an ncmER, we tested whether actions of BPA, DES, and E_2 were modified by the pure antiestrogen ICI182,780, which inhibits ER-mediated effects. Figure 4 shows that the effects of BPA and DES were not modified by ICI182,780. In fact, BPA completely suppressed $[Ca^{2+}]_i$ oscillations in all the cells tested (n = 8, four islets), and DES blocked low-glucose–induced $[Ca^{2+}]_i$ oscillations in 10 of 13 cells (five islets), and in the remaining three cells it largely reduced the frequency of $[Ca^{2+}]_i$ oscillations (Figure 4C). ICI182,780 did not modify the frequency of low-glucose–induced $[Ca^{2+}]_i$ oscillations

(Figure 4C): ICI182,780 controls ICI1–ICI3 range from 0.8/min to 1.4/min, well within the range described for 0.5 mM glucose (Figure 2E). This, along with the experiment described in Figures 2 and 3, strongly suggests that EDC effects are mediated by a membrane ER with a different pharmacologic profile than that of the classical ERs.

cGMP and PKG mediate rapid effects of BPA and E_2. The experiments described to this point suggested that the actions of BPA and DES are mediated through the same receptor as is 17β-E_2 in α-cells. For that reason, we used BPA as the paradigmatic EDC in this system to further analyze the molecular pathway involved. Because we have previously described that cGMP and PKG were implicated in the case of the natural hormone (Ropero et al. 2002), we sought to study their effects on the action of BPA. Application of 10 µM 8-bromo-guanosine 3′,5′-cyclic monophosphate (8Br-cGMP) mimicked the effects of BPA, DES, and E_2 on $[Ca^{2+}]_i$ oscillations (Figure 5A), as previously demonstrated (Ropero et al. 2002; Sugino et al. 2002). Given that the action of cGMP was probably mediated by PKG, we tested the action of 8Br-cGMP in cells pretreated with the membrane-permeable PKG inhibitor KT-5823 for 1 hr. Pretreatment with the PKG inhibitor did not have any effect on low-glucose–induced $[Ca^{2+}]_i$ oscillations (Figure 5C), but it almost completely blocked 8Br-cGMP action (Figure 5B,C).

When we used KT-5823 pretreated islets to test the effect of BPA, we found that the PKG inhibitor completely blocked BPA action (Figure 6A,B). Thus, KT-5823 does not alter $[Ca^{2+}]_i$ oscillations but prevents the suppression of low-glucose–induced $[Ca^{2+}]_i$ oscillations by BPA, indicating that BPA's effect is exerted by a cGMP/PKG-mediated mechanism, as has been demonstrated for the natural hormone 17β-E_2 (Figure 6C).

Involvement of soluble guanylate cyclase and NOS in BPA and E_2 actions. In many cell types, increases in cGMP levels are due to the

Figure 3. Binding assays with E-HRP and HRP and competition with E_2, BPA, DES, and DDT. (*A–C*) Transmission images of nonpermeabilized pancreatic cells with E-HRP or HRP developed using Co-DAB: (*A*) cells incubated with E-HRP as control, (*B*) competition of 30 µM DES for the binding site of E-HRP, and (*C*) background staining with 100 nM HRP. (*D–F*) Glucagon-secreting cells identified by immunocytochemistry: (*D*) identification of the α-cells present in (*A*), (*E*) the same α-cell as in (*B*), and (*F*) the same cell as in (*C*) stained with antiglucagon antibody. (*G*) Competition for E-HRP binding site at the plasma membrane by 30 µM E_2, BPA, DES, and DDT. Background binding obtained with 100 nM HRP has been subtracted. Data are from three duplicate experiments, expressed as mean + SE. Bars = 5 µm.
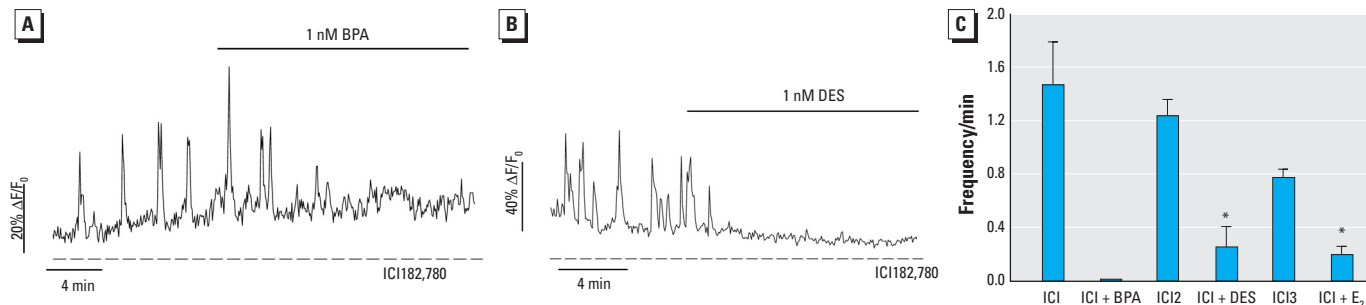

