

## ROCK1 Translocates From Non-Caveolar to Caveolar Regions Upon KCl Stimulation in Airway Smooth Muscle

B. SOMMER<sup>1</sup>, L. M. MONTAÑO<sup>2</sup>, J. CHÁVEZ<sup>1</sup>, V. CARBAJAL<sup>1</sup>, L. M. GARCÍA-HERNANDEZ<sup>2</sup>, C. IRLES<sup>4</sup>, A. M. JIMÉNEZ-GARDUÑO<sup>3</sup>, A. ORTEGA<sup>3</sup>

<sup>1</sup>Department of Bronchial Hyperreactivity Research, National Institute of Respiratory Diseases, Mexico City, Mexico, <sup>2</sup>Department of Pharmacology, Faculty of Medicine, National Autonomous University of Mexico, Mexico City, Mexico, <sup>3</sup>Department of Biochemistry, Faculty of Medicine, National Autonomous University of Mexico, Mexico City, Mexico, <sup>4</sup>Department of Physiology, Faculty of Medicine, National Autonomous University of Mexico, Mexico City, Mexico

Received March 25, 2013

Accepted September 17, 2013

On-line January 8, 2014

### Summary

Airway smooth muscle (ASM) membrane depolarization through KCl opens L-type voltage dependent Ca<sup>2+</sup> channels (Ca<sub>v</sub>1.2); its opening was considered the cause of KCl contraction. This substance is used to bypass intracellular second messenger pathways. It is now clear that KCl also activates RhoA/Rho kinase (ROCK) pathway. ROCK isoforms are characterized as ROCK1 and ROCK2. Because ROCK1 seems the most abundant isotype in lung, we studied its participation in KCl stimulated bovine ASM. With methyl-β-cyclodextrin (MβCD) we disrupted caveolae, a membrane compartment considered as the RhoA/ROCK assembly site, and found that KCl contraction was reduced to the same extent (~26 %) as Y-27632 (ROCK inhibitor) treated tissues. We confirmed that KCl induces ROCK activation and this effect was annulled by Y-27632 or MβCD. In isolated plasmalemma, ROCK1 was localized in non-caveolar membrane fractions in Western blots from control tissues, but it transferred to caveolae in samples from tissues stimulated with KCl. Ca<sub>v</sub>1.2 was found at the non-caveolar membrane fractions in control and MβCD treated tissues. In MβCD treated tissues stimulated with KCl, contraction was abolished by nifedipine; only the response to Ca<sub>v</sub>1.2 opening remained as the ROCK component disappeared. Our results show that, in ASM, the KCl contraction involves the translocation of ROCK1 from non-caveolar to caveolar regions and that the proper physiological response depends on this translocation.

### Key words

Bovine airway smooth muscle • Rho-kinase1 • Caveolae • L-type Ca<sup>2+</sup> channels

### Corresponding author

B. Sommer, Bronchial Hyperreactivity Research Department, National Institute of Respiratory Diseases, Calzada de Tlalpan 4502, Mexico City, D.F. CP 14080, Mexico. Fax: (52-55)5666-5868. E-mail: bsommerc@hotmail.com

### Introduction

For a long time, KCl induced airway smooth muscle (ASM) contraction has been considered to be developed solely by Ca<sup>2+</sup> influx through L-type voltage-dependent Ca<sup>2+</sup> channels (Ca<sub>v</sub>1.2). In vascular smooth muscle, it has been demonstrated that KCl causes Ca<sup>2+</sup> sensitization *via* Rho-activated kinase (ROCK) activation (Mita *et al.* 2002, Urban *et al.* 2003). Ca<sup>2+</sup> sensitization involves monomeric G protein RhoA and its downstream effector, ROCK, responsible of phosphorylating myosin light chain phosphatase (MLCP) on its targeting subunit, diminishing its activity, and therefore favoring contraction without detectable changes in [Ca<sup>2+</sup>]<sub>i</sub> (Somlyo and Somlyo 2003). In ASM, RhoA translocation to the membrane is indispensable for the signaling pathway activation (RhoA/ROCK), and it is thought to be depolarization and Ca<sub>v</sub>1.2 dependent (Janssen *et al.* 2012). Additionally, it is known that KCl stimulation

promotes cytosolic ROCK translocation to specialized membrane domains called caveolae in vascular smooth muscle (Urban 2003). However, Nuno *et al.* (2012) published recently that, in mice aortic tissues, ROCK moves from caveolar to non-caveolar regions after 5-HT stimulation, showing that ROCK translocation develops in the membrane domains and not only from the cytosol to caveolae. ROCK isoforms have been characterized as ROCK1 and ROCK2. It has been documented that their distribution is tissue related: isotype 1 is abundant in lung, liver, spleen, kidney and testis, whereas isotype 2 is present mainly in heart, vascular and brain (Noma *et al.* 2006, Nuno *et al.* 2012). Therefore, we found it noteworthy to define if ROCK1 localization in ASM membrane domains is related to its function during depolarization-induced contraction.

By the other hand,  $Ca_v1.2$  localization in lipid rafts has been well documented, and their proper functionality seems independent of caveolar integrity. In rat cerebral artery, methyl- $\beta$ -cyclodextrin (M $\beta$ CD) treatment, a pharmacological agent commonly used to remove membrane cholesterol and disrupt caveolae (Sommer *et al.* 2009), did not modify the  $Ca_v1.2$  currents (Löhn *et al.* 2000). In this same context, using tail artery from rats or rat ureter smooth muscle, KCl tonic responses were not altered by caveolae disruption after M $\beta$ CD treatment (Dreja *et al.* 2002, Babychuk *et al.* 2004). Furthermore,  $Ca_v1.2$  showed partial co-localization with caveolin-1 (Cav-1) at the cell membrane of the intestinal smooth muscle cells, interstitial cells of Cajal, and lower esophageal sphincter and skeletal muscle cells of mouse (Cho and Daniel 2005). In canine airway smooth muscle, Darby *et al.* (2000) found that  $Ca_v1.2$  are enriched in caveolae, but proposed that it is more likely that they are located throughout the plasma membrane.

