Nongenomic Regulation of the Kinetics of Exocytosis by Estrogens

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ABSTRACT

The role of nongenomic action of estrogens on elicited catecholamine secretion and exocytosis kinetics was studied in perfused rat adrenals and in cultured bovine chromaffin cells. 17β-Estradiol as well as the estrogen receptor modulators raloxifene and LY117018, but not 17α-estradiol, inhibited at the micromolar range the catecholamine output elicited by acetylcholine or high potassium. However, these agents failed to modify the secretion elicited by high Ca^{2+} in glands treated with the ionophore A-23187 (calcimycin), suggesting that estrogens did not directly act on the secretory machinery. At the single cell level, estrogens modified the kinetics of exocytosis at nanomolar range. All of the drugs tested except 17α-estradiol produced a profound slowing down of the exocytosis as measured by amperometry. LY117018 also reduced the granule content of catecholamines. 17β-Estradiol reduced the intracellular free Ca^{2+} but only at micromolar concentrations, whereas nanomolar concentrations increased the cAMP levels. These effects were reproduced with the nonpermeable drug 17β-estradiol-horseradish peroxidase and antagonized with nanomolar concentrations of the antiestrogen ICI 182,780 (fulvestrant). Our data suggest the presence of membrane sites that regulate both the exocytotic phenomenon and the total catecholamine release with high and low affinity, respectively.

The nongenomic actions of estrogens are receiving a renewed interest. It is now becoming clear that the rapid onset of cellular responses upon drug application, together with the insensitivity of these responses to blockers of transcription or translating, cannot be attributed to genomic activity (Wehling, 1997).

The naturally occurring steroid 17β-estradiol, its α-isomer, and other molecules with estrogen activity have been widely studied in a variety of tissues including chromaffin cells (López et al., 1991; Park et al., 1996; Dar and Zinder, 1997) and PC-12 cells (Chen et al., 1998; Kim et al., 2000; for a recent review, see Falkenstein et al., 2000b).

A number of drugs with estrogenic activity have been synthesized; some of them exhibited agonist effects in some tissues whereas they behaved as antagonists in others. This observation has motivated the coining of the term "estrogen modulator" for tamoxifen and other compounds like raloxifene or LY117018, which are currently under investigation (Fig. 1). The reasons explaining their different tissue selectivity and specific activity are still obscure and cannot be satisfactorily explained by the simple α- and β-estrogen receptor affinity (Nadal et al., 2000).

The sites of action responsible for the rapid action of steroids are a source of controversy. In an attempt to provide an assessment of the nongenomic receptors, the so-called Mannheim classification was published (Falkenstein et al., 2000a); however, it says very little about the nature of receptors involved in the rapid cellular responses to steroids. One of the candidates for being a receptor for estradiol is the β-subunit of the maxi-K potassium channel (Valverde et al., 1999). However, the presence of functional classical nuclear receptors on cell membrane has been described (Watson et al., 1999; Razandi et al., 2000; Wyckoff et al., 2001), although they are far from explaining all of the membrane-mediated effects thus far described for estrogens.

Several second messenger routes were implicated in the cellular signaling occurring upon nongenomic estrogen stimulation. These routes included cAMP (Minami et al., 1990; Gu and Moss, 1996), inositol 1,4,5-trisphosphate formation

ABBREVIATIONS: ACh, acetylcholine; CA, catecholamine; $[Ca^{2+}]_c$, cytosolic calcium concentration; DMPP, 1,1-dimethyl-4-phenylpiperazinium; HRP, horseradish peroxidase; IBMX, 3-isobutyl-1-methylxanthine; ICI 182,780, fulvestrant; A-23187, calcimycin.
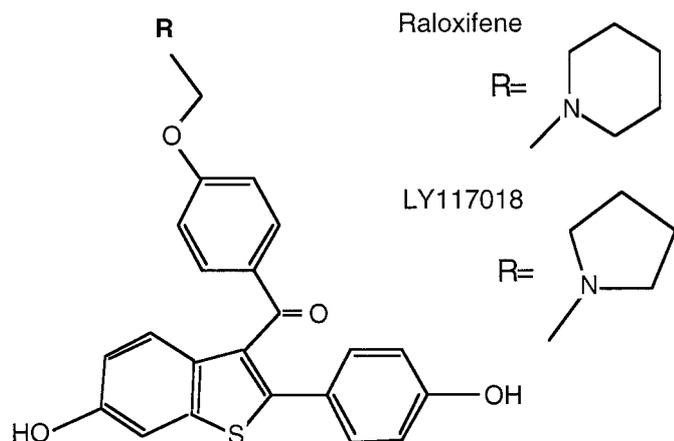
Fig. 1. Structures of raloxifene and LY117018.

