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Depolarization of the Membrane Potential by Hyaluronan

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ABSTRACT

The membrane potential is mainly maintained by the K^+ concentration gradient across the cell membrane between the cytosol and the extracellular matrix. Here, we show that extracellular addition of high-molecular weight hyaluronan depolarized the membrane potential of human fibroblasts, human embryonic kidney cells (HEK), and central nervous system neurons in a concentration-dependent manner, whereas digestion of cell surface hyaluronan by hyaluronidase caused hyperpolarization. This effect could not be achieved by other glycosaminoglycans or hyaluronan oligosaccharides, chondroitin sulfate, and heparin which did not affect the membrane potential. Mixtures of high-molecular weight hyaluronan and bovine serum albumin had a larger depolarization effect than expected as the sum of both individual components. The different behavior of high-molecular weight hyaluronan versus hyaluronan oligosaccharides and other glycosaminoglycans can be explained by a Donnan effect combined with a steric exclusion of other molecules from the water solvated chains of high-molecular weight hyaluronan. Depolarization of the plasma membrane by hyaluronan represents an additional pathway of signal transduction to the classical CD44 signal transduction pathway, which links the extracellular matrix to intracellular metabolism. J. Cell. Biochem. 111: 858–864, 2010.

KEY WORDS: HYALURONAN; MEMBRANE POTENTIAL; EXTRACELLULAR MATRIX; PROTEOGLYCANS; DONNAN EFFECT

The cellular response to hyaluronan has mostly been explained by the interaction of hyaluronan with its cell surface receptor CD44 and the signaling cascade triggered by this interaction. In addition to signaling mediated by CD44, hyaluronan must exert a Donnan effect that influences the osmotic pressure as well as the ionic distribution across the plasma membrane. While many studies investigated the osmotic behavior of glycosaminoglycans in solution [Ogston, 1966; Maroudas, 1975; Maroudas and Venn, 1977; Urban et al., 1979; Maroudas and Bannon, 1981; Urban and Maroudas, 1981; Maroudas et al., 1985; Reed and Rodt, 1991; Peitzsch and Reed, 1992; Knepper et al., 2003], the ionic distribution across plasma membranes has been neglected so far. Thus, the presence of hyaluronan on the cell surface should have profound effects on the membrane potential, which in turn influences cellular behavior. Since, extracellular glycosaminoglycans are often locally concentrated on the cell surface by receptor binding and since the membrane potential is formed by the charge distribution in the direct vicinity of both membrane sides, the precise effect of exogenous glycosaminoglycans on the membrane potential has to be determined experimentally. This proposition holds true particularly for high-molecular weight hyaluronan that shows anomalous osmotic behavior in the presence of other macromolecules due to steric exclusion [Laurent and Ogston, 1963]. Therefore, we measured the effect of several glycosaminoglycans on the membrane potential of different cell lines and acutely isolated central neurons.

The membrane potential is regulated by K^+ currents in fibroblasts [Chilton et al., 2005] and endothelial cells [Adams and Hill, 2004], which is mainly determined by the inward rectifying channel K_{ir}. In central neurons the resting potential is assumed to be determined by channels active below firing threshold, with TASK channels, HCN channels, and inward rectifying channel playing major roles [Meuth et al., 2006]. Furthermore, the contribution of Cl^- channels, the Na^+/

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K^+ pump, and secondary active ion carriers have been taken into account [Hille, 2001]. The possible influence of hyaluronan on the membrane potential of neurons has yet not been explored.

MATERIALS AND METHODS

MATERIALS

Bis-(1,3-dibutylbarbituric acid) (Di-BAC4(3)) was from Invitrogen (Eugene, OR) and other chemicals were from Sigma Chemical, Co. Non-inflammatory hyaluronan (Na^+-salt) with a weight–average molecular weight of 1.6×10^6 Da was a gift from Genzyme Corp. [Shiedlin et al., 2004]. It was dissolved and equilibrated with medium at a concentration of 2 mg/ml by slow turning of the tube for 24 h at room temperature. Hyaluronan oligosaccharides were prepared by incubation of a solution of 1 mg/ml hyaluronan from rooster comb (Sigma Chemical Co.) in 0.15 M NaCl, 10 mM sodium acetate buffer pH 5.5 at 37°C with 0.1 mg/ml testicular hyaluronidase for 24 h. The enzyme was inactivated by heating to 100°C for 10 min and the precipitated protein was removed by centrifugation at 14,000*g* for 2 min. Chondoitin sulfate (Na^+-salt) from shark cartilage and heparin (Na^+-salt) from bovine intestinal mucosa were from Sigma Chemical, Co. The serum free complete Quantum medium 333 for fibroblasts containing growth factors was from PAA Laboratories.

