ION CHANNELS, RECEPTORS AND TRANSPORTERS

cAMP–PKA inhibition of SK3 channel reduced both Ca^{2+} entry and cancer cell migration by regulation of SK3–Orai1 complex

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Abstract SK3 channel mediates the migration of various cancer cells. When expressed in breast cancer cells, SK3 channel forms a complex with Orai1, a voltage-independent Ca^{2+} channel. This SK3–Orai1 complex associates within lipid rafts where it controls a constitutive Ca^{2+} entry leading to cancer cell migration and bone metastases development. Since cAMP was found to modulate breast cancer cell migration, we hypothesized that this could be explained by a modulation of SK3 channel activity. Herein, we study the regulation of SK3 channel by the cAMP–PKA pathway and the consequences for SK3-dependent Ca^{2+} entry and cancer cell migration. We established that the beta-adrenergic receptor agonist, isoprenaline, or the direct adenylyl cyclase activator forskolin alone or in combination with the PDE4 inhibitor, CI-1044, decreased SK3 channel activity without modifying the expression of SK3 protein at the plasma membrane. Forskolin and CI-1044 reduced the SK3-dependent constitutive Ca^{2+} entry and the SK3-dependent migration of MDA-MB-435s cells. PKA inhibition with KT 5720 reduced: (1) the effect of forskolin and CI-1044 by 50 % on Ca^{2+} entry and (2) SK3 activity by inhibiting the serine phosphorylation of SK3. These cAMP-elevating agents displaced Orai1 protein outside lipid rafts in contrast to SK3, which remained in the lipid rafts fractions. All together, these results show that activation of the cAMP–PKA pathway decreases SK3 channel and SK3–Orai1 complex activities, leading to a decrease in both Ca^{2+} entry and cancer cell migration. This work supports the potential use of cAMP-elevating agents to reduce cancer cell migration and may provide novel opportunities to address/prevent bone metastasis.

Keywords cAMP · PKA · SK3 channel · Breast cancer · Calcium entry · Migration

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Introduction

Based on their single channel conductance, Ca^{2+}-activated potassium channels (KCa channels) are divided into three families that include large or big KCa (BKCa), intermediate KCa (IKCa/KCa3.1) and small conductance KCa (SKCa) channels. There are three isoforms of SKCa subunits, named SK1/KCa2.1, SK2/KCa2.2 and SK3/KCa2.3, which associate to form homo- or hetero-tetramers [19, 25]. If KCa channels regulate neuronal and smooth muscle excitabilities in a physiological context, this is not what is observed in a tumour context where the physiological function of KCa is hijacked in

the cancer cell to drive essential biological functions for tumour development [1, 10, 38]. Among them, we have demonstrated a pivotal role of SK3 channel as a mediator of cancer cell migration [14]. When expressed in cancer cells, this channel associates with the voltage-independent Ca^{2+} channel Orai1 to form a SK3–Orai1 complex within lipid rafts where it triggers a constitutive Ca^{2+} entry leading to cancer cell migration [7].

The levels of cAMP are determined through the concerted action of adenylyl cyclases (AC) which synthesize this second messenger and cyclic nucleotide phosphodiesterases (PDE) that degrade it. cAMP–PKA signalling has been shown to regulate cell migration by exerting both negative and positive effects depending on many factors such as gradients of [cAMP] and level of PKA activity [18]. Indeed, in breast cancer cells, [cAMP] elevation modulates cell migration [28–30] depending on the localization of [cAMP] variation which provides a spatial and temporal PKA activity, subsequently influencing differentially Rac and RhoA functions [28].

An important mechanism for ion channel modulation is cAMP-dependent phosphorylation as observed following β-adrenergic receptor increases of the L-type voltage-gated Ca^{2+} current [43]. Among KCa, [cAMP] elevation increases BKCa channels activity through the activation of PKA or PKG [9, 11, 34]. Increase in [cAMP] has been shown to enhance [13, 16, 33] or to inhibit IKCa channel activity [8, 26], likely by indirect or direct PKA phosphorylation of the channel at PKA consensus sites respectively. Surprisingly, [cAMP] modulation of SK1 and SK3 channels activities have never been described, whereas some publications report that cAMP–PKA decreased the surface localization of SK2 channels, leading to a reduction of the number of SK2 channel expressed at the plasma membrane and an increase in excitatory postsynaptic potentials underlying long-term potentiation [12, 21, 23, 37].

In the present work, we have studied the regulation of SK3 channel by cAMP-elevating agents (isoprenaline, a beta-adrenergic receptor agonist; forskolin, an AC activator and CI-1044, a PDE4 inhibitor) and the consequence on breast cancer cell migration depending on Ca^{2+} entry through lipid rafts SK3–Orai1 complex. We demonstrate, for the first time, that the activation of the cAMP–PKA pathway reduces SK3 channel activity, as well as the constitutive, SK3–Orai1 complex-dependent Ca^{2+} entry and the SK3-dependent migration of the MDA-MB-435s cancer cells. cAMP elevation had no effect on the quantity of SK3 protein either expressed at the plasma membrane or on its localization in lipid rafts, but displaces Orai1 outside of lipid rafts. Thus, we propose that the activation of the cAMP–PKA pathway decreases breast cancer cell migration by inhibiting SK3 channel activity and by displacing Orai1 outside of lipid rafts, leading to a reduction of constitutive Ca^{2+} entry.

Materials and methods

Cell lines

Human breast cancer cell line MDA-MB-435s and human embryonic kidney 293T cells (HEK-293T) were obtained from the American Type Culture Collection (LGC Promochem, Molsheim, France) and cultured as already described [15]. HEK-293T and MDA-MB-435s cells were transduced using lentivectors carrying rat SK3 channels cDNA to generate HEK293T-rSK3 and shRNA specific to SK3 or a nontargeting shRNA to respectively generate SK3$^-$ and SK3$^+$ cells as previously described [15].

Live cell cAMP measurements using fluorescence resonance energy transfer

Cells were plated in 2-cm diameter glass bottom petri dishes and infected with an adenovirus encoding the fluorescence resonance energy transfer (FRET)-based cAMP sensor Epac2-camps at a multiplicity of infection of 100 viral particles/cell. This sensor contains a single cAMP-binding domain of the exchange factor Epac2 fused to YFP and CFP fluorophores [27]. In the absence of cAMP, when the CFP fluorophore is excited at 440 nm, the energy is transferred to the YFP by FRET, resulting in light emission at 545 nm, the maximum of YFP emission. Increasing intracellular cAMP concentrations leads to a conformational change in Epac2-camps which abrogates the FRET between CFP and YFP, so that CFP excitation results in CFP emission at 480 nm. Thus, the CFP/YFP ratio of Epac2-camps is proportional to intracellular [cAMP] [27].

For FRET measurements, cells were maintained physiological salt solution (PSS) solution containing (in millimoles per litre): NaCl, 140; MgCl$_2$, 1; KCl, 4; CaCl$_2$, 2; D-glucose, 11.1 and HEPES, 10, adjusted to pH 7.4 with NaOH. Control or forskolin-containing solutions were applied by placing the cell at the opening of a 250-μm (inner diameter) capillary tube. Images were captured every 5 s using the 40× objective of a Nikon TE 300 inverted microscope connected to a software-controlled (Metafluor, Molecular Devices) cooled charge-coupled camera (Sensicam PE, PCO). CFP was excited during 300 ms by a Xenon lamp (Nikon) using a 440/20BP filter and a 455LP dichroic mirror. Dual emission imaging of CFP and YFP was performed using an Optosplit II emission splitter (Cairn Research) equipped with a 495LP dichroic mirror and BP filters 470/30 and 535/30, respectively. Average fluorescence intensity of the entire cell was measured. Background was subtracted and YFP intensity was corrected for CFP spillover before calculating the ratio.

