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Biochimica et Biophysica Acta 1667 (2004) 190–199

BIOCHIMICA ET BIOPHYSICA ACTA BBA

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Description and role in proliferation of iberiotoxin-sensitive currents in different human mammary epithelial normal and cancerous cells

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Received 14 June 2004; received in revised form 28 September 2004; accepted 13 October 2004
Available online 27 October 2004

Abstract

Several studies suggested that potassium channels are involved in the proliferation of cancer cells but the involvement of the large conductance Ca^{2+}-activated K^+ channels (BK_{Ca}) in the cancerous phenomenon is still controversial. In the present study, we used iberiotoxin, a specific blocker of BK_{Ca}, and report the activity of an iberiotoxin-sensitive current in various human breast cancer cell lines (MCF-7, MDA-MB-231, MDA-MB-468 and MDA-MB-435s) as well as in normal mammary epithelial cells (HME).

Iberiotoxin and NS1619, an activator of BK_{Ca}, did not interfere with either cell proliferation or with the invasive properties of the cells, under normal culture conditions. However, extracellular pulses of ATP, which induced transient increases in intracellular Ca^{2+} concentration, revealed a significant reduction effect of iberiotoxin on cell proliferation.

We conclude that the iberiotoxin-sensitive current is not involved in cell proliferation in basal conditions but participates when the intracellular Ca^{2+} concentration is increased. These experiments also suggest that BK_{Ca} channels are not involved in the cancerous transformation and are probably a relic from normal cells.

Keywords: Proliferation; Iberiotoxin; Cancerous

1. Introduction

Increased K^+ channel activity has been reported to be associated with increased proliferation rates in several breast cancer cell lines [1,2]. A variety of different K^+ channels involved in mammary cancer cell proliferation have been described. These K^+ channels include ATP-sensitive channels [1], Ca^{2+}-activated channels [3, 4], voltage-activated Kv1.1 channels [5] and EAG K^+ channels [2]. Very recently, two Ca^{2+}-sensitive potassium channels have been described in MCF-7 cells [6,7]. The mechanisms of action of these K^+ channels are unknown but it has been proposed that they simply act through the changes in membrane potential [8]. The activation of Ca^{2+}-activated K^+ channels leads to a hyperpolarisation of the membrane and could lead to regulation of the transition from G1 to S phase of the mitosis [9]. Ca^{2+}-activated K^+ currents have been observed in breast cancer cell lines but their nature was not clearly established in these original experiments [3,4]. The high incidence of these currents in actively proliferating cells, as observed by Wegman et al. [3], initiated the hypothesis that they could play a role at the G1/S transition [9,10]. In accordance with this observation, the intermediate conductance (IK_{Ca} or hIK1) has been involved in cell proliferation [6]. In other studies, the use of specific Ca^{2+}-activated K^+ channel blockers such as apamin (blocking the small conductance, SK_{Ca}), charybdotoxin (blocking the intermediate conductance, IK_{Ca}, and the large conductance, BK_{Ca}) and iberiotoxin (blocking BK_{Ca}) did not have any effect on cell proliferation [1,2]. These studies have been performed in MCF-7 cells and it is not known whether the activity of Ca^{2+}-activated K^+ current is specific of some cancer cell lines.

The aims of the present study were (1) to characterise the presence of a BK_{Ca} current, in various cancer and normal

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doi:10.1016/j.bbamem.2004.10.002

cell types, using the patch-clamp technique and pharmacological tools; (2) to evaluate its participation in cell proliferation in standard conditions and in conditions where intracellular Ca^{2+} is increased; and (3) to determine if this conductance is altered in cancer cells.

2. Materials and methods

2.1. Cell culture

The human breast cancer cell lines MCF-7 (HTB-22, weakly metastatic), MDA-MB-468 (HTB-132, metastatic), MDA-MB-231 (HTB-26, strongly metastatic) and MDA-MB-435S (HTB-129, strongly metastatic) were purchased from the American Type Culture Collection (Rockville, MD, USA) at passages 151, 340, 28 and 240, respectively. All experiments were carried out within 20 additional passages. Cells were grown in Dulbecco's Modified Eagle's Medium (DMEM, 4.5 g/L glucose, 584 mg/L glutamine and 3.7 g/L $NaHCO_3$) supplemented with 5% foetal bovine serum (FBS).

The normal human mammary epithelial cells (HME cells) were obtained from Cambrex Bio Science (Walkersville, MD, USA) at passage 7. All the experiments were carried out within six additional passages. These cells were grown with the optimised medium: Clonetics MEGM® BulletKit (Cambrex Bio Science, Walkersville, MD, USA) containing mammary epithelial cell basal medium (MEBM) supplemented with 4% bovine pituitary extract, 0.1% human recombinant EGF, 0.1% hydrocortisone, 0.1% insulin and 0.1% antibiotics from a mixture of gentamicin sulfate and amphotericin-B.

Cells were grown at 37 °C in an atmosphere containing 5% CO_2 and saturated with humidity.

2.2. Electrophysiology

For electrophysiological analyses, cells were seeded into 35-mm Petri dishes at 2500 cells/cm^2. Before patch-clamping, the growth medium was discarded and replaced with a physiological saline solution (PSS, see Solutions). Patch pipettes were pulled from non-heparinized haematocrit tubes to a resistance of 3–5 MΩ. Currents were recorded under voltage-clamp mode at room temperature using an Axopatch 200 B patch clamp amplifier (Axon Instrument, Burlingame, CA, USA). Membrane potentials were obtained in current clamp mode (I=0) and/or from the voltage at null current after junction potentials were compensated. Analogue signals were filtered at 5 kHz, using a five-pole low-pass Bessel filter, and sampled at 10 kHz using a 1322-A Digidata converter. PClamp software (v8.1, Axon Instrument) was used for generation of voltage commands, acquisition and analysis of whole-cell currents. Cell capacitance and series resistances were electronically compensated by about 60%. The cell under investigation was continuously superfused with PSS or test solutions. The superfusion was performed by positioning the cell under study at the tip of a conical microcapillary that received the outlet of six microcapillaries connected to 20-ml syringes. Solutions were selected with solenoids allowing flowing or not (flow rate 500 μl/min).

