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RESEARCH PAPER

Role of connexin 43 in the maintenance of spontaneous activity in the guinea pig prostate gland

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BACKGROUND AND PURPOSE

To investigate the role of connexin 43 in the maintenance of spontaneous activity in prostate tissue from young and old guinea pigs.

EXPERIMENTAL APPROACH

Conventional intracellular microelectrode and tension recording techniques, coupled with Western blot analysis and immunohistochemistry for connexin 43 (CX43) were used. The effects of three gap junction uncouplers, 18β glycyrrhetinic acid (10 μM, 40 μM), carbenoxolone (10 μM, 50 μM) and octanol (0.5 mM, 1 mM), were studied in cells displaying slow wave activity and on spontaneously contracting tissue from prostate glands of young (2–5 months) and old (9–16 months) guinea pigs.

KEY RESULTS

18β Glycyrrhetinic acid (40 μM), carbenoxolone (50 μM) or octanol (0.5 mM) abolished slow wave activity in prostate tissue from young and old guinea pigs and depolarized membrane potential by approximately 5 mV. These treatments also abolished all contractions in both sets of prostate tissue. These effects were reversed upon washout. Western blot analysis and CX43 immunohistochemistry showed that there was no age-related difference in the expression and distribution of CX43 in prostate tissues.

CONCLUSION AND IMPLICATIONS

When gap junctional communication via CX43 was disrupted, spontaneous activity was abolished at a cellular and whole tissue level; CX43 is therefore essential for the maintenance of spontaneous slow wave activity and subsequent contractile activity in the guinea pig prostate gland.

Abbreviations

18β GA, 18β glycyrrhetinic acid; BPH, benign prostatic hyperplasia; CX43, connexin 43; IP_3, inositol trisphosphate; PICs, prostatic interstitial cells; PSS, physiological salt solution

Introduction

Benign prostatic hyperplasia (BPH) is an age- and androgen-related condition affecting approximately 50% of men by the age of 60 (Isaacs and Coffey, 1989). BPH is characterized by an overall enlargement of the prostate, accompanied by an increase in smooth muscle tone. Due to the anatomical position of the prostate, the enlarged gland occludes the proximal portion of the urethra, obstructing urine flow. Voiding problems are further exacerbated by the increase in prostatic smooth muscle tone which also compresses the urethra. Pharmacological intervention has long been used to treat BPH patients, although is associated with many side effects (Larson, 2003), which has encouraged the discovery

of alternative treatments. However, in order to identify more specific drug targets, it is crucial to have a better understanding of the aetiology of this disease.

Structural studies in BPH specimens have revealed that the hyperplasia associated with this condition occurs in the stromal and epithelial tissue (Berry *et al.*, 1984). The increase in stromal mass is thought to be responsible for the observed increase in smooth muscle tone. The prostate resembles, in many ways, the gastrointestinal tract where smooth muscle tone is maintained by phasic contractions which are associated with cyclic depolarizations of the membrane potential referred to as 'slow waves' (Horowitz *et al.*, 1999). Slow waves have also been previously recorded within the guinea pig prostate (Exintaris *et al.*, 2002; 2006) and are thought to also be responsible for maintaining overall tone of the organ. Slow wave activity is generated by neighbouring prostatic interstitial cells (PICs) providing a depolarizing pulse to the smooth muscle cells via gap junctions (Exintaris *et al.*, 2002).

Gap junctions are macromolecular structures that enable intercellular communication to occur between cells. They allow free transfer of otherwise non-permeable molecules of up to 1000 Da including second messengers [inositol trisphosphate (IP_3), cAMP, cGMP] and ions (K^+, Ca^{2+}) (Spray and Bennett, 1985). They are formed by connexin (CX) proteins in the cell membrane which are assembled as hexamers to form connexons that align with connexons in adjacent cells to form intercellular pathways (Geiger *et al.*, 1995). CX proteins appear to be expressed in a tissue-specific manner and form channels of specific permeabilities (Ruch, 1994), which together regulate cell-to-cell communication (Risek *et al.*, 1992; 1994). Within the human prostate gland, connexin 43 (CX43) has been identified on interstitial cells (Van der Aa *et al.*, 2003) and the presence of connexin 32 (CX32) has also been noted (Habermann *et al.*, 2001). A change in levels of expression of gap junction proteins has also been linked to certain disease states such as BPH, where there was an increase in CX43, prostate cancer with a decrease in CX43 (Habermann *et al.*, 2001), Hirschprung's disease with an absence of CX43 (Nemeth *et al.*, 2000) and overactive bladder syndrome (increase in CX43). Studies on human BPH specimens suggested that an increase in CX levels was an indication of increased metabolic activity in the stromal, basal and luminal cell compartments during hyperplasic tissue growth. It was also suggested that gap junctional intercellular communication was increased in epithelial and stromal cells and that this might have a role in the pathogenesis of BPH (Habermann *et al.*, 2001; Christ *et al.*, 2003).

Previous studies on the guinea pig prostate gland showed that there was an age-related change in the proportion of cells exhibiting the different types of electrical activity (Dey *et al.*, 2009). Thus, with increasing age there was a decrease in the proportion of cells exhibiting pacemaker like activity and an increase in cells displaying spike potential activity. One possible explanation for these observations may be that there is an age-related change in intercellular communication (Dey *et al.*, 2009).

The objective of this study, therefore, was to investigate the role of gap junctions, specifically the gap junction protein CX43, in maintaining spontaneous activity at the cellular as well as whole tissue level in the guinea pig prostate. We have chosen to investigate CX43 *specifically* because this CX has been identified on human (Van der Aa *et al.*, 2003) and guinea pig (Kusljic *et al.*, 2007) PICs and is therefore likely to be involved in the transmission of electrical activity from the PIC to the smooth muscle cells. Furthermore, CX32 has been identified in the epithelium of the human prostate and is therefore less likely to be involved in the regulation of spontaneous activity (Habermann *et al.*, 2001). We investigated the effects of gap junction inhibitors on the spontaneous slow wave activity, as well as spontaneous contractions in prostate gland tissue from young (2–5 months) and old (9–16 months) guinea pigs. The gap junctional agents used were specific for reversibly inhibiting the transmission of activity via CX43. 18β Glycyrrhetinic acid (18β GA) and carbenoxolone are known to affect the dephosphorylation of CX43 (Guan *et al.*, 1996). Octanol, however, is thought to exert its effects by inhibiting the conductance of gap junctions containing CX43 proteins (Sakai *et al.*, 1992). Immunohistochemical and Western blot techniques were also used to identify the presence of CX43 within both young and old groups of animals.

Methods

Animal model

All animal care and experimental procedures complied with the Guidelines approved by Monash University Animal Experimentation Ethics Committee in accordance with the Australian code of practice for the care and use of animals for scientific purposes (7th Edition 2004). Male guinea pigs of two weight ranges were used for all experiments. Young animals were 2–5 months in age, weighing 200–500 g with a mean weight of 408.6 ± 0.5 g. Old animals (9–16 months in age) were 800+ g with a mean weight of 937.2 ± 1.8 g. Animals were killed by stunning and exsanguination. Dorsal prostate

glands were removed through an abdominal incision. The number of animals used for each experimental series (*n*) is indicated in the figure legend and/or table for each age group where each cell represents a recording from one animal in the electrophysiological studies, and each preparation is representative of one animal in the contractile studies. The number of preparations used for the contractile experiments and the number of cells impaled for electrophysiological experiments are indicated in the legends.

Electrophysiology

Individual lobes (5 mm × 5 mm) of the dorsal gland were pinned firmly to the bottom of an organ bath (volume 1 mL) mounted on a stage of an inverted microscope and perfused with physiological salt solution [PSS; composition (in mM): NaCl 125, KCl 5, $CaCl_2$ 2.5, $MgCl_2$ 1, NaH_2PO_4 1, $NaHCO_3$ 25 and glucose 11, bubbled with a 95% O_2 : 5% CO_2 gas mixture to establish a pH of 7.3–7.4] at a rate of 3–4 mL·min^{-1} and held at a temperature of 37°C. Membrane potential recordings were made using a standard gain preamplifier (Axoclamp®, Union City, CA, USA) and microelectrodes with resistances of 60–80 MΩ when filled with 2 M KCl. Changes in the membrane potential were digitized and stored using a TL1 DMA analogue to digital interface and Axotape® software (Axon Instruments, Union City, CA, USA) (Exintaris *et al.*, 2002)

Contractile studies

Five to six saccular glands were dissected away from the prostate mass; each end of the glands was tied using suture cotton. The tied preparation was placed between two hooks of an organ bath (1 mL volume), which was perfused (4–5 mL·min^{-1}) with PSS (37°C; bubbled with carbogen). The right hook was attached to a force transducer connected to a bridge amplifier (PowerLab®, ADInstruments, Bella Vista, NSW, Australia). Tissue preparations were allowed to equilibrate for 30–60 min, after which approximately 1 mN of tension was applied. Changes in tension were recorded using Chart Pro® v5.5.6 (ADInstruments, Bella Vista, NSW, Australia) and stored on a personal computer for further analysis.