Figure 4. Effects of the pure antiestrogen ICI182,780 (1 µM) on 1 nM BPA (*A*) and 1 nM DES (*B*) action on low-glucose–induced $[Ca^{2+}]_i$ oscillations. ICI182,780 was perfused for at least 20 min before stimuli were added, and it was maintained during the whole period. (*C*) Mean frequencies in $[Ca^{2+}]_i$ oscillations in the presence of ICI182,780 (ICI) for each experiment (ICI1–ICI3) and in the presence of each stimulus. Results are representative of at least eight cells from four different islets, expressed as mean ± SE.
*$p < 10^{-5}$, Student *t*-test comparing each stimulus with its own control.

activation of soluble guanylate cyclase (GC) after NO generation by NOS. To test if this pathway was responsible for the BPA and E_2 actions described above, we started by testing the effect of the NO donor sodium nitroprusside (SNP). In cells that oscillate as usual in the presence of 0.5 mM glucose, SNP abolishes these $[Ca^{2+}]_i$ oscillations (Figure 7A,C). This effect resembles those produced by 8Br-cGMP and the estrogenic compounds BPA and E_2. To check whether the NO effects on $[Ca^{2+}]_i$ oscillations were mediated by an increase in the intracellular levels of cGMP, we applied SNP after blocking GC activity with the selective inhibitor ODQ (Garthwaite et al. 1995). As shown in Figure 7, B and C, ODQ did not modify the average oscillatory frequency of $[Ca^{2+}]_i$ but completely blocked SNP action.

Thus, blockade of GC activity prevented the inhibitory effect of SNP, suggesting that the action of NO is probably mediated by an increase in the intracellular levels of cGMP.

To prove that the cGMP/PKG-mediated actions of BPA and E_2 described in Figure 6 involved the upstream activation of a GC, we tested the effects of these compounds in ODQ-treated islets. In Figure 8, we demonstrate that the BPA and E_2 inhibitions of low-glucose–induced $[Ca^{2+}]_i$ oscillations are completely suppressed in the presence of ODQ. Therefore, we have demonstrated that BPA and E_2 actions involve the activation of a GC, probably by NO that generates cGMP, which in turns activates PKG.

To assess whether NOS is responsible for the generation of NO, we used the specific

antagonist N-nitro-L-arginine methyl ester (L-NAME), which is known to block NOS activation in pancreatic islets (Akesson et al. 1999; Novelli et al. 2004; Salehi et al. 1996). As shown in Figure 9, L-NAME completely reversed the effect of BPA and E_2 (Figure 9A,C,E), demonstrating that NOS activation and the concomitant NO increase are involved in the rapid actions of EDCs and E_2 in pancreatic α-cells.

BPA and E_2 effects involve a G-protein–coupled receptor. To asses the role of G-proteins in the actions of EDC and E_2 on $[Ca^{2+}]_i$ signals in α-cells, we used pertussis toxin (PTX), an inhibitor of $G\alpha_i$ and $G\alpha_o$. After a 4 hr preincubation with 100 ng/mL PTX, no inhibitory effect of BPA and E_2 was observed (Figure 10A). Figure 10B shows the effect of
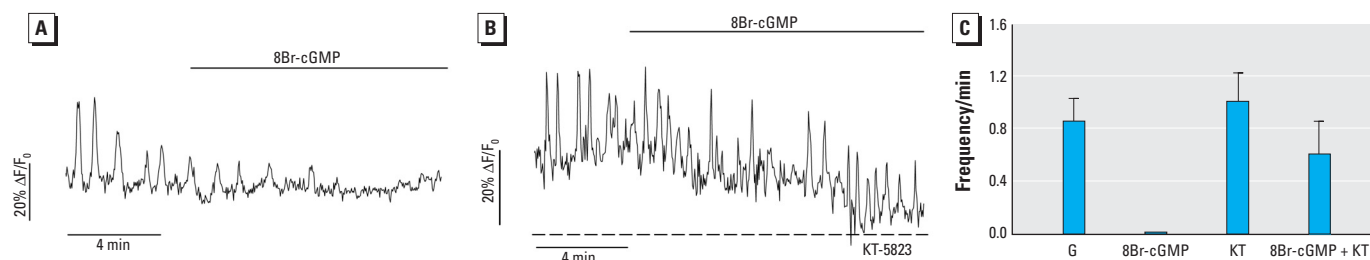