The ramp protocol used to evaluate the cell current–voltage features was a voltage ramp of 0.34 V s^{-1} from a holding potential of −100 mV.

The +60-mV depolarising protocol was as follows: from a holding potential at −70 mV, the membrane was depolarised and held at +60 mV for 500 ms at a stimulation frequency of 0.5 Hz. This protocol was used in order to evaluate the effect of pharmacological agents on the current.

The pulse protocol used to build the current–voltage (I–V) curve was as follows: from a holding potential at −70 mV, the membrane was depolarised and held for 100 ms at potentials between −70 and +90 mV, with 10-mV increments, at a stimulation frequency of 0.125 Hz. The Iberiotoxin-sensitive current was defined as the net difference between the current measured in the PSS condition and the one measured in the presence of Iberiotoxin. Current amplitudes were measured by averaging the last 50 ms of the voltage pulse and were normalised on cell capacitance to take into account differences in cell sizes and expressed as current density (pA/pF).

2.3. Fluorescence measurements

Intracellular levels of Ca^{2+} were studied using the ratio fluorescent dye fura-2. Cells were cultured at 5×10^4 cells per dish in WillCo-dish® glass bottom dishes (WillCo Wells, The Netherlands). Cells were incubated for 60–75 min at room temperature in PSS (see Solutions) containing 5 μM fura-2 AM, the membrane-permeant acetoxymethyl ester form of fura-2. Excess dye was removed by rinsing the cells four times with 1-ml PSS. The cells were then incubated for an additional 45–60 min in PSS to allow complete hydrolysis of the acetomethyl ester.

The dish was then placed on the stage of a Nikon Eclipse TE2000-S inverted epi-illumination microscope (Nikon, France). The excitation light source was a 75-W Xenon arc lamp. Excitation light at the two excitation wavelength maxima of fura-2 (340/380 nm) was chopped by a monochromator (Cairn Optoscan, UK). The excitation protocol was a 50-ms excitation at each wavelength every 2 s. Excitation light was directed through a 60× oil immersion objective with a numerical aperture of 1.4 (Nikon Plan Apo, France). Fluorescence emission at 510±20 nm was detected by a photomultiplier tube (PMT) placed on the side of the microscope. The analogic signal of the PMT was digitized by a Digidata 1322 A converter (Axon Instrument) at a sampling frequency of 2 kHz and further analysed using Clampex 8.2 (Axon Instrument). Autofluorescence was negligible (less than 5% of the total emission signal at the two excitation wavelengths). Background fluorescence was determined at 340 and 380 nm from an area of the coverslip free of cells

after the loading period and wash. These values were routinely subtracted. Intracellular calcium concentration was calculated as described previously using in situ calibration [11]. A Kd of 150 nM was used for these calculations, according to the supplier information on this batch of fura-2 (Molecular Probes, USA).

2.4. Solutions

The physiological saline solution (PSS) had the following composition (in mM): NaCl 140, KCl 4, $MgCl_2$ 1, $CaCl_2$ 2, D-glucose 11.1, and HEPES 10, adjusted to pH 7.4 with 1 M NaOH.

The pipette solutions were named based on their calculated free Ca^{2+} concentration [12]: pCa X indicates a free Ca^{2+} concentration of $[Ca^{2+}]=10^{-X}$ M.

The pCa 7 pipette solution had the following composition (in mM): K-glutamate 125, KCl 20, $CaCl_2$ 0.37, $MgCl_2$ 1, Mg-ATP 1, EGTA 1, HEPES 10, adjusted to pH 7.2 with 1 M KOH; the pCa 6.4 pipette solution: K-glutamate 125, KCl 20, $CaCl_2$ 0.7, $MgCl_2$ 1, Mg-ATP 1, EGTA 1, HEPES 10, adjusted to pH 7.2 with 1 M KOH.

Iberiotoxin (IbTx) and NS1619 (1-(2′ -hydroxy-5′ -trifluoromethylphenyl)-5-trifluoromethyl-2(3*H*)benzimidazolone) were added to the PSS at the concentrations indicated in the figure legends. All drugs and chemicals were purchased from Sigma-Aldrich (St Quentin, France).

2.5. Cell survival and proliferation

Cells were seeded at 4×10^4 cells per well in six wells of a 24-well plate for a given condition on three separate experiments. Medium and the different substances tested were changed every other day for experiments assessing the effect of blockade, or activation, of BK_{Ca} channels on cell proliferation and survival (at the concentrations indicated in figure legends).

For the experiments which tested the effect of 100 μM extracellular ATP on the MDA-MB-231 cells proliferation, cells were seeded at 4×10^4 cells per well in eight wells of a 24-well plate for a given condition, on three separate experiments. Three times a day, cells were submitted to a 30-min-long ATP pulse, separated by incubations in ATP-free medium. This condition was compared to a control condition without ATP, and to a condition in which ATP pulses were performed, in the continuous presence of 100 nM IbTx, during and after ATP pulses. To assess a possible influence of ATP incubation on enzymatic reduction of the tetrazolium salt (which is used to evaluate proliferation, see below), 24 wells were seeded at subconfluence and allowed to adhere to the surface of the wells. The day after, the cells in 12 wells were treated with 100 μM ATP for 45 min while the cells in the other 12 wells were treated with the solvant only, before performing the MTT assay. No difference was found, indicating that the ATP treatment did not interfere with the MTT assay.

Growth and viability of cells were measured as a whole, after a total of 5 days, by the tetrazolium salt assay [13]. Cells were incubated at 37 °C with the tetrazolium salt (3-[4,5-dimethyltiazol-2-yl]-2,5-diphenyl tetrazolium bromide) and metabolically active cells reduced the dye to purple formazan. Formazan crystals were dissolved with DMSO. The absorbance was measured at 570 nm. Identical numbers of living cells of the different cell lines yielded weaker or stronger formazan absorbances, indicating different reducing capacities among the different cell lines. Cell proliferation was expressed as formazan 570-nm absorbance and not converted to cell numbers since there always was a control condition for each cell line on each day of experiment. Standard curves for the absorbance of formazan were made for each cell line in order to determine the MTT incubation times in the linear part of the curves which allowed to use the test.

