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Cell Membrane Patches are Supported by Proteoglycans

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Abstract. A cell membrane patch isolated on a patch clamp pipette incorporates in addition to the phospholipid bilayer, an extracellular matrix and cytoskeletal components. The significance of the extracellular matrix for the patch formation was studied in aortic smooth muscle and cerebellar granule cells grow in the presence of an inhibitor of proteoglycan synthesis, β-D-xyloside. The xyloside improved the seal success rate, and after patch excision membrane vesicles were formed instead of inside-out patches. When amphotericin B was included in the pipette solution, perforated outside-out vesicles were formed in 96% of cells. The findings suggest, that membrane patches are supported by the extracellular matrix or by structures that relate to this matrix.

Key words: β-D-xyloside — extracellular matrix — seal success rate — membrane elasticity — vesicle formation — perforated outside-out vesicles

Introduction

It was probably a surprise in the early patch clamp days that excision of a cell-attached patch did lead to formation of an isolated inside-out patch. Now electrophysiologists use the procedure daily, but little is known about the structures that may support the patch and keep it from rounding up to form membrane vesicles.

Most cells have an extracellular matrix coating the phospholipid bilayer, and the matrix components are primarily synthesized by the cell itself. In the vascular wall the smooth muscle cells are the major producers of proteoglycans, which cross-link to many extracellular proteins and are pivotal for the deposition of the matrix (Hamati, Britton & Carey, 1989). Here, the effect was studied of an inhibitor of proteoglycan assembly, β-D-xyloside, and it was found, that membranes of cells grown in the presence of the inhibitor became very elastic and generally formed vesicles upon excision.

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Materials and Methods

Cell Culture

Bovine aortic smooth muscle cells were isolated from calf aorta, and explant cells were cultured from minced medial layer. The cells were subcultured to passages 5–9, plated on glass coverslips and used on day 1–7 after passage (Olesen et al., 1994).

The granule cells were isolated from 7-day-old mouse cerebellum, plated on polylysine-coated glass coverslips and maintained in vitro until day 1–5 (Drejer, Larsson & Schousboe, 1983).

All cells were cultured in Dulbecco's MEM medium + 10% fetal calf serum. The xyloside-treated group received 1 mM 4-methylumberiferyl-β-D-xyloside (Sigma, St. Louis, MO) in the culture medium immediately after replating, and the xyloside remained there until the cells were used for experiment.

Patch Clamp Recordings

The experiments were conducted with heat polished borosilicate glass pipettes of 4–6 MΩ tip resistance. A seal was considered successful when it had a resistance larger than 1 GΩ. Inside-out and outside-out patches were verified by the presence of 250–300 pS BK channels, the activity of which was stimulated by depolarizing the membrane potential, i.e., negative pipette potentials in inside-out patches and positive pipette potentials in outside-out patches. Membrane vesicles were detected as described below. The treatment of the cells was not known to the experimentator. The number of seal attempts was larger than 15 in all experimental groups and up to 50 in some groups. The data were statistically analyzed by a chi-square test ($n = 15$), and significant effects are indicated by $*(P < 0.05)$ and $**(P < 0.0005)$ in the figures.

The ion composition of the intra- and extracellular solutions was as follows (in mM): 146 K^+, 144 Cl^-, 1 Ca^{2+}, 1 Mg^{2+}, 2 EGTA, 10 HEPES. Amphotericin B, which was used in one series of experiments, was dissolved at 5 mg/100 μl DMSO and diluted 2000-fold in the pipette solution. The experiments were conducted at room temperature using a HEKA EPC-9 amplifier.

Results

The aortic smooth muscle cells attached well to the glass coverslips and flattened out after a few hours in culture. The xyloside-treated cells were slightly more rounded during the first three days after passage, but after this time there was no visible difference in cell shape.

The cells expressed classical BK channels (maxi-K or large-conductance Ca^{2+}-dependent K^+ channels), and in the patch clamp recordings 1–5 channels were found per patch (~10 μm^2 membrane surface). The BK channels in these cells had a unit conductance of 278 ± 24 pS (mean ± SD; n = 25), were blocked by external charybdotoxin and TEA^+, and activated by internal Ca^{2+} and depolarizing voltage (Olesen et al., 1994). In the cell-attached patches BK channels were activated at pipette potentials of −10 to −60 mV. When membrane vesicles were formed, the shape of these big channels became distorted, and the membrane potentials necessary to activate them increased to ±70 to 150 mV (Fig. 1). The apparent reduction in single-channel conductance was due to the voltage division across the two membranes of the vesicle, and the distorted shape was caused by charging of the vesicle interior by the single channel current. The vesicles were broken by air exposure or by brief voltage pulses in excess of 200 mV.