Western blots and immunohistochemistry

Collection of tissue. Prostate lobes were carefully dissected out, weighed and placed in Eppendorf tubes for Western blot analysis or embedded in OCT medium (Tissue-Tek®, Sakura Finetek, Torrance, CA, USA) and frozen in liquid nitrogen-cooled isopentane for immunohistochemistry.

Western blot analysis. Western blot experiments were performed to examine the expression of gap junction proteins. Frozen prostate tissues were homogenized in buffer (20 mM Tris-HCl, 100 mM NaCl, 1% Triton X-100) containing a protease inhibitor cocktail by sonication. Tissue lysates were clarified by centrifugation at 14 000 g for 15 min. The protein concentration of the supernatant was determined by the Bradford method. Aliquots of tissue lysates (equivalent to 50 µg protein) were resolved by electrophoresis on 10–12% SDS-polyacrylamide gel and transferred to PVDF membrane (Millipore, Billerica, MA, USA). The membranes were blocked with 5% non-fat dry milk in buffer solution (25 mM Tris-HCl, 500 mM NaCl) for 60 min at room temperature to block non-specific binding sites. The membranes were subsequently incubated overnight at 4°C on a rotating platform with anti-CX43 (1:1000, Invitrogen Corporation, Carlsbad, CA, USA) or β-tubulin (1:500, Abcam, Cambridge, UK) in buffer solution (25 mM Tris-HCl, 500 mM NaCl, 0.1% Tween-20). After washes in buffer solution, membranes were incubated with matching horse-radish peroxidase (HRP)-conjugated secondary antibodies, developed using enhanced chemiluminescent (ECL) reagent kit and exposed to ECL film. Signal intensities were normalized to the β-tubulin protein signal.

Connexin 43 immunohistochemistry. For detection of CX43 using immunofluorescence, staining was performed on acetone-fixed sections. Cryostat sections 8 µm in thickness were mounted on gelatin-coated glass slides and washed three times in 0.1 M phosphate buffer (PB), pH 7.5. The sections were subsequently incubated with a mouse monoclonal antibody against CX43 (2 h at room temperature; 1:200, Chemicon International, Temecula, CA, USA), appropriately diluted in PB containing 2% BSA and 0.3% Triton X-100. Following washes in PB, sections were incubated with biotinylated anti-mouse IgG (1:200, Vector Laboratories, Burlingame, CA, USA) and streptavidin-linked Texas Red® (1:200, Vector Laboratories). Following immunohistochemical procedures, the sections were examined with the Olympus BX60 microscope illuminated at 575 nm.

Data analysis

Various parameters of the spontaneous activity were measured.

Analysis of spontaneous electrical activity. The membrane potential (mV) 1 s before the onset of each

slow wave, the total amplitude (mV), number of superimposed spikes on the depolarizing transient, half amplitude duration (ms) and frequency of slow wave activity (min^{-1}).

Analysis of spontaneous contractions. Basal tension (mN); defined as the resting tension of the preparation after equilibration and subsequent application of 1 mN tension, total amplitude ($N{\cdot}g^{-1}$) expressed as force generated per gram of tissue (prostate weights were weighed in mg after each experiment), half amplitude duration (s), slope ($mN{\cdot}s^{-1}$) and frequency of contractions (min^{-1}).

For both activities, indicated parameters of a total of five responses of control responses were analysed and averaged and then subsequently compared to five responses on the addition of a 'test' drug.

Data was exported to GraphPad Prism5 (GraphPad Software Inc., San Diego, CA, USA). Statistical analysis of variance was performed using two-way ANOVA and Bonferroni *post hoc* tests, accepting a significant effect when $P < 0.05$, when comparing between two age groups. Student's paired and unpaired *t*-tests were also used where appropriate, accepting a significant effect when $P < 0.05$.

Materials

The drugs used in the current study were: 18β GA, carbenoxolone, phenylephrine (1 μM) (Sigma Chemical Co., St Louis, MO, USA) and octanol (0.5 mM and 1 mM) (Chem Supply, Gillman, South Australia, Australia). 18β GA was dissolved in dimethyl sulfoxide and carbenoxolone and phenylephrine in distilled water to make a final stock solution of between 0.1 mM and 10 mM. Octanol was dissolved directly into PSS. The final concentration of all drugs was achieved by diluting in PSS, which was subsequently bubbled with 95% O_2 : 5% CO_2, before adding to the preparation, to restore any changes to pH. Drug and receptor nomenclature follows Alexander *et al.* (2009).

Results

Spontaneous activity in the young guinea pig prostate

Contractile activity. Of a total of 145 preparations of the prostate tissue from young (2–5 months) guinea pigs, 54% displayed spontaneous activity after equilibration of approximately 60 min (Table 1). Other variables measured are listed in Table 1 and illustrated by the experimental record in Figure 1A.

Electrical activity. Slow wave activity accounted for 78% of all electrical recordings (Table 2). This consisted of a depolarizing transient followed by nifedipine sensitive spikes superimposed on top of the depolarizing transient (Figure 2A). Frequency and other characteristics of the electrical activity measured are summarized in Table 2.

Spontaneous activity in the old guinea pig prostate

Contractile activity. Out of a total of 81 preparations of prostate tissue from old (9–16 months) guinea pigs, 51 displayed spontaneous contractions after equilibration for 60 min (63%). A representative experimental trace is shown in Figure 1B and data are summarized in Table 1.

Electrical activity. Slow wave activity accounted for 46% of all electrical recordings. A typical trace is shown in Figure 2B and other data are given in Table 2.

Effects of 18β GA on spontaneous activity

Contractile activity. Two concentrations of 18β GA (10 μM and 40 μM) were used to study its effects on spontaneous contractions, in prostate tissues from young and old guinea pigs. At the lower concentration (10 μM), 18β GA reduced amplitudes of contraction in tissues from both age groups, relative to the control values (0.38 ± 0.2 $N{\cdot}g^{-1}$ to 0.16 ± 0.02 $N{\cdot}g^{-1}$ in young, and 0.51 ± 0.2 $N{\cdot}g^{-1}$ to 0.10 ± 0.02 $N{\cdot}g^{-1}$ in old), although this treatment did not

Table 1

Parameters of the spontaneous contractile activity recorded in prostate tissue from young and old guinea pigs

	Basal tension (mN)	Amplitude ($N{\cdot}g^{-1}$)	Slope ($mN{\cdot}s^{-1}$)	Duration (s)	Frequency (min^{-1})	Number of preparations (*n*)
Young	4.7 ± 0.6*	0.3 ± 0.03	0.1 ± 0.009	3.0 ± 0.01***	3.6 ± 0.2	78
Old	6.0 ± 0.3	0.4 ± 0.05	0.1 ± 0.01	3.8 ± 0.2	3.9 ± 0.3	51

*$P < 0.05$, ***$P < 0.001$; Student's unpaired *t*-test.

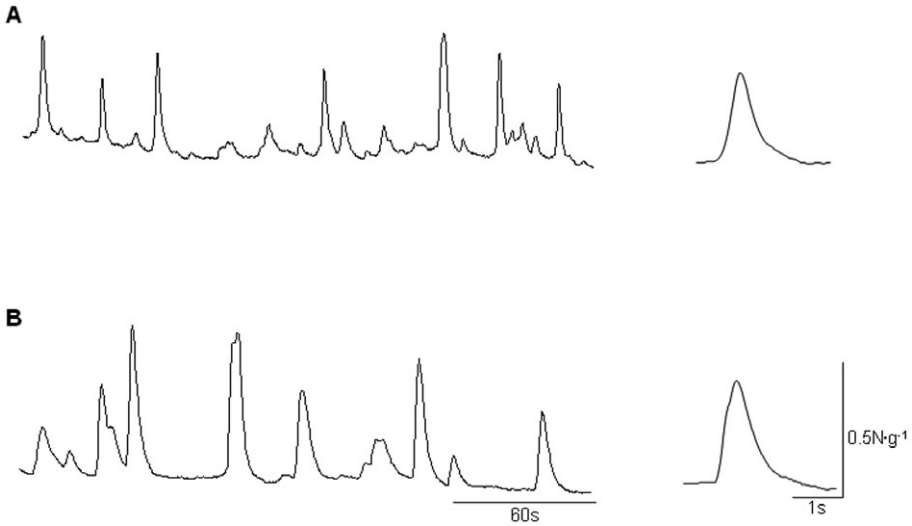

Figure 1

Spontaneous contractile activity in the young (A) and old guinea pig prostate (B). Spontaneous contractions occurred at irregular amplitudes in both age groups of animals. See Table 1 for more details.

