Quantitative analysis of voltage-gated potassium currents from primary equine (Equus caballus) and elephant (Loxodonta africana) articular chondrocytes

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Mobasher, A., T. C. Gent, M. D. Womack, S. D. Carter, P. D. Clegg, and R. Barrett-Jolley. Quantitative analysis of voltage-gated potassium currents from primary equine (Equus caballus) and elephant (Loxodonta africana) articular chondrocytes. Am J Physiol Regul Integr Comp Physiol 289: R172–R180, 2005.—In this comparative study, we have established in vitro models of equine and elephant articular chondrocytes, examined their basic morphology, and characterized the biophysical properties of their primary voltage-gated potassium channel (Kv) currents. Using whole cell patch-clamp electrophysiological recording from first-expansion and first-passage cells, we measured a maximum Kv conductance of 0.15 ± 0.04 pS/pF (n = 10) in equine chondrocytes, whereas in elephant chondrocytes, this was significantly larger (0.8 ± 0.4 pS/pF, n = 4, P ≤ 0.05). Steady-state activation parameters of elephant chondrocytes (V1/2 = −22 ± 6 mV, k = 11.8 ± 3 mV, n = 4) were not significantly different from those of horse chondrocytes (V1/2 = −12.5 ± 4.3 mV, k = 12 ± 2, n = 10). This suggests that there would be slightly more resting Kv activation in elephant chondrocytes than in their equine counterparts. Kinetic analysis revealed that both horse and elephant chondrocyte Kv currents had similar activation and inactivation parameters. Pharmacological investigation of equine chondrocyte Kv currents showed them to be powerfully inhibited by the potassium channel blockers tetraethylammonium and 4-aminopyridine but not by dendrotoxin-I. Immunohistochemical studies using polyclonal antibodies to Kv1.1–Kv1.5 provided evidence for expression of Kv1.4 in equine chondrocytes. This is the first electrophysiological study of equine or elephant chondrocytes. The data support the notion that voltage-gated potassium channels play an important role in regulating the membrane potential of articular chondrocytes and will prove useful in future modeling of electromechanotransduction of fully differentiated articular chondrocytes in these and other species.

cartilage; dendrotoxin; potassium channel; membrane potential; immunohistochemistry

ARTICULAR CHONDROCYTES are specialized mesenchyme-derived cells that play a key role in the synthesis, maintenance, and degradation of the extracellular matrix of cartilage in load-bearing synovial joints (1, 3). The bioenergetic and metabolic state of chondrocytes is robustly influenced by mechanical loading (11) and the extracellular ionic and osmotic environment of the cell (22). The mechanotransduction and subcellular signaling pathways that link mechanical signals to cellular responses of chondrocytes are poorly understood. However, a number of biophysical factors have been implicated in the mechanotransduction process. A nonexhaustive list includes J deformation, strain, and physical distortion of the plasma and nuclear membranes (11, 12, 36); 2 changes in fluid volumes and composition (22, 36); 3 osmotic pressures (36); and finally, 4 changes in membrane potential (36, 40). Indeed, chondrocytes express a variety of ion channels, ion pumps, and transporters (19, 22, 23), and the physiological importance of chondrocyte ion channels is becoming increasingly apparent. Furthermore, ion channel blockers have been shown to influence chondrocyte proliferation (38). Therefore, studying ion channel expression and regulation in chondrocytes will lead to an enhanced understanding of the biophysical and mechanical properties of these cells. This may reveal new therapeutic targets for modulating the behavior and metabolism of chondrocytes; such knowledge may ultimately be valuable for the treatment of osteoarthritis and related osteoarticular disorders in humans and companion animals (19, 39).