ELISA cAMP assays

To evaluate cAMP levels in MDA-MB-435s cells, the Monoclonal anti-cAMP Antibody Based Direct cAMP ELISA Kit has been used (NewEast Biosciences, USA), according to manufacturer's instructions. Briefly, cells were plated in 6-well plates (10 mm^2) the day before experiment, and cells were treated with forskolin (FSK) for 30 min. Then, cells were lysed using a 1 % triton X100 and 0.1 M HCl lysis buffer. The 96-well plate of the kit was coated with goat antigens to allow fixation of anti-goat antibodies, which will recognize anti-cAMP antibodies. cAMP-horseradish peroxidase (HRP) conjugated was placed in each well to compete with cell endogenous cAMP production. After a 2-h incubation with all antibodies and cAMP-HRP, the HRP substrate was placed in each well. To determine cAMP concentration, a standard curve was built by plotting the mean absorbance for each standard cAMP concentration.

Electrophysiology

Electrophysiological recordings were performed in the whole-cell configuration of the patch clamp technique as already described [15]. Briefly, patch pipettes (2.0–4.0 MΩ) were filled with a pipette solution contained (in millimoles per litre): KCl, 145; MgCl$_2$, 1; Mg-ATP, 1; HEPES, 10; CaCl$_2$, 0.87 and EGTA, 1, adjusted to pH 7.2 with KOH, pCa6. The effects of tested compounds on HEK293T-rSK3 and MDA-MB-435s cells were measured using a ramp protocol from +100 to −100 mV with a holding potential of 0 mV (500-ms duration; 4-s intervals). Current amplitudes of SK3 channels were analysed at 0 mV to minimize chloride currents (E_{Cl}^{-} = 0 mV). For MDA-MB-435s cells, external media contains 100 nM Iberiotoxin (IbTx) to fully inhibit BK$_{Ca}$ channels.

Constitutive Ca^{2+} entry measurements

This protocol has been already validated in MDA-MB-435s cells [7]. Briefly, cells were loaded with Fura2-AM (5 μM), and immediately after centrifugation, cells were re-suspended at 1×10^6 cells in 2 mL PSS Ca^{2+}-free solution containing (in millimoles per litre): NaCl, 140; MgCl$_2$, 1; KCl, 4; EGTA, 1; D-glucose, 11.1 and HEPES, 10, adjusted to pH 7.4 with NaOH. The validation of a constitutive Ca^{2+} entry control by SK3–Orai1 complex has been validated in SK3$^+$ cells silencing for STIM1 (supplementary Fig. S3 of [7]).

Cell viability and cell migration

Cell viability was determined using the tetrazolium salt reduction method, and cell migration was analysed as previously described [36]. Briefly, 4×10^4 cells were seeded in the upper compartment with medium culture supplemented with 5 % of foetal bovine serum (FBS ± drugs). The lower compartment was filled with medium culture supplemented with 5 % FBS (±drugs). After 24 h, stationary cells were removed from the topside of the membrane, whereas migrated cells in the bottom side of the inserts were fixed and nuclei were stained and automatically counted [3].

Proteinase K digestion

HEK293T-rSK3 and MDA-MB-435s cells were both pretreated with CI-1044 either with isoprenaline or FSK for 30 min. Next, proteinase K digestion was performed as described previously for SK3 protein [35, 42].

Drugs and antibodies

FSK (AC activator), isoprenaline (beta-adrenergic agonist), IbTx (BK$_{Ca}$ blocker), apamin (SK3 blocker), KT 5720 (a PKA inhibitor) and KT 5823 (a PKG inhibitor) were added to the PSS or culture media at the concentrations indicated in the figure legends. All drugs were purchased from Sigma-Aldrich (St. Quentin, France), except for KT inhibitors (R&D Systems, UK) and CI-1044 ([(R)-N-[9-amino-3,4,6,7-tetrahydro-4-oxo-1-phenylpyrrolo[3,2,1-j,k] [1, 4] benzodiazepin-3-yl]-3-pyridinecarboxamide]), a selective inhibitor of PDE4 with a purity of 98.5 % which was obtained from Pfizer R&D (Amboise, France). IC$_{50}$ of CI-1044 for the PDE4 isozyme is 0.5 ± 0.2 μM, whereas it is greater than 100 μM for PDE3, PDE1 and PDE5 [5]. The antibodies used were the following : rabbit anti-Orai1 (H-46, Santa Cruz Biotech., dilution 1/500), mouse anti-GAPDH (G8795, Sigma-Aldrich, dilution 1/10,000), rabbit anti-SK3 (P0608, Sigma-Aldrich, dilution 1/250) for proteinase K experiments, goat anti-SK3 (SantaCruz sc-16027, dilution 1/250) for immunoprecipitation experiments, rabbit anti-phosphoserine (Life Technologies #618100), rabbit anti-caveolin (D46G3, Cell Signaling Tech, dilution 1/200), goat anti-β-adaptin (sc 6425, Santa Cruz Biotech., dilution 1/1,000) and horseradish peroxidase conjugated anti-rabbit, anti-goat or anti-mouse (Jackson Immuno-Research Laboratories).

Membrane fractionation

Membranes were fractionated on sucrose gradient (90, 45, 35 and 5 %) as described [6]. Upon centrifugation, a total of 12 fractions were collected. Caveolin 1 was used as a marker for the identification of caveolae fractions, and β-adaptin was used as a marker for non-lipid rafts fractions.

Immunoprecipitation

Proteins were extracted with RIPA buffer (150 mM NaCl, 50 mM Tris–HCl, pH 8,1 % Nonidet P-40, 1 % Triton

X-100) containing a mixture of protease and phosphatase inhibitors. Extracted protein (1 mg) was incubated with 5 μg of anti-phosphoserine antibody or rabbit control IgG overnight at 4 °C. Rabbit IP matrix (ImmunoCruz™ IP/WB Optima A System, Sc-45038) was then added, followed by a further incubation at 4 °C for 3 h. The immunoprecipitates were washed, boiled in Laemmli buffer, resolved on 10 % SDS-PAGE gel and transferred onto polyvinylidene difluoride membranes (Amersham Biosciences).

Statistics

Statistical analyses have been performed using SigmaStat Software (version 3.0.1a, Systat Software, Inc.). Unless otherwise indicated, data were expressed as mean±standard error of the mean (N, number of experiments and n, number of cells from independent experiments). For comparison between more than two means, we used Kruskal–Wallis one-way analysis of variance followed by Dunn's or Dunnet's post hoc tests as appropriate. Comparisons between two means were made

using Mann–Whitney. Differences were considered significant when $p < 0.05$.