Figure 5. Effects of 8Br-cGMP on EDCs and E_2 via PKG. (*A*) Exposure to 10 µM 8Br-cGMP dramatically reduces the frequency of low-glucose–induced $[Ca^{2+}]_i$ oscillations. (*B*) After incubation with the specific PKG inhibitor KT-5823 (1 µM), 8Br-cGMP fails to evoke the marked reduction in $[Ca^{2+}]_i$ oscillations shown in (*A*). (*C*) Mean frequency values collected in the presence of 0.5 mM glucose (G), 8Br-cGMP plus 0.5 mM glucose (8Br-cGMP), KT-5823 (KT), and 8Br-cGMP plus KT-5823 (8Br-cGMP + KT).Results are representative of at least five cells in four different islets, expressed as mean ± SE.
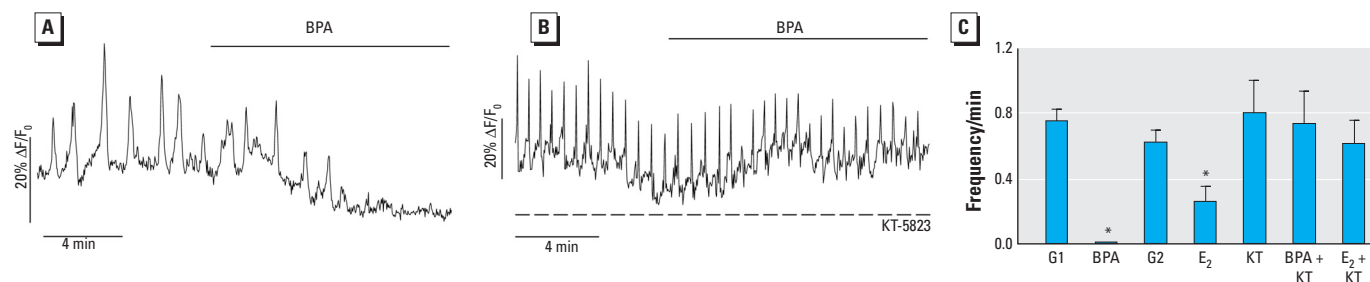

Figure 6. Effects of BPA on $[Ca^{2+}]_i$ oscillations through a PKG-mediated mechanism. (*A*) Low-glucose–induced $[Ca^{2+}]_i$ oscillations blocked by 1 nM BPA. (*B*) Frequency of $[Ca^{2+}]_i$ oscillations were not reduced by BPA in an islet from the same preparation and maintained in the same conditions but pretreated with and exposed to the PKG inhibitor KT-5823 (1 µM). (*C*) Mean frequency values of 0.5 mM glucose before application of either BPA (G1) or E_2 (G2), in the presence of 1 nM BPA or 1 nM E_2, as in (*A*), or in the presence of 1 µM KT-5823 plus 0.5 mM glucose (KT); KT plus 1 nM BPA (BPA + KT), and KT plus 1 nM 17β-E_2 (E_2 + KT) as in (*B*). Results are representative of at least 12 cells from nine different islets, expressed as mean ± SE.
*$p < 0.0001$, Student t-test comparing E_2 with glucose or BPA with glucose.
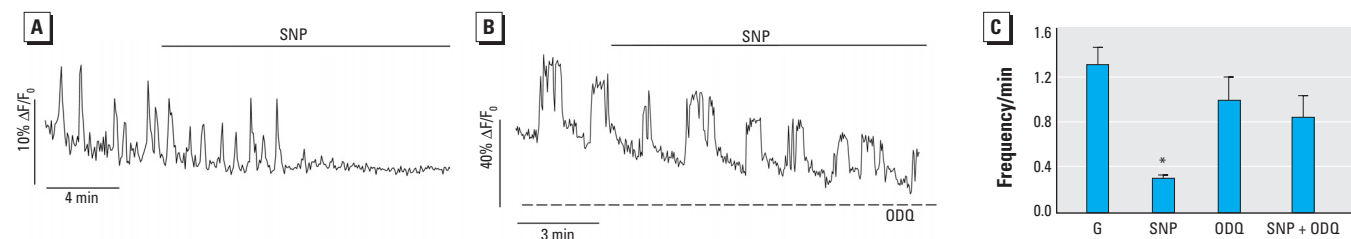

Figure 7. Effects of ODQ on SNP action. (*A*) SNP (100 µM) produces a rapid blockade of low-glucose–induced $[Ca^{2+}]_i$ oscillations. (*B*) When SNP (100 µM) is applied in the presence of ODQ (10 µM), the frequency of $[Ca^{2+}]_i$ oscillations does not vary compared with that observed in the presence of ODQ alone. (*C*) Mean frequency values of 0.5 mM glucose (G) and 100 µM SNP as in (*A*), and 0.5 mM glucose plus ODQ (ODQ), and ODQ plus SNP (SNP + ODQ) as in (*B*). Results are representative of at least four cells in four different islets, expressed as mean ± SE.
*$p < 10^{-5}$, Student t-test comparing E_2 with G1 and BPA with G2.

