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Hyaluronan activates calcium-dependent chloride currents in *Xenopus* oocytes

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Abstract Hyaluronan (5 mg/ml) activated an oscillatory inward current of up to 200 nA in oocytes held voltage-clamped at −60 mV. The reversal potential of the current was −25 ± 1 mV ($n = 5$), close to the Cl^- equilibrium potential for oocytes. The response was dose-dependent over the concentration range 0.2–10 mg/ml, independent of external Ca^{2+}, but abolished by injection of EGTA, a Ca^{2+} chelator. In addition, hyaluronan sensitivity in oocytes was seasonal with 91% of the responses occurring between June and December. Pre-heating hyaluronan to >50°C reduced the response; at 70–85°C the response was lost. Cooling the heated hyaluronan, however, restored the activity. Hyaluronan which had been previously digested with 10 mg/ml hyaluronidase for 1–7 h (at 37°C) activated smaller currents: for digestions >7 h the current-activating ability of hyaluronan was abolished. The results are discussed in relation to possible signal transduction pathways involving hyaluronan.

Key words: Hyaluronan; *Xenopus* oocytes; Two electrode voltage-clamp; Ca^{2+} mobilization; Ion channel activation; Reproduction

1. Introduction

Hyaluronan [D-glucuronic acid (β1→3) *N*-acetyl-D-glucosamine (β1→4)]$_n$ (HA) is an extremely ubiquitous member of the glycosaminoglycan extracellular matrix molecule family. Although many proteins have been shown to bind HA within the extracellular matrix (see [1]), cells also possess specific hyaluronan binding proteins (HABPs) which can act as structural components of the extracellular matrix and/or as cell surface receptors [1,2]. Indeed, the cell surface receptor protein, CD44 and receptors for hyaluronate-mediated motility (RHAMM) together account for a wide range of cellular actions of HA, including cell motility, lymphocyte homing, cell adhesion, and cell migration/cancer metastasis [1,3].

The membrane mechanisms by which HA elicits its wide range of functions are poorly understood. It has been suggested that HA binding to its receptor sites may lead to the activation of intracellular signalling pathways [4,5]. In fact, elevation of intracellular Ca^{2+} by HA binding to CD44 has been reported [4]. In addition, there is homology between the tail segment of the CD44 class of HA binding proteins and members of the G-protein family [6], and CD44 has both GTP-binding and GTPase activity in vitro, possibly indicating a role in G-protein coupled intracellular signalling [6].

Xenopus oocytes are large (>1 mm) single cells that have been used extensively as a model cellular system to probe intracellular signalling pathways. Both the oocyte membrane and the surrounding follicular cells possess a wide variety of endogenous signalling molecules including neurotransmitter/hormone receptors, ion channels, ion pumps and active second messenger pathways [7,8]. In the present study, *Xenopus* oocytes were used to investigate the possible intracellular signalling mechanisms associated with HA.

2. Materials and methods

Protocols for maintenance and dissection of *Xenopus* frogs and the preparation of oocytes have been described previously [7,9]. In summary, frogs were either bought from captively bred stock or imported directly from South Africa. Excised lobes of ovary were placed in modified Barth's medium with the composition (in mM): NaCl 88, KCl 1, $CaCl_2$ 0.41, $NaHCO_3$ 2.5, $MgSO_4$ 0.82, $Ca(NO_3)_2$ 0.33, Tris 7.5, pH 7.6. (Penicillin and streptomycin were added at 10 μg/ml each, and gentamycin sulphate was added at 0.1 mg/ml.) Oocytes at maturation stages V–VI were mechanically teased out of the lobes using Pasteur pipettes with tips of about 1.5 mm. Collagenase (Sigma, Type 2) at a concentration of 2 mg/ml in low-Ca^{2-} Ringer was applied to some oocytes for 1–2 h. Separated oocytes were washed thoroughly and stored in sterile, modified Barth's incubation medium. Both incubation medium and the Petri dish were changed daily in a flow cabinet.