In the present work we explored the possibility that KCl activates RhoA/ROCK pathway and promotes ROCK1 translocation from non-caveolar to caveolar membrane region. We also studied the membrane localization of the  $Ca_v1.2$  in airway smooth muscle.

## Materials and Methods

### *Organ baths experiments*

Bovine tracheal tissues were obtained from a local slaughterhouse and immersed in ice-cold Krebs solution for its transportation to the laboratory. Tissues were dissected free of cartilage, epithelium and

connective tissue, and smooth muscle strips (2-2.5 mm wide, 5 mm long) were cut and suspended in 5 ml organ baths containing Krebs solution (in mM): 118 NaCl, 25 NaHCO<sub>3</sub>, 4.6 KCl, 1.2 KH<sub>2</sub>PO<sub>4</sub>, 1.2 MgSO<sub>4</sub>, 11 glucose, and 2 CaCl<sub>2</sub>. Preparations were maintained at 37 °C and bubbled with 5 % CO<sub>2</sub> in oxygen at pH 7.4. Tissues were attached to an isometric force transducer (model FT03; Grass Instruments, West Warwick, RI, USA) connected to a system of signal conditioner (CyberAmp 380, Axon Instruments, Foster City, CA, USA) plus an analog-digital interface (Digidata 1200A; Axon). Recordings were stored in a microcomputer and analyzed using data acquisition and analysis software (AxoScope version 9.0; Axon). Preparations were equilibrated for 30 min under a resting tension of 1-1.5 g before testing. Tissues were then stimulated three times with 60 mM KCl (lasting 20 min each) until maximum stable responses were obtained. Tissues were washed 3 times with Krebs solution and, once the basal tone was reached, a fourth KCl stimulation was done. Afterwards, some tissues were incubated with (+)-(R)-trans-4-(1-aminoethyl)-N-(4-pyridyl) cyclohexane carboxamide (Y-27632, 10  $\mu$ M), a ROCK inhibitor, and stimulated again with KCl. In some experiments, tissues were contracted with KCl and during the maximal contraction responses 1  $\mu$ M nifedipine was added, followed later by 10  $\mu$ M Y-27632; in this context, similar experiments were carried out but adding Y-27632 first. In other set of experiments tissues were preincubated with 40 mM methyl- $\beta$ -cyclodextrin (M $\beta$ CD) for 3 h, washed and then stimulated with 60 mM KCl. Other tissues, after the incubation with M $\beta$ CD, were stimulated with KCl and when the maximum response was reached, nifedipine was added. In order to verify a possible additive effect among M $\beta$ CD and Y-27632, bovine tracheal smooth muscle was incubated with both drugs and stimulated with KCl. Control tissues without M $\beta$ CD were stimulated with KCl after 3 h.

### *Isolation of plasmalemma preparation*

Microsomal membranes were obtained from previously frozen bovine tracheal smooth muscle stimulated or not with 60 mM KCl. Another set of tissues was treated with 40 mM M $\beta$ CD for 3 h. Plasmalemma was isolated from the microsomal fraction by differential centrifugation and a discontinuous sucrose gradient in the absence of any reducing agent in a buffer medium containing in mM: 20 tris-malate, 100 KCl, 300 sucrose (10 %), pH 7.0. A first sucrose gradient of 24, 35, 43 and

55 % was centrifuged at 95,200g (Beckman SW28, CA, USA) for 2 h, to separate the plasmalemma. We found that the interface between 10 to 24 % contains the highest proportion of Cav-1 and the Ca<sub>v</sub>1.2, molecular markers for plasma membrane. This fraction was washed in a buffer containing: 20 mM Tris-maleate pH 7.0 and centrifuged at 180,000g (Beckman 45Ti, CA, USA)/45 min. The collected membranes were treated with a solution containing 20 mM Tris-maleate and 1 % Triton X-100 (Calbiochem, Darmstadt, Germany), pH 7.0, at 4 °C for 30 min. The membranes were diluted 1:1 with a solution containing 20 mM Tris-maleate, 80 % sucrose to give a final sucrose concentration of 40 %, pH 7.0. Triton X-100 treated plasmalemma was placed at the bottom of a second gradient: 40, 30 and 5 %. This gradient was centrifuged at 250,000g (Beckman-SW55T1, CA, USA) overnight, and afterwards nine fraction of equal volume (0.5 ml) were collected.

Caveolar and non-caveolar membrane fractions were then recognized following a modification of the methods described previously (Blank *et al.* 2002). Low-density fractions (1 to 5), designated as membrane caveolar fractions, are not soluble with Triton X-100 and high-density fractions (6 to 9), called non-caveolar membrane fractions, are enriched in Triton X-100. To corroborate that the low-density fractions correspond to lipid rafts, the expression of GM1 (a characteristic caveolar ganglioside, Parton 1994) was evaluated. From each fraction (9 in total) 2 µl were submitted to a dot blot in a nitrocellulose membrane that was blocked with 1 % non fat dry milk in TBS tween (Tween 20, 0.1 %, TBST) for 1 h. Afterwards, the membrane was incubated with cholera toxin B conjugated to FITC (1:10,000, Sigma). Dots were revealed with an ECLTM chemiluminescence kit and photographic paper (Amersham Hyperfil, ECL, Cat. 28-9068-35, Buckinghamshire, UK). Caveolar fractions 3-4 and non-caveolar fractions 8-9 respectively, were pooled as published elsewhere (Sommer *et al.* 2009). Total protein content was determined using Coomassie Plus Protein Assay Reagent (Pierce, Rockford, IL USA) with BSA as the standard. Samples were then adjusted to a protein content of 10 µg and boiled for 2 min. These samples were loaded in different lanes of an 8 % SDS-polyacrylamide gel and subjected to electrophoresis. Afterwards, proteins were transferred to a nitrocellulose membrane and blocked with 1 % non fat dry milk in TBST for 1 h. Membranes were incubated overnight at 4 °C with 1:1000 mouse anti-Cav-1 (monoclonal, cat # 610406, Becton Dickinson, CA, USA),