Table 2

Electrophysiological characteristics of the spontaneous slow wave activity recorded in prostate cells from young and old guinea pigs

	Membrane potential (mV)	Overall amplitude (mV)	Duration (ms)	Frequency (min^{-1})	Number of cells (*n*)
Young	–55.4 ± 0.8	51.5 ± 1.6	1007 ± 60	5.2 ± 0.3	70
Old	–55.1 ± 0.8	52.6 ± 1.5	933 ± 64	5.1 ± 0.4	67

There was no significant difference across any of the measured parameters between the age groups (Student's unpaired *t*-test $P > 0.05$).

have an age-related effect (ANOVA, $P > 0.05$). In contrast, the frequency of contractions was significantly reduced in old prostate tissue from 4.6 ± 0.6 min^{-1} to 2.0 ± 0.7 min^{-1} (ANOVA, $P < 0.01$), but not in tissue from young guinea pigs. No significant effect of 18β GA was observed on the responses to phenylephrine, for both age groups [Figure 3A(a) and B(a); five preparations for both age groups].

At the higher concentration of 18β GA (40 μM), spontaneous contractile activity was abolished in four of five preparations across both age groups (ANOVA, $P < 0.05$), although the basal tension was not affected in either age group (ANOVA, $P > 0.05$). The responses to phenylephrine were again unaffected by 18β GA at this higher concentration, in tissues from young and old guinea pigs [Figure 3A(b) and B(b)].

Electrical activity. 18β GA (40 μM) was added to cells, exhibiting slow wave activity, taken from prostate glands of young guinea pigs. Under the control conditions, the amplitude of depolarizing transient was 11.3 ± 1.2 mV and the number of spike potentials was 2.3 ± 0.2 ($n = 9$). Other variables measured in these cells under control conditions are summarized in Table 3.

The addition of 18β GA caused a time-dependent decrease in slow wave activity, such that after 2 min of exposure to the drug, activity was abolished (Figure 4A). The membrane potential was significantly depolarized from that under control conditions (Table 3; Student's paired *t*-test, $P < 0.01$). On addition of phenylephrine (1 μM) in the presence of 18β GA, slow wave activity was reinstated. Slow wave activity in the presence of both of these drugs occurred at a significantly higher frequency and at a more depolarized membrane potential (Table 3; Student's paired *t*-test, $P < 0.01$) than control values. Upon washout of phenylephrine as well as 18β GA, slow wave activity slowly returned to that observed under control conditions (Figure 4A).

Similarly, 18β GA was added to 11 cells exhibiting slow wave activity, from prostate tissue taken from old guinea pigs. As shown in Figure 4B, slow

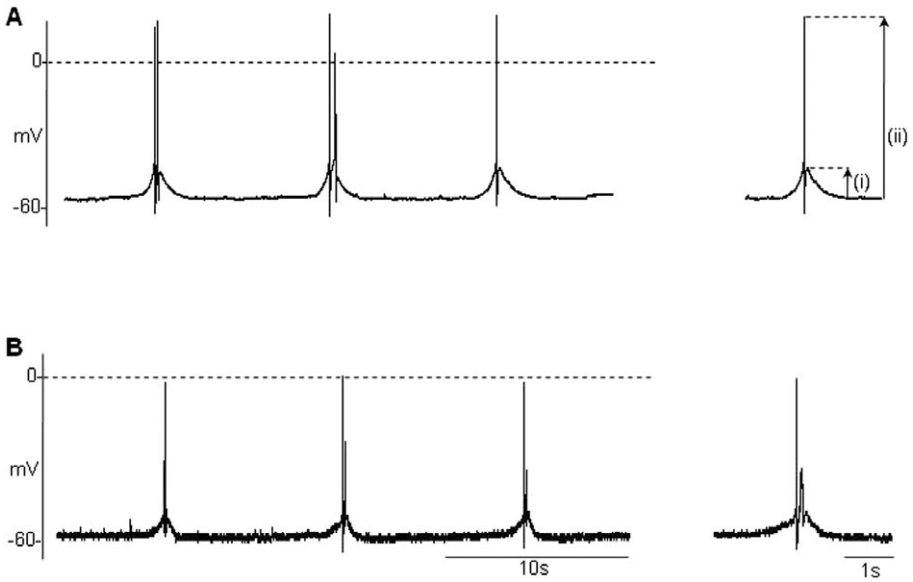

Figure 2

Spontaneous slow wave activity in the young (A) and old guinea pig prostate (B). Slow wave activity in both age groups occurred at a similar frequency, amplitude, membrane potential and half amplitude duration. (i) Represents amplitude of the depolarizing transient. (ii) Represents total amplitude. See Table 2 for more details.

wave activity in these cells was abolished in a time-dependent manner upon addition of 18β GA, as described for cells from young animals. Upon cessation of activity, the membrane potential rested at a significantly more depolarized membrane potential than that under control conditions (Table 3; Student's paired *t*-test, $P < 0.05$). On addition of phenylephrine in the presence of 18β GA, activity again returned and upon washing out both, activity slowly returned to control values (Figure 4Bc). Comparisons between the young and old prostates using two-way ANOVA showed that there were no significant differences between the young and old tissue in the response to 18β GA (Figure 4B). However, the membrane potential was more depolarized in the cells from old prostates (ANOVA, $P < 0.01$), compared to the cells from young prostates (ANOVA, $P < 0.05$).

Effects of carbenoxolone on spontaneous activity

Contractile activity. 10 μM carbenoxolone was used on six preparations of prostate tissue from young guinea pigs and five similar preparations from old guinea pigs [Figure 5A(a) and B(a)]. Parameters measured including basal tension, amplitude of contraction, slope and duration were not significantly affected on the addition of the drug compared to the control values for each age group (ANOVA, $P > 0.05$). However, the frequency of spontaneous contractions in the prostate tissue from old guinea pigs was significantly reduced from control values; 4.3 ± 0.6 min^{-1} to 1.8 ± 0.4 min^{-1} (ANOVA, $P < 0.01$), although in the young guinea pig prostate, frequency remained at control levels. No significant difference was observed on the addition of phenylephrine in the presence of carbenoxolone, compared to the phenylephrine values alone for both age groups. A higher concentration of carbenoxolone (50 μM) was tested in seven preparations from young guinea pigs and five from old guinea pigs. This concentration of carbenoxolone abolished spontaneous contractions in five of seven preparations in young tissues and four of five preparations in old tissues. In addition, carbenoxolone significantly reduced frequency from 3.1 ± 0.7 min^{-1} to 0.7 ± 0.4 min^{-1} (ANOVA, $P < 0.05$) in young tissue, although in old prostates, frequency was more markedly reduced to 0.12 ± 0.1 min^{-1} from 3.4 ± 0.4 min^{-1} in control (ANOVA, $P < 0.01$). No difference was observed on the addition of phenylephrine in the presence of carbenoxolone, compared to the phenylephrine values alone for both age groups [Figure 5A(b) and B(b)].