(Favit et al., 1991; Shears, 1991), and Ca^{2+}-current inhibition (Ruehlmann et al., 1998; Kim et al., 2000). Recently, Wyckoff et al. (2001) demonstrated the presence of a coupling between the α-receptor subtype and a nitric oxide synthase, but there are no studies available for other second messenger cascades and their relationship with secretion.

It is important to make a distinction between effects on secretion, i.e., the total amount of neurotransmitter release after a stimulus, and effects on exocytosis, i.e., kinetics at single event level. Both are closely related processes involved in the release of neurotransmitters and other substances, but the exocytotic kinetics can be drastically changed even when the total amount of products secreted results unchanged (Machado et al., 2000). In addition, exocytosis can also occur without secretion (Borges et al., 1997; Tabares et al., 2001).

In this study, we have addressed the question of whether the role of estrogens in the regulation of secretory responses is related to changes in the kinetics of exocytosis or granule content of catecholamine (CA). We have shown that estrogens, through a nongenomic mechanism, can modulate both processes, but with very different sensitivities. To our knowledge, this is the first report describing the effects of estrogens at the level of the single event of exocytosis.

Experimental Procedures

Materials. Urografin was obtained from Schering España (Madrid, Spain). Culture plates were purchased from Corning (Palo Alto, CA). LY117018 and LY139481 (raloxifene) were a gift of Eli Lilly & Co. SA (Madrid, Spain). ICI 182,780 was a gift of Zeneca Farma, SA (Madrid, Spain). All other drugs, culture media, and sera were purchased from Sigma-Aldrich (Madrid, Spain). All salts used for buffer preparation were reagent grade.

Perfused Rat Adrenals. Male Sprague-Dawley rats, weighing 200 to 300 g, were anesthetized with 50 mg/kg sodium pentobarbitone i.p. Adrenal glands were perfused retrogradely in vitro at 1 ml/min, as described previously (Borges, 1993), with a Krebs-bicarbonate solution containing 119 mM NaCl, 4.7 mM KCl, 1.2 mM $MgSO_4$, 1.2 mM KH_2PO_4, 2.5 mM $CaCl_2$, 25 mM $NaHCO_3$, and 11 mM glucose. The pH was kept at 7.4 by continuous bubbling with 95% O_2 and 5% CO_2. The CA release was measured fluorimetrically by the trihydroxyindol method without further alumina purification (Anton and Sayre, 1962). Glands received three consecutive stimuli with ACh (30 μM) or K^+ (35.4 mM) of 1-min duration separated by 30 min. All experiments were done at 37°C. All animal procedures were made in strict accordance with the National Institutes of Health

Guide for the Care and Use of Laboratory Animals and approved by The Ethical Committee of La Laguna University (Tenerife, Spain).

To establish the possible role of estrogens on the secretory machinery, we performed some experiments in A-23187 permeabilized tissues. Glands were perfused with Krebs' solution lacking Ca^{2+} ($CaCl_2$ was isosmolarly replaced by $MgCl_2$) for 15 min, then a 10-μM solution of A-23187 was perfused for 10 min. Glands were perfused in Ca^{2+}-lacking solution, and the secretion was triggered by risen $CaCl_2$ in the perfusate to 5 mM in 1-min duration pulses.

Ten minutes before and during the second stimulus, the glands received 1 μM estrogen; this concentration was raised to 10 μM in the third stimulus. Comparisons were made with the second and third secretory response in the absence of drug. Statistical analysis was performed with Dunnett's paired t test.

Culture Chromaffin Cells. Bovine adrenal chromaffin cells were isolated as described previously (Moro et al., 1990) and plated on glass coverslips 12-mm in diameter at an approximate density of 5×10^4 cells/coverslip. Cells were used at room temperature between 1 and 4 days of culture.

Amperometric Detection of Exocytosis. Carbon fibers of 5-μm radius (Thornel P-55; Amoco Corp., Greenville SC) were used to make the microelectrodes (Kawagoe et al., 1993). Electrochemical recordings were performed using an Axopatch 200B (Axon Instruments, Union City, CA) (see Machado et al., 2000 for details).

Glass coverslips with adhering adrenal cells were washed in Krebs-HEPES buffer solution containing 140 mM NaCl, 5 mM KCl, 1.2 mM $MgCl_2$, 2 mM $CaCl_2$, 11 mM glucose, and 10 mM HEPES, brought to pH 7.35 with NaOH. Cells were placed in a perfusion chamber positioned on the stage of an inverted microscope. Amperometric measurements were performed with the carbon fiber microelectrode gently touching the cell membrane. Catecholamine release was stimulated by 5-s pressure ejection of 5 mM Ba^{2+}, 10 μM DMPP, or 59 mM K^+ from a micropipette placed 40 μm away from the cell.