Results

cAMP-elevating agents are inhibitors of SK3 channel activity in HEK-293T cells

To examine functional effect of cAMP on SK3 channel properties, we developed HEK-293T cells expressing SK3 channel and exposed the cells to a specific PDE4 inhibitor (CI-1044) used alone or with isoprenaline (Iso, a beta-adrenergic receptor agonist), two conditions known to induce a stable increase of [cAMP] in HEK-293T cells [44]. Note that CI-1044 was used because PDE4 is one of a major PDE in HEK-293T cells known to potentiate cAMP response [24]. Figure 1a shows whole-cell SK3 currents recorded at membrane potentials varying from −100 to +65 mV for 500 ms before and after application of 100 μM CI-1044 alone, then with the addition
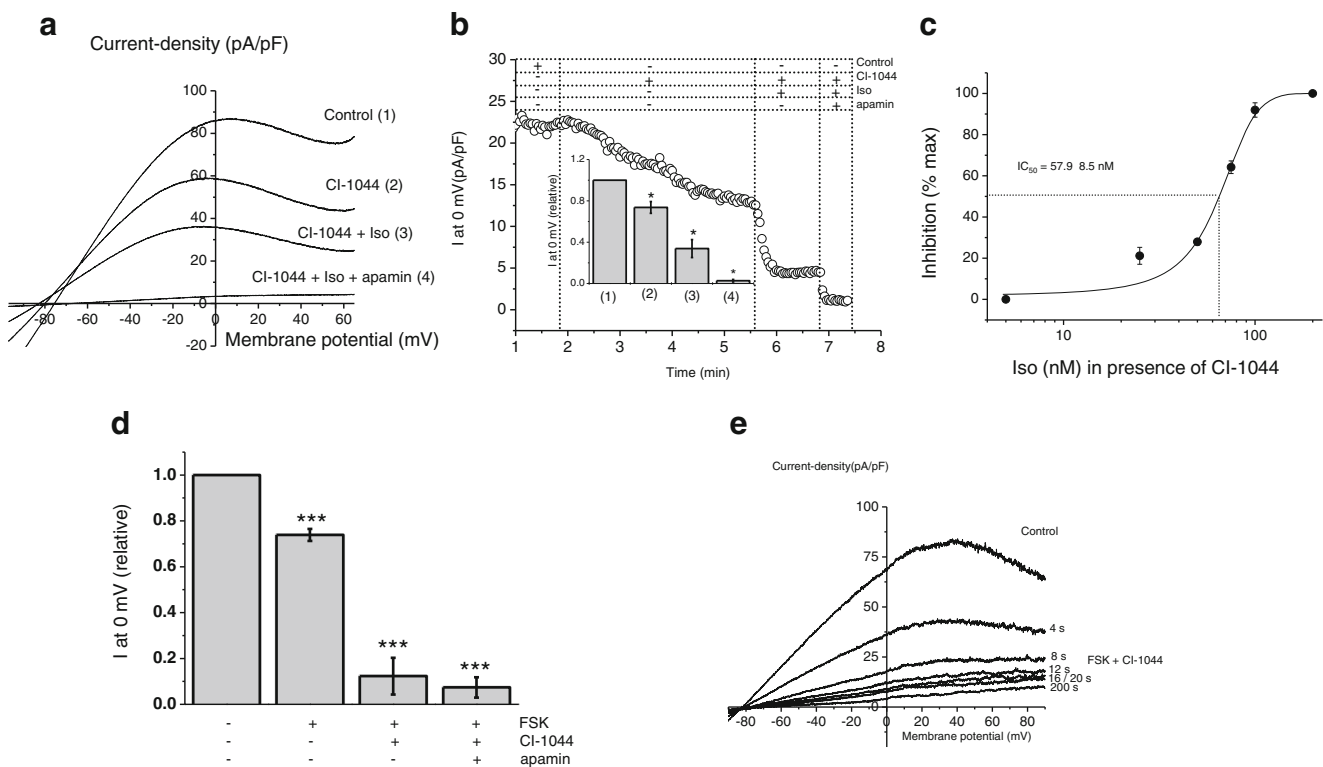

Fig. 1 [cAMP] increase reduced SK3 currents in HEK-293T-rSK3 cells. Currents were generated by ramp protocol from −100 to +100 mV (only currents recorded from −100 to +65 mV are shown in **a** and from −90 to +90 mV are shown in **e**) in 500 ms from a constant holding of 0 mV and with a pCa 6. **a** Example of whole-cell K⁺ currents recorded in HEK293T cells expressing recombinant rat SK3 in control condition and after the sequential addition of 100 μM CI-1044, 100 nM Isoprenaline (Iso) and 100 nM apamin. **b** Representative time response inhibition of 100 μM CI-1044, 100 nM Iso and 100 nM apamin on SK3 currents recorded at 0 mV. The *inset* represents histograms showing the effect of 100 μM CI-1044, 100 nM Iso and 100 nM apamin on SK3 current amplitudes. Means, *bars*,

SEM, $n=4$, *$p < 0.05$ Kruskal–Wallis test and Dunnet's post hoc. **c** Dose–response inhibition of SK3 currents amplitude by Iso recorded with 100 μM CI-1044. The IC$_{50}$ is the concentration of Iso where the amplitude of SK3 current is reduced by half ($n=5$, IC$_{50}=57.9\pm8.5$ nM). **d** Histograms showing the effect of sequential addition of 10 μM FSK, 100 μM CI-1044 and then 100 nM apamin on SK3 current amplitudes recorded at 0 mV. Means, *bars*, SEM, $n=4$, ***$p < 0.001$ Kruskal–Wallis test and Dunnet's post hoc. **e** Example of whole-cell K⁺ currents recorded in HEK293T-rSK3 before and after application of 10 μM FSK plus 100 μM CI-1044

of 100 nM Iso. When the steady-state inhibition was reached, 100 nM apamin was applied to completely inhibit residual SK3 currents. The inhibitory effects of CI-1044 and Iso were voltage independent (data not shown). The effect of cAMP-elevating agents-induced inhibition of the SK3 current was analysed at 0 mV, and the entire time course of the experiment is depicted in Fig. 1b. CI-1044 reduced the amplitude of the current by about 25 % after 200 s, and then, the addition of Iso further decrease SK3-current amplitude by 50 % after 30 s. In addition, application of apamin fully blocked the SK3 current. We then examined the dose-dependent effect of Iso in the presence of CI-1044. Iso reduced SK3 current amplitude in a concentration-dependent manner with a concentration required to evoke half-maximal inhibition (IC_{50}) of 57.9 ± 8.5 nM ($n=5$, Fig. 1c). We next evaluated the effect of 10 μM FSK alone and in the presence of 100 μM CI-1044 on SK3 current amplitude in HEK-293T cells. FSK alone decreased SK3 current by 25 % while the addition of CI-1044 dramatically reduced SK3 current by about 90 % after 200 s (Fig. 1d, e). These data show, for the first time, that cAMP-elevating agents, either by indirectly or directly stimulating AC or by inhibiting cAMP degradation, inhibit SK3 channel activity.

cAMP elevation reduces SK3 current, cancer cell migration and constitutive Ca^{2+} entry in MDA-MB-435s cells

We have shown that SK3 channel is a mediator of MDA-MB-435s cancer cell migration [14, 36], a critical step in bone metastasis outgrowth [7]. Prior to test, the effect of cAMP-elevating agents on SK3 channel activity of MDA-MB-435s cells and on MDA-MB-435s cancer cells migration, we checked the efficiency of FSK to increase [cAMP] in these cancer cells. FSK (10 μM) for 30 min increased [cAMP] 1.5 times compared to control, corresponding to concentration from 24.0 ± 7.8 to 33.6 ± 9.0 pmol/mL ($n=5$), respectively, in control and after stimulation with FSK (ELISA cAMP assays). This result was confirmed by measuring [cAMP] in real time in living MDA-MB-435s cells using the FRET-based cAMP sensor Epac2-camps [27]. Exposure of MDA-MB-435s cells to FSK produced a change in the probe's FRET response consistent with an increase of [cAMP] (Fig. 2a). Then, we assessed the effect of cAMP-elevating agents (10 μM FSK plus 100 μM CI-1044) on SK3 currents of MDA-MB-435s. IbTx (100 nM) was added to fully block BKCa channels (SK3 and BKCa are the main K^+ currents in these cells), and currents were recorded at a membrane potential of 0 mV to minimize chloride currents ($E_{Cl}=0$ mV). Figure 2b shows that, in 2 min, FSK plus CI-1044 induces a 60 % decrease of SK3 current amplitude in MDA-MB-435s cells. Then, we tested the effect of cAMP-elevating agents on MDA-MB-435s cells migration and on their constitutive Ca^{2+} entry. To exclude a possible cell viability effects in the

calculation of the number of cells that migrated, MTT assays were performed. Treatment with 10 μM FSK or 100 μM CI-1044 for 24 h had no significant effect on MDA-MB-435s cell viability, while the combined application of both cAMP-elevating agents seemed to increase their proliferation (data not shown). Although the treatment with FSK plus CI-1044 increased the number of cells, it dramatically reduced MDA-MB-435s cell migration by 81.9 % (Fig. 3a). SK3 action on cancer cell migration/bone metastasis proved to be mediated through an association with Orai1 channel, a voltage-independent Ca^{2+} channel that forms a complex with SK3 and regulates a constitutive Ca^{2+} entry independently of STIM1 [7]. Treatment with 10 μM FSK plus 100 μM CI-1044 for 30 min reduced by 51.4 % the constitutive Ca^{2+} entry of MDA-MB-435s cells (Fig. 3b, c).

