Estradiol binding to cell surface raises cytosolic free calcium in T cells

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Abstract The Fura-2 method is used to examine a possible action of 17β-estradiol (E_2) on $[Ca^{2+}]_i$ of splenic T cells isolated from female C57BL/10 mice. E_2 concentrations between 10 fM and 10 nM induce a rapid and dose-dependent increase in $[Ca^{2+}]_i$ due to Ca^{2+} influx and release of Ca^{2+} from intracellular stores. Ca^{2+} influx is mediated by Ca^{2+} channels which are completely blockable by Ni^{2+} and partly by nifedipine. The antiestrogen tamoxifen does not inhibit the E_2-induced rise in $[Ca^{2+}]_i$. Ca^{2+} influx and Ca^{2+} release from intracellular stores is also inducible by plasma membrane impermeable E_2 conjugated to BSA. E_2-BSA-FITC binds to the surface of T cells of both the $CD4^+$ and $CD8^+$ subset. Our data suggest a novel E_2-signalling pathway in T cells which is not mediated through the classical nuclear estrogen receptor response but rather through putative plasma membrane receptors for E_2.

Key words: Estradiol; Ca^{2+}; Estrogen receptor; Membrane receptor; T cell

1. Introduction

Estrogens, like other steroids, exert their major long-term effects on cell growth, differentiation, and function through nuclear estrogen receptors (ER), which belong to the steroid receptor superfamily, by activating specific estrogen-responsive genes [1–6]. The ER contains several domains for estrogen binding, nuclear localization, dimerization, DNA binding, and transactivation [7]. However, there is now also increasing evidence for a non-genomic action of steroid hormones including estrogens which is not mediated through the classical nuclear receptor response (for reviews, see e.g. [8,9]). For instance, 17β-estradiol (E_2) can induce a fast rise in the cytosolic free Ca^{2+} concentrations $[Ca^{2+}]_i$ of different cell types. This rise in $[Ca^{2+}]_i$ may be due to an influx of extracellular Ca^{2+} as in uterine endometrial cells [10], in rat osteoblasts [11], in LNCaP human prostate cancer cells [12], in intestinal mucosal cells [13], and/or due to a mobilization of Ca^{2+} from intracellular Ca^{2+} stores as in chicken granulosa cells [14] and rat osteoblasts [11]. The intracellular Ca^{2+} store involved in this mechanism is the endoplasmic reticulum since E_2 enhances the formation of inositol 1,4,5-trisphosphate via the activation of a phospholipase Cβ2 linked to a pertussis-sensitive G-protein [11,15].

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Abbreviations: $[Ca^{2+}]_i$, intracellular free Ca^{2+} concentration; E_2, 17β-estradiol; BSA, bovine serum albumin; EGTA, ethylene glycol-bis-(2-aminoethyl ether)-*N*,*N*,*N*′,*N*′,-tetraacetic acid; FITC, fluorescein isothiocyanate; ConA, concanavalin A; PE, phycoerythrin

T cells play a central role in the regulation of immune responses and thus the outcome of infectious diseases [16,17]. There is some circumstantial evidence that E_2 is able to affect T cells [16–19]. However, the action of E_2 on T cells is not yet understood. In particular, there is a controversial debate on whether or not T cells contain ER [17]. The current evidence indicates that only T cells of the $CD8^+$ subset contain classical ER whereas $CD4^+$ T cells have no ER [20]. Here, we reveal that T cells of both the $CD4^+$ subset and the $CD8^+$ subset possess E_2 binding sites on their cell surfaces and that binding of E_2 induces both Ca^{2+} influx through Ca^{2+} channels and release of Ca^{2+} from the endoplasmic reticulum.

2. Materials and methods

2.1. Isolation of T cells

Female mice of the inbred strain C57BL/10 were obtained from our animal facilities. Spleens were aseptically removed and total nucleated spleen cells were isolated as detailed previously [21]. T cells were then prepared using the nylon-wool procedure [22]. These contained about 90–95% $Thy1^+$ T cells with about 55–60% $CD4^+$ T cells and about 35–40% $CD8^+$ T cells as routinely examined by FACScan analysis.

