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Research article

Roles of syndecan-4 and relative kinases in dorsal root ganglion neuron adhesion and mechanotransduction

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HIGHLIGHTS

- DRG neuronal density, neurite length, and neurite branching were lower in PDMS.
- The expression of SDC-4 was relative low in PDMS substrate.
- pPKCα-FAKpTyr397-pERK1/2 was increased after stretching on PDMS substrate.

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ABSTRACT

Mechanical stimuli elicit a biological response and initiate complex physiological processes, including neural feedback schemes associated with senses such as pain, vibration, touch, and hearing. The syndecans (SDCs), a group of adhesion receptors, can modulate adhesion and organize the extracellular matrix (ECM). In this study, we cultured dorsal root ganglia (DRG) on controlled polydimethylsiloxane (PDMS) substrates coated with poly-L-lysine (poly) or fibronectin (FN) to investigate cell adhesion and mechanotransduction mechanisms by mechanical stretching on PDMS using DRG neurons. Our results demonstrated that neuronal density, neurite length, and neurite branching were lower in the PDMS group and could be further reversed through activating SDC-4 by FN. The expression of the SDC-4 pathway decreased but with increased pPKCα in the PDMS-poly group. After mechanical stretching, pPKCα-FAKpTyr397-pERK1/2 expression was increased in both poly- and FN-coated PDMS. These results indicate that SDC4-pPKCα-FAKpTyr397-pERK1/2 may play a crucial role in DRG adhesion and mechanotransduction.

1. Introduction

Mechanical stimulation alters cell morphology, function, and composition of living tissues [1]. Complicated mechanical forces constitute daily function and therefore, may sense different forces inside or outside the body [2]. For example, compressive or extensive forces are delivered on bones and cartilage during motion [3]. In mammals, the somatosensory system detects mechanical forces via primary afferent neurons cell bodies of which are located mainly in trigeminal ganglia and DRG [4]. Several proteins that serve mechanosensitive functions have been identified, but questions remain unclear between physical stimulation and biochemical response [5]. Accordingly, the ways in which cells respond to force deserved to be examined [6]. The physical environment is a critical component of the nervous system's ability to convert mechanical inputs into electrical signals [7,8]. Substrate interactions and stiffness, in particular, have recently received considerable attention as a mean of influencing cell responses in areas including mechanobiology, motility, and stem cell differentiation [9–11].

The role of the ECM in cell adhesion and neuronal outgrowth through integrin receptors has been investigated [12,13]. Focal adhesions are one of the macromolecular complexes formed

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with transmembrane receptors, structural molecules, and signaling molecules [14]. The ECM is a complex, interlocking mixture of proteins and glycosaminoglycans (GAGs) that has a diverse range of functions [15]. Important ECM proteins including collagen, elastin, fibronectin (FN), and laminin are also included [15]. The cell surface receptors, integrins and SDCs, are two gene families that mediate cell – ECM adhesion, and ECM molecules contain binding sites for both receptors [16]. These two receptors comprise focal adhesions for FN-adherent cells [14]. The integrins, which are the primary receptors for ECM ligands, not only have roles in tissue structure and cell migration but also participate in adhesive events in many pathophysiological processes such as wound healing [17–19]. Syndecan-4 (SDC-4) is one of the four types of SDCs in mammals, which is expressed in most tissue [20]. Although SDC-4 is expressed extensively [17,21] and is enriched in focal adhesions, the mechanisms and role of SDC-4 in DRG neurons are unclear.

SDC-4 is advantageous in that it is expressed in the focal adhesions of several cell types [17,21–24]. SDC-4 is the only syndecan group in mammals associated with integrins in focal adhesions [23]. It binds to FN to promote the formation of focal adhesions and stress fibers [17,25] and binds phosphatidylinositol 4 and 5-bisphosphate (PIP2) together to regulate protein kinase Cα (PKCα) activity [26]. PKC activation is necessary for the formation of focal adhesions and spreading in the integrin-SDC-4 signaling cascade [27,28] and is regulated by Rho-family GTPase and FAK [21]. FAK is activated during integrin activation [29], and SDC-4 appears to play a vital role in regulating the phosphorylation of FAK [12,30]. However, there studies are, as yet, limited regarding DRG neurons in the literature.

In this study, our hypothesis is that DRG neurons could sense environmental stiffness through mechanical receptors including SDC-4 and relevant signaling pathway. Accordingly, we showed that DRG neurons have higher neuronal density, neurite growth, and branches on an FN-coated glass substrate. We also showed that the expression of SDC-4 was low but with increased pPKCα in the PDMS-poly group. The expression of pPKCα-FAKpTyr397-pERK1/2 was augmented after mechanical stretching. Aforementioned data showed that SDC4-pPKCα-FAKpTyr397-pERK1/2 pathway is crucial in DRG adhesion and mechanotransduction.

