Molecular Pharmacology Fast Forward. Published on December 12, 2006 as doi: 10.1124/mol.106.028290
This article has not been copyedited and formatted. The final version may differ from this version.

The antiestrogen tamoxifen activates BK channels and stimulates proliferation of MCF-7 breast cancer cells

Guyllaume Coiret, Anne-Sophie Borowiec, Pascal Mariot, Halima Ouadid-Ahidouch and Fabrice Matifat

Laboratoire de Physiologie Cellulaire et Moléculaire: G.C., AS. B., H.OA, F.M.

EA 2086

Université de Picardie Jules Verne

Faculté des Sciences

33 rue Saint-Leu

80039 Amiens - France

Laboratoire de Physiologie Cellulaire: P.M.

INSERM U800

Université des Sciences et Technologies de Lille

59655, Villeneuve d'Ascq Cédex, France

Molecular Pharmacology Fast Forward. Published on December 12, 2006 as DOI: 10.1124/mol.106.028290
This article has not been copyedited and formatted. The final version may differ from this version.

Running title: Tamoxifen stimulates BK channels and proliferation of MCF-7 cells

Corresponding author:

Fabrice Matifat

Laboratoire de Physiologie Cellulaire et Moléculaire, EA 2086

Université Picardie Jules Verne, Faculté des Sciences

33, Rue St Leu 8000 Amiens, France

Fax: (33)322827644

E-mail: fabrice.matifat@u-picardie.fr

Number of:

- text pages: 29
- tables: 0
- figures: 7
- references: 27 < 40
- of words in the:
  - abstract: 223 < 250
  - introduction: 544 < 750
  - discussion: 1498 <1500

Abbreviations :

E_2: 17 β-estradiol; FCS: Fetal Calf Serum; DCCFCS: Dextran-Coated Charcoal-Treated FCS; IbTx: Iberiotoxin; ChTx: Charybdotoxin; ER: Estrogen Receptor; HRT: Hormone Replacement Therapy; E_2BSA: β-Estradiol 6-(O-carboxymethyl)oxime: BSA; SMC: smooth muscle cells.

Molecular Pharmacology Fast Forward. Published on December 12, 2006 as DOI: 10.1124/mol.106.028290
This article has not been copyedited and formatted. The final version may differ from this version.

Abstract

In the present study, we have investigated the effect of the antiestrogen compound tamoxifen on BK channels by the use of the patch-clamp technique. The perfusion of 10 nM tamoxifen significantly increased the magnitude of a voltage-dependent K^+ current by 22.6 ± 10.6 % (n=23). The effect of tamoxifen was always obtained in the first minute, peaked at 5.9 ± 2.2 min (n=23) and was abolished by the perfusion of TEA (0.5 mM), Charybdotoxin (50 nM) or Iberiotoxin (100 nM). The stimulatory effect of 10 nM tamoxifen was the same at low (50 nM) and high (700 nM) internal calcium concentration and was not additive to that of E_2 or its membrane impermeant form E_2BSA. Furthermore, the effect of tamoxifen was still recorded in the presence of the selective estrogen receptor (ER) antagonist ICI-182,780 (1 μM). At the single channel level, tamoxifen significantly increased NPo of the BK channel by 46.2 ± 10.1 % (n=4) without changing its unitary conductance. Moreover, we show here that the stimulation of BK channel activity by tamoxifen is involved in MCF-7 cell proliferation. Taken together, these results permitted us to identify the BK channel as the molecular target of tamoxifen that probably acts at the same extracellular molecular level as E_2. The site of action of tamoxifen is probably the channel itself or the auxiliary β subunits.

Molecular Pharmacology Fast Forward. Published on December 12, 2006 as DOI: 10.1124/mol.106.028290
This article has not been copyedited and formatted. The final version may differ from this version.

Breast cancer is generally believed to arise when dividing cells undergo mutations and these genetically damaged cells become susceptible to unrestrained division. Thus, female hormones and other hormones that affect growth of the mammary gland are potential risk factors for breast cancer. In contrast, factors that induce differentiation in the mammary gland, such as pregnancy and lactation, are likely to reduce the risk of breast cancer. In the case of breast cancer, estrogens are still one of the most important risk factors. Indeed, whilst 17 β-estradiol (E_2) is a key growth regulator in the normal mammary gland, clinical and experimental data have clearly established that exposure to this steroid hormone is also the leading cause of sporadic female breast cancer. Thus, the utility of antiestrogens and/or chemo preventives is closely associated with antagonizing the activity of E_2. The fact that estrogens may affect carcinogenesis by acting either as initiators (i.e. directly damage DNA, Liehr and Ricci, 1996) or as promoters (i.e. promoting the growth and/or survival of initiated cells, ESHRE, 2004; Veronesi et al., 2005) has justified the use of antiestrogenic compounds such as tamoxifen in estrogenic Hormone Replacement Therapy (HRT). However, in addition to their key role in female reproductive functions, estrogens have beneficial effects on unrelated tissues, as demonstrated by the effects of HRT on postmenopausal women (Mitlak and Cohen, 1997; Cosman and Lindsay, 1999). Indeed, estrogens can prevent osteoporosis by inhibiting bone resorption and partially reduce the incidence of coronary heart disease through effects on the hepatic lipid metabolism and on vascular smooth muscle cells (SMC). The use of tamoxifen relies on its ability to act as an estrogen receptor (ER) antagonist in the breast, where it prevents the carcinogenic effect of E_2 and as partial agonist of the ER in the bone, where it mimics the beneficial effect of E_2 on the maintenance of bone density (Zidan al., 2004).

Numbers of recent works (for reviews see Clarke et al., 2001; Lösel et al., 2003) show that estrogens and xenoestrogens, like tamoxifen, have many cellular effects that are not

mediated by the stimulation of ER. Among all the various alternative effects of tamoxifen, the modulation of numerous ion channels (Sahebgharani et al., 2001, Chesnoy-Marchais, 2005), including calcium-activated BK channels (Dick et al., 2001; Dick and Sanders, 2001) have been reported.

The aim of the present study was to investigate the acute effect of tamoxifen on K^+ channels in MCF-7 cells and the consequence of the potential modulation of these channels on cell proliferation. We show here that tamoxifen activates calcium- and voltage-dependent BK channels. The effect of tamoxifen is not additive to that of E_2 or its membrane impermeant form β-Estradiol 6-(O-carboxymethyl)oxime: BSA (E_2BSA) and is still recorded in the presence of the pure ER antagonist ICI-182,780. Furthermore, unitary patch-clamp data revealed that tamoxifen stimulates BK channels at the single channel level. We also show that auxiliary β_1 and β_4 subunits are co-expressed with α subunit in MCF-7 cells. Altogether, these results permit us to hypothesize that tamoxifen and E_2 act at the membrane level on an extracellular binding site as the BK channel pore or auxiliary β subunits. Furthermore, we show that the positive effect of tamoxifen on BK channels is responsible for an increase in breast cancer cell proliferation.

Molecular Pharmacology Fast Forward. Published on December 12, 2006 as DOI: 10.1124/mol.106.028290
This article has not been copyedited and formatted. The final version may differ from this version.

Materials and Methods

The breast cancer cell line MCF-7 was purchased from the American Type Culture Collection (ATCC, LGC Promochem, Molsheim France). Cells were grown in Eagle's Minimal Essential Medium (EMEM) supplemented with 5 % fetal calf serum (FCS), 2 mM L-Glutamine, 0.06 % HEPES Buffer and a mixture of penicillin (50 UI/ml) / streptomycin (50 µg/ml). The EMEM solution was renewed every two days. Cells were grown in an atmosphere saturated with humidity at 37 °C and 5 % CO_2. MCF-7 cells were tested between passages 150 and 210.

For electrophysiological analysis, cells were cultured in 35 mm Petri dishes at a density of 5.10^4 cells 2 days before patch-clamp experiments. Several minutes before recording, cells were washed with the saline solution that was used for the voltage-clamp experiment. Whole-cell currents were recorded in voltage-clamp mode, using an Axopatch 200 B patch-clamp amplifier (Axon Instruments, Burlingame, CA) and a Digidata 1200 interface (Axon Instruments). PClamp software (v. 6.0.3, Axon Instruments) was used to control voltage, as well as to acquire and analyze data. The whole-cell mode of the patch-clamp technique was used with 3-5 MΩ resistance borosilicate fire-polished pipettes (Hirschmann®, laborgerate). Seal resistance was typically in the 10-20 GΩ range. The maximum uncompensated series resistance was < 10 MΩ during whole-cell recordings, so the voltage error was < 5mV for a current amplitude of 500 pA. Recordings where series resistance resulted in errors greater than 5 mV in voltage commands were discarded. Whole cell currents were allowed to stabilize for 5 min before K^+ currents were measured. In order to evaluate the effect of pharmacological agents on the current, the cells were voltage-clamped at -40 mV and membrane potential was successively stepped to -100 and +100 mV for 200 ms and 300 ms respectively.

Molecular Pharmacology Fast Forward. Published on December 12, 2006 as DOI: 10.1124/mol.106.028290
This article has not been copyedited and formatted. The final version may differ from this version.