1 nM BPA in an α-cell within an islet obtained from the same mouse and in the same conditions but in the absence of PTX. The results obtained from different experiments are summarized in Figure 10E. These results support the involvement of either a $G\alpha_i$- or $G\alpha_o$-coupled membrane receptor in the $[Ca^{2+}]_i$ regulation by BPA and E_2.

Discussion

The findings described in this article demonstrate that the endocrine disruptors BPA and DES imitate the circulating hormone 17β-E_2 in suppressing low-glucose–induced $[Ca^{2+}]_i$ oscillations in pancreatic α-cells within intact islets of Langerhans. This rapid effect is observed in a preparation that is almost identical to the physiologic situation: intact islets of Langerhans used directly after their isolation. The $[Ca^{2+}]_i$ oscillatory pattern in β-cells observed *in vivo* (Fernandez and Valdeolmillos 2000; Sanchez-Andres et al. 1995) are identical to those described with the present method (Nadal et al. 1999; Santos et al. 1991). We assume by these experiments performed in β-cells that both preparations behave in a similar manner.

It has been demonstrated that an ncmER is involved in the rapid nongenomic effects of estrogens in pancreatic α- and β-cells within intact islets of Langerhans (Nadal et al. 2000; Ropero et al. 2002). This ncmER is pharmacologically different from classical ERs because it is insensitive to antiestrogens such as tamoxifen and ICI182,780 (Nadal et al. 2004). Moreover, in pancreatic β-cells, BPA and DES bind to

ncmER at doses similar to those of 17β-E_2, inducing the potentiation of $[Ca^{2+}]_i$ oscillations (Nadal et al. 2000). In this article, we have shown that a common binding site exists for BPA, DES, and E_2 in immunocytochemically identified α-cells. This binding site has a different pharmacologic profile than classical ERs: it binds BPA, DES, and E_2 at a similar dose. Moreover, the action of these agents is triggered by remarkably low concentrations, 10^{-9} M. However, BPA is considered a weaker estrogen about 1,000- to 2,000-fold less potent than 17β-E_2 when interacting with classical ERs located in the cell interior (Krishnan et al. 1993; Kuiper et al. 1997). This result, along with the absence of effect of the pure antiestrogen ICI182,780, strongly indicates an action via a receptor other than the classical ER. In addition, the ncmER involved in the regulation of $[Ca^{2+}]_i$ by 17β-E_2 in α-cells uses a cGMP/PKG-mediated pathway, the same one demonstrated here for BPA. Therefore, the receptor involved is very likely to be the same ncmER responsible for the 17β-E_2 actions previously described in α- and β-cells (Nadal et al. 2000; Ropero et al. 2002).

In other types of cells E_2 induced rapid cGMP/PKG-mediated actions. Upstream of cGMP/PKG, NOS is activated, producing NO and activating a GC that in turn increases cGMP levels (Chambliss and Shaul 2002; Rosenfeld et al. 2000; Xia and Krukoff 2004). In the present study, the suppression of low-glucose–induced $[Ca^{2+}]_i$ oscillations by E_2 and BPA was reproduced by the NO donor SNP. This effect was completely blocked when the

GC inhibitor ODQ was used, which indicates that NO is exerting its effect by activating a GC. When the GC inhibitor was used, E_2 and BPA had no effect on low-glucose–induced $[Ca^{2+}]_i$ oscillations. Moreover, when the NOS inhibitor L-NAME was applied, E_2 and BPA produced no effect on $[Ca^{2+}]_i$. This demonstrates that the actions of E_2 and BPA are mediated by NOS activation, which produces NO and activates a GC. This is responsible for the increase in cGMP and the downstream activation of PKG that will likely block ion channels (Figure 11).