2.2. Determination of $[Ca^{2+}]_i$

$[Ca^{2+}]_i$ was measured in T cell suspension as detailed previously [19]. In brief, 10^7 T cells/ml were loaded with 3 μM Fura-2/acetoxymethylester (Amersham, Les Ulis, France) at 37°C in HEPES buffer [19]. Aliquots of the loaded cells (5×10^6/ml) were measured at 37°C in a quartz cuvette placed in a Hitachi F-2000 spectrofluorometer after adding reagents under continuous stirring. E_2 (17β-estradiol), E_2-BSA (17β-estradiol 6-(*O*-carboxymethyl)oxime:BSA), nifedipine and tamoxifen were purchased from Sigma (St. Quentin, Fallavier, France). Cell suspensions were excited alternatively at 340 nm and 380 nm and the fluorescence was measured at 510 nm. Graphic representations of $[Ca^{2+}]_i$ were computed using the equation $[Ca^{2+}]_i = 224\times(R-R_{min})/(R_{max}-R)\times Sf380/Sb380$ as previously described [23]. R_{min} and R_{max} were evaluated from measurements using 25 μM digitonin, 4 mM EGTA and Tris base to raise the pH to or above 8.3 [24].

2.3. Confocal laser scanning microscopy (CLSM)

Intact T cells were diluted to 10^7 cells/ml in PBS^+ (140 mM NaCl, 2.7 mM KCl, 6.4 mM $Na_2HPO_4{\cdot}2H_2O$, 1.4 mM KH_2PO_4, 0.5 mM $MgCl_2{\cdot}6H_2O$, 0.9 mM $CaCl_2$, pH 7.2). Aliquots of 150 μl were centrifuged and the cell pellets labelled with 200 μl of E_2-BSA-FITC (17β-estradiol 6-(*O*-carboxymethyl)oxime:BSA-fluorescein isothiocyanate conjugate) (1.5×10^{-5} M; Sigma, Deisenhofen, Germany) for 1 h at room temperature. BSA-FITC was used in corresponding control experiments. For colocalization analysis, the plasma membrane was stained for 15 min with ConA-rhodamine (Vector, Burlingame, CA, USA) at a working dilution of 1:50. After two washes, the cells were fixed in 0.5% paraformaldehyde and allowed to adhere onto polylysine-coated glass coverslips for 15 min, which were then rinsed with PBS^+. Samples were mounted on microscope slides in a 1:1 (v/v) mixture of glycerol and Vectashield (Vector) containing 2% (w/v) 1,4-diazabicyclo[2,2,2]octane (DABCO, Merck, Darmstadt, Germany). The specimens were analyzed with a Leica confocal laser scanning microscope unit (Leica Lasertechnik, Heidelberg, Germany) mounted on a Zeiss IM 35 microscope (Zeiss, Oberkochen, Germany).

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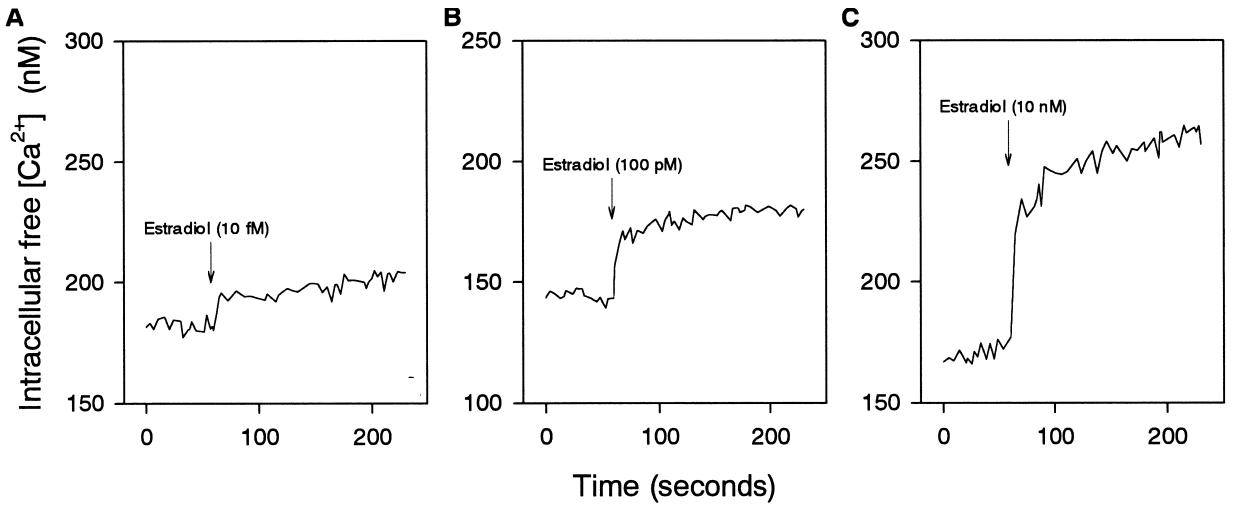

