INVOLVEMENT OF THE TREK-1 CHANNEL IN HUMAN ALVEOLAR CELLS MEMBRANE POTENTIAL AND ITS REGULATION BY INHIBITORS OF THE CHLORIDE CURRENT

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Abstract

K^+ channels of the alveolar epithelium control the driving force acting on the ionic and solvent flow through the cell membrane, contributing to the maintenance of cell volume and the constitution of epithelial lining fluid (ELF).

In the present work we analyze the effect of the Cl^- channels inhibitors: (4-[(2-Butyl-6,7-dichloro-2-cyclopentyl-2,3-dihydro-1-oxo-1H-inden-5-yl) oxy] butanoic acid DCPIB and 9-anthracenecarboxylic acid (9-AC) on the total current in a type II pneumocytes (A549 cell line)model by patch clamp, immunocytochemical and gene knockdown techniques,

We noted that DCPIB and 9-AC promote the activation of a K^+ conductance. In fact, they significantly increase the intensity of the current and shift its reversal potential to values more negative than the control. By silencing ORCC in its ANO6 portion, we excluded a direct involvement of Cl^- ions in modulation of I_K and, by means of functional tests with its specific inhibitor spadin, we identify the TREK-1 channel as the presumable target of both drugs.

Since the activity of TREK-1 has a key role for the correct functioning of the alveolar epithelium, the identification of DCPIB and 9-AC molecules as its activators, suggests their possible use to build new pharmacological tools for the modulation of this channel.

Keywords:

K^+ channels, ANO-6, DCPIB, 9-AC, pneumocytes, A549 cells.

Introduction

The epithelial cells of the pulmonary alveoli are involved in the physiological processes of the respiratory tract. In particular, second type pneumocytes (IITP) main function is to synthesize surfactant (Epithelial Lining Fluid, ELF) allowing to lower the surface tension in the alveoli for a more efficient gas exchanges. The functionality of ELF depends on its ionic composition which is strictly controlled by the IITP (Chilvers and O'Callaghan, 2000). The control of ionic movements through the membrane is carried out by means of specific and non-specific channels, ionic pumps and exchangers.

Many studies have tried to identify the channels involved in the regulation of the ELF ionic composition and to understand their functioning mechanisms (Boucher, 1994a; Boucher, 1994b; Berthiaume and Matthay, 2007; Hamacher et al., 2018). Great interest has been reserved to the channels that control the movements of sodium and chloride ions due to their essential role in alveolar fluid clearance (Boucher, 2003). On the other hand, potassium channels have been showed to play the main role in the maintenance of membrane potential and ~~therefore~~ the driving force able to maintain cellular homeostasis. In addition, the involvement of K^+ channel in mucosal defense and adaptation to oxygen concentration has also been demonstrated (Jovanović, 2003).

As elegantly summarized by Bardou et al., (Bardou et al., 2009) at least 40 different types of potassium channels in airway cells have been identified, and classified into three main categories: 1) voltage-dependent K^+ channels (K_v) and calcium-dependent channels (K_{Ca}); 2) potassium two-pore-domain (K_{2P}) family, and 3) inward-rectifier potassium channels (K_{ir}). The contribution of the different K^+ channels involved in the ability of the cell to recover its volume and to rebalance the ionic concentrations is not yet completely clarified. ------

Based on what described above, the aim of our study was to investigate the electrophysiological properties and molecular identity of K^+ channels expressed in IITP by an electrophysiological and biomolecular approach. -------

By patch clamp technique we were able to characterize the total current crossing the cell membrane when it was subjected to voltage steps of 20 mV, from -90 to +70 mV. This current is mainly the sum of K^+, Cl^- and Na^+ currents (I_K, I_{Cl}, I_{Na}), with a little contribution of Ca^{2+} ions (Choi et al., 2014) together with alther pumps and exchangers, althugh both, potassium and chloride currents are the widest. So we tried to confine, for what was possible, to only potassium current by treating the cells with chloride current inhibitor, 9-anthracenecarboxylic acid (9-AC; Jentsch, 2002) observing a significant increase in the total membrane current. A previous work by Minieri et al. (Minieri et al. 2013) has shown that 4-[(2-Butyl-6,7-dichloro-2-cyclopentyl-2,3-dihydro-1-oxo-1H-inden-5-yl) oxy] butanoic acid (DCPIB), the more specific inhibitor of Cl-outward rectifier channel (ORCC, Verkman and Galietta, 2009), is also able to activate the total membrane current in astrocytes, and they identified in TREK-type channels the

responsible of the current increase and the target of this inhibitor. Our results were in accordance with this study and we even found as additive effect when the cells were treated with both 9-AC and DCPIB.

The ability of the DCPIB to block the chloride current in A549 cells has been previously demonstrated (Canella et al., 2017), but the possibility of modulating other types of ion channels in these cells has not yet been investigated, nor the effect of 9-AC has been analyzed on non-Cl^- channels.

We decided to investigate whether the observed effect was linked to an increase in potassium current and if it was caused by the inhibition of chloride flow, or by the direct effect of DCPIB and 9-AC drugs on one of the different types of K^+ channels present on the IITP cell membrane.

Eggermont et al. (2001) indicated the involvement of Cl^- and K^+ channels in the ability of the cell to recover its volume and to rebalance the ionic concentrations. Pasantes-Morales et al. (2006) suggested TREK channels, belonging to the two-pore channel family domain K_{2P}, as possible candidates for this function, in astrocytes. They generate an outwardly rectifyer K^+ current, activated by swelling and cell stretching, by phosphorylation, polyunsaturated fatty acids, volatile anesthetics and acid pH (Patel and Honore, 2001; Berrier et al., 2013), with a stabilizing function on the membrane potential. The wide variety of mechanisms able to control the gating of the TREK channels, indicates their possible involvement in pathological conditions that cause an increase in polyunsaturated fatty acids or cellular swelling.

In the literature, there are contradictory results on the presence of these channels on lung epithelial cells. For example, Bardou et al. (2009), indicated the presence of TREK-1 in H441 and Calu3, and TREK-2 cells in H441, but not in A549, while Roan et al. (2014) was able to silence the TREK-1 gene in A549 cells and to suggest a role of this channels in the regulation of cytokines secretion.