The results for the mechanism of action of E_2 and BPA discussed above reinforce the idea that environmental estrogens imitate 17β-E_2 actions not only through the classical pathway but also via other alternative pathways that do not necessarily involve classical ERs (Nadal et al. 2005). Alternative effects are triggered by classical and nonclassical ERs located in the cytosol and the plasma membrane (Nadal et al. 2001). Two main molecular pathways implicated are Src/PI3-Kinase/Akt and Src/Ras/ERKs, which subsequently may activate NOS, as well as other enzymes (Alexaki et al. 2004; Cardona-Gómez et al. 2003; Guerra et al. 2004; Migliaccio et al. 2002; Viso-Leon et al. 2004). A number of studies have shown that both classical ERs associated with the plasma membrane and other unique membrane ERs activate G-proteins, initiating different signaling cascades that, in some cases, include the activation of NOS (Doolan and Harvey 2003; Qiu et al. 2003; Razandi et al. 1999; Wyckoff et al. 2001). In the present
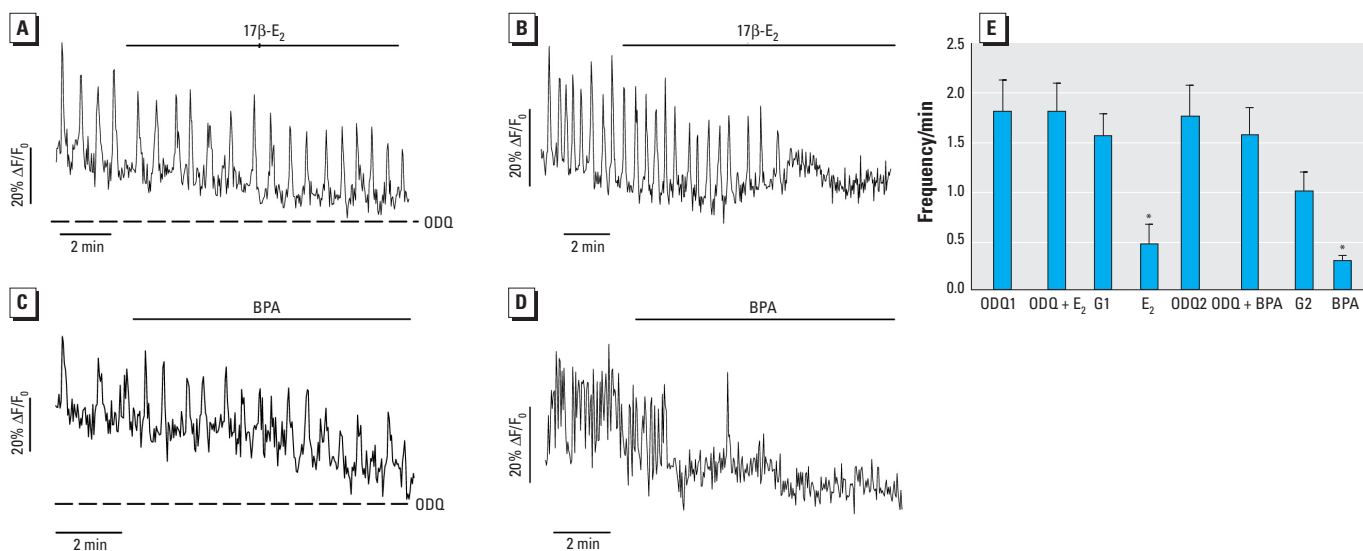

Figure 8. Effect of E_2 and BPA involving a GC. (*A*) When 1 nM 17β-E_2 is applied in the presence of 10 µM ODQ, the $[Ca^{2+}]_i$ oscillatory pattern is not modified. (*B*) Control experiment performed with an islet from the same preparation and maintained in the same conditions as in (*A*) but without ODQ. (*C*) Treatment with 10 µM ODQ prevents the effect of 1 nM BPA. (*D*) Control experiment performed with an islet from the same preparation and maintained in the same conditions as in (*C*) but without ODQ. (*E*) Mean frequency values for experiments as in (*A*): 0.5 mM glucose and 10 µM ODQ (ODQ1), 1 nM 17β-E_2 in the presence of 10 µM ODQ (ODQ + E_2); experiments as in (*B*): 0.5 mM glucose (G1), 1 nM 17β-E_2; experiments as in (*C*): 0.5 mM glucose and 10 µM ODQ (ODQ2), 1 nM BPA in the presence of ODQ (ODQ + BPA); experiments as in (*D*): 0.5 mM glucose (G2), 1 nM BPA. Results are representative of at least six cells from five different islets, expressed as mean ± SE. *$p < 10^{-5}$, Student *t*-test comparing E_2 with G1 and BPA with G2.

study, the suppression of low-glucose–induced $[Ca^{2+}]_i$ oscillations is blocked by the action of PTX, an inhibitor of $Gα_i$ and $Gα_o$, indicating that a G-protein–coupled receptor (GPCR) is involved. All known GPCRs have seven transmembrane–spanning domains, whose intracellular domains propagate the signal from the receptor to the G-protein. It is possible that the ncmER found in the endocrine pancreas may be a seven-transmembrane domain GPCR, as is the case with the orphan receptor GPR30, which behaves as an estrogen membrane receptor in breast cancer cells (Thomas et al. 2005), and the new progestin receptor discovered in teleosts (Zhu et al. 2003). Another possibility is that a receptor with a different structure from GPCRs is coupled to a classical GPCR that activates Gα, which has been proposed for ER-α in endothelial cells (Wyckoff et al. 2001).

