British Journal of Pharmacology

DOI:10.1111/j.1476-5381.2011.01802.x
www.brjpharmacol.org

RESEARCH PAPER

17β-Oestradiol inhibits doxorubicin-induced apoptosis via block of the volume-sensitive Cl^- current in rabbit articular chondrocytes

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Keywords
chondrocyte; osteoarthritis; volume-sensitive Cl^- current; 17β-oestradiol; apoptosis; caspase; doxorubicin

Received
21 June 2011
Revised
31 October 2011
Accepted
15 November 2011

BACKGROUND AND PURPOSE

Chondrocyte apoptosis contributes to disruption of cartilage integrity in osteoarthritis. Recent evidence suggested that the volume-sensitive organic osmolyte/anion channel [volume-sensitive (outwardly rectifying) Cl^- current ($I_{Cl,vol}$)] plays a functional role in the development of cell shrinkage associated with apoptosis (apoptotic volume decrease) in several cell types. In this study, we investigated the cellular effects of 17β-oestradiol on doxorubicin-induced apoptotic responses in rabbit articular chondrocytes.

EXPERIMENTAL APPROACH

Whole-cell membrane currents and cross-sectional area were measured from chondrocytes using a patch-clamp method and microscopic cell imaging, respectively. Caspase-3/7 activity was assayed as an index of apoptosis.

KEY RESULTS

Addition of doxorubicin (1 μM) to isosmotic bath solution rapidly activated the Cl^- current with properties similar to those of $I_{Cl,vol}$ in chondrocytes. Doxorubicin also gradually decreased the cross-sectional area of chondrocytes, followed by enhanced caspase-3/7 activity; both of these responses were totally abolished by the $I_{Cl,vol}$ blocker DCPIB (20 μM). Pretreatment of chondrocytes with 17β-oestradiol (1 nM) for short (approximately 10 min) and long (24 h) periods almost completely prevented the doxorubicin-induced activation of $I_{Cl,vol}$ and subsequent elevation of caspase-3/7 activity. These effects of 17β-oestradiol were significantly attenuated by the oestrogen receptor blocker ICI 182780 (10 μM), as well as the phosphatidyl inositol-3-kinase (PI3K) inhibitors wortmannin (100 nM) and LY294002 (20 μM). Testosterone (10 nM) had no effect on the doxorubicin-induced Cl^- current.

CONCLUSIONS AND IMPLICATIONS

17β-Oestradiol prevents the doxorubicin-induced cell shrinkage mediated through activation of $I_{Cl,vol}$ and subsequent induction of apoptosis signals, through a membrane receptor-dependent PI3K pathway in rabbit articular chondrocytes.

Abbreviations

AVD, apoptotic volume decrease; C_m, cell membrane capacitance; DCPIB, 4-(2-butyl-6,7-dichloro-2-cyclopentyl-indan-1-on-5-yl) oxybutyric acid; DMEM, Dulbecco's modified Eagle's medium; DPI, diphenylene-iodonium chloride; E_{Cl}, equilibrium potential for Cl^-; ICI 182780, 7α,17β-[9-[4,4,5,5,5-pentafluoropentyl)sulfinyl]estra-1,3,5(10)-triene-3,17-diol; $I_{Cl,vol}$, volume-sensitive (outwardly rectifying) Cl^- current; NAC, *N*-acetyl-cysteine; OA, osteoarthritis; PI3K, phosphatidyl inositol-3-kinase; ROS, reactive oxygen species; RVD, regulatory volume decrease

Introduction

Chondrocyte apoptosis is a functionally important phenomenon in the development and growth of articular cartilage under physiological conditions. It is well known that in the processes of normal bone growth and endochondral ossification, terminally differentiated chondrocytes are removed from the calcified cartilage by apoptosis prior to the transition to bone (Adams and Shapiro, 2002). On the other hand, evidence is accumulating that an increased incidence of chondrocyte apoptosis during aging is responsible for hypocellularity associated with degradation and/or pathological remodelling of cartilage matrix, and exaggerates the risk of degenerative joint diseases such as osteoarthritis (OA) (Blanco *et al.*, 1998; Hashimoto *et al.*, 1998; Mobasheri, 2002). Although the specific molecular switch that promotes chondrocyte apoptosis under physiological conditions remains to be fully elucidated, several cellular events have been implicated in chondrocyte apoptosis under pathological conditions. These include the release of pro-apoptotic cytokines (such as IL-1 and TNF-α) from the surrounding tissues, loss of prosurvival signals (such as β1-integrin-mediated cell-matrix interaction) (Cao *et al.*, 1999; Goggs *et al.*, 2003) and enhanced production of nitric oxide and reactive oxygen species (ROS) such as hydrogen peroxide (Blanco *et al.*, 1995; Lo and Kim, 2004).

Previous studies using electron microscopy, terminal deoxynucleotidyltransferase-mediated dUTP nick-end labeling staining and/or flow cytometry assay have characterized several morphological and biochemical processes associated with chondrocyte apoptosis during the progression of OA. These include cell shrinkage, nuclear condensation, deposition of cell remnants (apoptotic bodies) and internucleosomal DNA fragmentation (Blanco *et al.*, 1998; Hashimoto *et al.*, 1998). In the early stages of apoptosis, a progressive cell shrinkage under normosmotic conditions, referred to as apoptotic volume decrease (AVD), is a commonly observed feature, that is not dependent on cell type (Bortner and Cidlowski, 1998; Saraste and Pulkki, 2000; Bortner and Cidlowski, 2007). Because the prevention of cell shrinkage has been reported to be effective at rescuing cells from apoptosis, AVD can be regarded as a prerequisite step preceding key biochemical events for apoptosis, such as the release of cytochrome *c* from mitochondria, DNA fragmentation and caspase activation (Maeno *et al.*, 2000). Whereas it is generally accepted that water and ions such as K^+ and Cl^- are transported across the plasma membrane during AVD, less is understood about the changes in the activity of membrane ion channels and transporters during chondrocyte apoptosis. Evidence has been presented to indicate that oestrogen activates prosurvival signals through a non-genomic action and thereby prevents apoptotic responses in some cell types including osteoblasts and osteocytes (Honda *et al.*, 2001; Kousteni *et al.*, 2001; Kim *et al.*, 2006). Importantly, the incidence of OA is markedly greater in women than in men after the age of 50 years, thus suggesting that the postmenopausal decrease in oestrogen levels somehow facilitates the OA-associated chondrocyte death (Gokhale *et al.*, 2004; Takano *et al.*, 2007; Sniekers *et al.*, 2008; Roman-Blas *et al.*, 2009).

The volume of articular chondrocytes rapidly decreases following the development of hyposmotic cell swelling (referred to as regulatory volume decrease, RVD), and several ion channels and transporters including the taurine transport pathway have been implicated in the chondrocyte RVD (Hall, 1995; Bush and Hall, 2001). Our previous electrophysiological studies have shown that the volume-sensitive organic osmolyte/anion channel [Alexander *et al.*, 2011; also referred to as volume-sensitive (outwardly rectifying) Cl^- current ($I_{Cl,vol}$)] is functionally expressed in rabbit articular chondrocytes and is involved in inducing RVD (Isoya *et al.*, 2009; Okumura *et al.*, 2009). In contrast to its physiologically important role in the homeostatic regulation of cell volume, $I_{Cl,vol}$ activation has also been suggested to contribute to the cell shrinkage associated with induction of apoptosis in several cell types such as cardiomyocytes and neurons (Okada *et al.*, 2006). In fact, various blockers of $I_{Cl,vol}$ effectively prevent AVD and subsequent cell death induced by ischaemia-reperfusion stress or apoptotic inducers such as staurosporine, doxorubicin, Fas ligand, TNF-α or sphingolipids (Maeno *et al.*, 2000; Souktani *et al.*, 2000; d'Anglemont de Tassigny *et al.*, 2004; Shimizu *et al.*, 2004; Wang *et al.*, 2005). However, it is not known whether chondrocyte apoptosis is mediated through the activation of $I_{Cl,vol}$.

The present investigation was therefore undertaken to examine (i) the functional role of $I_{Cl,vol}$ in the development of AVD and subsequent elevation of caspase activity during exposure to doxorubicin, and (ii) the cellular basis underlying the anti-apoptotic effect of 17β-oestradiol in rabbit articular chondrocytes. Our data provide experimental evidence to indicate (i) that the doxorubicin-induced chondrocyte apoptosis is mediated through the activation of $I_{Cl,vol}$, and (ii) that 17β-oestradiol totally suppresses the activation of $I_{Cl,vol}$ and subsequent apoptotic responses by stimulating a receptor-mediated non-genomic pathway including phosphatidyl inositol-3-kinase (PI3K) activation.

Methods

Isolation of rabbit articular chondrocytes

All animal care and experimental protocols conform to *The Guide for the Care and Use of Laboratory Animals* published by the US National Institutes of Health (NIH Publication no. 85-23, revised 1996) and were approved by the Institution's Animal Care and Use Committee (Shiga University of Medical Science, no. 2009-3-3). Articular chondrocytes were isolated from 32 of adult male Japanese White rabbits (body weight, 2 to 3 kg) using an enzymatic dissociation procedure similar to that described previously (Wilson *et al.*, 2004), with some modifications (Okumura *et al.*, 2009). In brief, rabbits were deeply anaesthetized with an i.m. injection of ketamine (70 mg·kg^{-1}) and xylazine (5 mg·kg^{-1}) and then killed by i.v. injection of sodium pentobarbital (70 mg·kg^{-1}). Articular cartilages were removed from bilateral knee, hip and shoulder joints and were washed with PBS (ICN Biomedicals Inc., Aurora, OH, USA). The sliced cartilages were incubated in plastic culture dishes containing Dulbecco's modified Eagle's medium (DMEM; Gibco BRL, Grand Island, NY, USA) supplemented with 10% fetal calf serum and antibiotics in a humidified atmosphere of 95% air plus 5% CO_2 at 37°C for 1 to 3 days. On the day of experiments, cartilages were minced

into small pieces (~1 mm^3) and were digested with 0.5% collagenase (Type 2; Worthington Biochemical Corp., Lakewood, NJ, USA) for 4 h. Dispersed chondrocytes were washed three times, resuspended in DMEM supplemented with 40 mM mannitol (~360 mosmol·L^{-1}) and used for experiments within 8 h after isolation, eliminating monolayer (two-dimensional) culture. In some experiments, chondrocytes were resuspended in the same solution containing 1 nM 17β-oestradiol (Sigma Chemical Company, St. Louis, MO, USA) and incubated for 24 h.

Solutions and chemicals

The isosmotic external solution used for patch-clamp experiments contained (in mM): mannitol 150, NaCl 100, sodium aspartate 40, $MgCl_2$ 2.0, $BaCl_2$ 2.0, glucose 5.5, HEPES 10 (pH adjusted to 7.4 with NaOH). The hyposmotic external solution was prepared by simply omitting 70 mM mannitol from the isosmotic solution, while the hyperosmotic external solution was made by adding 70 mM mannitol to the isosmotic solution, keeping extracellular Cl^- concentration constant (108 mM). In some experiments, Cl^- concentration in the isosmotic external solution was altered by replacing NaCl with an equimolar concentration of sodium aspartate. The osmolarity of these isosmotic, hyposmotic and hyperosmotic external solutions, measured using a freezing point depression osmometer (FISKE, Burlington, MA, USA), averaged 360, 290 and 430 mosmol·L^{-1} (approximately 20% decrease and increase), respectively (Hall, 1995; Bush and Hall, 2001). The standard pipette solution contained (in mM): caesium aspartate 135, CsCl 30, tetraethylammonium chloride 20, $MgCl_2$ 2.0, Tris-ATP 5.0, Li_2-GTP 0.1, EGTA 5.0, HEPES 5.0 (pH adjusted to 7.2 with CsOH), yielding a Cl^- concentration of 54 mM. The concentration of free Ca^{2+} and Mg^{2+} in the pipette solution was calculated to be approximately 1.5×10^{-10} M (pCa = 9.8) and 5.1×10^{-5} M (pMg = 4.3), respectively (Fabiato and Fabiato, 1979; Tsien and Rink, 1980). The isosmotic external solution used for examining RVD and AVD contained (in mM): mannitol 180, NaCl 90, KCl 5.4, $CaCl_2$ 1.8, $MgCl_2$ 0.5, NaH_2PO_4 0.33, glucose 5.5, HEPES 5.0 (pH adjusted to 7.4 with NaOH). The hyposmotic external solution for RVD was made by simply omitting mannitol. The osmolarity of these isosmotic and hyposmotic external solutions averaged 360 and 180 mosmol·L^{-1}, respectively.

Various test compounds were added to the isosmotic, hyposmotic and/or hyperosmotic external solutions, as denoted by horizontal bars in the figures. These included: doxorubicin (Sigma), 4-(2-butyl-6,7-dichloro-2-cyclopentyl-indan-1-on-5-yl) oxybutyric acid (DCPIB; Tocris, Ellisville, MO, USA), arachidonic acid (Sigma), 17β-oestradiol (Sigma), ICI 182780 (Tocris), *N*-acetyl-cysteine (NAC; Sigma), diphenylene-iodonium chloride (DPI; Sigma), hydrogen peroxide (Wako Pure Chemical Industries, Osaka, Japan), wortmannin (Wako), LY294002 (Sigma) and testosterone (Sigma). Concentrated stock solution was made for doxorubicin (1 mM) in distilled water, and DCPIB (10 mM), arachidonic acid (30 mM), 17β-oestradiol (1 μM), ICI 182780 (10 mM), DPI (20 mM), wortmannin (0.1 mM), LY294002 (20 mM) and testosterone (10 μM) in dimethyl sulphoxide. These were stored in aliquots at –20°C.