cAMP-elevating agents reduce SK3-dependent cancer cell migration and constitutive Ca^{2+} entry

To investigate whether the inhibitory effect of FSK plus CI-1044 on cell migration was dependent on SK3 channel, we tested cAMP-elevating agents on the migration capacity of MDA-MB-435s cells that do not express SK3, SK3$^-$ cells, and compared the effect to that observed in SK3$^+$ cells. Figure 3c shows that the suppression of SK3 decreases the migration capacity of MDA-MB-435s cells to approximately 46 % (924 cells/2,022 cells). Treatment with FSK and CI-1044 further reduced cell migration of SK3$^-$ cells by 66 % that represent a 30.4 % reduction of SK3$^-$ cell migration. Thus, combination of two cAMP-elevating agents reduced by 51.5 % (81.9–30.4) the SK3-dependent part of cancer cell migration. Note that as observed in SK3$^+$ cells, FSK and CI-1044 increased SK3$^-$ cell viability (data not shown). We next evaluated the SK3-sensitive part of the Ca^{2+} entry that was inhibited by FSK and CI-1044. Figure 3d shows that the suppression of SK3 decreases the constitutive Ca^{2+} entry of MDA-MB-435s cells to approximately 40 %. Treatment with FSK and CI-1044 further reduced the constitutive Ca^{2+} entry of SK3$^-$ cells by 61 %. Thus, combination of two cAMP-elevating agents reduced by 27 % (51.4–24.4) the SK3-dependent part of the constitutive Ca^{2+} entry of MDA-MB-435s cells. In conclusion, these experiments demonstrated that increasing [cAMP] reduced by 51.5 and 27 % the SK3-dependent cancer cell migration and constitutive Ca^{2+} entry, respectively.

cAMP-elevating agents have no effect on SK3 channel expression and localization but moved Orai1 outside of lipid rafts

The results above showed that increasing [cAMP] reduced cancer cell migration by inhibiting a constitutive Ca^{2+} entry that depends on SK3 channel activity. To determine if the

Fig. 2 [cAMP] measurements using FRET assays and effect of [cAMP] increase on SK3 currents of MDA-MB-435s cells. **a** The graph illustrates a representative cAMP measurement with Epac2-camps in a single MDA-MB-435s cell. Stimulation with 10 μM FSK strongly increased the CFP/YFP ratio, and the effect was rapidly reversed upon washout of the drug. The histogram shows the percent increase of the ratio, normalized to baseline, in response to 10 μM FSK (Mean, bars, columns, $n=10$, Wilcoxon test **$p<0.002$). **b** *Left*, representative time course of current recorded at 0 mV in control condition and in the presence of 10 μM FSK plus 100 μM CI-1044. The *inset* represent a current–voltage relation (+60, +20 mV, HP 0 mV) in control condition and in the presence of 10 μM FSK plus 100 μM CI-1044. To fully block BKCa currents , 100 nM IbTx was added in the external solutions. *Right*, histograms represent the mean of relative currents recorded at 0 mV with or without 10 μM FSK plus 100 μM CI-1044 (Mean, bars, SEM, $n=6$, **$p<0.01$ Mann–Whitney test)
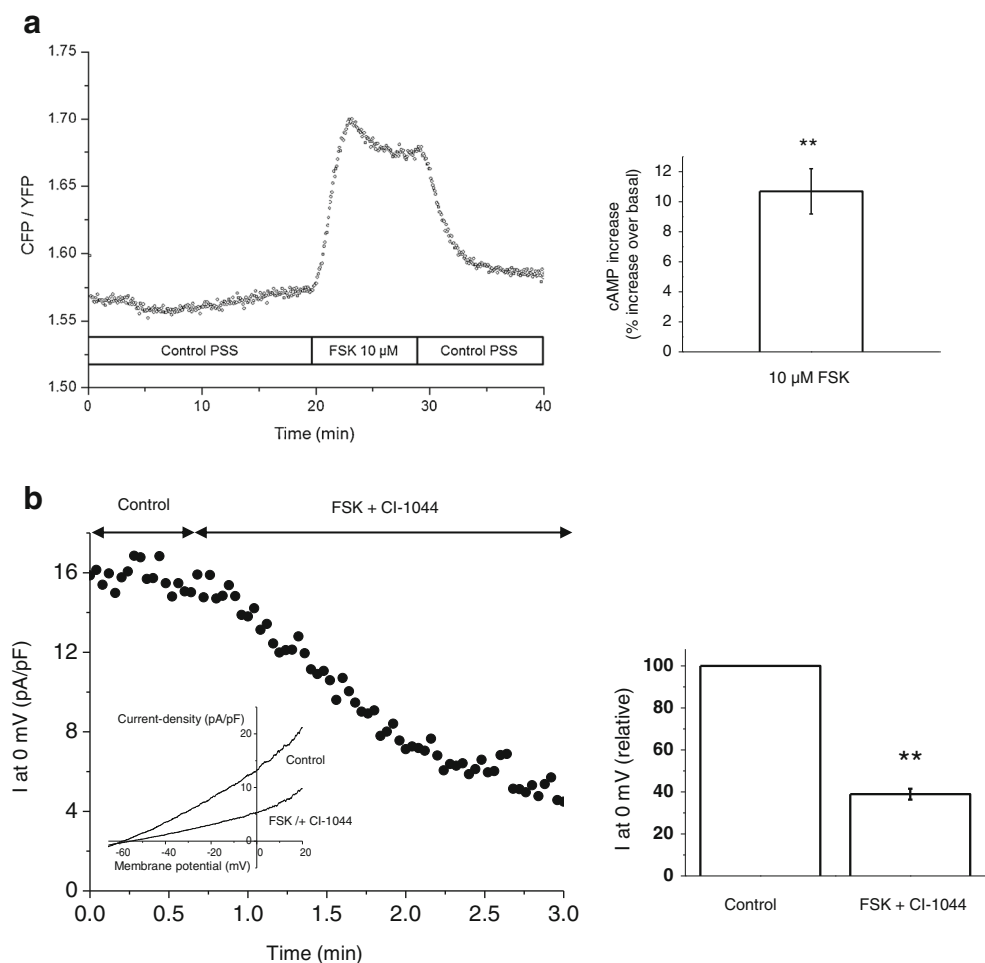
decrease in SK3 channel activity observed following cAMP elevation was due to a decrease in plasma membrane SK3 protein expression, we assessed cell-surface protein expression using a proteinase K cleavage assay as described previously [35, 42]. In the absence of proteinase K, SK3 ran at an apparent molecular mass of 75 kDa, consistent with the full-length protein in HEK293T-rSK3 and MDA-MB-435s cells (Fig. 4a, b). Treatment with 10 μM FSK and 100 μM CI-1044 for 30 min did not change the expression level nor the size of the SK3 protein (Fig. 4a, b). Following proteinase K treatment, almost all of this 75-kDa band was converted to a product with an apparent molecular mass of 45 kDa both in control untreated and cAMP-elevating agent-treated cells. Same results were observed in HEK293T-rSK3 cells treated with 100 nM Iso plus 100 μM CI-1044 (data not shown). This demonstrates that the majority of the SK3 proteins were expressed at the plasma membrane and that increasing [cAMP] did not change SK3 protein expression and localization at the plasma membrane. Membrane fractionation experiments confirm the localization of SK3 protein in plasma membrane and more precisely into lipid rafts (Fig. 4c). Same results were obtained for Orai1 protein (Fig. 4c).

consistent with a co-localization of a SK3–Orai1 complex in lipid rafts [7].

