17 β -Oestradiol increases intracellular Ca²⁺ concentration in rat enterocytes

Potential role of phospholipase C-dependent store-operated Ca²⁺ influx

Gabriela PICOTTO, Guillermo VAZQUEZ and Ricardo BOLAND¹

Departamento de Biología, Bioquímica y Farmacia, Universidad Nacional del Sur, San Juan 670, 8000 Bahía Blanca, Argentina

The involvement of the phospholipase C (PLC) pathway in the non-genomic regulation of duodenal cell Ca²⁺ concentration by 17 β -oestradiol was investigated. The PLC inhibitors neomycin (0.5 mM) and U-73122 (2 μ M) suppressed the stimulatory effect of 0.1 nM 17 β -oestradiol on the ⁴⁵Ca²⁺ influx into enterocytes isolated from rat duodenum. The hormone (1 pM to 10 nM) increased the formation of 1,2-diacylglycerol in a biphasic pattern, characterized by an early peak at 45 s (+82 %) and a later peak at 5 min (+46 %). Both PLC inhibitors suppressed the first peak but were unable to block the 17 β -oestradiol effect at 5 min. 17 β -Oestradiol also increased the generation of inositol 1,4,5-trisphosphate within 15 s, with maximal stimulation at 30 s. 17 β -Oestradiol induced a rapid (30 s) and sustained (up to 5 min) increase in the intracellular Ca²⁺ concentration ([Ca²⁺]_i) of fura 2-loaded enterocytes. The fast rise in [Ca²⁺]_i was specific because other sex steroid hormones were without effect and

could be blocked to a great extent by U-73122 (by 86 % at 1 min). The effects of 17 β -oestradiol on enterocyte [Ca²⁺]_i were decreased significantly (by 75 %) in a Ca²⁺-free extracellular medium but a pronounced increase in [Ca²⁺]_i was obtained after readmission of Ca²⁺ to the medium. The latter change was suppressed by 10 μ M La³⁺, whereas nitrendipine (1 μ M) and verapamil (10 μ M) separately were without effect. The permeability of the 17 β -oestradiol-induced Ca²⁺ influx pathway to Mn²⁺ was increased 2.8-fold by treatment with oestrogen. These results suggest the operation of a PLC-dependent store-operated Ca²⁺ channel mechanism in 17 β -oestradiol regulation of enterocyte extracellular Ca²⁺ influx.

Key words: intestine, non-genomic effects, steroid hormones, store-operated Ca²⁺ channels.

INTRODUCTION

Parathyroid hormone, 1,25-dihydroxyvitamin D₃ [1,25(OH)₂D₃] and calcitonin regulate extracellular Ca²⁺ homeostasis by the modulation of Ca²⁺ fluxes in intestine, bone and kidney [1]. Oestrogens are not usually considered calciotropic hormones even though they affect bone resorption and oestrogen deficiency has been established as a major aetiological factor in post-menopausal osteoporosis [2,3]. The existence of a putative oestrogen receptor in duodenal mucosal cells of rats has been reported, raising the possibility that oestrogen might have a direct physiological role in the regulation of intestinal Ca²⁺ absorption [4]. Furthermore recent work suggests the presence in rat duodenum of oestrogen receptor-like proteins encoded by a variant oestrogen receptor gene [5].

Although it is widely accepted that the mechanism of action of sex steroid hormones involves binding to cytosolic receptors leading to the transcriptional regulation of protein synthesis, several non-genomic actions of oestrogens affecting important second-messenger signalling systems have been reported [6,7]. We have demonstrated rapid, direct and specific actions of 17 β -oestradiol in isolated duodenal cells, initiated on the cell surface and resulting in the activation of the cAMP pathway and Ca²⁺ influx through dihydropyridine-sensitive Ca²⁺ channels [8]. Oestrogens have been shown to stimulate phosphoinositide metabolism rapidly in rat female osteoblasts involving the participation of phospholipase C (PLC) [9]. The present study was undertaken to investigate the involvement of the PLC pathway in the non-genomic modulation of intestinal cell Ca²⁺ homeostasis by oestrogens. We characterized the effects of 17 β -

oestradiol on the generation of both 1,2-diacylglycerol (DAG) and inositol 1,4,5-trisphosphate (IP₃), as well as its effect on intracellular Ca²⁺ concentration ([Ca²⁺]_i), in rat duodenal cells.

MATERIALS AND METHODS

Materials

17 β -Oestradiol, progesterone, dihydrotestosterone, verapamil, fura 2 acetoxymethyl ester (fura 2/AM) and pluronic F127 were purchased from Sigma Chemical Co. (St. Louis, MO, U.S.A.). ⁴⁵CaCl₂ was provided by New England Nuclear (Chicago, IL, U.S.A.). Nitrendipine was from Bayer Leverkusen (Leverkusen, Germany). [³H]Arachidonic acid and [³H]IP₃ assay kit were obtained from Diagnostic Products Co. (Los Angeles, CA, U.S.A.) and Dupont NEN (Chicago, IL, U.S.A.) respectively. 1-(6-[[17 β -3-Methoxyestra-1,3,5(10)-trien-17-yl]amino]hexyl)-1H-pyrrole-2,5-dione (U-73122) and 1-(6-[[17 β -3-methoxyestra-1,3,5(10)-trien-17-yl]amino]hexyl)-2,5H-pyrrolidine-dione (U-73343) were from Biomol Research Laboratories (Plymouth Meeting, PA, U.S.A.). Other chemicals used were of analytical grade.

Animals

Wistar female rats (3–5 months-old) were fed with standard rat food (1.2 % Ca/1.0 % phosphorus), given water *ad libitum* and maintained on a 12 h: 12 h light/dark cycle. Animals were killed by cervical dislocation.