For easier comparison between conditions, results obtained for proliferation were normalised: cells measured in wells, in a given condition, were added and the ratio of these sums (total number of cells in presence of drug/total number of cells in control experiments) calculated for each experiment. The means were then calculated on these daily calculated ratios. The mean of each triplicate was used to create data points for comparing cell growth in different conditions.

2.6. Cell migration and invasion in vitro

Migration and invasion were evaluated as published previously [14]. Migration was analysed in BD Falcon™ 24-well plates receiving 8-μm pore size polyethylene terephtalate membrane BD Biocoat™ cell culture inserts (Becton Dickinson, France). For all the four breast cancer cell lines, the upper compartment was seeded with 4×10^4 viable cells in DMEM with 5% FBS and the lower compartment was filled with DMEM supplemented with 10% FBS as a chemoattractant. For HME cells, the upper compartment was seeded at the same cell density in MEGM® medium and the lower compartment received MEGM® supplemented with 10% FBS. After 24 h at 37 °C, cells remaining on the upper side of the membrane were removed with a cotton swab, and cells that had migrated to the lower side were stained with haematoxylin for 2 min and counted in five different randomly taken fields using a light microscope at ×200 magnification. In vitro invasion was assessed as above but with the membrane covered with a film of Matrigel®. Migration and invasion assays were performed in triplicate in two separate experiments for all the cell types. For easier comparison between cell lines, results obtained for migration and invasion were normalised: cells counted on inserts, in the different conditions, were added and the ratio of these sums (total number of cells in presence of drug/total number of cells in control experiments) calculated for each day. The means were then calculated on these daily calculated ratios.

2.7. Statistics

Data are described as mean ± standard error of the mean (n=number of cells). Cell capacitances, membrane potentials and membrane resistances were compared by an Kruskal–Wallis ANOVA on ranks followed by the Dunn's test. Kruskal–Wallis ANOVA on ranks followed by a Student–Neumann–Keuls test was used to compare proliferation results. $P<0.05$ was considered as significant.

3. Results

3.1. Electrophysiological characterisation of BK_{Ca} current in normal and cancer cell types

In the whole-cell configuration of the patch-clamp technique, with a clamped intracellular Ca^{2+} concentration of 100 nM (pCa 7), we found, in the MCF-7 cell line, an outward-rectifying outward current which is sensitive to