Amperometry Data Analysis. Amperometric records were low-pass filtered at 1 KHz, sampled at 4 KHz, and collected using a locally written software using LabVIEW for Macintosh (National Instruments, Austin, TX). To analyze the exocytotic events, a series of kinetics parameters were extracted from each spike. Data analysis was carried out using locally written macros for IGOR (Wavemetrics, Lake Oswego, OR). These macros allowed the automatic digital filtering, secretory spike identification, and data analysis (Segura et al., 2000). All the above macros and their user instructions can be downloaded free from the following web address: http://webpages.ull.es/users/rborges/

In this study, significant differences were observed between the untreated cells, used as controls, from different days and electrodes. For instance, the average I_{max} from untreated cells ranged from 17.4 to 88.1 pA. For this reason, effects of drugs on secretory spikes were always compared with control experiments carried out along the same day and using the same electrode. To avoid misinterpretation of data, amperometric spike characteristics were not pooled but grouped by individual cells (Colliver et al., 2000). Statistical analysis was carried out by Mann-Whitney test.

Measurement of Cytosolic-Free Ca^{2+} Concentrations. Glass coverslips with adhering cells were washed twice in Krebs' buffer solution and incubated with 2 μM fura-2/acetoxymethyl ester (stock solution dissolved in 20% pluronic F-127 gel in dimethyl sulfoxide) and 0.1% fetal calf serum for 60 min at room temperature. Then, cells were washed twice to remove extracellular dye and placed in a perfusion chamber. Intracellular Ca^{2+} was measured using a computer-operated monochromator (TILL Photonics, Munich, Germany) controlled by a locally written software using LabVIEW. Fluorescence signals were low-pass filtered at 510 nm and detected by a photomultiplier.

Data of $[Ca^{2+}]_c$ time courses were collected at 1 Hz and expressed as fluorescence ratio (F_{340}) and (F_{380}). Statistical analysis was carried out by Student's t test.

cAMP Measurements. Cells were cultured on 24-well plates at 5×10^5/well for 48 h. Cells were preincubated in Krebs-HEPES buffer containing 500 μM 3-isobutyl-1-methylxanthine (IBMX) for 15 min. Testing drugs were incubated for another 15 min, always in the presence of IBMX. Cyclic AMP measurements were done with the cAMP enzyme immunoassay (RPN225) kit (Amersham Biosciences, Cerdanyola, Spain). Data were expressed in femtomoles per microgram of total protein measured by the bicinchoninic acid method. Statistical analysis was carried out by a two-way analysis of variance followed by Tukey's test.

Results

Estrogens Inhibited Secretion from Perfused Rat Adrenals in the Micromolar Range. Adrenal secretory responses to all of the secretagogues used were stable and reproducible along control experiments. Control experiments consisted of three repetitive stimuli with ACh (30 μM; $n = 8$), high K$^+$ (35.4 mM; $n = 10$), or high Ca^{2+} (5 mM, in glands pretreated with 10 μM Ca^{2+} ionophore A-23187; $n = 7$).

A series of estrogen molecules, 17α-estradiol, 17β-estradiol, raloxifene, and LY117018, were assayed on ACh-evoked responses. None of these agents inhibited secretion at concentrations of 10^{-7} M or lower (data not shown). Inhibition of secretion became evident over 10^{-6} M, although it was only significant for LY117018. Raloxifene and 17β-estradiol significantly attenuated the responses when given at 10 μM; however, 17α-estradiol failed to reduce the CA output (Fig. 2). No differences in the degree of the blockade of secretion were observed when 10 μM was perfused without a previous incubation with lower concentrations of drugs (data not shown).

Subsequent experiments were carried out to elucidate the possible cellular targets for estrogens. The LY117018 inhibited the secretion elicited with ACh or K$^+$ in a similar extent whereas it failed to modify the Ca^{2+}-evoked responses (Fig. 3). These data suggest that estrogen did not directly act on the secretory machinery but through a step situated between cell membrane depolarization and the activation by Ca^{2+} of the secretory machinery.

The compound LY117018 also reduced the secretion of CA from isolated bovine chromaffin cells when they were stimulated with 5-s stimuli separated by 5 min with high K$^+$ (35 mM) or DMPP (10 μM). As in rat adrenals, this effect was

Fig. 3. Comparison of the effects of LY117018 on CA release evoked by various agents on rat adrenals. Experiments were performed as described under *Materials and Methods*. Data (mean $\pm$ S.E.M.) were compared with the second or third stimuli (normalized as 100%) obtained in the absence of drug. ⋆, $p < 0.05$; ⋆⋆, $p < 0.01$ (Dunnett's paired t test). Data were from 7 to 10 different glands of each group. No significant (n.s.) differences were found between the inhibition observed on ACh and K$^+$ stimulations.

only observed at 1 μM (12% inhibition) and 10 μM (38% inhibition) (data not shown).