In the control cells the seal success rate varied between 12 and 26% during the first five days after passage with no evident time dependence (Fig. 2*A*). The xyloside treatment improved the sealing to success rates of: 6% on day 1; 26% on day 2; 67% on day 3; 81% on day 4; and 40% on day 5. On day 6–7 the seal rate was reduced to 21–25%, i.e., comparable to the control cells. The characteristics of the BK channels recorded in the two groups did not differ in terms of unit conductance, substates, number of channels, and voltage-sensitivity.

Retraction of the pipette from the cell resulted in formation of inside-out patches in the control cells and rarely in formation of membrane vesicles (2–12% of cell-attached patches; Fig. 2*B*). In the xyloside-treated cells the frequency of vesicles increased, particularly on day 3–4: 2% on day 1; 10% on day 2; 60% on day 3; 73% on day 4; and 25% on day 5. When the vesicles were exposed to air, most of them survived this treatment and a large part formed inside-out patches, but outside-out patches were also formed: 20 inside-out and 12 outside-out patches were formed from 32 vesicles on day four. The xyloside-treated membranes appeared very elastic and often sealed over to reform vesicles, e.g., one membrane sealed over three times and could each time be ruptured to form an inside-out patch.

To take advantage of the high frequency of vesicle formation an attempt to routinely make perforated outside-out vesicles was undertaken by including amphotericin B in the pipette solution (Horn & Marty, 1988; Levitan & Kramer, 1990). The cells incorporated amphotericin B well (R_s < 20 MΩ within less than 10 min), and out of 24 seals 23 resulted in perforated outside-out vesicles containing 280 pS BK channels (day 4).

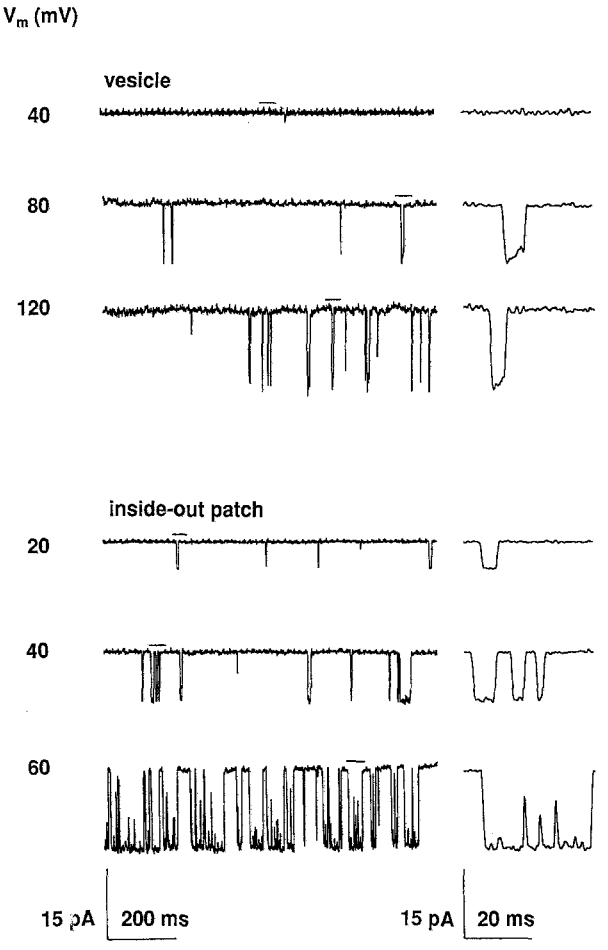

Fig. 1. BK channel in membrane vesicle and in inside-out patch. The bovine aortic smooth muscle cells expressed large BK channels, and the vesicles were easily detected by the size and shape of the single channel currents. The BK channel in the vesicle had an apparent unit conductance of about 120 pS, and showed a characteristic decay of the open state current. Following air exposure the outer membrane disrupted and a BK channel of 280 pS conductance, which was activated by depolarizing membrane potential in the range 20–60 mV, appeared. The single openings, which are marked by bars above the tracings, are shown at a tenfold expanded time scale in the right panel. The vesicle currents decayed with an average time constant of 11 msec. Symmetric 146 mM $[K^+]$; $[Ca^{2+}]_i$ = 100 nM

The effects of the xyloside on the membrane characteristics was also studied in a primary culture of mouse cerebellar granule cells. The control cells sealed well (70–84%) and rarely formed vesicles (2–4%) during the first three days after isolation. In the xyloside-treated cells, the seal rates were 75%, 67%, and 88% and the vesicle rates were 23%, 47%, and 57% on day 1, 2, and 3, respectively. The effect on vesicle formation was significant at $P < 0.0005$ on each of the three days. The results from day three are shown in Fig. 3.