Fig. 1. Action of E_2 on $[Ca^{2+}]_i$ of T cells. Arrows indicate addition of different E_2 concentrations to T cell suspensions.

FITC and rhodamine fluorescence were excited by the 488 nm argon laser line and the 568 nm krypton laser line, respectively. Z-series optical sections were taken at 0.5 μm intervals and evaluated using AVS software (Advanced Visual Systems Inc., Waltham, MA, USA) on an Indigo 2 Unix workstation (Silicon Graphics Inc., Mountain View, CA, USA) as described elsewhere [25].

2.4. Flow cytometry

T cells were labelled with E_2-BSA-FITC or BSA-FITC as above and with monoclonal antibody to mouse Ly-2 conjugated with phycoerythrin (CD8-PE) (Boehringer, Mannheim, Germany) and anti-mouse L3T4 PE (CD4-PE) (Becton Dickinson, Heidelberg, Germany) as described previously [21]. Cells were analyzed in a FACScan (Becton Dickinson, Sunnyvale, CA, USA) with a sample size of 10 000 cells gated on the basis of forward and side scatter, and the data were stored and processed using the FACScan software as described previously [21].

3. Results

E_2 induced a rapid increase of $[Ca^{2+}]_i$ in T cells which always occurred as a prolonged elevation. These E_2 effects were dose-dependent: concentrations as low as 10 fM E_2 induced an increase by about 15–20 nM Ca^{2+} (Fig. 1A), 0.1 nM E_2 raised $[Ca^{2+}]_i$ by about 30–35 nM (Fig. 1B) and 10 nM E_2 by about 80–90 nM Ca^{2+} (Fig. 1C). The E_2-induced increase in $[Ca^{2+}]_i$ resulted from two mechanisms: the release of Ca^{2+} from the endoplasmic reticulum and the influx of extracellular Ca^{2+} through the plasma membrane [19]. The Ca^{2+} influx is specific, i.e. it is not a simple diffusion, but rather it is channel-mediated. Indeed, the E_2-induced Ca^{2+} increase can be gradually decreased and almost completely inhibited with increasing doses of the specific Ca^{2+} channel blocker Ni^{2+} (Fig. 2A). Moreover, nifedipine, a blocker of L-type voltage-gated Ca^{2+} channels, reduced the E_2-induced Ca^{2+} influx by about 50% (Fig. 2B). In contrast, depolarization of T cells with high K^+ concentrations did not affect the E_2-induced Ca^{2+} influx at all (Fig. 2C).

Preincubation of T cells for 2 h with 0.5 μM of the anti-estrogen tamoxifen did not prevent the increase in $[Ca^{2+}]_i$ induced by only 0.1 nM E_2 (Fig. 3A). Moreover, a rise in $[Ca^{2+}]_i$ could be also induced by E_2 conjugated to bovine serum albumin (E_2-BSA), which did not enter the cells (Fig. 3B). Subsequent addition of the same concentration of 100 nM E_2-BSA to the same cell suspension resulted in a higher,