Electrical activity. Carbenoxolone (50 μM) was added to seven cells exhibiting slow wave activity, taken from young guinea pig prostates (Table 3). In the control with no drug present, the amplitude of depolarizing transient was 11.8 ± 0.9 mV and the number of spike potentials was 2.3 ± 0.3 ($n = 9$). Other variables measured are summarized in Table 3. On the addition of carbenoxolone, activity

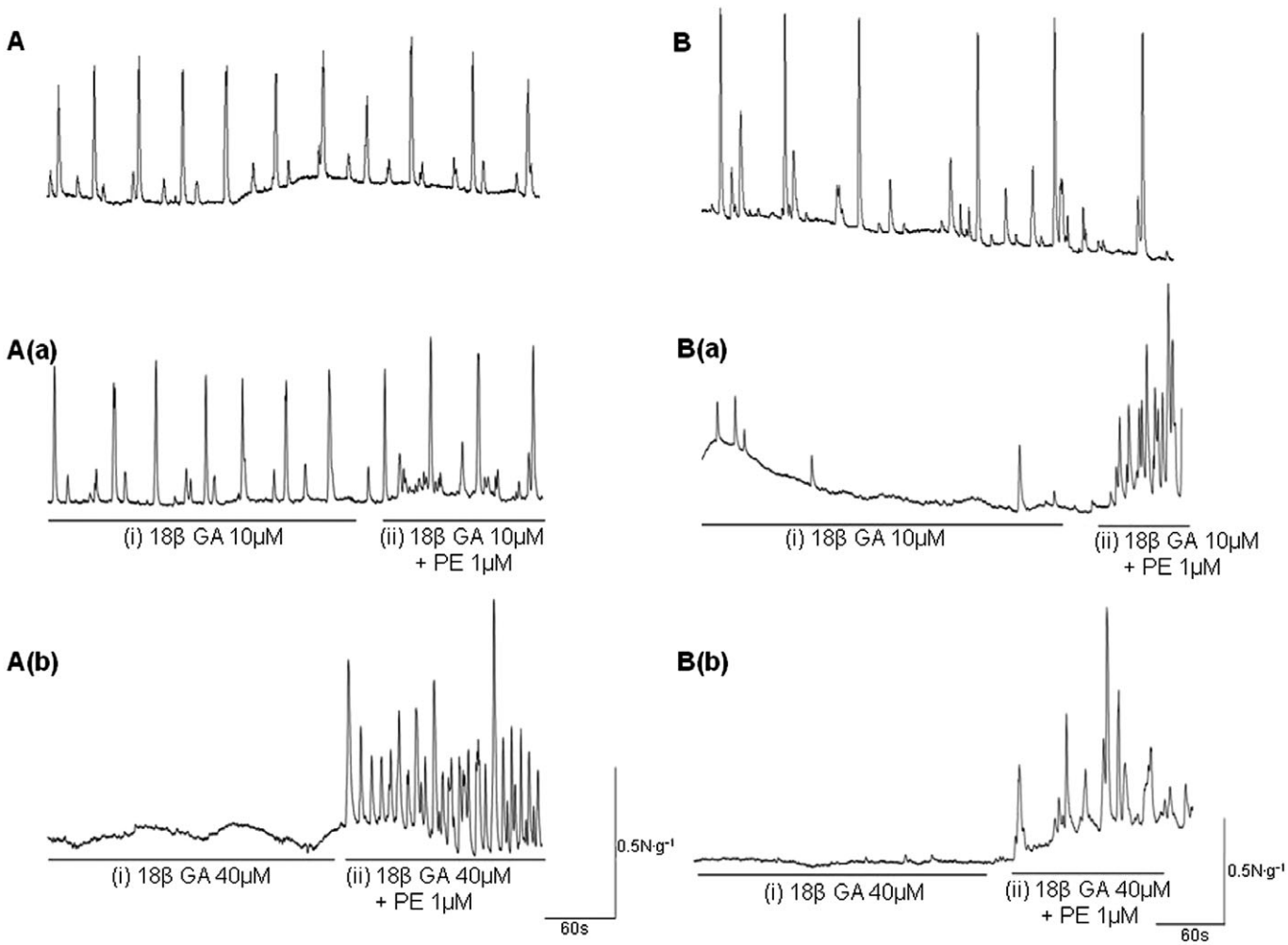

Figure 3

Effect of 10 μM [A(a), B(a), five preparations both age groups] and 40 μM [A(b), B(b), five preparations both age groups] 18β glycyrrhetinic acid (18β GA) on the spontaneous contractile activity in tissues from young (A) and old (B) guinea pig prostate. Application of 10 μM significantly reduced the frequency of contractions in tissues from old guinea pigs ($P < 0.01$) but not in those from the young animals. Application of 40 μM abolished contractions in both age groups ($P < 0.001$). Application of phenylephrine (PE) in the presence of both concentrations of 18β GA restored activity.

was abolished after 2 min exposure (Figure 6A) and the membrane potential was significantly depolarized from that of control values (Table 3; Student's paired *t*-test, $P < 0.05$). When phenylephrine was added in the presence of carbenoxolone, activity returned and upon washout of both these drugs, activity slowly returned to 4.2 ± 0.3 min^{-1} and the membrane potential repolarized (Figure 6A).

With prostatic smooth muscle cells displaying slow wave activity from old guinea pigs, the amplitude of depolarizing transient was 7.8 ± 1.3 mV and the number of spikes 2.6 ± 0.7 ($n = 5$); other variables measured are summarized in Table 3. In contrast to its effects on prostate tissue from young guinea pigs, in tissues from old animals, carbenoxolone abolished activity immediately it was added to the tissues (Figure 6B). The membrane potential was again significantly depolarized from the control value (Table 3; Student's paired *t*-test, $P < 0.05$). Upon addition of phenylephrine, activity was reinstated and upon washout with PSS, slow wave activity slowly returned to that of the control values, and the membrane potential repolarized to near control values. Two-way ANOVA revealed the membrane potential appeared to be significantly more depolarized in cells from the young prostates (ANOVA, $P < 0.01$) than in those from the old tissue (ANOVA, $P < 0.05$).

Effects of octanol on spontaneous activity

Contractile activity. 0.5 mM octanol was used on five prostate preparations from the young, as well as

Table 3

Comparisons of all the parameters of slow wave activity in the young and old prostate tissue on application of the gap junction inhibitor indicated, and on addition of phenylephrine

	Membrane potential (mV)	Total amplitude (mV)	Duration (ms)	Frequency (min^{-1})	Number of cells (*n*)
Young:					9
Control	−57.6 ± 2.9	50.2 ± 2.0	928 ± 14	6.1 ± 0.8	
18β GA (40 μM)	−49.1 ± 3.3**†	–	–	–	
+phenylephrine (1 μM)	−48.7 ± 5.8**	41.4 ± 6.2	954 ± 28	10.9 ± 2.3*	
Old:					11
Control	−53.7 ± 1.2	47.5 ± 3.7	902 ± 86	5.1 ± 0.8	
18β GA (40 μM)	−47.7 ± 2.5*††	–	–	–	
+phenylephrine (1 μM)	−48.8 ± 3.1*	22.1 ± 2.1	671 ± 78	6.9 ± 0.4	
Young:					7
Control	−57.1 ± 2.0	61.3 ± 3.6	1088 ± 92	6.1 ± 0.7	
CBX (50 μM)	−42.1 ± 4.1*††	–	–	–	
+phenylephrine (1 μM)	−47.0 ± 6.1	48.7 ± 7.6	888 ± 53	8.7 ± 0.8**	
Old:					5
Control	−60.8 ± 3.8	57.0 ± 6.8	1019 ± 16	5.1 ± 1.3	
CBX (50 μM)	−49.5 ± 3.6*†	–	–	–	
+phenylephrine (1 μM)	−52.5 ± 12.4	40.2 ± 13.5	991 ± 88	17.1 ± 4.2**	
Young:					5
Control	−55.8 ± 3.1	56.7 ± 3.0	1120 ± 40	4.2 ± 0.9	
Octanol (0.5 mM)	−50.1 ± 2.5*††	–	–	–	
+phenylephrine (1 μM)	−45.7 ± 2.4*	44.5 ± 6.9	704 ± 104	9.7 ± 3.2*	
Old:					5
Control	−61.2 ± 2.6	54.6 ± 3.4	1146 ± 45	4.8 ± 0.4	
Octanol (0.5 mM)	−53.2 ± 1.0*††	–	–	–	
+phenylephrine (1 μM)	−53.5 ± 0.1*	55.5 ± 3.9	996 ± 76	5.7 ± 3.6	

*$P < 0.05$, **$P < 0.01$; Student's paired *t*-test.
†$P < 0.05$, ††$P < 0.01$; two-way ANOVA, Bonferroni *post hoc* test.
18β GA, 18β glycyrrhetinic acid; CBX, carbenoxolone.

the old guinea pigs (Figure 7). Octanol effectively abolished all contractile activity within both age groups (ANOVA, $P < 0.01$). Spontaneous contractions were restored on the addition of phenylephrine in the presence of octanol. The force of contraction generated was not dissimilar to that of the phenylephrine controls within both age groups (ANOVA, $P > 0.05$).

Octanol (1 mM) was also used on old as well as young guinea pig prostates and abolished all spontaneous contractions in both age groups (ANOVA, $P > 0.01$). Spontaneous contractions occurred at a frequency of 3.4 ± 0.7 min^{-1} in the young and 4.4 ± 0.8 min^{-1} in old prostates. On addition of phenylephrine in the presence of 1 mM octanol, activity was returned to that of the control phenylephrine values (Figure 7).

