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Activation of endothelial BK_{Ca} channels causes pulmonary vasodilation

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ABSTRACT

Background: Large-conductance Ca^{2+}-activated K^+ (BK_{Ca}) channels cause hyperpolarization and can regulate vascular tone. In this study, we evaluated the effect of endothelial BK_{Ca} activation on pulmonary vascular tone.
Methods: The presence of BK_{Ca} channels in lung microvascular endothelial cells (LMVEC) and rat lung tissue was confirmed by RT-PCR, immunoblotting and immunohistochemistry. Isolated pulmonary artery (PA) rings and isolated ventilated-perfused rat lungs were used to assay the effects of BK_{Ca} channel activation on endothelium-dependent vasodilation.
Results: Immunoblotting and RT-PCR revealed the presence of BK_{Ca} channel α- and β_4-subunits in LMVEC. Immunohistochemical staining showed BK_{Ca} channel α-subunit expression in vascular endothelium in rat lungs. In arterial ring studies, BK_{Ca} channel activation by NS1619 enhanced endothelium-dependent vasodilation that was attenuated by tetraethylammonium and iberiotoxin. In addition, activation of BK_{Ca} channels by C-type natriuretic peptide caused endothelial-dependent vasodilation that was blocked by iberiotoxin, L-NAME, and lanthanum. Furthermore, BK_{Ca} activation by NS1619 caused a dose-dependent reduction in PA pressures that was attenuated by L-NAME. *In vitro*, BK_{Ca} channel activation in LMVEC caused hyperpolarization and increased NO production.
Conclusions: Pulmonary endothelium expresses BK_{Ca} channels. Activation of endothelial BK_{Ca} channels causes hyperpolarization and NO mediated endothelium-dependent vasodilation in micro- and macrovasculature in the lung.

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1. Introduction

The vascular endothelium is composed of a single layer of cells situated between circulating blood and vessel wall. This cell monolayer is responsible for regulating many different physiological functions, such as angiogenesis, metabolism, synthesis and degradation of the extracellular matrix, and vascular tone and permeability (Aird, 2007). Endothelial membrane potential (E_m) plays an important role in the regulation of many of these functions especially vascular tone (Nilius and Droogmans, 2001; Nilius et al., 1997). Endothelial cell hyperpolarization can increase the gradient for extracellular Ca^{2+} entry via non-specific cation channels or trp channels (Adams and Hill, 2004). Increased $[Ca^{2+}]_i$ stimulates the release of nitric oxide (NO), prostacyclin, and endothelium-derived hyperpolarizing factors (EDHF) that act on vascular smooth muscle cells resulting in vasodilation (Himmel et al., 1993). In addition, the presence of myo-endothelial gap-junctions facilitates the hyperpolarization of vascular smooth muscle cells causing vasorelaxation (Chaytor et al., 1998; Larson and Sheridan, 1985; Ungvari et al., 2002; Yamamoto et al., 1999).

Large-conductance calcium activated potassium (BK_{Ca}) channels are comprised of a pore-forming α-subunit that may be associated with a regulatory β-subunit (Ghatta et al., 2006). Each channel exists as a tetramer, composed of 4 α-subunits either alone or in association with β-subunit pair (Garcia-Calvo et al., 1994; Knaus et al., 1994). The α-subunit in BK_{Ca} channels opens in response to depolarization and Ca^{2+} binding, causing efflux of K^+ ions and hyperpolarization (Eichhorn and Dobrev, 2007). The β-subunits play important regulatory roles in modulating the activity of the channel (Brenner et al., 2000b; Cox, 2005; Ungvari et al., 2002). There have been several reports on the presence and role of vascular smooth muscle BK_{Ca} channels in the regulation of pulmonary vascular tone (Barman et al, 2004; Bonnet et al., 2003a,b; Dubuis et al., 2005); however, there is no evidence yet supporting the role of endothelial BK_{Ca} channels affecting pulmonary vascular tone.

BK_{Ca} channels have been observed in other endothelial cells (Faehling et al., 2001; Frieden et al., 2002; Kawasaki et al., 2004; Kim et al., 2006; Kuhlmann et al., 2005; Papassotiriou et al., 2000; Wang et al., 2005; Wrzosek et al., 2009) and we recently demonstrated that C-type natriuretic peptide (CNP) activated BK_{Ca} channels in

Abbreviations: BK_{Ca}, Large-conductance Ca^{2+}-activated K^+ channel; LMVEC, Lung microvascular endothelial cells; PA, Pulmonary artery; NO, Nitric oxide; EDHF, Endothelium-derived hyperpolarizing factors; CNP, C-type natriuretic peptide; IbTx, Iberiotoxin; TEA, Tetraethylammonium.

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pulmonary microvascular endothelial cells causing hyperpolarization (Simon et al., 2009a). However, controversy still exists regarding their expression and role in the vascular endothelium (Gauthier et al., 2002; Sandow and Grayson, 2009). The activation of endothelial BK_{Ca} channels can lead to endothelial hyperpolarization, enhance endothelial-dependent vasodilation (Ledoux et al., 2006), and improve endothelial dysfunction (Félétou, 2009; Feletou and Vanhoutte, 2006). This is important in pulmonary vasculature since diseases such as pulmonary hypertension are associated with impaired endothelial function (Budhiraja et al., 2004). However, the effects of BK_{Ca} channel activation on pulmonary endothelial function have not yet been studied.

The objective of this study was to demonstrate the activity and expression of BK_{Ca} channels in pulmonary endothelium *in vitro* and *ex vivo*. Furthermore, we sought to evaluate the effects of endothelial BK_{Ca} channel activation in the regulation of pulmonary vascular tone.