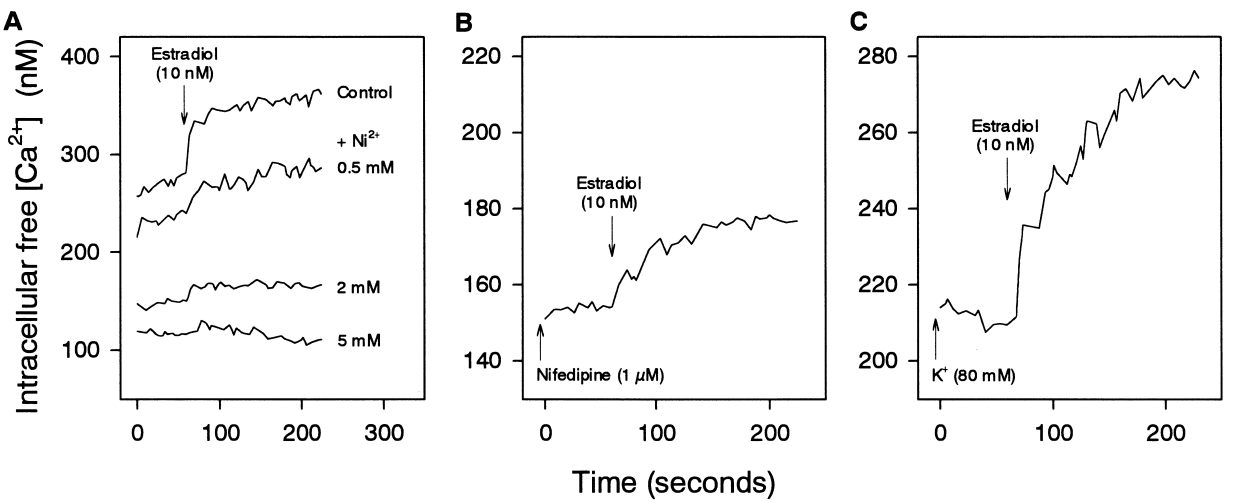

Fig. 2. Effects of different substances on cytosolic free calcium concentration in T cells. A: T cells were incubated for 5 min with various concentrations of Ni^{2+}, before adding E_2. B: T cells were incubated for 1.5 min with nifedipine, before addition of E_2. C: T cells were pretreated for 1 min with KCl before adding E_2.

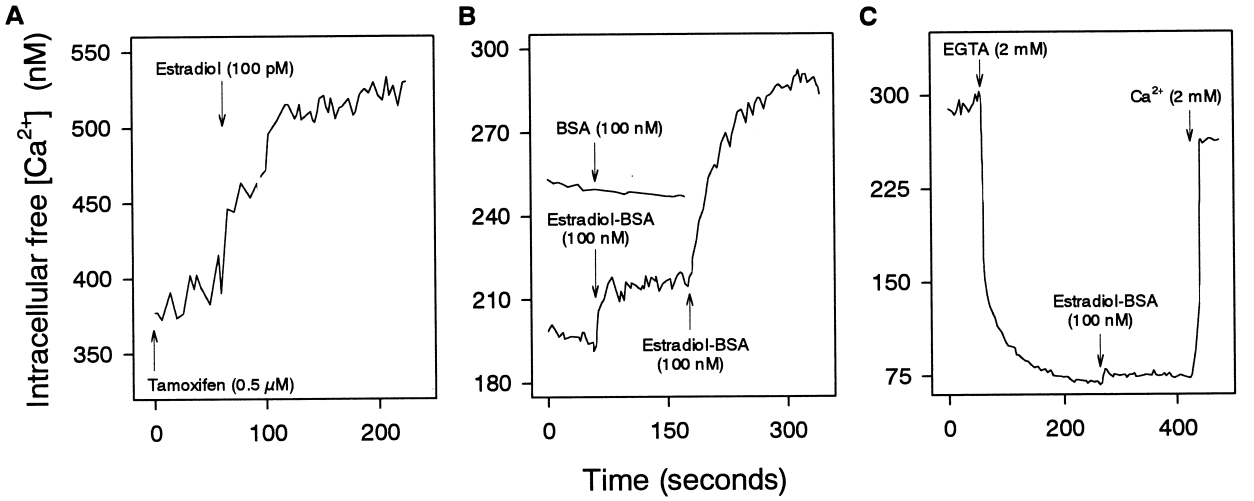

Fig. 3. Action of E_2 on $[Ca^{2+}]_i$ via non-genomic surface receptors. A: T cells were pretreated with tamoxifen for 4 h before adding E_2. B: Effects of E_2 conjugated to BSA (estradiol-BSA), and BSA alone on T cells. C: After removal of external Ca^{2+} by EGTA, estradiol-BSA induces intracellular Ca^{2+} release in T cells.

i.e. approximately 2–3-fold increase in $[Ca^{2+}]_i$. In contrast, BSA without conjugated E_2 had no effect (Fig. 3B). The impermeable E_2-BSA like unconjugated E_2 [19] increased the $[Ca^{2+}]_i$ through Ca^{2+} influx and release from intracellular Ca^{2+} stores. Indeed, when extracellular Ca^{2+} was completely removed by EGTA, E_2-BSA was still able to induce a small increase in $[Ca^{2+}]_i$ exclusively due to mobilization of intracellular Ca^{2+} (Fig. 3C).