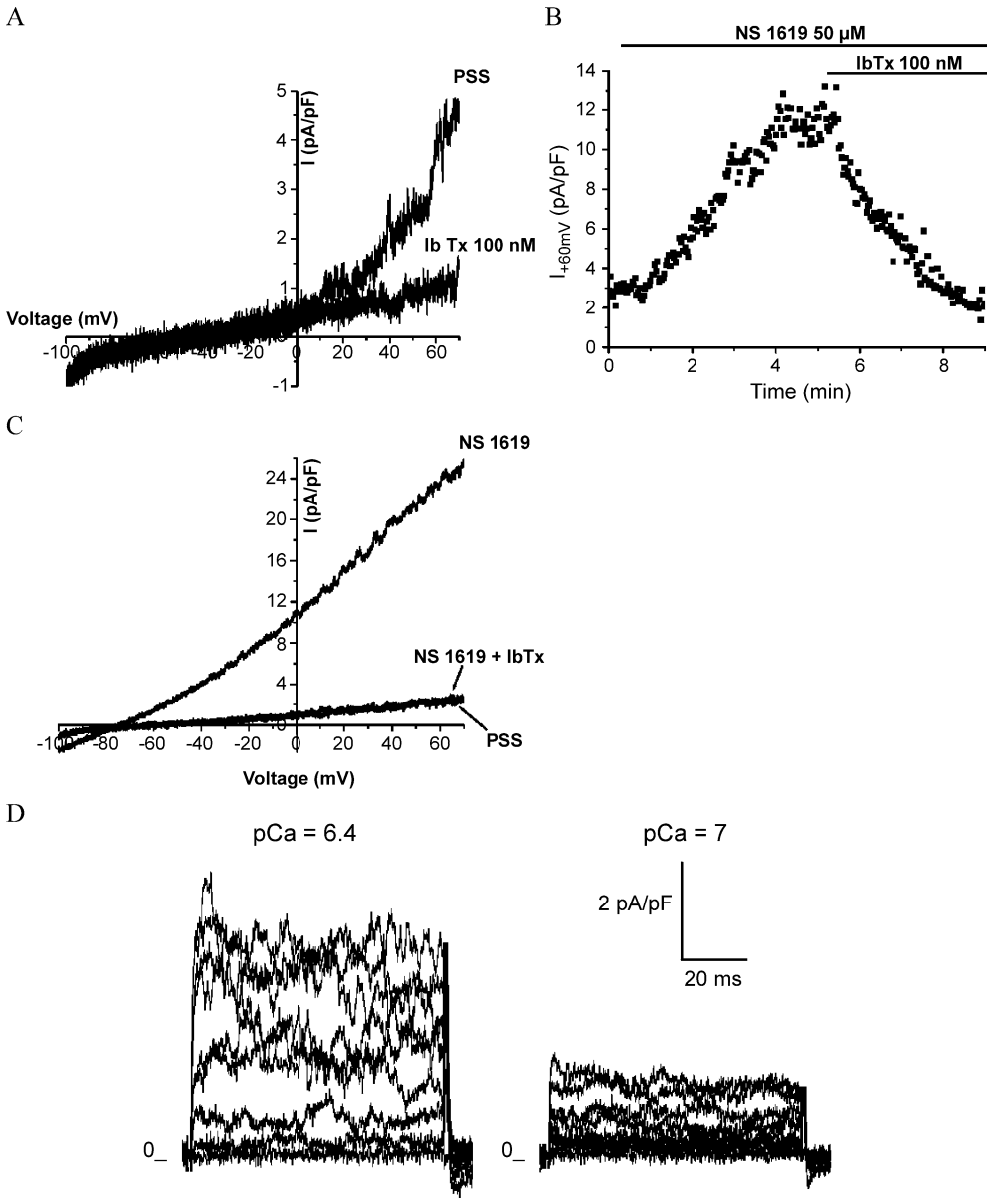

Fig. 1. Identification of an IbTx-sensitive outward current in MCF-7 and MDA-MB-231 breast cancer cell lines. (A) Representative recording of a current elicited by a voltage ramp protocol (see METHODS) in MCF-7 cells, before and after application of 100 nM IbTx in the external solution. The pCa of the pipette solution was 7. (B) Effect of IbTx on the amplitude of the end-pulse outward current elicited by a +60-mV depolarisation from a holding potential of −70 mV and activated by the BK_{Ca} activator NS1619, on a representative MCF-7 cell. The pipette solution used had a pCa 7. NS1619 and IbTx were externally applied to the cell at the times and concentrations indicated on top. (C) Examples of instantaneous currents elicited by the ramp protocol, in a representative MDA-MB-231 cell, by a continuous depolarisation from a holding potential of −100 to +70 mV, in 500 ms. Three successive solutions were used: external perfusion of PSS, then PSS with 50 µM NS1619, and finally PSS with 50 µM NS1619 plus 100 nM IbTx. The pipette solution had a pCa 7. (D) Examples of IbTx-sensitive currents elicited, in two representative MDA-MB-231 cells, by 100-ms step potentials ranging from −70 to +90 mV, every 20 mV. Recordings were obtained with the two pipette solutions, pCa 6.4 and pCa 7, as indicated above the traces.

IbTx, a specific blocker of BK_{Ca} [15] (Fig. 1A). This current can be further activated by the Ca^{2+}-sensitive BK_{Ca} activator NS1619 (50 μM) [16] and then selectively blocked by 100 nM IbTx (Fig. 1B). In the MDA-MB-231 cell line, we previously reported that there were no open K^+ channels, using an uncontrolled intracellular free Ca^{2+} concentration solution [17]. Identical results were obtained using pCa 7 pipette solution. However, when the Ca^{2+}-sensitive BK_{Ca} activator NS1619 was superfused, a large outward current was elicited (Fig. 1C). Activation of this outward current led to a significant membrane potential hyperpolarisation, from −35.3±1.5 to −64.7±1.8 mV (n=9). This outward current