Microscopy and image analysis

An aliquot of cell (chondrocyte) suspension was transferred to a recording chamber (0.5 mL in volume) mounted on the stage of a Nikon eclipse TE2000-U inverted microscope (Tokyo, Japan) and was allowed to adhere lightly to the glass bottom for at least 5 min. The chamber was continuously perfused at a constant rate of 2 mL·min^{-1} with an external solution at 36 ± 1°C, and the external solution was exchanged by switching the perfusates at the inlet of the chamber, with a complete bath solution change taking 15 to 20 s. All of cell size measurements and patch-clamp experiments were conducted on round-shaped healthy chondrocytes. Light microscopy images of chondrocytes were consecutively (at 1 min intervals) captured at a 2560 × 1920 pixel resolution using a charge-coupled device digital camera (DS-Fi1, Nikon) equipped with a DS-L2 control unit (Nikon). The cross-sectional area of each chondrocyte was measured by counting the pixels contained within the cell image, using Image-J public domain software (NIH, Bethesda, MD, USA) and was normalized to its respective initial isosmotic size obtained 1 min before switching to test solutions. In the present study, the cross-sectional area of cell image was used as an index of the cell volume, and percentage RVD (Figure 4E), and AVD (Figure 5A) were calculated as follows: (peak relative area – relative area at test time)/(peak relative area – 1) × 100, where test time is 60 and 30 min, respectively.

Whole-cell patch-clamp technique and data analysis

Whole-cell membrane currents (Hamill *et al.*, 1981) were recorded from isolated chondrocytes using an EPC-8 patch-clamp amplifier (HEKA, Lambrecht, Germany). Fire-polished pipettes pulled from borosilicate glass capillaries (Narishige Scientific Instrument Lab., Tokyo, Japan) had a resistance of 2.0 to 4.0 MΩ when filled with the standard pipette solution. Either square step or voltage ramp protocols were used to record the whole-cell current. Voltage ramps were used to monitor the time course of changes in membrane currents during various interventions, while the steady-state effects were recorded using square voltage steps, unless otherwise stated. The voltage ramp protocol ($dV \cdot dt^{-1} = \pm 0.25$ V·s^{-1}) was repeated every 6 s and consisted of three phases: an initial +80 mV depolarizing phase from a holding potential of –30 mV, a second hyperpolarizing phase of –150 mV and then a third phase returning to the holding potential. The current – voltage (*I–V*) relationship was measured during the second hyperpolarizing phase. Changes in the swelling-induced membrane conductance were evaluated from a linear least-squares fit to the *I–V* curves at potentials within approximately 20 mV range centred on the reversal potential (Lewis *et al.*, 1993; Sakaguchi *et al.*, 1997). Voltage-clamp protocols and data acquisition were controlled with a Patchmaster software (v. 1.03, HEKA), and current records were filtered at 1 kHz, digitized at 5 kHz through an LIH-1600 interface (HEKA), and stored on a Macintosh computer. Cell membrane capacitance (C_m) was calculated from the capacitive transients elicited by 20 ms voltage-clamp steps (±5 mV) from a holding potential of –30 mV, using the following relationship (Bénitah *et al.*, 1993): $C_m = \tau_c \ I_0/\Delta V_m \ (1–I_\infty/I_0)$, where τ_c is the time constant of the capacitive transient, I_0 is

the initial peak current amplitude, ΔV_m is the amplitude of voltage step (5 mV), and I_∞ is the steady-state current value. The sampling rate for these measurements of C_m was 50 kHz with a low-pass 10 kHz filter. The average C_m for rabbit chondrocytes used in the present study was 7.08 ± 0.17 pF (n = 105, N = 27). Membrane current amplitude and slope conductance were normalized to C_m in each cell and expressed as pA·pF^{-1} and pS·pF^{-1}, respectively. The zero-current level is indicated by an arrowhead to the left of the current traces in the figures.

Caspase-3/7 activity measurement

Caspase-3/7 activity was measured in chondrocytes treated with 1 μM doxorubicin for 24 h without or with various test compounds. Briefly, cells were lysed and the supernatant was collected for the measurement of caspase-3/7 activity using the Caspase-Glo 3/7 assay system (Promega, Madison, WI, USA) following the manufacturer's instructions. The luminescent signal was measured with a luminometer (Infinite M200, Tecan, Männedorf, Switzerland).

Statistical analysis

Data values are expressed as means ± SEM, with the number of animals (cell isolations) and cells from which measurements were made indicated by N and n, respectively. Statistical comparisons were evaluated using either Student's t-test or ANOVA followed by a *post hoc* Newman–Keuls test, and differences were considered significant at $P < 0.05$.

Results

Doxorubicin-induced activation of Cl⁻ current in rabbit articular chondrocytes

Figure 1 shows a representative experiment examining the effect of bath application of doxorubicin (1 μM) on membrane currents in rabbit articular chondrocytes. Membrane currents were recorded using both square step or voltage ramp protocols. During superfusion with control isosmotic solution, membrane currents recorded during square steps applied from a holding potential of –30 mV to test potentials between +80 and –100 mV were of small amplitude and almost time-independent (Figure 1A, a and B, a), indicating that the voltage-gated Na^+ (a holding potential of –30 mV), Ca^{2+} (removal of Ca^{2+} from the external solution) and K^+ currents (omission of K^+ from internal solution and addition of $BaCl_2$ to the external solution; Vittur *et al.*, 1994; Sugimoto *et al.*, 1996; Martina *et al.*, 1997; Wilson *et al.*, 2004) were almost, if not totally, abolished under the present isosmotic conditions. Bath application of 1 μM doxorubicin under isosmotic conditions (360 mosmol·L^{-1}) gradually activated the membrane current, which reached a steady level about 10 min after the drug application (Figure 1A). This doxorubicin-induced current, obtained by digital subtraction of membrane currents recorded before and during exposure to doxorubicin using square step protocol (Figure 1B), exhibited a marked inactivation at potentials positive to +50 mV (Figure 1D, b–a) and an outward rectification with a reversal potential of –18.7 ± 0.3 mV (n = 5, N = 5, Figure 1E, b–a), close to the equilibrium potential for Cl^- (E_{Cl} = –18.4 mV) under the present experimental conditions. It should be noted that this increase in membrane current was not accompanied by an appreciable change in cell size (a, diameter, 14.2 ± 0.3 μm; b, 14.2 ± 0.3 μm, n = 5, N = 5), as assessed by measuring cross-sectional area of microscopic cell images (Figure 1A, inset). Subsequent exposure to hyperosmotic solution (430 mosmol·L^{-1}) rapidly and completely reversed the doxorubicin-induced increase in membrane current even in the presence of this drug (Figure 1A, B, c and C, c), with a modest but significant decrease in cell size (12.6 ± 0.3 μm).

To confirm that this doxorubicin-induced current was indeed a Cl^- current, the reversal potential was measured at three different extracellular Cl^- concentrations (18, 54 and 108 mM). Figure 2A summarizes the relationship between the reversal potential of the doxorubicin-induced current and extracellular Cl^- concentrations. The linear line that was fitted to the data points according to a regression analysis with least-squares method had a slope of 47.9 mV per 10-fold change in extracellular Cl^- concentrations, supporting that Cl^- ions are the main charge carrier of this current component. Thus, rabbit articular chondrocyte was found to possess a class of Cl^- current that is activated by doxorubicin in isosmotic conditions (designated as $I_{Cl,Dox}$ in the present study).

We further characterized the electrophysiological and pharmacological properties of $I_{Cl,Dox}$ and compared them with those of the volume-sensitive Cl^- current ($I_{Cl,vol}$) that is typically activated during hyposmotic conditions. One of the typical electrophysiological properties associated with $I_{Cl,vol}$ is the presence of prominent outward rectification even under equivalent concentrations of Cl^- inside and outside the cell (Hume *et al.*, 2000). In the experiment illustrated in Figure 2B, a chondrocyte was exposed to doxorubicin (1 μM) under equivalent Cl^- conditions ($[Cl^-]_i = [Cl^-]_o$ = 54 mM), leading to the activation of $I_{Cl,Dox}$, and its I–V relationship was found to exhibit an outward rectification; the slope conductances measured at potential ranges of +60 to +80 mV and –100 to –80 mV averaged 990 ± 68 and 370 ± 43 pS·pF^{-1}, respectively (n = 3, N = 3).

Figure 2C and D illustrate the response of $I_{Cl,Dox}$ to DCPIB (20 μM) and arachidonic acid (30 μM), both of which have an inhibitory effect on $I_{Cl,vol}$ in rabbit articular chondrocytes (Isoya *et al.*, 2009; Okumura *et al.*, 2009). It should also be noted that DCPIB is a novel selective inhibitor for $I_{Cl,vol}$ (Decher *et al.*, 2001). $I_{Cl,Dox}$ was gradually and almost totally inhibited by subsequent exposure to DCPIB (88.8 ± 21.9% inhibition; n = 3, N = 3) or arachidonic acid (86.4 ± 9.1% inhibition; n = 6, N = 3, respectively). The pharmacological effects of these compounds on $I_{Cl,Dox}$ were almost the same as those on $I_{Cl,vol}$ in the same cell types (rabbit articular chondrocyte, Isoya *et al.*, 2009; Okumura *et al.*, 2009).

$I_{Cl,Dox}$ was thus found to have electrophysiological (high Cl^- selectivity, progressive inactivation at strong depolarized potentials and outward rectification in symmetrical Cl^- condition) and pharmacological (inhibition by DCPIB and arachidonic acid) properties, similar to those of $I_{Cl,vol.}$ (Hume *et al.*, 2000; Decher *et al.*, 2001; Isoya *et al.*, 2009; Okumura *et al.*, 2009).

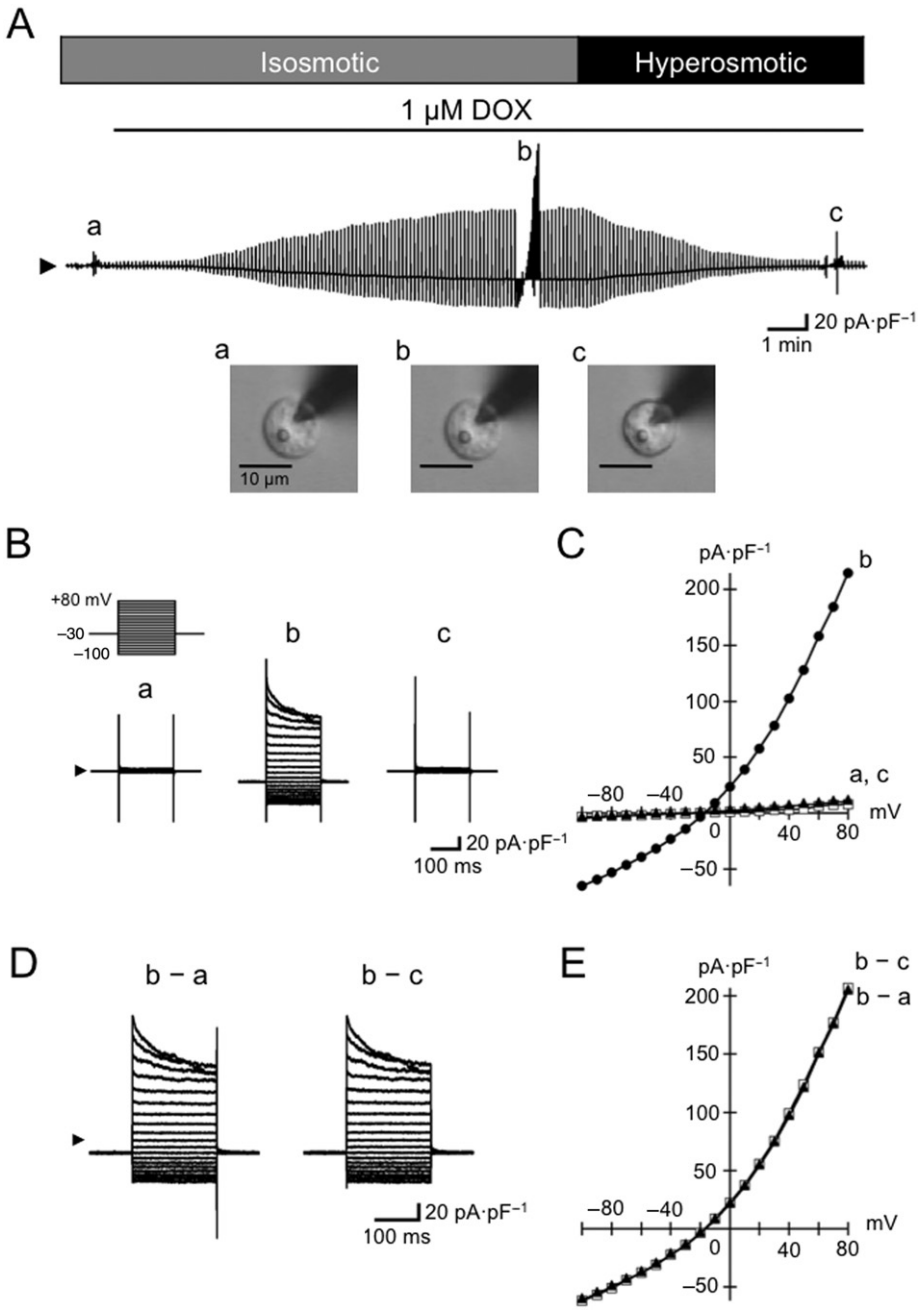

Figure 1

Whole-cell membrane currents evoked by doxorubicin in rabbit articular chondrocytes. (A) Time course of changes in whole-cell currents recorded from a chondrocyte, initially exposed to doxorubicin (DOX, 1 μM) in isosmotic solution (360 mosmol·L^{-1}) and then to hyperosmotic solution (430 mosmol·L^{-1}) during the continued presence of doxorubicin, as denoted above the current traces. The vertical deflections of current trace reflect the imposition of either voltage ramps or square steps. Microscopic images of whole-cell clamped chondrocyte taken before (a) and during (b) exposure to doxorubicin in isosmotic solution and after switching to hyperosmotic solution in the presence of doxorubicin (c). The calibration bar represents 10 μm. The diameter of the chondrocyte was measured to be 14.1 (a), 14.1 (b) and 12.7 μm (c), respectively, in each condition. (B) Superimposed current traces in response to 200 ms square-steps applied from a holding potential of –30 mV to test potentials of +80 through –100 mV in 10 mV steps, at the time points identified by letters (a, b and c) in (A). (C) *I–V* relationships of whole-cell currents recorded at each time point (a, b and c) shown in (B). (D) Membrane currents activated by doxorubicin in isosmotic solution (b–a) and those inhibited by hyperosmotic solutions in the presence of doxorubicin (b–c), obtained by digital subtraction of current traces, as indicated. (E) *I–V* relationships for the doxorubicin-induced currents (b–a) and hyperosmotic solution-inhibited currents (b–c), shown in (D).