Current information on ion channels in chondrocytes is limited compared with other well-studied cell types (22). Interestingly, for these nonexcitable cells, the list of ion channels so far identified includes voltage-gated Na+ and voltage-gated K+ channels (16, 29, 35), stretch-activated K+ channels (17, 33, 34), and voltage-gated Cl− channels (29, 33). Pharmacological and molecular evidence has also been obtained for the presence of N- and L-type voltage-gated Ca2+ channels (17, 18) and stretch-activated nonglycoprotein cation channels (13, 21, 32, 40, 41). In articular and growth plate chondrocytes. Despite this information, many of these recent studies have not been carried out with authentic, “phenotypically stable” chondrocytes (i.e., primary chondrocytes that have maintained their differentiated phenotype). For example, many of the recent electrophysiological studies of these proteins have been conducted on secondary and tertiary cultures of avian (16, 35) or mammalian (24, 25, 29) chondrocytes. Although the information provided from work with chondrocyte cell lines and chondrocyte-like cells is invaluable, it is important that it be followed up by studies of fully differentiated, phenotypically stable chondrocytes in a range of mammals. A particularly interesting recent paper that examined the physiologies of delayed-rectifier K+ channels in freshly dissociated canine chondrocytes (37) provided data to strongly support the notion that canine chondrocyte voltage-gated K+ channel (Kv) current activity is important to chondrocyte function.

Accordingly, the aim of the present study was to exploit a unique opportunity to characterize Kv currents in articular chondrocytes of fully developed horses (Equus caballus) and the world’s largest land-dwelling mammal, the African elephant (Loxodonta africana). To our knowledge, this represents the first ever patch-clamp analysis of equine chondrocytes and elephant cells of any kind. We sought to measure the sensitiv-

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ity of horse chondrocyte Kv currents to the commonly used K+ channel blockers tetraethylammonium (TEA), 4-aminopyridine (4-AP), and dendrotoxin-I. We also used polyclonal antibodies to five members of the Kv1.x family of K+ channels (Kv1.1, Kv1.2, Kv1.3, Kv1.4, and Kv1.5) in immunohisto-chemical experiments to identify the Kv channel subtype expressed in equine articular chondrocytes. Knowledge of the properties and pharmacological profile of these authentic chondrocyte Kv currents will prove useful in future modeling and analysis of electromechanical coupling.

EXPERIMENTAL PROCEDURES

Cartilage source. Normal equine articular cartilage was obtained from the femoropatellar, carpal, and metacarpophalangeal joints of skeletally mature horses (E. caballus) euthanized for welfare and unrelated clinical reasons at the Philip Leverhulme Large Animal Hospital, University of Liverpool. The study was conducted with local ethical approval in strict accordance with national guidelines (no animals were euthanized for the purpose of this study). Equine cartilage from the same joints was also obtained from a local abattoir (Nantwich, UK). Elephant cartilage was obtained from hip joints of two skeletally mature female African elephants (L. africana) euthanized for unrelated clinical reasons (Knowsley Safari Park, Merseyside, UK).

Preparation of dissociated cells. Equine and elephant cartilage shavings (not full depth; see Fig. 1, E and F) were rinsed with phosphate-buffered saline (PBS), cut into small slices, and incubated overnight with type I collagenase (EC 3.4.24.3, from Clostridium histolyticum; 100 collagen digestion units/ml) in serum-free Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 1,000 mg/l glucose and 1% penicillin/streptomycin solution. The filtered cell suspension was washed three times in fresh DMEM, and the cells were grown in monolayer culture with 4% fetal calf serum for no more than two passages. Recent molecular studies on tyrosine-phosphorylated intracellular signaling and cytoskeletal proteins that are known to be involved in the maintenance of chondrogenic potential have demonstrated that acutely isolated chondrocytes will lose their differentiated functions if cultured in two-dimensional monolayer culture beyond the fourth passage (27). For this reason, electrophysiological studies were carried out using freshly isolated chondrocytes, first-expansion and first-passage cells grown in monolayer culture with 4% fetal calf serum for no more than two passages. Recent molecular studies on tyrosine-phosphorylated intracellular signaling and cytoskeletal proteins that are known to be involved in the maintenance of chondrogenic potential have demonstrated that acutely isolated chondrocytes will lose their differentiated functions if cultured in two-dimensional monolayer culture beyond the fourth passage (27). For this reason, electrophysiological studies were carried out using freshly isolated chondrocytes, first-expansion and first-passage cells grown in low serum (4%) growth medium.

Histology. Full-depth equine and elephant cartilage samples from each batch of fresh tissue were fixed for 24 h in 10% neutral buffered formalin, decalcified in EDTA for a further 72 h, embedded in paraffin wax, and processed for routine histological staining (alcian blue, hematoxylin and eosin). Stained slide preparations were examined with a Nikon Microphot-FX microscope and photographed using a Nikon DXM1200 digital camera.

