MOL PHARM

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, EA 2086, Université de Picardie Jules Verne, Faculté des Sciences, Amiens, France (G.C., A.-S.B., H.O.-A., F.M.); and Laboratoire de Physiologie Cellulaire, Institut National de la Santé et de la Recherche Médicale U800, Université des Sciences et Technologies de Lille, Villeneuve d'Ascq Cédex, France (P.M.)

MOLECULAR PHARMACOLOGY

ABSTRACT

In the present study, we 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 $22.6 \pm 10.6\%$ ($n = 23$). 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 tetraethylammonium (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 17-β-estradiol (E_2) or its membrane-impermeant form, β-estradiol 6-(O-carboxymethyl)-oxime:bovine serum albumin. Furthermore, the effect of tamoxifen was still recorded in the presence of the selective estrogen receptor antagonist faslodex (ICI-182,780; 1 μM). At the single-channel level, tamoxifen significantly increased the open probability of the BK channel by $46.2 \pm 10.1\%$ ($n = 4$) 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.

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, whereas 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 usefulness of antiestrogens and/or chemopreventives 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 Capri Workshop, 2004; Veronesi et al., 2005) has justified the use of antiestrogenic compounds such as tamoxifen in estrogenic hormone replacement therapy. However, in addition to their key role in female reproductive functions, estrogens have beneficial effects on unrelated tissues, as demonstrated by the effects of hormone replacement therapy on postmenopausal women (Mitlack 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 a partial agonist of the ER in the

ABBREVIATIONS: E_2, 17-β-estradiol; FCS, fetal calf serum; DCCFCS, dextran-coated charcoal-treated fetal calf serum; IbTx, iberiotoxin; ChTx, charybdotoxin; ER, estrogen receptor; NPo, open probability; E_2BSA, β-estradiol 6-(O-carboxymethyl)oxime:bovine serum albumin; SMC, smooth muscle cells; EMEM, Eagle's minimal essential medium; BAPTA, 1,2-bis(2-aminophenoxy)ethane-N,N,N′,N′-tetraacetic acid; TEA, tetraethylammonium; PCR, polymerase chain reaction; PBS, phosphate-buffered saline; ANOVA, analysis of variance; ICI-182,780, faslodex.

bone, where it mimics the beneficial effect of E_2 on the maintenance of bone density (Zidan et al., 2004).

A number of recent works (Clarke et al., 2001; Lösel et al., 2003) show that estrogens and xenoestrogens, such as 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 and Sanders, 2001; Dick et al., 2001), has been reported.

The aim of the present study was to investigate the short-term 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 coexpressed 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.

Materials and Methods

The breast cancer cell line MCF-7 was purchased from the American Type Culture Collection (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 IU/ml)/streptomycin (50 μg/ml). The EMEM solution was renewed every 2 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 (Molecular Devices, Sunnyvale, CA) and a Digidata 1200 interface (Molecular Devices). PClamp software (version 6.0.3; Molecular Devices) was used to control voltage and to acquire and analyze data. The whole-cell mode of the patch-clamp technique was used with 3 to 5 MΩ resistance borosilicate fire-polished pipettes (Hirschmann Laborgerate, Eberstadt, Germany). Seal resistance was typically in the range of 10 to 20 GΩ. The maximum uncompensated series resistance was <10 MΩ during whole-cell recordings, so the voltage error was <5 mV for a current amplitude of 500 pA. Recordings in which 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. 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 and 300 ms, respectively.

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 Corporation, Long Island, NY) low-pass-filtered at 3 kHz, and stored online on the hard drive of the computer. Electrodes were pulled in two stages on a PIP5 puller (HEKA) from borosilicate glass capillaries (PG52151; World Precision Instruments, Aston, UK) to a tip diameter giving a pipette resistance of 5 to 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 intrapipette calcium concentration of 300 nM. Single-channel analysis and open probability (NPo) determination were carried out using WinEDR software (version 2.6.9; J. Dempster, University of Strathclyde, Glasgow, Scotland, UK). 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 extracellular 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 nonreversibility of tamoxifen and the poor reversibility of iberiotoxin (IbTx) and charybdotoxin (ChTx), no more than one cell per Petri dish was recorded.