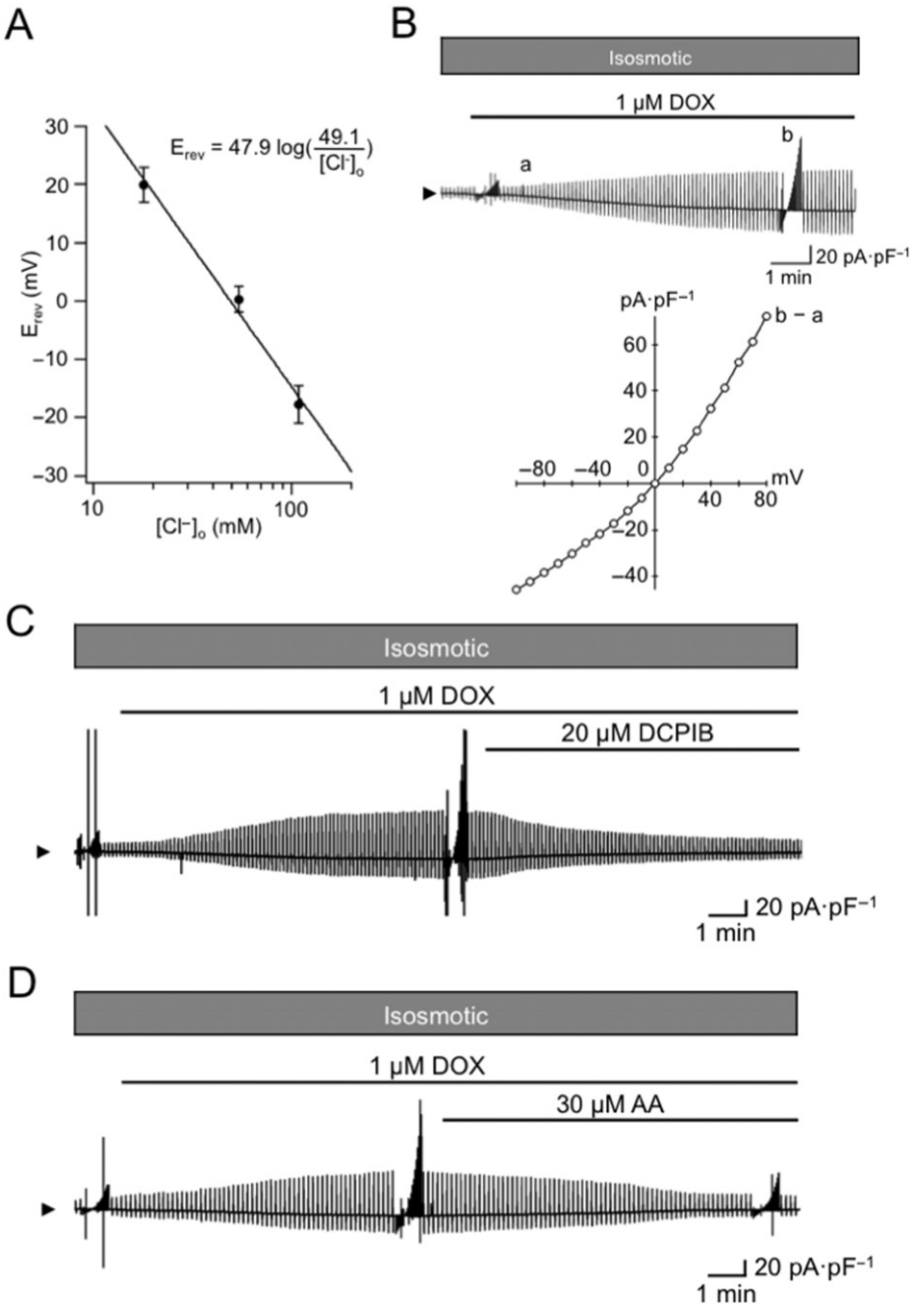

Figure 2

Electrophysiological and pharmacological properties of $I_{Cl,Dox}$. (A) The relationship between the reversal potential (E_{rev}) of the doxorubicin (1 μM)-activated current and extracellular Cl^- concentrations ($[Cl^-]_o$), obtained with an internal solution containing 54 mM Cl^-. Data points represent mean ± SEM ($n = 5$). The straight line represents a linear regression fit to the data points. (B) *I–V* relationship of $I_{Cl,Dox}$ in the presence of an equivalent concentration of Cl^- inside and outside the cell ($[Cl^-]_i = [Cl^-]_o = 54$ mM). The inset shows the changes in membrane current during exposure to 1 μM doxorubicin in isosmotic conditions in equivalent Cl^- (54 mM). (C) and (D) Chondrocyte was initially exposed to 1 μM doxorubicin, and then to 20 μM DCPIB (C) or 30 μM arachidonic acid (AA, D) during the continued presence of 1 μM doxorubicin, as indicated. Whole-cell current was recorded during repetitive imposition of voltage ramps and in part by square voltage-steps (B–D).

Inhibitory action of 17β-oestradiol on $I_{Cl,Dox}$ via its plasma membrane receptor

We next examined the effect of bath application of 17β-oestradiol (1 nM) on $I_{Cl,Dox}$ in rabbit articular chondrocytes (Figure 3). As demonstrated in Figure 3A, $I_{Cl,Dox}$ was gradually but almost totally inhibited by subsequent application of 1 nM 17β-oestradiol (90.6 ± 5.4% inhibition by 10 min exposure, $n = 6$, $N = 6$; refer to Figure 3C). It should be noted that activation of $I_{Cl,Dox}$ by doxorubicin was almost stable for a period of approximately 20 min (data not shown), which indicates that rundown of $I_{Cl,Dox}$ was relatively small for such

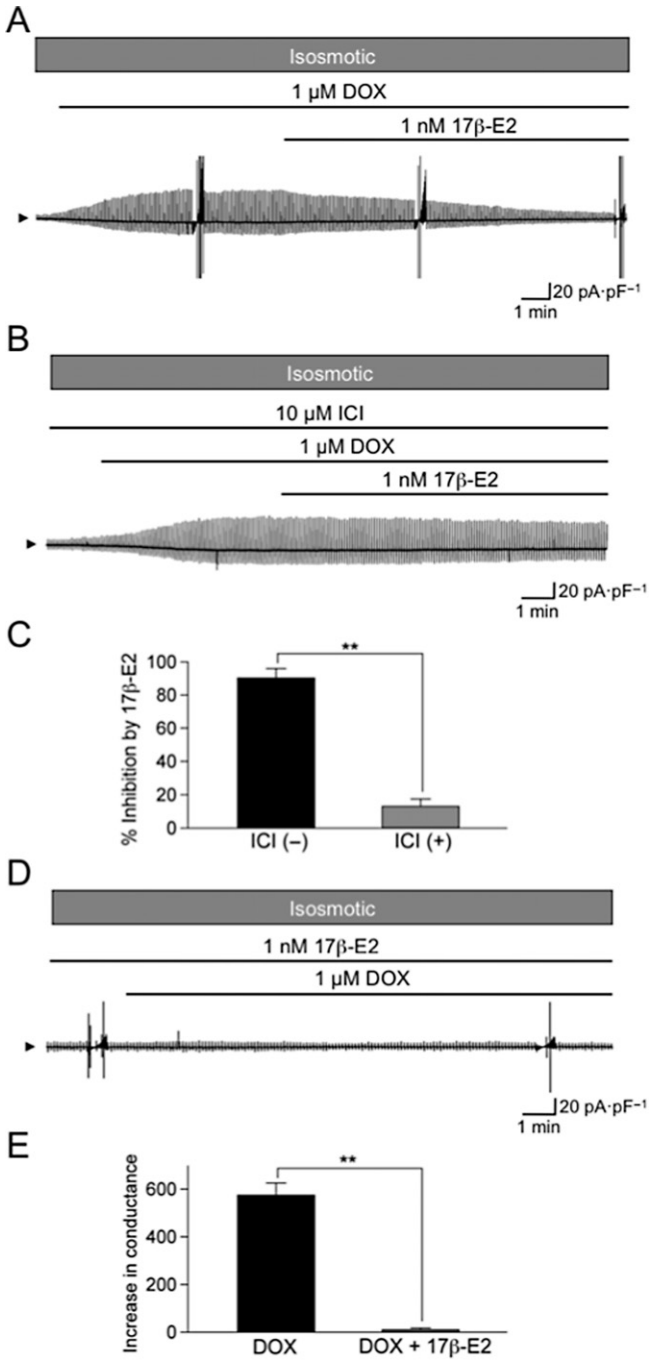

Figure 3

Inhibitory action of 17β-oestradiol on $I_{Cl,Dox}$ mediated via its plasma membrane receptor. (A) Inhibition of doxorubicin (DOX, 1 μM)-induced activation of $I_{Cl,Dox}$ by subsequent application of 17β-oestradiol (17β-E2, 1 nM). (B) Effect of pre-exposure to ICI 182780 (ICI, 10 μM) on 17β-oestradiol-induced inhibition of $I_{Cl,Dox}$. (C) % inhibition of $I_{Cl,Dox}$ by 17β-oestradiol in the absence and presence of ICI 182780 (10 μM). $^{**}P < 0.01$ (Student's unpaired *t*-test). (D) Effect of pre-exposure to 17β-oestradiol (1 nM) on the activation of $I_{Cl,Dox}$. ICI 182780 (B) or 17β-oestradiol (D) was added approximately 10 min before the application of doxorubicin (DOX, 1 μM) and was present throughout the experiments. (E) Slope conductance of $I_{Cl,vol}$ activated by doxorubicin (1 μM) without and with pre-exposure to 17β-oestradiol. $^{**}P < 0.01$ (Student's unpaired *t*-test).

a duration and means it is unlikely that the observed inhibitory action of 17β-oestradiol is due to the rundown of $I_{Cl,Dox}$. It was thus found that 17β-oestradiol almost fully inhibited $I_{Cl,Dox}$ with a relatively rapid time course (on the order of minutes), which strongly suggests that the inhibitory action of 17β-oestradiol on $I_{Cl,Dox}$ was mediated through an acute action rather than through an altered transcriptional process that usually takes place over a longer period of time (in the order of hours or days). It is generally accepted that the non-genomic action of oestrogen is mediated through the plasma membrane oestrogen receptor (Falkenstein *et al.*, 2000; Kousteni *et al.*, 2001; Björnström and Sjöberg, 2005; Kim *et al.*, 2006). As illustrated in Figure 3B, the inhibitory action of 17β-oestradiol was markedly attenuated by the presence of the oestrogen receptor blocker ICI 182780. The inhibition of $I_{Cl,Dox}$ induced by 17β-oestradiol was significantly smaller in the presence of ICI 182780 than in its absence (13.3 ± 4.1% inhibition, $n = 5$, $N = 3$ vs. 90.6 ± 5.4% inhibition, $n = 6$, $N = 6$, $P < 0.01$; Figure 3C), suggesting that 17β-oestradiol primarily acted on its plasma membrane receptor to inhibit $I_{Cl,Dox}$ in articular chondrocytes. Doxorubicin (1 μM) also failed to activate $I_{Cl,Dox}$ when chondrocytes were pre-exposed to 17β-oestradiol (Figure 3D). Figure 3E summarizes the degree of $I_{Cl,Dox}$ activation by doxorubicin (1 μM) without and with pre-exposure to 17β-oestradiol, confirming that $I_{Cl,Dox}$ activation was almost completely abolished by pre-exposing chondrocytes to 17β-oestradiol.

Lack of effect of 17β-oestradiol on the $I_{Cl,vol}$ activation by cell swelling

We also investigated whether 17β-oestradiol affects $I_{Cl,vol}$ in rabbit articular chondrocytes, which contributes to a RVD process during hyposmotic challenge (Okumura *et al.*, 2009). As illustrated in Figure 4A, exposure of chondrocytes to hyposmotic external solution (290 mosmol·L^{-1}) during the continued presence of 17β-oestradiol (1 nM) caused the cells to swell (a, diameter, 14.1 ± 0.3 μm; b, 17.2 ± 0.3 μm; $P < 0.01$; $n = 4$, $N = 4$), which was accompanied by the activation of $I_{Cl,vol}$, characterized by a prominent inactivation at strong depolarized potentials (Figure 4B) and an outward rectification with a reversal potential (–18.4 ± 0.3 mV, $n = 8$, $N = 5$) near E_{Cl} (Figure 4C). There was no significant difference in the degree of $I_{Cl,vol}$ activation between the control cells (561.0 ± 100.6 pS·pF^{-1}, $n = 9$, $N = 5$; Figure 4D) and those in the presence of 17β-oestradiol (551.9 ± 91.5 pS·pF^{-1}, $n = 8$, $N = 5$) ($P = 0.50$), thus indicating that 17β-oestradiol had little effect on the activation of $I_{Cl,vol}$ during exposure to hyposmotic solution.