Electrophysiological recordings. Freshly isolated first-expansion and first-passage equine and elephant chondrocytes were allowed to adhere to uncharged glass coverslips for at least 30 min in serum-free DMEM before being transferred to a recording chamber and superfused with the following bath solution (in mM): 153 NaCl, 5 KCl, 2 CaCl2, 1.4 MgCl2, 131 Cl−, 26 HCO3−, 10 glucose, and 1.2 H2PO4−, in 5% CO2-95% O2. Patch pipettes were fabricated from Clark GC150F capillary glass (Harvard Apparatus) and had resistances of 5–10 MΩ when filled [pipette solution (in mM): 155 KCl, 1.4 MgCl2, 134 Cl−, 5 EGTA, and 10 HEPES, pH 7.2]. We calculated, for these solutions, a junction potential of −4.4 mV. Patch-clamp experiments were performed with an Axon 200B Axopatch amplifier (Axon Instruments, Union City, CA), and data were filtered at 5 kHz and digitized (100-μs sample interval) with a Digidata 1200B interface attached to a Windows 98 personal computer running the AXGOX suite of Axobasic programs written by Dr. Noel Davies (University of Leic-}

ester, Leicester, UK) or the WCP program by John Dempster (University of Strathclyde, Glasgow, UK). Leak subtraction of current-voltage data was performed using 10 pulses of P/12 between each command voltage step; over this range, the current-voltage relationship was linear. Current density-voltage curves were calculated by dividing each current point by the cell capacitance (yielded by the amplifier’s capacitance subtraction circuitry). Mean whole cell capacitance of elephant and equine cells was not significantly different (horse: 23.8 ± 2.8 pF; elephant: 18.6 ± 6.4 pF; Student’s two-tailed t-test).

Data were transformed from current to conductance density plots according to the following equation:

$$G = \frac{I}{(V_c - E_K)}$$

where G is the conductance density, I is the current density, Vc is the command potential, and EK is the equilibrium potential for potassium. These transformed data were then fit with the standard Boltzmann equation:

$$G = G_0 + \frac{G_{\text{max}}}{1 + \exp(-\left(V_c - V_{0.5}\right)/k)}$$

where G0 and Gmax are the minimum and maximum conductance densities, respectively, V0.5 is the midpoint parameter, and k is the slope. These transformations were performed on each cell recorded from and were then averaged together to provide the mean and SE data reported in the text.

Dissociation constants Kd for inhibition of K+ current by drugs were calculated from the simplest form of the Hill equation, in the form:

$$K_d = \frac{[\text{drug}]}{(1 - F)} - [\text{drug}]$$

where [drug] is the concentration of drug and F is the fraction of current remaining in the presence of drug.

Experiments were performed at an elevated room temperature of 24–26°C, and results are expressed as means ± SE (n = number of experiments). Data fitting was performed using Microsoft Excel, and figures were prepared using SigmaPlot 8.0 (SPSS, Chicago, IL). We used two-tailed Student’s t-tests and two-way ANOVA with Bonferroni post hoc modifications where appropriate (and stated in the text). These were either included with or written in Microsoft Excel 97. The null hypothesis was rejected if its probability was less than or equal to 0.05.