Abbreviations used: [Ca²⁺]_i, intracellular Ca²⁺ concentration; DAG, 1,2-diacylglycerol; fura 2/AM, fura 2 acetoxymethyl ester; IP₃, inositol 1,4,5-trisphosphate; 1,25(OH)₂D₃, 1,25-dihydroxyvitamin D₃; PKA, protein kinase A; PKC, protein kinase C; PIP₂, phosphatidylinositol 4,5-bisphosphate; PLC, phospholipase C; SOC, store-operated Ca²⁺ channels; U-73122, 1-(6-[[17 β -3-methoxyestra-1,3,5(10)-trien-17-yl]amino]hexyl)-1H-pyrrole-2,5-dione; U-73343, 1-(6-[[17 β -3-methoxyestra-1,3,5(10)-trien-17-yl]amino]hexyl)-2,5H-pyrrolidine-dione; VDCC, voltage-dependent Ca²⁺ channels.

¹ To whom correspondence should be addressed (e-mail rboland@criba.edu.ar).

Duodenal cell isolation

Duodenal cells were isolated as described previously [8]. The method employed yields preparations containing only highly absorptive epithelial cells and are devoid of cells from the upper villus or crypt [10,11]. The duodenum was excised, washed with 0.9 % NaCl and trimmed of adhering tissue. The intestine was slit lengthways and cut into small segments (2 cm length) and placed into solution A containing (in mM): 96 NaCl, 1.5 KCl, 8 KH_2PO_4 , 5.6 Na_2HPO_4 , 27 Na citrate, pH 7.3, for 10 min at 37 °C. The solution was discarded and replaced with solution B (isolation medium) containing (in mM): 154 NaCl, 10 NaH_2PO_4 , 1.5 EDTA, 0.5 dithiothreitol, 5.6 glucose, pH 7.3, for 15 min at 37 °C with continuous shaking (87 oscillations/min). The cells were sedimented by centrifugation for 10 min at 750 *g*, washed twice with 154 mM NaCl/10 mM NaH_2PO_4 /5.6 mM glucose (pH 7.3), then resuspended in solution D (incubation medium) containing (in mM): 154 NaCl, 5 KCl, 1 Na_2HPO_4 , 1 MgCl_2 , 2.5 glutamine, 10 Mops sodium salt, 5.6 glucose, 1 CaCl_2 , 0.5 % BSA, pH 7.3. All of the above-mentioned steps were performed under an O_2/CO_2 (19:1) atmosphere. Cell viability was assessed by the Trypan Blue technique. Exclusion of the dye in more than 90 % of the cells was observed for at least 90 min after isolation. Morphological characterization was performed by phase-contrast microscopy. Enterocytes isolated by this procedure have been shown to possess functional characteristics of intestinal cells [10,11].

Ca^{2+} uptake

Cell suspensions were equilibrated for 20 min in the incubation medium before hormone treatment and measurement of Ca^{2+} uptake. Duodenal cells were then incubated in medium D with 17 β -oestradiol in the presence of $^{45}\text{CaCl}_2$ (0.2 $\mu\text{Ci}/\text{ml}$; 1 mM). Ethanol (less than 0.1 %) or distilled water were added to the corresponding control cell suspensions. When used, PLC inhibitors (neomycin and U-73122) were added during the equilibration period, before hormone addition. Immediately after treatment, aliquots of the cell suspension were diluted 1:25 in ice-cold unlabelled medium (in mM: 140 NaCl, 10 Tris/HCl, pH 7.4, 1 LaCl_3 , 1 CaCl_2) and centrifuged quickly for 45 s at 1500 *g*, and the pellet was solubilized in 1 M NaOH/0.1 % SDS. Under these conditions, extracellularly bound $^{45}\text{Ca}^{2+}$ is removed completely. Aliquots were taken for measurement of radioactivity and protein determination by the method of Lowry et al. [12] with BSA as standard.

Formation of DAG

Isolated duodenal mucosa was prelabelled with [^3H]arachidonate (0.1 $\mu\text{Ci}/\text{ml}$) at 37 °C for 90 min under O_2/CO_2 (19:1), followed by extensive washing with the incubation medium. After hormonal treatment, the incubation was finished by adding chloroform/methanol (2:1, v/v) and lipids were extracted by using the Folch procedure [13]. Neutral lipids were isolated by one-dimensional TLC on silica gel G plates developed with hexane/diethyl ether/acetic acid (75:25:4, by vol.) as the mobile phase. This system completely separates free fatty acids, monoacylglycerol, DAG and triacylglycerol [14,15]. Lipids were detected by staining with iodine and were identified by comparison with appropriate standards. Labelled DAG was scraped off and counted for radioactivity by liquid scintillation. When used, PLC inhibitors were added to the incubation medium 10 min before hormonal treatment.

Measurements of IP_3

After hormonal treatment, aliquots of cell suspension were transferred to ice-cold trichloroacetic acid [20 % (w/v) final concentration], mixed thoroughly, left on ice for 15 min and centrifuged at 1000 *g* for 10 min at 4 °C. The precipitate was used for protein determination, and trichloroacetic acid from the supernatant was extracted four times with 5 vol. of water-saturated diethyl ether [16]. The extract (final pH 4.5) was then used for IP_3 measurements with a radioreceptor assay provided as a commercially available kit [17]. Defined as the mass corresponding to twice the S.D. of the zero binding, the sensitivity of the system was found to be approx. 0.1 pmol.