Electrical activity. Octanol (0.5 mM) was added to five cells from the young guinea pig prostate. Before addition of octanol, the amplitude of the depolarizing transient was 12.6 ± 1.8 mV and the number of spikes was 3.0 ± 0.4. Other parameters of slow wave activity are summarized in Table 3. On the addition of octanol, slow wave activity was abolished (Figure 8A) and the membrane potential was significantly depolarized from that of the control values (Table 3; Student's paired *t*-test, $P < 0.05$). On the addition of phenylephrine, in the presence of octanol, the activity was restored and upon washout of both phenylepherine and octanol, the slow wave activity slowly returned to that of the control values (Figure 8A).

In prostatic smooth muscle cells displaying control slow wave activity from old guinea pigs, the

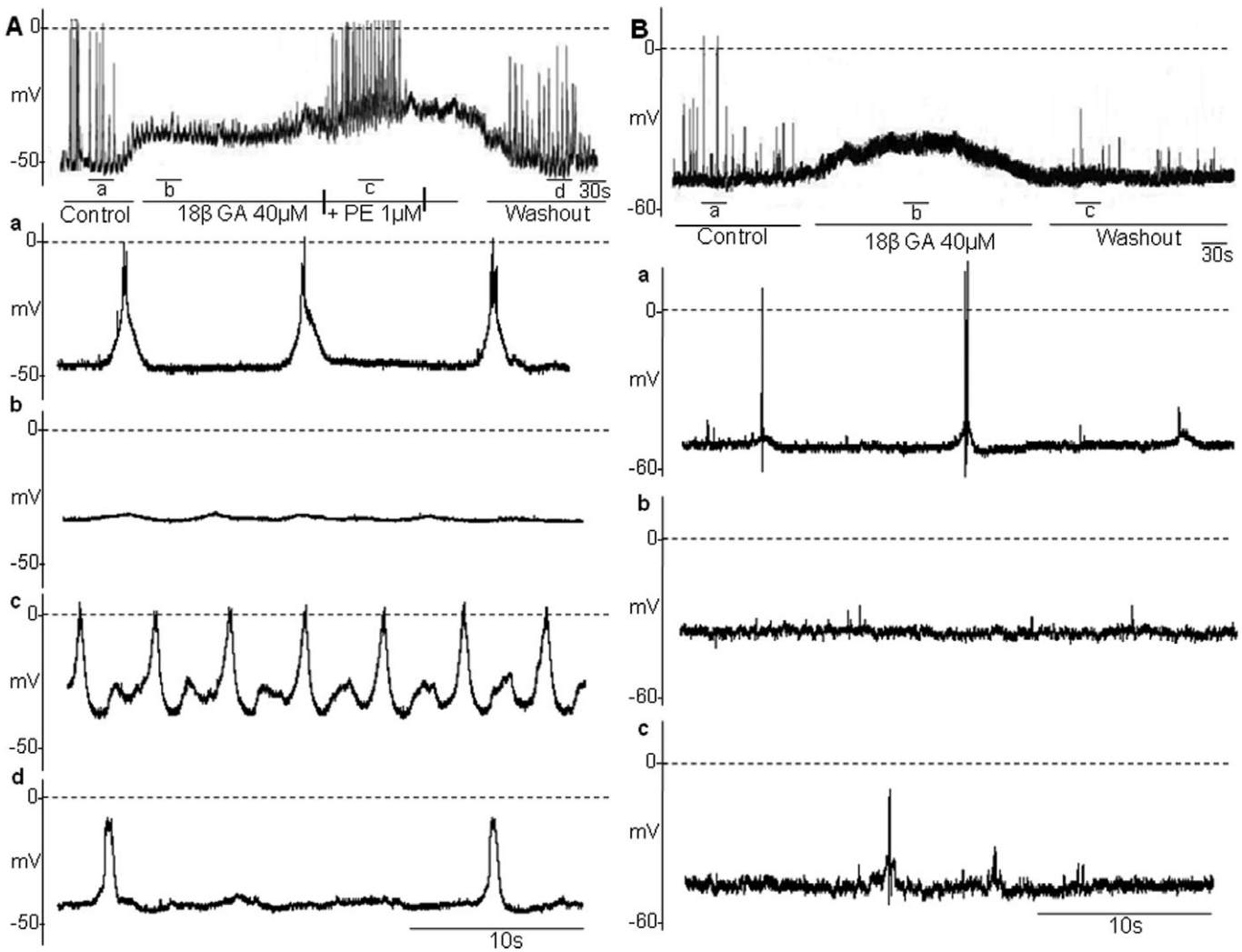

Figure 4

Effect of 40 μM 18β glycyrrhetinic acid (18β GA) on the spontaneous slow wave activity in cells from young (A, from 9 cells) and old (B, from 11 cells) guinea pig prostate. Control slow wave activity occurred regularly in both age groups (Aa, Ba) and on the addition of 18β GA, activity was abolished (Ab, Bb); the membrane potential was significantly depolarized in both age groups. The addition of 1 μM phenylephrine (PE) initiated activity and upon washout (Ad, Bc) of phenylephrine and 18β GA, spontaneous activity returned.

amplitude of the depolarizing transient was 12.6 ± 1.8 mV and the number of spikes was 3.1 ± 0.9 ($n = 5$), with other values as shown in Table 3. When octanol was added to the tissues, activity was abolished (Figure 8B) and the membrane potential was again depolarized significantly (Table 3; Student's paired t-test, $P < 0.05$). On the addition of phenylephrine in the presence of octanol, activity was restored. On washout with PSS, slow wave activity slowly returned to that of the control values (Figure 8B).

Western Blot and CX43 immunohistochemistry

The presence of CX43 in the young and old guinea pig prostate was confirmed by Western blot analysis for CX43. An immunoreactive band was observed near the 43 kDa position of the SDS gel in the prostate lysate from both young and old guinea pigs (Figure 9). However, there was no statistical difference in the expression of CX43 between young and old guinea pigs. When expressed in arbitrary units, mean data values for the young group was 0.8 ± 0.1 and for the old group was 0.7 ± 0.06.

Microscopic examination of preparations of guinea pig prostate taken from five animals revealed the presence of CX43 immunoreactive cells within the border between the smooth muscle and glandular layers in young and old guinea pigs (Figure 10). CX43 immunoreactive cells were also sparsely distributed within the glandular layer but not within the smooth muscle layer itself. CX43 immunoreac-

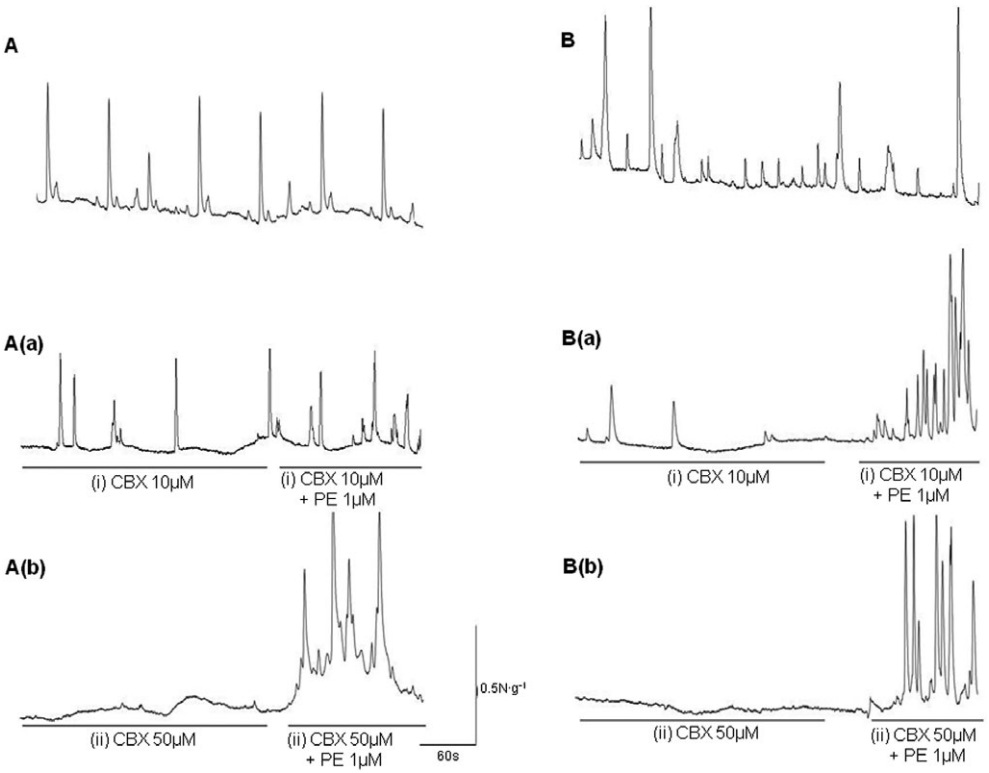

Figure 5

Effect of 10 μM [A(a), $n = 6$, B(a), $n = 5$ preparations] and 50 μM [A(b), $n = 7$, Bb, $n = 5$ preparations] carbenoxolone (CBX) on the spontaneous contractile activity in the young (A) and old (B) guinea pig prostate tissue. Panels A and B depict the control activity in the young and old guinea pig prostate respectively. Application of 10 μM significantly reduced the frequency in the old guinea pig prostate ($P < 0.01$) although not in the young. Application of 50 μM abolished contractions in both age groups ($P < 0.01$). Application of phenylephrine (PE) in the presence of both concentrations of CBX initiated activity. Low amplitude contractions were abolished.

tivity appeared as dots usually forming clusters encircling particular structures. Sections in which the primary antibody was omitted, providing a negative control, showed little staining (data not shown). Qualitative analysis of the CX43 immunoreactivity showed that there was no change in the distribution of CX43 positive cells with age.