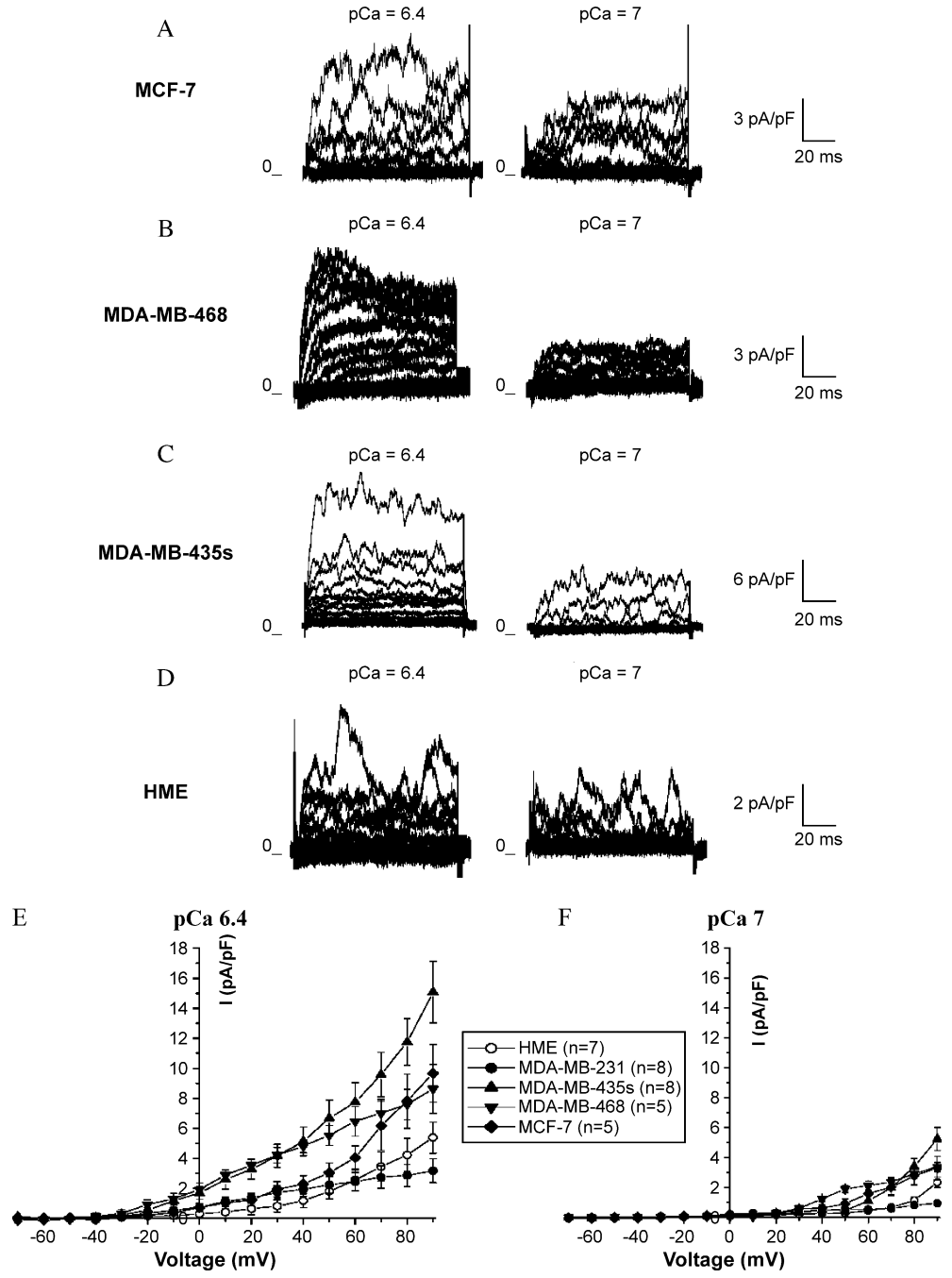

Fig. 2. Identification of an IbTx-sensitive outward current in MCF-7, MDA-MB-435S, MDA-MB-468 breast cancer cell lines and HME cells. Identification of IbTx-sensitive currents elicited, in representative cells, by 100-ms step potentials ranging from −70 to +90 mV, every 10 mV. Recordings were obtained, with the two pipette solutions, pCa 6.4 (left traces) and pCa 7 (right traces), in (A) MCF-7, (B) MDA-MB-468 and (C) MDA-MB-435S breast cancer cells and (D) in representative normal HME cells. Mean current–voltage relation for the IbTx-sensitive (100 nM) current obtained from the normal human mammary epithelial cells (HMEC) and from the human breast cancer cell lines MCF-7, MDA-MB-231, MDA-MB-435S and MDA-MB-468. From a holding potential of −70 mV, the cell membrane was depolarised and held for 100 ms at potentials between −70 and +90 mV, with 10-mV increments, at a stimulation frequency of 0.125 Hz, with the (E) pCa 7 or the (F) pCa 6.4 pipette solution. The number of investigated cells is indicated in brackets.

Table 1
Passive membrane properties of the different cell types studied

	Cell capacitance (pF)	pCa 7		pCa 6.4	
		MP (mV)	Rm (MΩ)	MP (mV)	Rm (MΩ)
HMEC	58.2±2.8† (*n*=24)	−32.0±2.1* (*n*=11)	860±57* (*n*=11)	−47.6±3.1 (*n*=13)	660±36 (*n*=13)
MCF-7	38.1±1.9 (*n*=59)	−29.3±1.9* (*n*=11)	750±36 (*n*=11)	−51.9±2.9 (*n*=26)	710±39 (*n*=26)
MDA-MB-468	41.2±1.7 (*n*=48)	−34.2±0.6* (*n*=13)	840±56* (*n*=13)	−62.9±1.6 (*n*=28)	450±21 (*n*=28)
MDA-MB-231	37.3±1.4 (*n*=40)	−35.4±1.8* (*n*=22)	1 020±36* (*n*=22)	−46.0±3.7 (*n*=14)	800±35 (*n*=14)
MDA-MB-435S	39.4±1.2 (*n*=57)	−30.6±1.6* (*n*=21)	830±29* (*n*=21)	−64.0±1.5 (*n*=27)	530±27 (*n*=27)

Summary of electrophysiological data comparing the five cell types for their cell capacitances, their membrane potentials (MP) and their membrane resistances (Rm) at the two concentrations of intracellular Ca^{2+}, pCa 7 and pCa 6.4. Data are expressed as mean±S.E. (*n*=number of cells).

* $P<0.05$ when comparing MP or MR at pCa 6.4 vs. pCa 7.

† $P<0.05$ when comparing cell capacitances.

activated by 50 μM NS1619 was fully blocked by the addition of 100 nM IbTx (Fig. 1C).

To confirm the nature of this current, and to electrophysiologically characterise it, we used the current–voltage relationship protocol in the absence and presence of 100 nM IbTx, using two different pCa in the pipette solutions: pCa 7 and pCa 6.4. Even in the MDA-MB-231 cell line, which has very few functional outward K^+ currents, it is possible to unveil the presence of a small amplitude IbTx-sensitive current, by the accurate control of the intracellular free Ca^{2+} concentration (Fig. 1D).

Fig. 2A shows typical recordings of this current in two representative MCF-7 cells. A larger current was observed in the presence of the pipette Ca^{2+} solution, pCa 6.4. This current was not only observed in MDA-MB-231 (Fig. 1D) and MCF-7 cells (Fig. 2A), but also in MDA-MB-468 (Fig. 2B) and MDA-MB-435S (Fig. 2C) breast cancer cells. In these four cell lines, the IbTx-sensitive current density was increased when intracellular Ca^{2+} was elevated (pCa 6.4).

The presence of this conductance in all the cell lines tested raised the question of its link to cancer. Therefore, we searched for the activity of BK_{Ca} in long-term primary cultures of HME cells, and found it as shown in Fig. 2D.

In order to characterise this current electrophysiologically, mean current–voltage curves have been constructed for each cell type, and with the two pCa pipette solutions (Fig. 2E and F). With the pCa 7 intracellular condition (Fig. 2F), the activation threshold of this current is between −20 and −10 mV and the current exhibits an apparent outward rectification, for all the cell types. Fig. 2E shows that at a higher intracellular free Ca^{2+} concentration (pCa 6.4), the apparent activation threshold is shifted towards hyperpolarised membrane potentials. This Ca^{2+}-sensitive current activates around −40 mV and reaches a current density two to three times higher than with the pCa 7 pipette solution. This was observed in all the cell types tested. The apparent activation threshold is identical in all five cell types.

At the two intracellular Ca^{2+} concentration, MDA-MB-435S cells exhibit the highest current density and MDA-MB-231 cells, the lowest. However, there is a tendency for a lower current density in HME cells in comparison to three of the four other cancer cell lines. This suggests that the level of expression and functioning of BK_{Ca} could be a marker of cancer cells.

Table 1 summarises the passive electrophysiological properties of the five epithelial cell types studied by the patch-clamp technique. There was no significant difference between the cell capacitances, a cell size indicator, of the four cancer cell lines. In contrast, HME cells have a significantly higher capacitance, indicating that they are bigger than the four breast cancer cell lines tested. MDA-MB-231 cells exhibited the highest membrane resistance, both at pCa 7 and at pCa 6.4. This suggests that they have only few, functional, open ionic channels and is in agreement with the observation that they displayed the lowest current density.