Kv immunohistochemistry. Equine cartilage tissue microarrays (TMAs) were prepared using an Abcam Tissue Micro Array builder (ab1802; Cambridge, UK). TMAs permit parallel processing of multiple paraffin-embedded tissue samples. With the use of this basic TMA preparation kit, 24 equine cartilage samples were arranged in a 6 × 4 grid array on a single charged microscope slide, increasing the throughput for screening cartilage samples for Kv channel expression using the immunohistochemical technique. All samples were fixed in 10% neutral buffered formalin and decalcified in EDTA before paraffin wax embedding and immunohistochemical processing. Immunohistochemistry was carried out essentially as described recently (20). TMA slides were deparaffinized in xylene for 20 min to remove embedding medium and washed in absolute ethanol for 3 min. The TMAs were gradually rehydrated in a series of alcohol baths (96, 85, 70, 50, 40, 30, 20, 10%) and placed in distilled water for 5 min. Endogenous peroxidase activity was blocked for 1 h in a solution of 97% methanol, 3% hydrogen peroxide, and 0.01% sodium azide. The TMAs were then incubated for 1 h at room temperature with protease-free bovine serum albumin (BSA) in Tris-buffered saline (TBS) containing 1% BSA and 0.01% sodium azide to block nonspecific antibody binding. Slides were incubated overnight at 4°C with affinity-purified rabbit polyclonal antibodies to Kv1.1, Kv1.2, Kv1.3, Kv1.4, and Kv1.5.
Antibodies were diluted to working concentrations of 0.3 mg/ml in TBS containing 1% BSA. After 24 h at 4°C, the slides were washed three times for 5 min each in TBS containing 0.05% Tween 20 (TBS-T) before incubation with horseradish peroxidase-labeled polymer conjugated to affinity-purified goat anti-rabbit immunoglobulins (code no. K4010 Dako) for 30 min at room temperature. The sections were washed three times for 5 min in TBS-T before application of liquid DAB chromogen (3,3′-diaminobenzidine solution Dako). The development of the brown-colored reaction was stopped by rinsing in TBS-T. The stained slides were immersed for 5 min in a bath of aqueous hematoxylin (code no. S3309 Dako) to counterstain cell nuclei. Finally, the slides were washed for 5 min in running water and dehydrated in a series of graded ethanol baths before being rinsed in three xylene baths and mounted in 1,3-diethyl-8-phenylxanthine (BDH Laboratories, Atherstone, UK). Control experiments were performed by incubating equine cartilage TMAs with nonimmune serum and by omitting primary antibody. Additional control experiments were carried out with competing peptides (CP) provided by the antibody manufacturer, which served as the most appropriate antibody blocking antigens (working concentration: 1 mg/ml). A monoclonal pan antibody to the α-subunits of Na+/K+-ATPase (MAb 9A7; a gift from Dr. Masami Takahashi, Kitasato University School of Medicine, Kanagawa, Japan) was used as an internal positive control (4).

Chemicals. Unless otherwise stated, all chemicals used in this study were of molecular biology or ACS grade and supplied by Sigma-Aldrich (Poole, UK).

RESULTS

To establish a chondrocyte model of equine and elephant chondrocytes for our patch-clamp studies, we used previously established protocols in our laboratory for isolation of primary populations of equine, bovine, and ovine articular chondrocytes. It is important to point out, however, that to consistently remove the pericellular matrix and produce isolated chondrocytes receptive to gigaseal formation and patch-clamp experimentation, use of a different type of collagenase (type I collagenase) was necessary. Apart from the type of collagenase, no major modifications to the isolating medium, the period of tissue digestion, or collagenase concentrations were required.

**Morphological characteristics of the equine and elephant chondrocytes.** Low-power light micrographs of the superficial, middle, and deep zones of equine stifle and elephant hip cartilage sections shown in Fig. 1, C–F, highlight the hypocellularity of the tissue and the low abundance of chondrocytes in fully developed cartilage from these two species. The isolated elephant and equine chondrocytes are shown in Fig. 1, G and H. Primary cultured equine and elephant cells both exhibited a polymorphic morphology characteristic of early passage chondrocytes grown in monolayers.

**Voltage activated K+ currents in equine chondrocytes.** From a holding potential of −80 mV, depolarizing voltage steps of +40 mV or more evoked time-dependent outward K+ currents in equine chondrocytes (Fig. 2A). There was no evidence of any voltage-gated Na+ channel activity. Outward currents rectified (outwardly) in the range of −40 mV to approximately +20 mV (Fig. 2B) and were generally sustained throughout the course of ~100- or 200-ms depolarizing voltage steps. Recovery from inactivation, however, was rather slow, because repeating voltage pulses at a frequency of >1/2 Hz led to a rundown of the peak current. Our current-voltage protocol covered a range of command pulses from −120 mV to +60 mV, and peak current amplitude (at Vc +60 mV) was in the range of ~100–1,300 pA. In terms of current density, this corresponded to an average of 25.0 ± 6.8 A/F (n = 10).

Boltzmann transformation of the current density-voltage data (Eqs. 1 and 2; Fig. 3C) yielded a peak chord conductance density of 0.15 ± 0.04 S/F, V½ of −12.5 ± 4.3 mV, and slope k of 12 ± 2 mV (n = 10).