Discussion

The results from the current study, for the first time represent a comparative investigation of the spontaneous activities in tissues from the young (2–5 months) and old (9–16 months) guinea pig prostate. Our investigations demonstrated that slow waves recorded within the guinea pig prostatic stroma had similar frequencies to that of the spontaneous contractions in both age groups of animals, such that each slow wave was associated with a contraction, as has been previously suggested (Exintaris *et al.*, 2002). In addition, tissues from old prostates were observed to have a higher basal tension of 6.0 ± 0.3 mN than the younger prostates, 4.7 ± 0.66 mN, which suggests that older prostates had an increased level of smooth muscle tone. This is in accordance with the investigation in the old guinea pig prostate which found that spikes may be firing independently as smooth muscle bundles, giving rise to the increase in smooth muscle tone observed (Dey *et al.*, 2009). This also parallels some aspects of BPH, in which the prostate has a higher level of smooth muscle tone resulting in many of the voiding problems experienced by patients (Yamada *et al.*, 1987).

Although the proportion of cells exhibiting slow wave activity was different in the two age groups (young: 78% and old: 46%), the measured parameters were the same, which is consistent with previous reports (Dey *et al.*, 2009), suggesting that the channel populations responsible for the generation and maintenance of slow wave activity have not changed. The configuration of the prostatic slow wave depends on Ca^{2+} ions and its importance has been demonstrated in several experiments whereby electrical activity was abolished several minutes after Ca^{2+} was removed from external solutions, indicating that this ion was essential for

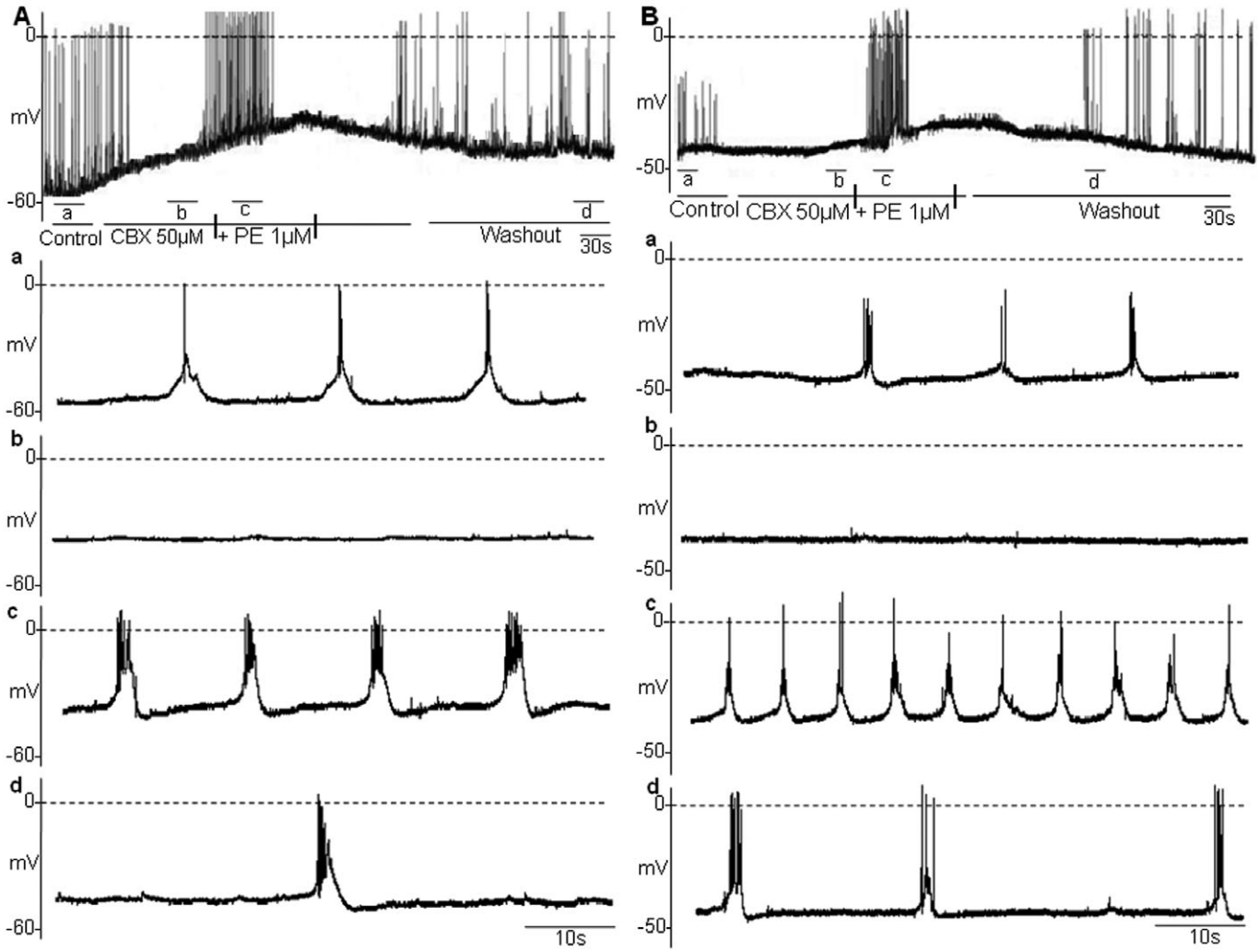

Figure 6

Effect of 50 μM carbenoxolone (CBX) on the spontaneous slow wave activity in cells from young (A, from 7 cells) and old (B, from 5 cells) guinea pig prostate. Control slow wave activity occurred regularly within both age groups (Aa, Ba) and on the addition of CBX, activity was abolished (Ab, Bb); the membrane potential was significantly depolarized in both age groups. The addition of phenylephrine (PE) initiated activity (Ac, Bc) and upon washout (Ad, Bd) spontaneous activity returned.

maintaining contractility (Exintaris *et al.*, 2002). The regulation of regenerative activity in the guinea pig prostate gland has been shown to be dependent on Ca^{2+} entry through L-type Ca^{2+} channels, non-selective cation channels, as well as Ca^{2+} cycling through intracellular compartments (Nguyen *et al.*, 2009), including IP_3-dependent Ca^{2+} stores and the buffering of Ca^{2+} from mitochondria (Exintaris *et al.*, 2009). Slow wave activity is spread from cell-to-cell via gap junctions, propagating the mechanical responses to individual smooth muscle cells and thus the entire prostate.

