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Original Article

Stimulation of β_2-adrenergic receptor increases CFTR function and decreases ATP levels in murine hematopoietic stem/progenitor cells

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Abstract

Background: The chloride channel CFTR (Cystic Fibrosis Transmembrane Conductance Regulator) is expressed by many cell types, including hematopoietic stem/progenitor cells (HSPCs). In this study, we sought to better comprehend the regulation of CFTR activity in HSPCs, namely by beta-adrenergic stimuli.
Methods: The expression of β_2-adrenergic receptor (β_2-AR) in murine Sca-1$^+$ HSPCs was investigated by immunofluorescence/confocal microscopy and flow-cytometric analysis. Association with CFTR was assessed by immunoprecipitation. HSPCs were evaluated for ATP content and CFTR activity by means of luminometric and spectrofluorometric methods, respectively, upon stimulation with salbutamol.
Results: HSPCs express β_2-AR over the whole plasma membrane and are associated in cellula with both the immature and mature forms of CFTR. β_2-AR was predominantly expressed by HSPCs with bigger size. CFTR channel activity was increased by salbutamol treatment and this activation was inhibited by either a specific CFTR inhibitor ($CFTR_{inh172}$) or a β_2-AR receptor inhibitor (ICI 118,551). Intracellular ATP levels were reduced by salbutamol stimulation and this effect was reversed when ICI 118,551 or CFTR inhibitors were present. A trend in the increase of extracellular ATP upon salbutamol stimulation was observed.
Conclusions: In HSPCs, CFTR is regulated by β_2-adrenergic receptor stimulation determining intracellular ATP depletion.

Keywords: Adrenergic receptor; ATP; CFTR; Hematopoietic stem/progenitor cells

1. Introduction

The CFTR (Cystic Fibrosis Transmembrane Conductance Regulator) gene encodes for a chloride channel whose absence/dysfunction causes cystic fibrosis (CF). Although initially shown to be expressed in epithelia of various organs with secretory/absorptive function, further studies have demonstrated CFTR expression and/or function in cells of hematopoietic origin, particularly in polymorphonuclear leukocytes [1], macrophages [2,3], and platelets [4]. Recently, we have shown that CFTR is expressed at the mRNA and protein levels in hematopoietic stem/progenitor cells (HSPCs) and that its activation is inhibited by

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oligomycin treatment, indicating a relationship between energetic metabolism and its function [5]. Furthermore, our data show that CFTR is expressed at low levels in a subset of HSPCs with bigger size, which is characterized also by low mitochondrial content [5].

CFTR is a member of the ATP binding cassette family and functions as a cAMP-activated chloride channel [6]. In airway epithelial cells, CFTR activity is physiologically regulated by β-adrenergic stimuli. The activation of β_2-adrenergic receptors (β_2-AR) is able either to stimulate CFTR channel activity [7] or to increase its expression on the apical surface [8]. Since this aspect of CFTR regulation has not been studied in cells of hematopoietic origin, we sought to evaluate CFTR activity in HSPCs upon β-adrenergic stimulation and correlate this with the intracellular ATP content.

2. Materials and methods

2.1. HSPC isolation

The experiments were performed using 6–8 weeks old male C57Bl/6 mice (Charles River Laboratories, Calco, Italy) with a body weight of 23–27 g. They were housed and handled according to institutional guidelines which comply with Italian legislation and were given food and water ad libitum. HSPCs were isolated from femurs and tibiae of C57Bl/6 mice as previously described [5]. The animals were killed by sodium pentothal hyperanaesthesia, both ends of the femur and tibia were removed and the bone marrow was flushed out with PBS using a needle. The recovered total bone marrow cells were filtered (CellTrics 100 mm, Partec, Münster, Germany), centrifuged, washed and re-suspended in PBS supplemented with 2% FBS and 1 mM EDTA. The total number of cells was determined with a Bürker's cytometer. Positive selection of bone marrow stem/progenitor cells by EasySep (Mouse Sca1 selection kit; StemCell Technologies) was done according to the manufacturer's instructions. Briefly, recovered total bone marrow cells were incubated with the PE-conjugated anti-Sca-1 antibody. Then tetrameric antibody complex, which recognizes both PE and dextran, was added, which was followed by addition of dextran coated magnetic nanoparticles. Magnetically labeled target cells were then separated from unlabeled unwanted cells using the EasySep magnet by pouring the unlabelled cells off. 3–4 mice were used to obtain a pool of HSPCs.

2.2. Immunostaining and cytofluorimetric analysis of β_2-adrenergic receptor

Aliquots of 2×10^5 Sca-1^+ HSPCs were pelleted by centrifugation at 337 ×*g* for 10 min at +4 °C, fixed in PBS containing 3% paraformaldehyde and 2% sucrose for 5 min at room temperature, permeabilized with ice cold Triton Hepes buffer (20 mM HEPES, 300 mM sucrose, 50 mM NaCl, 3 mM $MgCl_2$, 0.5% Triton X-100, pH 7.4) for 5 min at room temperature and then were incubated with rabbit polyclonal anti β_2-AR antibody [clone: H-20] (Santa Cruz Biotechnology, CA) at +4 °C overnight. The cells were then pelleted and washed with PBS to remove excess unbound primary antibody prior to incubation with a FITC-conjugated secondary antibody (anti-rabbit IgG, used at 1:80, Sigma-Aldrich, Milan, Italy) for 90 min at room temperature. An isotype control antibody (normal rabbit IgG, Santa Cruz Biotechnology, CA) was also used as negative control. After incubation, the cells were pelleted, washed twice with PBS and analyzed by fluorescence-activated cell sorting (FACS) with a EPICS XL MCL flow cytometer (Beckman Coulter Fullerton, CA, USA) to determine the percentage of cells expressing β_2-AR.