It is of interest to note that the membrane potential was significantly hyperpolarised in all cell types, and the membrane resistance was significantly reduced in all cell types but MCF-7, when the intracellular Ca^{2+} concentration was increased from pCa 7 to pCa 6.4. These data are in agreement with the activation of a Ca^{2+}-activated hyper-

Table 2
Effects of IbTx on the membrane potential of different cell types studied using two intracellular pCa

	pCa 7		pCa 6.4	
	Control	IbTx	Control	IbTx
HMEC	−32.0±2.0 mV (*n*=7)	−31.0±1.9 mV (*n*=7)	−48.5±1.9 mV (*n*=4)	−47.2±1.7 mV (*n*=4)
MCF-7	−26.6±4.0 mV (*n*=5)	−26.0±4.0 mV (*n*=5)	−48.5±4.4 mV (*n*=11)	−44.5±5.0 mV (*n*=11)
MDA-MB-468	−37.6±1.7 mV (*n*=5)	−35.8±1.6 mV (*n*=5)	−61.2±4.3 mV (*n*=8)	−58.5±4.6 mV (*n*=8)
MDA-MB-231	−36.0±4.6 mV (*n*=8)	−35.0±4.3 mV (*n*=8)	−44.1±5.0 mV (*n*=9)	−43.3±4.8 mV (*n*=9)
MDA-MB-435S	−37.5±3.5 mV (*n*=8)	−36.1±3.4 mV (*n*=8)	−65.1±2.8 mV (*n*=8)	−62.3±3.3 mV (*n*=8)

Summary of electrophysiological data comparing the effects of 100 nM IbTx on membrane potential, in five cell types at the two concentrations of intracellular Ca^{2+}, pCa 7 and pCa 6.4. Data are expressed as mean±S.E. (*n*=number of cells).

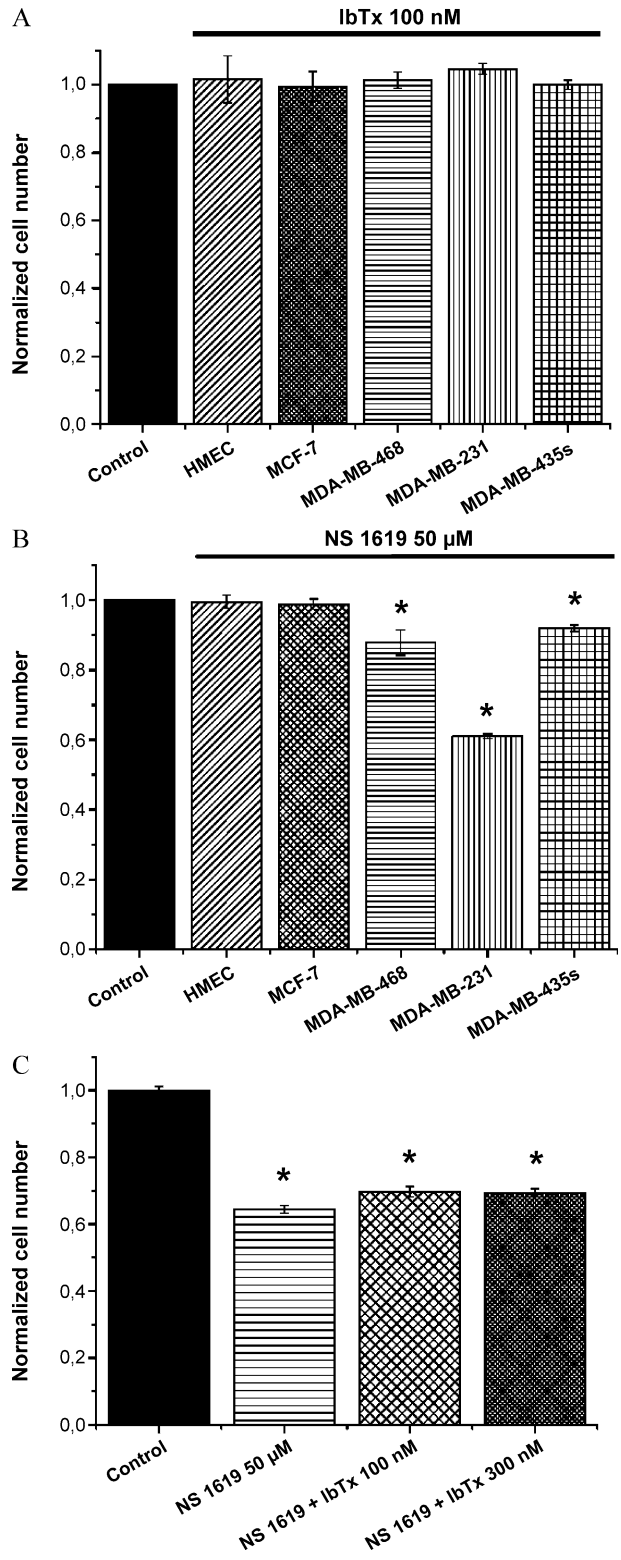

Fig. 3. Influence of the IbTx-sensitive currents on cell proliferation and survival. (A) Relative effects of 100 nM IbTx on cell proliferation and survival of the five different cell types. No significant difference was found. (B) Effect of 50 µM NS1619 in the different cell lines, as compared to a control condition (vehicle of the drugs). (C) Effect of 50 µM NS1619 without or with 100 or 300 nM IbTx on the proliferation and survival of MDA-MB-231 cells, as compared to control conditions (vehicle of the drugs). * $p<0.05$ vs. control conditions.