Interestingly, FSK plus CI-1044 treatment displaced Orai1 protein outside of lipid rafts in contrast to SK3 that remained in the lipid rafts fractions (Fig. 4c).

cAMP-elevating agents trigger PKA-mediated SK3 phosphorylation

To test a potential role of PKA or PKG in the inhibitory effect of cAMP on SK3 channels, we used KT 5720 and KT 5823 which are PKA and PKG inhibitors, respectively. Cells were treated for 30 min with these compounds before testing FSK and CI-1044 on SK3 currents and on constitutive Ca^{2+} entry. Figure 5a shows that FSK with CI-1044 still reduced SK3 currents amplitude of HEK293T-rSK3 cells in the presence of KT 5823 as observed in control condition (without KT 5823). Surprisingly, in the presence of KT 5720, SK3 currents amplitude was not reduced but increased by FSK plus CI-1044. Most of this increase was transient although SK3 current amplitude remained slightly higher at steady state compared to control condition (Fig. 5a). Same experiments were
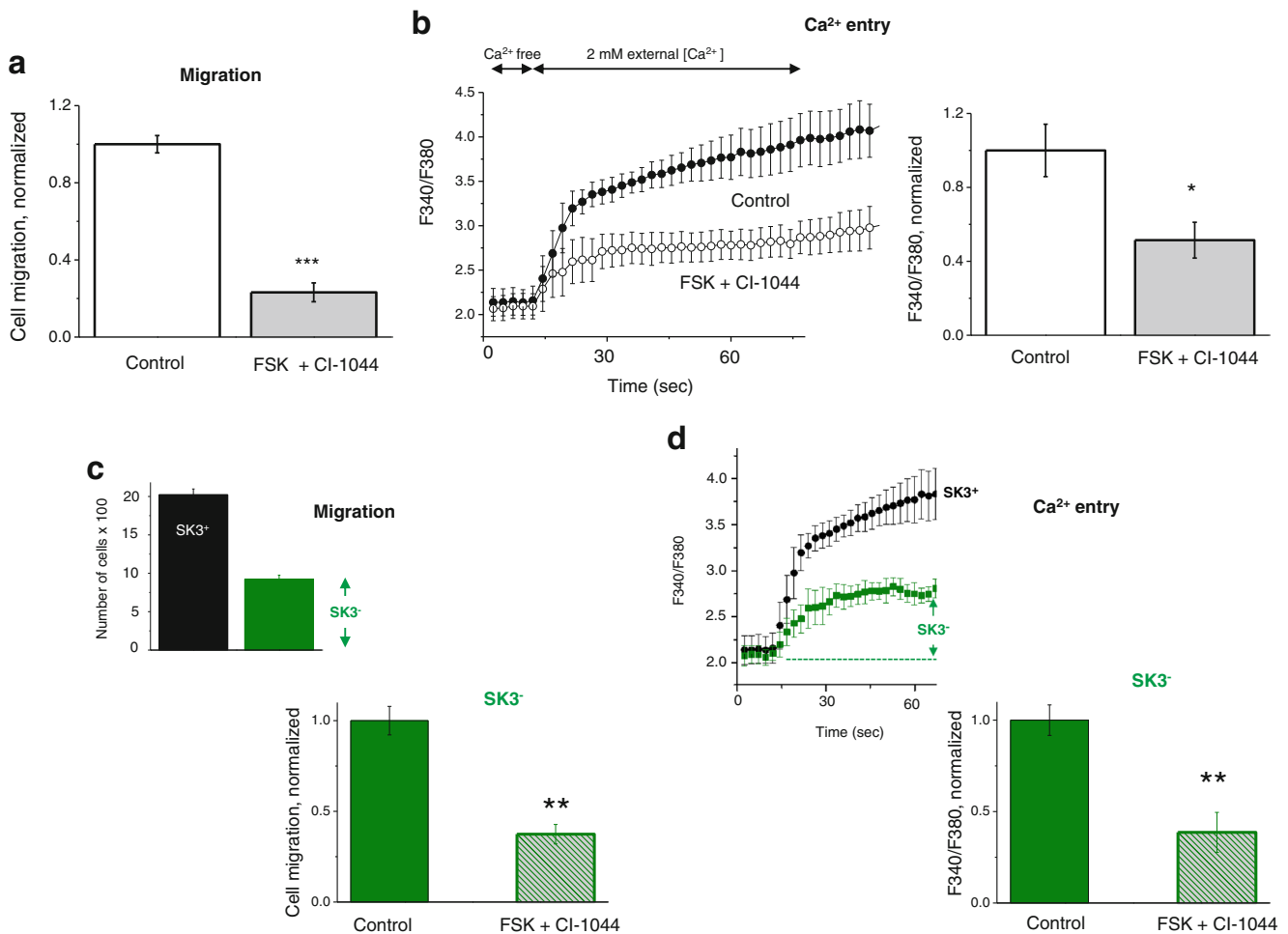
Fig. 3 cAMP-elevating agents reduced SK3-dependent cell migration and constitutive Ca^{2+} entry in MDA-MB-435s cells. **a** Histograms showing the effect of 10 µM FSK plus 100 µM CI-1044 on the migration of MDA-MB-435s cells. The normalized cell number corresponds to the ratio of total number of migrating cells in presence of drugs/total number of migrating cells in control experiments. Results are expressed as mean ± SEM. ***p=0.001, significantly different from control at this level (N=3, n=9, Mann–Whitney test). **b** *Left*, time-dependent fluorescence measurement of constitutive Ca^{2+} entry recorded in MDA-MB-435s cells. *Right*, histograms showing steady-state relative (to control) fluorescence to Ca^{2+} entry in control condition or in the presence of 10 µM FSK plus 100 µM CI-1044. Data are means ± SEM. *p<0.05, significantly different from

control at this level (N=5, Mann–Whitney test). **c** *Top*, histograms showing the number of migrated MDA-MB-435s cells that expressed (SK3^{+} cells) or not SK3 (SK3^{-} cells). *Bottom*, histograms showing the effect of 10 µM FSK plus 100 µM CI-1044 on the migration capacity of SK3^{-} cells (N=3, n=9; data normalized to SK3^{-} cells in control condition). **d** *Top*, time-dependent fluorescence measurement of constitutive Ca^{2+} entry recorded in SK3^{+} and SK3^{-} cells. *Bottom*, histograms showing the effect of 10 µM FSK plus 100 µM CI-1044 on steady-state relative fluorescence to Ca^{2+} entry in SK3^{-} cells (N=3, n=9; data normalized to SK3^{-} cells in control condition). **p<0.01 in **c** and **d** indicated a significant difference from control at this level, Mann–Whitney test

performed on constitutive Ca^{2+} entry of MDA-MB-435s cells (Fig. 5b). Whereas KT 5823 had no significant effect compared to control conditions, KT 5720 reduced by 50 % the inhibitory effect of FSK plus CI-1044 on MDA-MB-435s Ca^{2+} entry (Fig. 5b). All of these results strongly suggest that cAMP-elevating agents reduced SK3 current amplitude and Ca^{2+} entry by activating PKA but not PKG.