2. Materials and methods

2.1. Materials

Iberiotoxin was purchased from Anaspec (Anaspec, Fremont, CA) while all other chemicals were purchased from Sigma (Sigma Inc., St. Louis, MO).

2.2. Animals

All procedures and protocols were approved by the Animal Care and Use Committee at the Providence VA Medical Center and conformed to the Guidelines for the Care and Use of Laboratory Animals published by the National Institutes of Health. Adult Male Sprague–Dawley rats were purchased from Charles River Laboratories (Wilmington, MA).

2.3. Cell culture

Pulmonary microvascular endothelial cells (LMVEC) used for all experiments were isolated from rat lung and characterized as endothelial cells by VEC Technologies (Rochester, NY). We confirmed the cells as endothelial cells by demonstrating the uptake of acetylated LDL and expression of von-Willebrand factor. Also, the LMVEC stained with the lectins, *Helix pomatia* and *Griffonia* (*Bandeiraea*) *simplicifolia*, in a pattern consistent with that previously reported, with a greater level of *Griffonia* (*Bandeiraea*) *simplicifolia* staining, relative to *Helix pomatia* (Simon et al., 2009a,b). The cells were used at *passages 3–8* for all experiments.

2.4. Immunoblot analyses

Antibodies were purchased from Alomone Laboratories (Alomone Laboratories, Jerusalem, Israel). Equivalent amounts of LMVEC lysate were resolved by SDS-PAGE and immunoblotted as previously described (Klinger et al., 2007; Simon et al., 2009a). The films were scanned and quantitative densitometric analysis was performed using NIH ImageJ. Equal protein loading was verified by Ponceau S staining and by stripping and reprobing the membrane for Vinculin (Sigma Inc.).

2.5. Polymerase chain reaction

RNA was extracted from rat lungs or cells grown on 60 mm dishes using Trizol reagent (Sigma Inc.) following the manufacturer's protocol. Two step RT-PCR was performed on extracted RNA using BioRad iScript cDNA Synthesis kit, BioRad iTaq DNA polymerase, iTaq Buffer, and 100 nM primers (Table 1) (IDT Inc.). The temperature profile of amplification consisted of a 2 min activation at 95 °C, followed by 35 cycles of 30 s denaturation at 95 °C, 30 s annealing at 55 °C, and 30 s extension at 72 °C. Electrophoresis was performed using a 1.5% agarose gel to confirm the presence or absence of the PCR product using ethidium bromide staining.

2.6. Immunohistochemistry

Rat lungs were removed from the chest and infused with normal buffered formalin. Intratracheal pressure was fixed at 23 cm H_2O and PA pressure at 60 cm H_2O. Immunohistochemical staining was performed on paraffin-embedded transverse sections. Tissue sections were deparafinized and hydrated by standard methods. Antigen retrieval was done by 20 μg/ml in PBS of Proteinase K (Sigma). Endogenous peroxidase activity was quenched with 3% hydrogen peroxide (Sigma). Immunohistochemical staining was performed via the Vectastain Elite ABC immunoperoxidase system (Vector Laboratories, Burlingame, CA) in accordance with the manufacturer's instructions. Briefly, tissue sections were blocked with normal serum (NS) in PBS, sections incubated with primary antibody (Alomone Labs, Jerusalem, Israel) diluted in NS in PBS. Sections were then incubated with biotinylated secondary antibody, washed, and incubated with Vectastain Elite ABC Reagent. IHC signal was visualized with Vector VIP Substrate Kit for Peroxidase (Vector Labs). Tissue sections were counterstained with Methyl Green (Vector Labs), dehydrated, and cleared with Citrisolv. Slides were permanently mounted with Cytoseal 60 (Richard-Allan Scientific, Kalamazoo, MI).

2.7. Arterial ring studies

Male Sprague Dawley rats weighing 250–350 g were anaesthetized with pentobarbital (100 mg/kg ip) after which the heart and lungs were removed en bloc and the proximal intrapulmonary artery was carefully dissected out from the lungs. Subsequently, 2–3 mm ring segments were suspended between two tungsten wires (32 μm) in a tissue bath containing Earle's balanced Salt solution maintained at 37 °C and aerated with 5% CO_2/95% O_2, and attached to a Grass Force displacement transducer (FT03). The vessels were suspended at 0.5 g optimal active tension generation for 30 min to establish a stable baseline tension. Subsequently, the rings were preconstricted with phenylephrine (10^{-6} M). An intact endothelium was defined as a vasodilatory response >50% of preconstricted tension in response to 10^{-5} M acetylcholine. The PA rings were then washed three times every 10 minutes. Next, the rings were again preconstricted with phenylephrine after which a cumulative dose–response curve was generated using incremental dose of Ach (10^{-9} M to 10^{-5} M) or C-type natriuretic peptide (10^{-9} M to 10^{-6} M) every five minutes. The dose response curves were performed both in the presence and the

Table 1
Primers used for polymerase chain reaction.

	Forward primer	Reverse primer	Product size
BK_{Ca} α-subunit	CAC AGA ATA TCT TTC CAG TGC CTTC	ACC TTC TTG GGT CTT AAG GTG GTT	178
BK_{Ca} β_1-subunit	GTA TCA CAC AGA AGA CAC TCG GGA	AAG AAG GAG AAG AGG AGG ATT TGG G	216
BK_{Ca} β_2-subunit	ACT GGG AAT CAC ACT GCT GCG	ACT CAC AAG GGA CAT GGA CTC CTC	289
BK_{Ca} β_4-subunit	ATG GCG AAG CTC AGG GTG TC	ACT CGA ACA CCT CGC CGA TC	202