2.3. Confocal microscopy

For β_2-AR detection, Sca-1^+ HSPCs were labeled with β_2-AR antibody following the same protocol as for cytofluorimetry. An aliquot (1×10^5 cells/ml) of the preparation was cytospun onto glass slides and incubated with propidium iodide (diluted at 1:5000 of 1 mg/ml stock solution) for 5 min at 37 °C, and washed. The slides were mounted and observed through the FITC (excitation 488 nm, emission 516 nm) and TRITC (excitation 544 nm, emission 620 nm) filters. Cells were analyzed using Nikon TE2000 microscope coupled to a Radiance 2100 confocal dual-laser scanning microscopy system (Bio-Rad, Segrate, Italy). Specimens were viewed through a 60× oil immersion objective and zoomed at 2.0×. Digital images were processed using the program Laser Sharp 2000 (Bio-Rad).

For CFTR detection, 1×10^6 Sca-1^+ HSPCs were pelleted by centrifugation at 337 ×*g* for 10 min at +4 °C, fixed in PBS containing 3% paraformaldehyde and 2% sucrose for 5 min at room temperature, permeabilized with ice cold Triton Hepes buffer (20 mM HEPES, 300 mM sucrose, 50 mM NaCl, 3 mM $MgCl_2$, 0.5% Triton X-100, pH 7.4) for 5 min at room temperature and then were incubated with Ab anti-CFTR (MAb 25031 mouse IgG2a, R&D Systems, Milan, Italy) at +4 °C for 60 min, at dilution of 1:500. The cells were then pelleted and washed with PBS to remove excess unbound primary antibody prior to incubation with a FITC-conjugated secondary antibody (anti-mouse IgG, used at 1:100, Sigma-Aldrich, Milan, Italy) for 45 min at +4 °C. As negative control, cells were incubated only with the secondary antibody. An aliquot (1×10^5 cells/ml) of the preparation was cytospun onto glass slides, observed through the FITC and TRITC filters, and analyzed as described before.

2.4. Immunoprecipitation and Western blotting

Sca-1^+ HSPCs and Calu-3 cells were lysed with RIPA buffer (Sigma-Aldrich), and 500–1000 μg of cell lysate was immunoprecipitated overnight at +4 °C on a mixer using protein A agarose beads and an appropriate dilution of anti β_2-AR antibody (2 μg) or anti-CFTR antibody (2 μg). After washing, the immunoprecipitated proteins were electrophoresed through 4–12% or 8–16% polyacrylamide gel (mini-protean TGX gels, Bio-Rad), and transferred onto blotting membrane (trans-Blot Transfer Medium pure nitrocellulose cat 162-0115, Bio-Rad). The blots were incubated with β_2-AR antibody (1:100) or anti-CFTR antibody (1:500). The primary Abs were counter-stained by a HRP-conjugated anti-IgG Ab (anti-rabbit and anti-mouse 1:10,000, Bio-Rad) for 60 min at room temperature.

Proteins were visualized by chemiluminescence (Immun-Star Western C Kit, Bio-Rad).

2.5. CFTR chloride channel activity

Chloride efflux was measured using the method implemented in [9]. Cells seeded on 0.1% gelatin coated glass coverslips were loaded overnight in culture medium containing 5 mM MQAE; after 12 h, coverslips were inserted into a perfusion chamber and superfused continuously and fluorescence was recorded with a Cary Eclipse Varian spectrofluorometer. In all the experiments, cells were perfused at 37 °C using alternatively two different HEPES-buffered bicarbonate-free media, (i) a Cl^- medium (135 mM NaCl, 3 mM KCl, 1.8 mM $CaCl_2$, 0.8 mM $MgSO_4$, 20 mM HEPES, 1 mM KH_2PO_4, 11 mM glucose), and (ii) a NO_3 medium (135 mM $NaNO_3$, 3 mM KNO_3, 0.8 mM $MgSO_4$, 1 mM KH_2PO_4, 20 mM HEPES, 5 mM $Ca(NO_3)_2$, 11 mM glucose). The measurement of chloride efflux was carried out after replacement of the Cl^- medium by the NO_3^- medium. The rates of chloride efflux were calculated by linear regression analysis of the first 30 points taken at 4 s intervals while the change of fluorescence was still linear. CFTR-dependent chloride efflux was evaluated as the difference in fluorescence intensity induced by 1 μM salbutamol (Sigma-Aldrich, Milan, Italy) plus IBMX (500 μM) or salbutamol alone, in the absence and presence of either of the CFTR inhibitors, glibenclamide (Sigma-Aldrich; 100 μM) or $CFTR_{inh172}$ (Calbiochem; 5 μM). The effect of β_2-AR inhibition on CFTR-dependent chloride efflux was studied by adding ICI 118,551 hydrochloride (Sigma-Aldrich, 10 μM) for 5 min before salbutamol plus IBMX addition.