Single-channel recordings were performed in the outside-out configuration in asymmetrical K^+ using a RK-300 patch-clamp amplifier (Biologic, Grenoble, France). The patch-clamp amplifier was driven by Pulse 9.1 software (HEKA Elektronik, Lambrecht, Germany). Membrane currents were digitized at 20 kHz using an ITC16 computer interface (Instrutech Corp., Long Island, NY, USA) low-pass filtered at 3 kHz and stored on-line on the hard-drive of the computer. Electrodes were pulled in two stages on a PIP5 puller (HEKA, Germany) from borosilicate glass capillaries (PG52151, World Precision Instruments, Aston, UK) to a tip diameter giving a pipette resistance of 5-7 MΩ. Outside-out patches were obtained by making a GΩ seal, rupturing the membrane to gain whole-cell access, and then pulling the pipette from the cell. BK currents were recorded using an intra-pipette calcium concentration of 300 nM. Single channel analysis and NPo determination were carried out using WinEDR software (Version 2.6.9; J. Dempster, University of Strathclyde). The unitary conductance of BK channels was calculated from current-voltage relationships from -60 to +80 mV with an increment of +10 mV in control conditions and after perfusion with 10 nM tamoxifen.

Cells were allowed to settle in Petri dishes placed at the opening of a 250-µm-inner diameter capillary for extra-cellular perfusions. The cell under investigation was continuously superfused with control or test solutions. All electrophysiological experiments were performed at room temperature (20-22 °C). Because of the non-reversibility of tamoxifen and the poor reversibility of IbTx and ChTx, no more than one cell per Petri dish was recorded.

External and internal solutions had the following compositions (in mM): external: NaCl 145, KCl 5, $CaCl_2$ 2, $MgCl_2$ 1, and Hepes 10 at pH 7.4 (NaOH); Internal: KCl 150, Hepes 10, EGTA 0.1, $MgCl_2$ 2 at pH 7.2 (KOH). In some experiments needing a change in the internal calcium concentration, EGTA was replaced by BAPTA (5 mM) and free calcium concentration was adjusted with different amounts of $CaCl_2$ calculated with MaxChelator software (C. Patton, Stanford University).

Molecular Pharmacology Fast Forward. Published on December 12, 2006 as DOI: 10.1124/mol.106.028290
This article has not been copyedited and formatted. The final version may differ from this version.

Charybdotoxin (ChTx) and Iberiotoxin (IbTx) were made up in 1% BSA, 5 mM Hepes (pH 7.2). β-Estradiol 6-(O-carboxymethyl)oxime: BSA (E_2BSA) contained 35 moles of steroid per mole of BSA and was dissolved in water. E_2, tamoxifen and ICI-182,780 were made up in ethanol. Final concentrations were obtained by appropriate dilution in an external control solution and the concentration of the solvent never exceeded 1/10·000. All the products were from Sigma (France) unless otherwise stated.

The qualitative detection of the expression of α, β_1 and β_4 subunits of the BK channel was realized as follows. Total RNA was extracted from approximately $1x10^6$ cultured cells using the Trizol method (Invitrogen) according to the manufacturer's instructions. RNA samples were treated with 1U of DNase I (Promega, France) at 37°C for 30 min. A phenol/chloroform (vol/vol) extraction was performed and RNA was precipitated with ethanol and dissolved in 20 µl of sterile distilled water. The RNA level was measured by spectrophotometry (OD at 260 nm) and was reverse-transcribed into cDNA using an SSII kit (Invitrogen) following the manufacturer's instructions. Complementary DNA was stored at -20°C. The PCR primers used to amplify the RT-generated KCNMA1, KCNMB1 and KCNMB4 cDNAs were designed on the basis of established GenBank sequences. The primers for KCNMA1 cDNA were: 5'-TGTTATGGTGATCTGTTCTGC-3' and 5'-ACCAACTGGGGAAATGAGTG-3' (nucleotides 3255-3673, GenBank accession number U13913). The primers for KCNMB1 cDNA were: 5'-CTCTACCAGAAAAGCGTGTG-3' and 5'-CCATGGCGATAATGAGGAG-3' (nucleotides 271-691, GenBank accession number U25138). The primers for KCNMB4 cDNA were: 5'-GTGTCGCTCTTCATCTTCG-3' and 5'-CAATGCAGGAGGACAATCTC-3' (nucleotides 87-518, GenBank accession number AF207992). The expected amplified DNA length is 418 bp, 412 bp and 431 bp for KCNMA1, KCNMB1 and KCNMB4, respectively. Primers were synthesized by Invitrogen. PCR was performed on the RT-generated cDNA using a Biorad iCycler thermocycler. PCR reaction

Molecular Pharmacology Fast Forward. Published on December 12, 2006 as DOI: 10.1124/mol.106.028290
This article has not been copyedited and formatted. The final version may differ from this version.

mixtures contained 1 µl cDNA, 1 µl of dNTPs (10 mM; Invitrogen), 2.5 µl of sense and antisense primer (both 5 µM), 0.2 µl of Taq DNA polymerase (1U) (Invitrogen), 5 µl of PCR Buffer and 1.5 µl of $MgCl_2$ (50 mM) in a final volume of 50 µl. Samples were first incubated for 5 min at 95°C, followed by 45 cycles of 30 s at 95°C, 30 s at 55°C, 58°C and 55°C for α, β_1 and β_4 respectively, and 40s at 72°C, followed by a final extension at 72°C for 4 min. PCR products (15 µl) were analyzed by electrophoresis in a 1.2 % agarose gel in 0.5x TBE and stained with ethidium bromide. The following primers were used to amplify a 210 bp of β-actin cDNA fragment: 5'-CAGAGCAAGAGAGGCATCCT-3' and 5'-GTTGAAGGTCTCAAACATGATC-3'. The PCR conditions were 5 min at 95°C, followed by 25 cycles of 30 s at 95°C, 30 s at 58°C and 40 s at 72°C, and then by a final extension at 72°C for 4 min.

For cell proliferation assays, cells were seeded in 96-well plates in EMEM with 5 % FCS. After 48 h, cells were incubated with EMEM without FCS for a 24 h starvation period. Cells were then washed and incubated with 5 % steroid-free FCS (dextran-coated charcoal treated FCS; DCCFCS) in phenol red-free EMEM, and incubated with varying doses of tamoxifen or E_2, alone or in association with IbTx, ChTx and TEA. The medium was changed every other day. After 2 days of treatment, the cell number was determined by a colorimetric method (CellTiter 96 Aqueous Non-Radioactive Cell Proliferation Assay, Promega France). The potential toxicity of the various drugs was assessed by comparing the proliferation rate of the cells in FCS-free culture medium in the absence or in the presence of the drugs. Furthermore, flow cytometry experiments were carried out and it appeared that the percentage of events in sub-G_1 was unchanged and always under 1.4 % in all experimental conditions.

For the flow cytometry experiments, cells were grown in 5 % FCS in a 75 cm^2 flask until a confluence of over 50 % was reached. After a 24 h starvation period, cells were treated with 5 % DCCFCS medium supplemented with tamoxifen (10 nM), IbTx (100 nM) and

Molecular Pharmacology Fast Forward. Published on December 12, 2006 as DOI: 10.1124/mol.106.028290
This article has not been copyedited and formatted. The final version may differ from this version.

tamoxifen plus IbTx (10 and 100 nM respectively). After 48 h, cells were rinsed twice with Phosphate Buffered Saline (PBS) and harvested using PBS-EDTA (5 mM). $6x10^5$ cells in 300 µl PBS-EDTA were then fixed using 700 µl of ice-cold absolute ethanol under vortexing. Aliquots were kept at + 4 °C until flow cytometric analysis. After centrifugation, fixed cells were treated with RNase A (100 µg/ml) in PBS solution for 30 min at room temperature, followed by staining with propidium iodide (100 µg/ml) in PBS. The distribution of the cells in the different phases of the cell cycle (G_1, S and G_2/M) was acquired for 10,000 events using an Elite Beckman/Coulter flow cytometer.

Results were expressed as mean ± S.E.M. Experiments were repeated at least three times. The Student's paired and unpaired t-test and one way analysis of variance (ANOVA) with Bonferroni post-hoc analysis were used to compare treatment means with control means, as appropriate. The experimental group median marked by asterisks is significantly different from the control median (∗ $P < 0.05$; ∗∗ $P < 0.01$; ∗∗∗ $P < 0.001$).

Molecular Pharmacology Fast Forward. Published on December 12, 2006 as DOI: 10.1124/mol.106.028290
This article has not been copyedited and formatted. The final version may differ from this version.

Results

MCF-7 cell membrane was held at -40 mV and successively stepped at -100 mV for 200 ms and at +100 mV for 300 ms every 30 s with an increment of 20 mV. Typical current traces obtained in these control conditions are depicted in Figure 1Aa. The perfusion of 10 nM tamoxifen significantly increased the magnitude of the K^+ current measured at +100 mV by 22.6 ± 10.6 % ($P<0.001$, paired t-test, n=23) compared to control (Figure 1Ab). Current-voltage relationships were obtained by measuring the maximum amplitude of K^+ currents at the end of the depolarization. Analysis of these curves clearly shows that a significant effect of tamoxifen was observed for membrane potentials above 20 mV (Figure 1B). The time course for the effect of tamoxifen was then investigated and is presented in Figures 2A and B. The effect of tamoxifen was generally initiated within the first minute and peaked at 5.9 ± 2.2 min (n=23). Furthermore, the action of tamoxifen could not be reversed even after 20 minutes of continuous washout (data not shown, n=6).

Dose-response curves were then realized (Figure 2C) and showed that the activation threshold appeared for tamoxifen concentrations around 0.3 nM with an EC_{50} value of 0.71 ± 0.12 nM determined by curve fitting. The maximum effect was obtained for 10 nM and this latter concentration was thus chosen for all further experiments.