In the epithelial cells it is not easy to identify the channel responsible for a pharmacological effect, because, for each ionic species that might be involved, there are many types of channels that could be responsible for that effect. Therefore, it is difficult to find selective inhibitors of the single type of channel and to separate the pure current carried by “that” channel from the total current.

Nevertheless, we demonstrated the involvement of K^+ current in the DCPIB and 9-AC enhancement of the total current, performing experiments with the not diffusible ions N-Methyl-D-glucamine (NMDG) instead of K^+ ions in the internal solution, and performing experiments with, $BaCl_2$, a K^+ current inhibitor (Ma et al., 2011), in the bath solution.

In addition, by silencing the ORCC channel gene (ANO6, Martins et al., 2011), we excluded a direct cross-talk between the Cl^- ions and I_K, confirming the results already observed for DCPIB in astrocytes.

In order to identify the channel responsible for the current increase, we followed the literature and verified the involvement of the TREK type channels. We observed, through immunocytochemistry technique, that TREK-1 protein is expressed in the A549 cells and that it is active. In fact, patch clamp experiments with its specific activator (arachidonic acid, AA, Minieri et al., 2013) and the specific blocker spadin (Djillani et al., 2017) showed its functionality.

Overall, our work gives strong indications of the existence of a conductance due to the TREK-1 channel in A549 cells, and that this conductance can be modulated by 9-AC and DCPIB drugs that can play a protective role if the cell undergoes to non-physiological volume changes.

MATERIALS AND METHODS

Cell culture and treatments

The A-549 (A549: ATCC, Manassas, VA) was cultured in Dulbecco's modified Eagle's medium high glucose (DMEM, Lonza®, Milan, Italy) supplemented with 10% fetal bovine serum (FBS, EuroClone, Milan, Italy), L-glutamine at 1%(Lonza) and antibiotics penicillin (100 U/ml) and streptomycin (100 µg/ml) at 1% (Lonza). The cells were then kept in an incubator at a temperature of 37 °C with 95% air and 5% CO_2 until the ideal confluence was reached as previously described (Valacchi et al., 2011).

Western blot analysis

Total cell lysates were extracted in RIPA buffer containing protease and phosphatase inhibitor cocktails (Sigma–Aldrich Corp.), as described before (Benedusi et al., 2018). Cells were harvested by centrifugation and protein concentration was determined by the method of Bradford (Biorad Protein assay, Milan, Italy). After protein quantification, 60 µg boiled proteins were loaded into 10% sodium dodecyl sulphate-polycrylamide electrophoresis gels and separated by molecular size. Gels were electro-blotted onto nitrocellulose membranes and then blocked for 90 min in Tris-buffered saline, pH 7.5, containing 0.5% Tween 20 and 5% (w/v) milk powder. Membranes were incubated overnight at 4 °C with the appropriate primary antibody: ANO6 Polyclonal Antibody (Cat. Nr. PA5-35240, Thermofisher Scientific) diluted 1:1000 (Millipore, Billerica, MA). The membranes were finally incubated with the peroxidase-conjugated secondary anti-Rabbit antibody (1:5000) for 1 hr. The bound antibodies were detected by chemiluminescence (Bio-Rad). β-actin was used as loading control. Images of the bands were digitized using an Epson Stylus SX405 scanner and the densitometry analysis was performed using ImageJ software as previously reported (Benedusi et al., 2018).

Immunocytochemistry

A549 cells were grown on coverslips at a density of 1 × 105 cells/ml, and after treatment fixed in 4% paraformaldehyde for 30min at room temperature (RT) as previously described (Canella et al., 2018). Cells were then permeabilized for 5 min at RTwith PBS containing 0.2% Triton X-100, then the coverslips were blocked in PBS containing 1% BSA at RT for 1 hr. Coverslips were then incubated with the appropriate primary antibodies (TREK-1 (F-6): SC-398449; TREK-2 (1C1): SC-293332, Santa Cruz TX, USA) (1:200) in PBS containing 0.5% BSA at 4 °C overnight.

After washing, coverslips were incubated with appropriate secondary antibody (linked to a fluorophore, FITC), (1:100) for 1 hr at RT. Nuclei were stained with 1 µg/ml DAPI (Sigma–Aldrich) for 1 min. Coverslips were mounted onto glass slides using anti-fade mounting medium 1,4 diazabicyclooctane (DABCO) in glycerine and examined by the Leica light microscope equipped with epifluorescence at × 40 magnification.

Negative controls for the immunostaining experiments were performed by omitting primary antibodies. Images were acquired and analyzed with Leica software.

siRNA transfections

A549 cells were seeded in 6-well plates at a density of 2 X 105 cells/well; cells to approximately 50% confluency were transfected with 75 or 100 pmol negative control#1 (Scrambled) siRNA (AM4611 Lot# ASO26WKF, Ambion Austin, TX), or Silencer Select® Pre-designed human Anoctamine 6 siRNA (Cat. Nr. AM16708; ID: 279842, Ambion Austin), using Lipofectamine™ 2000 (Invitrogen) and serum-free medium OptiMEM according to the manufacturer's instructions. After about 6 hours the medium was added with 10% FBS to avoid cell suffering and A549 were incubated for 24 hours until analyses. To test the Efficiencies of siRNAs used, after 24 hours from the transfection, the total cell lysates were analyzed by immunoblotting with the related antibody (ANO6 polyclonal Antibody) as described in Western Blot section of Materiale and Method.

Patch Clamp Technique

Patch pipettes were pulled from glass capillaries with 1.0 mm outer diameter using a micropipette puller (NARISHIGE Instruments, Japan, mod PP-830), fire-polished (tip resistance between 2 and 5 MOhm) and filled with an intracellular solution.

In order to characterize the overall membrane current response, the following solutions were employed:

Control intracellular solution containing (in mM): 145 KCl, 1 $MgCl_2$, 10 HEPES, and 5 EGTA; the pH was adjusted with KOH up to the value of 7.4;

N-Methyl-D-glucamine intracellular solution containing (in mM): 145 NMDG, 1 $MgCl_2$, 10 HEPES, and 5 EGTA; the pH was adjusted with HCl up to the value of 7.4;

Control extracellular solution containing (in mM) 145 NaCl, 1.8 CaCl2, 1 $MgCl_2$, 5.4 KCl, 10 glucose, and 10 HEPES; pH was adjusted with NaOH up the value of 7.35.