To test the occurrence of putative receptors on the cell surface, we have used the impeded ligand E_2-BSA labelled with FITC in confocal laser scanning microscopy. E_2-BSA-FITC was localized only at the cell surface of T cells (Fig. 4). In control experiments, BSA-FITC did not result in any fluorescence at the cell surface. When the cells were stained with ConA-rhodamine, a general marker for the plasma membrane, the fluorescence pattern was identical to that observed with E_2-BSA-FITC. FACScan analysis showed that binding of E_2-BSA-FITC occurred on both $CD4^+$ T cells and $CD8^+$ T cells (Fig. 5).

The E_2-induced rise in $[Ca^{2+}]_i$ obviously had consequences for the responsiveness of T cells to antigens. Stimulation of T cells by the monoclonal anti-CD3 antibody, which binds to the 25 kDa ε chain of the T cell receptor-associated CD3 complex, caused an immediate rise of $[Ca^{2+}]_i$ in T cells (Fig. 6A). The profile of the $[Ca^{2+}]_i$ response to anti-CD3 antibody was modified when T cells were preincubated with E_2 (Fig.

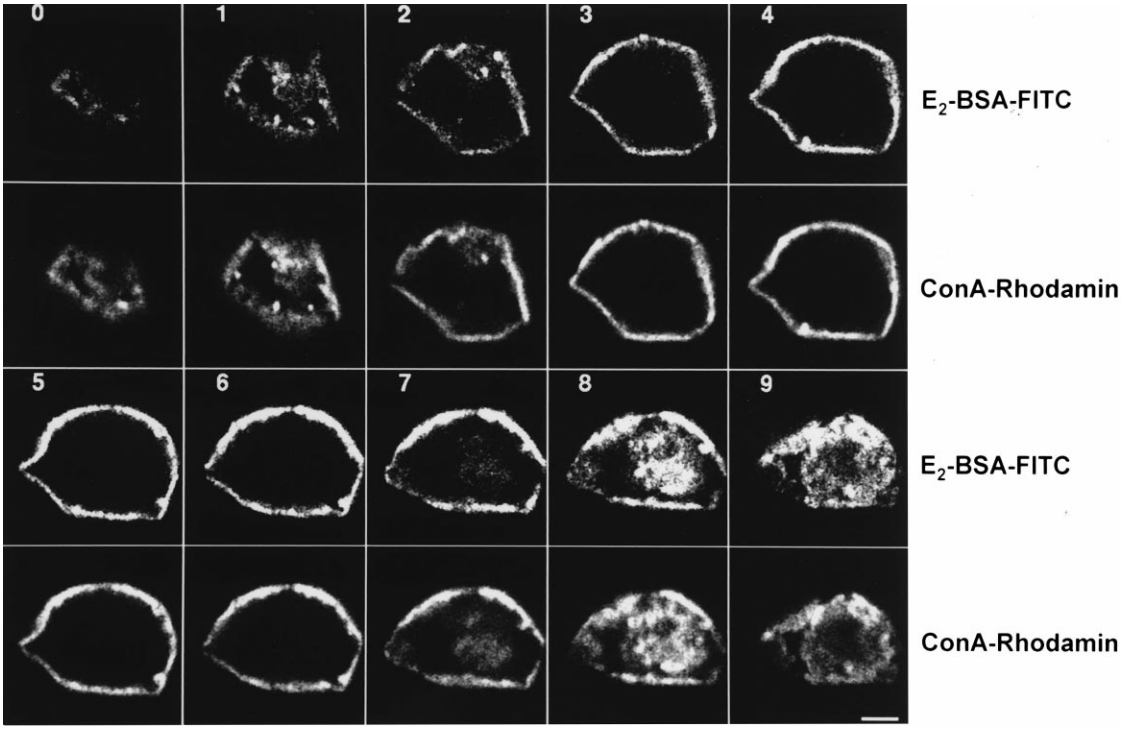

Fig. 4. Corresponding confocal optical slices of a T cell incubated with E_2-BSA-FITC and ConA-rhodamine. Note the obvious colocalization of the two different fluorescences. Bar indicates 2 μm.