**Voltage activated K+ currents in elephant chondrocytes.** Incremental depolarizing voltage steps to patch-clamped elephant chondrocytes again resulted in the activation of a large outward K+ current (Fig. 3A). These currents were generally similar to those of equine chondrocytes, activating at approximately −40 mV and outwardly rectifying but were somewhat larger in amplitude, with peak currents ranging from −900 to 3,500 pA (at Vc +60 mV). Boltzmann transformation of the current density-voltage data (Eqs. 1 and 2; Fig. 3C) yielded a
peak chord conductance density of 1.05 ± 0.33 S/F, which was significantly larger than that of equine chondrocytes (Student’s t-test, \( P < 0.05 \)). The \( V_{50} \) of \(-22.2 \pm 6.4 \text{ mV} \) and slope \( k \) of \( 11.8 \pm 3.0 \text{ mV} \) (\( n = 4 \)) were not significantly different from those of equine chondrocytes (Student’s t-tests).

Kinetic analysis of chondrocyte \( K^+ \) channels. The next stage of our analysis of elephant and equine chondrocytes was to measure the kinetics of voltage-gated activation and inactivation. Each current waveform was fitted with a double exponential (Fig. 4). The first component of this curve represents the activation time constant (\( \tau_{\text{act}} \)), and the second represents the inactivation time constant (\( \tau_{\text{inact}} \)). Activation and inactivation time constants for command potentials of +30 mV make useful cross-channel comparison parameters (6). There was no significant difference in activation or inactivation parameters between the two species under study (\( \tau_{\text{act}} \) elephant: \( 3.1 \pm 0.9 \text{ ms} \), \( n = 6 \), Student’s t-test, \( P > 0.05 \); \( \tau_{\text{inact}} \) elephant: \( 1.396 \pm 10.6 \text{ ms} \), \( \tau_{\text{inact}} \) horse: \( 710 \pm 256 \text{ ms} \), \( n = 6 \), Student’s t-test, \( P > 0.05 \)). Further statistical analysis shows no significance between horse and elephant activation or inactivation parameters (Fig. 4B) with any (measured) \( V_c \) between -30 and +60 mV (\( P > 0.05 \) for each comparison).

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Fig. 2. Current-voltage curves of equine chondrocytes. A: a representative family of (leak subtracted) whole cell outward currents recorded from equine chondrocytes. Top: voltage protocol. From a holding potential of \(-80 \text{ mV} \), cells were stepped to command voltages in the range of \(-120 \text{ to } +60 \text{ mV} \) (in 10-mV increments). Bottom: leak-subtracted currents. B: mean peak current voltage data, calculated as current density by dividing peak current in each cell by the whole cell capacitance. \( V_m \), membrane potential.

Fig. 3. Current-voltage curves of elephant chondrocytes. A: a representative family of (leak subtracted) whole cell outward currents recorded from elephant chondrocytes. Top: voltage protocol. From a holding potential of \(-80 \text{ mV} \), cells were stepped to command voltages in the range of \(-120 \text{ to } +60 \text{ mV} \) (in 10-mV increments). Bottom: leak-subtracted currents. B: mean peak current voltage data, calculated as current density by dividing peak current in each cell by the whole cell capacitance. C: combined Boltzmann curves for equine and elephant chondrocytes. Smooth lines represent fits to the data with maximum conductance density (\( G_{\text{max}} \)) and midpoint parameters, as given in text. \( G \), conductance density. Note that the time scale is different from that shown in Fig. 2.
2-way ANOVA with Bonferroni post hoc modification for multiple comparisons.

**Pharmacological sensitivity of equine chondrocytes.** To provide a preliminary pharmacological identification of the primary K+ conductance, we tested equine chondrocytes for sensitivity to three well-known K+ channel blockers: TEA, 4-AP, and dendrotoxin-I. We first activated K+ currents by stepping from a holding potential of -80 mV to a V_c of +40 mV and applied TEA at increasing concentrations by bath perfusion. In the presence of 1 mM TEA, there was a large inhibition of evoked K+ currents (Fig. 5B). Comparison of current survival in the presence of 0.1, 1, and 10 mM TEA (Fig. 5E) indicates that these channels are sensitive to TEA with a dissociation constant of 2.6 ± 0.6 mM (n = 6; Eq. 3). In similar experiments, we measured the fraction of K+ current surviving in 1 mM 4-AP (Fig. 5, C and E) and, finally, 100 nM dendrotoxin (Fig. 5, D and E). In the case of 4-AP, we calculated a dissociation constant of 1.3 ± 0.7 mM (n = 5). We failed to detect significant block by 100 nM dendrotoxin-I, a concentration that blocks Kv1.1/2/6 (see DISCUSSION).