1:500 rabbit anti-Ca<sub>v</sub>1.2 (cat # ACC-003, Alomone, Jerusalem, Israel) or 1:500 rabbit anti-ROCK1 (cat # ab45171, Abcam, MA, USA). We used ROCK1 antibody since its mRNA is preferentially expressed in lung, whereas ROCK2 mRNA is highly expressed in the heart and brain (Noma *et al.* 2006). The secondary antibody (HRP conjugated goat anti-mouse, Amersham Biosciences, Buckinghamshire, UK; or anti-rabbit IgG, Millipore-Upstate, CA, USA; 1:1000), was incubated during 1.5 h, at room temperature. Immunoblots were developed using an enhanced chemiluminescent reactant (LumiGLO; Cell Signaling, MA, USA) and sheets of photographic paper (Amersham Hyperfilm, ECL, Cat. 28-9068-35).

To confirm that proteins were properly transferred to the nitrocellulose membrane, Ponceau red was added and the membranes were gently shaken for 15 min until the bands were seen.

To evaluate the presence of ROCK1, Ca<sub>v</sub>1.2 and Cav-1 in caveolar and non-caveolar fractions, a densitometric analysis was carried out using ImageJ NIH software (<http://rsb.info.nih.gov/ij/>).

The research protocol was approved by the Scientific and Ethical committee from the National Institute of Respiratory Diseases.

#### *Assay for ROCK activity*

ROCK mediated phosphorylation of the myosin phosphatase targeting subunit (MYPT1) by 60 mM KCl, 10 µM Y-27632 + 60 mM KCl or 40 mM MβCD + 60 mM KCl was determined (Sommer *et al.* 2009). Briefly, bovine tracheal smooth muscle strips were placed in organ baths and submitted to the previously described protocol. Afterwards, they were collected and frozen in liquid nitrogen. Tissues were homogenized (Polytron PT 1200 E, Kinematica, Luzern, Switzerland) in 500 µl lysis solution containing 50 mM Tris-HCl, pH 7.5, 0.1 mM EDTA, 0.1 mM EGTA, 750 mM NaCl, 1 % Triton X-100, 50 mM MgCl<sub>2</sub>, 1 mM Phenylmethylsulfonyl fluoride (PMSF), 2 mM sodium orthovanadate, protease inhibitor cocktail (Set III, Calbiochem, Cat. No. 539134, La Jolla, CA, USA). A commercial kit (DC Protein Assay, Catalog 500-0116, Bio-Rad, Hercules, CA, USA) was used to determine total protein content. Forty µg of the sample were loaded in different lanes of an 8 % SDS-polyacrylamide gel and subjected to electrophoresis. Afterwards, proteins were transferred to a nitrocellulose membrane and blocked with 5 % non fat dry milk in TBS-Tween (Tween 20, 0.1 %) for 1.5 h, at room

temperature. Membranes were incubated overnight at 4 °C with anti-phospho- MYPT1 (1:500, Thr696, rabbit polyclonal antibody, Cat. 07-251, Millipore, CA, USA). Afterwards, membranes were incubated with a HRP conjugated goat anti-rabbit IgG secondary antibody (1:1000, Cat. 12-348, Millipore, CA, USA), incubated during 1 h, at 37 °C. Immunoblots were developed using an enhanced chemiluminescent reactant (Super Signal West Femto Maximum Sensitivity Substrate, Cat. 34096, Thermo Scientific, Rockford, IL, USA). Phospho-MYPT1 immunoblots were analyzed by densitometry using a Molecular Imaging ChemiDoc XRS (Bio-Rad, Hercules, CA, USA). Mouse monoclonal  $\alpha$ -actin antibody (1:1000, Cat. A5228, Sigma Chem. Co., St. Louis, MO, USA) was blotted as a control for protein load; membranes were incubated with a horseradish peroxidase-conjugated sheep anti-mouse IgG secondary antibody (1:2000, Cat. NA9310V, Amersham, Buckinghamshire, UK).

### Drugs

Nifedipine, (+)-(R)-trans-4-(1-aminoethyl)-N-(4-pyridyl) cyclohexane carboxamide (Y-27632) and methyl-

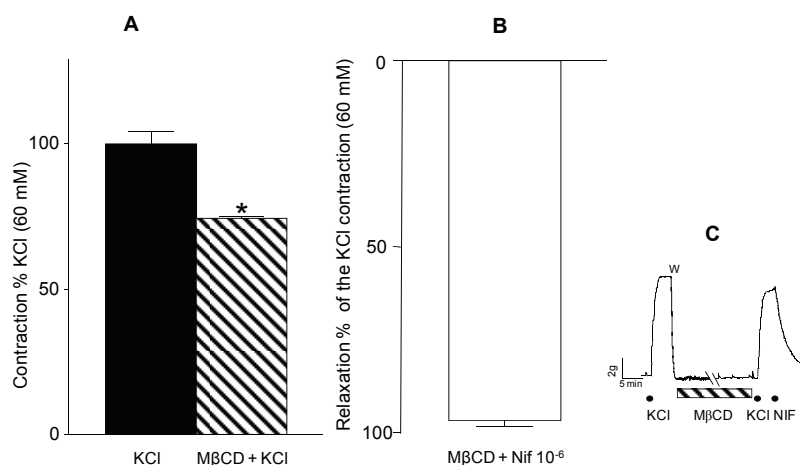
$\beta$ -cyclodextrin were all purchased from Sigma-Aldrich.