There are no extensive studies of endocrine disruptors acting through the same alternative pathways as the natural hormone 17β-E_2. Nonetheless, evidence has accumulated in
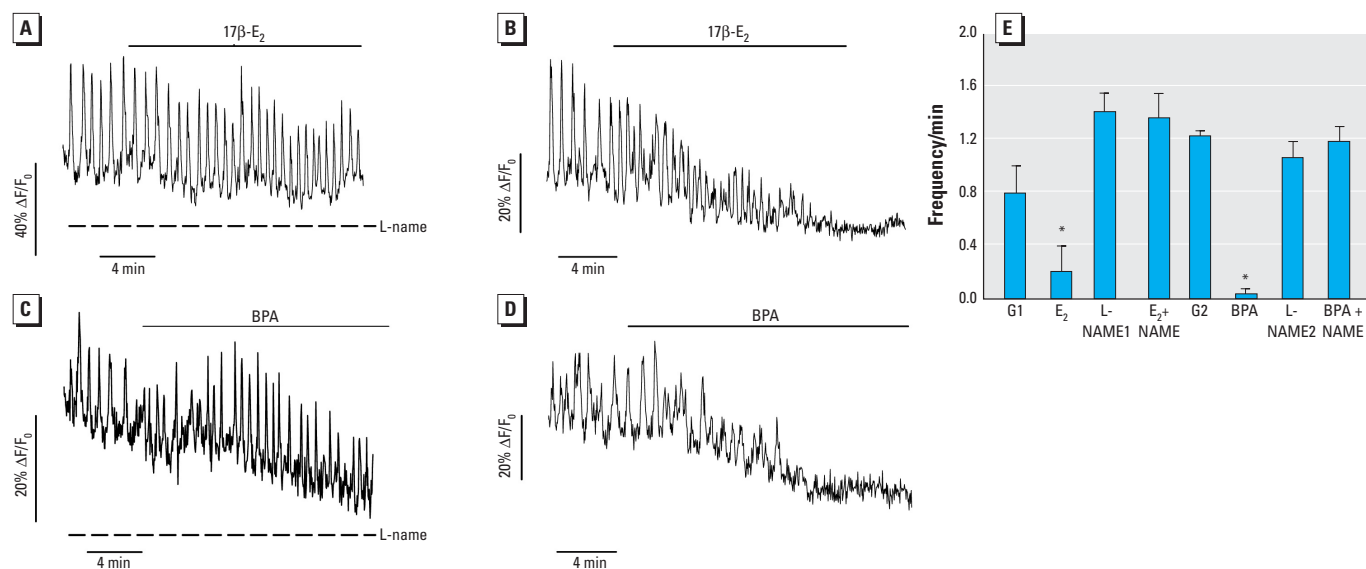

Figure 9. The NOS blocker L-NAME inhibits E_2 and BPA actions. (*A*) When 1 nM 17β-E_2 is applied in the presence of 100 μM L-NAME, the $[Ca^{2+}]_i$ oscillatory pattern is not modified. (*B*) Control experiment performed with an islet from the same preparation and maintained in the same conditions as in (*A*) but without L-NAME. (*C*) Treatment with 100 μM L-NAME prevents the effect of 1 nM BPA. (*D*) Control experiment performed with an islet from the same preparation and maintained in the same conditions as in (*C*) but without L-NAME. (*E*) Mean frequency values for experiments as in (*A*): 0.5 mM glucose and 100 μM L-NAME (L-NAME1), 1 nM 17β-E_2 in the presence of 100 μM L-NAME (E_2 + NAME); experiments as in (*B*): 0.5 mM glucose (G1), 1 nM 17β-E_2; experiments as in (*C*): 0.5 mM glucose and 100 μM L-NAME (L-NAME2), 1 nM BPA in the presence of L-NAME (BPA + NAME); experiments as in (*D*): 0.5 mM glucose (G2), 1 nM BPA. Results are representative of at least 10 cells from eight different islets, expressed as mean ± SE.
*$p < 10^{-5}$, Student *t*-test comparing E_2 with G1 and BPA with G2.
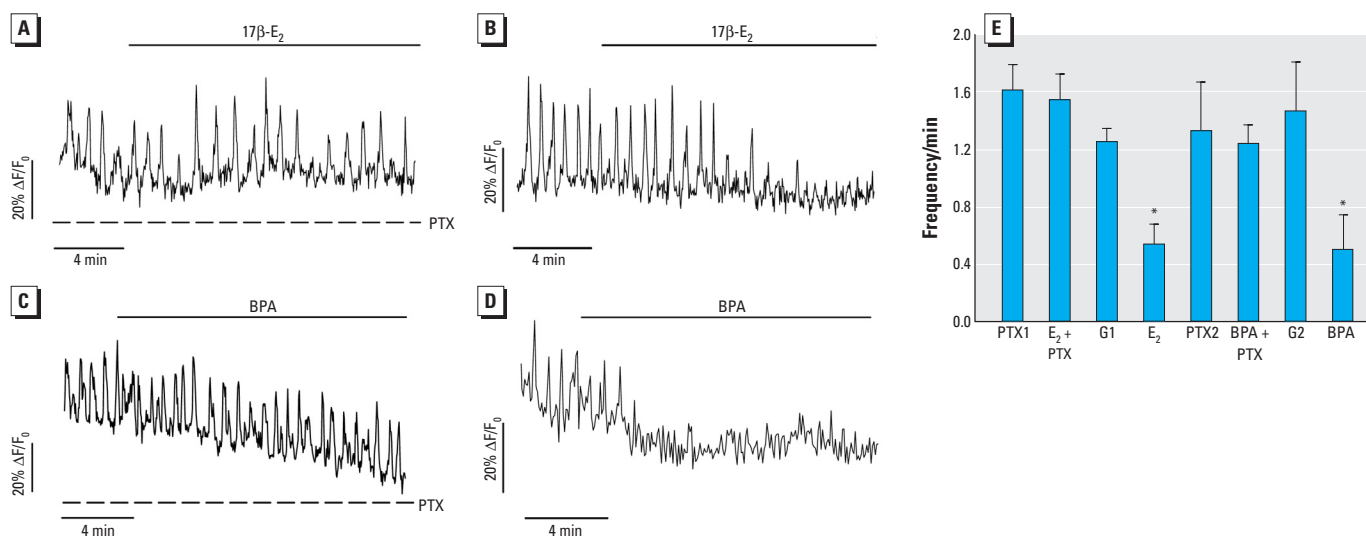