2.6. ATP levels

To study intracellular ATP level, 1×10^4 HSPCs were incubated with salbutamol (1 μM) for 20 min after pre-treatment or not with ICI 118,551 hydrochloride (10 μM) for 20 min or glibenclamide (100 μM) or $CFTR_{inh172}$ (5 μM) for 10 min. The incubation with DMSO for 40 min at the final dilution of 1:10 was used as control condition. All the incubations were performed at 37 °C.

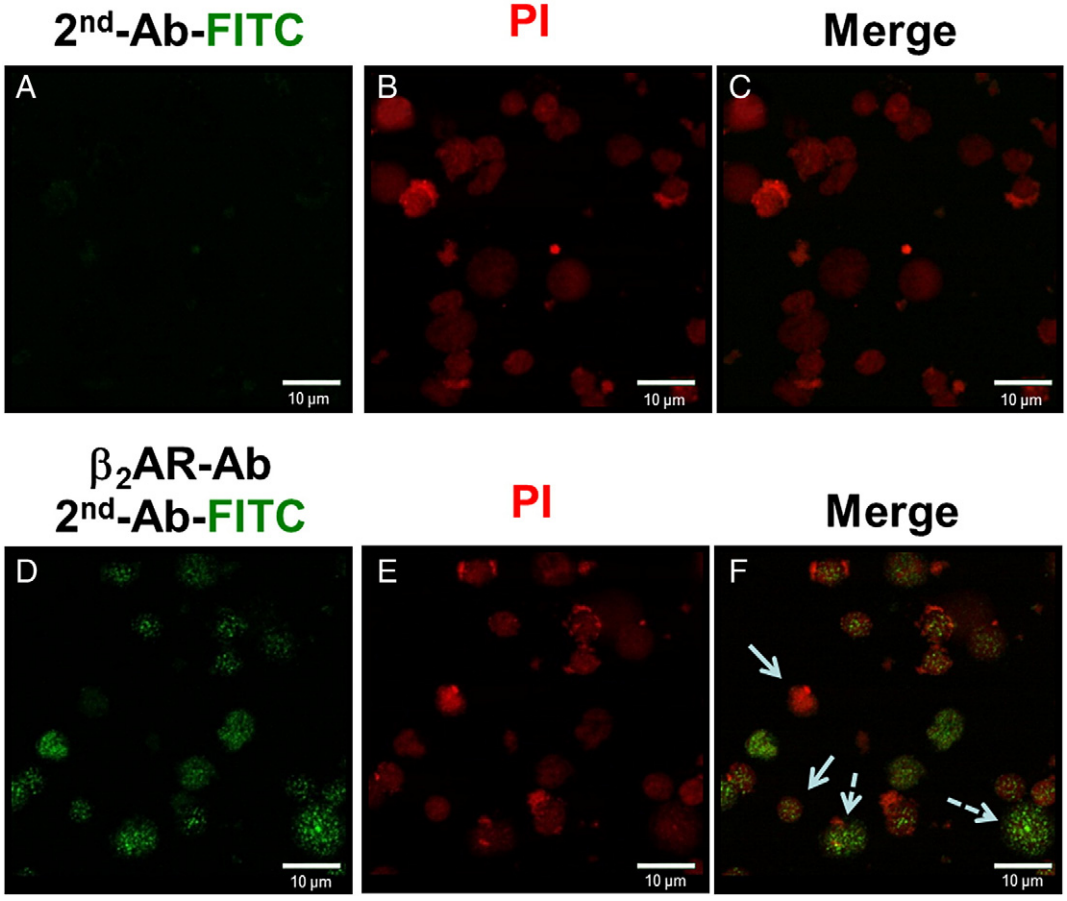

Fig. 1. Immunofluorescence and confocal microscopy of β_2-AR in HSPCs. Sca-1^+ HSPCs were labeled with anti β_2-AR antibody and then with FITC-conjugated anti-rabbit antibody. Finally, cells were labeled with propidium iodide (PI). Panel A represents negative control without primary antibody. Panel B shows Sca-1^+ HSPCs without labeling with anti β_2-AR antibody and panel C merge. Panel D shows the staining with anti β_2-AR antibody and in panel F merge with the red channel (Panel E) is shown. The figure represents images of the overlapped confocal planes and is representative of three different experiments carried out with as many HSPC different samples. Continue and dotted white arrows point to smaller and bigger cells respectively. Panels have been obtained with 2.0× zoom. Bar: 10 μM.