CELLS AND CELL CULTURE

Cells were grown in Dulbecco's medium supplemented with streptomycin/penicillin (100 units of each/ml), 10% fetal calf serum or in serum free Quantum medium supplemented with streptomycin/penicillin (100 units of each/ml), kanamycin (100 units/ml) on 96-well microtiter plates.

DETERMINATION OF THE MEMBRANE POTENTIAL

Changes in membrane potential responses were assessed in the fluorometric plate reader as described earlier [Gopalakrishnan et al., 2003] using the bisoxonol dye Di-BAC4(3), an anionic potentiometric probe which partitions between cellular and extracellular fluids in a membrane potential-dependent manner. Briefly, cells were grown in Dulbecco's medium in 96-well microtiter plates to near confluency. They were rinsed with 100 μl Quantum medium containing 1 μg/ml DiBAC4(3) and incubated with the same medium containing varying concentrations of glycosaminoglycans. The glycosaminoglycans were dissolved at concentrations of 2 mg/ml and this concentration was applied to the first culture wells. Serial 1:1 dilutions with medium were made for the following wells. Changes in fluorescence were monitored from the bottom of the wells at excitation and emission wavelengths of 488 and 520 nm, respectively. Depolarizations and hyperpolarizations were reflected by an increase and decrease in fluorescence, respectively. The resting potential was determined by the method of Krasznai et al. [1995]. The fluorescence values were converted into membrane potentials using the Nernst equation $E_t = E_0 - 61.5 \times \log\ f_t/f_0$, where E_0 is the resting potential, f_t the measured fluorescence, and f_0 the fluorescence of resting cells at 37°C.

PATCH CLAMP MEASUREMENTS

All animal procedures were approved by local authorities. Rats (P15–P25) were deeply anesthetized using isoflurane and decapitated. Brains were removed and placed in cold, oxygenated artificial cerebrospinal fluid (ACSF) containing (mM): sucrose, 210; PIPES, 20; KCl, 2.4; $MgCl_2$, 10; $CaCl_2$, 0.5; dextrose, 10; pH 7.25 with NaOH. Thalamic slices (500 μm) were obtained from coronal vibratome sections (Model 1000; Ted Pella, Redding, CA) and dLGN tissue was transferred to a spinner flask and incubated for 25–30 min at 30°C in an oxygenated solution containing trypsin (0.5–1 mg/ml; Sigma, Taufkirchen, Germany) and (mM): NaCl, 120; KCl, 5; $MgCl_2$, 3; $CaCl_2$, 1; PIPES, 20; dextrose, 25; pH adjusted to 7.35 with NaOH. Single neurons of the dorsal lateral geniculate nucleus of the thalamus were obtained by trituration and whole-cell recordings were performed at room temperature using borosilicate glass pipettes (GC150TF-10; Clark Electromedical Instruments, Pangbourne, UK) connected to an EPC-10/2 amplifier (HEKA Lamprecht, Germany). The typical electrode resistance was 3–5 MΩ, while access resistance was in the range of 3–8 MΩ. Current clamp experiments were governed by PatchMaster software and started 5 min after establishing the whole cell configuration and lasted for 15–20 min. The following recording solutions were used: (i) extracellular solution (mM): NaCl, 155; KCl, 3; HEPES, 10; dextrose, 10, $MgCl_2$, 1; $CaClain_2$, 2; TTX, 0.001; pH 7.35 with NaOH. (ii) Intracellular solution: K-gluconate, 88; K_3-citrate, 20; NaCl, 10; BAPTA, 3; $CaCl_2$, 0.5; $MgCl_2$, 1; HEPES, 10; Mg-ATP, 3; Na_2-GTP, 0.5, phosphocreatin 15; pH 7.25 with NaOH. Osmolarity of internal and external solution were kept at 295 and 305 mOsm, respectively. Addition of hyaluronan did not alter the measurable osmolarity of the external solution. In one set of experiments KCl was increased to 15 mM resulting in a calculated shift in K^+ equilibrium potential of +40 mV. NaCl was decreased accordingly. A custom-made multibarrel application pipette with a tip diameter of about 100 μm was used for test substance application. Recorded cells were kept in the gravity driven laminar flow generated by this system. Results are shown as mean ± SD. Since a Gaussian distribution of measured resting potentials was found, statistical significance was calculated using a modified *t*-test for small samples [Dixon and Massey, 1969].