### Statistical analysis

Differences in responses of bovine tracheal smooth muscle strips to KCl were evaluated through paired Student's *t* test. For Western blot analysis we used non paired Student *t* test and when more than two groups were evaluated, one-way analysis of variance followed by Dunnett's test. Statistical significance was set at two-tailed  $p < 0.05$ . Data are expressed in the text and illustrations as mean  $\pm$  SEM, and *n* represents the number of animals used.

## Results

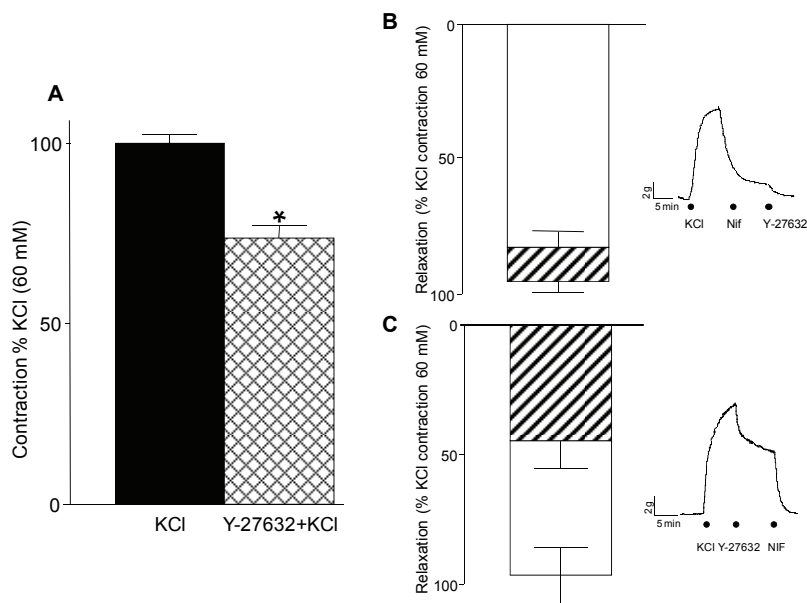
We confirmed an earlier finding (Sommer *et al.* 2009) that airway smooth muscle responses to 60 mM KCl were reduced by incubation with M $\beta$ CD (Fig. 1A,  $30.22 \pm 7.02$  %, *n*=6). When tissues were incubated with M $\beta$ CD during 3 h, nifedipine almost completely relaxed the KCl-induced contraction (Fig. 1B,C). Contrastingly, time control showed no differences in the contractile response to KCl ( $6.37 \pm 0.45$  vs  $6.19 \pm 0.52$  g, *n*=5).



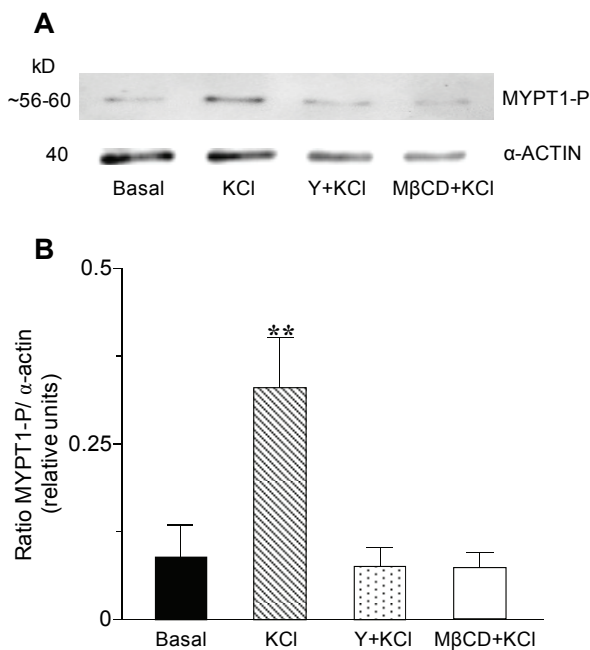
**Fig. 1.** Effect of methyl- $\beta$  cyclodextrin (M $\beta$ CD) and nifedipine (Nif) on KCl stimulated tissues. **(A)** Black bar represents KCl (60 mM) before M $\beta$ CD. Crossed lined bar represents KCl induced contraction after M $\beta$ CD (40 mM, 3 h, *n*=6) **(B)** Open bar represents relaxation induced by Nif (1  $\mu$ M) on KCl stimulated bovine tracheal smooth muscle previously incubated with M $\beta$ CD (40 mM, 3 h, *n*=4). **(C)** Representative trace of this experimental protocol. W: wash. \* $p < 0.05$ , using paired Student *t* test.