External and internal solutions had the following compositions: external: 145 mM NaCl, 5 mM KCl, 2 mM $CaCl_2$, 1 mM $MgCl_2$, and 10 mM HEPES at pH 7.4 (NaOH); internal: 150 mM KCl, 10 mM HEPES, 0.1 mM EGTA, and 2 mM $MgCl_2$ 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, Stanford, CA).

ChTx and IbTx were made up in 1% bovine serum albumin and 5 mM HEPES, pH 7.2. E_2BSA contained 35 mol of steroid per mole of bovine serum albumin 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 of the products were from Sigma-Aldrich (Lyon, 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 1×10^6 cultured cells using the TRIzol method (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions. RNA samples were treated with 1 U of DNase I (Promega, France) at 37°C for 30 min. A phenol/chloroform (v/v) 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 (optical density at 260 nm) and was reverse-transcribed into cDNA using an SSII kit (Invitrogen) by following the manufacturer's instructions. Complementary DNA was stored at −20°C. The PCR primers used to amplify the reverse-transcription-generated KCNMA1, KCNMB1, and KCNMB4 cDNAs were designed on the basis of established GenBank sequences. The primers for KCNMA1 cDNA were 5′-TGTTATGGTGATCTGTTCTG-C-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′-CAATGCAGGAGGA-CAATCTC-3′ (nucleotides 87–518, GenBank accession number AF207992). The expected amplified DNA length is 418, 412, and 431 base pairs for KCNMA1, KCNMB1, and KCNMB4, respectively. Primers were synthesized by Invitrogen. PCR was performed on the reverse-transcription-generated cDNA using a Bio-Rad iCycler thermocycler (Bio-Rad, Hercules, CA). PCR reaction mixtures contained 1 μl of 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 (1 U) (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 40 s 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 0.5× Tris borate-EDTA and stained with ethidium bromide. The following primers were used to amplify a 210-base pair β-actin cDNA fragment: 5′-CAGAGCAA-GAGAGGCATCCT-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, 40 s at 72°C, and then 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 Nonradioactive Cell Proliferation Assay; Promega France, Charbonnieres, 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 seemed 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 more than 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 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). Cells (6 × 10^5) in 300 μl of 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$_o$, G$_1$, S, and G$_2$/M) was acquired for 10,000 events using an Elite Beckman/Coulter flow cytometer (Beckman Coulter Inc., Fullerton, CA).

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 (∗, $P < 0.05$; ∗∗, $P < 0.01$; ∗∗∗, $P < 0.001$).

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 Fig. 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 with control (Fig. 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 greater than 20 mV (Fig. 1B). The time course for the effect of tamoxifen was

then investigated and is presented in Fig. 2, A 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 min of continuous washout (data not shown, $n = 6$).

Dose-response curves were then realized (Fig. 2C) and showed that the activation threshold appeared for tamoxifen concentrations of approximately 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 of 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 Fig. 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 inhibited previously by the 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 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 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).

Because both E$_2$ and tamoxifen regulate BK channels, we investigated whether their effects were additive. We first increased the BK current by the perfusion of 10 nM tamoxifen, and then we applied E$_2$ at the same concentration (Fig. 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, Fig. 4B). Statistical analysis revealed that even though the perfusion of tamoxifen or E$_2$ alone significantly enhanced the BK current amplitude compared with 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, Fig. 4C). Furthermore, it seemed that the effect of tamoxifen was also not additive to that of the membrane-impermeant form of E$_2$ (E$_2$BSA; Fig. 4, D and E). Indeed, whereas the perfusion of E$_2$BSA (Fig. 4D) or tamoxifen (Fig. 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 then tested the effect of tamoxifen in the presence of the pure ER antagonist ICI-182,780 (1 μM) to assess whether tamoxifen regulates the BK channel through the activation of the ER pathway. Results are depicted in Fig.