In consistence with these results, we showed that cAMP-elevating agents trigger PKA-mediated SK3 phosphorylation (Fig. 6). PKA substrates were immunoprecipitated using anti-phophoserine antibody, and the presence of SK3 in the immunocomplexes was detected by western blotting. Cell

treatment with FSK and CI-1044 triggered a strong phosphorylation of SK3 channel, while a pre-treatment with PKA inhibitor KT 5720 prevented this event.

Discussion

Although cAMP is known to regulate a large number of ion channels, this study is the first to demonstrate that stimulation of cAMP–PKA pathway reduces the activity of SK3 channels. In addition, the stimulation of these pathway disrupted SK3–Orai1 complexes and impaired the constitutive Ca^{2+} entry and

a

HEK293T-rSK3
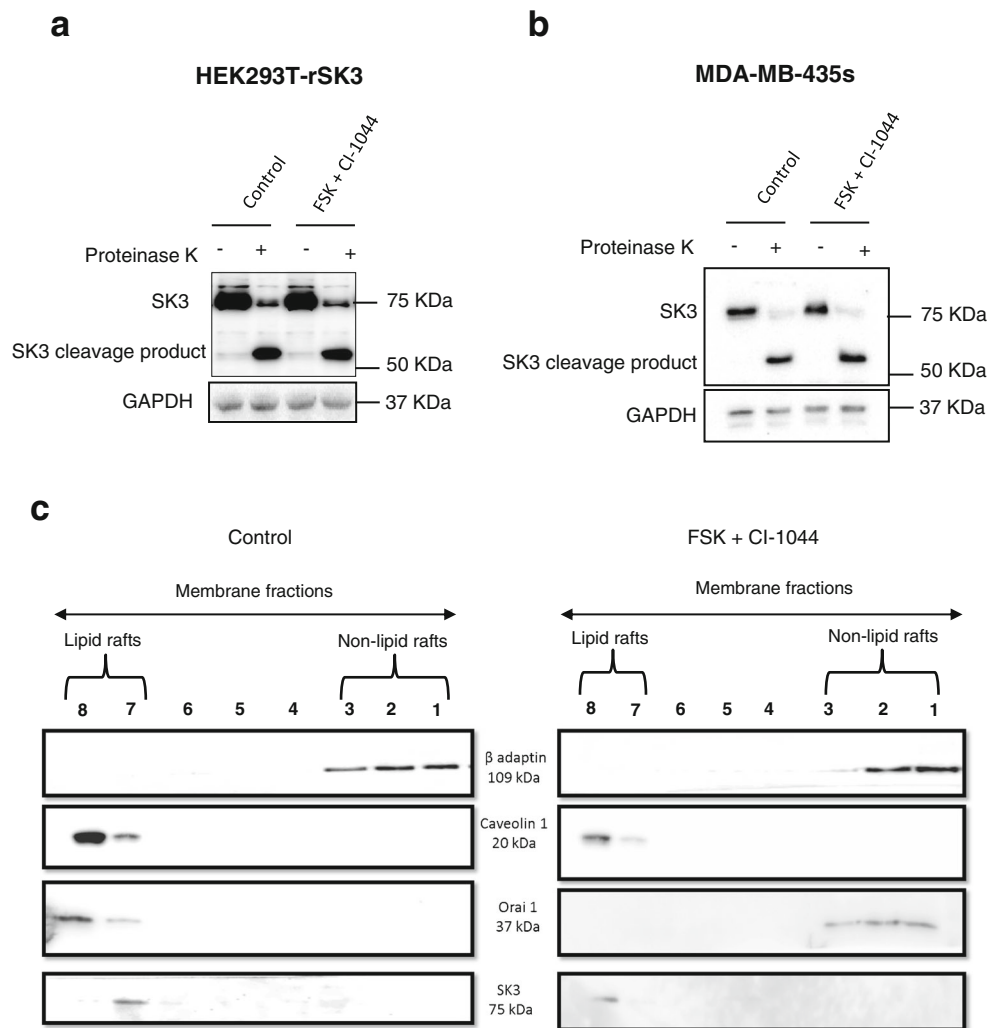

b

MDA-MB-435s

c

Fig. 4 An increase of the cAMP production had no effect on SK3 channel cell surface expression nor on its lipid rafts localization but moved Orai1 outside of lipid rafts. Representative immunoblots of rSK3 channel overexpressed in HEK-293T (**a**) or endogenous hSK3 channel expressed in MDA-MB-435s (**b**) following incubation in the absence (*minus sign*) or presence (*plus sign*) of proteinase K. GAPDH was used as loading control. HEK293T-rSK3 and MDA-MB-435s cells were treated with 100 μM CI-1044 plus 10 μM FSK for 30 min before

proteinase K digestion (30 min of incubation). Proteinase K digestion permit to evaluate the level of SK3 channel expressed in the plasma membrane with detection of SK3 cleavage product (~45 kDa). **c** Immunoblots representing membrane fractionation on a sucrose gradient of MDA-MB-435s cells treated or not with 10 μM FSK plus 100 μM CI-1044 for 45 min. Beta adaptin is a protein used as non-lipid rafts marker and caveolin-1 is a marker of lipid raft fractions

cell migration. Taken together, our results reveal a hitherto unknown regulation of SK3 channel by cAMP–PKA and its consequence in regulation of cancer cell migration.

Isoprenaline, forskolin and PDE4 inhibitors alone or in combination are well-known conditions that induce robust increases of [cAMP] in various cells including HEK cells [44]. These conditions remarkably reduced the amplitude of SK3 current recorded in HEK-293T-rSK3 cells and inMDA-MB-435s cells. The mechanism by which cAMP causes a decrease in SK3 channel activity may involve direct binding of cAMP to SK3 channel or an intermediate protein activated by cAMP such as PKA and PKG phosphorylations. A direct binding of cAMP is unlikely because SK3 channels do not possess a cyclic nucleotide binding domain like that observed