For electrophysiological recording the oocyte was held in a small perfusion chamber (about 0.01 ml) and perfused with frog Ringer (at 4 ml/min). The excess solution was drawn off via a pipette attached to a vacuum pump. The recording system included a two-electrode voltage clamp set-up (Biologic CA-100 clamp amplifier; Biologic VF-180 micro-electrode amplifier; power supply PS-215). Voltage and current recordings were made on a chart recorder (W+W model 314). The intracellular micro-electrodes (GC100F-10, Clark Electromedical Instruments) were pulled on a vertical puller (Narishige) and had tip resistances of 1–3 MΩ when filled with 2.5 M KCl. All recordings were performed at room temperature (~20°C).

The frog Ringer solution contained (in mM): NaCl 115, KCl 2.5, $CaCl_2$ 1.8, HEPES 5, the pH was adjusted to 7.3 with 1 M NaOH. The nominally Ca^{2+}-free Ringer contained no added Ca^{2+}. Sodium hyaluronate (protein contamination ≤5%; Lowry), heparin sulphate and chondroitin sulphate (70% chondroitin sulphate A, 30% chondroitin sulphate C) were dissolved in frog Ringer by repeated sonication and vortexing and were applied by pipetting 100 μl of each solution into the perfusion chamber with the saline flow turned off. The pH of the glycosaminoglycan solutions was 7.3. Washing was achieved by restarting the perfusion. Unless otherwise stated drugs and chemicals were purchased from Sigma. Over the years of study, three different batches of sodium hyaluronate were purchased. Each displayed the ability to generate oscillatory currents in *Xenopus* oocytes. Data in the text have been presented as means ± standard errors. Numbers of measurements (n) are from different oocytes. Comparison of means was performed with Student's t-test.

3. Results

In oocytes held voltage-clamped at −60 mV, 5 mg/ml hyaluronan (HA) activated an oscillatory, inward membrane current of up to 200 nA (Fig. 1a). Under identical recording

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conditions, two other glycosaminoglycans, heparin sulphate and chondroitin sulphate (both at 10 mg/ml), produced small, predominantly smooth, inward membrane currents of 43 ± 16 nA and 13 ± 4 nA, in 66% ($n = 15$) and 33% (n=15) of oocytes tested, respectively (Fig. 1a). The responses to heparin sulphate and chondroitin sulphate could be detected in oocytes not responsive to HA, and vice versa, but their response waveforms have not been further characterized in the present study.

The current to 5 mg/ml HA displayed an average onset latency of 166 ± 34 s ($n = 44$), and was associated with a conductance increase. The response to HA showed a linear current-voltage relationship (over the −60 mV to +10 mV range), with a reversal potential of −25 ± 1 mV ($n = 5$; Fig. 1b). This value is close to the E_{Cl} of the oocyte and suggested involvement of the well known Ca^{2+}-dependent Cl^- current of oocytes [10]. In fact, pre-injection of 20 nl 50 mM EGTA into oocytes which had previously shown a large (> 100 nA) response to HA completely abolished the oscillatory current ($n = 6$; not shown), whilst application of 5 mg/ml HA, in low-Ca^{2+} Ringer, to an oocyte perfused in low-Ca^{2+} Ringer resulted in a current of the same size to that produced by