4F and clearly show that tamoxifen is still able to stimulate BK current by $17.5 \pm 3.8\%$ ($P < 0.05$, $n = 5$, 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. 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 (top trace) and after the perfusion of tamoxifen at a concentration of 10 nM (bottom 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 (Fig. 5B; $\gamma = 140.9 \pm 14.5$ pS, $n = 4$, versus 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 $46.2 \pm 10.1\%$ ($P < 0.01$, $n = 4$, paired t test; Fig. 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 Fig. 6 and clearly show that these subunits are coexpressed 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. To eliminate the eventual participation of native steroids contained in fetal calf serum (FCS), all of the following experiments were performed in culture medium complemented with steroid-free 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 ($10.4 \pm 3.7\%$, $P < 0.05$, $n = 8$, one-way ANOVA; Fig. 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 ($15.8 \pm 4.6\%$, $P < 0.05$, $n = 8$, one-way ANOVA; Fig. 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, because flow cytometry analysis showed that the number of cells in the sub-G_1 phase of the cell cycle was not significantly modified compared with control conditions (data not shown). We then compared the ability of tamoxifen, alone or in combination with E_2 to modulate cell proliferation. We
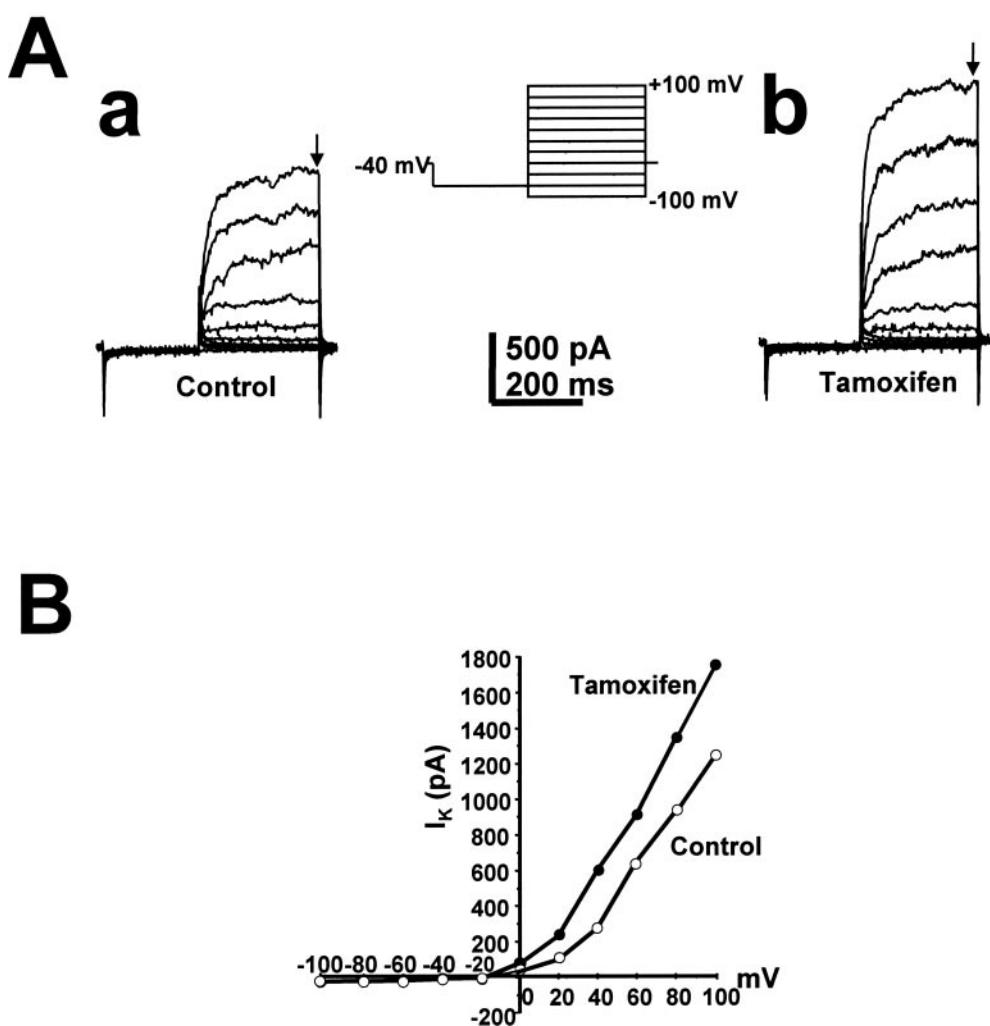