EFFECT OF HYALURONIDASE TREATMENT

Human fibroblasts were seeded into 96-well microtiter plates and grown to confluency. The cells were washed with 100 μl of 50 mM sodium acetate buffer pH 5.0, 0.9% NaCl and incubated with 100 μl of a serial dilution of hyaluronidase from *Streptomyces hyaluronolyticus* starting with 22.5 units for 20 min at 37°C. The cells were washed with 100 μl Quantum medium containing 1 μg/ml DiBAC4(3), incubated with the same medium for 30 min at 37°C and fluorescence was measured as described above.

DETERMINATION OF THE MEMBRANE POTENTIAL BY EQUILIBRIUM DIALYSIS

Hyaluronan was dissolved in Quantum medium at different concentrations and subjected to equilibrium dialysis against hyaluronan-free Quantum medium. The hyaluronan solutions (1 ml) were transferred into an Eppendorf tube. A hole was punched into the tube cap and covered with a dialysis membrane. The Eppendorf

tube was sealed and placed into a glass tube containing 1 ml of hyaluronan-free Quantum medium. The two solutions separated by a dialysis membrane were shaken overnight at 37°C and the pH of the solutions were measured. The membrane potential (E) was calculated from the pH-difference (ΔpH) of the hyaluronan-containing and hyaluronan-free solutions by the following equation:

$$E = 61.5 \times \Delta pH.$$

RESULTS

MEMBRANE POTENTIAL OF HUMAN FIBROBLASTS

To eliminate any interfering interactions of hyaluronan and serum components, the experiments were performed in serum free Quantum medium. Since it was known that growth factors in media hyperpolarize cells due to Ca^{2+} signals [Moolenaar et al., 1986], the alteration of the membrane potential after medium change was determined in a preliminary experiment by fluorescence of DiBAC4(3). Figure 1 shows that medium replacement caused a transient hyperpolarization that leveled off to the resting potential after 120 min. Therefore, the subsequent measurements were performed 120 min after the change of the medium.

In order to convert the fluorescence readings into membrane potentials, the resting potential of human fibroblasts and embryonic kidney cells (HEK) was determined in another preliminary experiment. They were found to be −17 mV for human fibroblasts and −30 for HEK cells, respectively, in agreement with previous measurements [Voets et al., 1996; Kamkin et al., 1999].

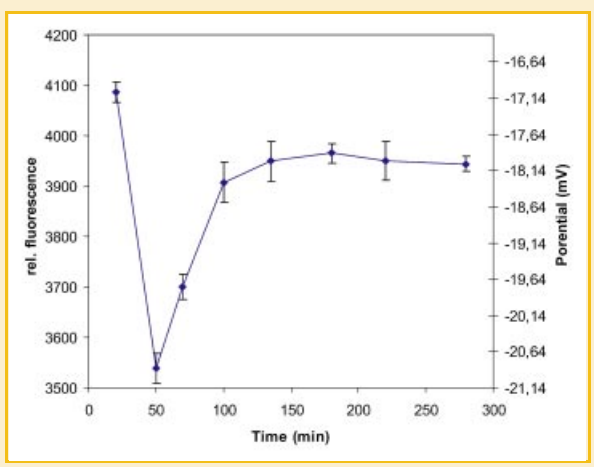

Fig. 1. Response of the membrane potential to media change. Fibroblasts were grown in Dulbecco's medium containing 10% fetal calf serum to near confluency and changed to serum free Quantum medium containing the fluorescent dye Di-BAC4(3). The fluorescence was recorded at the time points indicated. The fluorescence values were converted into membrane potentials using the Nernst equation with the resting potential of −17 mV for cells 20 min after medium change. The error bars indicate the SD of three determinations.