for cyclic nucleotide-gated channels and hyperpolarization-activated cyclic nucleotide-gated channels [17]. In contrast, several amino acid residues were predicted as candidate for PKA phosphorylation sites in the amino-terminal and carboxyl-terminal regions of SK3 channel alpha subunit (found using www.cbs.dtu.dk/services/NetPhosK/). PKA phosphorylation was found to modulate KCa channels, either increasing or decreasing their activities. [cAMP] elevation increases the open probability of BKCa channel, a mechanism that depends not only on PKA [9, 11, 34] but also on PKG through the cross-activation of PKG by cAMP [2]. Here, a cross-activation of PKG by cAMP is unlikely since the inhibition of PKG by KT 5823 did not affect the efficiency of cAMP-elevating agents to reduce SK3 channel activity and
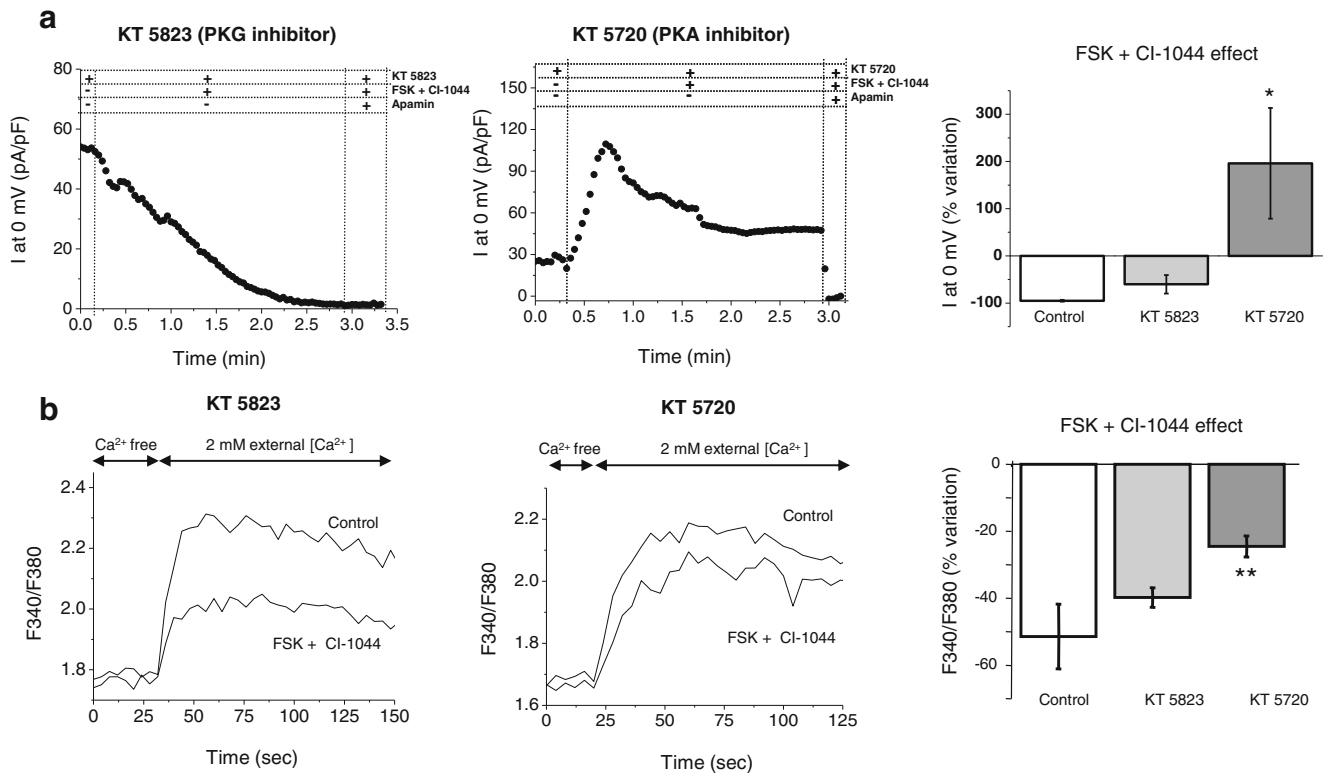
a

b

Fig. 5 cAMP-elevating agents reduced SK3 current and constitutive Ca²⁺ entry by activating PKA but not PKG. Cells were treated for 30 min with KT5823 and KT5720 before patch clamp and Ca²⁺ entry recordings and were continuously perfused with these drugs during SK3 currents and Ca²⁺ entry recordings. **a** Time response measurements of whole-cell SK3 currents recorded in HEK-293T cells expressing rat SK3 at membrane potential 0 mV in the presence of 1 μM KT5823 (*left*) or 5 μM KT5720 (*middle*) and after the sequential addition of 10 μM FSK plus 100 μM CI-1044 and 100 nM apamin. *Right*, histograms showing the percent effect of 10 μM FSK plus 100 μM CI-1044 on SK3 current amplitudes recorded at 0 mV without KT pre-treatment (control)

or after pre-treatment with 1 μM KT5823 or 5 μM KT5720. **b** Time-dependent fluorescence measurements of constitutive Ca²⁺ entry recorded in MDA-MB-435s cells in the presence of 1 μM KT5823 (*left*) or 5 μM KT5720 (*middle*) and with the addition of 10 μM FSK plus 100 μM CI-1044. *Right*, histograms showing the percent effect of 10 μM FSK plus 100 μM CI-1044 on Ca²⁺ entry without KT pre-treatment (control) or after pre-treatment with 1 μM KT5823 or 5 μM KT5720. Means, *bars*, SEM, $N=5$ (Ca²⁺ entry) and $n=6$ (patch-clamp). Significantly different from control at *$p<0.05$ or **$p<0.01$ Kruskal–Wallis test and Dunnet's post hoc

Ca²⁺ entry. In addition, we found no putative PKG phosphorylation sites within the SK3 amino acid sequence (www.cbs.dtu.dk/services/NetPhosK/). IKCa channels activity was found to be

Fig. 6 cAMP-elevating agents trigger PKA-mediated SK3 phosphorylation. HEK-293T-rSK3 cells were treated with either 10 μM FSK plus 100 μM CI-1044 for 7 min alone or following a 30 min pre-treatment with 5 μM KT 5720. *Upper panel*, serine-phosphorylated proteins (P-ser) were immunoprecipitated, and the presence of SK3 in the immunocomplexes was detected by Western blotting. Immunoglobulin (IgG) is used as negative control. *Lower panel*, western blot analysis of SK3 expression in lysates used for immunoprecipitation experiments

inhibited following [cAMP] elevation by direct PKA phosphorylation of the channel [26]. Here, we observed a major role of PKA since the inhibition of PKA by KT 5720 reduced by 50 % the efficiency of cAMP-elevating agents to inhibit the constitutive Ca²⁺ entry and abolished or even reversed to some extent their effect on SK3 channel activity (small activation instead of inhibition). Concerning SKCa channels, [cAMP] elevation was only found to reduce SK2 activity by decreasing the plasma membrane localization of SK2 through PKA phosphorylation on the three amino acid residues, Ser568, Ser569 and Ser570 [37]. Note that none of the PKA phosphorylation sites described in the SK2 carboxyl-terminal domain (RX1–2(S/T)X are found conserved in the amino acid sequence of SK3 channel alpha subunit. To our knowledge, a direct effect of [cAMP] on the open probability of SKCa has never been described. It is unlikely that cAMP acts on SK3 channel activity by decreasing its plasma membrane localization since proteinase K and fractionation experiments demonstrate that [cAMP] elevation did not change the expression of SK3 protein nor its localization in lipid raft domains of the plasma membrane. Since
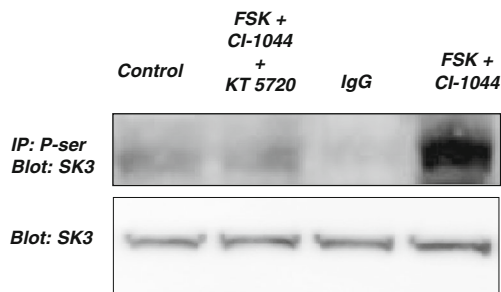
we have shown that FSK plus CI-1044 enhances the serine phosphorylation of SK3, an effect that was blocked by the PKA inhibitor KT 5720, our data strongly suggest that cAMP decreases SK3 activity by a PKA-mediated serine phosphorylation of the channel.