Fig. 1. Tamoxifen activates a voltage-dependent K^+ current in MCF-7 breast cancer cells. A, whole-cell K^+ currents were recorded in the MCF-7 cells with a physiological K^+ gradient (5 mM K^+ outside and 150 mM K^+ inside). Cells were held at -40 mV, and membrane potential was successively stepped at -100 mV for 200 ms and then at $+100$ mV for 300 ms every 30 s 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 ($\bigcirc$) and after the perfusion of tamoxifen ($\bullet$) at a concentration of 10 nM. These results clearly show that K^+ current was affected for all membrane potentials superior to $+20$ mV.

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, $n = 5$, Fig. 7B). 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 seemed that whereas 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 (Fig. 7C).
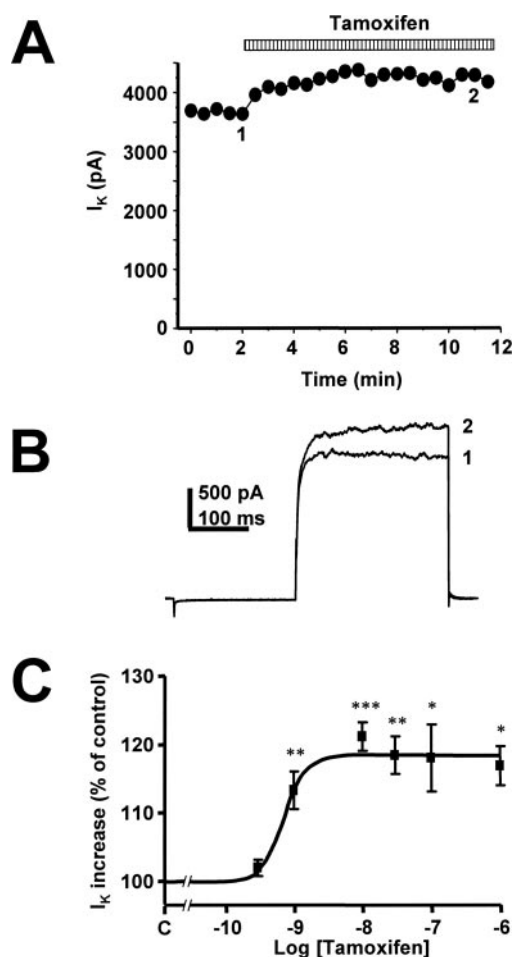
Discussion