ROCK inhibition with 10  $\mu$ M Y-27632 significantly diminished the KCl-induced contraction (Fig. 2A,  $26.34 \pm 3.70$  %, *n*=6). No additive effect among M $\beta$ CD and Y-27632 on KCl-induced contraction was found ( $38.43 \pm 7.12$  %, *n*=8). When 1  $\mu$ M nifedipine was added during the plateau of the contraction induced by KCl, we observed a partial relaxation of about 82 %. The addition of Y-27632 produced an additional relaxation (~12 %) which almost reached the resting basal level (Fig. 2B). In those experiments where Y-27632 was added first to a KCl precontracted tissue, we observed

a relaxation about ~45 % and the remaining contraction was abolished by nifedipine (~55 %, Fig. 2C). When we compared each antagonist under the different experimental conditions, we found that they (nifedipine vs nifedipine,  $p = 0.05$ ; and Y-27632 vs Y-27632  $p = 0.025$ ) were statistically different (Fig 2B,C). One possible explanation to these results would be that ROCK activation is sensitive to intracellular  $Ca^{2+}$  concentration. Therefore, when the  $Ca^{2+}$  entrance is not diminished by nifedipine, the amount of activated ROCK is more efficiently inhibited by 10  $\mu$ M Y-27632.

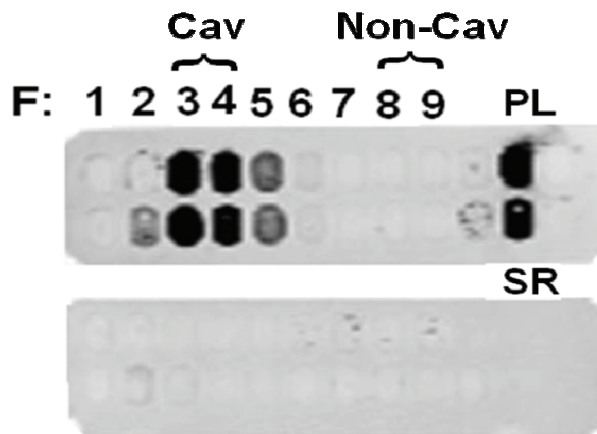


**Fig. 2.** Effect of Y-27632 and nifedipine on KCl induced contraction. **(A)** Black bar shows KCl (60 mM) contraction before incubation with Y-27632 (10  $\mu$ M, n=6, Rho kinase inhibitor). Crossed lined bar depicts KCl contraction after the incubation with Y-27632. **(B)** Open bar illustrates the relaxation induced by nifedipine (Nif, 1 $\mu$ M, n=6) in control tissues. Crossed patterned rectangle indicates the further relaxation induced on the same preparation by the addition of Y-27632. **(C)** Crossed patterned bar shows the Y-27632 induced relaxation and the open bar, the nifedipine response. Right, a representative trace of the experiments graphed in (B) and (C) is shown. \*, significant at  $p < 0.01$  by paired Student  $t$  test.



**Fig. 3.** ROCK activity through MYPT1 phosphorylation (MYPT1-P) assay in bovine tracheal smooth muscle. **(A)** Representative western blots for MYPT1-P (basal, n=4) induced by KCl stimulation (60 mM, n=3) by Y-27632 (Y, 10  $\mu$ M, n=3) and M $\beta$ CD (40 mM, 3 h, n=4). **(B)** Densitometry analysis showing that KCl stimulation significantly increased MYPT1-P and its diminution by Y-27632 or M $\beta$ CD. \*\* $p < 0.01$  by analysis of variance and Dunnett tests.

In bovine tracheal smooth muscle, ROCK activity was significantly increased by KCl stimulation ( $p < 0.01$ ); this augmentation was annulled when tissues were pre-incubated with Y-27632 or M $\beta$ CD (Fig. 3). This last result points out that caveolae are required for the proper activation of the ROCK component.



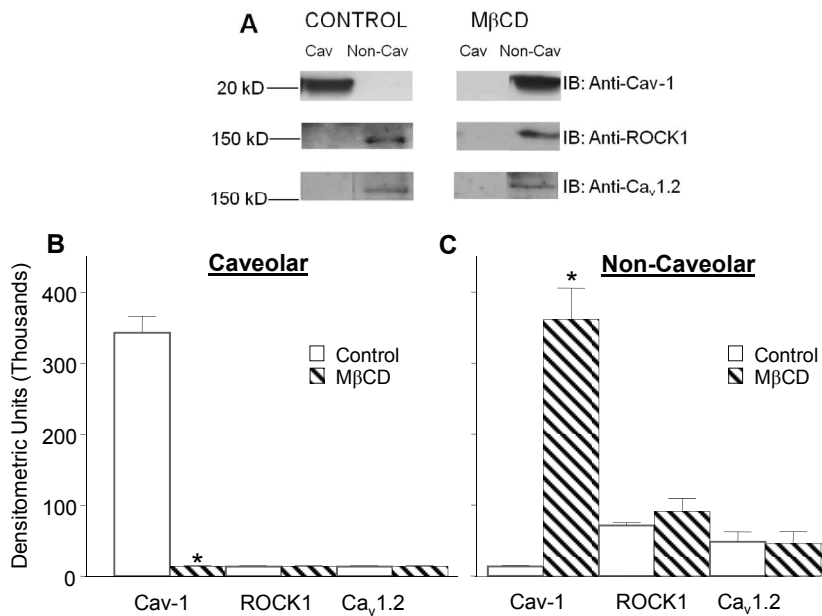
**Fig. 4.** Dot blot from bovine tracheal smooth muscle membrane fractions to identify caveolar ganglioside GM1, through FITC coupled cholera toxin B. Fractions 3-4 were recognized as caveolar (Cav) fractions and 8-9 as non-caveolar (Non-Cav). F – membrane fraction from gradient top (1) to bottom (9). PL – plasmalemma. SR – sarcoplasmic reticulum. Top blot shows results in duplicate for fractionated PL, while lower blot shows negative results from SR only. The n values for these experiments were 3.

Dot blot results showed that GM1 (a characteristic caveolar ganglioside) was mainly present in membrane fractions 3-4 from bovine tracheal smooth muscle, corresponding to caveolar regions (Cav). No staining was seen in non-caveolar (Non-Cav) fractions 6-9. We corroborated that this GM1 was not present in the sarcoplasmic reticulum from the same preparation (Fig. 4).