17β-Estradiol Reduced the Ca^{2+} Entry in the Micromolar Range. Figure 4a shows a typical trace of the effect of DMPP (10 μM), applied for 5 s, on [Ca^{2+}]$_c$ in an isolated bovine chromaffin cell. The nicotinic agonist applications were repeated three times at 3-min intervals. Under control conditions, desensitization occurred and the response fell down to 78 and 54%, respectively, on the second and third stimuli. Figure 4b showed the average responses from six different control cells or after 10-min incubation with 17β-estradiol at 10 nM or 10 μM.
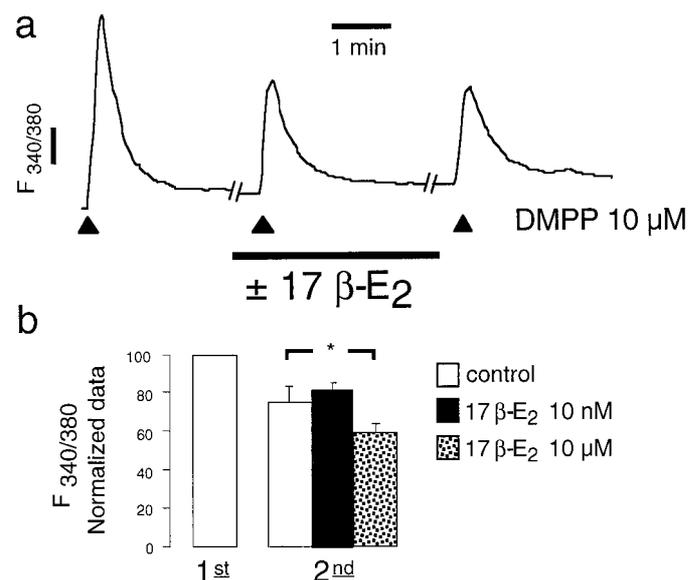
Fig. 4. The effects of low and high concentrations of 17β-estradiol on [Ca^{2+}]$_c$. Bovine chromaffin cells were loaded with fura-2, as described under *Materials and Methods*, and stimulated with 5-s pulses of DMPP (filled triangles) every 3 min. The estrogen was incubated from 10 min prior to the second pulse and remained bathing cells along the rest of the experiment. a, typical fluorescent trace of 340:380 nm ratio; b, means $\pm$ S.E.M. of six different cells from each group. ⋆, $p < 0.05$ (Student's t test).
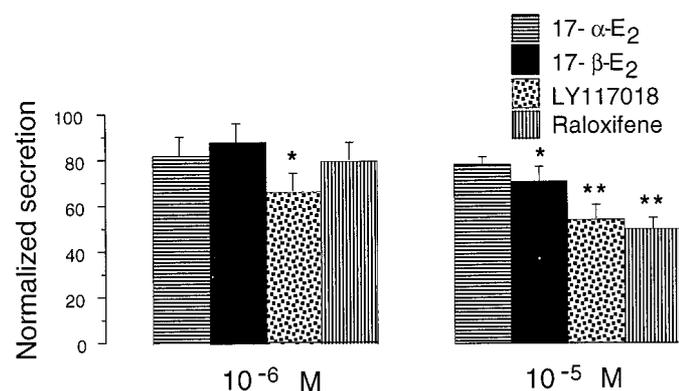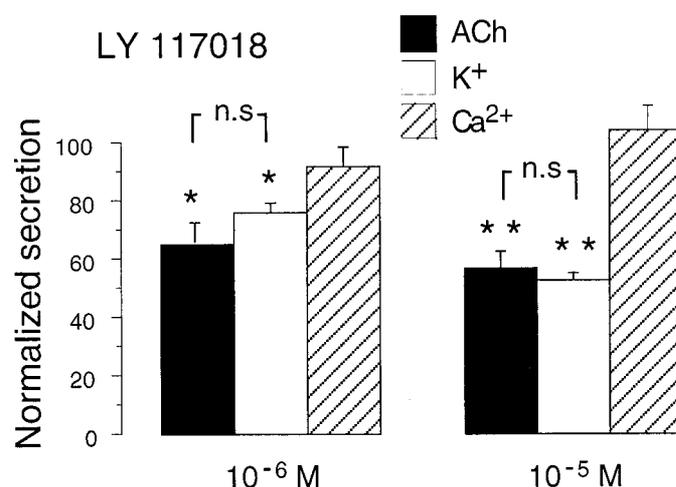
Fig. 2. The effects of estrogens on acetylcholine-evoked responses. Estrogens were applied along the second and third ACh stimuli at the concentrations shown. Data (mean $\pm$ S.E.M.) were compared with the second or third stimuli (normalized as 100%) obtained in the absence of drug. Data were pooled from 6 to 10 different glands of each group. ⋆, $p < 0.05$; ⋆⋆, $p < 0.01$ (Dunnett's paired t test).