Based on previous studies showing the modulation of BK channels by E_2, we tested whether this channel type can also be the target for tamoxifen. To this end, we used three different inhibitors of the BK channel: IbTx, ChTx and TEA. All these compounds are known to obstruct BK channel pore and at the concentrations used in this study, block only these channels in MCF-7 cells. As shown in Figure 3, IbTx (100 nM, 3A), ChTx (50 nM, 3B), and TEA (0.5 mM, 3C) always reversed the enhancement of K^+ current induced by the perfusion of 10 nM tamoxifen. In the same way, when the K^+ current was previously inhibited by the

Molecular Pharmacology Fast Forward. Published on December 12, 2006 as DOI: 10.1124/mol.106.028290
This article has not been copyedited and formatted. The final version may differ from this version.

perfusion of IbTx (100 nM, 3D) or TEA (0.5 mM, 3E), the perfusion of tamoxifen (10 nM) was ineffective until the drugs had been washed out.

The modulatory effect of tamoxifen on BK channel activity has been suggested to be dependent on the internal calcium concentration (Pérez, 2005). We thus tested the effect of 10 nM tamoxifen on MCF-7 cell in which free calcium concentration was adjusted to a low (50 nM) and to a high level (700 nM). As expected, the mean amplitudes of K^+ currents recorded in both experimental conditions were significantly different (2645 ± 446 pA (n=6) and 3834 ± 530 pA (n= 4) for 50 nM and 700 nM internal Ca^{2+}, respectively). Tamoxifen enhanced the BK current amplitude by 17.2 ± 2.6 % (n=6) and 16.4 ± 1.7 % (n=4) for 50 and 700 nM internal calcium, respectively (data not shown).

Since both E_2 and tamoxifen regulate BK channels, we investigated whether their effects were additive. We firstly increased the BK current by the perfusion of 10 nM tamoxifen and then we applied E_2 at the same concentration (Figure 4A). Then, we repeated the same experiment on another cell, except that we first perfused E_2 (10 nM) before the application of tamoxifen (10 nM, Figure 4B). Statistical analysis revealed that even though the perfusion of tamoxifen or E_2 alone significantly enhanced the BK current amplitude compared to the control (19.8 ± 4.7 %, $P<0.01$ n=7 and 14.8 ± 3.8 %, $P<0.001$, n=4 paired t-test, for tamoxifen and E_2 respectively), no significant difference could be observed between the effect of tamoxifen and E_2 when they were applied in combination (one-way ANOVA, Figure 4C). Furthermore, it appeared that the effect of tamoxifen was also not additive to that of the membrane impermeant form of E_2 (β-Estradiol 6-(O-carboxymethyl)oxime: BSA, E_2BSA, Figure 4D-E). Indeed, whilst the perfusion of E_2BSA (Figure 4D) or tamoxifen (Figure 4E) resulted in a net augmentation of the K^+ current amplitude (18.1 ± 5.1 %, $P<0.05$ n=4 and 19 ± 4.4 %, $P<0.05$, n=4, paired t-test, respectively), each compound was ineffective if the other one had previously been applied to the perfusion medium (one way ANOVA). We

Molecular Pharmacology Fast Forward. Published on December 12, 2006 as DOI: 10.1124/mol.106.028290
This article has not been copyedited and formatted. The final version may differ from this version.

then tested the effect of tamoxifen in the presence of the pure ER antagonist ICI-182,780 (1 μM) in order to assess whether tamoxifen regulates the BK channel through the activation of the ER pathway. Results are depicted in Figure 4F and clearly show that tamoxifen is still able to stimulate BK current by 17.5 ± 3.8 % ($P<0.05$, n=5, one way ANOVA) in these experimental conditions. This last result thus supports a direct effect at the level of the BK channel itself or at the level of one of its auxiliary β subunits. In order to verify this hypothesis, we carried out a unitary patch-clamp recording of the BK current in the absence and in the presence of tamoxifen at a concentration of 10 nM. Figure 5A presents typical recordings of single BK currents at a holding potential of 20 mV before (upper trace) and after the perfusion of tamoxifen at a concentration of 10 nM (lower trace). This current was reversibly blocked by 0.5 mM TEA (n=4, data not shown). Single channel analysis showed that whereas tamoxifen treatment did not change unitary conductance (Figure 5B; γ=140.9 ± 14.5 pS n=4 vs 140.6 ± 14.3 pS, n=4, paired t-test, in control conditions and after tamoxifen treatment, respectively), it significantly increased the open probability of the BK channel by 46.2 ± 10.1 % ($P<0.01$, n=4, paired t-test, Figure 5C).

Based on numerous studies showing that the presence of β_1 (Dick and Sanders, 2001; Dick et al, 2001) or β_4 (Behrens et al, 2000) auxiliary subunits is a prerequisite for tamoxifen or E_2 to stimulate BK channels, we searched for their expression in MCF-7 cells. Results are depicted in Figure 6 and clearly show that these subunits are co-expressed with the pore forming α subunit.

The proliferation of breast cancer cells is under the control of E_2 through genomic effects and through the enhancement of BK channel activity, and this fact justifies the use of tamoxifen as an antiproliferative agent. Nevertheless, we investigated whether the positive modulation of BK channel by tamoxifen may have a repercussion on cell proliferation. In order to eliminate the eventual participation of native steroids contained in Fetal Calf Serum

Molecular Pharmacology Fast Forward. Published on December 12, 2006 as DOI: 10.1124/mol.106.028290
This article has not been copyedited and formatted. The final version may differ from this version.

(FCS), all of the following experiments were performed in culture medium complemented with steroid-free FCS (Dextran-Coated, Charcoal-Treated FCS, DCCFCS). Dose-response curves revealed that, at low doses, tamoxifen was responsible for a significant stimulation of cell proliferation with a maximal effect measured at 10 nM (10.4 ± 3.7 %, $P<0.05$, n=8, one way ANOVA, Figure 7Aa). However, as expected, tamoxifen efficiently blocked MCF-7 cell proliferation at higher doses than 1 µM (data not shown). Similar results were obtained with E_2 (15.8 ± 4.6 %, $P<0.05$, n=8, one way ANOVA, figure 7Ab). The EC_{50} values, determined by curve fitting, are 0.18 ± 0.1 nM and 9.2 ± 3.8 pM for tamoxifen and E_2 respectively. The increase in the cell number by 10 nM tamoxifen was not due to an antiapoptotic effect of this compound, since flow cytometry analysis showed that the number of cells in the sub-G_1 phase of the cell cycle was not significantly modified compared to control conditions (data not shown). We then compared the ability of tamoxifen, alone or in combination with E_2, to modulate cell proliferation. We measured no additive effect of E_2 and tamoxifen at a 10 nM concentration, even though separately they were both able to elevate cell proliferation to a similar extent (15.1 ± 6.7 %, $P<0.05$, n=5, and 21.2 ± 5.6 %, $P<0.01$, one way ANOVA, n=5, Figure 7B). In order to determine whether the positive effect of tamoxifen on MCF-7 cell proliferation relies on BK channel modulation, we conducted pharmacological experiments using the three inhibitors that were used in the electrophysiological part of this work. It appeared that, while TEA (0.5 mM), ChTx (50 nM) and IbTx (100 nM) were all unable to inhibit basal proliferation measured in control conditions (DCCFCS), these three BK channel inhibitors significantly antagonized the proliferation induced by 10 nM tamoxifen (Figure 7C).

Molecular Pharmacology Fast Forward. Published on December 12, 2006 as DOI: 10.1124/mol.106.028290
This article has not been copyedited and formatted. The final version may differ from this version.

Discussion

Many types of K^+ channels have been shown to be involved in the progression of the cell cycle and proliferation of MCF-7 cell line (Ouadid-Ahidouch et al., 2000, 2001, 2004a and 2004b, Coiret et al. 2005). We show here that tamoxifen stimulates MCF-7 cell proliferation through a direct modulation of BK channels independently of its ER antagonist properties. The effect of tamoxifen is not additive to that of E_2 and recorded at the single channel level. We thus hypothesize that they both modulate the BK channel by the mean of a very similar mechanism.

Our electrophysiological analysis revealed that the effect of tamoxifen was obtained for membrane potentials superior to 20 mV, suggesting that the involved channel is voltage-dependent. Specific BK channel inhibitors, namely IbTx, ChTx and TEA at a low concentration, antagonized the effect of tamoxifen. It has to be noted, however, that in the experiment presented in Figure 3D, the effect of tamoxifen is weak after IbTx washout compared to the same effect recorded in control conditions or after the washout of 0.5 mM TEA (Figure 3E). This smaller effect may be attributed to the poor reversibility of IbTx in these cells as can be seen in Figure 3A.

The time course for the achievement of the tamoxifen effect was short and the maximum effect was observed only a few minutes after the application of tamoxifen. Moreover, it appears that the effect was irreversible even after 20 minutes of continuous washout and the stimulation of the K^+ current by tamoxifen was still recorded after the inhibition of ER by the pure anti-estrogen ICI-182,780. The rapidity of action and the insensitivity to this compound exclude the participation of the genomic transduction pathway and are in favor of a direct effect at the level of the plasma membrane independent of neither the classical ER_α and ER_β subtypes nor the recently postulated membrane ER (for review see Toran-Allerand, 2004). This latter is not expressed in MCF-7 cells and is much more sensitive to 17_α-E_2 (Toran-

Molecular Pharmacology Fast Forward. Published on December 12, 2006 as DOI: 10.1124/mol.106.028290
This article has not been copyedited and formatted. The final version may differ from this version.

Allerand et al, 2002), a stereoisomer that is scarcely able to stimulate BK channel when expressed in *Xenopus* oocytes (Valverde et al., 1999). We also show in this study that the effect of tamoxifen on the BK channel activity is not additive to that of E_2 or E_2BSA, thereby suggesting that these three compounds share the same molecular target.