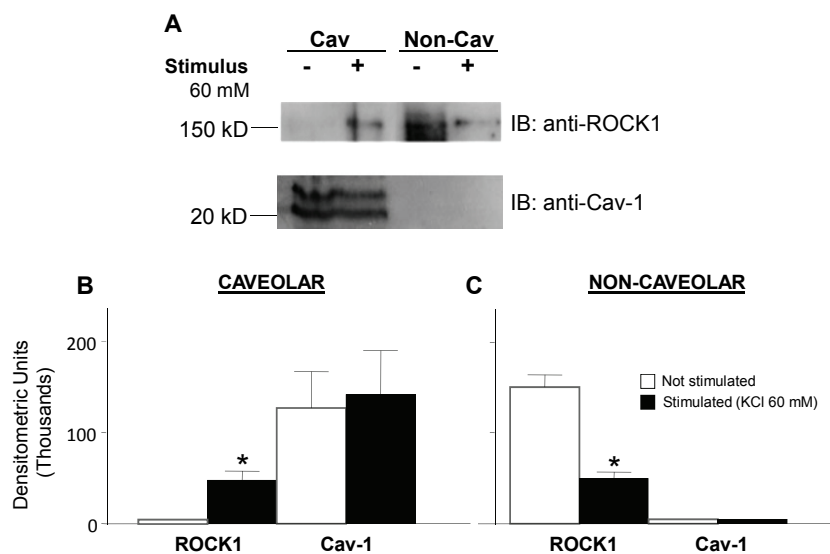
Western blot from pooled fractions (3-4: caveolar; 8-9: non-caveolar) showed that Ca<sub>v</sub>1.2 and ROCK1 are only present in non-caveolar membrane

fractions, while Cav-1 is located exclusively to the caveolar fraction (Fig. 5A). Two bands were recognized by the monoclonal antibody corresponding to isoforms Cav-1 $\alpha$  at 24 kD and Cav-1 $\beta$  at 21 kD (Fujimoto *et al.* 2000, Krasteva *et al.* 2006). Treatment with M $\beta$ CD did

not modify the non-caveolar localization of the Ca<sub>v</sub>1.2 nor ROCK1, but transferred Cav-1 from caveolar fraction to non-caveolar fractions as reported previously (Sommer *et al.* 2009). These findings were confirmed by densitometry analysis of the data (Fig. 5B,C).



**Fig. 5.** Densitometry data from Western blots (n=3, each) for Cav-1, ROCK1 and voltage dependent Ca<sup>2+</sup> channels (Ca<sub>v</sub>1.2), in caveolar (Cav) and non-caveolar (Non-Cav) membrane fractions from control and M $\beta$ CD treated bovine tracheal smooth muscles. **(A)** Representative western blots for caveolin-1 (Cav-1), Rho kinase 1 (ROCK1) and for Ca<sub>v</sub>1.2. **(B)** Densitometry data showed significant differences: \* p<0.01 with respect to its control group. Non paired Student *t* tests were used. IB = immunoblot.



**Fig. 6.** Densitometry data from Western blots for ROCK1 and Cav-1 (n=3, each) in caveolar (Cav) and non-caveolar (Non-Cav) fractions from plasmalemma obtained from KCl stimulated and not stimulated bovine tracheal smooth muscle. **(A)** Representative western blots for Rho kinase 1 (ROCK1) and for caveolin-1 (Cav-1) from KCl stimulated (+) and not stimulated (-) tissues. **(B)** Densitometry analysis showing that, in tissues stimulated with KCl, ROCK1 redistributed from non-caveolar to caveolar fractions. Cav-1 location was not modified by the KCl stimulus. Non paired Student *t* tests were used. \* p<0.05 with respect to its not stimulated group.

In not stimulated tissues ROCK1 was present only in non-caveolar membrane fractions, while, in membrane preparation from KCl stimulated tissues, ROCK1 was found in both caveolar and non-caveolar fractions. Cav-1 did not modify its localization after KCl stimulation (Fig. 6A). Further analysis of the data confirms that ROCK1 moves from non-caveolar to caveolar membrane fraction when tissues were stimulated with KCl (Fig. 6B,C).

## Discussion

In this manuscript we state that, in bovine airway smooth muscle, KCl stimulation activates RhoA/ROCK pathway and promotes ROCK1 translocation to caveolar regions. Although the detailed mechanisms of such translocation remain to be elucidated, it could be that this isotype is partially responsible for the KCl-induced contraction.

Airway smooth muscle excitation-contraction processes remain not fully understood. Our study illustrates this for the KCl-induced contraction. Membrane depolarization through relatively high KCl concentrations has routinely been used to trigger the opening of voltage dependent Ca<sup>2+</sup> channels and the consequent influx of extracellular Ca<sup>2+</sup> (Janssen *et al.* 1999, Flores-Soto *et al.* 2011). This increase in intracellular Ca<sup>2+</sup> concentration was long thought to be the main reason for airway smooth muscle contraction under these conditions. However, increasing evidences suggest that, in airway smooth muscle, the RhoA/ROCK signaling pathway is definitely involved in KCl induced contraction (Janssen *et al.* 2004, 2012). On the other hand, it has been shown in uterine and airway smooth muscle that stimuli that activate RhoA/ROCK augment both molecules translocation from the cytosol to the membrane (Taggart *et al.* 1999, Chiba and Misawa 2004). Later on, it was reported that these molecules move to a specific membrane compartment called caveolae (Taggart *et al.* 2000, Urban *et al.* 2003, Hunter and Dixon 2006). Recently, in mice aortic tissues, it was reported that RhoA/ROCK moves from caveolar to non-caveolar regions after stimulation with 5-HT (Nuno *et al.* 2012). These findings demonstrated that both molecules might move not only from the cytosol to the membrane, but from one membrane domain to another. In airway smooth muscle, it has been shown that, KCl induced Ca<sup>2+</sup> influx promotes RhoA/ROCK activation, although membrane potential could be also involved (Liu *et al.* 2005).