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,b; 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 is recorded at the single-channel level. We thus hypothesize that they both modulate the BK channel by the means 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
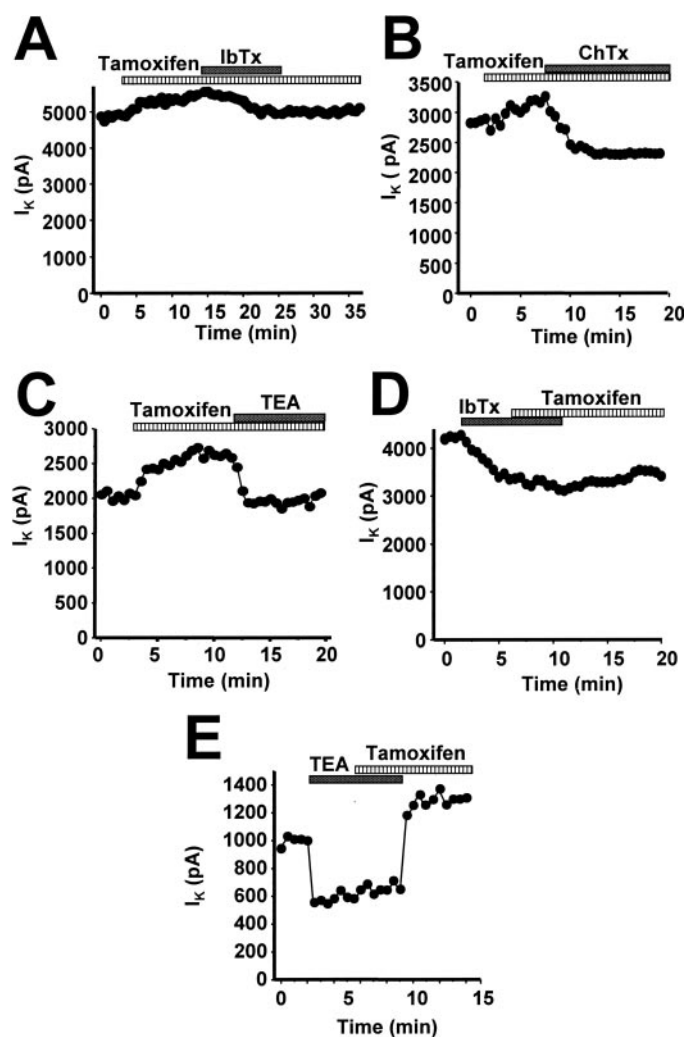

Fig. 2. The stimulatory effect of tamoxifen is dose-dependent and takes place rapidly. A, time course of the positive effect of 10 nM tamoxifen on the K^+ current. K^+ currents were elicited by stepping the membrane potential from −100 to +100 mV for 300 ms every 30 s. The maximal amplitude of K^+ current 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 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 experimental group median marked by asterisks is significantly different from the control median (one-way ANOVA with Bonferroni post hoc analysis).

Fig. 3. The molecular target of tamoxifen is the BK channel. A to E, time courses of K^+ currents elicited by stepping membrane potential from −100 to +100 mV for 300 ms every 30 s. A, the increase in K^+ current amplitude after bath application of tamoxifen (10 nM) was inhibited by the perfusion of the specific BK channel inhibitor IbTx (100 nM). B and C, the perfusion of ChTx (50 nM; B) or TEA (0.5 mM; C) inhibited the K^+ current that was enhanced previously by the application of 10 nM tamoxifen. D and 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.

tamoxifen. It has to be noted, however, that in the experiment presented in Fig. 3D, the effect of tamoxifen is weak after IbTx washout compared with the same effect recorded in control conditions or after the washout of 0.5 mM TEA (Fig. 3E). This smaller effect may be attributed to the poor reversibility of IbTx in these cells, as can be seen in Fig. 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 seems that the effect was irreversible even after 20 min of continuous washout, and the stimulation of the K^+ current by tamoxifen was still recorded after the inhibition of ER by the pure antiestrogen 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 classic ER_α and ER_β subtypes nor the recently postulated membrane ER (Toran-Allerand, 2004). This latter is not expressed in MCF-7 cells and is much more