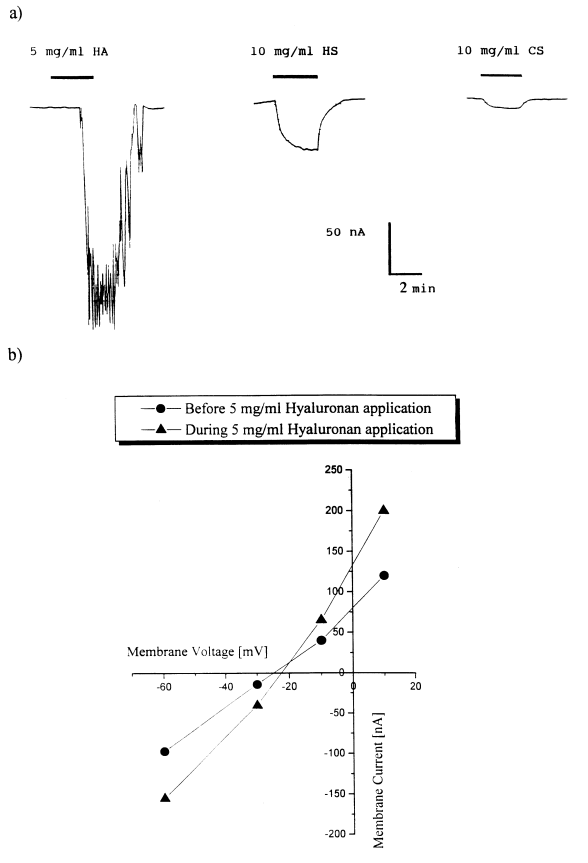

Fig. 1. Induction of membrane currents in *Xenopus* oocytes by application of hyaluronan (HA) and sulphated glycosaminoglycans. a: External application of 5 mg/ml HA to an oocyte results in an inward, oscillatory membrane current characterized by a slow onset. External application of 10 mg/ml heparin sulphate (HS) or 10 mg/ml chondroitin sulphate (CS) results in inward, smooth membrane currents. The horizontal bar denotes application of the drugs. The scale bar applies to all traces. b: Typical current-voltage (I-V) relationship for the oscillatory component of the current induced by 5 mg/ml HA. The data show that HA induced a conductance increase with a reversal potential of −20 mV.

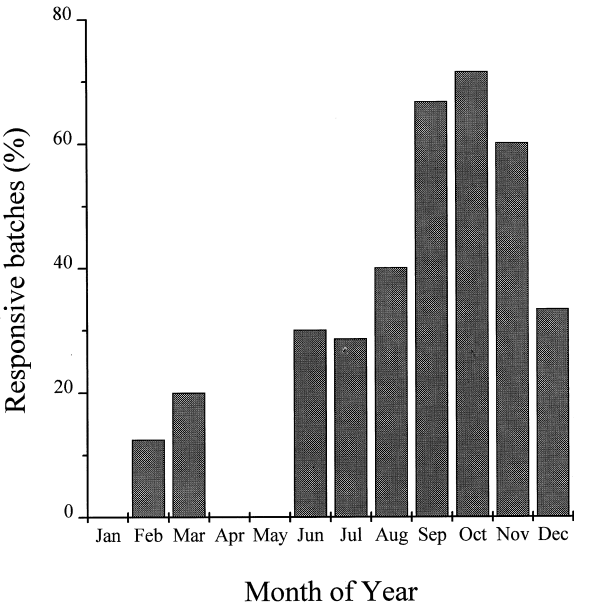

Fig. 2. The influence of season on the induction of membrane currents by hyaluronan (HA) in *Xenopus* oocytes. The histogram indicates the percentage of responsive batches per calendar month. Sixty-five different batches of oocytes were tested over a 3-year period with between three and eight batches being tested per month.

application of 5 mg/ml HA prepared in normal Ringer (67 ± 20 nA vs. 72 ± 19 nA, respectively; $n = 9$; $P = 0.69$).

Repeated application of 5 mg/ml HA to an oocyte showing an initial current larger than 40 nA reduced the observed response (by 54 ± 19% of the original response by the fourth application; $n = 4$). However, in oocytes which showed weak responses to 5 mg/ml HA (e.g. < 40 nA), a second application often did not elicit a further response ($n = 7$, not shown). In all cases, however, the response to HA disappeared within 3–4 days following isolation of the oocytes from the ovary. Importantly, oocytes which did not respond to HA demonstrated oscillatory, Ca^{2+}-dependent membrane currents to the muscarinic agonist carbachol ($n = 3$) or the G-protein activator AlF_4^- ($n = 6$; both not shown).