$[\text{Ca}^{2+}]_i$

Enterocytes obtained as described above were loaded with fura 2/AM for 10 min at 37 °C in the dark, in loading buffer containing (in mM) 138 NaCl, 5 KCl, 1 MgCl_2 , 5 glucose, 10 Hepes, pH 7.4, 1.5 CaCl_2 and also 0.1 % BSA, 0.012 % pluronic F127 and 4 μM fura 2/AM. The cell suspension was washed three times with measurement buffer containing (in mM) 138 NaCl, 5 KCl, 1 MgCl_2 , 5 glucose, 10 Hepes, pH 7.4, 1.5 CaCl_2 , to eliminate unloaded dye. Cells were maintained at room temperature for 15 min in the dark until use. For measurements of intracellular fura 2 fluorescence signals, cells were put into quartz cuvettes at a density of 700000/ml and then introduced into the sample compartment of a thermostatically controlled (37 °C) SLM Aminco 8100 spectrofluorimeter (Spectronics, Urbana, IL, U.S.A.) with constant low-speed stirring. Drugs and reagents were added directly into the cuvette at the indicated times. Fura 2 fluorescence intensity was monitored at an emission wavelength of 500 nm by alternating (300 Hz) the excitation wavelength between 340 and 380 nm with a dual excitation monochromator (DMX 1100). The signals at 340 and 380 nm were ratioed ($R_{360/390}$), and maximal (R_{max}) and minimal (R_{min}) intracellular dye fluorescences were determined by the addition of Triton X-100 [0.1 % (v/v) final concentration] and EGTA (5 mM, pH 8.3) respectively. The transformation of fluorescence signals into $[\text{Ca}^{2+}]_i$ was performed by the method of Grynkiewicz et al. [18]. The use of Triton X-100 in the calibration procedure did not interfere with the fluorescence signal or fura 2. Under the present conditions of measurement, the dissociation constant (K_d) for the fura 2/ Ca^{2+} complex was assumed to be 224 nM [18]. Some experiments were performed in Ca^{2+} -free extracellular medium, which was accomplished by preparing a nominally Ca^{2+} -free measurement buffer (calculated free Ca^{2+} concentration less than 20 nM).

Measurement of Mn^{2+} influx

For the measurement of membrane permeability to Mn^{2+} , fura 2-loaded cells were treated with vehicle or 17 β -oestradiol in an extracellular medium nominally free of Ca^{2+} , and variations in $[\text{Ca}^{2+}]_i$ were monitored from the fluorescence ratio $R_{360/390}$. When $[\text{Ca}^{2+}]_i$ had stabilized, Mn^{2+} influx was initiated (zero time for cation entry) by the addition of 1 mM MnCl_2 to the medium. When the rate of Mn^{2+} influx was measured, fluorescence quenching by Mn^{2+} precluded calibration of the signals to calculate $[\text{Ca}^{2+}]_i$. Mn^{2+} quenching of fura 2 was determined at the Ca^{2+} -insensitive isosbestic point of the dye (360 nm excitation wavelength). After 5 min, maximal quenching (100 %) at each condition was obtained by lysing the cells with 0.1 % (v/v) Triton X-100.

Hormones

Steroids were dissolved in ethanol. The final concentration of the vehicle (less than 0.1 %) was without effect on the experimental parameters measured in the present study.

Statistical evaluation

Data are presented as means $\pm$ S.D. and derived from at least two independently valid assays giving statistically homogeneous results. The significance of the results was evaluated by Student's *t* test, where $P < 0.050$ was considered significant. In addition, for multiple comparisons the Bonferroni test and ANOVA were employed [19].

RESULTS

To investigate the participation of PLC in the rapid stimulation of intestinal Ca^{2+} influx by oestrogen, studies were initially performed by testing whether known PLC inhibitors could block the hormone-induced increase in intestinal $^{45}\text{Ca}^{2+}$ uptake by rat duodenal cells. Neomycin is an aminoglycoside antibiotic that binds polyphosphoinositides, making them unavailable to PLC [20]; U-73122 is an aminosteroid compound that has a selective inhibitory effect on different agonist-induced PLC-mediated biological activities including the hydrolysis of phosphatidylinositol-4,5-bisphosphate (PIP_2), the mobilization of Ca^{2+} and the formation of DAG without affecting cAMP levels or the activities of protein kinase C (PKC) or phospholipase A_2 in several cell types [21–24]. As shown in Figure 1, neomycin (0.5 mM) and compound U-73122 (2 μM) separately suppressed the Ca^{2+} uptake response elicited by a 5 min exposure of enterocytes to 0.1 nM 17 β -oestradiol. The selectivity of U-73122 action was evidenced by the observation that compound U-73343, an inactive analogue of U-73122 [22], did not block the effects of 17 β -oestradiol on duodenal Ca^{2+} uptake (results not shown).

The phosphoinositide messenger system is closely linked to Ca^{2+} homeostasis. The hydrolysis of PIP_2 by PLC results in the

Figure 1 Effects of PLC inhibitors on the rapid increase of rat enterocyte Ca^{2+} uptake induced by 17 β -oestradiol

Rat duodenal cells were preincubated in the absence or presence of PLC inhibitors U-73122 (2 μM) or neomycin (0.5 mM) for 10 min and then exposed for 5 min to 17 β -oestradiol (0.1 nM) (filled bars) or vehicle (ethanol less than 0.1 %, control) (open bars) in the presence of $^{45}\text{CaCl}_2$ (0.2 $\mu\text{Ci/ml}$). $^{45}\text{Ca}^{2+}$ uptake was determined as described in the Materials and methods section. Results are means $\pm$ S.D. for three independent experiments. * $P < 0.001$ ($n = 5$).

Figure 2 Time course of 17 β -oestradiol stimulation of DAG release in rat duodenum

Isolated duodenal mucosa prelabelled with [^3H]arachidonate (0.1 $\mu\text{Ci/ml}$) for 90 min at 37 $^{\circ}\text{C}$ was exposed to either 17 β -oestradiol (0.1 nM) (●) or vehicle (ethanol less than 0.1 %, control) (○) for 30 s to 10 min. DAG was determined as described in the Materials and methods section. Results are means $\pm$ S.D. for four separate experiments. * $P < 0.025$, ** $P < 0.005$ ($n = 4$).