sensitive to 17_α-E_2 (Toran-Allerand et al., 2002), a stereoisomer that is scarcely able to stimulate BK channel when expressed in *Xenopus laevis* 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 NPo of *Slo* α expressed in human embryonic kidney 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. Further-
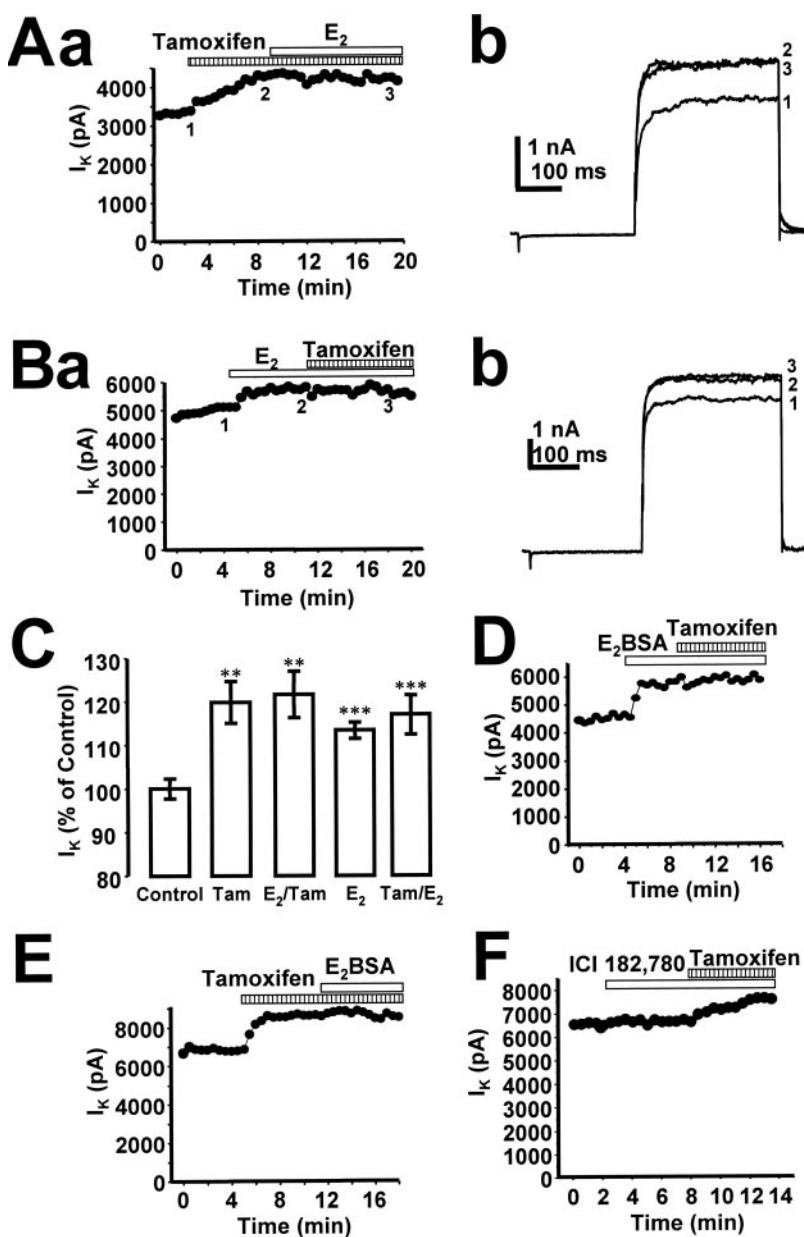
Fig. 4. The effect of tamoxifen on BK current is not additive to that of E_2. A and B and D to F, time course of K^+ currents elicited by stepping membrane potential from -100 to $+100$ mV for 300 ms every 30 s. E_2 at a concentration of 10 nM was ineffective if the BK current was activated previously 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 been enhanced previously by the membrane-impermeant form of E_2, E_2BSA (10 nM). In the same way, E_2BSA (10 nM) was ineffective after the previous perfusion of tamoxifen at the same concentration (E). F, the specific ER antagonist ICI-182,780 (1 μM) did not prevent the stimulatory effect of tamoxifen.