The ATP cytosolic content was measured by using the luciferin/luciferase (rL/L) reaction (Enliten ATP assay, Promega, Madison, WI) [10]. Briefly, HSPCs were spun at 12,000 ×*g* for 10 min, the supernatant was stored at −80 °C, while 200 μl of ATP-free boiling water was added to the pellet. Cell lysate was spun at 12,000 ×*g* per 5 min, and 20 μl of supernatant was added to 100 μl of the rL/L reagent and read at the luminometer TD-20/20 DL-Ready (Turner Design Instruments, Sunnyvale, CA) at the wavelength of 560 nm. Samples were compared to a standard curve obtained adding various amounts of ATP (from 1×10^{-7} to 1×10^{-11} M) to the rL/L reagent. To assess extracellular ATP levels, 1×10^4 HSPCs were treated with salbutamol (1 μM) 20 min after pre-treatment or not with ICI 118,551 hydrochloride (10 μM) for 20 min (or DMSO in control cells). HSPCs were spun at 12,000 ×*g* for 10 min and 20 μl of supernatant was used to measure the ATP content by the luciferin/luciferase (rL/L) reaction.

2.7. Statistical analysis

Data are shown as mean ± SEM. Statistical significance was studied by the ANOVA with Tukey's Multiple Comparison Test or paired Student's *t* test where appropriate. All data were analyzed using Prism 4 (GraphPad Software, Inc., La Jolla, CA). p values less than 0.05 were considered significant.

3. Results

3.1. β_2-Adrenergic receptor expression by HSPCs

Having demonstrated in a previous study that Lin^- $Sca\text{-}1^+$ HSPCs do express a functional CFTR on their plasma membrane [5], we confirmed the presence of the CFTR protein on the plasma membrane by immunofluorescence. Fig. S1 shows that the CFTR signal was localized onto the surface of HSPCs and overlapped that of the Sca-1 antigen. Then, we sought to determine whether these cells could also express β_2-AR. Confocal microscopy on cells labeled with double staining revealed that most of the HSPCs expressed β_2-AR. Analysis of the images reconstructed from the overlapped confocal planes displayed that the β_2-AR-related fluorescence was diffused over the whole plasma membrane (Fig. 1D). The staining was localized mostly in HSPCs with bigger size, while smaller cells showed low or null β_2-AR expression (Fig. 1F).

To investigate this in a more quantitative way, we analyzed β_2-AR expression by flow cytometry. As in our previous work, we identified two subpopulations, based on the size (forward

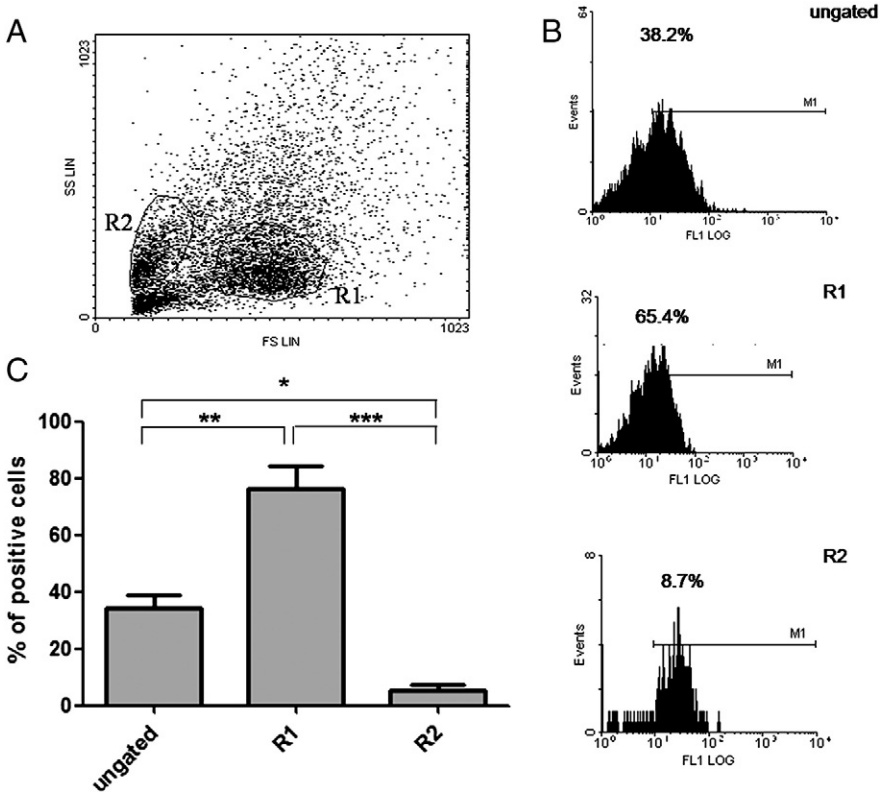

Fig. 2. Flow cytometry analysis of β_2-AR in HSPCs. $Sca\text{-}1^+$ HSPCs were labeled with anti β_2-AR antibody and then with FITC-conjugated anti-rabbit antibody, and analyzed by cytofluorimetry. Panel A depicts the dot plot analysis of the whole HSPC population, denoting two subsets of cells, distinguishable for their forward and scatter side and named R1 the biggest and R2 the smallest. Panel B shows representative histograms for the ungated, R1 and R2 subpopulations as concerning β_2-AR expression. Panel C shows the percentage of β_2-AR-positive cells in the whole population, R1 and R2 subsets obtained in three experiments. Data are represented as the mean ± SEM. *$p < 0.05$, **$p < 0.01$, and ***$p < 0.001$.

scatter) and granulosity (side scatter) (Fig. 2A). While HSPCs expressed β_2-AR in 34 ± 7.8% of the whole population, the expression of β_2-AR was found to differ in these two subsets of cells. The subpopulation with bigger size (R1) expressed more β_2-AR (in 76.2 ± 13.8% of cells) than the smaller R2 subpopulation (5.4 ± 3.5%) (Fig. 2B and C).

