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CA2+ HEMOSTASIS UNDER THE EFFECT OF BIOLOGICALLY ACTIVE SUBSTANCES ON SMOOTH MUSCLE CELLS

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Abstract. Protein kinase C (PKC) has been implicated in the regulation of smooth muscle cell (SMC) contraction and may contribute to airway hyperresponsiveness. Here, we combined optical and biochemical analyses of mouse lung slices to determine the effects of PKC activation on Ca^{2+} signaling, Ca^{2+} sensitivity, protein phosphorylation, and contraction in SMCs of small intrapulmonary airways. We found that 10 μM phorbol-12-myristate-13-acetate or 1 μM phorbol 12,13-dibutyrate induced repetitive, unsynchronized, and transient contractions of the SMCs lining the airway lumen. These contractions were associated with low frequency Ca^{2+} oscillations in airway SMCs that resulted from Ca^{2+} influx through L-type voltage-gated Ca^{2+} channels and the subsequent release of Ca^{2+} from intracellular stores through ryanodine receptors. Phorbol ester stimulation of lung slices in which SMC intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) was "clamped" at a high concentration induced strong airway contraction, indicating that PKC mediated sensitization of the contractile response to $[Ca^{2+}]$.

Keywords: Ca^{2+}, method, treatment, diagnosis.

INTRODUCTION

The small intrapulmonary airways are the major site of airflow limitation in both asthma and chronic obstructive pulmonary disease (Burgel, 2011; McDonough et al., 2011). However, the small size and peripheral location of these small distal airways, together with their mechanical interactions with the lung parenchyma, have made it challenging to determine the cellular mechanisms that regulate their diameter in normal and diseased lungs. Recent studies using customized imaging techniques to assess dynamic changes in airway diameter simultaneously with changes in smooth muscle cell (SMC) intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) in lung slices, however, have begun to provide insight into the processes that regulate small airway contraction (Sanderson, 2011).

MATERIALS AND METHODS

Most reagents were obtained either from Invitrogen, Life Technologies, and Gibco, or Sigma-Aldrich. The PKC activators PMA and phorbol 12,13-dibutyrate (PDBu) were purchased from LC Laboratories. GF-109203X and cyclopiazonic acid (CPA) were from Enzo Life Sciences, whereas Y-27632, nifedipine, and ryanodine were from Abcam. Thrombin, purified from bovine plasma, was from Sigma-Aldrich. Hanks' balanced salt solution was prepared from a 10× stock solution (Invitrogen), supplemented with 20 mM HEPES buffer, and adjusted to pH 7.4 (sHBSS). Ca^{2+}- free sHBSS was prepared from a 10× Ca^{2+}- and Mg^{2+}-free stock so- lution (Invitrogen) and supplemented with 20 mM HEPES, pH 7.4, 0.9 mM $MgCl_2$, and 1.0 mM EGTA. PMA and PDBu were prepared at 20 and 10 mM, respectively, in DMSO. Stock solutions were diluted in sHBSS to the final

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concentration on the same day of use; the concentration of vehicle (DMSO) never exceeded 0.1%.

RESULTS AND DISCUSSION

Lung slices were mounted at the center of a 22 × 40–mm cover glass in a custom-made perfusion chamber and held in place with a small sheet of nylon mesh. A small hole was cut in the mesh and centered over the selected airway. A second 11 × 30–mm cover glass, edged with silicone grease, was placed over the mounted lung slice to create a thin rectangular chamber. The lung slice was perfused by adding solution at one end of the chamber and removing it by suction at the opposite end by means of a gravityfed, computer-controlled perfusion system. The volume of the chamber (100 µl) and the perfusion rate (800 µl/min) were kept constant for the duration of each experiment. The chamber was placed on the stage of an inverted phase-contrast microscope (Diaphot TMD; Nikon), and lung slices were imaged with a 10× objective. Digital images (640 × 488 pixels) were recorded to a hard drive in time-lapse (0.5 Hz) using a CCD camera (KP-M1A; Hitachi), frame grabber (Picolo; Euresys), and image acquisition software (Video Savant; IO Industries).