Table 1 Dose profile of the early increase in DAG formation induced by 17 β -oestradiol in rat duodenum

Isolated duodenal mucosa was prelabelled with [^3H]arachidonate (0.1 $\mu\text{Ci/ml}$) for 90 min at 37 $^{\circ}\text{C}$ and then incubated for 45 s in the absence (ethanol less than 0.1 %, control) or presence of the indicated doses of 17 β -oestradiol. DAG was determined as described in the Materials and methods section. Results are means $\pm$ S.D. for four separate experiments, and are expressed as the percentage of the control value at each concentration. * $P < 0.025$, ** $P < 0.005$ ($n = 4$).

[17 β -Oestradiol] (M)	[^3H]DAG (% of control value)
10^{-12}	135*
10^{-11}	154*
10^{-10}	186**
10^{-9}	175*
10^{-8}	176*

generation of two second messengers, IP_3 and DAG, which respectively promote the release of Ca^{2+} from intracellular stores and the activation of PKC. As can be seen in Figure 2, the addition of 17 β -oestradiol (0.1 nM) to duodenal mucosa prelabelled with [^3H]arachidonic acid induced the rapid formation of [^3H]DAG. The hormone effect was biphasic, peaking at 45 s and 5 min (+82 % and +46 % respectively). No significant variations were detected in the baseline trace obtained in response to vehicle alone, which excludes the contribution of shear-induced artifacts to the 17 β -oestradiol-induced changes in DAG. Basal DAG levels oscillated between 4 % and 10 % of the total radioactivity found in labelled lipids, in agreement with resting DAG values frequently observed for cell systems both *in vitro* and *in vivo* [25–27]. The hormone effect was also dose-dependent (Table 1). At levels as low as 1 pM, 17 β -oestradiol was able to increase DAG formation at the early phase (45 s), with a maximal and sustained response obtained at a concentration of 0.1 nM. The same dose–response profile was detected in the delayed (5 min) DAG phase (results not shown).

Figure 3 Effects of PLC inhibitor U-73122 on 17β -oestradiol stimulation of DAG formation in rat duodenum

Isolated duodenal mucosa labelled with [3 H]arachidonate (0.1 μ Ci/ml, 90 min) was incubated in the absence or presence of U-73122 (2 μ M) for 10 min and then exposed for 45 s (A) and 5 min (B) to 17β -oestradiol (0.1 nM) (filled bars) or vehicle (ethanol less than 0.1%, control) (open bars). DAG formation was determined as described in the Materials and methods section. Results are means $\pm$ S.D. for three independent experiments. $^*P < 0.025$ ($n = 4$).

Figure 4 Time course of changes in IP_3 formation induced by 17β -oestradiol in rat duodenum

Rat enterocytes were incubated with 17β -oestradiol (0.1 nM) (●) or vehicle (0.1% ethanol, control) (○) for 15 s to 5 min. IP_3 was quantified by employing a [3 H] IP_3 radioreceptor assay as indicated in the Materials and methods section. Results are means $\pm$ S.D. for two separate experiments. $^*P < 0.001$ ($n = 4$).

To investigate the involvement of phosphoinositide hydrolysis in the biphasic production of DAG induced by 17β -oestradiol in rat duodenum cells, PLC inhibitors were used. Pretreatment of duodenum slices with U-73122 (2 μ M), followed by incubation with 17β -oestradiol (0.1 nM), abolished the hormone-dependent DAG production at 45 s (Figure 3A). However, U-73122 was unable to block diglyceride production at the delayed peak (Figure 3B). The same profile was obtained with 0.5 mM neomycin (results not shown).

To further study the stimulation of PIP_2 breakdown by 17β -oestradiol, the effects of the hormone on IP_3 generation were evaluated by a radioreceptor assay. Figure 4 shows that the oestrogen rapidly (within 15 s) increased IP_3 release, with maximal stimulation achieved at 30 s. As with DAG formation, the addition of vehicle alone did not affect basal IP_3 levels.

Figure 5 Effects of 17β -oestradiol on $[Ca^{2+}]_i$ in rat duodenal cells

Rat enterocytes loaded with fura 2 were exposed to 17β -oestradiol (1 nM, arrow) added directly into the cuvette under continuous stirring. $[Ca^{2+}]_i$ was calculated as indicated in the Materials and methods section. A time trace representative of four independent $[Ca^{2+}]_i$ recordings is shown.

Table 2 Specificity of 17β -oestradiol effects on $[Ca^{2+}]_i$ in enterocytes

Rat duodenal cells loaded with fura 2 were exposed to the hormones or ethanol vehicle (less than 0.1%) at the concentrations indicated. Compounds were added directly into the cuvette, with continuous stirring. $[Ca^{2+}]_i$ was calculated as described in the Materials and methods section. Results correspond to $[Ca^{2+}]_i$ at the sustained phase (3 min) of the response. $^*P < 0.001$ ($n = 5$).

Addition	Concentration (M)	$[Ca^{2+}]_i$ (nM)
Vehicle (control)	—	166 ± 10
17β -Oestradiol	10^{-9}	$284 \pm 12^*$
Progesterone	10^{-9}	168 ± 15
	10^{-6}	146 ± 23
Dihydrotestosterone	10^{-9}	165 ± 23
	10^{-6}	178 ± 18
Diethylstilboestrol	10^{-10}	$195 \pm 15^*$
	10^{-9}	$259 \pm 21^*$