TABLE 1

The effects of 17β-estradiol and raloxifene on secretory spike kinetics

Inset figure describes the kinetic parameters measured. I_{max} is the maximal current caused by the CA reaching the electrode; $t_{1/2}$ is the spike width at its half height; Q is the integrated area under the spike trace that indicates the total CA released during the exocytotic event; m is the ascending slope, horizontal ticks indicate the 25 and 75% of I_{max} where m is calculated; tP is the time need to reach the spike maximum. Secretory spikes from cells treated 10 min with 10 nM estrogens are compared with untreated control groups. Data are expressed in the units indicated. Data were calculated by averaging spike parameters from each cell (means ± S.E.M.). The number of spikes computed was not taken into account for statistic analysis.
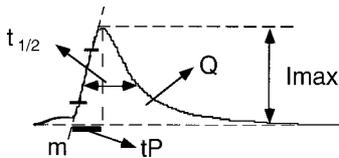
	I_{max}	$t_{1/2}$	Q	m	tP	n cells	n spikes
	pA	ms	pC	nA/s	ms		
Control	28.7 ± 4.2	31.7 ± 3.0	0.94 ± 0.1	11.5 ± 2.8	28.8 ± 5.1	12	1115
17β-Estradiol 10 nM	18.2 ± 2.4*	47.6 ± 5.9*	1.0 ± 0.1	4.5 ± 0.9*	38.9 ± 4.9	12	927
Control	36.6 ± 6.1	36.7 ± 4.4	1.35 ± 0.1	9.3 ± 3.3	29.4 ± 4.2	10	1533
Raloxifene 10 nM	22.7 ± 2.8*	54.4 ± 3.4*	1.38 ± 0.1	2.6 ± 0.4*	63.6 ± 8.4*	12	819

* Statistic differences ($p < 0.05$) from controls (Mann-Whitney tests).

This inhibitory effect of estrogen was only evident at micromolar concentrations. Similar effects were also observed with raloxifene and LY117018 (data not shown).

Estrogens Affected the Kinetics of Exocytosis at the Single Granule Level. It was only necessary for the brief application of nanomolar concentrations of estrogens to produce the slowing down of exocytosis. Table 1 shows the effects of 10 nM 17β-estradiol or raloxifene on the kinetic parameters of single exocytotic events. Since the electrode was placed onto the cell membrane, the slowing down of exocytosis drug was causing an average reduction of a 38% of the CA concentration reaching its surface. Several estrogens reproduced this effect (Fig. 5), which seems to be mediated by a membrane-associated receptor(s) since the cell-impermeable HRP-conjugated 17β-estradiol caused similar effects. It is important to stress that 17β-estradiol altered the kinetic parameters, $t_{1/2}$, I_{max}, m, and tP, but did not produce significant changes in net granule content of CA. All of these effects were observed within seconds of incubation.

In Table 2, we analyzed how these compounds affect the initial speed of granule emptying. The figure accompanying the table shows a spike with its first derivative trace. The ascending slope (m) was estimated between the 25 and 75% of the I_{max} (Table 1, figure inset) to avoid interference from

the presence of a foot (prespike feature) or the deceleration observed just before the tip of spikes. Note that the maximum releasing speed, in normal spikes, occurred at 52 ± 0.5% of the I_{max}, whereas the maximum acceleration took place at 15 ± 0.6%. Estrogens drastically reduced both the initial speed and its acceleration.

Cell incubation with low concentrations of raloxifene (10 nM) did not significantly modify the frequency of secretory spikes (90 ± 13, $n = 10$ versus 76 ± 4, $n = 12$ spikes). However, larger concentrations (1 and 10 μM) caused also a reduction in the number of exocytotic events. This reduction was similar to that observed in net CA released from perfused rat adrenals (Fig. 2).