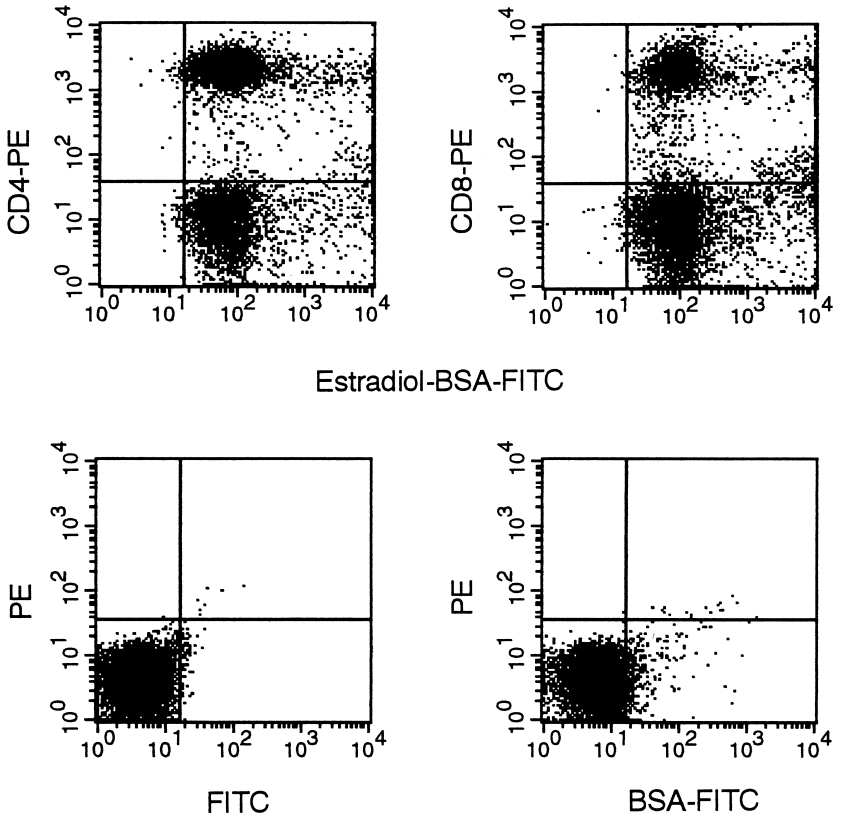

Fig. 5. Localization of estradiol-BSA-FITC on $CD4^+$ T cells and $CD8^+$ T cells by flow cytometry. The cells were simultaneouly labelled with estradiol-BSA-FITC and CD4-phycoerythrin (PE) or CD8-PE (upper figures). The lower figures show controls: on the left, red and green fluorescence of T cells incubated only with BSA; on the right, T cells incubated with BSA-FITC.

6B). Specifically, preincubation with 0.1 nM E_2 for 180 s delayed and diminished the slope of the initial $[Ca^{2+}]_i$ response to anti-CD3 antibody without apparently altering the sustained $[Ca^{2+}]_i$ plateau phase (Fig. 6A,B).

4. Discussion

This study provides evidence that E_2 exerts a direct and immediate effect on T cells. This action does not follow the classical nuclear ER response but rather is mediated through binding of E_2 to the cell surface. Indeed, the effect of E_2 manifests itself as an increase in $[Ca^{2+}]_i$ within seconds. Our data further show that this increase is due to both a specific influx of external Ca^{2+} through Ca^{2+} channels completely blockable by Ni^{2+}, and in part by nifedipine, and a release of Ca^{2+} from intracellular stores. Internal mobilization of Ca^{2+} appears to be a general mechanism used by E_2 [11,14], while the type of Ca^{2+} channels involved in the influx of

