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Electrophysiological Demonstration of Voltage-Activated H^+ Channels in Bovine Articular Chondrocytes

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Key Words

Cartilage• Patch-clamp • Whole-cell • pH • Proton channels

Abstract

Matrix synthesis by articular chondrocytes is sensitive to changes in intracellular pH (pH_i), so characterising the membrane transport pathways that determine pH_i is important for understanding how chondrocytes regulate the turnover of cartilage matrix. In the present study, the whole-cell patch-clamp technique has been employed to demonstrate the operation of voltage-activated H^+ channels (VAHC) in bovine articular chondrocytes. Using solutions designed to minimise the contribution of ions other than H^+, the application of step voltage-protocols elicited whole-cell currents. These currents were slow activating, observed only in the outward direction, dependent on both extracellular pH (pH_o) and pH_i, and inhibited by Zn^{2+}. The reversal potential values, measured by tail current analysis, over a range of different pH_o and pH_i values, were in good agreement with predicted values for membrane channels having a high selectivity for protons. The results presented here are consistent with the operation of VAHC in articular chondrocytes.

Introduction

Chondrocytes control matrix turnover through the synthesis of matrix macromolecules and of the enzymes (proteinases) which degrade them. There is a fine balance between the production of new elements and the breakdown of existing ones, which is essential to maintain the properties of the cartilage matrix and joint integrity. Identifying how this balance is regulated is central to understanding the processes underlying degenerative cartilage pathologies such as osteoarthritis.

Many studies have shown that changes to the intracellular composition of chondrocytes result in changes in matrix turnover so it is highly likely that the membrane transport proteins which regulate chondrocyte ionic composition will play an important role in maintaining matrix integrity [1]. It is therefore clearly important to under-

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stand the factors that modulate the activity of these transporters, and hence determine the ionic composition of chondrocytes. One variable that has received considerable attention in this context is intracellular pH (pH_i), since H^+ is a powerful regulator of matrix synthesis [2].

Described for the first time in snail neurons [3], voltage-activated H^+ channels (VAHC) have since been characterised in a variety of mammalian cell types [4]. Their role in pH homeostasis has been studied extensively [5-11] and, although their single-channel conductance is very small (in the region of 10 to 100 fS [12] when measured at a low pH_i), they can still provide a rapid route by which to raise pH_i. Little is known about the molecular identity of VAHC, although two recent reports have identified proteins that mediate voltage-gated H^+ conductances [13, 14].

The channels are activated principally by depolarization and are extremely selective for protons [6]. VAHC gating is regulated by both the extracellular pH (pH_o) and by pH_i, and one of the consequences of this regulation is that channel openings occur only when the electrochemical gradient is outward, which results in outward directed currents [10, 15, 16]. Both the gating and conductance of VAHC is very sensitive to temperature, which often leads to slow current activation times (in the order of seconds) when channel activity is measured at room temperature [8]. Furthermore, VAHC are inhibited by Zn^{2+} and other divalent cations [4].

Fluorescence recordings of pH_i in chondrocytes indicate that hypo-osmotic shock induces an alkalinisation of these cells. The osmotic shock activates mechanosensitive channels that cause membrane depolarisation, in turn promoting H^+ efflux through a pathway with properties consistent with those of VAHC [17]. The aim of the present study was to assess, using the whole-cell patch-clamp technique, whether VAHC operate in bovine articular chondrocytes.

Materials and Methods

Isolation of chondrocytes

Cells were isolated from bovine articular cartilage, as described previously [18]. Briefly, cells were isolated by type I collagenase digestion (2000 U/ml in Dulbecco's modified Eagle's medium (DMEM), supplemented with 2 mM glutamine, 0.1 mM penicillin and 0.1 mM streptomycin, for 18 h at 37 ºC) from the metacarphophalangeal joint of 18-36 month-old cattle obtained at abattoir slaughter. After filtration, the isolation solution was centrifuged and the cells were resuspended in fresh DMEM.

Electrophysiological recording

Aliquots of cells were resuspended in experimental medium in a recording chamber attached to an inverted microscope (Nikon, Tokyo, Japan) and standard whole-cell tight-seal voltage-clamp protocols were used to record membrane currents [19]. All experiments were performed at room temperature (20-22 °C). Patch pipettes (Clark PG150T glass (Harvard Apparatus, Kent, UK)) were pulled and polished on a DMZ-Universal Puller (Zeitz-Instrumente, Augsburg, Germany) to resistances of 4-7 MΩ. After seal formation (5-15 GΩ), brief, strong suction was applied to the pipette interior to rupture the membrane patch and attain the whole-cell configuration.