To examine the effect of 17β-oestradiol on a RVD process, the time course of changes in cell size during exposure to hyposmotic solution were assessed by measuring the cross-sectional area of cell images in the absence (Control) and presence of 17β-oestradiol (Figure 4E). In control cells, exposure to hyposmotic solution evoked a rapid increase in cell size (1.27 ± 0.02, $n = 15$, $N = 3$), which then gradually declined towards isosmotic baseline level (1.13 ± 0.01) over 60 min, thus showing a functional evidence for RVD in rabbit articular chondrocytes (Okumura *et al.*, 2009). In the presence of 1 nM 17β-oestradiol, exposure to hyposmotic solution was also accompanied by initial cell swelling (1.28 ± 0.02, $n = 18$, $N = 3$) and its subsequent decline

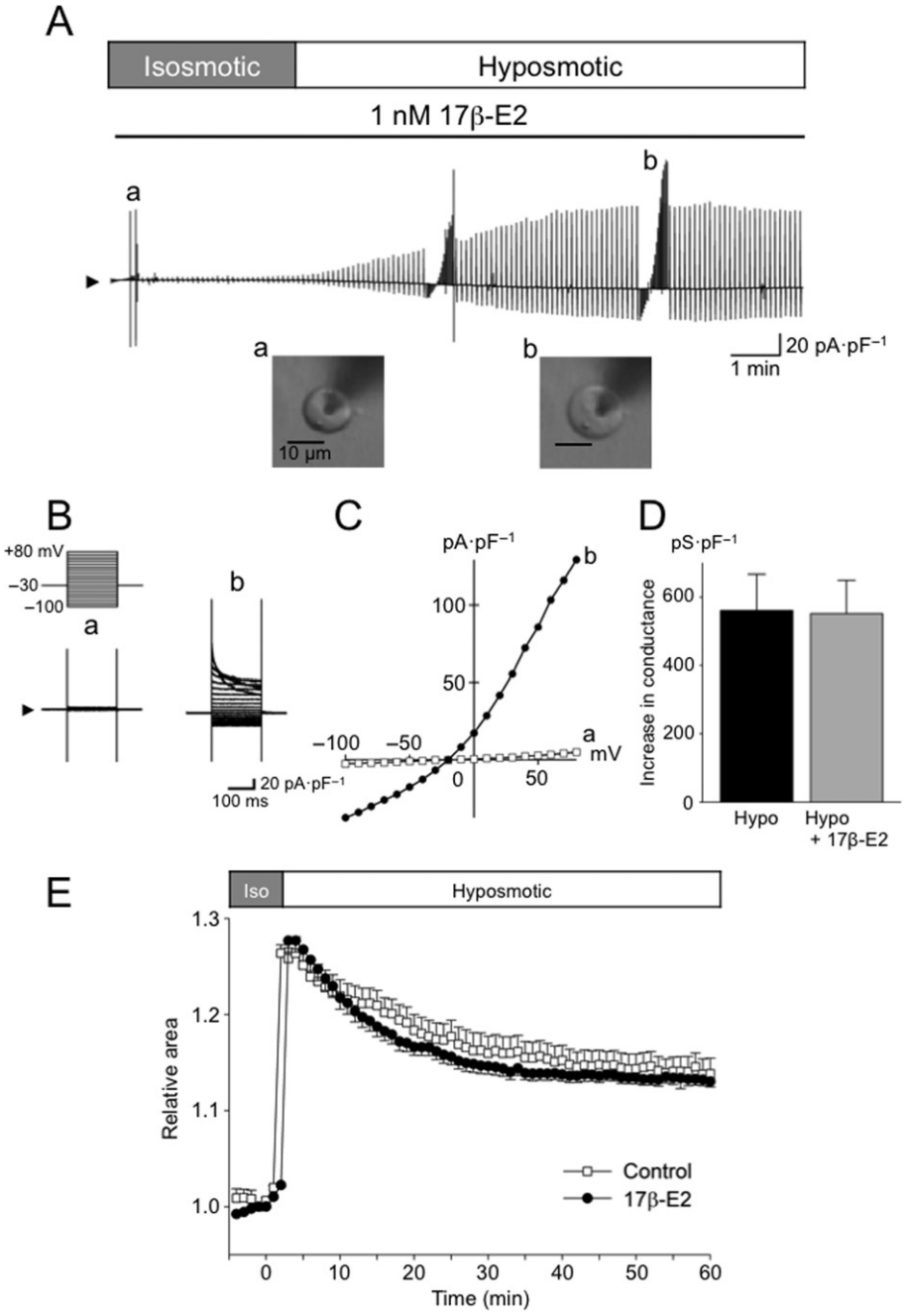

Figure 4

Lack of effects of 17β-oestradiol on the $I_{Cl,vol}$ activation by cell swelling. (A) Time course of changes in whole-cell current recorded from a chondrocyte during switching from isosmotic to hyposmotic external solution in the continuous presence of 17β-oestradiol (1 nM). 17β-oestradiol was added to the bath (isosmotic solution) approximately 10 min before switching to hyposmotic solution. Inset shows microscopic images of whole-cell clamped chondrocyte taken before (a) and after (b) switching to hyposmotic solution, and diameter in each condition was calculated to be 13.3 and 16.1 μm, respectively. (B) Superimposed current traces in response to 200-ms square steps applied from a holding potential of −30 mV to potentials of +80 through −100 mV in 10 mV steps, in isosmotic (a) and hyposmotic (b) solutions. (C) *I–V* relationships of membrane currents in isosmotic (a) and hyposmotic (b) solutions, shown in (B). (D) Increased conductance of $I_{Cl,vol}$ recorded without and with 17β-oestradiol pretreatment. There was no significant difference in the degree of $I_{Cl,vol}$ activation between these two groups (561.0 ± 100.6 pS·pF^{-1}, $n = 9$, $N = 5$ versus 551.9 ± 91.5 pS·pF^{-1}, $n = 8$, $N = 5$; $P = 0.50$). (E) Time course of changes in cross-sectional area during hyposmotic challenge without (Control) and with 17β-oestradiol (1 nM) pretreatment. Data points represent means and SEM of 15 ($N = 3$) and 18 ($N = 3$) chondrocytes without and with 17β-oestradiol pretreatment, respectively.

(1.13 ± 0.01). As is evident in the continuous measurement of cell size during exposure to a hyposmotic external solution, 17β-oestradiol had no appreciable effect on the time course and degree of RVD in rabbit articular chondrocytes (Figure 4E).

Doxorubicin-induced AVD and its inhibition by 17β-oestradiol

It has been demonstrated in various cell types that exposure to doxorubicin induces a decrease in cell size leading to apoptosis (designated as AVD), which is mediated through activation of $I_{Cl,Dox}$ (d'Anglemont de Tassigny *et al.*, 2004). To elucidate the functional significance of $I_{Cl,Dox}$ in articular chondrocytes, the effect of doxorubicin on cell size was examined in the absence and presence of DCPIB, a compound that potently blocks $I_{Cl,Dox}$ (Figure 2C). As illustrated in Figure 5A, addition of doxorubicin to isosmotic solution led to a gradual decrease in relative cell size (0.94 ± 0.01, $n = 6$, $N = 3$) over a period of 30 min, thus confirming that the size of the cell decreases in the presence of doxorubicin. The concomitant addition of 20 μM DCPIB completely abolished the doxorubicin-induced decrease in cell size (1.01 ± 0.01, $n = 6$, $N = 3$), suggesting that $I_{Cl,Dox}$ is primarily involved in mediating this effect of doxorubicin. Consistent with this view, 17β-oestradiol also completely inhibited the doxorubicin-induced decrease in cell size, and this effect of 17β-oestradiol was almost totally antagonized by the concomitant presence of ICI 182780. Taken together with the results shown in Figure 3, these observations indicate that 17β-oestradiol inhibited the activation of $I_{Cl,Dox}$ through its membrane receptor and thereby prevented the decrease in cell size induced by doxorubicin.

Previously it was demonstrated that caspase-3/7 activity, which is a dominant effector for final apoptotic cell death (Nicholson and Thornberry, 1997; Garcia-Calvo *et al.*, 1999), is appreciably elevated for some time (e.g. 24 h) after exposure to doxorubicin (Mukhopadhyay *et al.*, 2009). To examine whether the decrease in cell size induced by doxorubicin leads to apoptosis, caspase-3/7 activity was measured in chondrocytes exposed for 24 h to doxorubicin (1 μM) without and with DCPIB (20 μM), 17β-oestradiol (1 nM) and/or ICI 182780 (10 μM). As expected, caspase-3/7 activity was markedly elevated by exposure to doxorubicin (Figure 5B), indicating that the apoptotic signal was indeed evoked in chondrocytes by doxorubicin. This elevation of caspase-3/7 activity was completely abolished by DCPIB, indicating that activation of $I_{Cl,Dox}$ and resultant decrease in cell size are essential for the elevation of caspase-3/7 activity. Furthermore, addition of 17β-oestradiol also completely eliminated the elevation of caspase-3/7 activity, an effect almost totally antagonized by ICI 182780. These observations suggest that the inhibitory effect of 17β-oestradiol on $I_{Cl,Dox}$, through its membrane receptor (Figure 3), contributes its attenuation of the doxorubicin-induced elevation of caspase-3/7 activity.

ROS mediate doxorubicin-induced activation of $I_{Cl,Dox}$

Previous studies have demonstrated that doxorubicin induces intracellular production of ROS, such as superoxide anion and hydrogen peroxide in cardiomyocytes, endothelial cells and several cultured cell lines (Kalyanaraman *et al.*, 2002; Tsang *et al.*, 2003; Mizutani *et al.*, 2005; Wagner *et al.*, 2005). In addition, hydrogen peroxide was shown to activate $I_{Cl,vol}$ in some cell types, such as rabbit ventricular myocytes and some cell lines (Browe and Baumgarten, 2004; Varela *et al.*, 2004; Deng *et al.*, 2010). We therefore examined the possibility that ROS are involved in mediating the activation of $I_{Cl,Dox}$ in chondrocytes. As demonstrated in Figure 6A, pretreatment with the ROS scavenger NAC (Bernard *et al.*, 1984; Chen *et al.*, 1995) totally eliminated the activation of $I_{Cl,Dox}$, indicating that activation of $I_{Cl,Dox}$ is mediated through intracellular ROS formation.

Since sources of ROS may include membrane-associated nicotinamide adenine dinucleotide phosphate (NAD(P)H) oxidase, we checked the functional role of NAD(P)H oxidase in the activating process of $I_{Cl,dox}$. As shown in Figure 6B, pretreatment with the potent NAD(P)H oxidase inhibitor DPI 20 μM (Hampton and Winterbourn, 1995) completely eliminated the activation of $I_{Cl,Dox}$, suggesting that the NAD(P)H oxidase system is involved in the activation of $I_{Cl,Dox}$, through the production of ROS. Taken together, it is reasonable to assume that NAD(P)H oxidase-derived ROS mediates the activation of $I_{Cl,Dox}$.

17β-Oestradiol affects the PI3K signalling pathway that is upstream of ROS production to inhibit $I_{Cl,Dox}$

We next addressed the question as to whether 17β-oestradiol affects signalling pathways upstream or downstream of ROS production to inhibit the activation of $I_{Cl,Dox}$ in rabbit chondrocytes. As demonstrated in Figure 7A, bath application of hydrogen peroxide (100 μM) gradually increased the membrane current despite the continued presence of 17β-oestradiol (1 nM), which had electrophysiological properties almost similar to those of $I_{Cl,Dox}$, such as an appreciable inactivation at strong depolarized potentials (Figure 7B) and an outwardly rectifying *I–V* relationship with a reversal potential near E_{Cl} (Figure 7C). The hydrogen peroxide-induced activation of $I_{Cl,Dox}$, as estimated by an increase in slope conductance near E_{Cl}, was not affected by pretreatment with 17β-oestradiol (control, 489.6 ± 50.1 pS·pF^{-1} $n = 4$, $N = 4$; 17β-oestradiol, 462.2 ± 62.9 pS·pF^{-1}, $n = 4$, $N = 4$; $P = 0.27$, Figure 7D). These observations clearly show that 17β-oestradiol fails to prevent the $I_{Cl,Dox}$ activation by exogenously applied hydrogen peroxide and indicate that 17β-oestradiol inhibits the activation of $I_{Cl,Dox}$ by affecting signalling steps prior to ROS generation.

There are a number of studies indicating that PI3K is involved in the prosurvival effect of 17β-oestradiol, mediated through oestrogen receptors (Castoria *et al.*, 2001; Björnström and Sjöberg, 2005; Kim *et al.*, 2006). To elucidate whether PI3K activity mediates the inhibitory action of 17β-oestradiol on $I_{Cl,Dox}$, we examined the effect of two structurally unrelated PI3K inhibitors, namely wortmannin and LY294002 (Feranchak *et al.*, 1998; Schliess *et al.*, 2001; Browe and Baumgarten, 2006; Olsen *et al.*, 2007). Figure 8A and B illustrate representative experiments examining the effect of 17β-oestradiol on $I_{Cl,Dox}$ in the presence of wortmannin (100 nM) and LY294002 (20 μM), respectively. As summarized in Figure 8C, the inhibition of $I_{Cl,Dox}$ by 17β-oestradiol