In view of the PLC-mediated fast stimulation of duodenal Ca^{2+} uptake and phosphoinositide hydrolysis induced by oestrogen, we studied the effects of the hormone on $[Ca^{2+}]_i$ in enterocytes. As observed in Figure 5, treatment with 0.1 nM 17β -oestradiol caused an almost immediate rise in $[Ca^{2+}]_i$. The hormonal response pattern of $[Ca^{2+}]_i$ shows an initial fast rise within 30 s, rapidly reaching a peak level that remained sustained for as long as cells were exposed to the stimulus. As shown in Table 2, progesterone and dihydrotestosterone did not induce any increase in $[Ca^{2+}]_i$ even when used at doses three orders of magnitude higher than that of 17β -oestradiol. Only diethylstilboestrol was able to elevate $[Ca^{2+}]_i$ to almost the same extent as that induced by 17β -oestradiol (+56% and +70% respectively at 1 nM). As observed in Table 3, pretreatment of rat enterocytes with 2 μ M U-73122 diminished the 17β -oestradiol-induced $[Ca^{2+}]_i$ response by 86% at 1 min and by 41% at 5 min. Basal $[Ca^{2+}]_i$ levels fluctuated from 30 to 350 nM. A similar broad distribution of resting $[Ca^{2+}]_i$ has been reported for other related and non-related cell systems [28–30]. Regardless of the absolute starting basal value, the magnitude of the 17β -oestradiol-dependent $[Ca^{2+}]_i$ increase was highly reproducible.

Experiments were designed to determine whether the action of oestrogen on enterocyte $[Ca^{2+}]_i$ was due to an influx of Ca^{2+} from extracellular medium and/or to the mobilization of Ca^{2+} from intracellular pools. When rat duodenal cells were incubated in an

Table 3 Partial suppression by PLC inhibitor U-73122 of the 17 β -oestradiol-induced increase of $[Ca^{2+}]_i$ in enterocytes

Rat duodenal cells loaded with Fura-2 were preincubated in the absence or presence of U-73122 (2 μ M) for 5 min and then exposed to 17 β -oestradiol (1 nM) or vehicle for 1 or 5 min. Values within parentheses represent percentage increases with respect to basal $[Ca^{2+}]_i$ levels. $[Ca^{2+}]_i$ was calculated as described in the Materials and methods section. * $P < 0.001$ ($n = 5$).

Time (min)	Addition	$[Ca^{2+}]_i$ (nM)	$\Delta [Ca^{2+}]_i$	Inhibition (%)
1	None (control)	295 $\pm$ 25	—	—
	17 β -Oestradiol	478 $\pm$ 32*	183	—
	U-73122	345 $\pm$ 33	—	—
	17 β -Oestradiol + U-73122	370 $\pm$ 36	25	86*
5	None (control)	295	—	—
	17 β -Oestradiol	503 $\pm$ 45*	208	—
	U-73122	345 $\pm$ 33	—	—
	17 β -Oestradiol + U-73122	468 $\pm$ 43	122	41*

Figure 6 Effects of Ca^{2+} -free medium on 17 β -oestradiol-induced changes in enterocyte $[Ca^{2+}]_i$

Rat duodenal cells loaded with fura 2 were exposed to 17 β -oestradiol (1 nM, left arrow) in a Ca^{2+} -free extracellular medium. The right arrow indicates the readdition of Ca^{2+} (1.5 mM) to the medium. Reagents or vehicle were added directly into the cuvette, with continuous stirring. $[Ca^{2+}]_i$ was calculated as described in the Materials and methods section. A time-trace representative of three independent $[Ca^{2+}]_i$ recordings is shown.

Figure 7 17 β -Oestradiol-induced Mn^{2+} influx into rat duodenal cells

Fura 2-loaded cells were treated with vehicle (ethanol less than 0.1 %, control) or 17 β -oestradiol (1 nM) in a Ca^{2+} -free extracellular medium. When $[Ca^{2+}]_i$ had been stabilized, Mn^{2+} influx was measured as described in the Materials and methods section. Time-traces representative of three independent recordings are shown.

Figure 8 Effect of Ca^{2+} channel blockers on the 17 β -oestradiol-induced $[Ca^{2+}]_i$ rise in duodenal cells

Duodenal cells loaded with fura 2 were exposed to 17 β -oestradiol (1 nM) in a Ca^{2+} -free extracellular medium and variations in $[Ca^{2+}]_i$ were monitored for 2 min. Then lanthanum (10 μ M), Ni^{2+} (2 mM), verapamil (10 μ M), nitrendipine (1 μ M) or vehicle (ethanol less than 0.1 %, control) was added to the cuvette 2 min before the readdition of Ca^{2+} (1.5 mM) to the medium. Reagents or vehicle were added directly into the cuvette, with continuous stirring. $[Ca^{2+}]_i$ was calculated as described in the Materials and methods section. Results are expressed as the amplitude of $[Ca^{2+}]_i$ change after readdition of Ca^{2+} compared with controls (100%). * $P < 0.001$ ($n = 5$).

extracellular medium nominally free of Ca^{2+} , the effects of the hormone on cytosolic Ca^{2+} levels were decreased significantly (by 75 %) but a pronounced increase in $[Ca^{2+}]_i$ was obtained after the readdition of Ca^{2+} to the external solution (Figure 6). The latter change was partly blocked (by 50 %; $P < 0.005$) by 2 mM Ni^{2+} but was completely sensitive to micromolar (10 μ M) doses of lanthanum (Figure 7). The addition of either 1 μ M nitrendipine or 10 μ M verapamil before the readmission of Ca^{2+} to the medium did not affect the magnitude of the hormone-induced cation entry (Figure 7). Using a fura 2 fluorescence-quenching technique, we evaluated the permeability of the 17 β -oestradiol-induced Ca^{2+} influx pathway to Mn^{2+} . As shown in Figure 8, the rate of Mn^{2+} entry was markedly increased by the steroid hormone treatment (2.8-fold with respect to the control).

DISCUSSION

The present work shows that the PLC transmembrane signalling system is also involved in the non-genomic action of oestrogen in intestinal cells. In fact, we observed that the PLC-inhibitors neomycin and compound U-73122 completely suppressed the rapid 17 β -oestradiol-induced $^{45}Ca^{2+}$ uptake, whereas the latter decreased to a great extent the hormone-dependent increase in $[Ca^{2+}]_i$ in rat enterocytes.