2. Materials and methods

2.1. Ethics statement

Experiments were carried out on ICR mice (aged 8–12 weeks) purchased from BioLASCO Co., Ltd., Taipei, Taiwan. After arrival, 12 h light dark cycle with sufficient water and food were given. All procedures were approved by the Institute of Animal Care and Use Committee of China Medical University (permit no. 101-116-N) and were in accordance with Guide for the use of Laboratory Animals by National Research Council.

2.2. Polydimethylsiloxane substrate

We bought the PDMS (Sylgard 184) from Dow Corning Corp. (Midland, MI, USA). We modified the ratio of elastomeric base to curing agent to modify stiffness as measured by Young's modulus. For instance, a PDMS base to curing agent ratio of 35:1 provided an elastic modulus of approximately 88 kPa [31]. We prepared PDMS ratio 35:1 substrates at room temperature [31,32]. To better control over the mechanical stimulation and the extracellular matrix interactions, we used PDMS that has a highly cross-linked three-dimensional structure. PDMS is composed of a silicone T-resin cross-linked by mixing with vinylterminated PDMS (base) and trimethylsiloxy-terminated polymethylhydroxosiloxane polymers (curing agent). PDMS can be modified to have various elasticity properties, which is useful when using force as an influential parameter to understand cell signaling [32].

2.3. DRG primary culture

We harvested DRGs from ICR mice (8–12 weeks old) for cell culture as previous described [31–33]. Mice were sacrificed using CO_2 to minimize suffering. DRGs cells were acutely dissociated and seeded on glass coverslips or PDMS ratio 35:1 substrates each coated with poly-L-lysine (poly; 0.1%; Sigma, St. Louis, MO, USA) and FN (10%; Sigma, St. Louis, MO, USA). Both glass coverslips and PDMS ratio 35:1 substrates with DRG neurons were then placed in petri dishes with Dulbecco's modified Eagle's medium containing 1% penicillin/streptomycin and 10% fetal calf serum, and incubated at 5% CO_2 at 37 °C for 48 h. To provide more specific understanding of protein expression in DRG neurons, we used cytosine β-D-arabinoside hydrochloride (0.3 μM, Sigma, St. Louis, MO, USA) to inhibit growth of Schwann cells and other glial cells [34].

2.4. Immunofluorescent microscopy and image analysis

Immunostaining of DRG neurons were employed according our previous publications [31–33]. The primary antibody used was anti-PGP9.5 antibody [13C4/I3C4] – neuronal marker (1:100, Sigma, St. Louis, MO, USA), mouse-anti-SDC-4 (1:100, Santa Cruz Biotechnology, Santa Cruz, CA, USA). F-actin observation was directly stained with rhodamine–phalloidin (1:200, Invitrogen, Carlsbad, CA). The secondary antibodies were Goat anti-Rabbit IgG DyLight488 (1:500, Jackson Immuno Research Laboratories), and Goat anti-Mouse IgG DyLight594 (1:500, Jackson Immuno Research Laboratories). The stained DRG neurons were sealed under the coverslips, and examined using an epi-fluorescent microscope (Olympus, BX-51, Japan) with a 40X high numerical aperture (NA = 1.4) objective for imaging the distribution of the structural proteins in DRG neurons. Further, all images were analyzed using NIH ImageJ software (Bethesda, MD, USA).

2.5. Polydimethylsiloxane substrate stretching

After 2 day of culturing, we cut out the PDMS membrane sections that were seeded with DRG neurons. The cell stretching devices could allow us to place the PDMS membrane on the top, and fix the PDMS gels using an adaptor. A pressure monitor was used to induce a mechanical force. We applied pressure (5 Ib/in^2) through the bottom of the PDMS gel controlling force with a pressure gauge, to stretch the elastomeric membrane as previous descripted [5]. It was critical that we ensured airtightness in the device to avoid any pressure imbalance. After 48 h of culturing, we stretched cells for 10 min and then harvested the cell lysates for Western blot analysis.

2.6. Western blot analysis

Total protein samples were prepared by homogenizing neurons in lysis buffer containing 50 mM Tris–HCl pH 7.4, 250 mM NaCl, 1% NP-40, 5 mM EDTA, 50 mM NaF, 1 mM Na3VO4, 0.02% NaN3 and 1X protease inhibitor cocktail (AMRESCO). The extracted proteins (30 μg per sample assessed by BCA protein assay) were subjected to 8% SDS-Tris glycine gel electrophoresis and transferred to a PVDF membrane. The membrane was blocked with 1% BSA in TBS-T buffer (10 mM Tris pH 7.5, 100 mM NaCl, 0.1% Tween 20) for 30 min, incubated with anti-PKCα (1:1000, Millipore), FAK [p Tyr397] (1:500, Novus Biologicals), FAK [p Tyr925] (1:500, Novus Biologicals), and ERK1/2 [p Thr202, p Tyr204] (1:500, Novus Biologicals) SDC-4 (1:200, Santa Cruz Biotechnology, Santa Cruz, CA, USA) in TBS-T with 1% bovine serum albumin at 4 °C