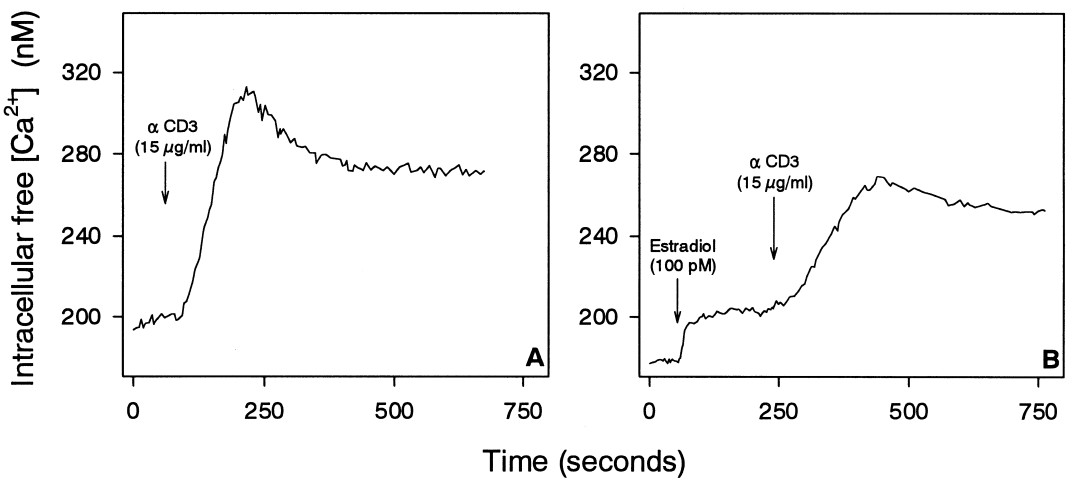

Fig. 6. Effects of E_2 and anti-CD3 on $[Ca^{2+}]_i$ of T cells. A: Increase in $[Ca^{2+}]_i$ by anti-CD3. B: Effect of E_2 on anti-CD3-induced increase in $[Ca^{2+}]_i$.

extracellular Ca^{2+} appears to depend on the cell type: Ni^{2+}-sensitive Ca^{2+} channels in T cells and voltage-gated Ca^{2+} channels as in osteoblasts [11].

An increase in $[Ca^{2+}]_i$ is also induced by E_2-BSA which is impermeable to the plasma membrane, but acts by the same mechanisms as E_2. This indicates that the plasma membrane of T cells contains putative receptors for E_2. This view is further substantiated by confocal laser scanning microscopy showing a fluorescent staining at the cell surface with the impeded ligand E_2-BSA-FITC. This corroborates recent data obtained in GH3/B6 rat pituitary cells with the same fluorescent estrogen-BSA conjugate [26]. In addition, our FACScan analysis reveals E_2 binding sites on the surface of both $CD4^+$ T cells and $CD8^+$ T cells. Although only T cells of the $CD8^+$ subset contain classical ER [17,20], both T cell subsets exhibit putative plasma membrane receptors for E_2. Moreover, the antiestrogen tamoxifen, which antagonizes estrogen action by inhibiting estradiol-induced activation of genes containing estrogen-responsive elements in their promoters to various extents [27,28], does not prevent the E_2-induced increase in $[Ca^{2+}]_i$ in T cells as in other cells [11,14]. Collectively, our data suggest that the putative plasma membrane receptors for E_2 on T cells are not the classical genomic ERs.

T cells also possess unconventional androgen receptors [19]. Binding of testosterone to these receptors increases $[Ca^{2+}]_i$ only by Ca^{2+} influx through Ca^{2+} channels. The present data indicate that the putative membrane receptors for E_2 are not identical to those of testosterone since only E_2 is able to mobilize Ca^{2+} from the endoplasmic reticulum. Moreover, impermeable E_2-BSA also mobilizes intracellular Ca^{2+} from endoplasmic reticulum. This suggests that the putative plasma membrane receptors for E_2 on T cells are coupled to an intracellular signalling pathway, which remains to be elucidated. In this context, it is noteworthy that the multiple second messenger Ca^{2+} is known to change gene expression via both Ca^{2+}-responsive elements and negative Ca^{2+}-responsive elements in gene promoters as well as via Ca^{2+}-sensitive transcription factors [29].

Finally, our data indicate that binding of E_2 to the surface interferes with the responsiveness of T cells to antigenic stimulation with anti-CD3. It remains to be seen in how far this contributes to E_2-sensitive immune responses and outcome of infectious diseases [15–17].

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