Additional evidence of the participation of the PLC pathway in the rapid effects of 17 β -oestradiol in intestinal cells was provided by the demonstration of an early (15–30 s) increase in IP_3 and neomycin/U-73122-sensitive DAG release in response to the hormone. Interestingly, the action of 17 β -oestradiol on the cAMP pathway [8,31] and the PLC cascade is similar to that observed for the secosteroid hormone 1,25(OH) $_2D_3$ acting on isolated rat enterocytes [25,32]. The activation of both messenger systems mediates the rapid stimulation by 1,25(OH) $_2D_3$ of Ca^{2+} influx into enterocytes, a process known as transcaltachia [33]. The contribution of 17 β -oestradiol to this phenomenon by interacting with 1,25(OH) $_2D_3$ -regulated signalling pathways should be investigated.

The delay (5 min) in the DAG peak that was insensitive to both PLC inhibitors and independent of IP_3 production, observed

after hormone treatment, suggests that it probably originated from phospholipids other than PIP_2 , e.g. phosphatidylcholine. In agreement with our interpretation, it has been shown that the early and late DAG phases induced by the secosteroid hormone $1,25(\text{OH})_2\text{D}_3$ in skeletal muscle cells are associated with PLC-mediated PIP_2 breakdown (PIP_2 -PLC) and with phospholipase D-catalysed phosphatidylcholine hydrolysis respectively [34]. However, the participation of phospholipase D in the delayed 17β -oestradiol-induced generation of DAG in enterocytes remains to be established. Lieberherr et al. [9] have reported that in rat osteoblasts, 17β -oestradiol stimulates the simultaneous biphasic production of IP_3 and DAG, which could be abolished by neomycin, indicating that the oestrogen-dependent formation of these second messengers in bone cells is linked only to PIP_2 -PLC breakdown. Taken together, these results suggest that the signalling pathways mediating the non-genomic actions of steroids might reflect the existence of tissue-specific mechanisms accounting for the above apparent discrepancies. Nevertheless this is the first evidence for oestrogen's acting on the production of DAG partly through a metabolic pathway that is independent of PLC-catalysed phosphoinositide cleavage. It remains to be elucidated whether this novel aspect of the non-genomic mechanism of action of 17β -oestradiol in enterocytes is relevant to the hormone's modulation of intestinal cell processes coupled to the sustained activation of PKC.

These investigations also indicate that 17β -oestradiol might act as an endocrine factor participating in the regulation of duodenal intracellular Ca^{2+} homeostasis, as suggested previously by $^{45}\text{Ca}^{2+}$ radiotracer uptake studies [8]. The effect of 17β -oestradiol on rat enterocyte $[\text{Ca}^{2+}]_i$ seems to be specific because only diethylstilboestrol had similar potency, whereas progesterone and dihydrotestosterone were devoid of activity even when used at significantly higher levels. Moreover, 17α -oestradiol, at concentrations 100-fold higher, has been shown to be only 40% as effective as 17β -oestradiol in rapidly stimulating Ca^{2+} influx [8]. It is unknown whether the oestradiol effect is sex-specific, because enterocytes from male rats were not tested. The sex-specificity of non-genomic actions has been shown to depend on the cell type and steroid under study [35–37].

Moreover our work provides information on the Ca^{2+} pools that mediate the regulation of intestinal $[\text{Ca}^{2+}]_i$ by 17β -oestradiol. The concentration of cytoplasmic Ca^{2+} is set by the balance between the passive influx of the cation from both extracellular and intracellular compartments and its active extrusion from the cytosol either to endogenous stores or out of the cell, requiring Ca^{2+} -ATPases. Ca^{2+} entry from the outside occurs through voltage-dependent channels (VDCC), ligand-gated Ca^{2+} channels and/or store-operated Ca^{2+} channels (SOC), whereas the release of Ca^{2+} from internal stores takes place through either IP_3 - or ryanodine-sensitive Ca^{2+} channels [38]. The contribution of extracellular Ca^{2+} influx to the oestrogen-induced increase in enterocyte $[\text{Ca}^{2+}]_i$ was revealed by the significant blockade of the hormone's effects detected in a Ca^{2+} -free medium. Under these conditions, the small increase (+25%) in $[\text{Ca}^{2+}]_i$ observed when cells were treated with 17β -oestradiol might be associated with the mobilization of Ca^{2+} from intracellular pools, an interpretation congruent with the rapid changes in IP_3 production measured in response to oestrogen. The fast elevation in $[\text{Ca}^{2+}]_i$ observed after the readdition of Ca^{2+} to enterocytes that had undergone such 17β -oestradiol-induced intracellular Ca^{2+} mobilization is evidence of Ca^{2+} influx from the outside of cells through a preactivated pathway. We have previously reported the inhibitory effects of verapamil and nitrendipine on 17β -oestradiol-induced $^{45}\text{Ca}^{2+}$ influx in enterocytes, implying thereby the participation of VDCC in the action of the hormone [8]. There are

also pharmacological data involving VDCC in the elevation by 17β -oestradiol of $[\text{Ca}^{2+}]_i$ in osteoblasts [9]. Under the present conditions, the contribution of VDCC to the Ca^{2+} influx observed by the Ca^{2+} readmission protocol can be ruled out because the incorporation of either nitrendipine or verapamil before the readdition of Ca^{2+} to the medium did not affect the magnitude of the hormone-induced cation entry. Moreover this 17β -oestradiol-dependent Ca^{2+} influx was significantly decreased by both lanthanum and nickel, which are known to block SOC influx in many non-excitable cell systems [39,40]. Furthermore 17β -oestradiol augmented the rate of quenching of fura 2 fluorescence by Mn^{2+} , reflecting an increased permeability to this cation. Mn^{2+} ion could be used as a selective tracer for non-VDCC-mediated Ca^{2+} entry because it is not pumped out of the cell and exhibits a very low permeability to voltage-operated Ca^{2+} channels [41,42]. Taken together, our results suggest that the oestrogen-dependent activation of SOC occurs as an alternative Ca^{2+} entry pathway in enterocytes. The notion therefore arises that a store-operated Ca^{2+} entry process might mediate the regulation of cell Ca^{2+} metabolism by 17β -oestradiol, not hitherto reported for any oestrogen target tissue. Although further experimental support is required, this concept might also apply to the non-genomic effects of other steroid hormones. In fact, the modulation of SOC in avian muscle cells by $1,25(\text{OH})_2\text{D}_3$ has recently been reported [43].