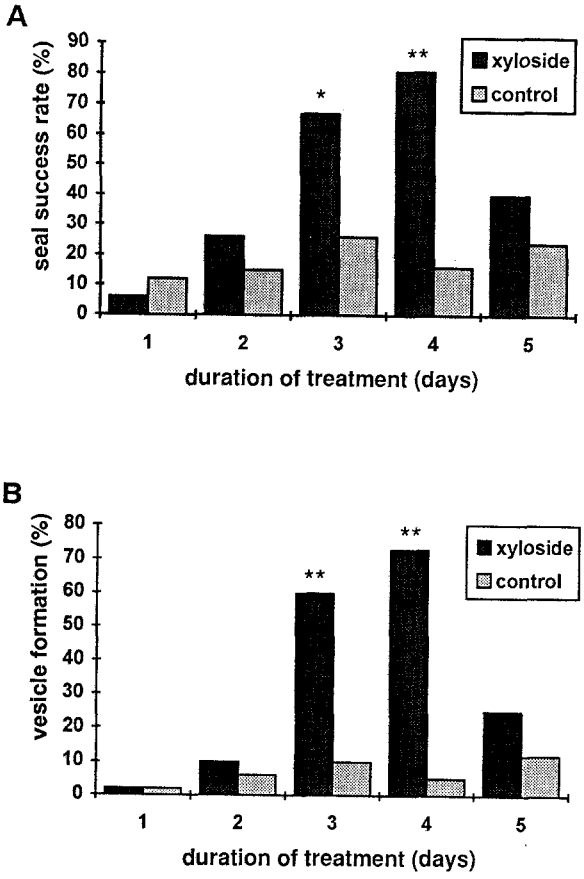

Fig. 2. Effects of xyloside on the formation of seals and vesicles in bovine aortic smooth muscle cells. *(A)* The presence of 1 mM xyloside in the cell culture medium slowly improved the seal success rate, which peaked 3–4 days after the cells were passaged. *($P < 0.05$), **($P < 0.0005$). *(B)* In the control group excision of the cell-attached patches generally resulted in inside-out patches, and less than 12% resulted in vesicles throughout the period. In the xyloside-treated group few vesicles were formed during the first days, but the frequency of vesicle formation increased dramatically on day 3–4 after isolation. The ordinate represents the percentage of the cell-attached patches in *A* resulting in vesicles after excision.

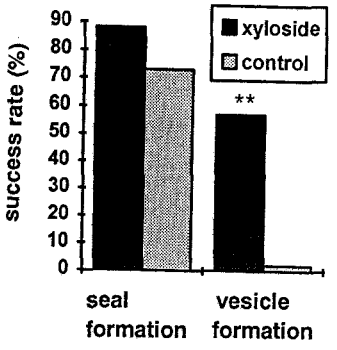

Fig. 3. Effects of xyloside on seal success and vesicle formation in cerebellar granule cells on day 3 after isolation. The seal success rate was high in the control group of these neuronal cells (73%) leaving little room for improvement by the xyloside treatment (88%) (nonsignificant change). However, the xyloside treatment significantly increased the frequency of vesicle formation ($P < 0.0005$). The ordinate shows the percentage of seal attempts resulting in cell-attached seals and in vesicles.

Discussion

Proteoglycans are essential for assembly of the extracellular matrix because these molecules with their multiple side chains serve as cross-linkers by binding to proteins such as fibronectin, laminin and collagen (Hamati et al., 1989). Deposition of the extracellular matrix is attenuated in many cell types by treatment with a xyloside, which inhibits the addition of proteoglycan side chains to the core protein (Hamati et al., 1989; Gressner, 1991; Hahn & Birk, 1992). Xyloside treatment was found in this study to significantly improve the sealing of a patch pipette to smooth muscle cell membranes. The effect, which is in good agreement with a reduced extracellular matrix, has also been described by Izu & Sachs (1991) in the renin-secreting cell line As4.1. In this original study, the cells were pretreated with xyloside for 3 days before they were passaged, and effect peaked on the first day after passage, which is in accordance with the delayed effect seen after 3–4 days treatment in the present study.

The xyloside significantly increased the number of vesicles formed after patch excision and apparently decreased the rigidity of the cell membrane, since patches easily sealed over. This effect could be caused by the reduction of extracellular matrix *per se,* but it could also be due to an effect on the cytoskeleton, since the organization of the latter depends on factors such as adhesion surface, anchor proteins, and the extracellular matrix. Thus, in several cell types assembly of α-actin filaments is inhibited by xyloside treatment (Hamati et al., 1989; Gressner, 1991). In this study, the xyloside changed the morphology of the SMC towards more rounded cells in contrast to the spindle-shaped control cells—a change in structure which could have been caused by modulation of intra- as well as extracellular proteins.

Perforated outside-out vesicles were easily obtained in xyloside-treated cells, and the procedure could be helpful when this configuration is desired. The near 100% success found here is very high for amphotericin B incorporation, which could be due to the lipid composition of the SMC membrane, but the reduction in extracellular matrix may also facilitate this process.

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