As we reported previously (Sommer *et al.* 2009), the KCl induced contraction in ASM partially depends on ROCK while caveolae remain intact. These membrane structures have been widely studied and, while they are indispensable for a proper KCl-induced contraction in ASM we now report that ROCK's inhibition through Y-27632, diminishes KCl induced contractions to the same extent as MβCD (both approximately 30 % of the KCl contraction, Fig. 1A). Our physiological data also demonstrated that, in MβCD treated tissues, the ROCK component completely disappeared, leaving only the response to Ca<sub>v</sub>1.2 (Fig. 1C). These results show that the KCl contraction is composed by the activation of Ca<sub>v</sub>1.2 and RhoA/ROCK signaling pathway and the latest is dependent of the caveolar integrity. We confirm that KCl induced ROCK activation and this response was abolished by Y-27632 and MβCD (Fig. 3). This drug also modified the membrane localization of Cav-1, transferring it from caveolar to non caveolar membrane

fractions (Fig. 4A) as previously reported (Sommer *et al.* 2009). These findings might explain the disappearance of ROCK participation during the KCl induced contraction when caveolae were disrupted.

To clarify further if ROCK's isotype 1 is implicated in the caveolar-dependent component of the KCl induced contraction, we performed western blots from membrane fractions (caveolar and non-caveolar, Fig. 5) from stimulated and not stimulated tracheal smooth muscle preparations. ROCK1 localization in the cytosol as well as in the cell membrane was already known (Sommer *et al.* 2009), but, at that time the precise membrane site where ROCK1 was located was not defined in an isolated plasmalemma. Our present results showed, in isolated airway smooth muscle plasma membrane, that ROCK1 can be found at the non-caveolar fractions in not stimulated tissues, and that it translocates to caveolar fractions upon KCl stimulation. It has been shown that in airway smooth muscle, KCl induced Ca<sup>2+</sup> influx promotes RhoA/ROCK activation, although membrane potential could be also involved (Liu *et al.* 2005). Thus, ROCK1 translocation from non-caveolar to caveolar membrane fractions upon KCl stimulation could be related to Ca<sup>2+</sup> influx and/or membrane depolarization. This result sharply contrasts with the findings in mice aortic tissues (Nuno *et al.* 2012), where the ROCK component of the KCl induced contraction is caveolae-independent. Because ROCK's predominant isotype in vascular smooth muscle is type 2, physiological and molecular differences among this tissue and airway smooth muscle could be due to this fact. Probably, ROCK's different isotypes imply different physiological responses. Further investigation in ASM is needed to clarify whether ROCK1 also translocates to caveolar regions after its activation through other pathway activating stimuli (i.e. histamine, 5-HT, etc.).

Additionally, we found that, under our experimental conditions, Ca<sub>v</sub>1.2 is not localized in caveolar membrane fractions, but is the sole membranal structure responsible for KCl induced contraction when caveolae were disrupted with MβCD. A possible explanation to this finding is that Ca<sub>v</sub>1.2 might move from non caveolar to caveolar domains as ROCK1 after KCl stimulation.

In conclusion, airway smooth muscle stimulation with KCl probably promotes ROCK1 translocation from non-caveolar to caveolar regions. This translocation seems to be indispensable for a complete KCl-induced contraction; when caveolae are disrupted through MβCD, a Ca<sub>v</sub>1.2-dependent partial contraction remains.

## Conflict of Interest

There is no conflict of interest.

## Acknowledgements

This study was supported by the National Institute of Respiratory Diseases.