EFFECT OF GLYCOSAMINOGLYCANS ON THE MEMBRANE POTENTIAL

Human fibroblasts and HEK cells were incubated with increasing concentrations of high-molecular weight hyaluronan, hyaluronan oligosaccharides, chondroitin sulfate or heparin, respectively, and alterations in the membrane potential were recorded. Figure 2 shows that solely high-molecular weight hyaluronan depolarized both cell lines in a concentration-dependent manner, while hyaluronan oligosaccharides, chondroitin sulfate, or heparin had no effect at all. The characteristics and the molecular size of the high molecular weight with 1.6×10^6 Da was described previously [Shiedlin et al., 2004]. The hyaluronan induced increase in fluorescence was linear with the log concentration scale and persisted for at least 24 h.

EFFECT OF HYALURONIDASE TREATMENT ON THE MEMBRANE POTENTIAL

If the influence of hyaluronan on the membrane potential has any biological importance, it should also occur on fibroblasts that naturally contain a hyaluronan surface coat. Human fibroblasts were incubated with a serial dilution of hyaluronidase from *Streptomyces hyalurolyticus* for 20 min. The enzyme was washed out and the alteration of the membrane potential was determined 30 min later. Figure 3 shows that the membrane potential increased with hyaluronidase in a concentration-dependent manner.

MEMBRANE POTENTIAL ON CENTRAL NEURONS

Identified thalamocortical relay (TC) neurons [Broicher et al., 2007] were used to monitor the effects of hyaluronan on the neuronal resting potential as an independent measure. Acutely isolated TC neurons were recorded under whole-cell current clamp conditions and resting membrane potentials averaged -60.9 ± 6.0 mV ($n = 14$). Following a stable control period of 2–3 min, application of 1 mg/ml hyaluronan resulted in fast and strong membrane depolarization amounting to $+11.2 \pm 3.6$ ($n = 5$; $P < 0.01$) which was associated with a slight decrease in membrane resistance from 490 ± 84 to 475 ± 68 MΩ (Fig. 4A, left panel). The magnitude of the depolarization increased with increasing hyaluronan concentration (Fig. 4B). The recovery from the hyaluronan-induced depolarization was slow and during the recording period of up to 20 min this effect was reversible in three out of nine cells. For comparison TC neurons were challenged by increasing the extracellular K^+ concentration from 3 to 15 mM (Fig. 4A, right panel). This maneuvre resulted in a fast and fully reversible shift of $+32.0 \pm 8.6$ mV ($n = 5$; $P < 0.01$) in membrane potential, which was close to the expected +40 shift calculated by the Nernst equation (Fig. 4B).

DETERMINATION OF THE MEMBRANE POTENTIAL BY EQUILIBRIUM DIALYSIS

It is possible that the effect of high-molecular weight hyaluronan on the membrane potential is mediated by cellular factors such as surface receptors (CD44). To demonstrate the Donnan effect of high-molecular weight hyaluronan and to exclude any cellular interference possibly mediated by hyaluronan CD44 interaction, we performed an equilibrium dialysis of hyaluronan in Quantum medium. Hyaluronan causes an uneven distribution of counterions including H^+ in the different compartments that can easily be