Because of the difficulties in regularly procuring elephant cartilage tissue, we were not able to repeat these pharmacological experiments with elephant chondrocytes.

**Immunohistochemical analysis of chondrocyte K+ channels.** The pharmacological investigation was extended with an immunohistochemical approach intended to identify the Kv1.x channel subtype present in equine articular chondrocytes. The results of the immunohistochemical data are summarized in Fig. 6. We found evidence for expression of Kv1.4 in custom-designed equine cartilage TMAs. No evidence was found for expression of other Kv1.x subtypes. Previous studies have shown that Na+-K+-ATPase is abundantly expressed in articular cartilage (31). Monoclonal pan antibodies to the α-units of Na+-K+-ATPase were included to show positive immunoreactivity for this abundant ATPase in the equine cartilage TMAs (Fig. 6). In control experiments, cartilage TMA slides were preincubated with control Kv antigen (molar equivalent competing peptide) in the presence of Kv primary antibody to demonstrate that the blocking peptides were able to inhibit Kv antibody immunoreactivity. An additional negative control was omission of primary antibody to show the absence of nonspecific secondary antibody immunostaining.

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**Fig. 5. Sensitivity of equine chondrocyte Kv currents to tetraethylammonium (TEA).** A: schematic representation of voltage protocol used in B–D. Holding potential was -80 mV, V_c was +40 mV. B: representative whole cell outward currents recorded from equine chondrocytes in the presence and absence of 1 mM TEA. C and D: equivalent representative traces in the presence and absence of 1 mM 4-aminopyridine (4-AP) or 100 nM dendrotoxin-1 (Dtx), respectively. E: average data from a number of experiments similar to those shown in A–D. Ordinate shows relative current remaining in the presence of 0.1, 1, and 10 mM TEA, 100 nM Dtx, and 1 mM 4-AP.
DISCUSSION

We established a primary in vitro model of equine and elephant chondrocytes suitable for patch-clamp electrophysiological recording. Having confirmed their purity, viability, and phenotypic characteristics, we proceeded to characterize the biophysical properties of their primary voltage-gated K$^+$ currents. These currents appeared to be a typical “delayed rectifier” type of K$^+$ current, with fast activation in the $-40$ to $+20$ mV range. Pharmacologically, these channels were sensitive to 4-AP and TEA but insensitive to dendrotoxin-I. Immunohistochemical experiments detected the presence of Kv1.4 subunits.

Histological studies of equine and elephant cartilage samples. The hypocellularity of the cartilage samples used in this study established that the joints were taken from skeletally mature animals. Microscopic evaluation of the samples also confirmed that the samples did not include any calcified cartilage (i.e., the deepest zone of cartilage adjacent to subchondral bone), which in some preparations will result in contamination of the isolated chondrocyte population with a percentage of bone cells (i.e., osteoblasts). Thus this was a genuine and highly pure preparation of articular chondrocytes.

Previous reports of K$^+$ currents in chondrocytes. In this work, we recorded exclusively from first-expansion and first-passage cells. Although this is the first report of voltage-gated K$^+$ currents in equine and elephant chondrocytes, there have been reports of K$^+$ currents in various preparations of chondrocytes from other animal species. First, Grandolfo et al. (8, 9) observed Ca$^{2+}$-activated K$^+$ current in cultured porcine chondrocytes. Subsequently, Walsh et al. (35) identified delayed rectifier-type K$^+$ currents in chondrocytes from the growth cone of chickens. In a study of cultured rabbit chondrocytes, Sugimoto et al. (29) identified similar delayed rectifier-type K$^+$ channels but also observed voltage-gated Na$^+$ channels. Perhaps more importantly, delayed rectifier K$^+$ currents also were observed by Wilson et al. (37) in a recent study of acutely isolated canine chondrocytes.

