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Short communication

Inhibiting synthesis of extracellular matrix improves patch clamp seal formation

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ABSTRACT: The ability to form gigaohm seals is essential for patch-clamp studies. Cells with otherwise useful properties must be abandoned for electrophysiological studies if seal formation is not possible. We have found that by inhibiting the growth of extracellular matrix with ß-D-xyloside, the success of forming gigaohm seals increased from near 0% to near 100%. Treated cells remained viable and appeared morphologically similar to untreated cells. Prototype treatment protocols are given for the renin secreting cell line As4.1.

Key words: patch clamp/ seal/ channel /renin

Introduction:
The As4.1 cell is a renin expressing cell line isolated from a mouse primary kidney tumor and ascites (Sigmund et al., 1990). To examine whether these cell had the predicted stretch sensitive response, we tried repeatedly to patch clamp the cells and were almost never able to form seals. Under high magnification phase optics, the membrane appeared to be covered with a thick layer of transparent material that might have prevented seal formation. We tried unsuccesfully to "clean" the cell surface with trypsin, collagenase, and pronase. We then tried inhibiting the synthesis of the extracellular matrix (ECM) using 4-methylumberiferyl-ß-D-xyloside, that has been shown to reduce the ECM deposition in rat aortic smooth muscle cells (Hamati et al., 1989). This treatment greatly enhanced the probability of forming gigaohm seals and allowed us to demonstrate the presence of ion channels. Similar results have been observed for other cell lines (J. Hidalgo, Univ. Chile, Santiago, personal communication)

Materials and Methods:
Cell Preparation: As4.1 cells (provided by Dr. K. W. Gross, Roswell Park Memorial Institute) were grown in a normal culture medium of DMEM + 10% fetal bovine serum and maintained at 37°C in a 5% CO_2/air incubator. The cells were passed every week and fed twice per week. For patch-clamp studies, we used 0.25% trypsin (+ 1mM EDTA) to isolate the cells, then plated them onto class cover slips in plastic culture dishes. To test the effect of different duration treatments, we created three protocols. Cells in the *control* group were cultured in normal medium before and after re-plating. Cells in the *post-isolation* group were cultured in normal medium before re-plating and in ß-D-xyloside (Sigma) medium after re-plating. The *pre-isolation* group was cultured in normal medium, but ß-D-xyloside was added to the medium three days before re-plating and maintained there for the remainder of the culturing. We anticipated that those cells that underwent the most growth in the presence of ß-D-xyloside would have the least extracellular matrix and be the easiest to patch. ***Blind Testing:*** To minimize bias in judging ease of patching, the person doing the experiment was unaware of the treatment condition of the cells. A seal was deemed successful if it had a resistance > 1GΩ. ***Pipettes:*** Pipettes were made from borosilicate glass (100λ Microcaps, Drummond Scientific, Broomal, PA) on a Sutter Instruments (San Raphael, CA) PC-84 micropipette puller with the filament block maintained at 50°C. The pipette tips had a diameter of about 1μm and a length of 5mm. After fire polishing, the pipette had a resistance of about 7MΩ. To maximize consistency, only one person performed the patch clamp experiments. For experiments the bath and the pipette were filled with normal saline containing, in mM: NaCl/137.5, KCl/4.2, $CaCl_2$/0.9, $MgCl_2$/0.5, Na-HEPES/6, HEPES/8, and pH7.5.

Results:
Figure 1 shows the dose-response relationship for seal formation for the *post-isolation* protocol. With no ß-D-xyloside (control group) we succeeded once in twenty one tries (4.8%). For cells in 0.25mM ß-D-xyloside, we succeeded six times out of sixteen tries (37.5%); for 0.5mM ß-D-xyloside, also six times out of sixteen tries (37.5%). For the *post-isolation* group in 1mM ß-D-xyloside, we got the highest success rate -- fifteen success out of thirty tries (50%), an improvement of 10 fold over the control group.