The effects of raloxifene on the kinetics of exocytosis were studied at three different concentrations. Surprisingly, the

TABLE 2

The effects of various drugs with estrogen activity on ascending slope (m) of spikes

Data (means ± S.E.M.) are expressed in nanoamperes per second obtained in the absence and presence of estrogens. % indicates the fall caused by drugs. Figure inset represents a spike with its first derivative (thinner trace) superimposed. Note the different scale.
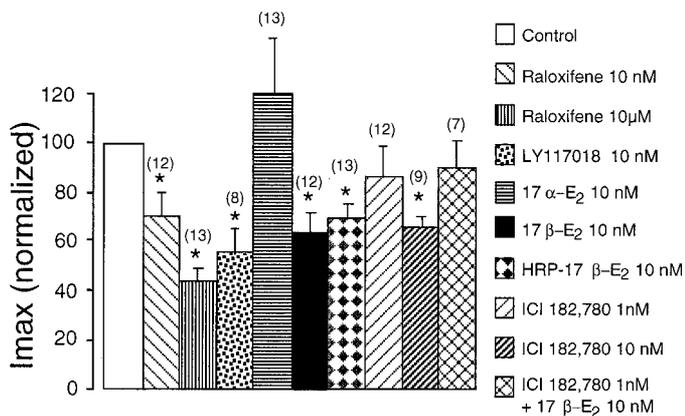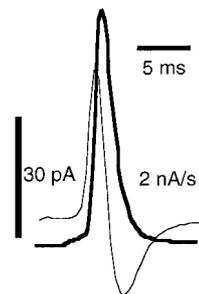

	m (Control)	m (Drug)	%
Raloxifene (10 nM)	9.3 ± 3.3	2.6 ± 0.4	28*
Raloxifene (100 nM)	4.1 ± 0.5	2 ± 0.2	49*
Raloxifene (1 μM)	5.1 ± 0.8	2.5 ± 0.4	45*
Raloxifene (10 μM)	4.1 ± 0.7	1.8 ± 0.5	44*
LY117018 (10 nM)	9.5 ± 3.3	3.6 ± 0.7	38*
17β-E$_2$ (10 nM)	11.5 ± 2.8	4.5 ± 0.9	39*
HRP-17β-E$_2$ (10 nM)	3.8 ± 0.6	2.3 ± 0.3	61*
ICI 182,780 (1 nM)	5.4 ± 1.1	4.1 ± 1.0	76
ICI 182,780 (10 nM)	5.4 ± 1.1	1.7 ± 0.2	32*
ICI 182,780 (1 nM) + 17β-E$_2$ (10 nM)	5.4 ± 1.1	4.2 ± 0.7	77

E$_2$, estradiol.
* $p < 0.05$ with respect to their own control (Mann-Whitney test).

Fig. 5. The effects of various estrogens on I_{max}. Normalized data (means ± S.E.M.) were compared with their own control group of untreated cells ($n = 12$–16). ★, $p < 0.05$ (Mann-Whitney test).
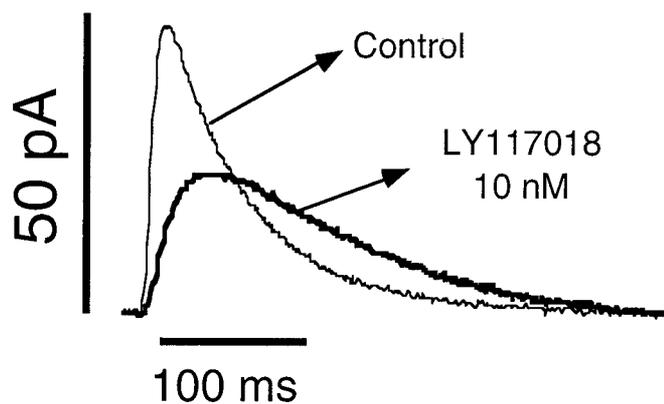
Fig. 6. The estrogen modulator LY117018 slowed down the time course of exocytosis. Spikes were plotted incorporating the average values of the kinetic parameters obtained from real data. Note the different ascending slope, the increase in the $t_{1/2}$ and tP, as well as a decrease in the CA concentration reaching the electrode. Net granule content of CA was, however, reduced.

effects on "m" did not exhibit a clear concentration dependence, and full effects could be obtained with 10 nM (Table 2). Similar results were obtained with 17β-estradiol and with LY117018 (Table 2 and Fig. 5). Conversely, 17α-estradiol did not produce changes in the kinetics of exocytosis nor in granule content. Surprisingly, the pharmacological profile of LY117018 was different than the other drugs tested, because it caused a reduction to 79% in the apparent charge of granules from 1.12 ± 0.1 to 0.85 ± 0.1 picocoulombs (Fig. 6), suggesting that this drug operates through an additional mechanism.