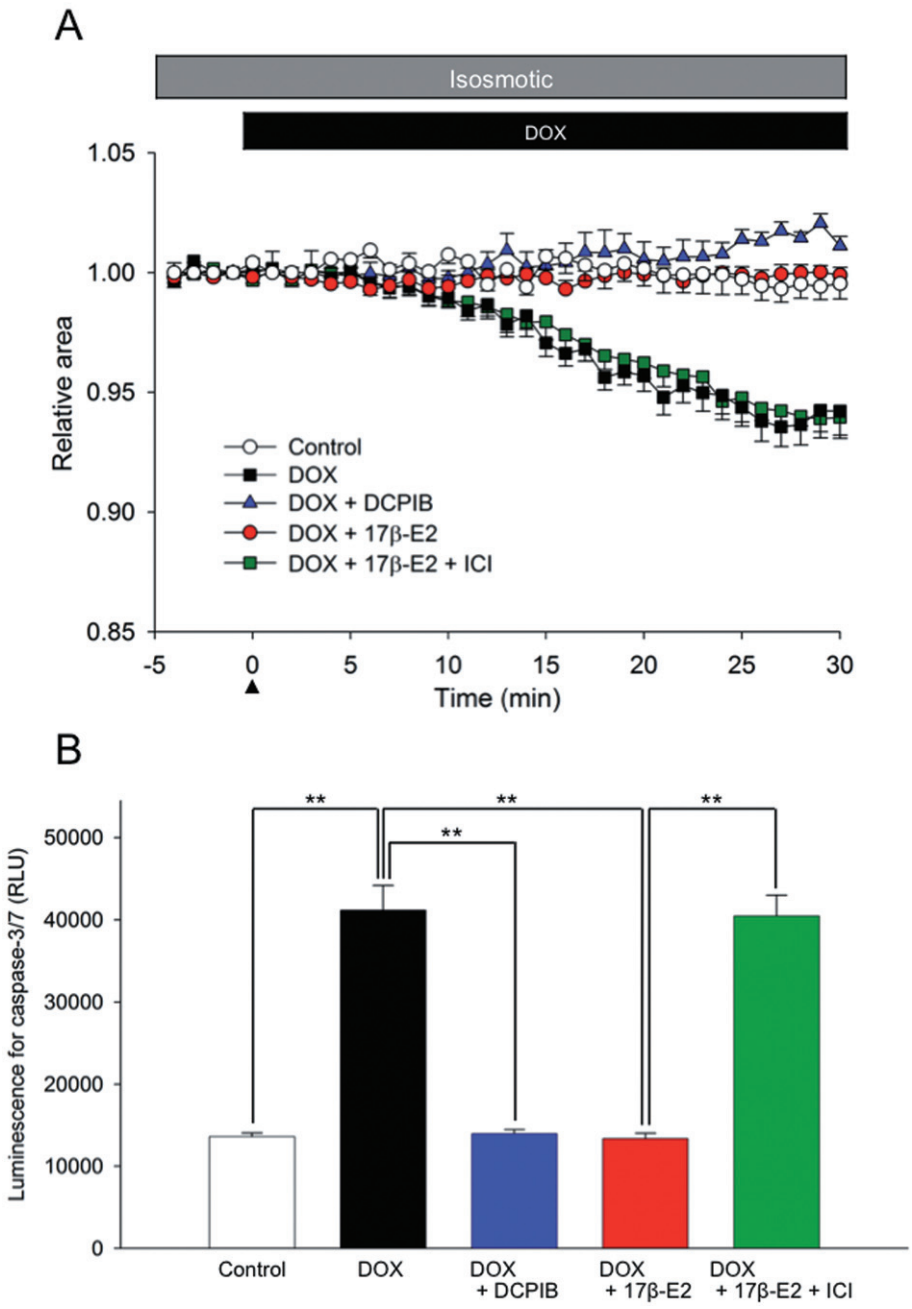

Figure 5

Doxorubicin-induced AVD and caspase-3/7 activity in chondrocytes. (A) Time course of changes in cross-sectional area of cell (chondrocyte) image in the absence (Control) and presence of doxorubicin (1 μM) applied without or with DCPIB (20 μM), 17β-oestradiol (1 nM) and/or ICI 182780 (10 μM), as indicated. All these compounds were added to the isosmotic solution at time 0. (B) Caspase-3/7 activity, measured from chondrocytes after 24 h exposure to doxorubicin (1 μM) without or with DCPIB (20 μM), 17β-oestradiol (1 nM) and/or ICI 182780 (10 μM). Asterisks represent *P* values according to Newman–Keuls multiple means comparison test (**$P < 0.01$).

(90.6 ± 5.4% decrease, $n = 6$, $N = 2$) was significantly attenuated by 100 nM wortmannin (31.4 ± 16.2% decrease, $n = 8$, $N = 2$, $P < 0.05$) or 20 μM LY294002 (56.9 ± 14.3% decrease, $n = 4$, $N = 2$, $P < 0.05$). These results indicate that PI3K is involved in mediating the inhibitory action of 17β-oestradiol on $I_{Cl,Dox}$.

We further checked the effect of wortmannin and LY294002 on caspase-3/7 activity in the presence of doxorubicin and 17β-oestradiol (Figure 8D). As expected, doxorubicin-induced increase in caspase-3/7 activity was completely abolished by the concomitant presence of 17β-oestradiol, and both wortmannin (100 nM) and LY294002

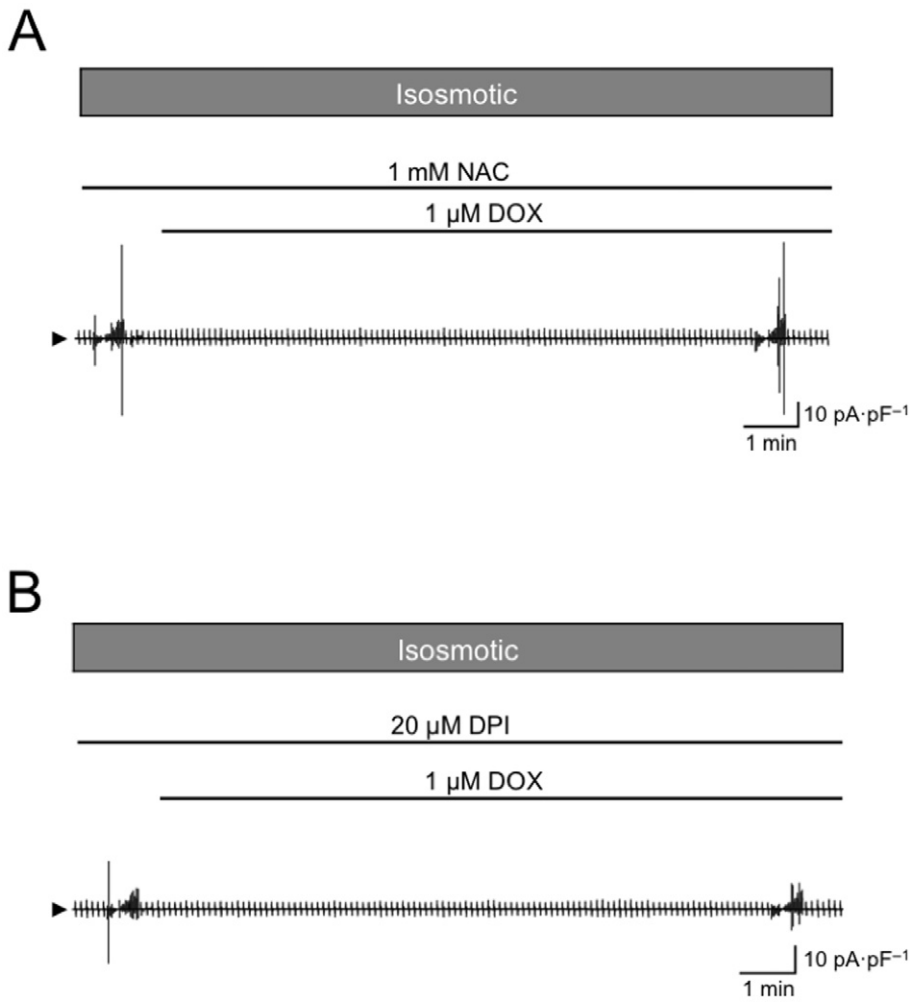

Figure 6

Involvement of reactive oxygen species in the activation of $I_{Cl,Dox}$. (A) and (B) Abolishment of $I_{Cl,Dox}$ activation by pretreatment with the ROS scavenger *N*-acetyl-cysteine (NAC) at 1 mM (A) or the general inhibitor of NAD(P)H oxidase diphenylene-iodonium chloride (DPI) at 20 μM (B). *N*-acetyl-cysteine (A) or diphenylene-iodonium chloride (B) was added approximately 10 min before the application of doxorubicin (DOX, 1 μM) and was present throughout the experiments.

(20 μM) significantly reversed this effect of 17β-oestradiol on doxorubicin-induced caspase-3/7 activity.

Because female hormones are normally present in the organism of either sex, it is important to examine the effect of a longer pre-incubation with 17β-oestradiol on the doxorubicin-induced changes in membrane currents, cell size and caspase-3/7 activity. In the experiments shown in Figures 9 and 10, chondrocytes were pre-incubated with 17β-oestradiol (1 nM) for 24 h and were then examined either during the continued presence of the hormone or after 1 h of washing out the hormone. As depicted in Figure 9A, doxorubicin (1 μM) failed to activate any $I_{Cl,Dox}$ during the continued presence of 17β-oestradiol (1 nM) in chondrocytes pre-incubated for 24 h. On the other hand, when 17β-oestradiol (1 nM) was washed out for 1 h after a 24 h of pre-incubation, doxorubicin (1 μM) activated $I_{Cl,Dox}$ (Figure 9B). As summarized in Figure 9C, the longer (24 h) pre-incubation with 17β-oestradiol was effective at preventing the activation of $I_{Cl,Dox}$, but the $I_{Cl,Dox}$ activation had recovered after 1 h of washing out the hormone to a level (552.5 ± 44.3 pS·pF^{-1}, $n = 5$, $N = 3$) equivalent to that of $I_{Cl,Dox}$ activation in chondrocytes without pre-incubation with 17β-oestradiol (576.1 ± 49.3 pS·pF^{-1}, $n = 15$, $N = 5$; refer to Figure 3E).

As demonstrated in Figure 10A, doxorubicin (1 μM)-induced decrease in cell size in isosmotic solution was eliminated by the continued presence of 17β-oestradiol (1 nM) in chondrocytes pre-incubated for 24 h (1.01 ± 0.01, $n = 10$, $N = 3$; DOX + 17β-E2). On the other hand, there was a gradual decrease in cell size over 30 min (0.94 ± 0.01, $n = 15$, $N = 3$) during exposure to doxorubicin in chondrocytes when 17β-oestradiol was washed out for 1 h after 24 h of pre-incubation with the hormone. Similarly, doxorubicin-induced elevation of caspase-3/7 activity was greatly reduced in chondrocytes pre-incubated with 17β-oestradiol for 24 h, and this inhibitory effect was reversed 1 h after washing out the hormone.

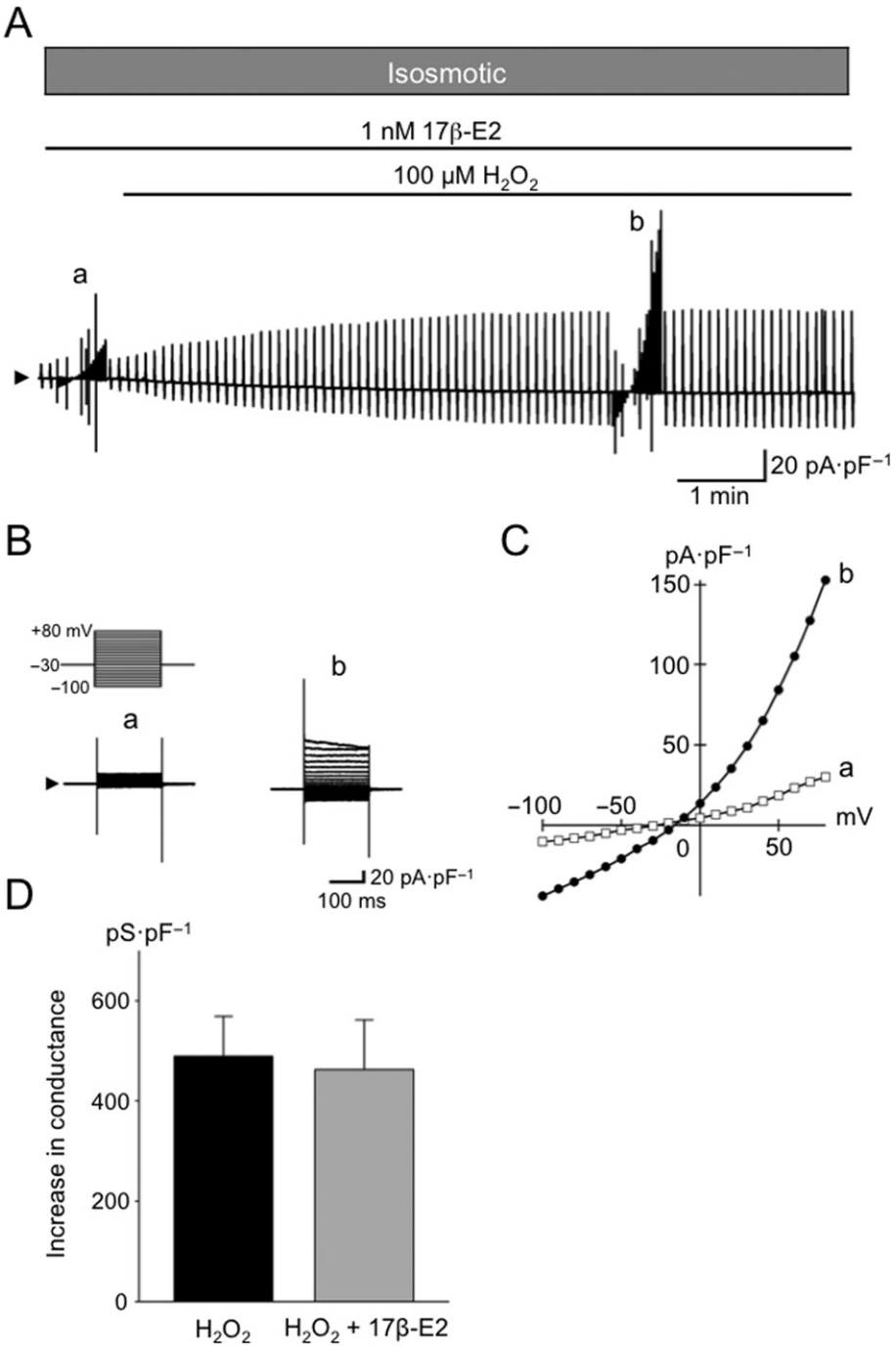

Figure 7

Lack of effects of 17β-oestradiol on $I_{Cl,vol}$ activation induced by hydrogen peroxide. (A) Time course of changes in whole-cell current during exposure to hydrogen peroxide (100 μM) in isosmotic solution in a chondrocyte pretreated with 17β-oestradiol (1 nM). 17β-Oestradiol was added to isosmotic solution approximately 10 min before exposure to hydrogen peroxide and was present throughout the experiments. (B) Superimposed current traces in response to 200-ms square steps applied from a holding potential of −30 mV to test potentials of +80 through −100 mV in 10 mV steps before (a) and during (b) exposure to hydrogen peroxide (100 μM) in the presence of 17β-oestradiol (1 nM). (C) *I–V* relationships for whole-cell currents recorded at each time points (a and b) in (B). (D) Maximal increase in the slope conductance for $I_{Cl,Dox}$ without and with pretreatment of 17β-oestradiol (1 nM). There was no significant difference between the control ($n = 4$, $N = 4$) and 17β-oestradiol ($n = 4$, $N = 4$) groups ($P = 0.27$).