18β Glycyrrhetinic acid

18β Glycyrrhetinic acid has been used extensively to examine the effects of gap junctional communication on the spread of electrical and mechanical activity throughout various smooth muscle tissues. In this study, 18β GA (40 μM) effectively blocked gap junctional communication in preparations of guinea pig prostate tissue in young as well as old animals. This decrease in mechanical activity could be attributed to the inhibition of currents through L-type Ca^{2+} channels (Takeda *et al.*, 2005). However, on the addition of the α_1-agonist phenylephrine in the presence of 18β GA, contractile force was not dissimilar to the effect of phenylephrine alone, suggesting that 18β GA had not compromised the ability of the smooth muscle to contract [Figure 3A(b) and B(b)]. At the lower concentration (10 μM), 18β GA reduced spontaneous contractile activity in old tissues only [Figure 3A(a) and B(a)],

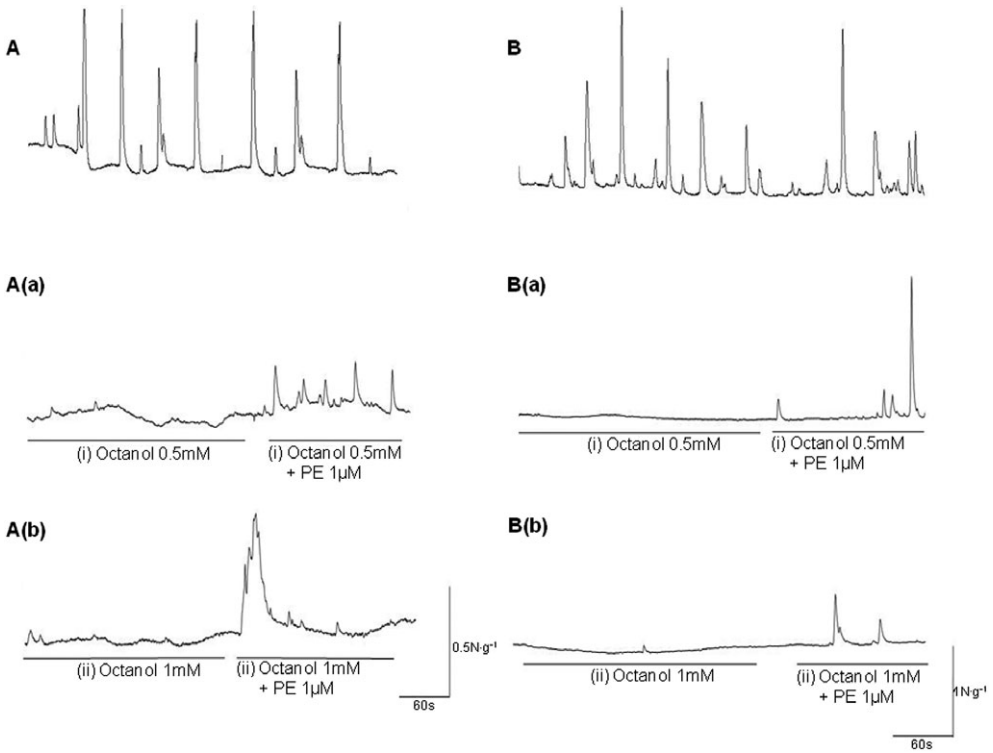

Figure 7

Effect of 0.5 mM [A(a), B(a), five preparations] and 1 mM [A(b), B(b), five preparations] octanol on the spontaneous contractile activity in prostate tissue from young (A) and old (B) guinea pigs. Panels A and B depict the control activity in the young and old guinea pig tissues respectively. Application of 0.5 mM octanol significantly reduced the frequency in the old guinea pig prostate ($P < 0.01$) although not in the young tissues. Application of 1 mM octanol abolished contractions in both age groups ($P < 0.01$).

which could be explained by an increase in CX43 expression, as shown in human BPH specimens (Habermann *et al.*, 2001), thereby increasing the overall sensitivity of old tissues to 18β GA. However, Western blot analysis indicated the clear presence of proteins expressing CX43 within the young as well as old guinea pig prostate (Figure 9), and no qualitative change in the expression of CX43. Furthermore, immunohistochemical staining within the young as well as old guinea pig prostate revealed the presence of CX43 between the smooth muscle and glandular layer of the prostate although not within the smooth muscle layer itself (Figure 10), indicating that PICs communicate with each other but not with smooth muscle via this CX protein. This is in agreement with other studies on the presence of CX43 within the human prostate (Habermann *et al.*, 2001; Van der Aa *et al.*, 2003).

In terms of the effects of 18β GA on the spontaneous electrical activity in the prostate gland (Figure 4), slow wave activity was effectively abolished within both age groups, which was consistent with the tension recordings performed within this study. In addition, the membrane potentials of both age groups of tissue in the presence of 18β GA were significantly depolarized from their control values, which could be expected if smooth muscles were uncoupled from the PICs that generate pacemaker potentials. An alternative explanation for the observed membrane depolarization in the presence of 18β GA (Figure 4) could be due to inhibition of an outward or activation of an inward, ionic conductance in smooth muscle cells (Takeda *et al.*, 2005). For example, 18β GA decreased outward K^+ currents in whole cell patch clamping experiments (Takeda *et al.*, 2005). It was also suggested that the specific membrane depolarization could be a consequence of 18β GA blocking effects on delayed rectifier K^+ currents in smooth muscle cells (Takeda *et al.*, 2005). 18β GA investigated in the rat liver showed a blockage of dye coupling and a dephosphorylation of CX43 causing a morphological disassembly of the gap junction, which was reversible (Guan *et al.*, 1996). Moreover, investigations into dye spreading with the use of Lucifer Yellow on primary cultures of gastrointestinal smooth muscle showed that the application of 18β GA in the presence of Lucifer Yellow greatly reduced the spreading of the dye as

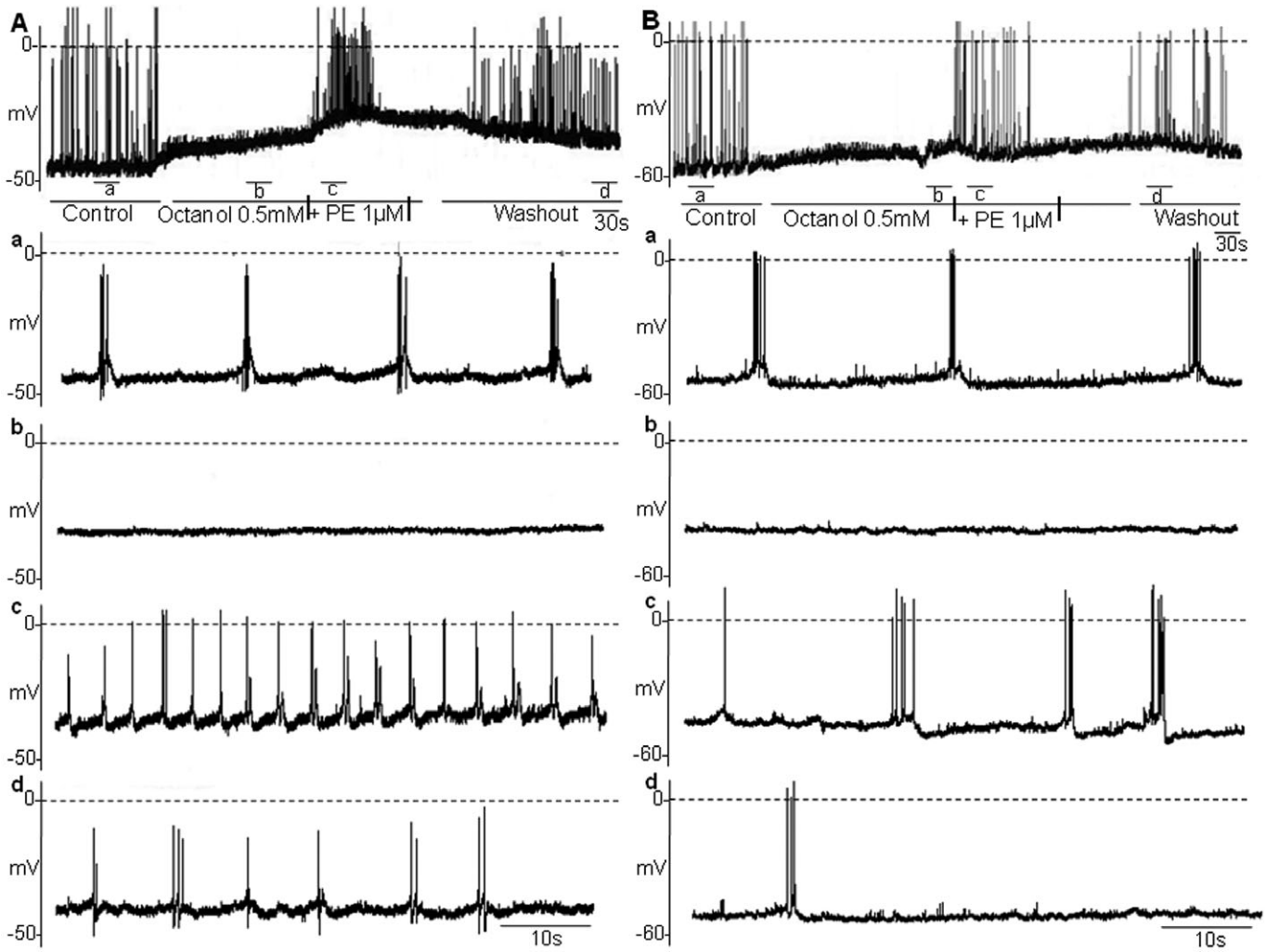

Figure 8

Effect of octanol on the spontaneous slow wave activity in cells from the young (A, from 5 cells) and old guinea pig prostate (B, from 5 cells). Control slow wave activity occurred regularly in both age groups (Aa, Bb). On the addition of 0.5 mM octanol, activity was immediately abolished and the membrane potential depolarized (Ab, Bb). On addition of phenylephrine (PE) in the presence of octanol activity was initiated (Ac, Bc) and upon washout, slow wave activity slowly returned to that resembling control values (Ad, Bd).