We investigated whether cAMP-elevating agents might alter Ca^{2+} entry, and more precisely a constitutive Ca^{2+} entry and the migration of cancer cells. The phosphorylation of SK3 channel and the displacement of Orai1 channel outside lipid rafts are two mechanisms that could explain the reduction of the constitutive Ca^{2+} entry and cell migration. Our recent findings revealed a novel signalling pathway in which the SK3–Orai1 complex elicited a constitutive and store-independent Ca^{2+}-signalling controlling SK3-dependent cancer cell migration [7]. A role for SK3

channel in this complex is to generate negative membrane potentials (K^+ efflux) that result in a stronger electrochemical driving force for Ca^{2+}, leading to an increase in constitutive Ca^{2+} entry through Orai1 channel (Fig. 7a). cAMP-elevating agents, by activating PKA, decrease SK3 channel activity and depolarize the plasma membrane, thus by itself is sufficient to reduce the constitutive Ca^{2+} entry and the SK3-dependent cancer cell migration. This would break the positive feedback loop that exists between Orai1 and SK3 channels: the decrease in Ca^{2+} entry through Orai1 reduces the activity of the Ca^{2+}-dependent SK3 channels, leading to a more positive membrane potential (Fig. 7b). In parallel, cAMP-elevating agents displaced Orai1 protein outside lipid rafts. We have shown that a delocalization of one of the two partners of SK3–Orai1 complex was sufficient to
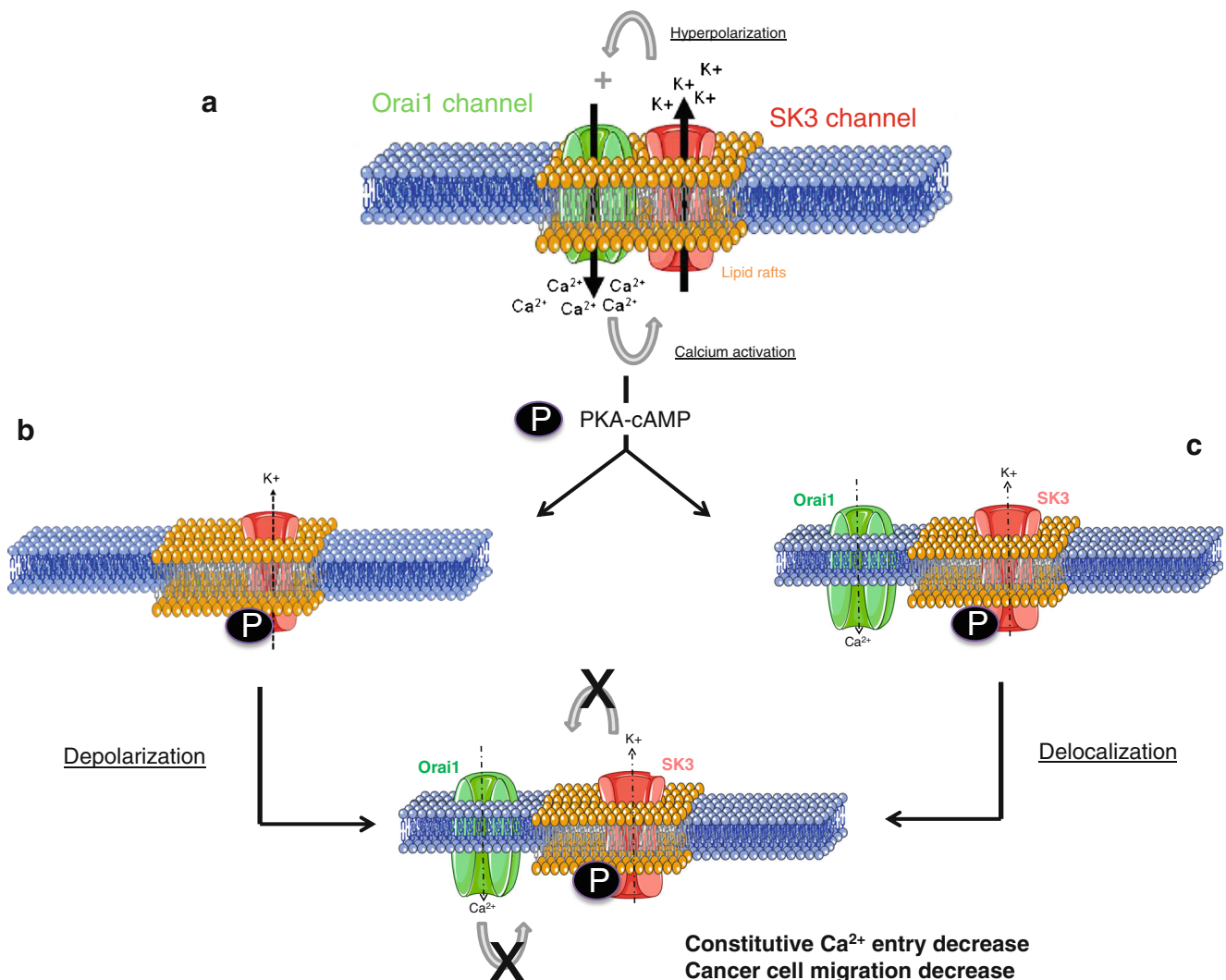

Fig. 7 Hypothetic mechanism for cAMP regulation of SK3–Orai1 complex activity. **a** SK3–Orai1 complex is located in lipid rafts domains in MDA-MB-435s cells resulting in plasma membrane hyperpolarization (by SK3) and constitutive Ca^{2+} entry (by Orai1). SK3 channel hyperpolarizes plasma membrane which increases electrochemical driving force for Ca^{2+} and enhances Ca^{2+} entry through the voltage-independent Ca^{2+} channels Orai1. A positive feedback loop exists in which Ca^{2+} entry further increases the activity of SK3 channel. The elevation of cAMP induces the PKA phosphorylation of SK3 protein that reduces the activity of SK3 channel (**b**) and allows Orai1 to move outside lipid rafts (**c**). Therefore, this decrease the SK3-dependent constitutive Ca^{2+} entry and SK3-dependent cancer cell migration

suppress SK3-dependent Ca^{2+} entry and SK3-dependent migration [7]. Thus, the delocalization of Orai1 by cAMP away from lipid rafts is another mechanism that could explains, by itself, the reduction of the Ca^{2+} entry and cancer cell migration regulated by SK3–Orai1 complex (Fig. 7c). These two mechanisms of action of cAMP-elevating agents are probably link. Indeed, [cAMP] elevation may reduce the interactions between SK3 and Orai1 channels probably through PKA phosphorylations of SK3 and/or Orai1 proteins leading to Orai1 moving outside lipid rafts. It is established that such interaction occurs between ion channel such as SK2 and Ca(v)1.3 channels or hERG and KCNQ1 channels [22, 31]. Interestingly, if direct interactions between hERG and KCNQ1 have been found, this interplay action was reduced by cAMP elevation [31]. Further studies are needed to delineate the contribution of PKA-mediated effects on SK3–Orai1 interaction. Nevertheless, the results described here represent the first description of a [cAMP] regulation of interplay between SK3 and Orai1 channels.

Results from previous studies reported both a reduction and a stimulation of breast cancer cell migration, including for MDA-MB-435s, following [cAMP] elevation [28–30, 32, 41]. This discrepancy could be explained by the localization of [cAMP] variations, providing a spatial and temporal cAMP–PKA activity that differentially influences Rac and RhoA function [28]. Indeed, PKA is required for Rac activation by chemo-attractants as well as beta1 integrins, a function that contrasts with its inhibition of RhoA [28]. Interestingly, membrane-associated PKA activity was found to be highest in the leading edge of migrating cells compared to the rear of migrating cells [20]. These data, in addition to those demonstrating that intracellular Ca^{2+} concentration is lower in the leading edge than in the rear of the migrating cells [4, 39], strongly suggest a spatial SK3 channel activity in migrating cancer cells with a higher activity in the back of the migrating cells compared to the front. A spatial KCa activity has already been observed for IKCa channels which are found to be more active at the rear part in cell migration than at the cell front [40].

In conclusion, cAMP–PKA activation reduces SK3 channel activity, leading to a decrease of the lipid raft SK3–Orai1 complex-dependent Ca^{2+} entry and cancer cell migration. As SK3–Orai1 complex affects the ability of cancer cell to form bone metastases, these results underscore an innovative opportunity to use [cAMP]-elevating agents to inhibit bone metastasis.

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