It was then studied if β_2-AR and CFTR were interacting in HSPCs. Calu-3, an epithelial cell line derived from submucosal gland, was used as positive control since it has been already shown that β_2-AR and CFTR interact with each other in this cell line [7]. Western blotting for CFTR showed a predominant mature form of CFTR in Calu-3 cells as compared with immature form of CFTR in agreement with previous studies [11,12] (not shown). HSPCs displayed both immature and mature forms, as previously demonstrated [5]. Immunoprecipitation analysis of protein cell extract with anti-β_2-AR-Ab revealed that β_2-AR and CFTR interacted *in cellula* in both Calu-3 and HSPCs (Fig. 3A). In HSPCs, both immature (band B) and mature (band C) forms of CFTR were associated with β_2-AR. Fig. 3B shows that the same results were obtained when cell lysates were immunoprecipitated with the anti-CFTR antibody and blotted with either the anti-β_2-AR or the anti-CFTR antibody. These data indicate that CFTR and β_2-AR are physically associated in HSPCs.

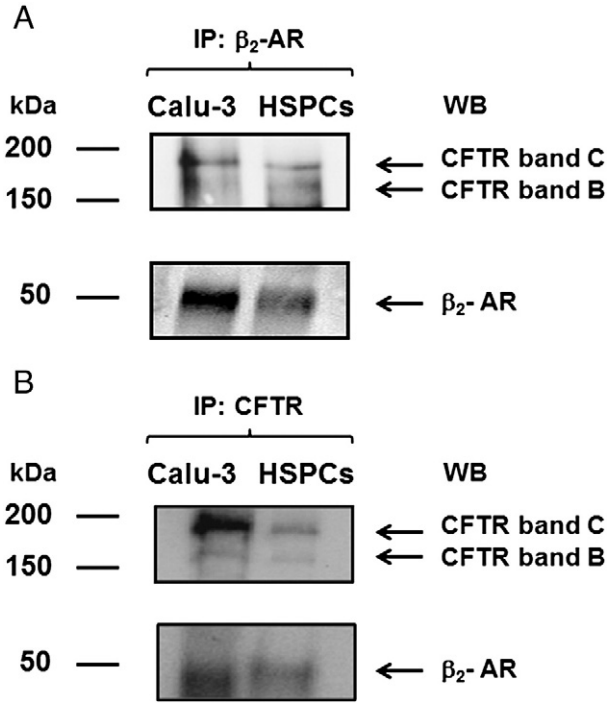

Fig. 3. Immunoprecipitation of β_2-AR and CFTR in Calu-3 and Sca-1$^+$ HSPCs. A) The cell lysates were incubated with a mixture of protein A agarose beads and the polyclonal anti β_2-AR antibody. The precipitates were resolved via SDS-PAGE and proteins were blotted to nitrocellulose membrane. Blots were then incubated with an anti-CFTR antibody (upper panel) or an anti β_2-AR antibody (lower panel) followed by incubation with the relevant secondary antibody. B) The immunoprecipitation was performed as in A) but with the anti-CFTR antibody. Blots were incubated with the anti-CFTR antibody (upper panel) or the anti β_2-AR antibody (lower panel) followed by incubation with the relevant secondary antibody. Blots were visualized by chemiluminescence. One out of three experiments is shown.

3.2. Activation of CFTR by β_2-AR stimulation

As in other cell types [9,13,14], Sca-1$^+$ HSPCs exhibited a basal chloride efflux when chloride was replaced by nitrate (first slope of the trace in Fig. 4A and C, and first bar in Fig. 4B and D). Moreover, we previously showed that Sca-1$^+$ HSPCs display CFTR-dependent chloride secretion upon activation by forskolin, an adenylyl cyclase activator, plus IBMX, a phosphodiesterase inhibitor [5]. Here we evaluated the ability of β_2-AR to activate CFTR in HSPCs using the β_2-AR agonist, salbutamol, either combined or not with IBMX. Fig. 4 illustrates a typical experiment (A and C) and the summary of several experiments (B and D) of chloride efflux in Sca-1$^+$ HSPCs seeded on glass coverslips. Stimulation of β2-AR with salbutamol (1 μM) plus IBMX (second slope of the trace in Fig. 4A and C, and second bar in Fig. 4B and D) significantly increased chloride efflux compared to basal levels. The addition of the specific CFTR inhibitor, $CFTR_{inh172}$ [15], 2 min before as well as in the presence of salbutamol plus IBMX in NO_3 medium (third slope of the trace in Fig. 4A, and the third bar in Fig. 4B), inhibited the chloride efflux salbutamol plus IBMX dependent increase. Similar results were obtained when glibenclamide, a less specific CFTR inhibitor [9], was used as CFTR inhibitor (data not shown). The amount of CFTR-dependent chloride efflux, defined as the difference between the rates of salbutamol plus IBMX-stimulated chloride efflux before and after $CFTR_{inh172}$ treatment, was similar to that previously observed by us in the same cells treated with forskolin plus IBMX [5]. To confirm that this effect was due to activation of the β_2-AR, we studied the reversibility of the salbutamol effect with ICI 118,551 hydrochloride, a highly specific β_2-AR antagonist. As can be seen from the representative trace of Fig. 4C and the summary in Fig. 4D the pre-incubation with ICI 118,551 hydrochloride (10 μM) for 5 min before salbutamol plus IBMX addition (third slope of the trace in Fig. 4C, and the third bar in Fig. 4D) inhibited the increase of chloride efflux. Importantly, these results indicate that the entity of the chloride efflux inhibition obtained after treatment with the CFTR blocking agent is comparable to that obtained after treatment with β_2-adrenergic receptor antagonist, thus suggesting that this receptor plays an important role in modulating the CFTR activity in Sca-1$^+$ HSPCs.