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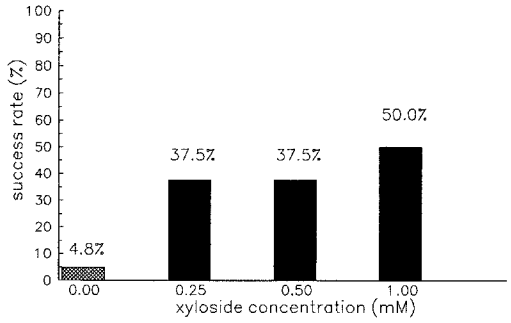

Figure 1. The dose responce relationship of seal formation in the post-isolation treatment group.

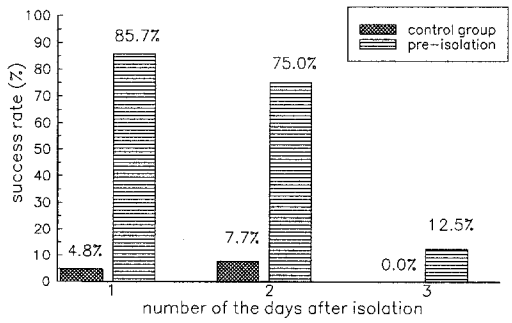

Figure3. The time responce of the ß-D-xyloside effect on the pre-isolation group using 1mM ß-D-xyloside. The efficiency of treatment falls as cells become confluent at three days in culture.

A more extensive treatment, the *pre-isolation* protocol, involved incubating the cells with ß-D-xyloside before and after re-plating. With this protocol, the seal formation rate was raised further to about 90%. Figure 2 shows the differences between the two experimental treatments and the control group. Although the results were so striking, we used Student's t-test to check the conclusions. We used a sample size of fourteen (the lowest sample size in the three groups). For the relative advantage of the post-isolation group over the control group, $z = 3.209$, a confidence level of more than 99%. For the *pre-isolation* group over the control group, $z = 7.290$, a confidence level of more than 99.999%. For the *pre-isolation* group over the post-isolation group $z = 2.291$, a confidence level above 95%.

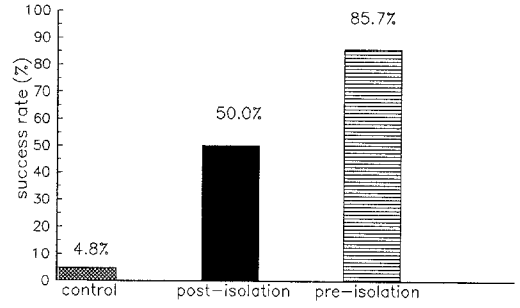

Figure 2. The success rate for different treatment protocols. Treating the cells with xyloside for three days before replating and then growing the cells for one day in xyloside medium is the most effective procedure.

The above data was obtained on the cells within the first day after plating. We also studied the effect of extended exposure to ß-D-xyloside to cells in the pre-isolation group. Figure 3 shows that while the treatment is effective for the first and second days, by the third day the effect has faded. One possible explanation is that the suppression of ECM formation by the ß-D-xyloside is incomplete and the ECM slowly builds up over three days.

Discussion:

Forming a good seal in patch clamp experiments depends on a number of factors including cell type, culture condition, and composition of the pipette glasses. Our results show that in the As4.1 line, treating cells with ß-D-xyloside dramatically improves the likelihood of forming good seals. The ease of making seals is important, not only for the efficiency in experimentation, but also for reducing stresses on the patch that may affect channel properties.