According to the present study, two major putative mechanisms could be considered for the role of PLC as a mediator of the regulation of duodenal cell Ca^{2+} levels by 17β -oestradiol: hormone-dependent SOC activation by the IP_3 -mediated Ca^{2+} release from internal stores and, although not specifically addressed here, the activation of PKC by DAG, thus promoting phosphorylation processes related to the regulation of VDCC [44] and/or SOC [38,45]. We have recently reported that the adenylate cyclase–cAMP–protein kinase A (PKA) cascade mediates the fast stimulation of Ca^{2+} influx by 17β -oestradiol in isolated rat duodenal cells [8]. It is then possible that the oestrogen-dependent stimulation of both PKC and PKA might contribute to the modulation of Ca^{2+} influx into the enterocyte. A comparison of the time courses of 17β -oestradiol effects on PLC (Figures 2 and 4) and adenylate cyclase [8] indicates that stimulation of the former is earlier than cAMP generation. Thus it is possible that cross-talk between the PLC–DAG–PKC and adenylate cyclase–cAMP–PKA pathways occurs as part of the non-genomic mechanism of action of the steroid. Interactions between DAG- and cAMP-generating systems have been observed for several agonists in a wide variety of cells [46].

The rapid and specific nature by which 17β -oestradiol activates the Ca^{2+} messenger system through the PLC signalling cascade in intestinal cells is compatible with the existence of a cell-surface resident receptor acting as the locus of these membrane-linked events. The anti-oestrogenic compounds tamoxifen and ICI 182,780 did not abolish the fast effects of 17β -oestradiol on Ca^{2+} influx (results not shown), suggesting that they are independent of the classical nuclear receptor. Experimental evidence supporting this concept has been obtained for other oestradiol target cells [47–49]. However, no membrane-associated oestrogen receptor has yet been chemically characterized in detail or cloned. Alternatively, 17β -oestradiol might stimulate phosphoinositide turnover and Ca^{2+} homeostasis via a classical cell-surface receptor, e.g. in a manner analogous to the mechanism of progesterone modulation of oxytocin receptor responses [50].

In summary, the present results suggest that 17β -oestradiol is an effective modulator of $[\text{Ca}^{2+}]_i$ in rat duodenal cells through a rapid, non-genomic mechanism that affects both extracellular Ca^{2+} influx and Ca^{2+} mobilization from intracellular stores. As

regards the physiological significance of this study, because it was performed with non-polarized cells it is not possible to distinguish between apical and basolateral Ca²⁺ transport. However, there is evidence that voltage-dependent L-type Ca²⁺ channels are located at the basolateral membranes of mammalian duodenal cells [51]. In addition, according to the currently accepted mechanism of transcaltachia [52], Ca²⁺ influx through duodenal basolateral membranes triggers Ca²⁺ transfer to the circulation by exocytosis. Again, it remains to be established to what extent the present actions of 17 β -oestradiol are related to 1,25(OH)₂D₃-dependent transcaltachia. Interestingly, a direct role of oestrogen in the regulation of intestinal Ca²⁺ absorption has been suggested by clinical and experimental evidence [3].

Experimental activities were supported by grants from the Consejo Nacional de Investigaciones Científicas y Técnicas (CONICET), Agencia Nacional de Promoción Científica y Tecnológica and the Comisión de Investigaciones Científicas de la Provincia de Buenos Aires (CIC), Argentina.