Notably, control experiments showed that IBMX was dispensable to elicit the salbutamol-mediated CFTR-linked chloride efflux (salbutamol + IBMX: 0.0338 ± 0.004 Δ(F/F0)/min, salbutamol: 0.0292 ± 0.0024 Δ(F/F0)/min, $n = 4$, $p > 0.05$). Moreover and consistently, IBMX alone did not cause any change of the CFTR-linked chloride efflux (IBMX (500 μM) 0.0224 ± 0.0035; IBMX + CFTR inhibitor 172 (5 μM) 0.0181 ± 0.0030 Δ(F/F0)/min, $n = 3$, $p > 0.05$). Thereby, we conclude that salbutamol is sufficient alone to stimulate CFTR-dependent efflux in HSPCs.

3.3. Reduction of intracellular ATP levels by salbutamol

Next, we sought to investigate the mechanism of the activation of CFTR by salbutamol. It has been already shown

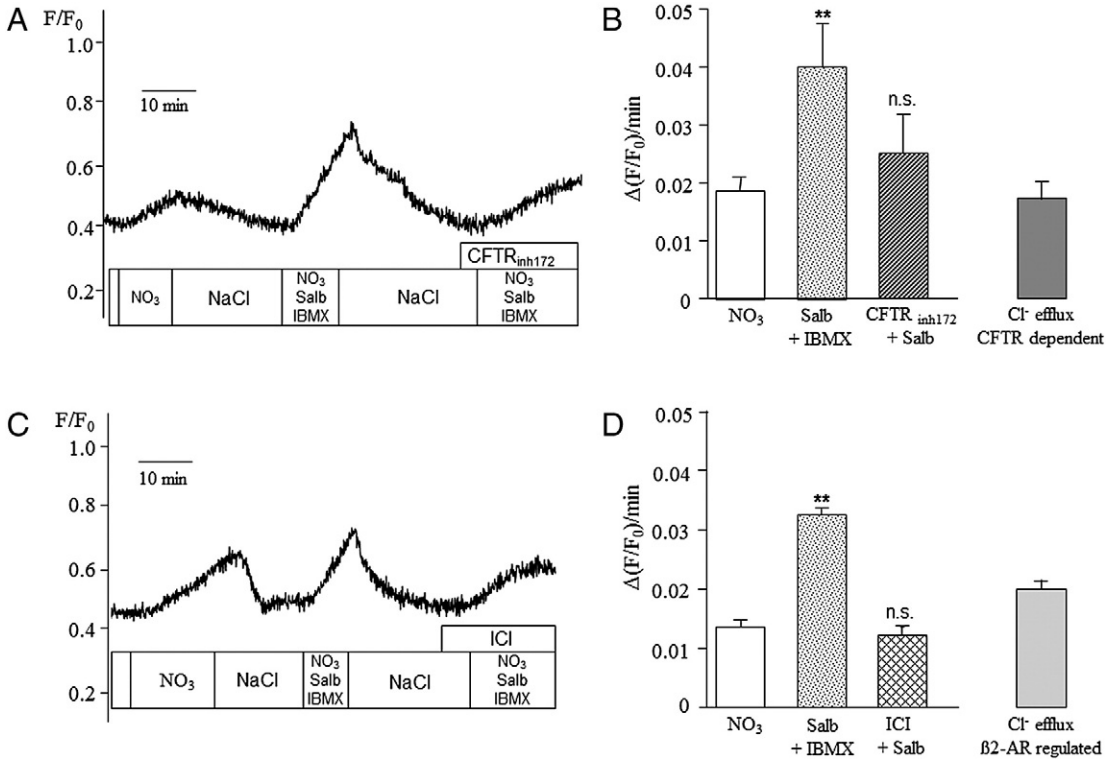

Fig. 4. Effect of salbutamol on the CFTR activity. (A) Typical recordings showing changes in intracellular Cl^- dependent MQAE fluorescence (expressed as the F/F0 ratio) when HSPCs were incubated in the presence of salbutamol (1 μM) and IBMX (500 μM), added without and with $CFTR_{inh172}$ (5 μM). (B) Summary of the data collected from 6 independent experiments. The first bar represents a basal chloride efflux under baseline conditions when chloride was replaced by nitrate. CFTR-dependent chloride efflux (fourth bar) was calculated as difference between fluorescence intensity obtained after treatment with salbutamol plus IBMX in the absence (second bar) and in presence of $CFTR_{inh172}$ (third bar). (C) Typical trace showing changes in intracellular Cl^- when the cells were treated with salbutamol (1 μM) and IBMX (500 μM), in the absence or presence of ICI 118,551 hydrochloride (10 μM). (D) Summary of the data collected from 3 experiments. β_2-AR regulated chloride efflux (fourth bar) was calculated as difference between fluorescence intensity obtained in the absence (second bar) and in presence of ICI 118,551 hydrochloride (third bar). Data are expressed as the mean ± SEM. Statistical comparison was made using paired Student's *t* test with respect to the basal chloride efflux when chloride was replaced by nitrate. In B) and D), Salb + IBMX vs NO_3: **$p < 0.01$.