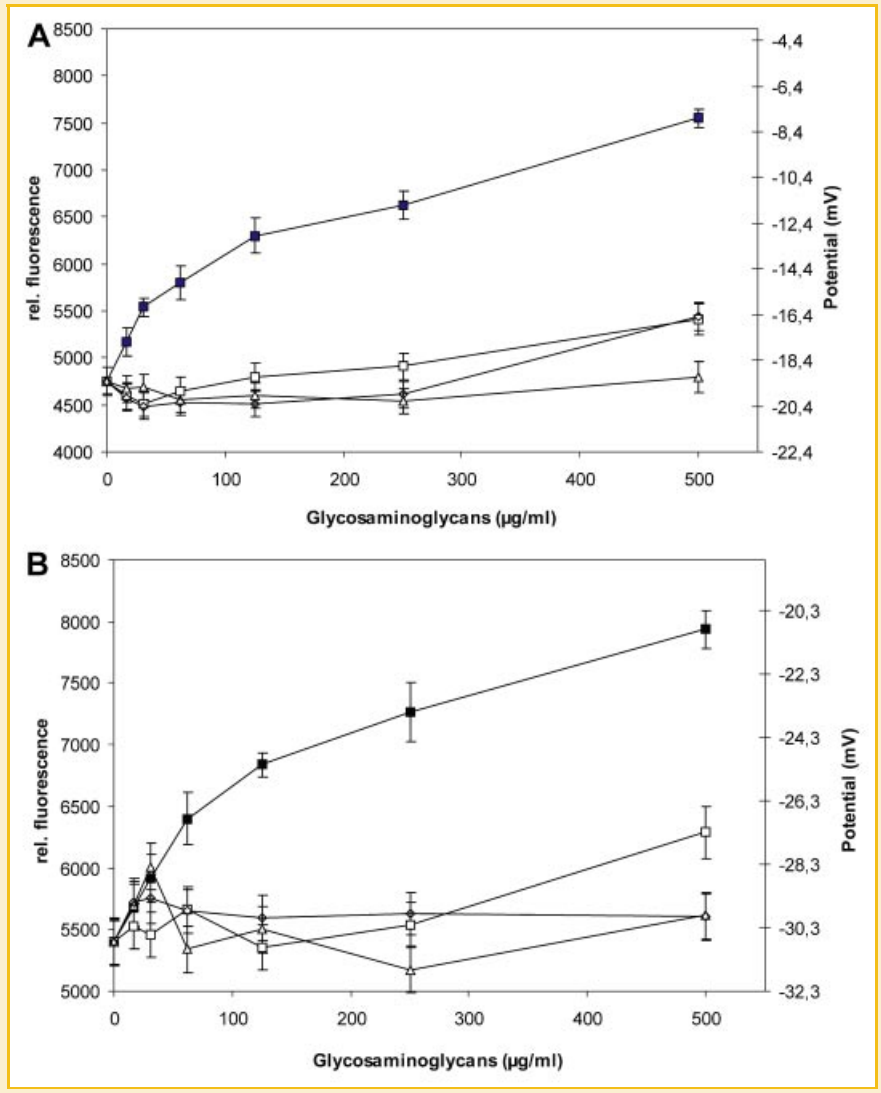

Fig. 2. Influence of exogenous glycosaminoglycans on the membrane potential. Human fibroblasts (A) or HEK cells (B) were incubated with increasing concentrations of hyaluronan (■), hyaluronan oligosaccharides (□), chondroitin sulfate (◇), or heparin (Δ) and the fluorescence was recorded after 2 h. The fluorescence values were converted into membrane potentials using the Nernst equation with the resting potential of −17 mV for fibroblasts and −30 mM for HEK cells, respectively, without any additive. The error bars indicate the SD of three determinations. ANOVA tests were performed for the concentrations above 100 µg/ml between hyaluronan and hyaluronan oligosaccharides and revealed $P < 0.0003$.

determined by pH-measurements. The different proton concentrations are related to the membrane potential by the following equation:

$$E = RT/F \times \ln H_a^+ / H_I^+$$

where R is the gas constant, T the absolute temperature, F the Faraday constant, H_a^+ the proton concentration of the hyaluronan-containing solution and H_I^+ the proton concentration of the hyaluronan-free solution. At 37°C the equation simplifies to $E = 61.5 \times \Delta pH$.

Figure 5 shows that hyaluronan indeed caused a pH difference in the two compartments in a concentration-dependent manner. The magnitude of the potential was higher than that observed for the potential alterations on living cells, because the ionic concentrations in the cytosol strongly influence the magnitude of the Donnan effect.