Osmolality was adjusted with sucrose to obtain values between 300 and 310 mOsm/Kg for all solution type.

4-(2-butyl-6,7-dichloro-2-cyclo pentyl-indan-1-on-5-yl) oxobutyric acid (DCPIB; Tocris) was dissolved in ethanol and added to the perfusion solution to achieve a final concentration of 10uM (Sato-Numata et al., 2016).

9-acetylanthracene (9-AC; Tocris) was dissolved in ethanol and added to the perfusion solution to achieve a final concentration of 1mM (Cronier et al., 1995; Arnaiz et al., 2013).

Barium Chloride ($BaCl_2$; Merk) was dissolved in the perfusion solution to achieve a final concentration of 4mM (Ma et al., 2011).

Arachidonic Acid (Sigma-Aldrich) was dissolved in DMSO and added to the perfusion solution to achieve a final concentration of 10uM (Minieri et al., 2013).

Spadin (Tocris) was dissolved in water and added to the perfusion solution to achieve a final concentration of 100nM (Moha ou Maati et al., 2011; Borsotto et al., 2015).

Cells were viewed through a TV monitor connected to a contrast enhanced video camera (T.I.L.L. Photonics, Planegg, Germany). The camera was coupled to an inverted microscope (Olympus IMT-2, Tokyo, Japan) equipped with a 40× Hoffman-modulation contrast objective.

After a gigaseal had been formed, intracellular access was established by suction.

Whole cell currents were elicited by voltage-clamp pulses (1,400 ms duration) between +70 and -90 mV in 20mV steps from a beginning holding potential of -30 mV.

The voltage protocol and data acquisition were performed with Digidata card 1322A and pClamp package (version 9.0). The currents were recorded with a commercial patch clamp amplifier (EPC-7; Consumer E-List, Darmstadt, Germany); the recordings were filtered at 5 kHz and acquired at 10 kHz and stored on disk.

Data analysis and statistical procedures

Data are reported in the text and figures as mean ± SEM. The control-treated comparisons were made with GraphPad Prism v.5, and the significance of P values was reported in the text and figures (Student's t test for means, and two-way ANOVA or one-way ANOVA for set of data; significant differences for $P < 0.05$).

RESULTS

DCPIB and 9-AC effect on membrane current

The total current passing through the alveolar cells membrane is mainly due to the movements of chloride and potassium ions (O'Grady and Lee, 2003). In order to better confine the potassium current, we treated the cells with 1mM of 9-AC, a known blocker of the Cl^- currents (Furukawa et al., 1998; Ponce et al., 2012), with 10uM of DCPIB, specific inhibitor of ORCC channels (Verkman and Galietta, 2009) alone or in combination drugs.

Figure 1A shows an example of the current traces that can be obtained when the cells are subjected to the voltage protocol described in the Materials and Methods, in control condition (Fig. 1A).

In Figure 1B is represented the effect 9-AC on a single example cell. Surprisingly, 9-AC causes a very important increase in the outward current range.

In Figure 1C it is possible to see the traces obtained from a cell treated with DCPIB, and in Figure 1D, the example of a possible drug interaction, obtained treating the cell with 9-AC and DCPIB simultaneously. In this last treatment it is possible to notice a further enhancement of the outward component of the current.

In Figure 1E it is possible to observe the average current-voltage curves (I-V) obtained from cell samples under the different experimental conditions confirming the increase of the current in all the different treatments (two-way ANOVA: $P_{CTL\text{-}9AC}<0.0001$; $P_{CTL\text{-}DCPIB}<0.001$; $P_{CTL\text{-}(DCPIB+9AC)}<0.001$).

Regarding the reversal potential of the total current (E_{CTL}), which under control conditions is around 0.74 ± 2.93mV (n = 19), the action of the drugs moves it towards values significantly more negative than the control ($E_{9\text{-}AC}$ = -8.67 ± 3.17, n = 6, P<0.05; E_{DCPIB} = -12.9 ± 5.1, n = 6, P<0.05; $E_{9\text{-}AC+DCPIB}$ = -14.1 ± 2.5, n = 9, P<0.01; Student's t test). These values are quite far from E_K because the Na^+ channels and numerous exchangers and pumps are active giving an important contribution to I_{tot}. Moreover, the effect of DCPIB and 9-AC is not strong at membrane potential less than +30mV (the Bonferroni test applied to ANOVA indicated that the significance for the difference between control and treated groups is given at potentials >= +30 mV) suggesting that the increase in I_K is scarce.

These data, together with the fact that the other main current involved in the genesis of the membrane potential (chloride current), is blocked by DCPIB and 9-AC, has made us suspect a voltage dependent effect of drugs on the potassium flow.

Involvement of K^+ current

In order to demonstrate that the drugs act on I_K, we have replaced the K^+ ions of the intracellular solution with the non-diffusible NMDG ions. In this condition, chloride flow is reduced by DCPIB and 9-AC and potassium current has a positive reversal potential.

Figure 2A shows that, in this experimental condition, the current of a cell sample treated with 9-AC is significantly lower than that of 9-AC treated cells patched with control intra solution (two-way ANOVA: $P_{NMDG\text{-}9AC}<0.0001$) and the reversal potential of the total current shifts to more positive values ($E_{NMDG\text{-}9\text{-}AC}$ = 24.7 ± 2.8, n = 4) consistently with the decrement in potassium current.

Similar results were also obtained when the cells were treated with DCPIB (Fig.2B; two-way ANOVA: $P_{NMDG\text{-}DCPIB}<0.0001$; $E_{NMDG\text{-}DCPIB}$ = 36.8 ± 2.5, n = 5) or both drugs simultaneously (Fig.2C; two-way ANOVA: $P_{NMDG\text{-}(DCPIB+9AC)}<0.0001$; $E_{NMDG\text{-}(DCPIB+9\text{-}AC)}$ = 51.0 ± 8.4, n = 5).

To confirm these results, the cells were treated (control intra and extra solution) with a specific inhibitor of potassium current: barium chloride ($BaCl_2$) (Nelson and Quayle, 1995; Ma et al., 2011).