Figure 10. E_2 and BPA action is PTX sensitive. (*A*) Treatment of cells with the G-protein inhibitor PTX (100 ng/mL) for 4 hr completely abolishes 1 nM 17β-E_2 action on $[Ca^{2+}]_i$ oscillations. (*B*) A control experiment performed with an islet from the same preparation and maintained in the same conditions as in (*A*) but without PTX. (*C*) Treatment with PTX prevents the effect of 1 nM BPA. (*D*) Control experiment performed with an islet from the same preparation and maintained in the same conditions as in (*C*) but without PTX. (*E*) Mean frequency values for experiments as in (*A*): 0.5 mM glucose and PTX (PTX1), 1 nM 17β-E_2 in the presence of PTX (E_2 + PTX); experiments as in (*B*): 0.5 mM glucose (G1), 1 nM 17β-E_2; experiments as in (*C*): 0.5 mM glucose and PTX (PTX2), 1 nM BPA in the presence of PTX (BPA + PTX); experiments as in (*D*): 0.5 mM glucose (G2), 1 nM BPA, Results are representative of at least 10 cells from five different islets, expressed as mean ± SE.
*$p < 10^{-5}$, Student *t*-test comparing E_2 with G1 and BPA with G2.

recent years in this direction (Nadal et al. 2005). The activation of ERKs (extracellular signal-regulated kinases) by several xenoestrogens has been described in GH3/B6 pituitary tumor cells (Bulayeva and Watson 2004). Recently, BPA and other xenoestrogens have been shown to mediate Ca^{2+} influx and prolactin release in GH3/B6 pituitary tumor cells at low concentrations (Wozniak et al. 2005). Evidence shows that BPA activates NO synthesis in endothelial cells (Noguchi et al. 2002), the release of dopamine in PC12 cells (Yoneda et al. 2003), caspase-3 in neurons (Negishi et al. 2003), and ERKs and protein kinase C in immune system cells (Canesi et al. 2004). Because all of these actions are blocked by the pure antiestrogen ICI182,780, a classical ER should be involved.

Other actions triggered by endocrine disruptors are not blocked by ICI182,780, and they probably do not involve classical ERs. These include rapid effects of environmental pollutants, such as 4-octylphenol, nonylphenol, and o,p'-DDT, that inhibit Ca^{2+} channels in smooth muscle cells (Ruehlmann et al. 1998); the activation of the nuclear factor of activated T-cell (NF-AT) signaling pathways (Lee et al. 2004); and the activation of AP-1–mediated gene expression in cells that do not express classic ERs (Frigo et al. 2002). In human breast cancer cells, BPA modifies Ca^{2+} signals in a rapid manner
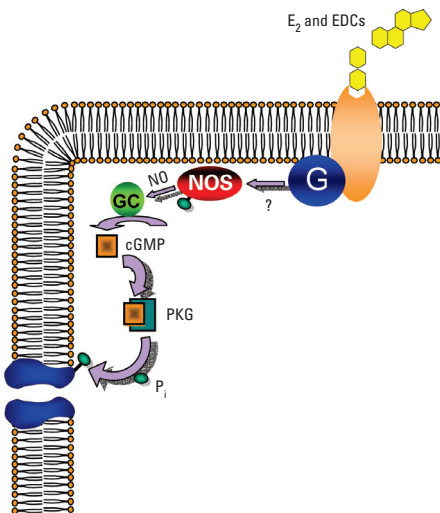