REFERENCES

- Nordin, B. E. C. (1988) Calcium in Human Biology, Springer-Verlag, Berlin
- Lindsay, R. (1991) *Am. J. Med.* **91** [Suppl. 5B], 10S–13S
- Heaney, R. P., Recker, R. R., Stezman, M. R. and Moy, A. J. (1989) *J. Bone Mineral Res.* **4**, 469–475
- Arjmandi, B. H., Salih, M. A., Damon, H. C., Sims, S. H. and Kalu, D. N. (1993) *Bone Mineral* **21**, 63–74
- Salih, M. A., Sims, S. H. and Kalu, D. N. (1996) *Mol. Cell. Endocrinol.* **121**, 47–55
- Aronica, S. M., Kraus, W. L. and Katzenellenbogen, B. S. (1994) *Proc. Natl. Acad. Sci. U.S.A.* **91**, 8517–8521
- Sanchez-Bueno, A., Sancho, M. J. and Cobbald, P. H. (1991) *Biochem. J.* **280**, 273–276
- Picotto, G., Massheimer, V. and Boland, R. (1996) *Mol. Cell. Endocrinol.* **119**, 129–134
- Lieberherr, M., Grosse, B., Kachkache, M. and Balsan, S. (1993) *J. Bone Mineral Res.* **11**, 1365–1376
- Walters, J. and Weiser, M. (1987) *Am. J. Physiol.* **252**, G170–G177
- Weiser, M. (1973) *J. Biol. Chem.* **218**, 2536–2541
- Lowry, O. H., Rosebrough, N. J., Farr, A. L. and Randall, R. J. (1951) *J. Biol. Chem.* **193**, 265–275
- Folch, J., Lees, M. and Sloane-Stanley, G. H. (1957) *J. Biol. Chem.* **226**, 497–509
- Mattheus, E. K. (1986) *Br. Med. Bull.* **42**, 391–397
- Korte, K. and Casey, M. L. (1982) *J. Chromatogr.* **232**, 47–53
- Challiss, R. A. J., Batty, I. H. and Nahorski, S. R. (1988) *Biochem. Biophys. Res. Commun.* **157**, 684–689
- Bredt, D. S., Mourey, R. J. and Snyder, S. H. (1989) *Biochem. Biophys. Res. Commun.* **159**, 976–981
- Gryniewicz, G., Poenie, M. and Tsien, R. Y. (1985) *J. Biol. Chem.* **260**, 3440–3450
- Snedecor, G. W. and Cochran, W. G. (1967) *Statistical Methods*, pp. 120–134, Iowa State University Press, Ames
- Prentki, M., Deeney, J. T., Matschinsky, F. M. and Joseph, S. K. (1986) *FEBS Lett.* **197**, 285–288
- Smallridge, R. C., Kiang, J. G., Gist, I. D., Fein, H. G. and Galoway, R. J. (1992) *Endocrinology (Baltimore)* **131**, 1883–1888
- Smith, R. J., Sam, L. M., Justen, J. M., Bundy, G. L., Bala, G. A. and Bleasdale, J. E. (1990) *J. Pharmacol. Exp. Ther.* **253**, 688–697
- Yule, D. I. and Williams, J. A. (1992) *J. Biol. Chem.* **267**, 13830–13835
- Bleasdale, J. E., Thakur, N. R., Gremban, R. S., Bundy, G. L., Fitzpatrick, F. A., Smith, R. J. and Bunting, S. (1990) *J. Pharmacol. Exp. Ther.* **255**, 756–768
- De Boland, A. R., Facchinetti, M. M., Balogh, G., Massheimer, V. and Boland, R. L. (1996) *Cell. Signal.* **8**, 153–157
- Facchinetti, M. M., Boland, R. L. and de Boland, A. R. (1998) *Mol. Cell. Endocrinol.* **136**, 131–138
- Facchinetti, M. M., Boland, R. L. and de Boland, A. R. (1998) *J. Lipid Res.* **39**, 197–204
- Brenner, B. M., Russell, S., Albrecht, R. J. and Davies, R. J. (1998) *Cancer Lett.* **127**, 43–53
- Klin, M., Smogorzewski, M., Khilnani, H., Michnowska, M. and Massry, S. (1994) *Am. J. Physiol.* **267**, G754–G763
- Benten, W., Lieberherr, M., Giese, G. and Wunderlich, F. (1998) *FEBS Lett.* **422**, 349–353
- Massheimer, V., Picotto, G., de Boland, A. R. and Boland, R. L. (1995) *Endocrinol. Metab.* **2**, 157–163
- Lieberherr, M., Grosse, B., Duchambon, P. and Drücke, T. (1989) *J. Biol. Chem.* **264**, 20403–20406
- De Boland, A. R. and Norman, A. W. (1990) *Endocrinology (Baltimore)* **127**, 39–45
- De Boland, A. R., Morelli, S. and Boland, R. (1994) *J. Biol. Chem.* **269**, 8675–8679
- Lieberherr, M. and Grosse, B. (1994) *J. Biol. Chem.* **269**, 7217–7223
- Benten, P., Lieberherr, M., Sekeris, C. and Wunderlich, F. (1997) *FEBS Lett.* **407**, 211–214
- Benten, P., Lieberherr, M., Giese, G. and Wunderlich, F. (1998) *FEBS Lett.* **422**, 349–353
- Birnbaumer, L., Zhu, X., Jiang, M., Boulay, G., Peyton, M., Vannier, B., Brown, D., Platano, D., Sadeghi, H., Stefani, E. and Birnbaumer, M. (1996) *Proc. Natl. Acad. Sci. U.S.A.* **93**, 15195–15202
- Pandolf, S. J., Schoeffield, M. S., Fimmel, C. J. and Muallem, S. (1987) *J. Biol. Chem.* **262**, 16963–16968
- Kwan, C. Y., Takemura, H., Obie, J. F., Thasturp, O. and Putney, Jr., J. W. (1990) *Am. J. Physiol.* **258**, C1006–C1015
- Suh, B. C., Song, S. K., Kim, Y. K. and Kim, K. T. (1996) *J. Biol. Chem.* **271**, 32753–32759
- Densmore, J. J., Haverstick, D. M., Szabo, G. and Gray, L. S. (1996) *Am. J. Physiol.* **271**, C1494–C1503
- Vazquez, G., de Boland, A. R. and Boland, R. (1997) *Biochem. Biophys. Res. Commun.* **239**, 562–565
- McDonald, T. F., Pelzer, S., Trautwein, W. and Pelzer, D. J. (1994) *Physiol. Rev.* **74**, 365–507
- Putney, J. W. and Bird, G. S. (1994) *Endocr. Rev.* **14**, 610–631
- Houslay, M. D. (1991) *Eur. J. Biochem.* **195**, 9–27
- Audy, M. C. and Dufy, P. V. (1996) *Eur. J. Endocrinol.* **135**, 367–373
- Pappas, T., Gametchu, B. and Watson, C. (1995) *FASEB J.* **9**, 404–410
- Watson, C., Pappas, T. and Gametchu, B. (1995) *Environ. Health Perspect.* **103**, 41–50
- Grazzini, G. E., Guillon, G., Mouillac, B. and Zingg, H. H. (1998) *Nature (London)* **392**, 509–512
- Homoidian, F. R., Donowitz, M., Weiland, G. A. and Sharp, G. W. (1989) *Am. J. Physiol.* **257**, G86–G93
- Nemere, I. and Norman, A. W. (1991) *Mol. Cell. Endocrinol.* **80**, C165–C169

Received 6 July 1998/30 November 1998; accepted 6 January 1999