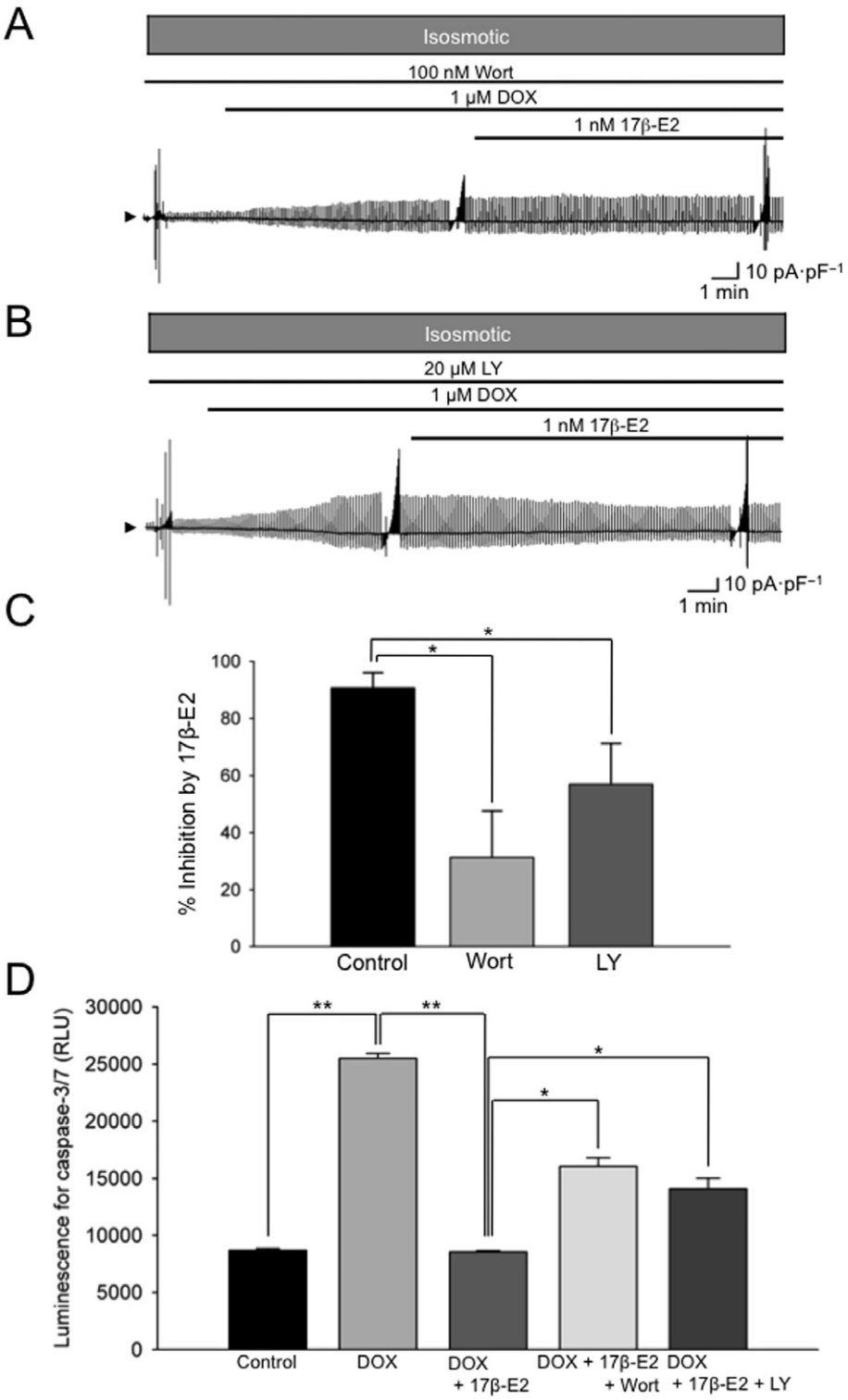

Figure 8

Involvement of PI3-kinase activity in the inhibitory effect of 17β-oestradiol on of $I_{Cl,Dox}$. (A) and (B) The effect of pretreatment with 100 nM wortmannin (Wort, A) or 20 μM LY294002 (LY, B) on the inhibitory effect of 17β-oestradiol (1 nM) on $I_{Cl,Dox}$. Various test compounds were added to isosmotic bath solutions, as indicated. (C) Percentage inhibition of $I_{Cl,Dox}$ by 17β-oestradiol without and with pretreatment with wortmannin and LY294002. (D) Caspase-3/7 activity was measured after 24 h treatment without (Control) and with doxorubicin in the absence and presence of 17β-oestradiol, wortmannin (100 nM) and/or LY294002 (20 μM). Asterisks represent *P* values according to Newman–Keuls multiple means comparison test (*$P < 0.05$, **$P < 0.01$).

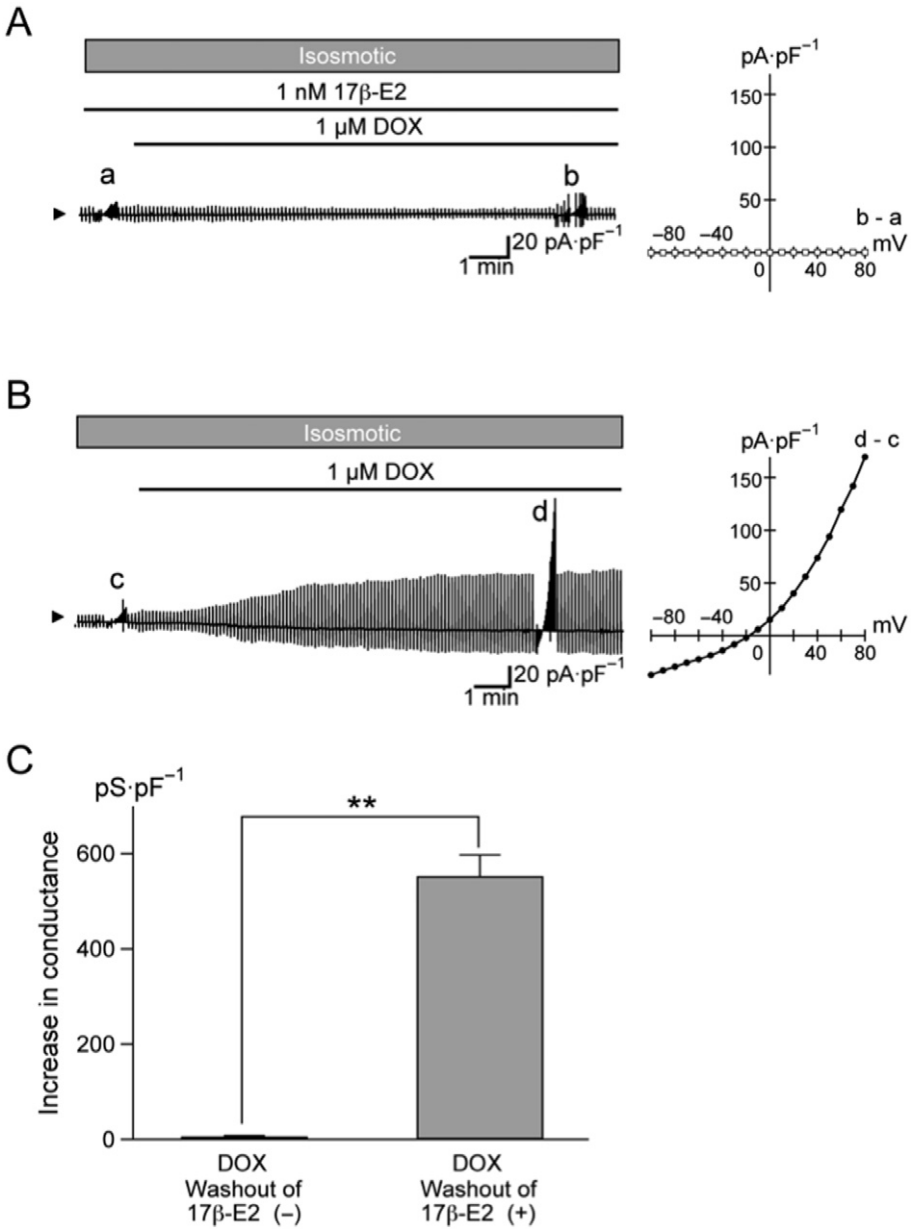

Figure 9

Effect of a longer pre-incubation with 17β-oestradiol on the activation of $I_{Cl,Dox}$ in chondrocytes. (A) Abolishment of $I_{Cl,Dox}$ activation in chondrocytes pre-incubated with 17β-oestradiol for 24 h without washing out (17β-oestradiol was present throughout the experiment). (B) Activation of $I_{Cl,Dox}$ in chondrocytes when 17β-oestradiol was washed out for 1 h after 24 h of pre-incubation. Insets (right) in (A and B) show the *I–V* relationship of difference current representing the doxorubicin (1 μM)-evoked current. (C) Increase in conductance of $I_{Cl,Dox}$ in chondrocytes pre-incubated with 17β-oestradiol for 24 h without (6.6 ± 1.7 pS·pF^{-1}, $n = 5$, $N = 3$) and with (552.5 ± 44.3 pS·pF^{-1}, $n = 5$, $N = 3$) washing out the hormone for 1 h. $^{**}P < 0.01$ between the two groups (Student's unpaired *t*-test).

We finally investigated whether the male hormone has a similar inhibitory action on $I_{Cl,Dox}$ in articular chondrocytes. In the experiments shown in Figure 11, the effect of testosterone 10 nM on $I_{Cl,Dox}$ was examined; this concentration has previously been shown to be effective at exerting a modulatory action on ion channel activity (Yang *et al.*, 2010). Activation of $I_{Cl,Dox}$ was not affected by subsequent application of 10 nM testosterone (Figure 11A, B and C); this was confirmed in five different chondrocytes (Figure 11D).

Discussion

Activation of $I_{Cl,vol}$ by doxorubicin mediated via ROS production and its contribution to AVD and subsequent caspase-3/7 elevation

Electrophysiological (high Cl^- selectivity, outward rectification in the presence of equivalent concentrations of Cl^-, inactivation at strong depolarizations and abolishment by

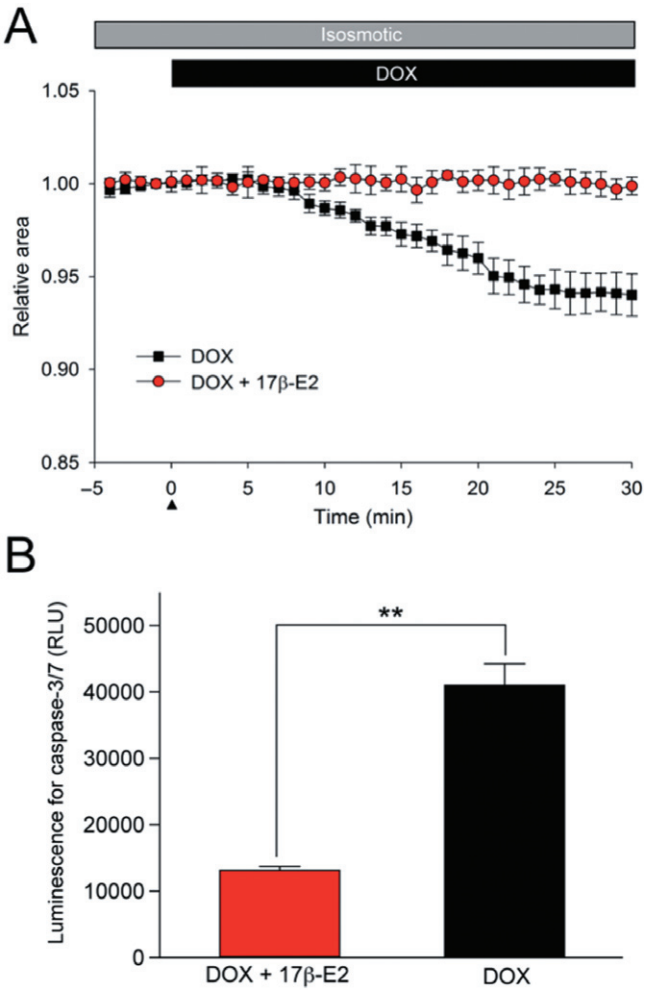

Figure 10

Effect of longer pre-incubation with 17β-oestradiol on the doxorubicin-induced changes in cell size and caspase-3/7 activity. (A) Changes in cell size (as assessed by relative area) during exposure to doxorubicin (1 μM) in chondrocytes pre-incubated with 17β-oestradiol for 24 h without washing out the hormone (17β-oestradiol was present throughout the experiment) or with washing out for 1 h (17β-oestradiol was absent during the measurement). (B) Caspase-3/7 activity in chondrocytes pre-incubated with 17β-oestradiol for 24 h without and with washing out the hormone for 1 h. $^{**}P < 0.01$ between the two groups (Student's unpaired *t*-test).

hyperosmolarity) and pharmacological properties (inhibition by DCPIB and arachidonic acid) of the doxorubicin-evoked current ($I_{Cl,Dox}$) were almost identical to those of the volume-sensitive Cl^- current ($I_{Cl,vol}$) found in the same cell types (Isoya *et al.*, 2009; Okumura *et al.*, 2009). The cystic fibrosis transmembrane regulator (CFTR) Cl^- channels are characterized by linear *I–V* relationship in symmetrical Cl^- (Hume *et al.*, 2000). The ClC Cl^- channel family is resistant to blockade by DCPIB at 10 μM (Decher *et al.*, 2001). It is thus unlikely that CFTR and ClC Cl^- channels are responsible for $I_{Cl,Dox}$. The intracellular Ca^{2+} was buffered to very low levels (in the order of 10^{-10} M) with the Ca^{2+} chelator EGTA at 5 mM in the pipette solution and with no added Ca^{2+} to the bath, which may rule out the possible involvement of the anoctamin/TMEM16 family that is proposed to form the Ca^{2+}-activated Cl^- channels (Hartzell *et al.*, 2009). Taken together, it appears most likely that $I_{Cl,vol}$ is activated by doxorubicin under isosmotic conditions without appreciable changes in cell size. Doxorubicin has also been shown to evoke activation of $I_{Cl,vol}$ under isosmotic conditions in rabbit ventricular myocytes (d'Anglemont de Tassigny *et al.*, 2004).