This effect of tamoxifen has already been described in numerous tissues but to our knowledge, we provide the first evidence that BK channels are modulated by tamoxifen in breast cancer cells. In fact, conflicting results were obtained in the case of SMC. For example, many authors report a crucial role of the presence of the β_1 subunit of the BK channel in mediating the effect of tamoxifen. Indeed, Dick et al. (2001) showed that tamoxifen activates BK current by increasing the open probability (NPo) of *Slo* α expressed in HEK cells only in the presence of the β_1 subunit. However, tamoxifen reduces the unitary conductance (γ) of the α subunit expressed alone, without any effect on NPo. Furthermore, a similar conclusion was obtained by Dick and Sanders (2001) who have shown that tamoxifen was unable to stimulate BK current in Murine colonic myocytes isolated from β_1 subunit knockout mice. In the same way, Duncan (2005) reported that tamoxifen modulates BK channels by mediating a conformation change in the α subunit and that this interaction is responsible for an alteration in the way that α and β subunits interact. This modulation results in an enhanced gating occurring without direct binding to the β subunit. Our results from outside-out patch-clamp experiments are in agreement with the assumption of a direct modulation of BK channel by tamoxifen. Indeed, we show here that tamoxifen significantly increased the NPo of BK channels by 46 % without affecting its unitary conductance. The discrepancy between tamoxifen effect in single channel and in whole-cell recordings (46 % vs 22 % change) should be due to the patch-clamp configuration. Indeed, in both experiments, the modulatory effect on BK channels should be the same but the percentage is probably minimized in whole cell recordings because of the measurement of other, tamoxifen-insensitive, K conductances. In

Molecular Pharmacology Fast Forward. Published on December 12, 2006 as DOI: 10.1124/mol.106.028290
This article has not been copyedited and formatted. The final version may differ from this version.

our study, there are three major differences with the current literature regarding xenoestrogen modulation of BK channels. First of all, BK channels in MCF-7 cells are stimulated by low doses of tamoxifen (in the nM range) whereas other authors have reported the same effect for concentrations of tamoxifen in the µM range (Dick et al., 2001; Dick and Sanders, 2001). Second, unlike other preparations (Dick, 2002; Liu et al., 2003), 1 µM ICI-182,780 is not able to modulate BK channel activity, and third, the effect of tamoxifen is not reversed even after 20 minutes of washout.

These discrepancies should rest on the subunit composition of BK channels that are expressed in MCF-7 cells and it is conceivable that tamoxifen interacts with α and/or β_1 or β_4 subunits in a different manner than in other preparations. This could explain the high sensitivity of BK channels to tamoxifen, as has already been reported for E_2 in a previous work (Coiret et al., 2005). In the same way, King et al. (2006) reported that the nature of β subunits that are co-expressed with α subunit in HEK 293 cells was able to influence the sensitivity of the BK channel to different kinds of steroids. Furthermore, such discrepancies have also been reported in cultured endothelial cells and SMC from human coronary arteries (Liu et al., 2003) and seem to be dependent on the cell type. Indeed, whereas ICI-182,780 reduces BK channel NPo without γ modification in endothelial cells, this compound regulates BK channel activity in a bell-shaped manner in SMC. In order to explain the non-reversibility of the modulatory action of tamoxifen, we would submit that its interaction with the BK channel is sufficiently strong to withstand washout. A non-reversible stimulation of BK channel has already been described by Dick (2002) for ICI-182,780 in SMC in which the stimulation of BK channel remains unaltered even after several tens of minutes of washout.

We thus postulate that tamoxifen activates BK channels by a direct interaction at the level of the channel itself or at the level of β auxiliary subunit. However, in opposition to this hypothesis, Pérez (2005) reported that the dual effect of tamoxifen is independent on the

Molecular Pharmacology Fast Forward. Published on December 12, 2006 as DOI: 10.1124/mol.106.028290
This article has not been copyedited and formatted. The final version may differ from this version.

presence of the auxiliary β_1 subunit, but relies on the internal calcium concentration in mouse arterial SMC. Indeed, whereas tamoxifen (5 µM) is able to stimulate BK channel activity when internal calcium concentration is below 500 nM, it inhibits BK current for higher internal calcium concentrations. This modulatory effect of calcium was not observed in our study. Indeed, whatever the internal free calcium concentration tested (50 nM or 700 nM) the stimulatory effect of tamoxifen at 10 nM was not significantly different.

An important result is that a low concentration of tamoxifen is able to stimulate MCF-7 cell proliferation and that this stimulation is sensitive to BK channel antagonists. Furthermore, this stimulatory effect of 10 nM tamoxifen on MCF-7 proliferation was not statistically different from that of E_2 at the same concentration and their effects were not additive. In addition, our results show that the effect of E_2 is not antagonized by the use of this low concentration of tamoxifen.

MCF-7 cell line expresses numerous K^+ channels involved in the control of its proliferation according to the « membrane potential model » (Ouadid-Ahidouch et al., 2004b). The insensitivity of the proliferation obtained in control conditions (i.e. steroid-free culture medium) to the BK channel antagonists probably resides in the inactivity of BK channels in these conditions and the fact that the proliferation is mainly supported in this case by the activity of other K^+ channels.

Such stimulatory effects of tamoxifen on the proliferation of breast cancer cells have already been reported and have been put forward as an explanation of the clinical resistance mechanism (for review see Clarke et al., 2001). For example, Keaton and Brown (2005) have shown that tamoxifen is able to stimulate the MCF-7 proliferation, in which the nuclear receptor co-repressor (N-CoR) and silencing mediator for retinoid and thyroid receptors (SMRT) have been silenced. This study shows that N-CoR and SMRT play a role in tamoxifen-bound ER_α action and that the relative level of ER co-regulators can influence the

cellular response to tamoxifen. Nevertheless, in our study, the stimulatory effect of tamoxifen does not seem to involve ER_α since it was obtained in the presence of ICI-182,780.

These findings give insight into BK channels' roles and function in breast epithelial cancer cells and into the side effects of tamoxifen. The stimulatory effect of tamoxifen at low concentrations has to be taken into account in the first few weeks of starting tamoxifen treatment and could be responsible for the « tamoxifen flare » and the apparent worsening of the disease seen in some patients until sufficiently high steady-state concentrations of tamoxifen and its metabolites have been accumulated.

Molecular Pharmacology Fast Forward. Published on December 12, 2006 as DOI: 10.1124/mol.106.028290
This article has not been copyedited and formatted. The final version may differ from this version.

Acknowledgments

Authors wish to thank Jean-François Lefebvre and Stéphanie Guénin from the "Centre de Recherche Régional de Biologie Moléculaire" for their help concerning molecular biology experiments.

Molecular Pharmacology Fast Forward. Published on December 12, 2006 as DOI: 10.1124/mol.106.028290
This article has not been copyedited and formatted. The final version may differ from this version.

References

Behrens R, Nolting A, Reimann F, Schwarz M, Waldschütz R, Pongs O (2000) hKCNMB3 and hKCNMB4, cloning and characterization of two members of the large-conductance calcium-activated potassium channel β subunit family. *FEBS Letters* 474:99-106

Chesnoy-Marchais D (2005) The estrogen receptor modulator tamoxifen enhances spontaneous glycinergic presynaptic inhibition of hypoglossal motoneurons. *Endocrinology* 146(10):4302-11.

Clarke R, Leonessa F, Welch JN and Skaar TC (2001) Cellular and molecular pharmacology of antiestrogen action and resistance. *Pharm Rev* 53:25-71.

Coiret G, Matifat F, Hague F and Ouadid-Ahidouch H (2005) 17-β-Estradiol activates maxi-K channels through a non-genomic pathway in human breast cancer cells. *FEBS Letters* 579:2995-3000.

Cosman F and Lindsay R (1999) Selective estrogen receptor modulators: Clinical spectrum. *Endocr Rev* 20:418-434.

Dick GM (2002) The pure anti-oestrogen ICI-182,780 (Faslodex) activates large conductance Ca(2+)-activated K(+) channels in smooth muscle *Br. J. Pharmacol.* 136:961-964

Dick GM and Sanders KM (2001) (Xeno)estrogen sensitivity of smooth muscle BK channels conferred by the regulatory beta1 subunit: a study of beta1 knockout mice. *J Biol Chem* 276:44835-44840.

Dick GM, Rossow CF, Smirnov S., Horowitz B and Sanders KM (2001) Tamoxifen activates smooth muscle BK channels through the regulatory β_1 subunit. *J Biol Chem* 276:34594-34599.

Duncan RK (2005) Tamoxifen alters gating of the BK alpha subunit and mediates enhanced interactions with the avian beta subunit. *Biochem Pharmacol* 70:47-58.

ESHRE Capri Workshop Group (2004) Hormones and breast cancer. *Human Reproduction Update* 10:281-293.

Keaton EK and Brown M (2005) Cell cycle progression stimulated by tamoxifen-bound Estrogen Receptor-α and promotor-specific effects in breast cancer cells deficient in N-CoR and SMRT. *Mol Endocrinol* 19:1543-1554.

King JT, Lovell PV, Rishniw M, Kotlikov MI, Zeeman ML and McCobb DP (2006) Beta2 and beta4 subunits of BK channels confer differential sensitivity to acute modulation by steroid hormones. *J. Neurophysiol.* 95:2878-2888.

Liehr JG and Ricci MR (1996) 4- hydroxylation of estrogens as a marker of human mammary tumours. *Proc Natl Acad Sci USA* 93:3294-3296.

Liu YC, Lo YC, Huang CW and Wu SN (2003) Inhibitory action of ICI-182,780, an estrogen receptor antagonist, on BK_{Ca} channel activity in cultured endothelial cells of human coronary artery. *Biochem Pharmacol* 66:2053-2063.

Lösel RM, Falkenstein E, Schultz MFA, Tillmann HC, Rossol-Haseroth K and Wehling M (2003) Non-genomic Steroid Action: Controversies, Questions, and Answers. *Physiol Rev* 83:965–1016.

