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Research Article

Phenotypic conversion leads to structural and functional changes of smooth muscle sarcolemma

*Katharina Matschke, Eduard B. Babiychuk, Katia Monastyrskaya, Annette Draeger**

Department of Cell Biology, Institute of Anatomy, University of Bern, Baltzerstrasse 2, 3012 Bern, Switzerland

ARTICLE INFORMATION

Article Chronology:
Received 18 May 2006
Revised version received
19 July 2006
Accepted 20 July 2006
Available online 26 July 2006

Keywords:
Smooth muscle
Contraction
Annexins
Atherosclerosis
Intima
5′ nucleotidase
Aminopeptidase N

ABSTRACT

Continuous changes in the length of smooth muscles require a highly organized sarcolemmal structure. Yet, smooth muscle cells also adapt rapidly to altered environmental cues. Their sarcolemmal plasticity must lead to profound changes which affect transmembrane signal transduction as well as contractility.

We have established porcine vascular and human visceral smooth muscle cultures of epithelioid and spindle-shaped morphology and determined their plasma membrane properties. Epithelioid cells from both sources contain a higher ratio of cholesterol to glycerophospholipids, and express a less diverse range of lipid-associated annexins. These findings point to a reduction in efficiency of membrane segregation in epithelioid cells. Moreover, compared to spindle-shaped cells, cholesterol is more readily extracted from epithelioid cells with methyl-β-cyclodextrin and its synthesis is more susceptible to inhibition with lovastatin. The inability of epithelioid cells to process vasoactive metabolites, such as angiotensin or nucleotides further indicates that contractile properties are impaired.

Phenotypic plasticity extends beyond the loss of smooth muscle cell marker genes. The plasma membrane has undergone profound functional changes which are incompatible with cyclic foreshortening, but might be important in the development of vascular disease.

Introduction

Smooth muscle cells are usually derived from local populations of mesenchymal origin or from the neural crest [1]. But they can also arise from circulating or resident populations of progenitor cells [2]. Hence, it is natural to ascribe differences in their characteristics to their site of origin, and their mode of recruitment, as well as to functional variables. Clonal variability in vascular smooth muscle cells has been implicated in the development of atherosclerotic lesions [3,4].

In the absence of reliable *in vivo* markers, the identification of phenotypically distinct clones *in situ* is technically difficult [5,6]. Therefore, clonally and morphologically distinct lines of vascular smooth muscle have been established from different species: spindle-shaped cells, in morphology and gene expression akin to native smooth muscle cells, and epithelioid cells, which display rudimentary contractile properties and possess an enhanced proliferative activity [5]. Recently, microarray experiments have confirmed the existence of clonal variations between plaque and medial smooth muscle cells, and have revealed a genetically distinct subset of smooth muscle cells to be responsible for the formation of vascular lesions [4].

A careful biochemical and functional characterization of these two vascular smooth muscle phenotypes has revealed

* *Corresponding author.*
E-mail address: draeger@ana.unibe.ch (A. Draeger).

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doi:10.1016/j.yexcr.2006.07.012

differences in their expression of cytoskeletal and contractile proteins [7–10], in their synthesis of extracellular matrix components [11,12] and in the growth-factor dependency of their proliferative activity [13,14].

Plaque cells have been shown to possess elevated levels of transcripts for enzymes involved in the formation of lipoproteins and glycosaminglycans [4].

It remains a moot question whether the observed phenotypic differences extend also to the lipid/protein components of the sarcolemma and if so, whether they have functional consequences. Smooth muscle sarcolemmal lipids are segregated into domains of cholesterol-rich lipid rafts and glycerophospholipid-rich non-raft regions. We have previously shown that membrane segregation is important for discriminating and conducting signals and essential for the directional translocation of proteins to the sarcolemma [15,16]. The segregation of membrane lipids is supported in a Ca^{2+}-dependent manner by the annexin protein family. The interaction of annexins with distinct plasma membrane regions promotes membrane segregation and permits a spatially confined, graded response to a large number of stimuli [15,16].

In the present study, we investigated the lipid-based process of membrane segregation in two phenotypically distinct smooth muscle cultures, both of vascular (porcine aorta) and of visceral (human myometrial) origin.