Bath and pipette solutions contained 80 mM tetramethylammonium methanesulphonate (impermeant ions), 2 mM $CaCl_2$, 1 mM $MgCl_2$, 10 mM glucose and 100 mM buffer. The buffers employed were 2-morpholinoethanesulphonic acid (MES; pH 6.0), piperazine-1,4-bis(2-ethanesulphonic acid (PIPES; pH 7.0) and 4-(2-hydroxyethyl)piperazine-1-ethanesulphonic acid (HEPES; pH 7.4 and 7.8). The pH was adjusted using either methanesulphonic acid or tetramethylammonium hydroxide.

Whole-cell currents (digitised at 10 kHz and filtered at 5 kHz with a 4-pole Bessel filter) were recorded using an Axopatch 200B amplifier and a CV203BU headstage, with voltage-commands generated and currents analysed using the pCLAMP software suite (Version 9, Axon Instruments Inc., USA). Cell capacitance (between 16 and 20 pF) was recorded before each experiment using the Membrane Test routine in pCLAMP.

Statistics

Results are presented as mean ± standard error of the mean (SEM), where *n* is the number of cells tested. Each experiment was repeated using cells isolated from at least three different animals. Differences between sample means were tested using Student's unpaired *t*-test and a probability level of less than 0.05 was considered significant.

Results

Activation of outward whole-cell currents in bovine articular chondrocytes

Using bath and pipette solutions designed to minimise the contribution of ions other than protons (see Methods) and applying 5 s test pulses in the range -100 to +100 mV, from a holding potential of -40 mV, Figures 1A, 1B and 1C show a family of whole-cell current recordings from a single articular chondrocyte, obtained at a pH_i of 6.0 and at a pH_o of 7.8, 7.0 and 6.0, respectively (similar currents were also evident at more physiological pH values (*i.e.* pH_o / pH_i = 7.4 / 7.1), data not shown). In each case, only slowly activating, outward currents were observed. In addition, the magnitude of the current recordings decreased as the pH_o was reduced from pH 7.8 to 7.0 to