EFFECT OF HYALURONAN AND ALBUMIN MIXTURES ON THE MEMBRANE POTENTIAL

The effect of hyaluronan mixtures with bovine serum albumin (BSA) as a representative of an abundant physiological protein on the membrane potential of fibroblasts was analyzed as well. The hyaluronan and BSA solutions were mixed immediately before the experiment, and stable fluorescent recording was reached only after an incubation period of 20 h indicating that hyaluronan and BSA underwent a slow interaction before reaching an equilibrium. Figure 6 shows that both hyaluronan and BSA alone decreased the membrane potential in a concentration-dependent manner.

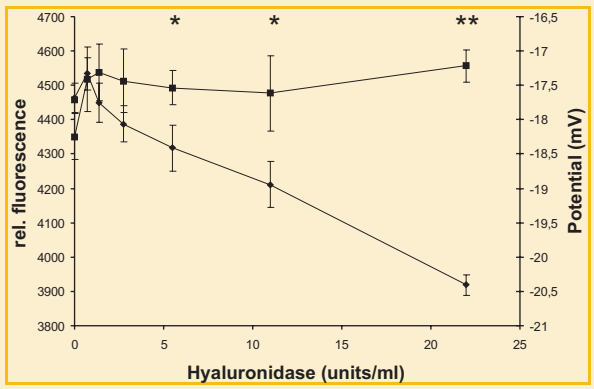

Fig. 3. Effect of hyaluronidase treatment on the membrane potential. Human fibroblasts were incubated in the absence (■) and presence (◆) of serial dilutions of hyaluronidase from *Streptomyces hyalurolyticus* for 20 min, and the membrane potential was measured in Quantum medium containing the fluorescent dye Di-BAC4(3) 30 min later. The error bars indicate the SD of four determinations. $^{*}P < 0.04$; $^{**}P < 0.0001$ (ANOVA test).

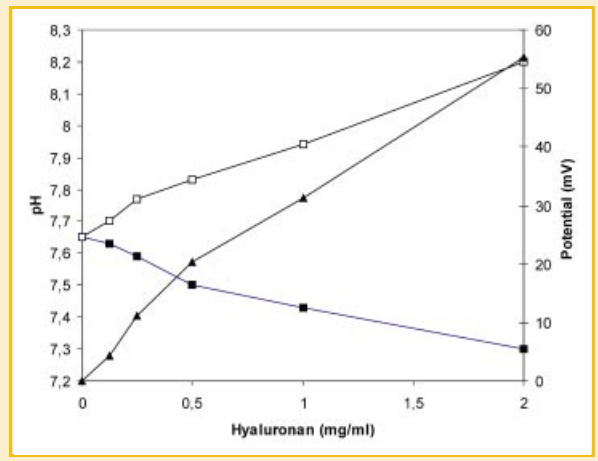

Fig. 5. Donnan effect on hyaluronan in equilibrium dialysis. Hyaluronan was dissolved in Quantum medium (■; 1 ml) at the concentrations indicated and subjected to equilibrium dialysis against hyaluronan-free Quantum medium (□). The pH in the two compartments was measured and converted to the respective membrane potential (▲). The SD of the measurements was <10%.

However, mixtures of hyaluronan and BSA decreased the potential more effectively than the sum of the individual components.

DISCUSSION

This is the first report to describe the effect of high-molecular weight hyaluronan on the membrane potential of eukaryotic cells. This effect was not cell type specific as it was observed in fibroblasts, epithelial cells and central neurons. The depolarization effect of the membrane potential was only related to the concentration of high-molecular weight hyaluronan, and not to the charge of glycosaminoglycans, as glycosaminoglycans with higher charge density and lower molecular weight such as chondroitin sulfate and heparin were inactive in depolarizing the membrane potential indicating that it is indeed a hyaluronan specific effect on the membrane potential. The intracellular fluorescence recordings increased almost linearly with the logarithm of extracellular hyaluronan concentrations. This observation is in agreement with the following theoretical consideration: first, the concentration change of K^+ caused by the Donnan effect is proportional to the hyaluronan concentration; second, fluorescence changes are directly proportional the K^+ difference quotient between the cytosol and the extracellular matrix; third, the membrane potential is proportional to the logarithm of intracellular K^+ concentration. Thus, our results indicated that high-molecular weight hyaluronan caused an influx of extracellular K^+. The classical model of the Donnan distribution of small permeable ions at cell membranes