Various cell types have been reported to produce hydrogen peroxide on exposure to doxorubicin and this is mediated, at least partly, through a mechanism involving NADPH oxidase activation (Kalyanaraman *et al.*, 2002; Tsang *et al.*, 2003; Mizutani *et al.*, 2005; Wagner *et al.*, 2005). The present findings are also consistent with the view that NADPH oxidase-mediated formation of hydrogen peroxide is primarily involved in isosmotic activation of $I_{Cl,vol}$ ($I_{Cl,Dox}$) in rabbit articular chondrocytes. A similar activation of $I_{Cl,vol}$ by NAD(P)H oxidase-derived hydrogen peroxide has been observed in several cell types, such as hepatoma tissue culture cells, HeLa cells and rabbit ventricular myocytes (Browe and Baumgarten, 2004; 2006; Varela *et al.*, 2004; Deng *et al.*, 2010). Several mechanisms have been proposed to account for the isosmotic activation of $I_{Cl,vol}$ by doxorubicin without cell swelling. It seems likely that hydrogen peroxide acts as a second messenger to mediate doxorubicin-induced activation of $I_{Cl,vol}$, probably by shifting the intracellular phosphorylation/dephosphorylation balance towards a more phosphorylated state (Varela *et al.*, 2004). Alternatively, a change in the threshold cell volume for the activation of $I_{Cl,vol}$ (termed the volume set point; Cannon *et al.*, 1998; Hoffmann, 2000) has been proposed to explain the isosmotic activation of $I_{Cl,vol}$ by other pharmacological interventions (Voets *et al.*, 1998). For example, evidence has been obtained suggesting that an enhanced tyrosine phosphorylation results in the decrease in the volume set point and thereby produces the isosmotic activation of $I_{Cl,vol}$ in Ehrlich ascites tumour cells (Hoffmann, 2000) and articular chondrocytes (Okumura *et al.*, 2009).

Doxorubicin evokes apoptosis in various cell types, as evidenced by an elevation in caspase activity and/or induction of DNA fragmentation (Kalyanaraman *et al.*, 2002; Tokudome *et al.*, 2002; Mizutani *et al.*, 2005). Although it is generally accepted that a crucial step for the induction of apoptosis involves a decrease in cell volume, referred to as AVD (Maeno *et al.*, 2000; Okada *et al.*, 2009), only a limited amount of experimental evidence has been obtained to show that doxorubicin actually causes a cell volume decrease associated with AVD (d'Anglemont de Tassigny *et al.*, 2004), eventually leading to an increase in caspase activity and cell apoptosis. The present study clearly demonstrates that doxorubicin rapidly decreases cell size, within minutes, and this is accompanied by a subsequent increase in caspase-3/7 activity; both of these effects were completely abolished by the $I_{Cl,vol}$ inhibitor DCPIB. These findings strongly suggest that the activation of $I_{Cl,vol}$ contributes to the doxorubicin-induced AVD in articular chondrocytes.

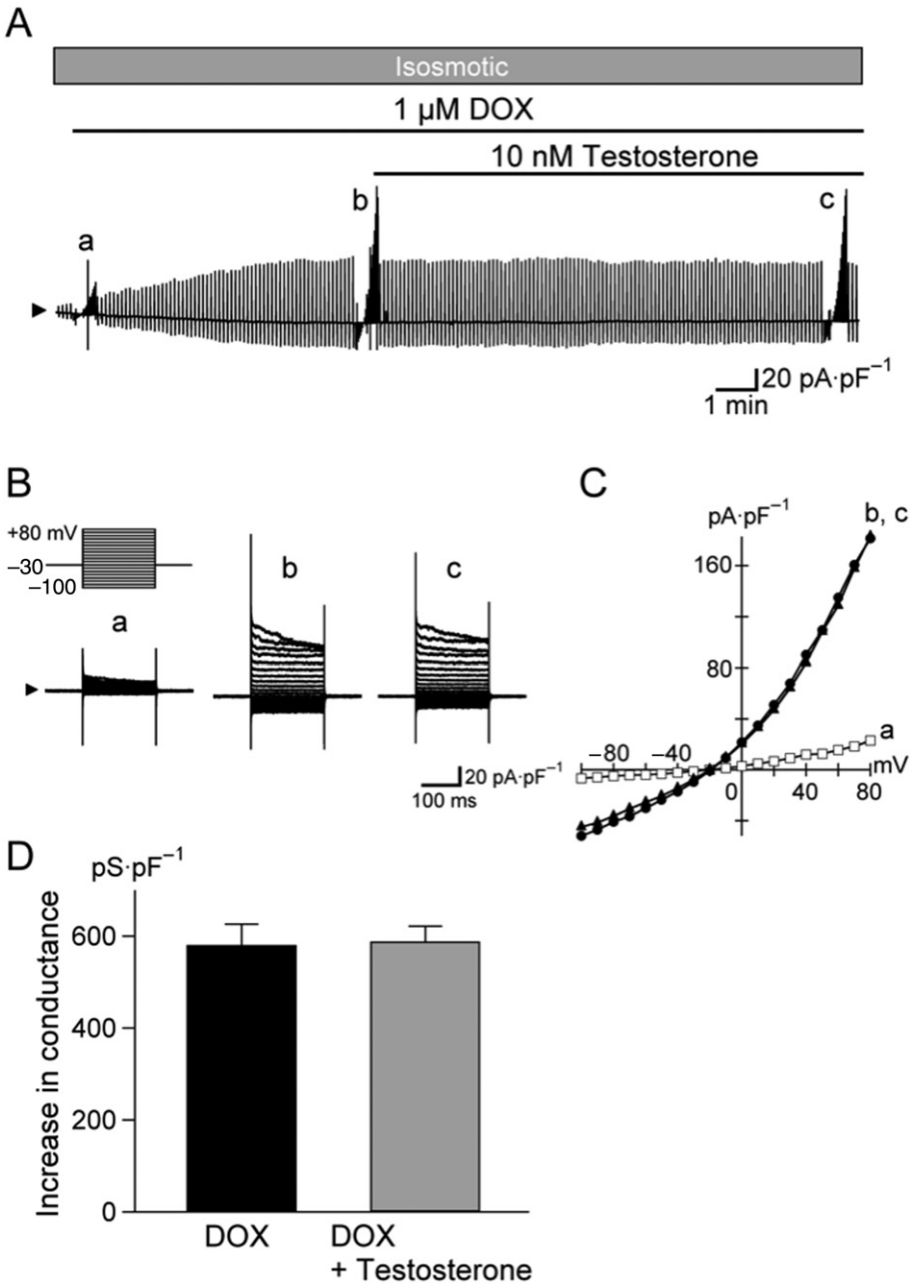

Figure 11

Lack of inhibitory effect of testosterone on $I_{Cl,Dox}$. (A) Time course of changes in whole-cell current recorded from a chondrocyte that was initially exposed to doxorubicin (DOX, 1 μM) and subsequently to doxorubicin plus testosterone (10 nM) in isosmotic solution. (B) Superimposed current traces in response to 200 ms square-steps applied from a holding potential of −30 mV to test potentials of +80 through −100 mV in 10 mV steps, at the time points identified by letters (a, b and c) in (A). (C) *I–V* relationships of whole-cell currents recorded at each time point (a, b and c) shown in (B). (D) Increased conductance of $I_{Cl,Dox}$ measured at a steady-state activation (DOX) and 10 min after subsequent addition of 10 nM testosterone (DOX + Testosterone). There was no significant difference in the degree of $I_{Cl,Dox}$ activation between these two conditions (580.9 ± 45.4 pS·pF^{-1} versus 588.4 ± 32.9 pS·pF^{-1}, $n = 5$, $N = 3$; $P = 0.84$).

17β-Oestradiol prevents the doxorubicin-induced apoptosis by blocking the activation of $I_{Cl,vol}$ via membrane receptors (non-genomic pathways)

Pretreatment with 17β-oestradiol for either short (approximately 10 min) or long (24 h) periods prevented the doxorubicin-induced elevation of caspase-3/7 activity; an effect which appears to be mediated through inhibition of the $I_{Cl,Dox}$ responsible for AVD. This anti-apoptotic action of 17β-oestradiol can also be ascribed to a receptor-mediated non-genomic pathway that includes PI3K activation. The signalling step at which 17β-oestradiol exerts its inhibitory action against doxorubicin-induced apoptosis is likely to be

upstream of hydrogen peroxide production. In neonatal rat cardiomyocytes it has been demonstrated that stimulation of plasma membrane oestrogen receptor suppresses ROS production mediated through PI3K activation, and thereby prevents subsequent apoptosis during ischaemia/reperfusion stress (Kim *et al.*, 2006), thus showing that PI3K activation is associated with ROS suppression. More recently, oestrogens have been found to suppress the oxidative stress in bone and bone marrow via a non-genomic action, and decrease the prevalence of mature osteoblast apoptosis (Almeida *et al.*, 2010).

On the other hand, 17β-oestradiol was found to have no appreciable effect on the hyposmotic activation of $I_{Cl,vol}$ or on the time course and degree of RVD in hyposmotic conditions. Articular chondrocytes are exposed *in vivo* to a continually changing osmotic environment, but can readily restore the normal cell volume through volume regulatory mechanisms, namely regulatory volume increase and RVD (Hall, 1995; Bush and Hall, 2001). Because $I_{Cl,vol}$ plays a functionally important role in the process of RVD (Okumura *et al.*, 2009), the possible inhibition of $I_{Cl,vol}$ activated during hyposmotic cell swelling would jeopardize the volume regulatory function of articular chondrocytes in response to hyposmotic stress. However, as judged from our results, hyposmotic activation $I_{Cl,vol}$ and resultant RVD are not expected to be inhibited by 17β-oestradiol. Future studies should elucidate the precise mechanism underlying these differential actions of 17β-oestradiol on isosmotic and hyposmotic activation of $I_{Cl,vol}$ in articular chondrocytes.

In recent years, much evidence has accumulated to strongly suggest that some of the biological actions of 17β-oestradiol are mediated through its receptors near or in the plasma membrane, referred to as non-genomic action (Falkenstein *et al.*, 2000; Kousteni *et al.*, 2001; Björnström and Sjöberg, 2005). These include modulation of ion channel function (Kurokawa *et al.*, 2008). However, whereas the genomic action of 17β-oestradiol takes place in the order of hours to days through gene transcription, its non-genomic action occurs rapidly, within minutes (Falkenstein *et al.*, 2000; Kousteni *et al.*, 2001; Björnström and Sjöberg, 2005). The present findings that the inhibitory action of 17β-oestradiol on the doxorubicin-induced $I_{Cl,vol}$ reaches a steady state within 10 min is also consistent with 17β-oestradiol acting on membrane receptors through a non-genomic effect. It is also important to note that a longer (24 h) pre-incubation with 17β-oestradiol also resulted in the same inhibitory effect on the activation of $I_{Cl,Dox}$ associated with development of AVD and elevation of caspase-3/7 activity. This suggests that the inhibitory action of 17β-oestradiol is preserved during the continued presence of 17β-oestradiol, such as in *in vivo* conditions.

Testosterone has also been shown to exhibit non-genomic actions, in addition to genomic actions, and to activate various signalling pathways (Kousteni *et al.*, 2001; Gatson *et al.*, 2006). However, we did not detect any effect of testosterone on $I_{Cl,Dox}$ activation, which suggests that testosterone does not have an antioxidant effect on chondrocytes.

Physiological and pathophysiological role for preventive action of 17β-oestradiol on $I_{Cl,vol}$

Evidence is accumulating that the most common degenerative joint disease OA is associated with chondrocyte apoptosis (Blanco *et al.*, 1998; Hashimoto *et al.*, 1998; Lotz *et al.*, 1999; Mobasheri, 2002), and the incidence of OA is increased in postmenopausal women and is associated with oestrogen deficiency (Gokhale *et al.*, 2004; Takano *et al.*, 2007; Sniekers *et al.*, 2008; Roman-Blas *et al.*, 2009). The present findings showing that 17β-oestradiol blocks $I_{Cl,vol}$ associated with AVD suggest that 17β-oestradiol could have a preventive effect against OA at least partly by suppressing chondrocyte apoptosis caused by AVD. This beneficial effect of 17β-oestradiol might be responsible for the low incidence of OA in women until menopause, but higher incidence after menopause. However, because in the present experiments, chondrocyte apoptosis was induced by pharmacological (doxorubicin) interventions, the results cannot be directly extrapolated to chondrocyte apoptosis associated with OA in humans. Future studies are needed to examine whether and how 17β-oestradiol has a favourable action against chondrocyte apoptosis evoked by pathophysiological stimuli such as IL-1 and TNF-α in humans.

In conclusion, the present findings suggest that 17β-oestradiol exerts an inhibitory action on Cl^- current associated with AVD, and thereby prevents apoptosis through a membrane receptor-mediated non-genomic pathway. This anti-apoptosis action of 17β-oestradiol in chondrocytes could account for the increased incidence of OA in postmenopausal women.

Acknowledgement

This study was supported by a Grant-in-Aid for Scientific Research from the Japan Society for the Promotion of Science.

Conflicts of interest

None.

References

Adams CS, Shapiro IM (2002). The fate of the terminally differentiated chondrocyte: evidence for microenvironmental regulation of chondrocyte apoptosis. Crit Rev Oral Biol Med 13: 465–473.

Alexander SPH, Mathie A, Peters JA (2011). Guide to Receptors and Channels (GRAC), 5th Edition. Br J Pharmacol 164 (Suppl. 1): S1–S324.

Almeida M, Martin-Millan M, Ambrogini E, Bradsher R 3rd, Han L, Chen X-D *et al.* (2010). Estrogens attenuate oxidative stress and the differentiation and apoptosis of osteoblasts by DNA-binding-independent actions of the ERα. J Bone Miner Res 25: 769–781.

d'Anglemont de Tassigny A, Souktani R, Henry P, Ghaleb B, Berdeaux A (2004). Volume-sensitive chloride channels ($I_{Cl,vol}$) mediate doxorubicin-induced apoptosis through apoptotic volume decrease in cardiomyocytes. Fundam Clin Pharmacol 18: 531–538.

Bénitah JP, Gomez AM, Bailly P, Da Ponte JP, Berson G, Delgado C *et al.* (1993). Heterogeneity of the early outward current in ventricular cells isolated from normal and hypertrophied rat hearts. J Physiol 469: 111–138.

