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Comparison of mechanical and electrical activity and interstitial cells of Cajal in urinary bladders from wild-type and *W/W^v* mice

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Background and purpose: *W/W^v* and wild-type murine bladders were studied to determine whether the *W/W^v* phenotype, which causes a reduction in, but not abolition of, tyrosine kinase activity, is a useful tool to study the function of bladder interstitial cells of Cajal (ICC).
Experimental approach: Immunohistochemistry, tension recordings and microelectrode recordings of membrane potential were performed on wild-type and mutant bladders.
Key results: Wild-type and *W/W^v* detrusors contained *c-Kit*- and vimentin-immunopositive cells in comparable quantities, distribution and morphology. Electrical field stimulation evoked tetrodotoxin-sensitive contractions in wild-type and *W/W^v* detrusor strips. Atropine reduced wild-type responses by 50% whereas a 25% reduction occurred in *W/W^v* strips. The atropine-insensitive component was blocked by pyridoxal-5-phosphate-6-azophenyl-2′,4′-disulphonic acid in both tissue types. Wild-type and *W/W^v* detrusors had similar resting membrane potentials of −48 mV. Spontaneous electrical activity in both tissue types comprised action potentials and unitary potentials. Action potentials were nifedipine-sensitive whereas unitary potentials were not. Excitatory junction potentials were evoked by single pulses in both tissues. These were reduced by atropine in wild-type tissues but not in *W/W^v* preparations. The atropine-insensitive component was abolished by pyridoxal-5-phosphate-6-azophenyl-2′,4′-disulphonic acid in both preparations.
Conclusions and implications: Bladders from *W/W^v* mice contain *c-Kit*- and vimentin-immunopositive ICC. There are similarities in the electrical and contractile properties of *W/W^v* and wild-type detrusors. However, significant differences were found in the pharmacology of the responses to neurogenic stimulation with an apparent up-regulation of the purinergic component. These findings indicate that the *W/W^v* strain may not be the best model to study ICC function in the bladder.
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Keywords: Interstitial cells; nerve-mediated contraction; immunohistochemistry; intracellular recordings

Abbreviations: ICC, interstitial cells of Cajal; EFS, electrical field stimulation; EJPs, excitatory junction potentials

Introduction

The functional role of interstitial cells of Cajal (ICC) in the physiology of the gastrointestinal tract that was suggested by numerous *in vitro* investigations of tissues and isolated cells, has been established by the use of mice that have mutations leading to deficiencies in their populations of ICC. Such animal models have been crucial in revealing the pacemaking and neurotransmission roles of several populations of ICC subtypes in different gastrointestinal tissues (Sanders *et al.*, 1999; 2006; Sanders and Ward, 2007). Commercially available animal models that have been employed by researchers interested in ICC have essentially included animals with defects in their Kit signalling pathways either at the Kit receptor (*W* mutants) or by affecting the Kit receptor ligand, stem cell factor (Steel mutants). The murine *W* locus, located on chromosome 5, has been identified as being allelic with the *c-Kit* gene (Chabot *et al.*, 1988; Geissler *et al.*, 1988; Bernstein *et al.*, 1990; Reith *et al.*, 1990) and mice with mutations at the *W* locus typically have severe macrocytic anaemia, loss of hair pigmentation, defects in haematopoietic stem cells (Russell, 1979) and gastrointestinal motility disorders (Maeda *et al.*, 1992). Homozygote *W* mutants (*W/W*) die before birth due to acute anaemia; however, heterozygotes with less severe anaemia from the *W/W^v* strain are used widely in research. *W^v*

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is a point mutation at amino acid 660 in *Kit* that causes a reduction in but not abolition of tyrosine kinase activity. W/W^v mice survive to adulthood although they have significant *c-Kit*-related defects, in particular the loss of some populations of ICC in the gut. Interestingly, other populations of gut ICC have apparently normal development (Burns *et al*., 1996).

The fact that W/W^v mice lack defined populations of ICC has allowed them to be an excellent model for studying the physiological function of these cells in specific gastrointestinal tissues.

The finding that ICC were absent in the myenteric plexus region of the small intestine (ICC-MY) in W/W^v animals was significant given that those tissues were electrically quiescent and normal slow wave (pacemaking) activity was absent (Ward *et al*., 1994; Huizinga *et al*., 1995), establishing a key pacemaker role for ICC-MY. Intramuscular ICC (ICC-IM), which are found in the smooth muscle layers in the gut, were found to be missing from the stomach and sphincteric regions of W/W^v mice. The significant defects in inhibitory and excitatory neurotransmission in these W/W^v tissues were explained by the loss of ICC-IM and not by changes in properties of neurons or smooth muscle cells (Burns *et al*., 1996; Ward *et al*., 1998; 2000; Beckett *et al*., 2002).