Estrogen Antagonist ICI 182,780 Also Exhibited Estrogen Activity. The compound ICI 182,780 has been proposed as a "pure estrogen antagonist" on the classical nuclear estrogen receptor. It means that its effects only will be evident in the presence of an agonist. However, on exocytotic kinetics it seemed that ICI 182,780, at concentrations of 1 nM or lower, behaved as an antagonist blocking estrogen action. However, over this concentration it exhibited estrogenic activity. Figure 5 shows the effects of ICI 182,780 on I_{max} when
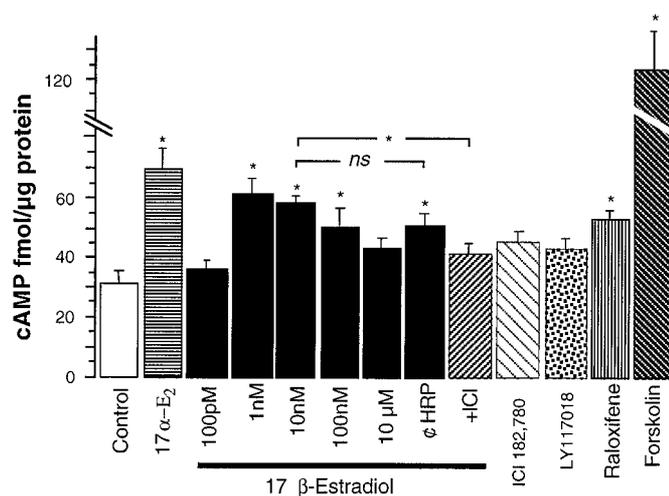

Fig. 7. Estrogens increased the intracellular cAMP levels. Cells were pretreated with 500 μM IBMX for 15 min prior to another 15-min incubation with Krebs' solution (Control), 17α-estradiol (17α-E$_2$), 17β-estradiol (at the indicated concentrations), 10 nM HRP-conjugated 17β-estradiol (¢-HRP), 10 nM 17β-estradiol + 1 nM ICI 182,780 (+ICI), 1 nM ICI 182,780, 10 nM raloxifene, 10 nM LY117018, or 1 μM forskolin. Data were the average of six different experiments. $p < 0.05$ (Tukey's test).

it was applied alone or in the presence of 17β-estradiol. This compound at 10 nM produced the slowing down of the exocytotic process, which was not accompanied by changes in the apparent granule content of CA.

Estrogens Increased the Intracellular cAMP Levels. To explain the action mechanism of estrogens on exocytotic kinetics, we analyzed the cell production of cAMP. These effects are resumed in Fig. 7. The natural isomer 17β-estradiol increased cAMP only in the range of concentrations from 1 to 100 nM. However, no significant change was observed at 10 μM. This effect of estrogen seems to be membrane-delimited as 17β-estradiol HRP-conjugated also increased cAMP. No significant differences were observed between free and conjugated drug. The preincubation of cells with ICI 182,780 abolished the effect of 17β-estradiol on cAMP production.

Even when some estrogens produced changes in cAMP production that were also observed in the time course of secretory spikes, this was not a general rule. Hence, 17α-estradiol, which was revealed to be inactive on secretion, increases the cAMP. Conversely, the LY117018 did not alter the cAMP levels but drastically affected exocytosis at the same concentration.

Note, however, that the cAMP rise over the basal level was modest when compared with 10 μM forskolin. It was only evident after 15 min of drug incubation and required the phosphodiesterase blockade with IBMX.

Discussion

We are far from understanding which are the membrane targets for estrogens despite the wide number of nongenomic effects of estrogens that have been described to date.

In the present study, we combined secretory experiments on perfused adrenals with experiments of exocytosis on isolated cells to explore responses in these different preparations as well as species differences. It was not only intended for two different species and preparations but also because culture maneuvers could cause alterations in the responses of a tissue like adrenal medullae presumably exposed to high levels of steroids. However, the degree of inhibition observed in secretion was similar in both preparations, suggesting that estrogenic transduction mechanisms were not highly affected by the isolation/culture processes.

Our results on perfused rat adrenal gland confirm and extend previous results, which showed that acute application of estrogens caused the inhibition of secretory responses in chromaffin tissues when they were applied at micromolar concentrations (López et al., 1991; Park et al., 1996; Dar and Zinder, 1997). Although the current study was not conducted to examine the target of estrogens to inhibit secretion, it seems that they affected the electrical properties of cell membrane. It could have occurred either by direct reduction of Ca^{2+} currents (Kim et al., 2000) or by increasing the K$^+$ conductivity (Minami et al., 1990; Valverde et al., 1999). Recently, Uki et al. (1999) described the inhibition of nicotinic currents with high estrogen concentrations in rat cervical superior ganglia neurons. However, in chromaffin tissue it is unlikely that estrogens act on neither the nicotinic receptor, because of the similar degree of inhibition observed between ACh and K$^+$, nor the secretory machinery, because LY117018 did not inhibit the Ca^{2+}-evoked secretion on A-23187-treated glands. The onset of the inhibition of CA

secretion was rapid both in perfused glands and in isolated bovine cells. The effect of 10 μM estrogen was not affected by a previous 1 μM incubation, reinforcing the idea of the nongenomic nature of the effect.