Mitlack BH and Cohen FJ (1997) In search of optimal long term female hormone replacement: the potential of selective estrogen receptor modulators. *Hormone Research* 48, 155-163.

Ouadid-Ahidouch H, Chaussade F, Roudbaraki M, Slomianny C, Dewailly E, Delcourt P and Prevarskaya N (2000) Kv1.1 K^+ channels identification in human breast carcinoma cells: involvement in cell proliferation. *Biochem Biophys Res Commun* 278 (2), 272-7.

Ouadid-Ahidouch H, Le Bourhis X, Roudbaraki M, Toillon RA, Delcourt P and Prevarskaya N (2001) Changes in the K^+ current-density of MCF-7 cells during progression through the

cell cycle: possible involvement of a h-*ether-a-gogo* K^+ channel. *Receptors Channels*. 7 (5), 345-56.

Ouadid-Ahidouch H, Roudbaraki M, Ahidouch A, Delcourt P and Prevarskaya N (2004a) Cell-cycle-dependent expression of the large Ca^{2+}-activated K^+ channels in breast cancer cells. *Biochem Biophys Res Commun* 316 (1), 244-51.

Ouadid-Ahidouch H, Roudbaraki M, Delcourt P, Ahidouch A, Joury N and Prevarskaya N (2004b) Functional and molecular identification of intermediate-conductance Ca^{2+}-activated K^+ channels in breast cancer cells: association with cell cycle progression. *Am J Physiol Cell Physiol* 287 (1), C125-34.

Pérez GJ (2005) Dual effect of tamoxifen on arterial K_{Ca} channels does not depend on the presence of the β_1 subunit. *J Biol Chem* 280:21739-21747.

Sahebgharani M, Hardy SP, Lloyd AW, Hunter AC and Allen MC (2001) Volume-activated chloride currents in HeLa cells are blocked by tamoxifen but not by a membrane-impermeant quaternary analogue. *Cell Physiol Biochem* 11:99-104.

Toran-Allerand CD, Guan X, MacLusky NJ, Horvath TL, Diano S, Singh M, Conolly ES, Nethrapalli IS and Tinikov AA (2002) ER-X: A novel, plasma membrane-associated, putative estrogen receptor that is regulated during development and after ischemic brain injury. *J. Neurosci.* 22:8391-8401.

Toran-Allerand CD (2004) A plethora of estrogen receptors in the brain: where will it end ? *Endocrinology* 145:1069-1074.

Valverde MA, Rojas P, Amigo J, Cosmelli D, Orio P, Bahamonde MI, Mann GE, Vergara C, and Latorre R (1999) Acute Activation of Maxi-K Channels (*hSlo*) by Estradiol Binding to the β Subunit. *Science* 285:1929-1931

Veronesi U, Boyle P, Goldhirsch A, Orecchia R and Viale G (2005) Breast cancer. *Lancet* 365:1727-1741.

Zidan J, Keidar Z, Basher W, Israel O (2004) Effects of tamoxifen on bone mineral density and metabolism in postmenopausal women with early-stage breast cancer. *Med Oncol.* 21(2):117-21.

Footnotes

This work was supported by La Ligue Contre Le Cancer, La Région Picardie, the Ministère de l'Education Nationale de l'Enseignement Supérieur et de la Recherche, and l'Association de la Recherche contre le Cancer (ARC, France). Financial support to AS Borowiec was from the Ministère de l'Education Nationale de l'Enseignement Supérieur et de la Recherche.

Molecular Pharmacology Fast Forward. Published on December 12, 2006 as DOI: 10.1124/mol.106.028290
This article has not been copyedited and formatted. The final version may differ from this version.

Legends for figures

Figure 1. Tamoxifen activates a voltage-dependent K^+ current in MCF-7 breast cancer cells. **A**, whole-cell K^+ currents were recorded in the MCF-7 cells with a physiological K^+ gradient (5 mM K^+ outside and 150 mM K^+ inside). Cells were held at -40 mV and membrane potential was successively stepped at -100 mV for 200 ms and then at +100 mV for 300 ms every 30 seconds in 20 mV increments. The typical current traces presented in A were recorded in the same cell in control conditions (Aa) and after the maximal effect of 10 nM tamoxifen (Ab). **B**, current/voltage relationships were obtained by measuring the maximum amplitude of the K^+ currents at the end of the depolarization as indicated by the arrow in current traces presented in A before (○) and after the perfusion of tamoxifen (●) at a concentration of 10 nM. These results clearly show that K^+ current was affected for all membrane potentials superior to +20 mV.

Figure 2. The stimulatory effect of tamoxifen is dose-dependent and takes place rapidly. **A**, time course of the positive effect of 10 nM tamoxifen on the K^+ current. K^+ currents were elicited by stepping the membrane potential from -100 to +100 mV for 300 ms every 30 seconds. The maximal amplitude of K^+ current was measured at the end of the depolarizing pulse and plotted versus time to show the rapidity with which the effect took place. The current traces presented in **B** represent currents obtained in control conditions (1) and after the maximal effect of 10 nM tamoxifen (2). **C**, K^+ currents were recorded after depolarizing voltage steps from -100 to +100 mV for 200 ms and 300 ms respectively from a holding potential of -40 mV. The dose-effect curve shows the percentage of K^+ current increase that was measured after the achievement of the maximal effect of each concentration of tamoxifen (n=5 for each concentration). Curve fitting gave an EC_{50} value of 0.71 ± 0.12 nM. The

Molecular Pharmacology Fast Forward. Published on December 12, 2006 as DOI: 10.1124/mol.106.028290
This article has not been copyedited and formatted. The final version may differ from this version.

experimental group median marked by asterisks is significantly different from the control median (one way ANOVA with Bonferroni post hoc analysis).

Figure 3. The molecular target of tamoxifen is the BK channel. **A-E**, Time courses of K^+ currents elicited by stepping membrane potential from -100 to +100 mV for 300 ms every 30 seconds. **A**, the increase in K^+ current amplitude following bath application of tamoxifen (10 nM) was inhibited by the perfusion of the specific BK channel inhibitor IbTx (100 nM). **B-C**, The perfusion of ChTx (50 nM; B) or TEA (0.5 mM; C) inhibited the K^+ current that was previously enhanced by the application of 10 nM tamoxifen. **D-E**, the application of tamoxifen (10 nM) was ineffective until the BK channel blockers IbTx (100 nM, D) or TEA (0.5 mM, E) had been washed out. Altogether, these results are in favor of a regulation of BK current by tamoxifen.

Figure 4. The effect of tamoxifen on BK current is not additive to that of E_2. **A-B** and **D-F**, time course of K^+ currents elicited by stepping membrane potential from -100 to +100 mV for 300 ms every 30 seconds. E_2 at a concentration of 10 nM was ineffective if the BK current was previously activated by the superfusion of 10 nM tamoxifen (Aa). On the other hand, 10 nM tamoxifen was also ineffective after the perfusion of E_2 at the same concentration (Ba). K^+ current traces presented in Ab and Bb were recorded at the time indicated by numbers in Aa and Ba, respectively. **C**, Bar graph showing the absence of significant difference between the effects of tamoxifen (Tam), E_2, E_2 after the previous application of tamoxifen (E_2/Tam), and tamoxifen after the previous application of E_2 (Tam/E_2) on the maximal amplitude of the K^+ current. **D**, the effect of tamoxifen (10 nM) was not recorded if BK current amplitude had previously been enhanced by the membrane impermeant form of E_2, E_2-BSA (10 nM). In the same way, E_2-BSA (10 nM) was ineffective after the previous perfusion of tamoxifen at the

Molecular Pharmacology Fast Forward. Published on December 12, 2006 as DOI: 10.1124/mol.106.028290
This article has not been copyedited and formatted. The final version may differ from this version.