The preparation of acutely isolated canine chondrocytes (37) is probably the most similar in terms of methodology to the cells used in the present investigation, and the data we have obtained are indeed very similar to those of Wilson et al. (37). In both studies, the chondrocytes were used as fresh as possible with little or no opportunity for them to dedifferentiate in

Fig. 6. Immunohistochemical analysis of Kv1.x expression in equine articular cartilage. Incubation of equine cartilage tissue microarrays (TMAs) with polyclonal antibodies to Kv1.1, Kv1.2, Kv1.3, and Kv1.4 did not produce any immunostaining with use of the extremely sensitive DakoCytomation EnVision+ Dual Link System-HRP (DAB+) kit. Positive staining was seen with polyclonal antibodies to Kv1.4. Representative images from TMA slides preincubated with primary antibody and control antigen [competing peptide (CP)] also are shown along with a negative control (primary antibody omitted) and a positive control (the α-subunits of Na$^+$-K$^+$-ATPase). Arrows indicate positively stained chondrocytes. Bars = 100 μm (except for the enlarged Kv1.4 image, where the bar = 10 μm).
culture. Furthermore, whole cell currents were recorded with 5 mM EGTA in the patch pipette, thus reducing likely contamination by the Ca$^{2+}$-activated K$^+$ channels observed by Grandolfo et al. (8) and Long and Walsh (16). In neither the present study nor the study by Wilson et al. (37) was there any evidence of a voltage-gated Na$^+$ conductance (for examples, see Figs. 2A and 3A in this work, and see Fig. 1A in Ref. 37).

Identity of the chondrocyte voltage-gated K$^+$ channel. Recent molecular biological and electrophysiological experiments have identified several families of voltage-gated K$^+$ channels in nature, and the most common of these are Kv1.x through Kv4.x. In the following paragraphs, we discuss which of these appear to be expressed in chondrocytes.

Steady-state activation parameters. Kv currents from equine and elephant chondrocytes appear broadly similar to those of canine chondrocytes in terms of their steady-state-activation parameters ($V_{1/2}$ and $k$). Interestingly, however, those measured in chondrocytes from the articular cartilage of elephants were larger than those of equine or canine chondrocytes. The physiological reason for this difference is unknown. It seems implausible that this is related to the remarkably large size of the elephant (or the predictably large mechanical loads experienced by elephant chondrocytes), because horse chondrocyte Kv currents were no larger than those of the dog. The midpoints ($V_{1/2}$) of steady-state activation of horse, dog, and elephant chondrocytes all lie in the range of $-12$ to $-25$ mV. These values are typical of K$^+$ channels of either the Kv1 or Kv4 families (6), suggesting that there would be significant Kv activity at the chondrocyte resting membrane potential (measured as $-15$ to $-42$ mV from canine and rabbit tissue, respectively; Refs. 33, 37, 40).

Activation and inactivation kinetics. The activation and inactivation kinetics of horse and elephant chondrocytes were very similar across the experimental range. The activation time constant we calculated ($\sim 3$ ms at $+30$ mV) is fast for a voltage-gated K$^+$ current. Similar values have been reported for several members of the Kv1 family but also for homomeric Kv3.4 (6). The activation time constant we measured (hundreds of milliseconds or seconds) is not unusual for voltage-gated K$^+$ channels and is typical for several members of the Kv1, Kv2, and Kv3 families (6). Homomeric Kv3.4, however, inactivates rather rapidly, leaving members of the Kv1 family as the most likely candidates.

Pharmacological identification. 4-AP and TEA are commonly used pharmacological inhibitors of K$^+$ currents. At sufficient concentration, TEA will inhibit most K$^+$ subtypes, albeit with a 1,000-fold difference in the dissociation constant between the least sensitive and the most sensitive voltage-gated K$^+$ channels (15). Furthermore, extracellular TEA inhibits Ca$^{2+}$-activated K$^+$ channels (maxiK, or BK channels) with a dissociation constant in the micromolar range (2). Generally, Kv3.x show similar TEA sensitivity to BK channels ($IC_{50} < 1$ mM), whereas homomeric Kv1.x and Kv2.x are $\sim 10 \times$ less sensitive (reviewed in Ref. 6). Kv4.x (A-type) currents often show little sensitivity to extracellular TEA at all (Ref. 30, reviewed in Ref. 6). 4-AP also blocks most voltage-gated K$^+$ channels, but there is a less striking difference in potencies between channel subtypes.