As it is shown in Fig.3A , when the cells were treated with 9-AC in the presence of $BaCl_2$, the total current was significantly lowers (two-way ANOVA, $P_{BaCl2\text{-}9\text{-}AC}$ <0.0001) respect the only 9-AC treated cell. In addition, the reversal potential of the total current shifted to more positive values ($E_{BaCl2\text{-}9\text{-}AC}$ = 15.6 ± 2.3, n = 13) consistently with the reduction of a current with negative reversal potential.

If the cells were treated with $BaCl_2$ and DCPIB (Fig.3B, two-way ANOVA: $P_{BaCl2\text{-}DCPIB}<0.0001$) or both inhibitors (DCPIB and 9-AC) simultaneously (Fig.3C, two-way ANOVA: $P_{BaCl2\text{-}(DCPIB+9\text{-}AC)}<0.0001$) we obtained similar results ($E_{BaCl2\text{-}DCPIB}$ = 18.2 ± 3.5, n = 12; $E_{BaCl2\text{-}(DCPIB+9\text{-}AC)}$ = 10.9 ± 4.9, n = 10).

Overall, these outcomes confirm that the potassium current is the target for both 9-AC and DCPIB.

Are Cl^- ions involved in potassium current regulation?

To explain the observed phenomenon, we formulated two hypotheses. The first considered a possible inhibiting action of the chloride ions on I_K. In this case, the use of two inhibitors 9-AC and DCPIB that limited the chloride flow, could remove the inhibition and allow an increase of the membrane current.

The second hypothesis considered a direct potentiating action of DCPIB and 9-AC on the potassium current.

To elucidate this issue, the protein ANO6 was silenced in A549 cells; since it is a functional part of the ORCC channel, responsible for most of the chloride current at depolarized potential.

The effect of siRNA on ANO6 protein expression level was evaluated after 24 hours. As shown in Figure 4A, after the ANO6 silencing, the protein level decreased significantly compared to the control; in particular, transfection with 75 pM of siRNA for 24 hours caused a decrease over 50% of ANO6 protein level. Moreover, A549 cells transfected with the scrambled siRNA showed no significant differences in ANO6 protein expression level. These data indicated that our experiment of siRNA-mediated gene knockdown was efficient and that the silencing effect achieved by ANO6-siRNA was gene-specific.

When the current in the silenced cells was analyzed (Fig. 4B) in the absence of further inhibitors, the observed I-V curve (n = 8) was significantly lower than that of the control (two-way ANOVA, $P < 0.01$), confirming the efficiency of the siRNA.

Furthermore, the addition of DCPIB and 9AC induced a further increase in total current similar to that observed in non-silenced cells.

These results lead us to conclude that the two drugs directly act on potassium current.

TREK-1 channel is present on cell membrane

The next step was to identify the K^+ channel that could be targeted by 9-AC and DCBIB. Based on the work of Minieri et al. (2013), our research was directed to analyze the involvement of TREK-1 and TREK-2 channels.

The expression of TREK-1, but not TREK-2 in A549 cell line was demonstrated by immunocytochemical experiment. Figure 5A shows positive staining for TREK-1 and the immunoreactivity was mainly localized at the perinuclear membrane, in the cytosol and at the cell membrane. Nuclei were visualized by staining with 4',6-diamidino-2-phenylindole (DAPI): the merged image of the two signals confirmed no nuclear staining for TREK-1 (no TREK-2 presence was detected; data not shown).

The final demonstration of the activity of the TREK-1 channels, was obtained through a functional approach. The cells were treated with 10um arachidonic acid (AA), a known TREK-1 channel activator (Minieri et al., 2013) and subjected to patch clamp. The results are shown in Figure 5B. When the cells are treated with AA, the total current results significantly increased respect to the control (two-way ANOVA: $P_{CTL-AA}<0.0001$). As a proof of concept, treating the cells with spadin a specific inhibitor of TREK-1 channels activity (Djillani et al., 2017), cancels the AA effect.

TREK-1 involvement in DCPIB and/or 9AC induced potassium current alteration

Unfortunately, it is not possible to compare the kinetics of the currents evoked by AA, DCPIB and 9-AC. In fact, in A549 cells, the AA also activates the ClC-2 chloride channels (Cuppoletti et al., 2001), while the 9-AC is a I_{Cl} inhibitor and the DCPIB is an ORCC chloride channel blocker. It is evident that, even if the control currents were subtracted from the treated ones, comparable curves could not be obtained.

Using spadin we demonstrated that a significant part of DCPIB and 9-AC elicited current is cancelled, as shown in Figure 6A and 6B, (two-way ANOVA, $P_{9AC-(spadin+9AC)}<0.0001$; $P_{DCPIB-(spadin+DCPIB)}<0.0001$).

We have interpreted this result as the functional demonstration that 9-AC and DCPIB influence the behavior of the TREK-1 channels.

DISCUSSION

The variation in cell volume is the consequence of various types of damage including trauma, oxidative stress etc. The cells react to the increase in volume, activating chloride and potassium conductance that allows the efflux of water guided by osmosis, and the recovery to the initial volume (Solc and Wine, 1991; Cowley and Lindsdell, 2002). Since cell volume changes are detrimental to cell survival, many studies have been performed to understand their mechanisms and to identify molecules that can counteract them. DCPIB is one of these drugs and its action is carried out by inhibiting the activity of ORCC channels in many cell types (Sato-Numata et al., 2016).

The action of this drug on ORCC channels has also been demonstrated on A549 cells, a model of human lung epithelial cells, when subjected to oxidative stress (Canella et al., 2017).

In this work we demonstrate that in this cell type, DCPIB also influences K^+ current, and that this action is replicated by another chloride current inhibitor: 9-AC. We also have presented several observations that their action is most likely carried out by enhancing the activity of the TREK-1 channel belonging to the large subfamily of the K_{2P} channels.

TREK-1 channel is normally activated by the stretching of the cell membrane and it plays important functions in regulating cytokines secretion, cell proliferation, protecting against hyperoxia induced alveolar cells damage (Bayliss and Barret, 2008; Schwingshackl et al., 2014; Schwingshackl et al., 2015).