## References

- BABIYCHUK EB, SMITH RD, BURDYGA T, BABIYCHUK VS, WRAY S, DRAEGER A: Membrane cholesterol regulates smooth muscle phasic contraction. *J Membr Biol* **198**: 95-101, 2004.
- BHATNAGAR A, SHEFFLER DJ, KROEZE WK, COMPTON-TOTH B, ROTH BL: Caveolin-1 interacts with 5-HT<sub>2A</sub> serotonin receptors and profoundly modulates the signaling of selected G<sub>αq</sub>-coupled protein receptors. *J Biol Chem* **279**: 34614-34623, 2004.
- BLANK N, GABLER C, SCHILLER M, KRIEGEL M, KALDEN JR, LORENZ HM: A fast, simple and sensitive method for the detection and quantification of detergent-resistant membranes. *J Immunol Methods* **271**: 25-35, 2002.
- CHIBA Y, MISAWA M: The role of RhoA-mediated Ca<sup>2+</sup> sensitization of bronchial smooth muscle contraction in airway hyperresponsiveness. *J Smooth Muscle Res* **40**: 155-167, 2004.
- CHO WJ, DANIEL EE: Proteins of interstitial cells of Cajal and intestinal smooth muscle, colocalized with caveolin-1. *Am J Physiol Gastrointest Liver Physiol* **288**: G571-G585, 2005.
- DARBY PJ, KWAN CY, DANIEL EE: Caveolae from canine airway smooth muscle contain the necessary components for a role in Ca<sup>2+</sup> handling. *Am J Physiol Lung Cell Mol Physiol* **279**: L1226-L1235, 2000.
- DREJA K, VOLDSTEDLUND M, VINTEN J, TRANUM-JENSEN J, HELLSTRAND P, SWÄRD K: Cholesterol depletion disrupts caveolae and differentially impairs agonist-induced arterial contraction. *Arterioscler Thromb Vasc Biol* **22**: 1267-1272, 2002.
- FLORES-SOTO E, CARBAJAL V, REYES-GARCÍA J, GARCÍA-HERNÁNDEZ LM, FIGUEROA A, CHECA M, BARAJAS-LÓPEZ C, MONTAÑO LM: In airways ATP refills sarcoplasmic reticulum via P2X smooth muscle receptors and induces contraction through P2Y epithelial receptors. *Pflugers Arch* **461**: 261-275, 2011.
- FUJIMOTO T, KOGO H, NOMURA R, UNE T: Isoforms of caveolin-1 and caveolar structure. *J Cell Sci* **113**: 3509-3517, 2000.
- HUNTER I, NIXON GF: Spatial compartmentalization of tumor necrosis factor (TNF) receptor 1-dependent signaling pathways in human airway smooth muscle cells. Lipid rafts are essential for TNF-alpha-mediated activation of RhoA but dispensable for the activation of the NF-kappaB and MAPK pathways. *J Biol Chem* **281**: 34705-34715, 2006.
- JANSSEN LJ: Airway smooth muscle electrophysiology in a state of flux? *Am J Physiol Lung Cell Mol Physiol* **302**: L730-L732, 2012.
- JANSSEN LJ, BETTI PA, NETHERTON SJ, WALTERS DK: Superficial buffer barrier and preferentially directed release of Ca<sup>2+</sup> in canine airway smooth muscle. *Am J Physiol* **276**: L744-L753, 1999.
- JANSSEN LJ, TAZZEO T, ZUO J, PERTENS E, KESHAVJEE S: KCl evokes contraction of airway smooth muscle via activation of RhoA and Rho-kinase. *Am J Physiol Lung Cell Mol Physiol* **287**: L852-L858, 2004.
- KRASTEVA G, PFEIL U, DRAB M, KUMMER W, KÖNIG P: Caveolin-1 and -2 in airway epithelium: expression and in situ association as detected by FRET-CLSM. *Respir Res* **7**: 108, 2006.
- LEUNG T, MANSER E, TAN L, LIM L: A novel serine/threonine kinase binding the Ras-related RhoA GTPase which translocates the kinase to peripheral membranes. *J Biol Chem* **270**: 29051-29054, 1995.
- LIU C, ZUO J, PERTENS E, HELLI PB, JANSSEN LJ: Regulation of Rho/ROCK signaling in airway smooth muscle by membrane potential and [Ca<sup>2+</sup>]<sub>i</sub>. *Am J Physiol Lung Cell Mol Physiol* **289**: L574-L582, 2005.
- LÖHN M, FURSTENAU M, SAGACH V, ELGER M, SCHULZE W, LUFT FC, HALLER H, GOLLASCH M: Ignition of calcium sparks in arterial and cardiac muscle through caveolae. *Circ Res* **87**: 1034-1039, 2000.
- MATSUI T, AMANO M, YAMAMOTO T, CHIHARA K, NAKAFUKU M, ITO M, NAKANO T, OKAWA K, IWAMATSU A, KAIBUCHI K: Rho-associated kinase, a novel serine/threonine kinase, as a putative target for small GTP binding protein Rho. *EMBO J* **15**: 2208-2216, 1996.



- 
- MITA M, YANAGIHARA H, HISHINUMA S, SAITO M, WALSH MP: Membrane depolarization-induced contraction of rat caudal arterial smooth muscle involves Rho-associated kinase. *Biochem J* **364**: 431-440, 2002.
- NOMA K, OYAMA N, LIAO JK: Physiological role of ROCKs in the cardiovascular system. *Am J Physiol Cell Physiol* **290**: C661-C668, 2006.
- NUNO DW, ENGLAND SK, LAMPING KJ: RhoA localization with caveolin-1 regulates vascular contraction to serotonin. *Am J Physiol Regul Integr Comp Physiol* **303**: R959-R967, 2012.
- PARTON RG: Ultrastructural localization of gangliosides; GM1 is concentrated in caveolae. *J Histochem Cytochem* **42**: 155-166, 1994.
- SOMLYO AP, SOMLYO AV: Ca<sup>2+</sup> sensitivity of smooth muscle and nonmuscle myosin II: modulated by G proteins, kinases, and myosin phosphatase. *Physiol Rev* **83**: 1325-1358, 2003.
- SOMMER B, MONTANO LM, CARBAJAL V, FLORES-SOTO E, ORTEGA A, RAMÍREZ-OSEGUERA R, IRLLES C, EL-YAZBI AF, CHO WJ, DANIEL EE: Extraction of membrane cholesterol disrupts caveolae and impairs serotonergic (5-HT<sub>2A</sub>) and histaminergic (H<sub>1</sub>) responses in bovine airway smooth muscle: role of Rho-kinase. *Can J Physiol Pharmacol* **87**: 180-195, 2009.
- TAGGART MJ, LEE YH, MORGAN KG: Cellular redistribution of PKC $\alpha$ , rhoA, and ROK $\alpha$  following smooth muscle agonist stimulation. *Exp Cell Res* **251**: 92-101, 1999.
- TAGGART MJ, LEAVIS P, FERON O, MORGAN KG: Inhibition of PKC $\alpha$  and rhoA translocation in differentiated smooth muscle by a caveolin scaffolding domain peptide. *Exp Cell Res* **258**: 72-81, 2000.
- URBAN NH, BERG KM, RATZ PH: K<sup>+</sup> depolarization induces RhoA kinase translocation to caveolae and Ca<sup>2+</sup> sensitization of arterial muscle. *Am J Physiol Cell Physiol* **285**: C1377-C1385, 2003.
-