more, 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
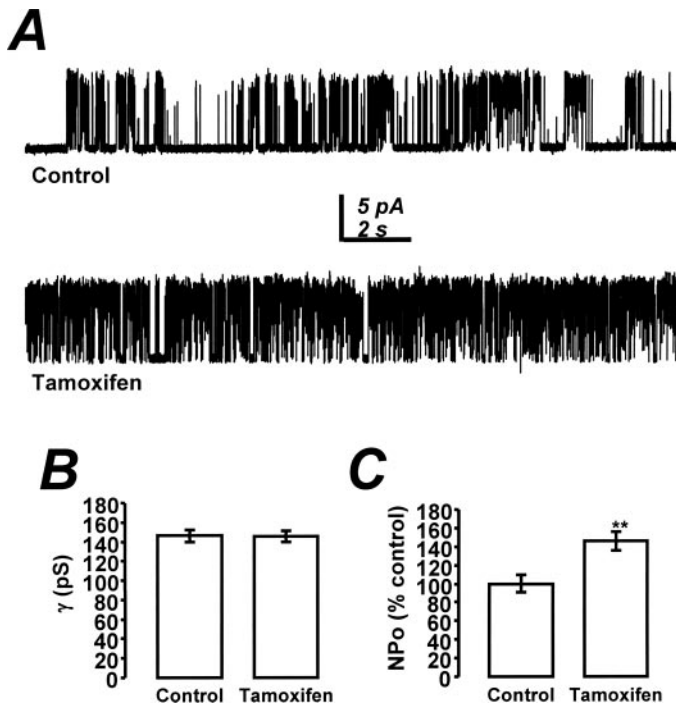

Fig. 5. Tamoxifen exerts its modulatory effect at the single channel level. A, time course of single BK currents recorded at a holding potential of 20 mV in the same patch of membrane before (control, top trace) and after (tamoxifen, bottom trace) the perfusion of tamoxifen at a concentration of 10 nM. 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 four patches. No significant difference could be observed between these two conditions ($\gamma = 140.9 \pm 14.5$ pS, $n = 4$, and 140.6 ± 14.3 pS, $n = 4$, for control and after tamoxifen treatment, respectively). C, NPo was calculated in control conditions (Control) and after the effect of 10 nM tamoxifen (tamoxifen). Tamoxifen stimulated NPo by $46.2 \pm 10.1\%$ ($P < 0.01$, $n = 4$, paired t test). The experimental group median marked by asterisks is significantly different to the control median (∗∗, $P < 0.01$).

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 versus 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 our study, there are three major differences with the current literature regarding xenoestrogen modulation of BK channels. First, BK channels in MCF-7 cells are stimulated by low doses of tamoxifen (in the nanomolar range), whereas other authors have reported the same effect for concentrations of tamoxifen in the micromolar range (Dick and Sanders, 2001; Dick et al., 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 min 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 coexpressed with α subunit in human embryonic kidney 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. To explain the nonreversibility of the modulatory action of tamoxifen, we submit that its interaction with the BK channel is sufficiently strong to withstand washout. A nonreversible 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
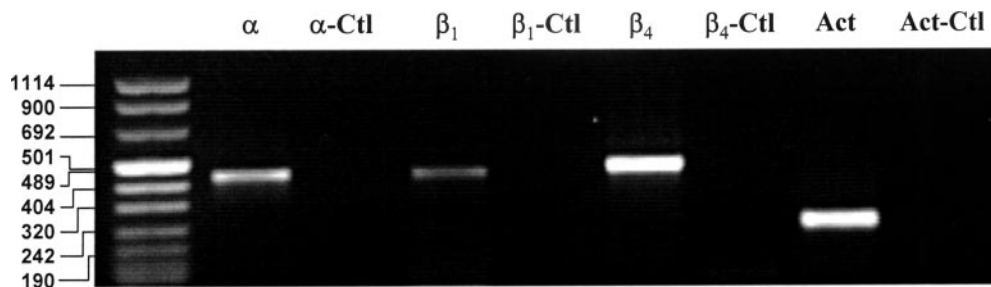
Fig. 6. BK channels' β_1 and β_4 auxiliary subunits are coexpressed with α in MCF-7 cells. cDNA samples were obtained by reverse-transcription of total RNAs from MCF-7 cells. PCR was then performed using specific primers given under *Materials and Methods* section. Amplified fragments were resolved by 1.2% agarose-gel electrophoresis and visualized by ethidium bromide staining. Numbers on the left indicate the molecular mass of the ladder (base pairs). α, β_1, β_4, and Act represent the fragments corresponding to BK channels subunits and β-actin, respectively. -Ctl suffix indicates the control conditions for each subunit subtype (i.e., water instead of cDNA).