The response to HA was dose-dependent over the concentration range 0.2–10 mg/ml. For example, for oocytes held voltage-clamped at −60 mV, 2 mg/ml HA resulted in a current of 25.0 ± 9.6 nA, whilst for 10 mg/ml HA the current increased to 161.7 ± 14.2 nA ($n = 3$ for both; $P < 0.01$). Collagenase treatment, to remove the follicular cells surrounding the oocyte, did not affect the membrane currents to HA demonstrating that the response is endogenous to the oocyte itself ($n = 9$). In addition, Ringer solution with added 10 mg/ml sucrose (to exceed the osmotic change produced by 10 mg/ml HA) did not produce any membrane currents, indicating that the response to HA was not due to the change in osmolarity (not shown).

There was a clear batch variability in the production of membrane currents to HA. 34% of the 65 frogs tested over a 3-year period had oocytes which responded to HA. From these responsive batches, HA application produced membrane currents in 72 ± 5% of oocytes tested ($n = 79$) whilst 91% of the responsive batches fell between June and December (Fig. 2).

The response to HA was abolished by prior digestion of the HA with 10 mg/ml hyaluronidase (HAse) at 37°C for 7 h

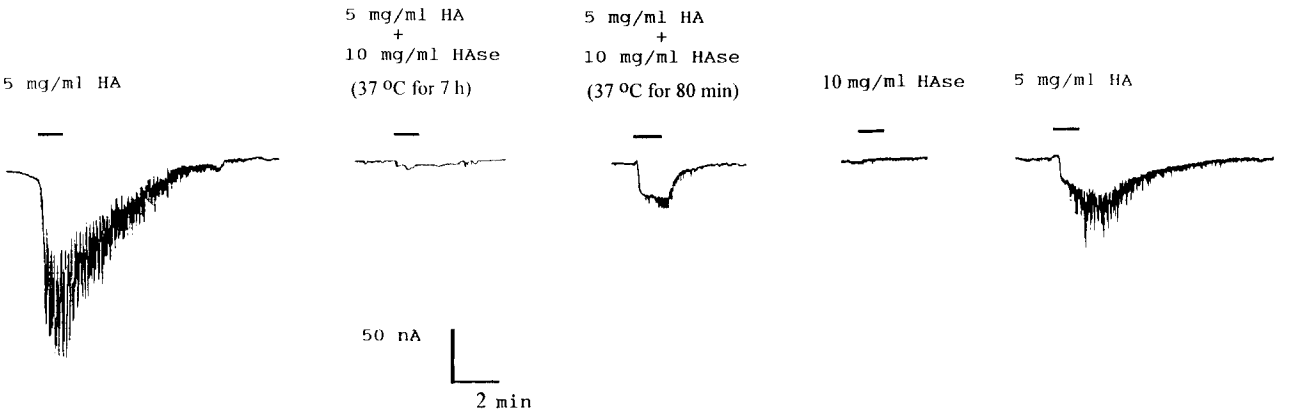

Fig. 3. The effect of hyaluronidase (HAse) on the membrane currents induced by hyaluronan (HA) in *Xenopus* oocytes. Application of 5 mg/ml HA results in a large inward oscillatory current which returns to baseline with washing. Application of 5 mg/ml HA (previously digested with 10 mg/ml HAse for 7 h at 37°C) results in little current activity. Application of 5 mg/ml HA (previously digested with 10 mg/ml HAse for 80 min at 37°C) results in a smaller inward oscillatory current which returns to the baseline with washing. Application of 10 mg/ml HAse does not result in a membrane current. Re-application of 5 mg/ml HA to the oocyte results in an inward oscillatory current which returns to baseline with washing. The traces represent sequential recordings from a single oocyte held voltage-clamped at −60 mV.

($n=7$; Fig. 3). HA which had been previously digested with 10 mg/ml HAse for shorter times (1–7 h) activated smaller currents with sizes which were proportional to the length of digestion. Importantly, 10 mg/ml HAse had little effect on the oocyte when applied by itself for short periods ($n=10$; Fig. 3) and prior heating of the HA to 37°C, by itself, did not affect the induction of membrane currents (not shown). However, pre-heating HA to increasing temperatures above 50°C for 5 min resulted in a gradual reduction of the induced membrane currents. When pre-heating reached 70–85°C the response to HA was lost ($n=6$; Fig. 4). However, cooling the heat-treated HA at 4°C for several days restored the responsiveness ($n=3$; Fig. 4).