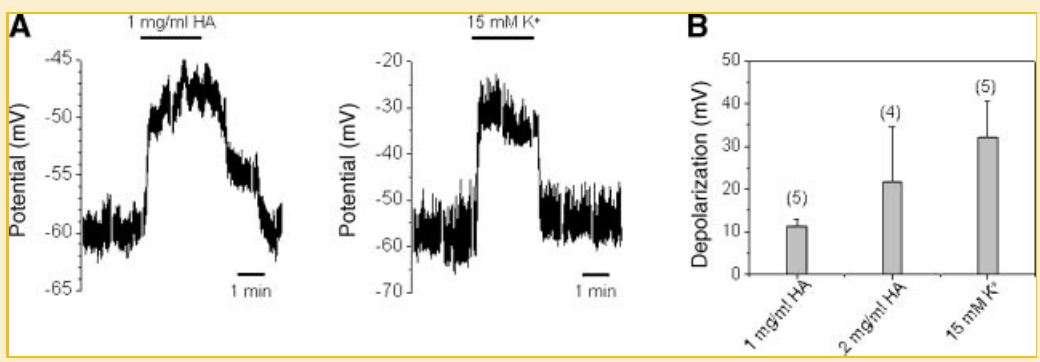

Fig. 4. Current clamp recordings in TC neurons. A: Visually identified TC neurons were maintained at zero current potential throughout the recording session in the presence of TTX. Continuous resting membrane potential recordings are shown for the application of 1 mg/ml hyaluronan (left panel) and an increase in the extracellular K^+ concentration to 15 mM (right panel). Horizontal bars indicate duration of substance application. Note that the onset of substance effects was very similar in both experimental conditions, thereby pointing to fast ion redistribution as the mechanistic basis. B: Mean bar graph representation of the depolarization induced by hyaluronan and K^+ as indicated. Results were presented as mean ± SD. The number determinations are shown in brackets.

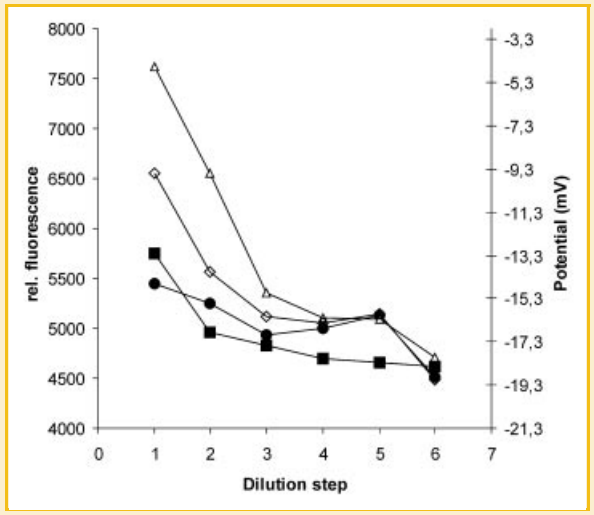

Fig. 6. Effect of hyaluronan and BSA mixtures on the membrane potential. Hyaluronan (500 μg/ml; ■), BSA (50 mg/ml; ●) or serial 1:1 dilutions mixtures of both (Δ) and were added to human fibroblasts in Quantum medium containing the fluorescent dye Di-BAC4(3). The fluorescence was recorded after 20 h. The fluorescence values were converted into membrane potentials using the Nernst equation with the resting potential of −17 mV for control cells without any additive (dilution step 6). The relative fluorescence of hyaluronan and BSA mixtures were larger the sum of the individual components (◇). ANOVA test first dilution between hyaluronan and hyaluronan-BSA mixture revealed $P < 0.001$.