For these analyses, we used lung slices that contained two or three well-preserved airways and no accompanying arteries and veins. The SMCs in these lung slices were almost exclusively airway SMCs, as confirmed by immunofluorescence with antibodies directed against SMC α actin (see below). Three slices persample were washed with sHBSS and incubated with the indicated drugs for the indicated times. Subsequently, the slices were transferred to 1.2-ml tubes (Eppendorf) containing 100 µl of stimulation solution and quickly frozen by adding 300 µl of dry ice-cold acetone supplemented with 10% TCA and 10 mM DTT. Samples were maintained at —20°C overnight before protein extraction. The next day, the samples were sonicated (three cycles) and then centrifuged in Eppendorf tubes at 13,000 *g* for 10 min at 4°C. The supernatant was discarded, and the pellets were washed two to three times with 400 µl of cold acetone (—20°C) containing 10 mM DTT to remove residual TCA. The remaining pellet was air dried, suspended in 50 µl Laemmli buffer (Bio-Rad Laboratories), and boiled for 10 min at 95°C. Samples were loaded (25 µl/well) in a 15% SDS/PAGE gel supplemented with 50 µM Phos-tag ligand (AAL-107; NARD Institute, Ltd.) and two equivalents of $MnCl_2$ according to the manufacturer's instructions. Electrophoresis was run at 120 V for 90 min. After electrophoresis, the gel was washed for 15 min with transfer buffer (25 mM Tris, 192 mM glycine, and 20% methanol, pH 8.3) supplemented with 100 mM EDTA (to remove the Mn^{2+}) and then with transfer buffer without EDTA (twice for 5 min each).

Phorbol esters induced airway SMC twitching

We characterized the contractile response of SMCs in small airways to PKC activation with phorbol esters (PMA and PDBu) and compared it to the responses to 5-HT and plasma membrane depolarization with KCl. A typical lung slice with a small airway in cross section before (rest) and after sequential stimulation with 5-HT, KCl, and PMA is shown. Superfusion of such lung slices with 10 µM PMA elicited transient and asynchronous contractions of the SMCs surrounding the airway lumen (SMC twitching). This PMA-elicited SMC twitching was accompanied by a small, slow decrease in airway lumen area that was equivalent to 8.8 ± 3.9% of the total lumen area at 30 min of PMA stimulation. 1

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μM PDBu also elicited SMC twitching and a small decrease in lumen area (11.4 ± 3.5%); however, the onset of SMC twitching was faster for PDBu (4.4 ± 1.5 min) than for PMA (18 ± 4.7 min). These PMA and PDBu responses persisted for up to 1 h after the phorbol esters were washed out by superfusion with sHBSS. Stimulation with 50 mM of isosmotic, KCl-containing sHBSS also resulted in SMC twitching and a small decrease in airway lumen area (10.9 ± 3.9%). In contrast, stimulation with 0.5 μM 5-HT produced a much larger decrease in airway lumen area (49.7 ± 7.3%) without initiating SMC twitching. Airway responses to KCl and 5-HT were rapidly reversed by washout with sHBSS. These results suggest that PKC activation induces SMC twitching in small airways.

CONCLUSION

In conclusion, we suggest that activation of PKC in small airways promotes Ca^{2+} influx into SMC via LVGCs and, subsequently, intracellular Ca^{2+} release via RyR to generate low frequency Ca^{2+} oscillations and SMC twitching. PKC activation also induces a strong Ca^{2+} sensitization mediated by CPI-17 and rMLC phosphorylation. Finally, PKC activation by specific molecules, such as thrombin, that are present in the airways in conjunction with inflammatory lung diseases, could conceivably sensitize the airway SMCs to local agonists and contribute to airway hyperresponsiveness.

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