The plasma membranes of epithelioid cells contain lower levels of glycerophospholipids but significantly higher concentrations of cholesterol and sphingomyelin than those of spindle-shaped cells. Moreover, in epithelioid cells cholesterol is more readily extracted, and its synthesis is more susceptible to inhibition. These changes in sarcolemmal lipid composition and cholesterol handling properties are associated with a reduced diversity in the range of membrane-binding annexins.

We have previously shown the contractile properties of smooth muscle cells to depend largely on the organization of their sarcolemma [17,18]. It is conceivable that the less sophisticated membrane organization in epithelioid cells is reflected in the greatly reduced activities of 5′-nucleotidase and aminopeptidase N. This deficiency in response to physiological stimuli might be of functional consequence in the development of vascular lesions.

Materials and methods

Cell cultures

Porcine aortae were collected from the local abbatoir. Primary cultures of vascular smooth muscle cells (PAo) were prepared according to the protocol by Ehler et al. [17]. Primary cultures of human myometrial smooth muscle cells (HU) were established using the same procedure [19]. Cells were passaged when subconfluent at a 1:3 ratio for HU and a 1:2 ratio for PAo.

The cells were cultured in DMEM containing penicillin and streptomycin and either 10% (v/v) heat-inactivated fetal calf serum (FCS) or lipoprotein-deficient serum (LPDS). LPDS was prepared from FCS using the absorbent PHM-L LIPOSORB (Calbiochem, Darmstadt, Germany), in accordance with the manufacturer's instructions. Spindle-shaped and epithelioid cells received identical treatment.

Population doubling time

The cells were seeded into 12-well plates at low density to permit their linear growth. During the first week of culturing, cells from 3 wells/day were trypsinized and counted and the average count was plotted against time. The population doubling time (PD) was calculated as $PD=(\log(N_1/N_0))/t$ (N_1 = cell count at the end of the experiment; N_0 = cell count at the onset of the experiment; t = number of days of culturing). Population doubling is expressed as the number of cell cycles/day, and the population doubling time (in days) = 1/PD.

For SDS-PAGE, Western blot analysis, thin-layer chromatography and enzyme activity measurements spindle-shaped cells after 12–14 population doublings (HU) or 5–6 population doublings (PAo) and epithelioid cells after 25–26 population doublings (HU) and 13–14 population doublings (PAo) were used.

Antibodies and reagents

The polyclonal antibodies against annexins 2 and 6 have been previously described [15,20]. Monoclonal antibodies against annexins 1, 2, 4, 6 and 7, a monoclonal antibody against S100A10,and a polyclonal one against caveolin were purchased from Transduction Laboratories (Lexington, USA). Monoclonal antibodies against vinculin, β-actin and smooth muscle myosin were obtained from Sigma (Buchs, Switzerland). Lovastatin (Mevinolin) was purchased from Fluka (Buchs, Switzerland). It was added to fresh culture medium at a concentration of 1 μM/day for 7 days. Methyl-β-cyclodextrin (MCD) (Sigma) was applied to cell cultures at a concentration of 30 mM (4% (w/v), diluted in Na^+-Tyrode's solution [(140 mM NaCl, 5 mM KCl, 1 mM $MgCl_2$, 10 mM Glucose, 10 mM HEPES; pH 7.4) containing 2 mM $CaCl_2$ for 1 h at ambient temperature.

SDS-PAGE, immunoblotting, thin-layer chromatography

For the detection of proteins by immunoblotting, confluent cells were washed, scraped, resuspended in SDS-sample buffer and sonicated. The protein concentration was measured using a bichinonic acid (BCA) assay kit (Sigma, Buchs, Switzerland), with bovine serum albumin as a standard. Identical amounts (10 μg) were subjected to SDS-PAGE according to Laemmli [21]. Western blotting was performed as previously described [22]. Thin-layer chromatography was performed as previously described [23]. Lipid standards of known concentration were run in parallel. The protein concentration was used to equalize cell amounts. Lipid standards of known concentration were run in parallel and spots were quantified using the ImageQuant TL software (v2003; Amersham Biosciences Europe GmbH). The share of each lipid in the total amount of lipids investigated was plotted in %. The cholesterol content was expressed as % of control. Each experiment was done in triplicate.

5′-nucleotidase and aminopeptidase N activity

5′-nucleotidase activity was monitored as P_i release, which was measured spectrophometrically at a wavelength of 820 nm (A_{820}) according to a modification of the method described by Fiske and Subarrow [24], using 5′-AMP as a substrate