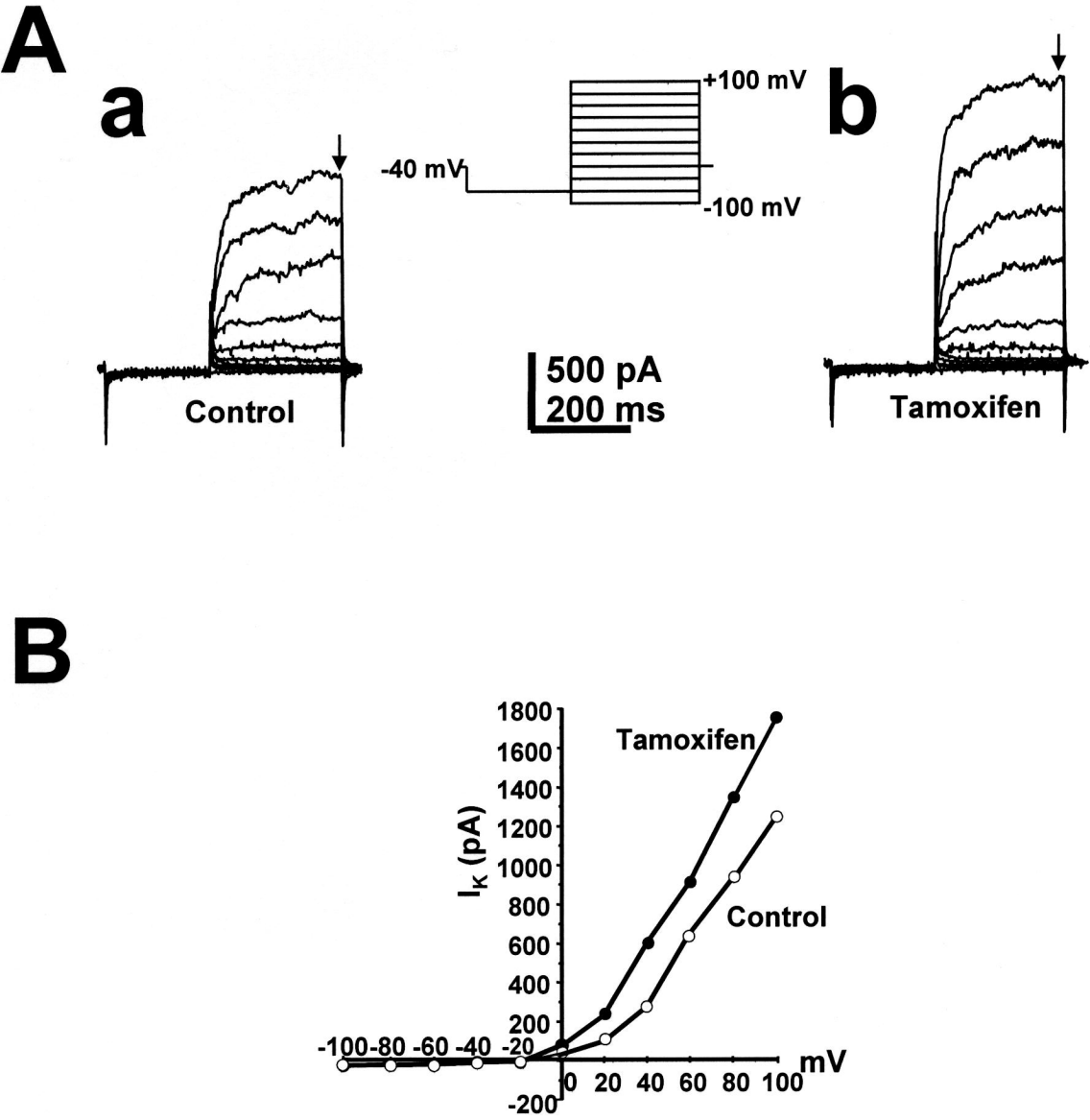
same concentration (E). **F**, the specific ER antagonist ICI-182,780 (1 μM) did not prevent the stimulatory effect of tamoxifen.

Figure 5. Tamoxifen exerts its modulatory effect at the single channel level. **A**, time course of single BK currents recorded at a holding potential of 20 mV in the same patch of membrane before (control, upper trace) and after (tamoxifen, lower trace) the perfusion of tamoxifen at a concentration of 10 nM. **B**, the unitary conductance in control conditions (control) and after the perfusion of 10 nM tamoxifen (tamoxifen) was calculated from I/V curves recorded in 4 patches. No significant difference could be observed between these two conditions (γ=140.9 ± 14.5 pS, n=4 and 140.6 ± 14.3 pS n=4 for control and after tamoxifen treatment, respectively). **C**, open probability (NPo) was calculated in control conditions (Control) and after the effect of 10 nM tamoxifen (tamoxifen). Tamoxifen stimulated NPo by 46.2 ± 10.1 % ($P<0.01$, n=4, paired t-test). The experimental group median marked by asterisks is significantly different to the control median (** $P < 0.01$).

Figure 6. BK channels' β_1 and β_4 auxiliary subunits are co-expressed with α in MCF-7 cells. cDNA were obtained by reverse-transcription of total RNAs from MCF-7 cells. PCR was then performed using specific primers given in the Materials and Methods section. Amplified fragments were resolved by 1.2% agarose-gel electrophoresis and visualized by Ethidium Bromide staining. Numbers on the left indicate the molecular weight of the ladder. α, β_1, β_4 and Act represent the fragments corresponding to BK channels subunits and β-Actin respectively. –Ctl suffix indicate the control conditions for each subunit subtype (i.e. water instead of cDNA).

Molecular Pharmacology Fast Forward. Published on December 12, 2006 as DOI: 10.1124/mol.106.028290
This article has not been copyedited and formatted. The final version may differ from this version.

Figure 7. Tamoxifen stimulates MCF-7 cell proliferation through the stimulation of BK channel. In order to eliminate the eventual participation of native steroids contained in Fetal Calf Serum (FCS), all of the experiments were performed in culture medium complemented with steroid-free FCS (Dextran-Coated, Charcoal-Treated FCS, DCCFCS). **A**, dose-effect curves of tamoxifen (Aa) and E_2 (Ab) on MCF-7 cell proliferation. Low concentrations of tamoxifen stimulated the proliferation with an EC_{50} determined at 0.18 ± 0.1 nM by curve fitting. **B**, the stimulatory effect of 10 nM tamoxifen was very similar and not additive to that of 10 nM E_2. **C**, effect of BK channel inhibitors TEA (0.5 mM), ChTx (50 nM) and IbTx (100 nM) on cell proliferation induced by 10 nM tamoxifen. Whilst these inhibitors did not significantly inhibit the proliferation in the control conditions, they all reduced the cell proliferation induced by 10 nM tamoxifen. The experimental group median marked by asterisks is significantly different from the control median (one way ANOVA).


A
a
+100 mV
-40 mV
-100 mV
b
Control
500 pA
200 ms
Tamoxifen

B
1800
1600
1400
1200
1000
800
600
400
200
-200
I_K (pA)
Tamoxifen
Control
-100 -80 -60 -40 -20
0 20 40 60 80 100
mV

Figure 1

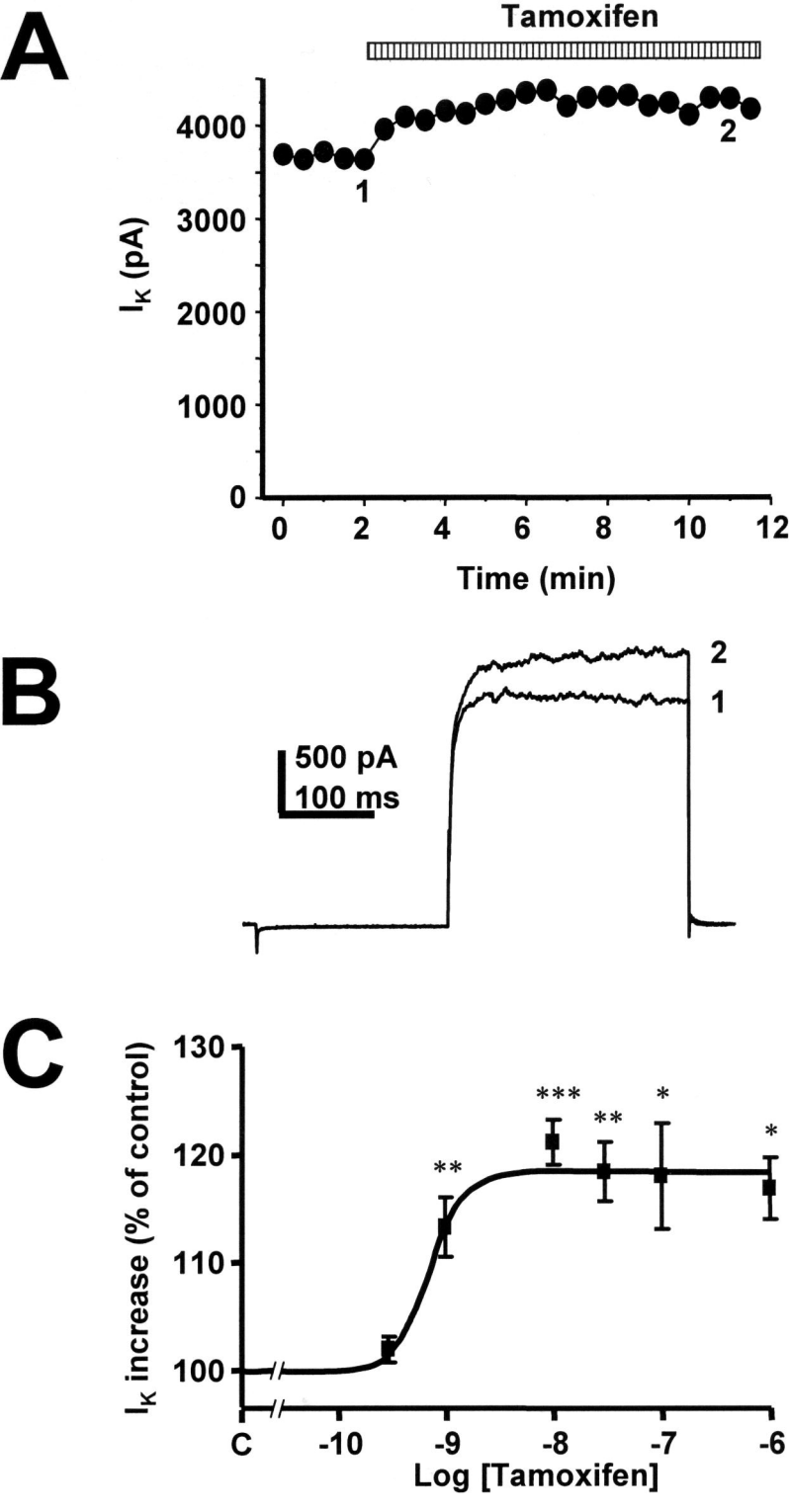

A
Tamoxifen
4000
3000
2000
1000
0
I_K (pA)
1
2
0
2
4
6
8
10
12
Time (min)
B
2
1
500 pA
100 ms
C
130
120
110
100
I_K increase (% of control)
**
***
**
*
*
C
-10
-9
-8
-7
-6
Log [Tamoxifen]