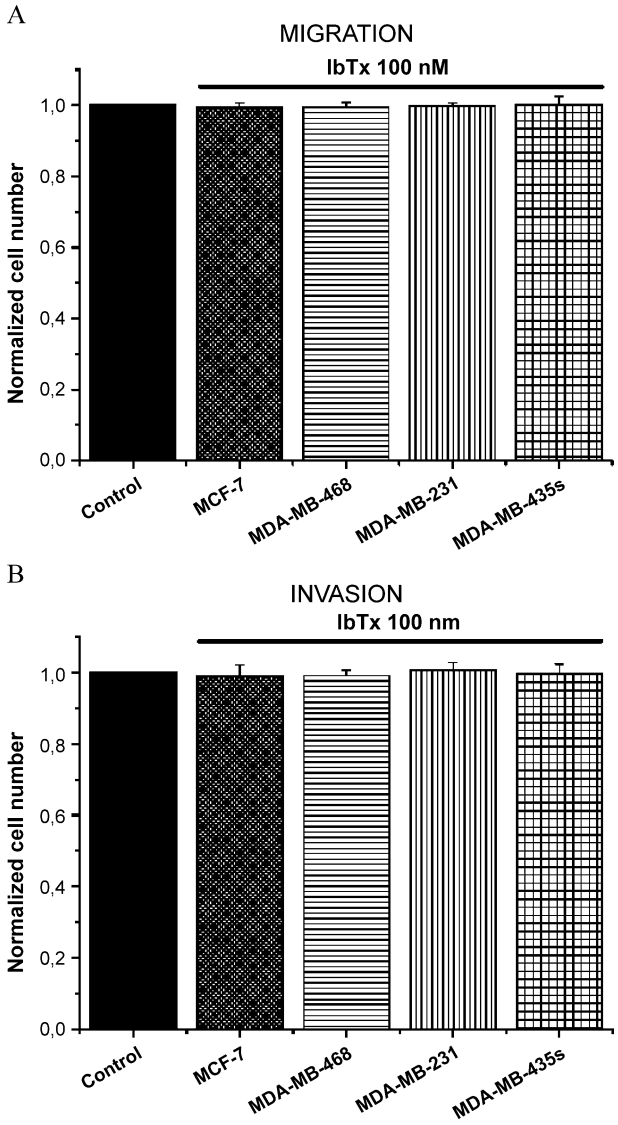

Fig. 4. Effect of IbTx on migration and invasion of four breast cancer cell lines: MCF-7, MDA-MB-468, MDA-MB-231 and MDA-MB-435s. (A) Migration and (B) invasion are expressed as the mean of the total number of cells observed in five different fields per insert. Results obtained using HME cells do not appear on the graph since these did not migrate.

polarising outward current and its participation in the membrane potential. However, when IbTx was applied on the different cells using pCa 7 or pCa 6.4 intrapipette solutions, it did not induce significant depolarisation of the cell membrane (see Table 2). This suggests that a Ca^{2+}-sensitive current, different from BK_{Ca}, is involved in the hyperpolarisation of the membrane [6].

3.2. Involvement of BK_{Ca} current in the proliferation and invasive properties of normal and cancerous cells

In order to evaluate a possible role of BK_{Ca} current on the growth of cancer and normal cells, we studied the effect of IbTx on cell proliferation and viability (Fig. 3A). The selective blockade of this K^+ current by 100 nM IbTx did not

modify cell proliferation. Higher concentrations of IbTx, up to 300 nM, were also tested and, likewise, did not modify cell proliferation (data not shown). It is, however, possible that in standard culture conditions, the IbTx-sensitive current is not activated and thus its participation in cell proliferation cannot be visible. To activate the current, we submitted the cells to a BK_{Ca} opener, NS1619. When cells were cultured in the presence of 50 μM NS1619, proliferation of MDA-MB-435S, MDA-MB-468 and MDA-MB-231 cells was significantly reduced, by approximately 8%, 12% and 40%, respectively (Fig. 3B). For the other cancer cell line, MCF-7, and the HME cells, proliferation was not statistically sensitive to the presence of NS1619. In order to evaluate the participation of BK_{Ca} currents in the decrease of cell proliferation, MDA-MB-231 cells (the proliferation of which was the most sensitive to NS1619) were cultured with 50 μM NS1619, in the presence or not of IbTx. Fig. 3C shows that treatment with IbTx (100 or 300 nM), which inhibits the NS1619-activated current, did not significantly reverse the reduction of cell proliferation induced by NS1619.

The role of BK_{Ca} in the migration and/or invasion capacities of the cells was assessed. The results in Fig. 4 show that IbTx had no effect on migration and invasion.

3.3. Involvement of BK_{Ca} current in proliferative properties of ATP-stimulated MDA-MB-231 cells

Another way to activate the current consists in increasing the level of intracellular calcium. MDA-MB-231 cells have been shown to express functional purinergic receptors [18,19]. These receptors are likely to be $P2Y_2$ receptors since these receptors have been observed in different mammalian breast cancer cell lines including MCF-7 and their activation leads to an intracellular calcium increase [20,21]. The stimulation of these receptors, by pulses of 100 μM extracellular ATP, led to a sustained increase in intracellular Ca^{2+} concentration in MDA-MB-231 cells (Fig. 5A) as already reported [19]. Fig. 5B shows that proliferation was increased by approximately 50% when MDA-MB-231 cells were treated for 5 days with 100 μM ATP pulses. In the same conditions, with the continuous presence of 100 nM IbTx, the proliferation-promoting effect of ATP was significantly reduced, by approximately 20%.