4. Discussion

The results indicated clearly the ability of HA to generate membrane currents in *Xenopus* oocytes through activation of the endogenous Ca^{2+}-dependent Cl^- channels. This response to HA was not dependent on extracellular Ca^{2+} and was abolished by EGTA injection, suggesting that the Ca^{2+} involved was released mainly from internal stores. Intracellular Ca^{2+} mobilization has been reported previously for the binding of HA to the CD44 receptor in T lymphoma cells, although the mechanism of action was not understood [4]. In oocytes, the mechanism of HA action is also unclear but it would appear that the response is a transient effect due to the binding (and/or unbinding) of the HA to a possible cell surface binding site/receptor on the oocyte. The surrounding follicular cells were clearly not involved, since collagenase treatment did not affect the response.

Application of HA which had been digested previously with 10 mg/ml HAse for 1–7 h at 37°C activated smaller currents, the sizes being proportional to the length of digestion: for pre-digestions longer than 7 h the ability of HA to activate currents was abolished. Similarly, heating HA above 50°C resulted in the graded reduction of the response. Conformational changes are known to be induced in HA by a variety of factors, including pH, Ca^{2+}, concentration and temperature [11–14]. Indeed, temperatures above 50°C induce a conformational change in HA but the original state can be achieved by cooling to 4°C [14]. The above experiments indicate that increasing temperature may indeed cause a reversible conformational change in HA which reduces membrane binding site recognition. Such conformational specificity has been noted for the binding of HA to CD44, where some cell lines bound immobilized, but not soluble HA [15].

The results indicating sensitivity of the HA response to both HAse digestion and temperature, coupled with the findings that responses to HA could be generated in oocytes insensitive to either 10 mg/ml CS or HS, would indicate that the response was specific to HA and not caused by contamination from either sulphated glycosaminoglycans or metal ions.

At present, the mechanism by which HA activates the membrane currents in oocytes is unclear. However, the response to HA is similar to those induced in *Xenopus* oocytes by AlF_4^- and the endogenous muscarinic receptor, both of which are known to activate G-proteins, lead to the formation of IP_3 and intracellular Ca^{2+} release [8,16,17]. There is evidence for homology between the tail segment of the CD44 class of HA binding proteins and members of the G-protein family and CD44 has both GTP-binding and GTPase activity in vitro [6]. The activation of an intracellular signalling pathway by HA in oocytes alludes to the importance of signal transduction mechanisms in other areas of HA functioning, including lymphocyte homing, cell adhesion, and cell migration/cancer metastasis [1,3] and also to the possible signalling capabilities of other extracellular matrix molecules.

In oocyte batches where there was no response to HA application it is not known whether the functional binding sites were present, but inactive, or absent. Such oocytes still responded to the muscarinic agonist carbachol and the G-protein activator AlF_4^- indicating the presence of functional Ca^{2+} stores and the IP_3 second messenger pathway. In addition, since the response to HA disappeared after several days (but the responses to carbachol and AlF_4^- remained), the HA binding site(s) must be either downregulated or, in some other way, lose the ability to respond to HA. In comparison, the binding of HA to membrane site(s) in mammalian cells has also been shown to be quite complex. For example, CD44 receptors are known to be present on cells that do not bind HA [18,19]. This lack of binding was not due to either the