Figure 2

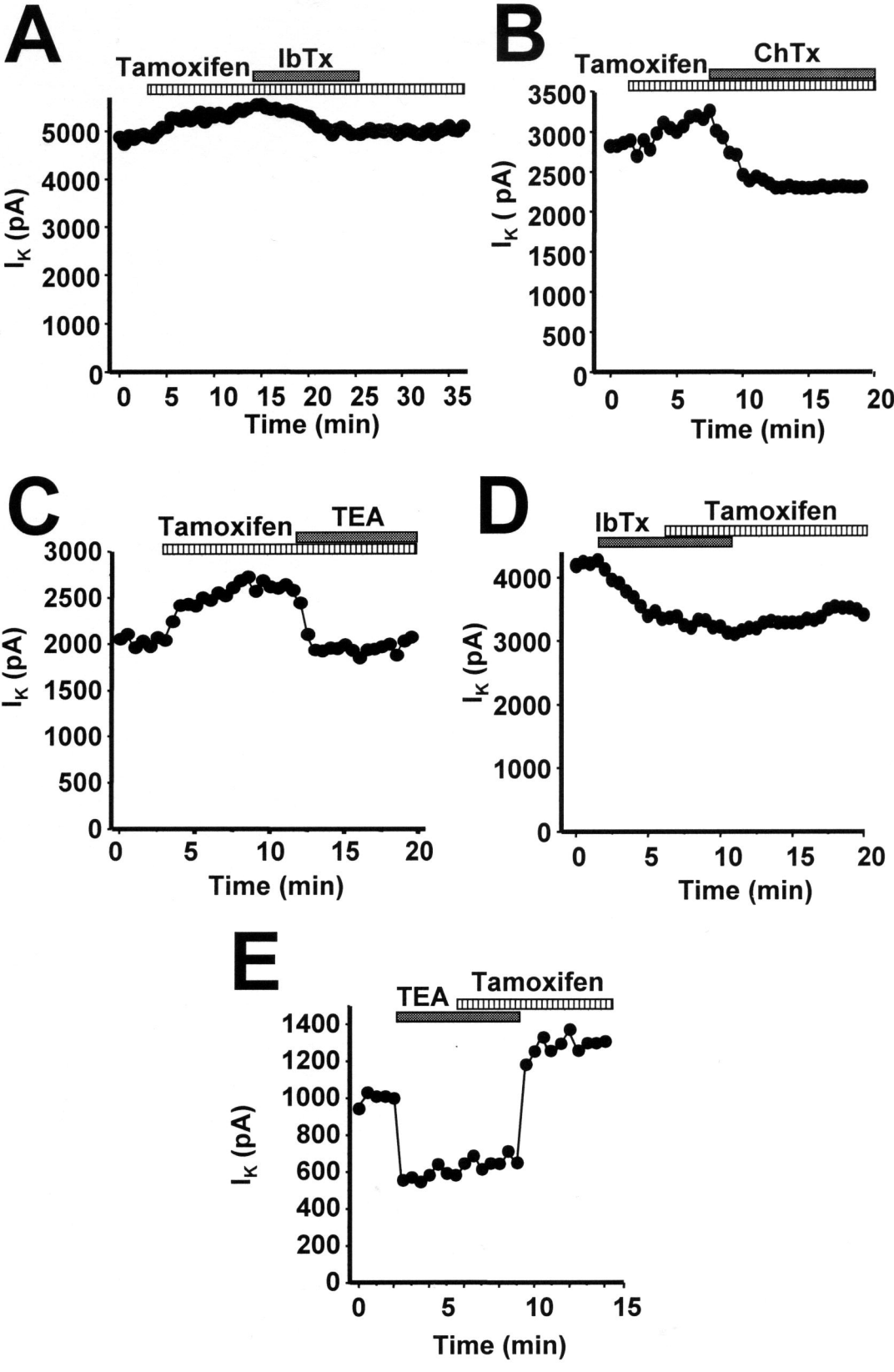

A
Tamoxifen
IbTx
I_K (pA)
Time (min)
B
Tamoxifen
ChTx
I_K (pA)
Time (min)
C
Tamoxifen
TEA
I_K (pA)
Time (min)
D
IbTx
Tamoxifen
I_K (pA)
Time (min)
E
TEA
Tamoxifen
I_K (pA)
Time (min)

Figure 3

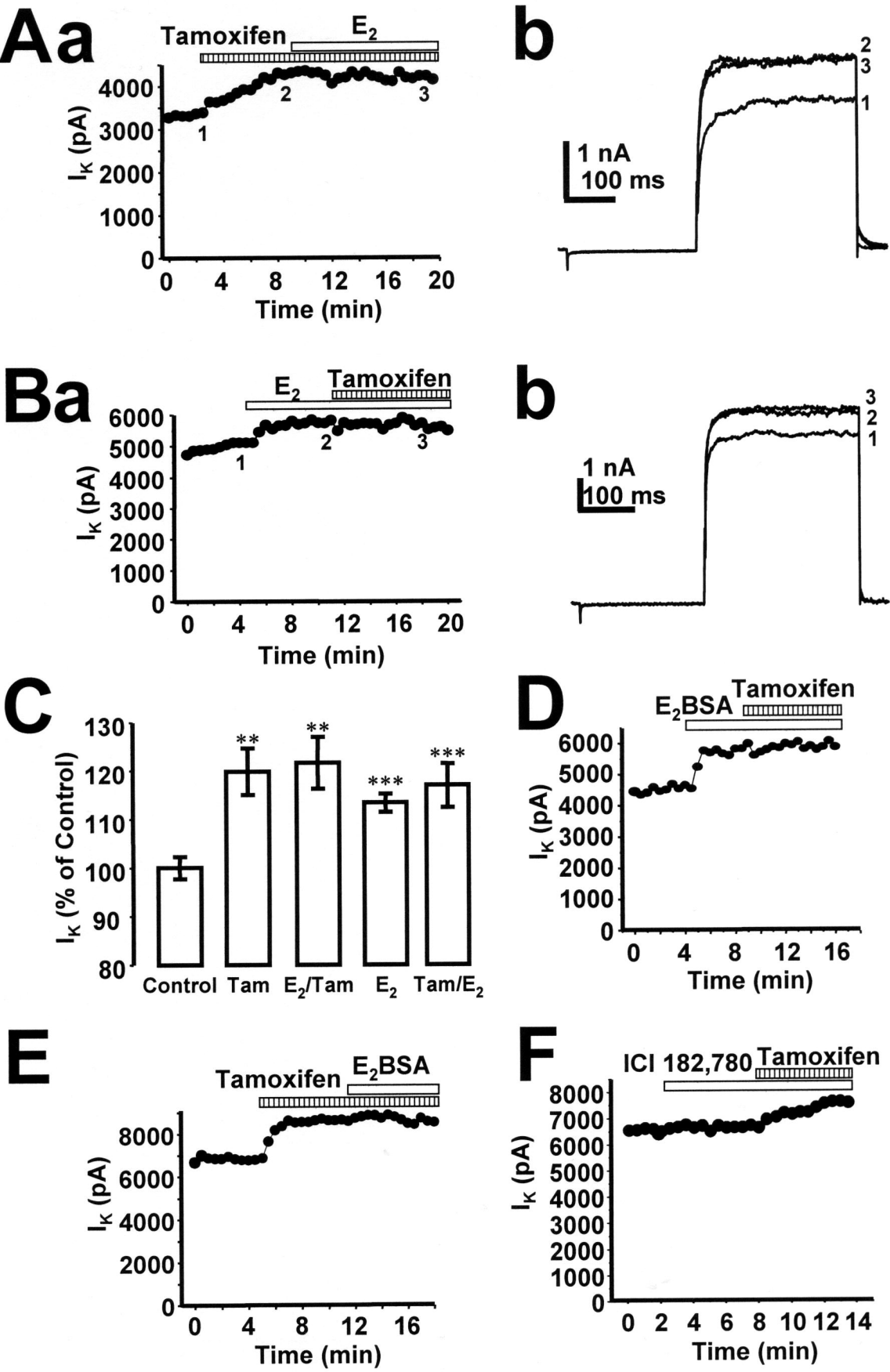

Aa
Tamoxifen
E2
IK (pA)
Time (min)
b
1 nA
100 ms
Ba
E2
Tamoxifen
IK (pA)
Time (min)
b
1 nA
100 ms
C
IK (% of Control)
Control
Tam
E2/Tam
E2
Tam/E2
D
E2BSA
Tamoxifen
IK (pA)
Time (min)
E
Tamoxifen
E2BSA
IK (pA)
Time (min)
F
ICI 182,780
Tamoxifen
IK (pA)
Time (min)