Figure 11. Hypothetical model of the molecular pathway involved in E_2-induced $[Ca^{2+}]_i$ regulation in α-cells. Abbreviations: G, G-protein; P_i, intracellular phosphate. 17β-E_2 and EDCs activate a PTX-sensitive pathway, indicating either that a G-protein–coupled receptor (GPCR) is involved or that a receptor with a different structure from GPCRs is coupled to a classical GPCR that activates a G-protein. This receptor is coupled in a yet undetermined manner with NOS, which generates NO. This activates soluble GC, which increases cGMP levels, which activates PKG, regulating ion channels and producing the abolishment of low-glucose–induced $[Ca^{2+}]_i$.

(Walsh et al. 2005). In pancreatic β-cells, low concentrations of BPA activate the transcription factor CREB via a calcium-dependent mechanism that is activated by an ncmER (Quesada et al. 2002). CREB phosphorylation activated by BPA will bind to CRE (cAMP-responsive element) and in turn modulate DNA transcription. This suggests that the activation of steroid membrane receptors can produce cellular effects through genomic mechanisms.

There is a current debate regarding the concentration of EDCs needed to produce biologic effects in both humans and animals (Kaiser 2000; Welshons et al. 2003), especially because BPA is highly unstable in water and undergoes biodegradation to a less active compound (Staples et al. 1998; Zalko et al. 2003). This would account for the BPA half-life of 2 days estimated in rivers (Zalko et al. 2003). Despite this, drinking water still has traces of BPA (Takahashi and Oishi 2000), which have cellular effects (Hunt et al. 2003; Quesada et al. 2002; Welshons et al. 2003). In the endocrine pancreas, only nanomolar concentrations of BPA are required to modify physiology of α- and β-cells. Furthermore, low concentrations of EDCs are enough not only to produce nongenomic effects but also to affect genomic pathways (Frigo et al. 2002; Quesada et al. 2002).

Ca^{2+} signals control many cellular processes, including secretion and gene expression. In pancreatic α-cells, glucagon release is Ca^{2+} dependent. In the absence of glucose, α-cells present an oscillatory $[Ca^{2+}]_i$ pattern that triggers glucagon secretion; this pattern is suppressed as the concentration of glucose is increased (Nadal et al. 1999), and a diminished glucagon secretion can be expected (Johansson et al. 1987). Unlike β-cells, α-cells are not synchronized (Nadal et al. 1999). Therefore, α-cells are subject to their individual sensitvity to stimuli, including low glucose. This may explain the heterogeneity in response to 17β-E_2 and environmental estrogens. Like for low glucose, some α-cells respond to estrogen and xenoestrogens with complete blockade of calcium oscillations, whereas other cells only decrease the frequency of such oscillations (Nadal et al. 1999). The reason may be different levels of ncmER or any of the steps along the signaling pathway responsible for this effect. The effect of BPA and DES we observed is similar to the one produced by glucose, the suppression of the $[Ca^{2+}]_i$ movements that, in turn, decrease glucagon release (Nadal et al. 1999).

A disruption of glucose signaling process in α-cells by endocrine disruptors may have important implications for normal physiology. Glucagon acts via a seven-transmembrane domain GPCR (Jelinek et al. 1993), which has been identified in multiple tissues including liver, brain, kidney, intestine, adipose tissue, and pancreas (Christophe 1996; Jiang and Zhang 2003). The main physiologic role of glucagon is to stimulate the hepatic glucose output, increasing glycemia. This provides the major counter-regulatory mechanism for insulin by maintaining the glucose homeostasis in blood. It also has an important effect in inducing lipolysis and the release of fatty acids from adipose tissue when blood glucose is low. In the present study, E_2, BPA, and DES all decrease α-cell signaling when glucose is low. This effect should diminish glucogenolysis as well as lipolysis and fatty acid release from adipocytes, contributing to a higher adiposity.

Therefore, the signaling pathway underlying the effects of EDCs described in this study reinforces the existence of a new scenario that explains some of the low-dose effects of EDCs: an action through new binding sites that rapidly activate different signaling cascades outside the cell nucleus. Furthermore, it describes the possibility that low doses of EDCs affect the normal physiology of the endocrine pancreas, altering the regulation of glucose and lipid metabolisms.

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