The inhibition caused by estrogens on elicited $[Ca^{2+}]_c$ was only observed at micromolar concentrations of estrogens as occurred with the secretion of CA (Figs. 2 and 4).

The most important observation of this study was, however, the effects of estrogens and related compounds at the level of single exocytotic events. This action of estrogens was fully observed at nanomolar concentrations and occurred within seconds (Fig. 6 and Table 2). To our knowledge, this is the first description of the role of estrogens in the regulation of kinetics of exocytosis.

It is not easy to address these effects of estrogens on exocytosis to an action mechanism. It can be discarded as a direct cytoplasmic effect of estrogens because the results with 17β-estradiol were reproduced using HRP-conjugated 17β-estradiol. In addition, the use of nanomolar estrogen concentrations makes improbable a direct effect on fluidity of lipid membranes. Also, these effects occurred within seconds. Therefore, estrogens should act on a membrane acceptor(s).

A few integral membrane proteins have been proposed as putative membrane receptors for estrogens, which include the classical α-receptor expressed on the plasmalemma (Razandi et al., 2000; Wyckoff et al., 2001). Another possible target might be the β-subunit of the maxi-K$^+$ channels, which directly produces hyperpolarization in the muscular cells of blood vessels (Valverde et al., 1999). However, we cannot find its consistent connection with the exocytosis. Recently, Nadal et al. (2000) showed that estrogens could act through the atypical γ-adrenoreceptor as a "nonclassical α- nor β-estrogen receptor"; however, the presence of these receptors has not been demonstrated so far in adrenomedullary and other secretory cells.

We have recently found that second messengers like cGMP (Machado et al., 2000) or cAMP (Machado et al., 2001) negatively modulated the kinetics of exocytosis. In the latter article, we found that even a very modest rise of intracellular cAMP slowed down exocytosis whereas strong elevations, like forskolin treatment, also caused an increase in the net granule content of CA (Machado et al., 2001). In other words, both cGMP and low cAMP concentrations caused the deceleration of exocytosis. Both cyclic nucleotides are usually inversely regulated (Soderling and Beavo, 2000). In addition to the increase in cAMP production, acute treatment with estrogens increased cGMP levels (and cGMP-dependent protein kinase activation) in pancreatic β-cells (Ropero et al., 1999). This overlapping action of both second messengers could explain the biphasic effect of 17β-estradiol observed on intracellular cAMP levels (Fig. 7), whereas the effects on exocytotic kinetics remained almost constant in a wide range of estrogen concentrations (Table 2). It is difficult to test the role of cGMP on type 2 phosphodiesterase in chromaffin cells because the low levels of both compounds obliged the use of IBMX, which strongly inhibits all phosphodiesterases.

Future investigations on nongenomic actions of estrogens should explain the different pharmacological profiles of LY117018 and raloxifene as well as why 17α-estradiol becomes inactive inhibiting rat adrenal secretion and the kinetics of exocytosis but increases cAMP production. This isomer is inactive on classical receptors but inhibits secretion in cat adrenals (López et al., 1991).

One effect that was difficult to explain was the behavior of "classical" antagonists on some nongenomic estrogen responses. In our hands, the ICI 182,780 antagonized the effects of 17β-estradiol on cAMP production (Fig. 7) and on the kinetics of exocytosis (Fig. 5). However, it behaved as an agonist when applied alone (Table 2). This latter observation was in agreement with Ruehlmann et al. (1998) who also found that acutely administered ICI 182,780 mimicked the inhibitory effects of estrogens on Ca^{2+} currents of vascular smooth muscle. The best explanation could be that this compound acts as a partial agonist with a higher activity but lower intrinsic activity than estradiol.

The pharmacological profile of estrogen modulators is still far from being understood, and several differences have been found between tamoxifen and raloxifene. The compound LY117018 also exhibited a different profile than raloxifene; it was more potent inhibiting secretion and slowing secretion, but it did not increase cAMP levels. These results prevent us from attributing all of the membrane-mediated effects of estrogens to the cAMP production.

The I_{max} reflects the concentration of CA reaching the electrode. The distance between the surface of a carbon fiber electrode and the cell membrane is similar to the synaptic cleft ($\approx$20 nM). It means that the kinetics of exocytosis could control the concentration of neurotransmitter reaching the postsynaptic cell using the same vesicle content. It suggests a new role for estrogens in the control of synaptic performance. Chromaffin granules and dense cored vesicles, found in noradrenergic and other synapses, are similar organelles (Winkler and Fisher-Colbrie, 1998). Estrogen can be continuously modulating the sympathetic nerve terminals like arteriolar nerve-muscle synapses. We hypothesize that part of the protective actions of estrogens on vascular diseases in premenopausal women could be mediated by this mechanism.

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