tamoxifen is independent of the 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 the internal calcium concentration is lower than 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 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 (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 corepressor and silencing mediator for retinoid and thyroid receptors have been silenced. This study shows that nuclear receptor corepressor and silencing mediator for retinoid and thyroid receptors play a role in tamoxifen-bound ER_α action and that the relative level of ER coregulators can influence the cellular response to tamoxifen. Nevertheless, in our study, the stimulatory effect of tamoxifen does not seem to involve ER_α because 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.
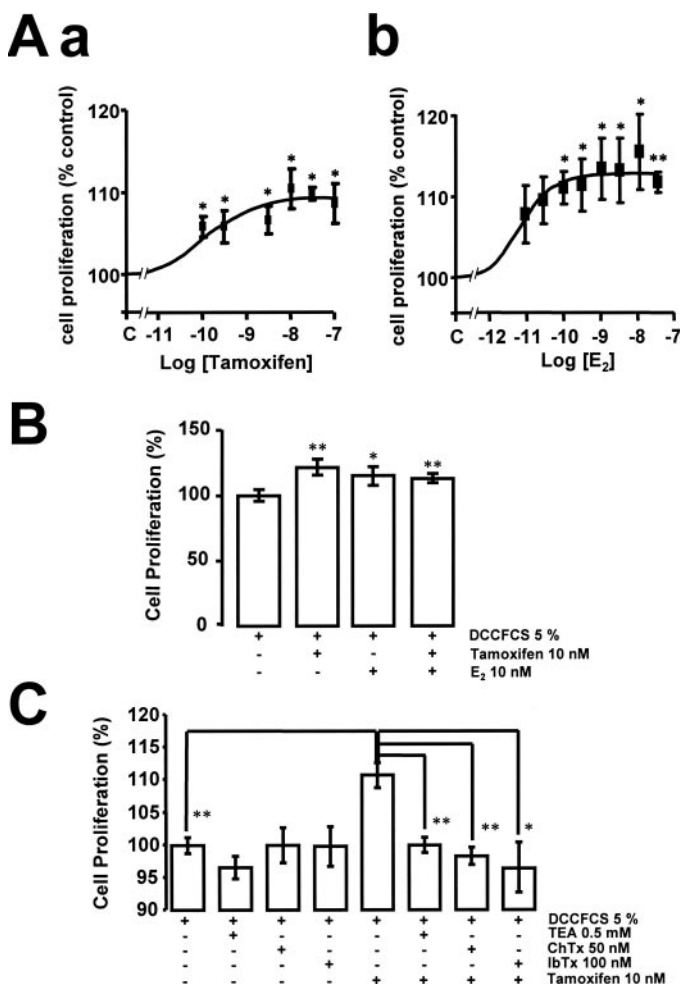
Fig. 7. Tamoxifen stimulates MCF-7 cell proliferation through the stimulation of BK channel. To eliminate the eventual participation of native steroids contained in FCS, all of the experiments were performed in culture medium complemented with steroid-free FCS (DCCFCS). A, dose-effect curves of tamoxifen (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. Whereas 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).

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Address correspondence to: Dr. Fabrice Matifat, Laboratoire de Physiologie Cellulaire et Moléculaire, EA 2086, Université Picardie Jules Verne, Faculté des Sciences, 33, Rue St Leu 80000 Amiens, France. E-mail: fabrice.matifat@u-picardie.fr

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