Figure 4

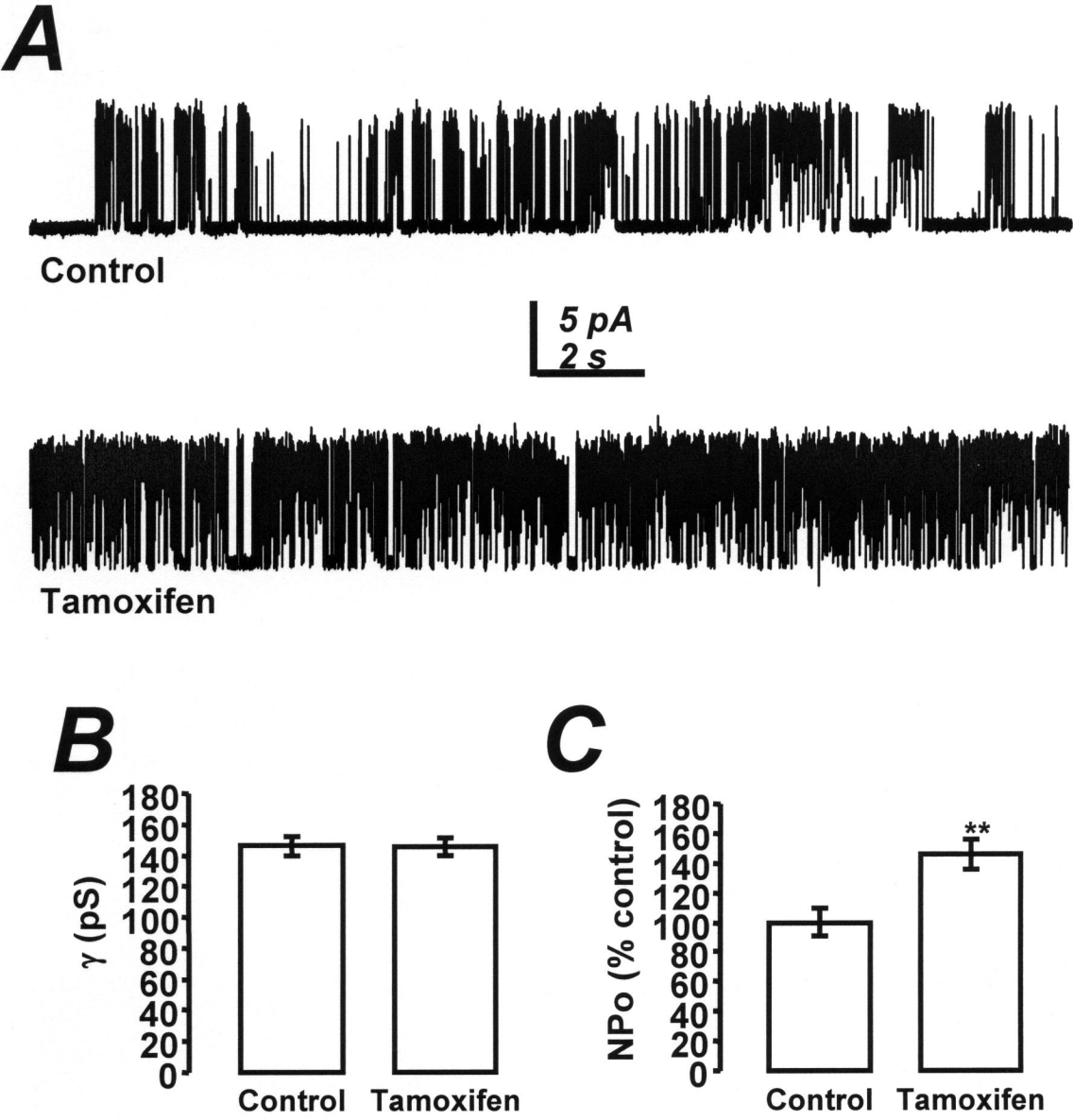

A
Control
5 pA
2 s
Tamoxifen
B
γ (pS)
180
160
140
120
100
80
60
40
20
0
Control
Tamoxifen
C
NPo (% control)
180
160
140
120
100
80
60
40
20
0
**
Control
Tamoxifen

Figure 5

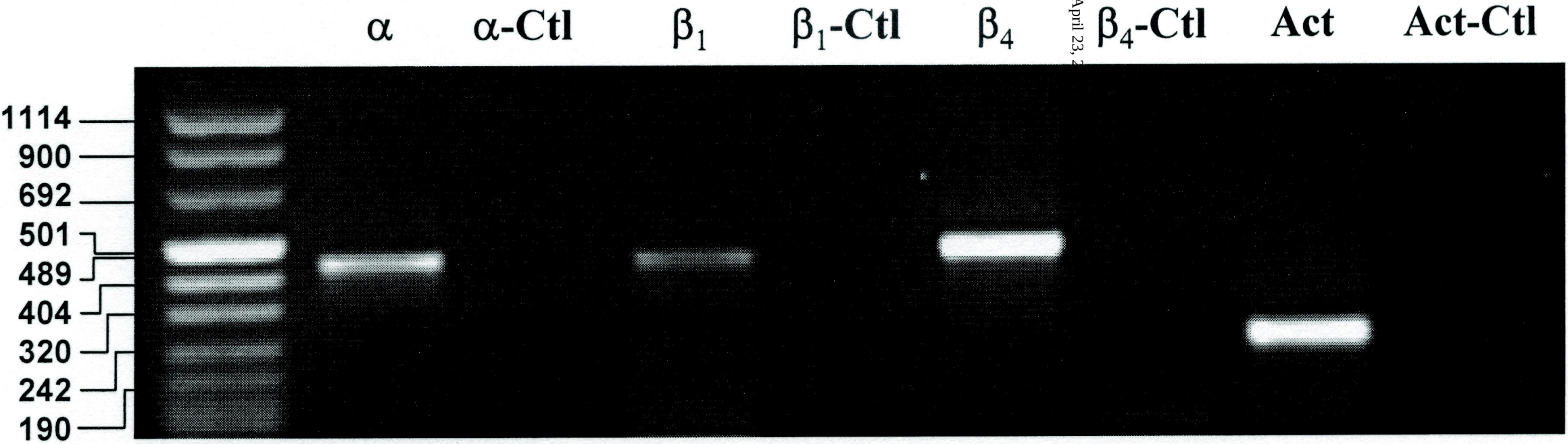

α
α-Ctl
β1
β1-Ctl
β4
β4-Ctl
Act
Act-Ctl
1114
900
692
501
489
404
320
242
190

Figure 6

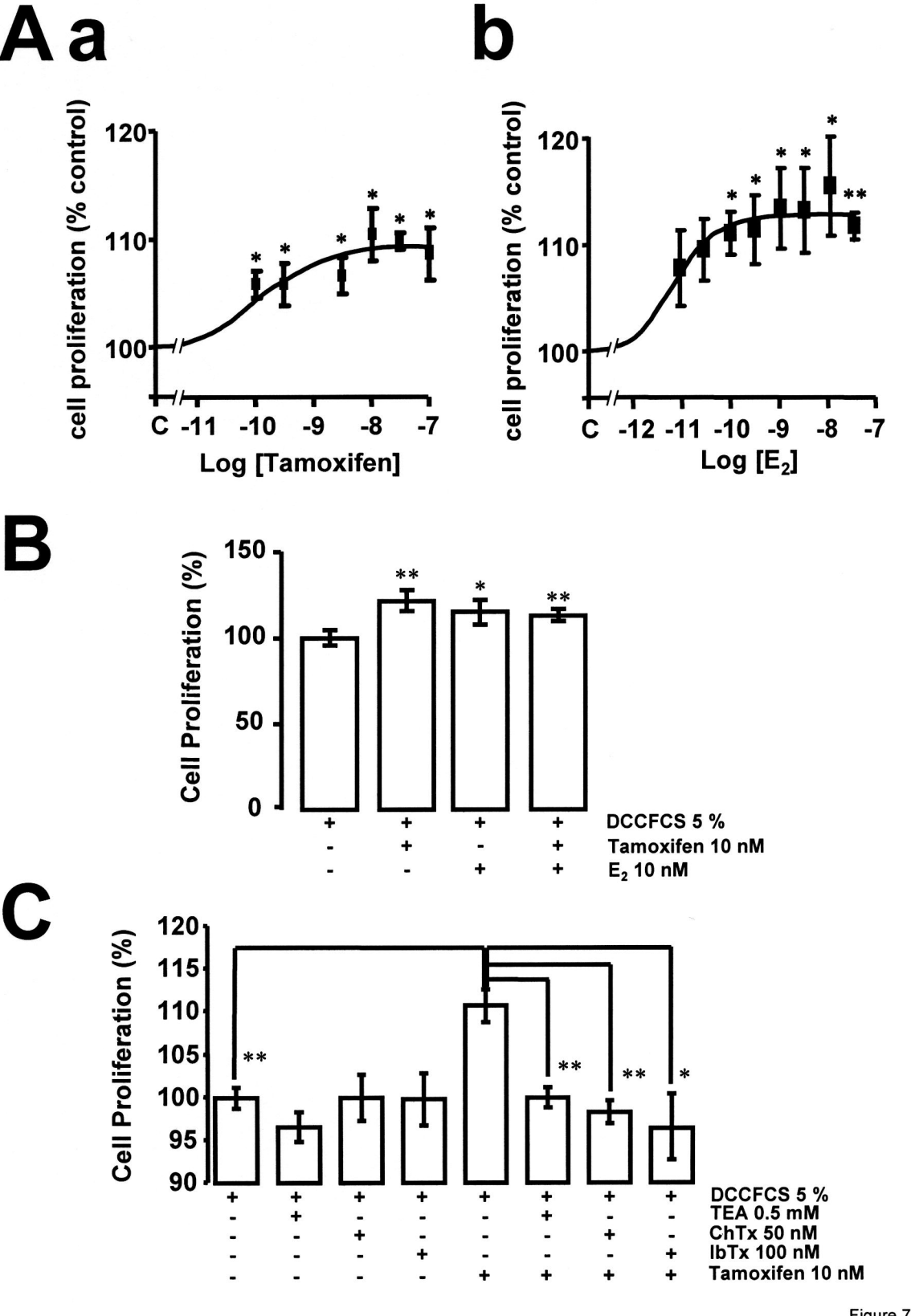

A a
cell proliferation (% control)
120
110
100
C -11 -10 -9 -8 -7
Log [Tamoxifen]
b
cell proliferation (% control)
120
110
100
C -12 -11 -10 -9 -8 -7
Log [E2]
B
Cell Proliferation (%)
150
100
50
0
DCCFCS 5 %
Tamoxifen 10 nM
E2 10 nM
C
Cell Proliferation (%)
120
115
110
105
100
95
90
DCCFCS 5 %
TEA 0.5 mM
ChTx 50 nM
IbTx 100 nM
Tamoxifen 10 nM

Figure 7