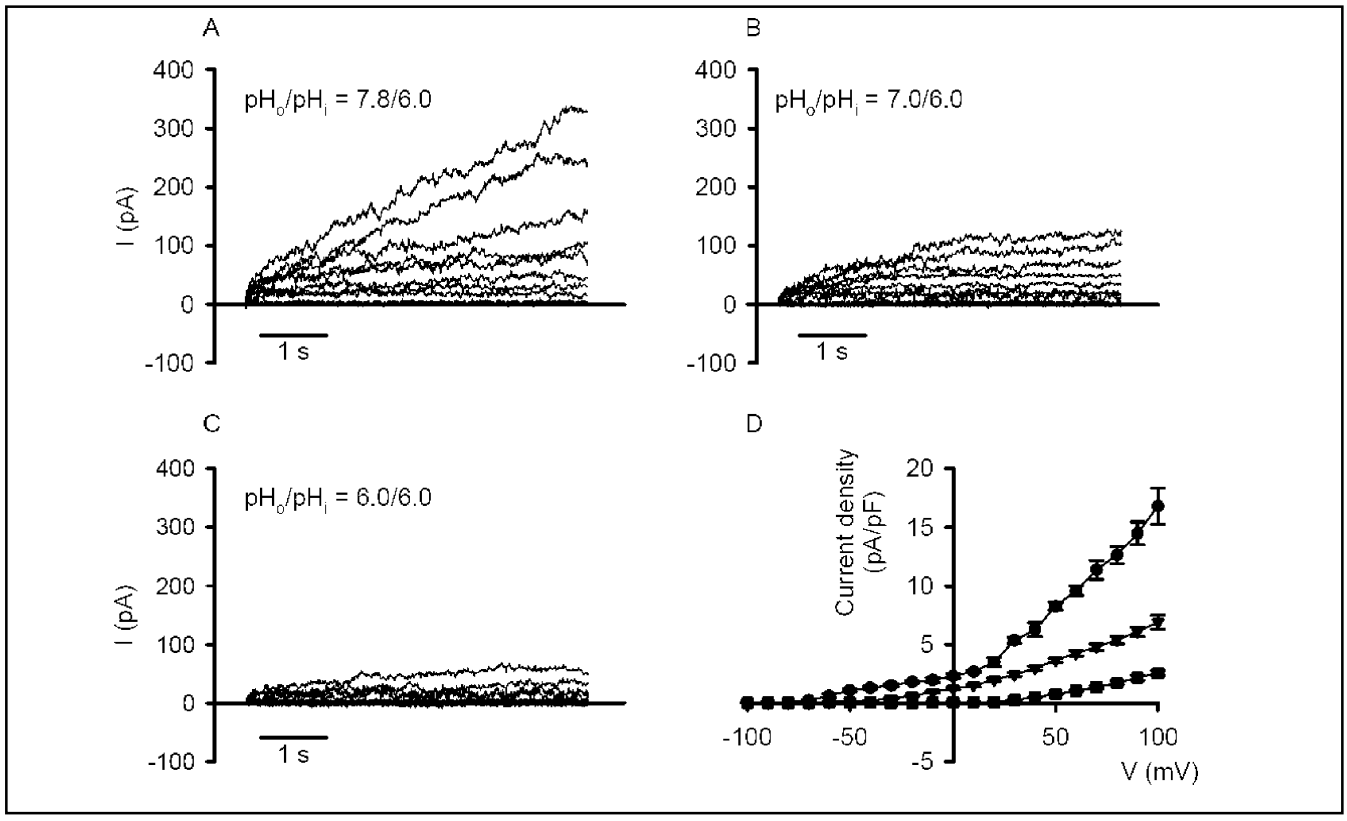

Fig. 1. The effect of altering pH_o on whole-cell currents in bovine articular chondrocytes. In each experiment, a step protocol was used in which a series of 5 s test potentials, at 20 s intervals, were applied to a cell increasing in 10 mV increments from -100 to +100 mV from a holding potential of -40 mV. A, B and C: Representative family of whole-cell currents measured at the pH_o/pH_i values indicated on each panel. D: *I-V* curves derived from the data in panels A (closed circles), B (closed inverted triangles) and C (closed squares), averaged with two additional experiments. To allow for variations in cell size, the currents were normalised per unit of membrane capacitance and are presented as current density. The data in panel D are shown as the mean ± SEM (n = 3) and, where not shown, error bars lie within the symbols.

6.0, with membrane slope conductances (corrected for capacitance) of 181 ± 30, 70 ± 10 and 40 ± 4 pS/pF (n = 3), respectively (measured between +70 and +100 mV).

The averaged current/voltage (*I-V*) curves from 4 separate experiments are shown in Figure 1D. These data show that decreasing the pH_o from 7.8 to 7.0 and then to 6.0 shifted the threshold voltage for current activation (defined as the most negative membrane potential at which currents are activated) from -70 to -30 to +30 mV, respectively. Decreasing the pH_o from 7.8 to 7.0 and then to 6.0 had no significant effect ($P > 0.14$) on the initial conductances, which were 31 ± 2, 26 ± 2 and 29 ± 3 pS/pF (n = 3), respectively (measured over a region 40 mV positive to the activation threshold).

The E_r of outward whole-cell currents in bovine articular chondrocytes

Given that the observed currents described above were always in the outward direction, further experiments were performed to determine their reversal potential (E_r), using tail current analysis. In each experiment, a step protocol was used in which a series of 5 s test potentials, at 20 s intervals, increasing in 20 mV increments from below to above the holding potential (set at the theoretical H^+ E_r, predicted by the Nernst equation for the particular conditions of the experiment in question) were applied to a cell after a 5 s +80 mV depolarising pulse (to activate outward currents). The maximal amplitude of the tail current transient at each potential was determined by