The first voltage-activated K$^+$ current reported in chondrocytes (chicken growth cone; Ref. 35) was somewhat distinctive in that it was insensitive to 10 mM TEA but sensitive to 4-AP ($IC_{50} < 1$ mM) and very sensitive to the peptide inhibitor charybdoxin ($IC_{50} < 10$ nM; Ref. 35). This pharmacology is most similar to that reported for certain members of the Kv1 family of voltage-gated K$^+$ channels (6, 7). Kv current sensitivity to TEA in the cultured rabbit chondrocyte (<5 nM; Ref. 29) and acutely dissociated canine chondrocyte (<1 nM; Ref. 37), however, was much greater and thus more typical of relatively TEA-sensitive channels of the Kv3 family. Canine chondrocytes have been shown to have TEA and 4-AP IC50 values of 0.66 mM and a little over 1 mM, respectively (37), again consistent with members of the Kv1 family such as homomeric Kv1.1 (6, 10).

In this report we calculate horse chondrocyte Kv currents to have 4-AP and TEA IC50 values of $\sim 1.3$ and 2.6 mM, respectively. These are similar to those of several members of both the Kv1 and Kv2 family. However, coupled with both the steady-state and kinetic activation data, the strongest suggestion is for the presence of members of the Kv1 family.

Far more selective blockers of voltage-gated K$^+$ channels are the snake dendrotoxins. This toxin family is highly selective for members of the Kv1 family (6, 10, 14), but dendrotoxin-I itself only has been shown to block Kv1.1/2/6 ($IC_{50} < 10$ nM; Ref. 14). We found that dendrotoxin-I did not block equine chondrocyte Kv currents. This would then point toward Kv1.3/4/5; however, all three of these Kv1 subtypes have a lower TEA sensitivity than we observed. Together, these data are not completely consistent with any individual homomeric Kv channel. Wild-type Kv1.x channels, however, are well known to form heteromultimers with other members of the Kv1 family, possibly confusing the biophysical and pharmacological properties (5, 26, 28). It is also possible that chondrocytes express more than one type of Kv channel. This led us to consider supplementing our pharmacological study with an immunohistochemical study.

Immunohistochemistry. Our electrophysiological data strongly suggest that chondrocytes express Kv channels of the Kv1 family, possibly in the form of a heteromultimer. Our immunohistochemical data, however, demonstrated the presence of Kv1.4 subunits. Taking these data together, our best estimation is that articular chondrocytes (equine at least) express Kv1.4 heteromultimer ion channels, including the Kv1.4 subunit and some other, as yet unidentified, Kv subunit(s).

Role of voltage-gated/delayed rectifier K$^+$ currents in chondrocytes. The role of delayed rectifier-type K$^+$ channels in the functioning of chondrocytes is unknown. In excitable tissue, the delayed rectifier current is responsible for repolarization of the membrane after each action potential, but no action potentials have ever been reported in chondrocytes, and we failed to see any evidence of a voltage-gated Na$^+$ conductance. Therefore, it seems possible that in chondrocytes, the voltage-gated K$^+$ channel may be important for determination of the resting membrane potential. Whole cell patch clamp is, perhaps, not the ideal means of measuring the resting membrane potential of cells in general, because it artificially controls the ionic composition of the cell, which in turn determines the resting membrane potential. Despite these difficulties, Wilson et al. (37) were recently able to demonstrate that the resting membrane potential of acutely isolated canine chondrocytes is significantly influenced by Kv channels. This is in marked contrast to the observation that the resting membrane potential of the rabbit cultured chondrocyte is almost entirely controlled
by a Cl\textsuperscript{−} conductance (33). The exact level of the resting membrane potential is likely to be of great importance to the in vivo physiology of chondrocytes, regulating, for example, the synthesis of key extracellular matrix macromolecules and modulating the activity of other voltage-sensitive membrane transporter proteins.

In conclusion, we have provided electrophysiological and immunohistochemical evidence for the presence of voltage-gated K\+ channels in articular chondrocytes from two large mammals. The data suggest that chondrocytes possess interesting electrical properties that may be important for their mechanobiology in vivo. Future experiments are needed to further characterize the molecular identity of voltage-gated K\+ channels in these cells and to examine the effects of blockers and modulators on signaling cascades and metabolic responses.

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