The effect of ß-D-xyloside treatment was studied by Hamati et al. (1989) using rat aortic smooth muscle cells. ß-D-xyloside inhibited synthesis of proteoglycans causing reduced ECM deposition, reduced proliferation and cytoskeletal reorganization. With the As4.1 line we found that cells cultured in normal medium will form multilayers after growing to confluence, but cells treated with ß-D-xyloside only formed monolayers. The treated cells recovered and grew to multilayers after being returned to normal culture medium. These observations are similar to those of Hamati et al. (1989). We did not see any obvious morphological difference between treated and untreated cells. (Figure 4)

It is not known what effects ß-D-xyloside may have on the electrophysiological properties of cells. This issue is particularly difficult to assess when the untreated population can't be examined. Our experiments were not sufficiently extensive to provide quantitative comparison of cultures at different stages of treatment, but we saw no distinct differences in channel activity. The ß-D-xyloside does affect cytoskeletal organization, tending to disrupt a-actin (Hamati, Britton and Carey, 1989). But interestingly, Hamati et al. showed that if their cells were cultured on dishes precoated with ECM, the cytoskeleton was unaffected by ß-D-xyloside. This suggests that a similar approach may be used for electrophysiology whereby the upper surface of the

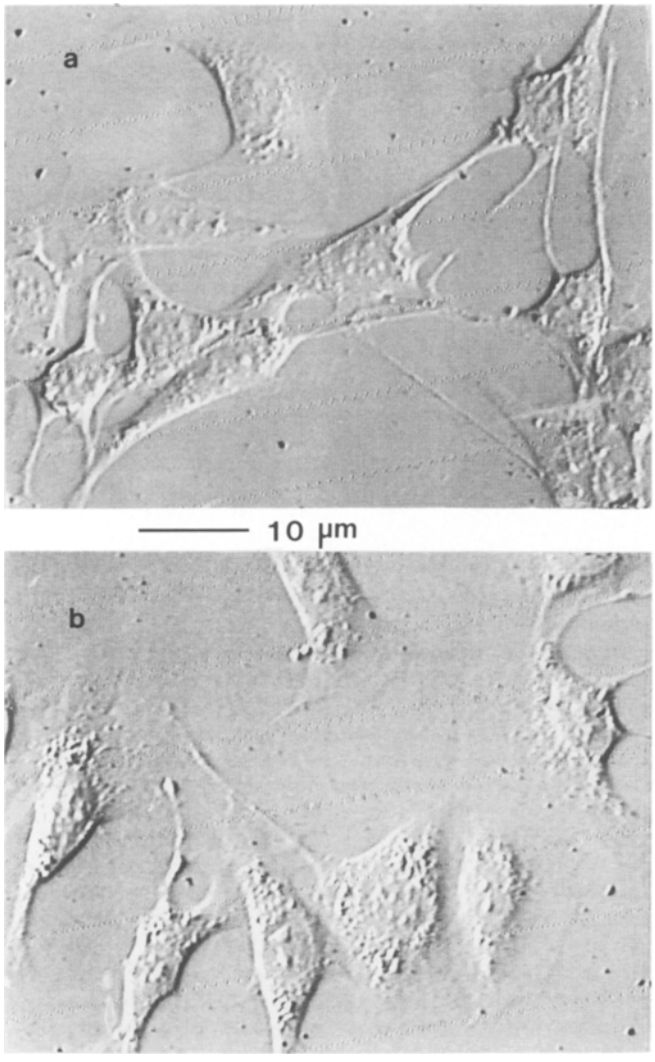

Figure 4. Morphology of As4.1 cells cultured on glass cover slips for two days. Cells incubated in the (a) normal culture medium, or (b) normal medium plus 1mM ß-D- xyloside.

cell has reduced amounts of endogenous ECM and the substrate ECM preserves intracellular structure.

We haven't yet tested the efficacy of ß-D-xyloside treatment on other cell lines, but J. Hidalgo (personal communication) has found similar results to those reported here using a newly developed human muscle cell line.

In summary, our results show that treating the cells with 1mM ß-D-xyloside under growth conditions substantially improves the likelihood of making gigaohm seals. We expect ß-D-xyloside treatment to work similarly on other cells. Cells must grow in the presence of the ß-D-xyloside. Simply adding it to the medium of a confluent culture has no effect. Because proteoglycans are an essential component of the ECM, inhibiting the synthesis of proteoglycans is expected to reduce the ECM and promote seal formation.

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