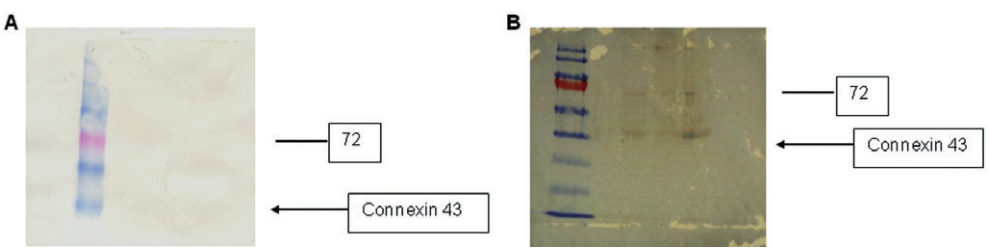

Figure 9

Western blot analysis for connexin 43 in the guinea pig prostate gland. Prostate gland proteins were separated by SDS-polyacrylamide gel and treated with an antibody to connexin 43. Numbers refer to molecular mass (kDa) based on protein standards. A indicates the negative control.

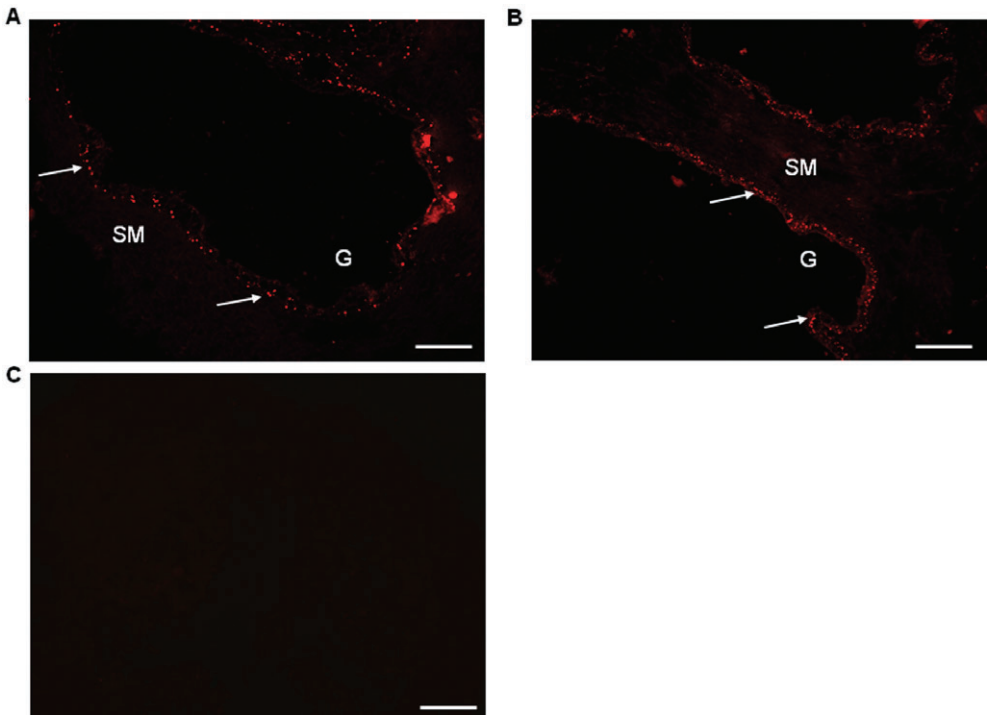

Figure 10

Representative photomicrographs of connexin 43 immunoreactivity in the guinea pig prostate gland. Note the presence of connexin 43 positive cells (arrows) within the border between the smooth muscle (SM) and glandular (G) layers in young (A) and old (B) guinea pig prostate gland. C represents the negative control. Scale bar represents 10 μm.

well as increasing the input cellular resistance and decreasing the capacitance (Takeda *et al.*, 2005).

Carbenoxolone

Carbenoxolone, a derivative of 18β GA, is more water soluble than 18β GA and is presumed to have similar effects on smooth muscle to its parent compound. Showing a similar trend to that of the experiments conducted with 18β GA, 50 μM carbenoxolone abolished spontaneous contractile activity recorded in both age groups of animals. Similarly, carbenoxolone reversibly abolished slow wave activity within both age groups of animals and significantly depolarized the membrane potential consistent with previous reports (Coleman *et al.*, 2001; Tare *et al.*, 2002). In addition, on the application of phenylephrine in the presence of carbenoxolone, slow wave activity resumed, indicating that the smooth muscle cell was still able to generate electrical activity.

Experiments conducted on Ca^{2+} waves within the guinea pig bladder have shown that carbenoxolone effectively inhibited the movement of Ca^{2+} between cells, indicating that propagation of Ca^{2+} waves resulted from the spread of spontaneous action potentials. It has also been suggested that the location of the interstitial cells within the bladder is essential in the spread of Ca^{2+} waves throughout the tissue (McCloskey and Gurney, 2002). Interstitial cells located on the boundary could be ideally situated to signal to the bulk of the smooth muscle and in addition isolated interstitial cells were able to generate their own spontaneous Ca^{2+} waves (McCloskey and Gurney, 2002). Within the guinea pig prostate gland, we have previously proposed that smooth muscle cells may be behaving as independent smooth muscle bundles (Dey *et al.*, 2009). Since pacemaker potentials were not recorded in the old guinea pig prostate, smooth muscle bundles may be electrically distant from the PICs perhaps explaining why older prostate tissues appear to be more sensitive to the effects of the gap junctional blockers.

Octanol

Octanol has been used extensively to examine the role of gap junctions in the spread of spontaneous activity (Daniel *et al.*, 1998; 2007; Lavoie *et al.*, 2007) and this compound exerts its effects by blocking the conductance of gap junctions composed of CX43 proteins (Sakai *et al.*, 1992). In the current study, octanol effectively abolished spontaneous

contractions within both age groups of animals at 0.5 mM and 1 mM concentrations. Investigations into in the guinea pig gallbladder revealed that on addition of octanol, spontaneous Ca^{2+} 'flashes' within the smooth muscle cells were eliminated, whereas they persisted in the interstitial cells of Cajal (ICC)-like cells (Lavoie *et al.*, 2007). This indicates that smooth muscle cells require pacemaker input from ICC-like cells, which play a role in the generation and propagation of electrical events.

In addition to gap junctional inhibition, octanol is known to have a multitude of effects. For example, experiments conducted on the mouse pancreas indicated that octanol was able to reversibly decrease gap junctional conductance at concentrations of 2 mM (Perez-Armendariz *et al.*, 1991), although voltage operated Ca^{2+} and K^+ currents were also depressed. Octanol has also been shown to inhibit L-type Ca^{2+} channels (Perez-Armendariz *et al.*, 1991; Serio *et al.*, 1991) which could be at least partly responsible for the inhibition of contractile activity observed in our experiments and the depolarization of membrane potential within both age groups. However, the maximal force generated in contractile activity in the presence of octanol as well as phenylephrine, was not significantly different to the values of phenylephrine alone on tissue (Figure 7). Similarly in electrophysiological experiments, octanol abolished all slow wave activity upon immediate exposure to the tissue. The addition of phenylephrine restored slow wave activity, which indicates that while smooth muscle cells were presumably uncoupled from their neighbouring PICs, when directly stimulated they were still able to generate activity (Figure 8).

In summary, we have demonstrated the presence of CX43 by immunostaining and Western blot analysis in the old as well as young guinea pig prostate. In addition, the results from this study reveal that intercellular communication via CX43 is essential in the propagation of spontaneous electrical activity and therefore spontaneous contractile activity throughout the prostate. A change in the proportion of cells exhibiting slow wave activity coupled with an increase in the basal tone of prostate tissue from the old guinea pigs may better explain the increase in smooth muscle tone that is observed in BPH. Furthermore the increased sensitivity to gap junction uncouplers shown by prostate tissue from old guinea pigs may reflect a change in the gap junction communication within the old prostate gland. Overall, this study has given an increased understanding of the changes that occur within the prostate gland with age and perhaps of the aetiology of age-related prostate specific conditions which may lead to the development of more specific and better targets to treat conditions such as BPH.

Acknowledgements

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Conflicts of interest

None to declare.

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