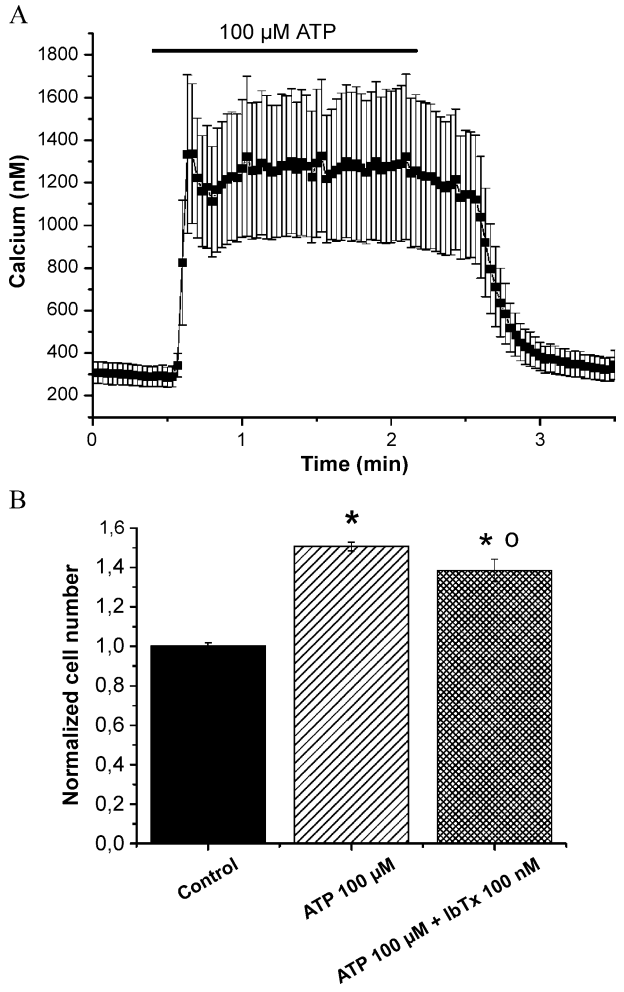

Fig. 5. Involvement of IbTx-sensitive currents on ATP-induced increase in cell proliferation. (A) Effect of extracellular ATP on intracellular calcium concentration of MDA-MB-231 cells. Intracellular calcium concentration was assessed using the ratiometric fluorescent dye fura-2. 100 μM ATP was extracellularly applied to the cells for 2 min as indicated by the bar above the graph. Mean±S.E. from 11 cells. (B) Effect of 100 μM external ATP, in the absence or continuous presence of 100 nM IbTx, on the proliferation and survival of MDA-MB-231 cells, as compared to control conditions (vehicle of the drugs). Results are normalised as explained in Materials and methods and presented as mean±S.E. Statistically different at $P<0.05$ when comparing: *, drug condition vs. control condition; ^{O}, ATP condition vs. ATP+IbTx condition.

4. Discussion

In this study, we describe the presence of an IbTx-sensitive and NS1619-activated current in four different breast cancer cell lines and a supposedly normal epithelial mammary primary culture. We evaluated its role on cell proliferation in standard conditions and following the BK_{Ca} current activation and inhibition. Some works suggest, based on the mere observation of such channels in MCF-7 cells [9,10], that they might be involved in the proliferation of breast cancer cells. Recently, Ouadid-Ahidouch et al. [6] showed that an intermediate conductance Ca^{2+}-activated channel (hIK1) is involved in cell proliferation while the BK_{Ca} is not [7] in MCF-7 cells. No other report exists concerning other cancer cell lines leaving unanswered whether Ca^{2+}-activated K^+ currents are a specificity of breast cancer cells. Up- or down-regulation of one single protein is never associated to all cases of breast cancers. For example, up-regulation of TASK-3 channels is merely found in nearly 50% of breast cancer [22]. In the present work, we bring more information in describing, for the first time, that BK_{Ca} is present in four different cancer cell lines and also in normal cells. This latter finding implies that this current is a souvenir from normality and its putative participation in

proliferation would not be associated to a cancerous transformation. We found that a Ca^{2+}-activated outward current, activated by 50 μM NS1619 and fully blocked by 100 nM IbTx (this concentration is reported to fully block BK_{Ca} channels, and we confirmed that there was no further effect with 300 nM; data not shown), is functionally active in the five breast epithelial cell types studied: four cancerous cell lines (MCF-7, MDA-MB-231, MDA-MB-435S and MDA-MB-468) and one control cell type (HME). The sensitivity of this current to both IbTx and intracellular Ca^{2+} concentration makes it very likely to be carried by BK_{Ca} [15].

Since the work of Wegman et al. [3], and their observation that the incidence of a 23-pS Ca^{2+}-activated K^+ current in excised patches was increased in rapidly proliferating cells, it has been postulated that such channels might participate in cell cycling probably at the beginning of the S phase [9]. This hypothesis had not been tested since a specific blockade of that kind of channels was not possible at that time [3,4]. The lack of effect of IbTx on basal proliferation in our present work and in that of others [1,2] rules out the necessary involvement of BK_{Ca} channels in cell cycling. A particular BK_{Ca} channel with increased sensitivity to calcium was found in glioma [23]. This channel was shown to be involved in the control of glial cell migration since its blockade by IbTx decreased migration [24]. Besides the fact that we found BK_{Ca} in normal HME cells which do not migrate, we searched for a possible involvement of this channel in migration and invasion of cancer cell lines. IbTx had no effect, suggesting that BK_{Ca} is not involved in the migration and invasion of these cells, unlike what was found in glioma.

If, in basal conditions, an IbTx-sensitive current has no effect on cell proliferation, enhancing its activation and hyperpolarising the cell membrane could lead to an increased proliferation [8]. NS1619 had no effect on cell proliferation in two out of five cell types. Unexpectedly, it blocked, partially, the proliferation of MDA-MB-468, MDA-MB-435S and MDA-MB-231 cells. In the latter cell line, this effect was more pronounced and proliferation was reduced by approximately 40%. The fact that this reduction was not reversed by IbTx up to 300 nM suggests that NS1619 affects proliferation through mechanisms which do not involve BK_{Ca} channels. Indeed, NS1619 was reported to have some nonspecific effects on ion channels. For example, it was shown to decrease Kv and K_{ATP} currents in vascular smooth muscle cells [25,26] and to affect Ca^{2+} current [26,27]. Its effects on proliferation can thus be mediated through these or not yet described other effects.

However, it is possible that such channels are involved in conditions where intracellular Ca^{2+} cycling is increased. To test this hypothesis, we submitted MDA-MB-231 cells to ATP pulses (three times a day) for 5 days. The increase of intracellular Ca^{2+} following stimulation of P2 purinergic receptors is one of the various effects caused by ATP in mammary epithelial cancer cells [18,19]. In our experiments, such a stimulation caused an increase in proliferation, 20% of which were prevented by 100 nM IbTx. This means that activation of BK_{Ca} channels is not a prerequisite for cell proliferation but when it occurs, proliferation is significantly increased through mechanisms which have still to be determined. The remaining 80% of the increase in proliferation could be due to other Ca^{2+}-activated K^+ currents such as IK_{Ca} [6] and/or other pathways activated by P2 receptors. However, this is beyond the scope of the present work.

In conclusion, in this study we describe, for the first time, the presence of an IbTx-sensitive Ca^{2+}-activated current in breast epithelial cancerous and normal cells. The activation of the current, following an increase in intracellular Ca^{2+}, increases cell proliferation. This current is observed in all the cell types studied including normal HME cells, which strongly suggests that this current is not a consequence of the cancerous transformation. This channel probably belongs to the primary, noncancerous cell, and as such should not be considered as a potential target of anti-cancer therapy.

Acknowledgements

We thank Dr. Eric DUBUIS for helpful discussion about experiments. This work was supported by a grant from the “Ligue contre le Cancer-Région Centre” and “INSERM”. Sébastien ROGER and Marie POTIER are recipients of fellowships from, respectively, the “Ministère de la Recherche et de la Technologie” and the “Ligue contre le Cancer”.

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