Previous research on the effect of DCPIB on K^+ channels has yielded conflicting results. Decher et al. (2001) showed that the drug does not modulate K^+ conductance in cardiomyocytes, while Best et al. (2004) observed activation of the K^+ ATP-dependent channel in the β-pancreatic cells in rat and Minieri et al. (2013) demonstrated the activation by DCPIB of TREK-1 and TREK-2 channels in astrocyte cell cultures. No studies have so far demonstrated a possible similar action by 9-AC.

In the present work, we studied the effect of DCPIB and 9-AC on the K^+ current under isotonic conditions and we observed total current enhancement by both drugs. Although, the effect of the drugs was significant mostly on the outward part of the current (two-way ANOVA, Bonferroni test), and considering that at potential values close to current reversal potential there is a contribution of several ionic species, DCPIB and 9-AC moved the total current E_{rev} towards significantly more negative potentials (E_{CTL} = 0.74 ± 2.39mV, n = 19; E_{DCPIB} = -12.9 ± 5.1; $E_{9\text{-}AC}$ = -8.67 ± 3.17mV; $E_{DCPIB+9\text{-}AC}$ = -14.11 ± 2.47mV;) in all the analyzed experimental conditions (for P and n see Results). These results made us hypothesize that the current increase was due mainly to I_K involvement.

Experiments in which the internal K^+ ions were substituted with the non-diffusible NMDG ions and experiments with the $BaCl_2$ to block the I_K, confirmed this hypothesis.

We further want to understand whether the inhibitory effect of DCPIB and 9AC on chloride current is via a direct inhibition of chloride channel. To verify this hypothesis we have performed siRNA for ORCC channel in its ANO6 portion, whose contribution to the chloride current is preeminent especially to the most

depolarized potentials. Interestingly our date have demonstrated that there was no relation between the movements of the Cl^- ions and the K^+ current.

The following step was to understand whether are the K^+ channels a target of our drugs also base on the literature that has suggested TREK channels being a direct target of DCPIB (Minieri et al., 2013)

By immunocytochemistry, we verified the presence of TREK-1, but not TREK-2 on the membrane of A549 cells and by patch clamp experiments using arachidonic acid as TREK-1 activator, we demonstrated that these channels are active on the cellular membrane. In addition, as a proof of concept, treatment of cells with spadin, a known inhibitor of TREK-1 activity, cancels the action of AA.

The comparison between the current evoked by DCPIB and that of cells treated with DCPIB during exposure to spadin, corroborates our hypothesis that the TREK-1 channel could be the target of this drug.

If this result was quite predictable on the basis of the literature, the same cannot be said for the 9-AC. Indeed, the same set of experiment using 9-AC in the presence of spadin, gave similar results.

The importance of the correct activity of TREK-1 in the alveolar epithelium has been demonstrated in several studies. Schwingshackl et al. (2014) showed that, in an in vivo model of Acute Lung Injury, the deficiency of TREK-1 leads to an increase in damage and apoptosis of alveolar cells and decreases the levels of cytokine in bronchoalveolar lavage (BAL) fluid. Roan et al. (2014) reported that the alveolar cells deficient in TREK-1 contain less F-actin and are more resistant to stretch damage. The same authors, in a previous study (Schwingshackl et al., 2012) showed that hyperoxia affects the expression of TREK-1 in pulmonary alveolar cells and indicated the involvement of this channel in the regulation of cell proliferation and cytokine secretion.

In conclusion, the present work provides biomolecular and functional evidences of the presence and the functionality of TREK-1 channel on A549. The effect of drugs mainly at depolarized potential, indicates their possible usefulness in the therapeutic field.

Further studies will be necessary to identify the interaction sites of these two molecules with the TREK-1 channel protein especially for lung diseases. It has already been hypothesized that molecules capable of opening these channels may play a protective role in heart and nervous tissue (Judge and Smith, 2009) therefore in the context of pulmonary diseases the potential therapeutic value of DCPIB, 9-AC and other related compounds, through the modulation of TREK's activity deserves further investigation.

CONFLICTS OF INTEREST

The authors declare that there are not any conflicts of interest

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ETHICAL STATEMENT:
Our protocols are applied on cell culture and therefore ethical approval is not required.

AUTHOR CONTRIBUTIONS:

Rita Canella, Mascia Benedusi and Giuseppe Valacchi were responsible for substantial contributions to the conception, design, analysis, drafting the work, revising the work, and reviewing of the manuscript. Marta Martini, Franco Cervellati and Carlotta Cavicchio performed experiments and assisted with the data gathering. Additionally, all authors provided final approval of the version to be published and agreed to be accountable for all aspects of the work in ensuring the accuracy and/or integrity of the work.