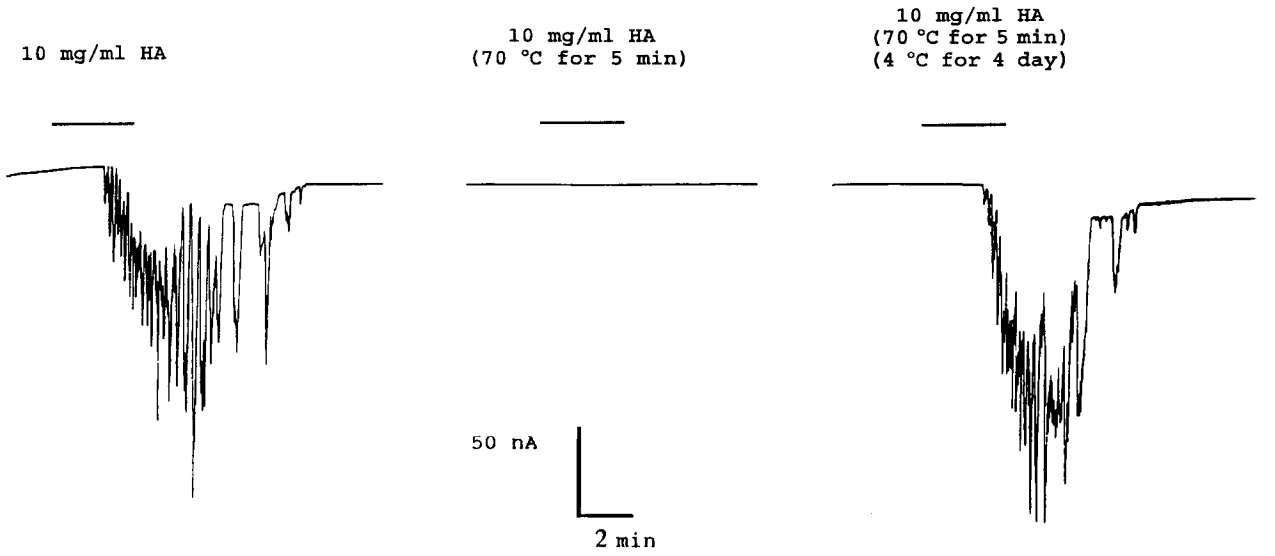

Fig. 4. Temperature dependence of the hyaluronan (HA)-induced membrane currents. Application of 10 mg/ml HA results in an inward oscillatory current which returns to baseline with washing. A subsequent application of 10 mg/ml HA (preheated to 70°C for 5 min) does not induce a membrane current. Application of 10 mg/ml HA (preheated to 70°C for 5 min and cooled to 4°C for 4 days) results in an inward oscillatory current which returns to baseline with washing. The oocyte was held voltage-clamped at −60 mV.

density of HA receptors or the masking of binding sites by previously bound HA [19,20]. HA binding, however, was induced by a variety of factors including phorbol esters or a monoclonal antibody to CD44 [15,18,20].

It is interesting to note that the response to HA was seasonal with the largest responses being recorded between June and December. These dates coincide with the breeding season (July to December) of *Xenopus* frogs in their native south Africa and for captively bred frogs (June) [21], and indicate a possible role for HA in *Xenopus* reproduction/development. In mammalian oocytes, cumulus cells are known to release HA [22]. In addition, sperm are thought to have a protein with HAse activity in their membrane [23] and can release soluble HAse from the acrosome in order to break through the oocyte membrane and fertilize the oocyte [24,25]. If a similar mechanism occurs in amphibians, HAse release and the subsequent activation of the HA response by fragmented HA may contribute to the rise in intracellular Ca^{2+} known to accompany fertilization [26–28]. Such Ca^{2+} release is known to result from a rise in IP_3 levels, possibly via a pertussis toxin-insensitive G-protein pathway [29]. The fertilization events in *Xenopus* oocytes can be mimicked by the expression of G-protein coupled neurotransmitter receptors [30] which show very similar membrane characteristics to those elicited by HA in oocytes.

In conclusion, the present results demonstrate the activation of an intracellular signalling pathway in *Xenopus* oocytes by HA resulting in a rise in intracellular Ca^{2+} and activation of a Ca^{2+}-dependent Cl^- current. Further work needs to be performed to elucidate the exact nature of the binding site(s) on the *Xenopus* oocyte, the mechanisms by which HA induces its response and the physiological importance of possible intracellular signalling by HA and other extracellular matrix molecules in both oocytes and other cell types.

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