that in airway epithelial cells, the β_2-AR agonist salmeterol both increased CFTR expression and cAMP levels [8]. To this end, we evaluated whether salbutamol have some effect on ATP levels. Salbutamol treatment reduced ATP content of HSPCs by ~33% (Fig. 5A). By exposing HSPCs to ICI 118,551 hydrochloride, together with salbutamol, ATP content was similar to that of control cells. Thus, we checked whether the channel activity of CFTR was involved in the ATP depletion. Our data show that both glibenclamide and $CFTR_{inh172}$ resumed intracellular ATP levels when added to HSPCs together with salbutamol (Fig. 5A). Assessment of the extracellular ATP content resulted in values approximately 75 fold lower than the intracellular ATP concentration when normalized to the same cell number (Fig. 5B). Salbutamol treatment increased extracellular ATP levels by ~23%, which was completely prevented by pre-incubation of cells with ICI 118,551 (Fig. 5B). The observed differences in the extracellular ATP level were at the border of statistical significance.

4. Discussion

There is an increasing evidence that CFTR can interact with a number of membrane-anchored and intracellular proteins [16]. Membrane-anchored protein interactions stabilize CFTR on the plasma membrane, allowing its main function of chloride channel, implicated in the fluid and ion homeostasis in absorbing/secreting epithelia. Intracellular protein interactions allow correct CFTR folding, trafficking and function. Thus, CFTR functions are believed to be more complex than thought before. Indeed, CFTR has been implicated in many cellular functions [17].

The β_2-adrenergic receptor is involved in CFTR activation by raising cAMP intracellular levels and mediating protein kinase A (PKA) activation. In airway epithelial cells, the interaction of β_2-AR with CFTR is mediated by scaffold proteins, such as NHERF1, allowing its interaction with PKA and stabilizing it on the plasma membrane [7]. We have previously shown that CFTR is expressed by murine HSPCs and that its expression is correlated with the mitochondrial content, i.e. the higher the mitochondrial content the higher the CFTR levels [5]. The biological significance of this correlation is unknown at the moment, thus in this work we have further explored the role of CFTR in HSPCs. We show that HSPCs express β_2-AR, confirming previously published results [18]. Moreover, similarly with airway epithelial cells, β_2-AR interacts with the mature form of CFTR. NHERF1 is expressed by c-kit^+/Lin^-/Sca-1^+ HSPCs and has been shown to be an intracellular ligand for