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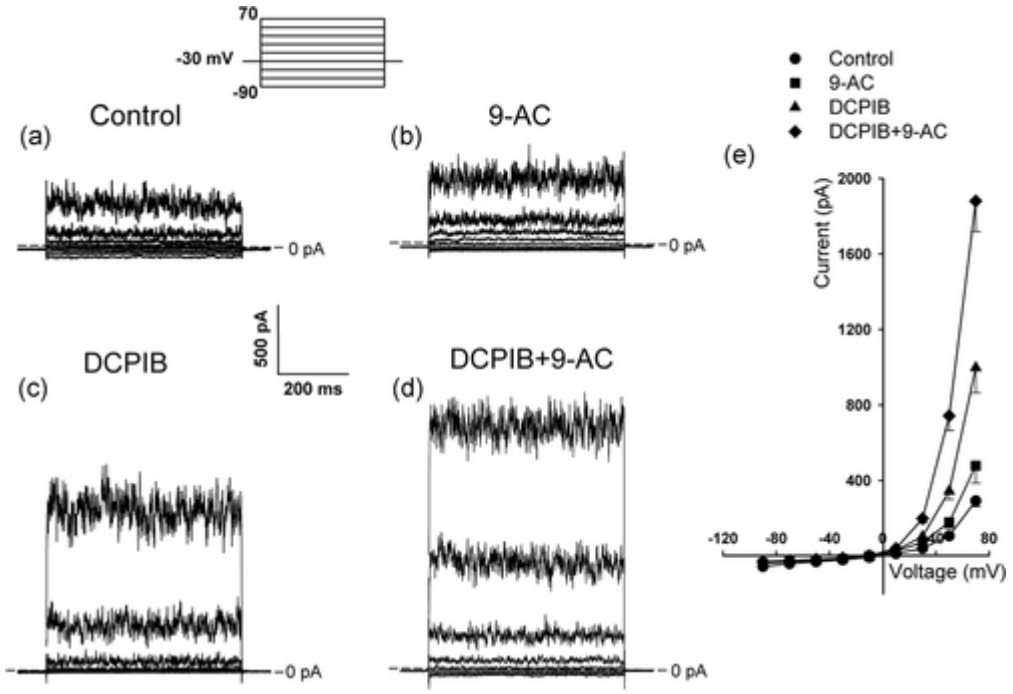
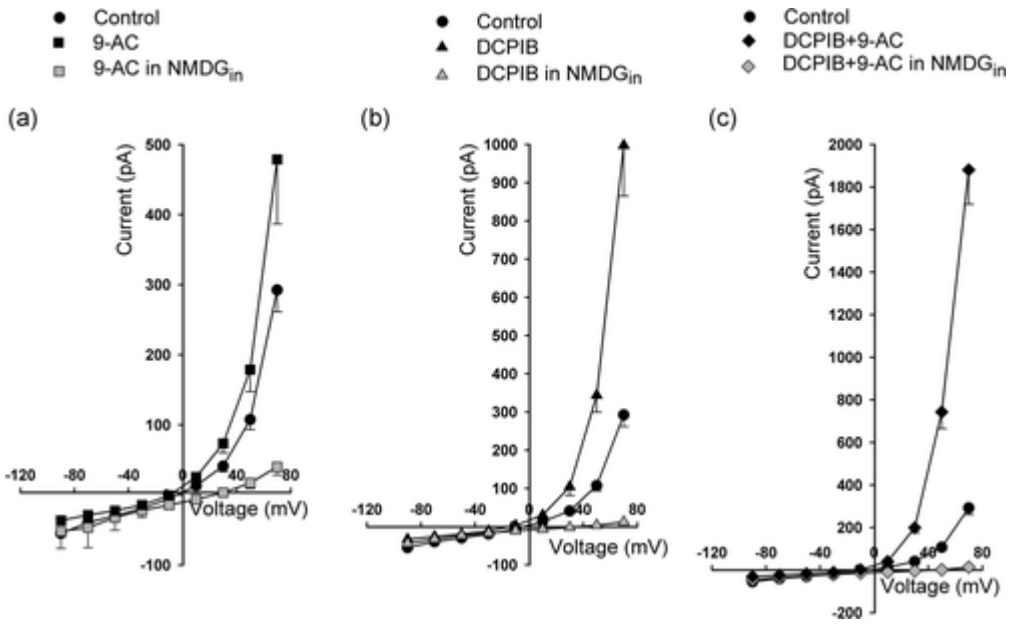
FIGURES

Figure 1



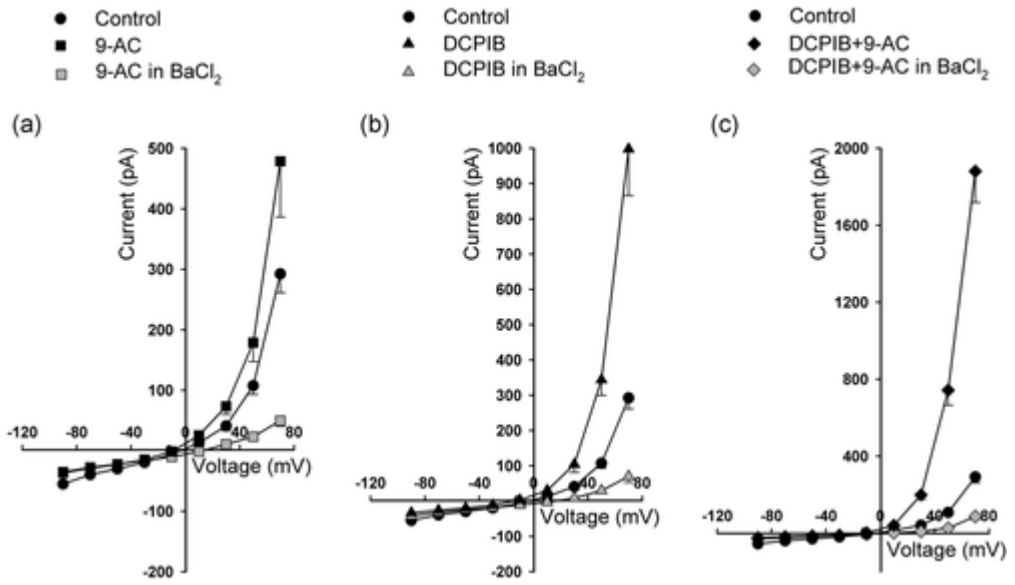
Representative families of total recording currents in control condition (a), after treatment with 9-AC (b), after DCPIB (c), and DCPIB + 9AC (d) treatments. Dashed line indicates zero-current level. (e) Current–voltage relationship of Control (n = 19), treated with 9-AC (n = 6), treated with DCPIB (n = 6) and with DCPIB + 9-AC (n = 9). The comparison between the Control group and the treated groups is significant (two-way ANOVA, p in the test for each comparison). 9-AC: 9-anthracenecarboxylic acid; ANOVA: analysis of variance; DCPIB: 4-[(2-butyl-6,7-dichloro-2-cyclopentyl-2,3-dihydro-1-oxo-1H-inden-5-yl) oxy] butanoic acid.