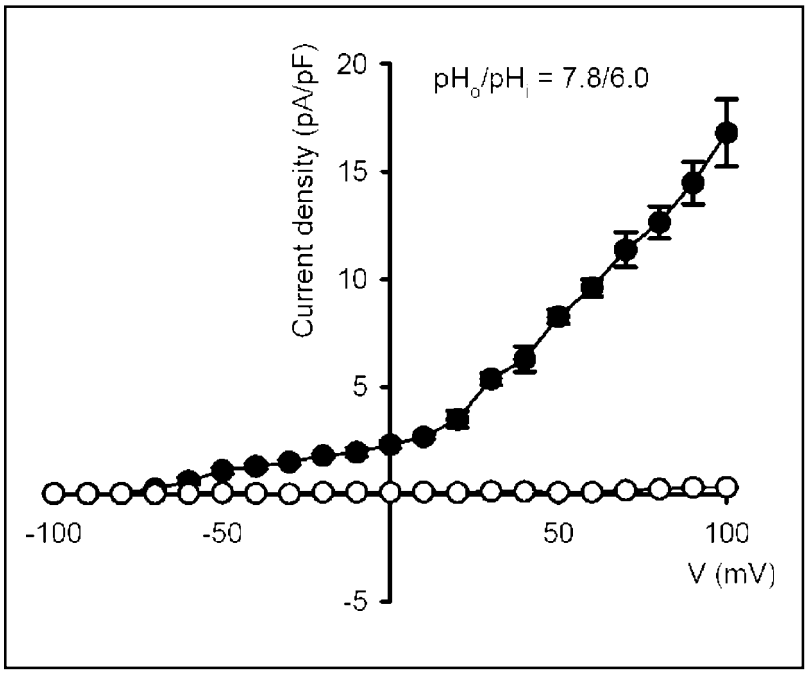

Fig. 2. The effect of Zn^{2+} on whole-cell currents in bovine articular chondrocytes. *I-V* curves were derived from whole-cell experiments in the absence (closed circles) and presence (open circles) of 0.1 mM Zn^{2+}, using the step protocol defined in the legend of Fig. 1, at the pH_o/pH_i values indicated on the panel. To allow for variations in cell size, the current was normalised per unit of membrane capacitance and is presented as current density. The data are shown as the mean ± SEM (n = 3) and, where not shown, error bars lie within the symbols. Note that the *I-V* curve derived from experiments in the absence of Zn^{2+} is also presented in Figure 1D.

fitting the decay with a single exponential. Using these data, *I-V* curves were constructed, from which the E_r was derived by extrapolation. Table 1 shows both the predicted and measured E_r values, which are in good agreement with each other, for a range of different pH_o and pH_i values.

Inhibition of outward whole-cell currents in bovine articular chondrocytes

Figure 2 shows the effect of Zn^{2+} on the outward currents observed in articular chondrocytes at a pH_o and a pH_i of 7.8 and 6.0, respectively. Outward currents were virtually abolished by the addition of 0.1 mM Zn^{2+} to the bath, with a current inhibition of > 95% at membrane potentials > 0 mV.

Discussion

Are the data consistent with the existence of H^+ currents in bovine articular chondrocytes?

Two lines of evidence support the proposal that the whole-cell currents described in the present study represent the movement of H^+ ions. First, as in previous reports characterising H^+ currents (e.g. [8]), the pipette and bath solutions were designed to minimise the contribution of other ions, although this alone does not rule out the possibility that the recorded currents are contaminated by either the major ions (tetramethylammonium and methanesulphonate, which are normally cell impermeant) or the minor ions (e.g. Cl^- or Ca^{2+}). Second, the E_r values derived for these currents - measured by tail current analysis - were in good accord with values predicted from the Nernst equation for the H^+ ion distributions employed (see Table 1). Taken together, these data are consistent with the notion that the membrane currents recorded here are H^+ currents.

Are the data consistent with the operation of VAHC in bovine articular chondrocytes?

The whole-cell currents presented here were activated by depolarization, were slow to develop, were in an outward direction only, were dependent on the pH gradient between the intracellular and extracellular solutions (with the threshold of current activation shifting to more positive potentials as pH_o was reduced) and were inhibited by Zn^{2+}. All these attributes are characteristic features of H^+ currents generated by VAHC [4, 6, 10, 15, 20]. However, the current traces shown here were much noisier than those reported in the majority of VAHC studies using other cell types (e.g. [20]), although similar H^+ current traces have been observed in HEK-293 cells after expression of NOX5 [21]. These H^+ currents were only activated in the presence of high internal Ca^{2+} levels, conditions which are similar to those used in this study (the pipette Ca^{2+} concentration used during experiments was 2 mM). It is also worth noting that the outwardly rectifying currents shown here are a consequence of the

Table 1. The effect of altering pH_o and pH_i on the E_r values of whole-cell currents in bovine articular chondrocytes (data are shown as the mean ± SEM).

pH_o/pH_i	Predicted E_r (mV)	Measured E_r (mV)	*n*
7.8/6.0	- 105	-97 ± 3	5
7.8/7.0	- 47	-39 ± 5	3
7.8/7.8	0	3 ± 2	3
6.0/6.0	0	6 ± 4	3
7.0/6.0	-58	-53 ± 2	3
7.4/7.1	-18	-15 ± 3	4

activation process rather than the behaviour of open channels, as the *I-V* curves derived from the tale current analysis were almost linear (data not shown), which, again, is a feature of H^+ currents generated by VAHC [20]. It is unlikely that H^+ efflux through VAHC plays a role in the regulation of steady-state pH_i in chondrocytes [22]. Although the currents we measure here are small, we have previously shown that pH_i alkalinises by 0.1 units or more in response to hypotonic shocks, and that this effect represents activation of VAHC [17], indicating that under certain circumstances these channels can have a major influence on chondrocyte pH.

Conclusions

The data presented here are the first electrophysiological demonstration of VAHC in bovine articular chondrocytes, albeit in experiments performed at room temperature. These channels constitute an additional pathway for H^+ efflux in articular chondrocytes, and may well play a role in pH_i modulation in response to osmotic challenges, as has been found in to occur in microglia [11]. Fluorescence experiments suggest that VAHC in articular chondrocytes exhibit an osmotic sensitivity [17]; electrophysiological characterisation of the osmotic sensitivity of these channels is now required.

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