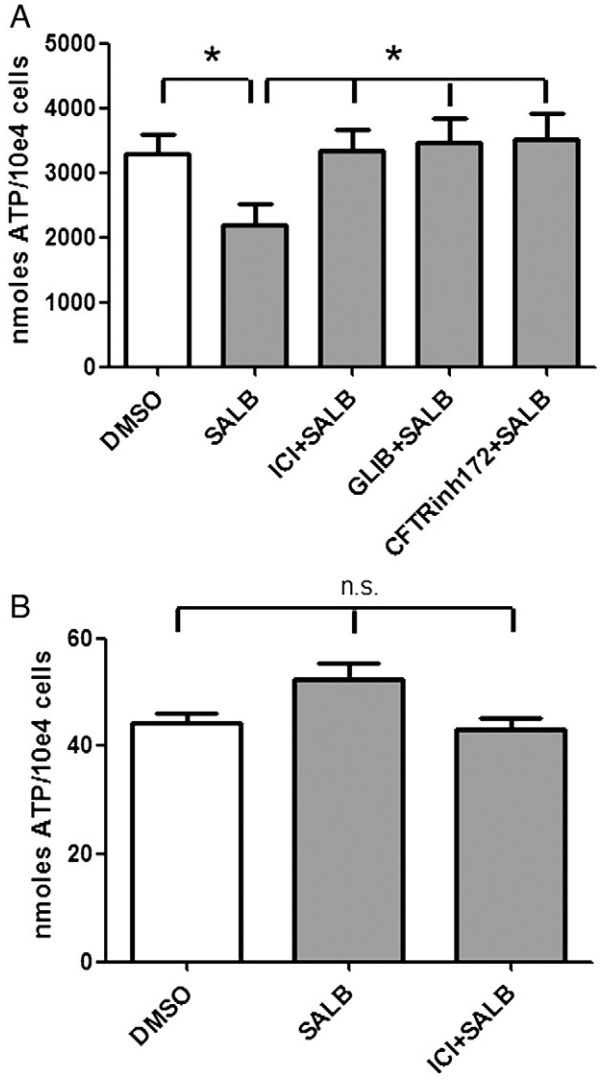

Fig. 5. Effect of salbutamol on ATP levels. A) HSPCs were treated with 1 μM salbutamol (or DMSO in control cells) in the absence or presence of ICI 118,551 hydrochloride, glibenclamide, or $CFTR_{inh172}$. Intracellular ATP levels were measured in total cell lysates and shown as nmoles per 10^4 HSPCs. Data are expressed as mean ± SEM of six experiments. *$p < 0.05$. B) HSPCs were treated with 1 μM salbutamol (or DMSO in control cells) in the absence or presence of ICI 118,551 hydrochloride. Extracellular ATP levels were measured in conditioned medium and shown as nmoles per 10^4 HSPCs. Data are expressed as mean ± SEM of six experiments. SALB vs DMSO: $p = 0.0723$; SALB vs ICI + SALB: $p = 0.0541$.

CD34-type proteins in the plasma membrane [19]. The role of NHERF1 in the β_2-AR–CFTR interaction is still to be explored in HSPCs. However, we have captured a glimpse of this interaction in HSPC cell biology. In fact, salbutamol, an agonist of β_2-AR, increases CFTR activity as CFTR-dependent chloride secretion (Fig. 4). Furthermore, salbutamol depletes ATP content and this effect is reversed by both β_2-AR and CFTR inhibitors (Fig. 5A). These data suggest that the intracellular ATP decrease upon β_2-AR stimulation by salbutamol could be due to CFTR activation. Indeed, to translocate Cl^- across plasma cell membrane, CFTR uses the energy generated by ATP binding and hydrolysis at its two cytoplasmic nucleotide-binding domains named NBD1 and NBD2 [20]. In airway epithelial cells, on agonist activation of the β_2-AR, adenylyl cyclase is stimulated through the Gs pathway [21], leading to an increase in highly compartmentalized cAMP. This increased local concentration of cAMP causes the activation of PKA, which is in close proximity to CFTR [22], inducing a compartmentalized and specific signaling of the channel. Phosphorylation of CFTR R domain disrupts the macromolecular complex involving CFTR-NHERF1-β_2-AR, leading to the receptor-based activation of CFTR [7]. However, the number of $CFTR^+$ HSPCs is lower than $CFTR^+$ airway epithelial cells (15.6% ± 1.91% as compared to 68.2% ± 6.30%) as judged by FACS analysis [5]. These results, together with the low level of mRNA expression found in HSPCs [5], indicate that the total amount of CFTR protein in HSPCs is low and not likely to give such an ATP consumption. This raises the question of whether the expression of CFTR protein in the whole HSPC population would be high enough to create a significant decrease in intracellular ATP levels upon CFTR activation. Moreover, activation of β_2-AR results in a variety of distinct signaling events besides activation of adenylyl cyclase, including notably the activation of Na,K-ATPase, which would result in further consumption of ATP. However, these metabolic processes have been found mainly in airway epithelial cells [23] and not, to the best of our knowledge, in HSPCs.

The reduction of intracellular ATP levels found with salbutamol stimulation may be explained differently. It has been suggested that CFTR functions as an ATP-conductive channel based on experiments comparing the ATP release of cells over-expressing P-glycoprotein or CFTR [24], although these results were not reproduced in native airway tissues [25]. Nevertheless, two studies have reported the depletion of intracellular ATP by ~30% coupled to ATP secretion upon CFTR activation in mammary adenocarcinoma C127 cells transfected with wild-type CFTR cDNA [26] and L6 myocytes [27]. Here we show that in HSPCs salbutamol stimulation increases the content of extracellular ATP, an effect which was blunted by the specific inhibitor of β_2-AR ICI 118,551. Thus, it might be possible that β_2-AR activation leads to ATP extrusion. However nucleotide hydrolyzing enzymes could have reduced the extracellular ATP concentration. CD39, known as ectonucleoside triphosphate diphosphohydrolase 1, which hydrolyzes extracellular ATP and ADP to AMP, is expressed by mesenchymal stem cells obtained from bone marrow and other various cell types [28]. Recently, it has been shown that functional CD39 is highly expressed also by a subset of murine and human HSPCs and is critical for chemotaxis and recruitment of these cells from bone marrow to the liver [29]. Notably, Prat and colleagues [26] have shown that steady state extracellular ATP levels result not only from ATP release but also from extracellular cleavage of ATP by ectoATPases. Future studies will demonstrate whether CD39 is involved in hydrolysis of extruded ATP and if this system is relevant to HSPC biology.

Interestingly, the two subpopulations identified express different levels of β_2-AR, which may have functional consequences. However, we could not investigate further this issue since CFTR function was assessed on the whole population. We have recently shown that the two subsets of HSPCs respond differently to an acute lung injury as concerning their expression

of adhesion molecules and chemotactic responses. The subset with bigger size responds better to chemotactic stimuli represented by SDF-1 [30]. Furthermore, the bigger cells displayed less Sca-1 expression than the smaller cells, an indication of their minor uncommitted status [5]. Further studies should elucidate whether β_2 agonists are involved in HSPC mobilization (in particular, bigger cells) from the bone marrow to the circulation in response to inflammatory stimuli.

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.jcf.2014.08.005.

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