Figure 2



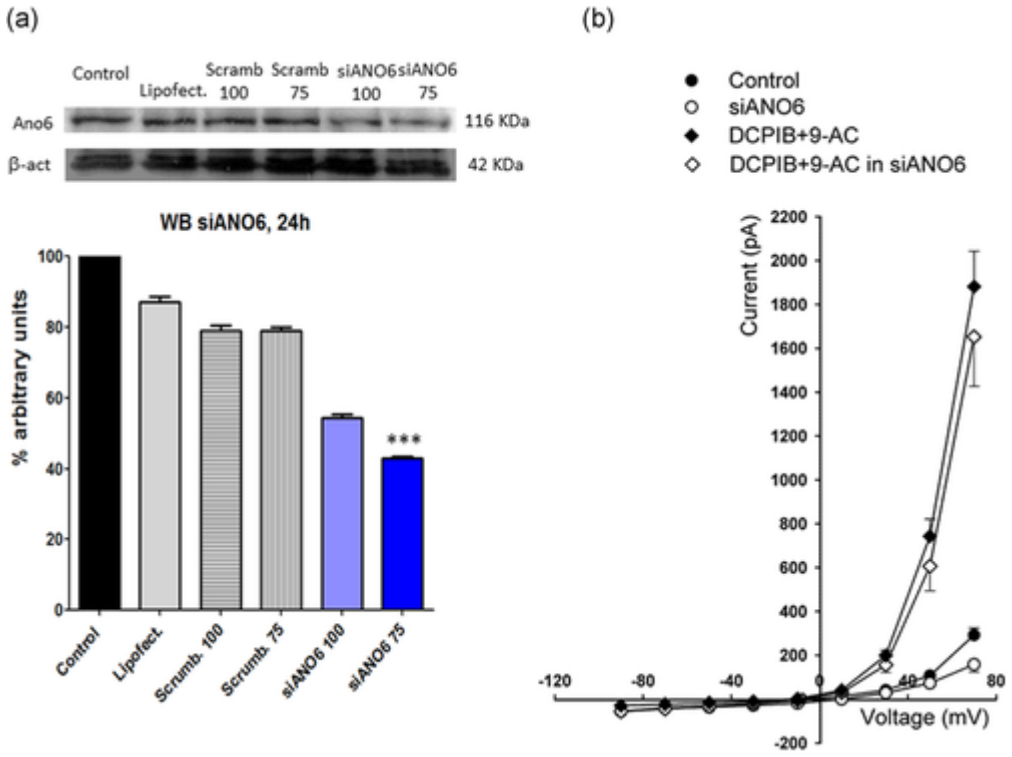
(a) Current–voltage relationship of control (n = 19), treated with 9-AC (n = 6), treated with 9-AC in NMDG (n = 4). (b) Current–voltage relationship of Control (n = 19), treated with DCPIB (n = 6), treated with DCPIB in NMDG (n = 5). (c) Current–voltage relationship of control (n = 19), treated with DCPIB + 9-AC (n = 9), treated with DCPIB + 9-AC in NMDG (n = 5). The comparisons between the 9-AC, DCPIB, and DCPIB + 9-AC groups and the corresponding groups in NMDG are always extremely significant (two-way ANOVA, $p < 0.0001$). 9-AC: 9-anthracenecarboxylic acid; ANOVA: analysis of variance; DCPIB: 4-[(2-butyl-6,7-dichloro-2-cyclopentyl-2,3-dihydro-1-oxo-1H-inden-5-yl) oxy] butanoic acid; NMDG, N-methyl-d-glucamine

Figure 3



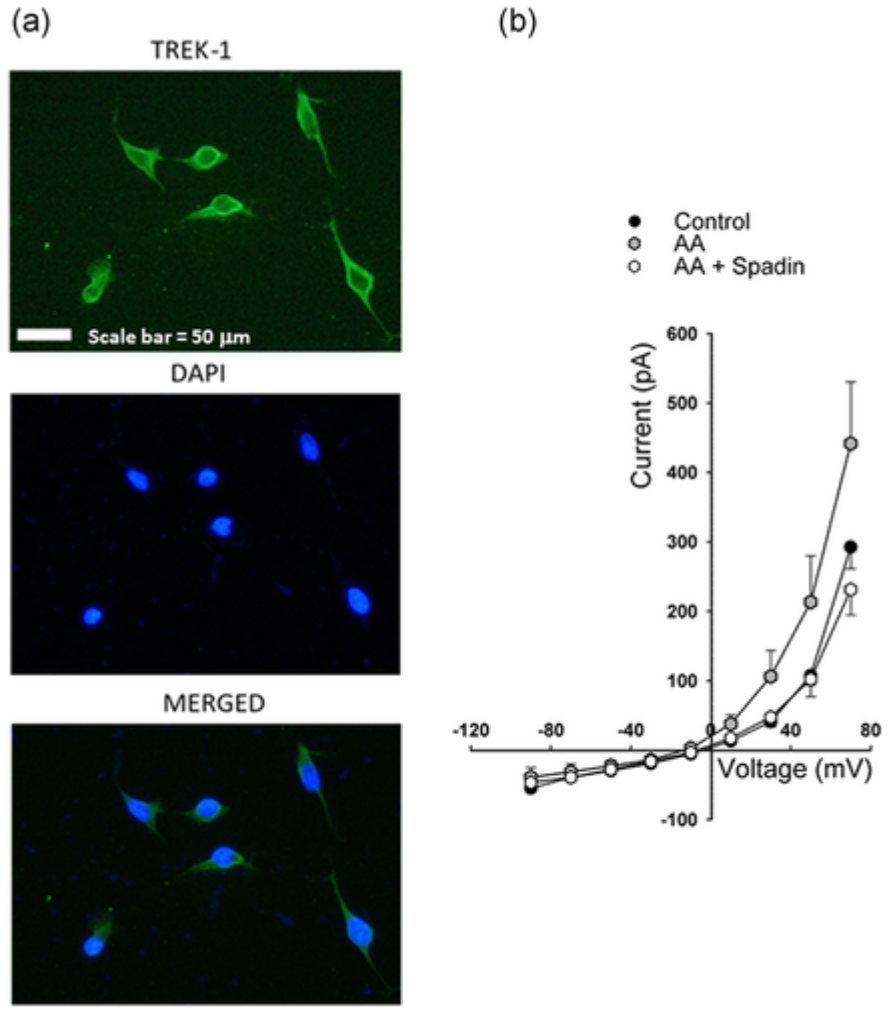
(a) Current–voltage relationship of Control (n = 19), treated with 9-AC (n = 6), and treated with 9-AC in BaClC2 (n = 13). (b) Current–voltage relationship of Control (n = 19), treated with DCPIB (n = 6), and treated with DCPIB in BaClC2 (n = 12). (c) Current–voltage relationship of control (n = 19), treated with DCPIB + 9-AC (n = 9), and treated with DCPIB + 9-AC in BaClC2 (n = 10). The comparisons between the 9-AC, DCPIB and DCPIB + 9-AC groups and the corresponding groups in BaClC2 are always extremely significant (two-way ANOVA, $p < 0.0001$). 9-AC: 9-anthracenecarboxylic acid; ANOVA: analysis of variance; DCPIB: 4-[(2-butyl-6,7-dichloro-2-cyclopentyl-2,3-dihydro-1-oxo-1H-inden-5-yl) oxy] butanoic acid