included negatively charged impermeable intracellular proteins, low-Na^+ permeability, and high-K^+ and Cl^- permeability [Boyle and Conway, 1941]. The addition of impermeable anionic high-molecular hyaluronan in our experiments to the extracellular site induced a redistribution of Cl^- and K^+ to the intracellular compartment and thus a reduction in extracellular K^+ concentration. Inwardly rectifying K_{ir} channels significantly contribute to setting the resting membrane potential of many cell types including fibroblasts [Chilton et al., 2005], endothelial cells [Adams and Hill, 2004], and TC neurons [Meuth et al., 2006]. An intriguing feature of K_{ir} channels is that the outward current through these channels decreases with decreasing extracellular K^+ concentrations [Stanfield et al., 2002; Dhamoon and Jalife, 2005]. Therefore, the hyperpolarizing influence of the current through K_{ir} channels is reduced and membrane depolarization occurs when the extracellular K^+ concentration is lowered [ten Eick et al., 1992].

In addition to these physico-chemical effects, the depolarizing effect of hyaluronan could also be mediated by cell surface receptors such as CD44 linked to intracellular pathways upon hyaluronan binding. The demonstration of the Donnan effect by equilibrium dialysis showed that the Donnan effect of hyaluronan existed also in a cell free system. However, it is possible that CD44 potentiates the Donnan effect. Human fibroblasts produce and deposit their own hyaluronan, whereas HEK cells do not synthesize hyaluronan. However, both cell lines express the receptor CD44 that retains and concentrates hyaluronan on the cell surface. It is thus probable that the depolarizing effect of hyaluronan is potentiated by CD44 binding. Indeed, we observed that HEK cells have a higher membrane potential and were more responsive to lower exogenous hyaluronan concentrations than fibroblasts that are already covered with their endogenous hyaluronan suggesting that clustering of hyaluronan on the cell surface by receptors contribute to the depolarizing effect. In contrast, membrane hyperpolarization was observed, when the hyaluronan surface coat was digested with hyaluronidase.

High-molecular weight hyaluronan binds large amounts of water that is not available for the solvation of other macromolecules resulting in steric exclusion of these macromolecules from the hyaluronan territory. Therefore, the effective hyaluronan concentrations needed to induce membrane current changes exceed that concentration measured analytically [Ogston and Preston, 1966]. This concept has explained the anomalous increase of the osmotic pressure that exceeded the sum of the individual components [Laurent and Ogston, 1963]. The same principle should also apply for the Donnan effect of mixtures of hyaluronan with other macromolecules on the membrane potential. Thus, we propose that high molecular weight hyaluronan caused membrane depolarization due to steric exclusion of other macromolecules, such as serum proteins or growth factors.

The magnitude of the Donnan effect exerted by physiological hyaluronan concentrations as measured by the pH difference in equilibrium dialysis was considerable and reached 59 mV for 2 mg/ml. Such large effects cannot be expected for the Donnan effect on living cells, because the intracellular ionic concentrations also influence this equilibrium. The Donnan effect of extracellular hyaluronan will not only drive K^+ into the cells, but also other ions including Na^+, Ca^{2+}, and H^+ and effect their equilibrium potentials. In the absence of extracellular hyaluronan, the resting potential is mainly reached by a balance between K^+ export driven concentration-dependent diffusion and retention due to intracellular negative charges. In the presence of extracellular hyaluronan, the Donnan effect forces extracellular ions including K^+ into the cells, until a new equilibrium is reached.

Our observation of the effect of high-molecular weight hyaluronan on the membrane potential fits well with other observations in which a striking coincidence of hyaluronan overproduction and altered membrane potential exists such as hypertension [Martens and Gelband, 1998; Aytekin et al., 2008], hypertrophy [Takada et al., 1999; Deiters and Prehm, 2008], proliferation [Nilius and Wohlrab, 1992; Monz et al., 2008], ischemia [Jennische et al., 1982; Gerdin and Hallgren, 1997], and apoptosis of osteoarthritic cartilage [Zhou et al., 2008; Grishko et al., 2009]. Recently the reduction of nocireceptor nerve activity of osteoarthritic knee joints by high-molecular weight hyaluronan was described [Gomis et al., 2008]. It remains to be shown, whether this phenomenon is related to hyaluronan induced membrane depolarization.

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