Bernard GR, Lucht WD, Niedermeyer ME, Snapper JR, Olgetree ML, Brigham KL (1984). Effects of N-acetylcysteine on pulmonary response to endotoxin in the awake sheep and upon in vitro granulocyte function. J Clin Invest 73: 1772–1784.

Björnström L, Sjöberg M (2005). Mechanisms of estrogen receptor signaling: convergence of genomic and nongenomic actions on target genes. Mol Endocrinol 19: 833–842.

Blanco FJ, Ochs RL, Schwarz H, Lotz M (1995). Chondrocyte apoptosis induced by nitric oxide. Am J Pathol 146: 75–85.

Blanco FJ, Guitian R, Vázquez-Martul E, de Toro FJ, Galdo F (1998). Osteoarthritis chondrocytes die by apoptosis. A possible pathway for osteoarthritis pathology. Arthritis Rheum 41: 284–289.

Bortner CD, Cidlowski JA (1998). A necessary role for cell shrinkage in apoptosis. Biochem Pharmacol 56: 1549–1559.

Bortner CD, Cidlowski JA (2007). Cell shrinkage and monovalent cation fluxes: role in apoptosis. Arch Biochem Biophys 462: 176–188.

Browe DM, Baumgarten CM (2004). Angiotensin II (AT1) receptors and NADPH oxidase regulate Cl^- current elicited by β1 integrin stretch in rabbit ventricular myocytes. J Gen Physiol 124: 273–287.

Browe DM, Baumgarten CM (2006). EGFR kinase regulates volume-sensitive chloride current elicited by integrin stretch via PI-3K and NADPH oxidase in ventricular myocytes. J Gen Physiol 127: 237–251.

Bush PG, Hall AC (2001). Regulatory volume decrease (RVD) by isolated and in situ bovine articular chondrocytes. J Cell Physiol 187: 304–314.

Cannon CL, Basavappa S, Strange K (1998). Intracellular ionic strength regulates the volume sensitivity of a swelling-activated anion channel. Am J Physiol 275: C416–C422.

Cao L, Lee V, Adams ME, Kiani C, Zhang Y, Hu W *et al.* (1999). β_1-Integrin-collagen interaction reduces chondrocyte apoptosis. Matrix Biol 18: 343–355.

Castoria G, Migliaccio A, Bilancio A, Di Domenico M, de Falco A, Lombardi M *et al.* (2001). PI3-kinase in concert with Src promotes the S-phase entry of oestradiol-stimulated MCF-7 cells. EMBO J 20: 6050–6059.

Chen W, Gabel S, Steenbergen C, Murphy E (1995). A redox-based mechanism for cardioprotection induced by ischemic preconditioning in perfused rat heart. Circ Res 77: 424–429.

Decher N, Lang HJ, Nilius B, Brüggemann A, Busch AE, Steinmeyer K (2001). DCPIB is a novel selective blocker of $I_{Cl,swell}$ and prevents swelling-induced shortening of guinea-pig atrial action potential duration. Br J Pharmacol 134: 1467–1479.

Deng W, Baki L, Baumgarten CM (2010). Endothelin signalling regulates volume-sensitive Cl^- current via NADPH oxidase and mitochondrial reactive oxygen species. Cardiovasc Res 88: 93–100.

Fabiato A, Fabiato F (1979). Calculator programs for computing the composition of the solutions containing multiple metals and ligands used for experiments in skinned muscle cells. J Physiol (Paris) 75: 463–505.

Falkenstein E, Tillmann HC, Christ M, Feuring M, Wehling M (2000). Multiple actions of steroid hormones – a focus on rapid, nongenomic effects. Pharmacol Rev 52: 513–555.

Feranchak AP, Roman RM, Schwiebert EM, Fitz JG (1998). Phosphatidylinositol 3-kinase contributes to cell volume regulation through effects on ATP release. J Biol Chem 273: 14906–14911.

Garcia-Calvo M, Peterson EP, Rasper DM, Vaillancourt JP, Zamboni R, Nicholson DW *et al.* (1999). Purification and catalytic properties of human caspase family members. Cell Death Differ 6: 362–369.

Gatson JW, Kaur P, Singh M (2006). Dihydrotestosterone differentially modulates the mitogen-activated protein kinase and the phosphoinositide 3-kinase/Akt pathways through the nuclear and novel membrane androgen receptor in C6 cells. Endocrinology 147: 2028–2034.

Goggs R, Carter SD, Schulze-Tanzil G, Shakibaei M, Mobasheri A (2003). Apoptosis and the loss of chondrocyte survival signals contribute to articular cartilage degradation in osteoarthritis. Vet J 166: 140–158.

Gokhale JA, Frenkel SR, Dicesare PE (2004). Estrogen and osteoarthritis. Am J Orthop 33: 71–80.

Hall AC (1995). Volume-sensitive taurine transport in bovine articular chondrocytes. J Physiol 484: 755–766.

Hamill OP, Marty A, Neher E, Sakmann B, Sigworth FJ (1981). Improved patch-clamp techniques for high-resolution current recording from cells and cell-free membrane patches. Pflugers Arch 391: 85–100.

Hampton MB, Winterbourn CC (1995). Modification of neutrophil oxidant production with diphenyleneiodonium and its effect on bacterial killing. Free Radic Biol Med 18: 633–639.

Hartzell HC, Yu K, Xiao Q, Chien L-T QZ (2009). Anoctamin/TMEM16 family members are Ca^{2+}-activated Cl^- channels. J Physiol 587: 2127–2139.

Hashimoto S, Ochs RL, Komiya S, Lotz M (1998). Linkage of chondrocyte apoptosis and cartilage degradation in human osteoarthritis. Arthritis Rheum 41: 1632–1638.

Hoffmann EK (2000). Intracellular signalling involved in volume regulatory decrease. Cell Physiol Biochem 10: 273–288.

Honda K, Shimohama S, Sawada H, Kihara T, Nakamizo T, Shibasaki H *et al.* (2001). Nongenomic antiapoptotic signal transduction by estrogen in cultured cortical neurons. J Neurosci Res 64: 466–475.

Hume JR, Duan D, Collier ML, Yamazaki J, Horowitz B (2000). Anion transport in heart. Physiol Rev 80: 31–81.

Isoya E, Toyoda F, Imai S, Okumura N, Kumagai K, Omatsu-Kanbe M *et al.* (2009). Swelling-activated Cl^- current in isolated rabbit articular chondrocytes: inhibition by arachidonic acid. J Pharmacol Sci 109: 293–304.

Kalyanaraman B, Joseph J, Kalivendi S, Wang S, Konorev E, Kotamraju S (2002). Doxorubicin-induced apoptosis: implications in cardiotoxicity. Mol Cell Biochem 234–235: 119–124.

Kim JK, Pedram A, Razandi M, Levin ER (2006). Estrogen prevents cardiomyocyte apoptosis through inhibition of reactive oxygen species and differential regulation of p38 kinase isoforms. J Biol Chem 281: 6760–6767.

Kousteni S, Bellido T, Plotkin LI, O'Brien CA, Bodenner DL, Han L *et al.* (2001). Nongenotropic, sex-nonspecific signaling through the estrogen or androgen receptors: dissociation from transcriptional activity. Cell 104: 19–30.

Kurokawa J, Tamagawa M, Harada N, Honda S, Bai C-X, Nakaya H *et al.* (2008). Acute effects of oestrogen on the guinea pig and human I_{Kr} channels and drug-induced prolongation of cardiac repolarization. J Physiol 586: 2961–2973.

Lewis RS, Ross PE, Cahalan MD (1993). Chloride channels activated by osmotic stress in T lymphocytes. J Gen Physiol 101: 801–826.

Lo MY, Kim HT (2004). Chondrocyte apoptosis induced by hydrogen peroxide requires caspase activation but not mitochondrial pore transition. J Orthop Res 22: 1120–1125.

Lotz M, Hashimoto S, Kuhn K (1999). Mechanisms of chondrocyte apoptosis. Osteoarthritis Cartilage 7: 389–391.

Maeno E, Ishizaki Y, Kanaseki T, Hazama A, Okada Y (2000). Normotonic cell shrinkage because of disordered volume regulation is an early prerequisite to apoptosis. Proc Natl Acad Sci USA 97: 9487–9492.

Martina M, Mozrzymas JW, Vittur F (1997). Membrane stretch activates a potassium channel in pig articular chondrocytes. Biochim Biophys Acta 1329: 205–210.

Mizutani H, Tada-Oikawa S, Hiraku Y, Kojima M, Kawanishi S (2005). Mechanism of apoptosis induced by doxorubicin through the generation of hydrogen peroxide. Life Sci 76: 1439–1453.

Mobasheri A (2002). Role of chondrocyte death and hypocellularity in ageing human articular cartilage and the pathogenesis of osteoarthritis. Med Hypotheses 58: 193–197.

Mukhopadhyay P, Rajesh M, Bátkai S, Kashiwaya Y, Haskó G, Liaudet L *et al*. (2009). Role of superoxide, nitric oxide, and peroxynitrite in doxorubicin-induced cell death in vivo and in vitro. Am J Physiol 296: H1466–H1483.

Nicholson DW, Thornberry NA (1997). Caspases: killer proteases. Trends Biochem Sci 22: 299–306.

Okada Y, Shimizu T, Maeno E, Tanabe S, Wang X, Takahashi N (2006). Volume-sensitive chloride channels involved in apoptotic volume decrease and cell death. J Membr Biol 209: 21–29.

Okada Y, Sato K, Numata T (2009). Pathophysiology and puzzles of the volume-sensitive outwardly rectifying anion channel. J Physiol 587: 2141–2149.

Okumura N, Imai S, Toyoda F, Isoya E, Kumagai K, Matsuura H *et al*. (2009). Regulatory role of tyrosine phosphorylation in the swelling-activated chloride current in isolated rabbit articular chondrocytes. J Physiol 587: 3761–3776.

Olsen H, ter Veld F, Herbrand U, Ahmadian MR, Kinne RK, Wehner F (2007). Differential regulation of cell volume and shape in confluent rat hepatocytes under hypertonic stress. Cell Physiol Biochem 19: 259–268.

Roman-Blas JA, Castaneda S, Largo R, Herrero-Beaumont G (2009). Osteoarthritis associated with estrogen deficiency. Arthritis Res Ther 11: 241.

Sakaguchi M, Matsuura H, Ehara T (1997). Swelling-induced Cl^- current in guinea-pig atrial myocytes: inhibition by glibenclamide. J Physiol 505: 41–52.

Saraste A, Pulkki K (2000). Morphologic and biochemical hallmarks of apoptosis. Cardiovasc Res 45: 528–537.

Schliess F, von Dahl S, Haussinger D (2001). Insulin resistance induced by loop diuretics and hyperosmolarity in perfused rat liver. Biol Chem 382: 1063–1069.

Shimizu T, Numata T, Okada Y (2004). A role of reactive oxygen species in apoptotic activation of volume-sensitive Cl^- channel. Proc Natl Acad Sci USA 101: 6770–6773.

Sniekers YH, Weinans H, Bierma-Zeinstra SM, van Leeuwen JP, van Osch GJ (2008). Animal models for osteoarthritis: the effect of ovariectomy and estrogen treatment – a systematic approach. Osteoarthritis Cartilage 16: 533–541.

Souktani R, Berdeaux A, Ghaleb B, Giudicelli JF, Guize L, Le Heuzey JY *et al*. (2000). Induction of apoptosis using sphingolipids activates a chloride current in *Xenopus laevis* oocytes. Am J Physiol 279: C158–C165.

Sugimoto T, Yoshino M, Nagao M, Ishii S, Yabu H (1996). Voltage-gated ionic channels in cultured rabbit articular chondrocytes. Comp Biochem Physiol C Pharmacol Toxicol Endocrinol 115: 223–232.

Takano H, Aizawa T, Irie T, Kokubun S, Itoi E (2007). Estrogen deficiency leads to decrease in chondrocyte numbers in the rabbit growth plate. J Orthop Sci 12: 366–374.

Tokudome T, Horio T, Yoshihara F, Suga S, Kawano Y, Kohno M *et al*. (2002). Adrenomedullin inhibits doxorubicin-induced cultured rat cardiac myocyte apoptosis via a cAMP-dependent mechanism. Endocrinology 143: 3515–3521.

Tsang WP, Chau SP, Kong SK, Fung KP, Kwok TT (2003). Reactive oxygen species mediate doxorubicin induced p53-independent apoptosis. Life Sci 73: 2047–2058.

Tsien RY, Rink TJ (1980). Neutral carrier ion-selective microelectrodes for measurement of intracellular free calcium. Biochim Biophys Acta 599: 623–638.

Varela D, Simon F, Riveros A, Jørgensen F, Stutzin A (2004). NAD(P)H oxidase-derived H_2O_2 signals chloride channel activation in cell volume regulation and cell proliferation. J Biol Chem 279: 13301–13304.

Vittur F, Grandolfo M, Fragonas E, Godeas C, Paoletti S, Pollesello P *et al*. (1994). Energy metabolism, replicative ability, intracellular calcium concentration, and ionic channels of horse articular chondrocytes. Exp Cell Res 210: 130–136.

Voets T, Manolopoulos V, Eggermont J, Elory C, Droogmans G, Nilius B (1998). Regulation of a swelling-activated chloride current in bovine endothelium by protein tyrosine phosphorylation and G proteins. J Physiol 506: 341–352.

Wagner BA, Evig CB, Reszka KJ, Buettner GR, Burns CP (2005). Doxorubicin increases intracellular hydrogen peroxide in PC3 prostate cancer cells. Arch Biochem Biophys 440: 181–190.

Wang X, Takahashi N, Uramoto H, Okada Y (2005). Chloride channel inhibition prevents ROS-dependent apoptosis induced by ischemia-reperfusion in mouse cardiomyocytes. Cell Physiol Biochem 16: 147–154.

Wilson JR, Duncan NA, Giles WR, Clark RB (2004). A voltage-dependent K^+ current contributes to membrane potential of acutely isolated canine articular chondrocytes. J Physiol 557: 93–104.

Yang PC, Kurokawa J, Furukawa T, Clancy CE (2010). Acute effects of sex steroid hormones on susceptibility to cardiac arrhythmias: a simulation study. PLoS Comput Biol 29: e1000658.