Figure 4



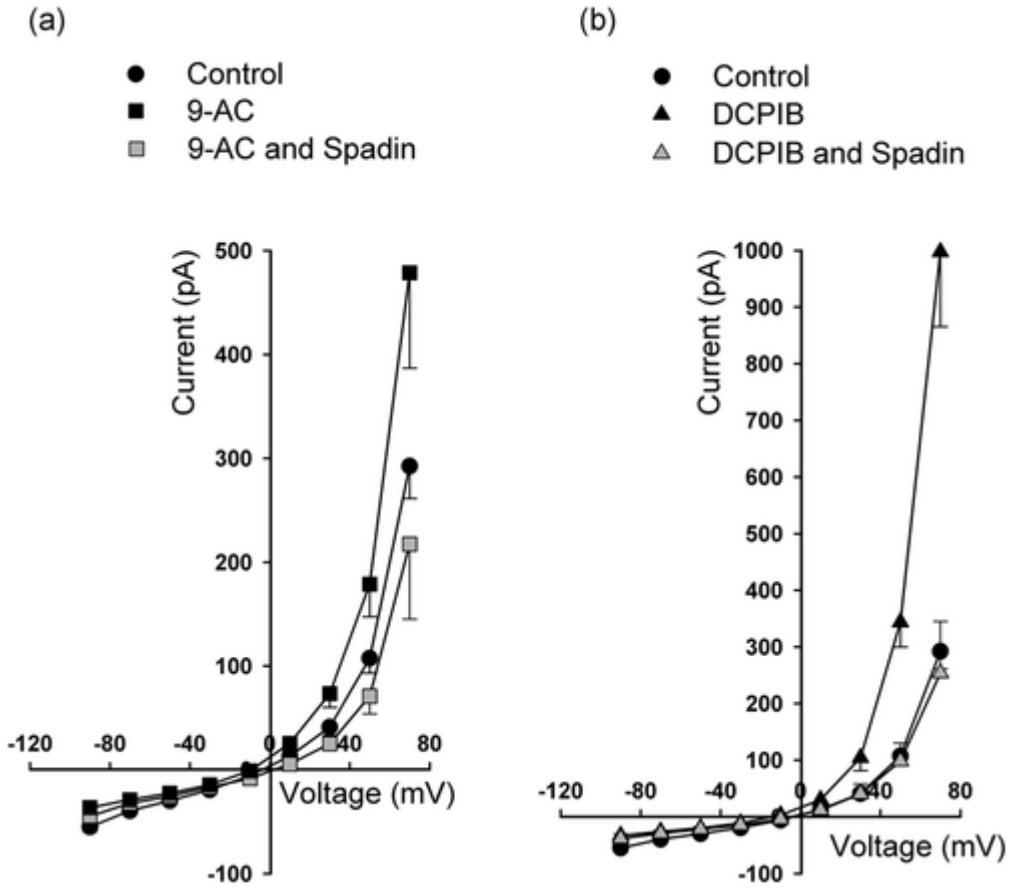
(a) Above: A549 cell-line was transfected with siRNA negative control (Scrambled 75 or 100 pmol) or siRNA for the knockdown of ANO6 (75 or 100 pmol) for 24 hr, and the level of anoctamine 6 protein was assessed by Western blot analysis with the relative antibody. A representative image is shown with β-actin as indicator of loaded proteins. Data are shown as the mean ± SEM of three independent experiments. Below: the quantification of the western blot bands is expressed as arbitrary unit versus control. Statistical test one-way ANOVA. (b) Current–voltage relationship of Control (n = 19), silenced ANO6 (n = 8), treated with DCPIB + 9-AC (n = 9), silenced ANO6 and treated with DCPIB + 9-AC (n = 9). The comparison between the Control group and the siANO6 group was very significant (two-way ANOVA, $p < 0.01$); the comparison between the DCPIB + 9AC group and DCPIB + 9AC in the siANO6 group was not significant (two-way ANOVA). 9-AC: 9-anthracenecarboxylic acid; ANO6; anoctamin 6; ANOVA: analysis of variance; DCPIB: 4-[(2-butyl-6,7-dichloro-2-cyclopentyl-2,3-dihydro-1-oxo-1H-inden-5-yl) oxy] butanoic acid; siRNA: small interfering RNA [Color figure can be viewed at wileyonlinelibrary.com]

Figure 5



(a) Representative image of immunocytochemistry for TREK-1 in A549 cell line: (left) FITC staining, (middle) DAPI staining of the same cells, (right) merge of the two previous images. Images are shown at 40X magnification. (b) Current–voltage relationship of Control (n = 19), treated with AA (n = 4), and treated with AA and Spadin (n = 7). The comparisons between Control group and AA group and between AA group and AA + Spadin group are extremely significant (two-way ANOVA, $p < 0.0001$). AA: arachidonic acid; ANOVA: analysis of variance; DAPI: 4′,6-diamidino-2-phenylindole; FITC: fluorescein isothiocyanate [Color figure can be viewed at wileyonlinelibrary.com]

Figure 6



(a) Current–voltage relationship of Control (n = 19), treated with 9-AC (n = 6), and treated with 9-AC + Spadin (n = 8). The comparison between the 9-AC group and the 9-AC + Spadin group was extremely significant (Two-way ANOVA, $p < 0.0001$); (b) Current–voltage relationship of Control (n = 19), treated with DCPIB (n = 6), and treated with DCPIB + Spadin (n = 4). The comparison between the DCPIB group and DCPIB + Spadin group was extremely significant (two-way ANOVA, $p < 0.0001$). 9-AC: 9-anthracenecarboxylic acid; ANOVA: analysis of variance; DCPIB: 4-[(2-butyl-6,7-dichloro-2-cyclopentyl-2,3-dihydro-1-oxo-1H-inden-5-yl) oxy] butanoic acid