Knowledge of the function of ICC in the urinary bladder is at a comparatively early stage. While there is an emerging body of evidence on the physiological properties of bladder ICC from the detrusor and lamina-propria regions, both from studies of isolated cells and *in situ* preparations, the precise physiological roles of ICC in normal bladder function have not yet been elucidated (McCloskey and Gurney, 2002; Sui *et al*., 2002; Sui *et al*., 2004; Wu *et al*., 2004; Hashitani *et al*., 2004; McCloskey 2005; 2006; Brading and McCloskey, 2005; Johnston *et al*., 2008). The aim of the present investigation was to study urinary bladders from wild-type and W/W^v mice: in particular, to examine their populations of ICC using specific labelling antibodies and confocal microscopy, to compare their electrical and contractile responses to neurogenic stimulation, to determine the pharmacological identity of the innervation responsible for evoking the responses, to compare their contractile responses to exogenously applied agonists and to investigate their spontaneous electrical activity.

Methods

Preparation of urinary bladders

All animal procedures were in accordance with United Kingdom Animal Scientific Procedures Act, Schedule 1 (1986) and as approved by local university animal welfare and ethics committee or as approved by Care of Animals in the University of Nevada, Reno. Wild-type and mutant animals were available in both the University of Strathclyde and the University of Nevada laboratories. Comparisons between wild-type and mutant tissues in all of the experimental protocols were carried out in the same laboratory.

Urinary bladders were removed from C57BL or W/W^v mice (Jackson laboratories), which had been killed by cervical dislocation. Bladders were opened longitudinally and pinned to the Sylgard silicone elastomer (Dow Corning Corp., Midland, MI, USA) base of Petri dissecting dish. The mucosa was removed by peeling, to leave the underlying detrusor.

Contractile responses to electrical field stimulation

For contractile studies, strips of detrusor (5 mm × 1 mm × 1 mm) were mounted in organ baths with one end tied by thread to a fixed hook and the other to a tension transducer. The initial tension was set to 500 mg. Tissues were constantly superfused with oxygenated Krebs' solution at 35°C. Electrical field stimulation (EFS) was delivered by a Grass stimulator, via two platinum electrodes positioned close to the tissue. The mechanical signals were digitized and recorded to a personal computer running Acknowledge software (BIOPAC Systems) with a computerized data acquisition and analysis system (MP100; BIOPAC Systems, Inc, Goleta, CA).

Immunohistochemistry

Whole-mount or flat-sheet preparations of detrusor were fixed in acetone (for anti-*c-Kit*) or 4% paraformaldehyde (for anti-vimentin) and washed in PBS for immunohistochemical labelling. After blocking with 1% BSA to prevent nonspecific binding, the tissues were incubated in primary antibody (anti-*c-Kit* 1:200, Santa Cruz; anti-vimentin 1:100, Sigma, anti-vesicular acetylcholine transferase 1:200, Santa Cruz) containing 0.05% Triton X-100 for at least 24 h. Excess antibody was removed by washing with PBS and tissues were then incubated in fluorescent secondary antibody (Alexa Fluor 488 anti-rabbit immunoglobulin G or Alexa Fluor 594 anti-goat immunoglobulin G, 1:200; Molecular Probes, Eugene, OR). After washing in PBS, tissues were mounted with cover slips and examined with epi-fluorescence microscopy. Slides were imaged using a confocal microscope (Bio-Rad Radiance) equipped with an argon ion laser and a green HeNe laser to excite the fluorophores at 488 or 543 nm respectively, and emission filters to enable collection to a photo-multiplier tube at 520 nm (green) or above 570 nm (red). Double-labelled specimens were imaged sequentially to minimize any bleed-through of fluorophores. Digital images were acquired using Bio-Rad Lasersharp software and reconstructed using Metamorph software (Universal Imaging Inc.) and Adobe Photoshop.

Controls

Omission of the primary antibody showed no significant fluorescence of the secondary antibody. There was minimal autofluorescence in tissues that had not been incubated in either primary or secondary antibodies; smooth muscle bundles were only just visible over background levels. Positive controls were carried out on preparations of guinea-pig bladder.

Intracellular recordings

Preparations of detrusor (6 mm × 4 mm) were made as described above and pinned to the base of a Sylgard recording chamber with the serosal surface facing downward. Detrusor cells were impaled with glass microelectrodes that were filled

with KCl (3 mol·L^{-1}) and had resistances of 80–120 MΩ. Transmembrane potentials were recorded with a standard electrometer (Intra 767; World Precision Instruments, Sarasota, FL, USA). Data were recorded to a personal computer running AxoScope 8.0 data acquisition software (Axon Instruments, Union City, CA, USA) and results were analysed using Clampfit analysis software (Axon Instruments). In all experiments, parallel platinum electrodes were placed on either side of the muscle strips and neural responses were elicited by square wave pulses of EFS (single pulse; 0.1–0.75 ms pulse duration, 15 V) using a Grass S48 stimulator (Quincy, MA, USA).

Data analysis

Data were analysed using Microsoft Excel, Prism (Graphpad) and Origin (Microcal, Northampton, MA, USA) software and are expressed as mean ± standard error of the mean. Statistical comparisons were made using the Student's paired or unpaired *t*-test with $P < 0.05$ considered to be significant. Drug/molecular target nomenclature conform to the *British Journal of Pharmacology*'s Guide to Receptors and Channels (Alexander *et al.*, 2008).

Drugs and solutions

Pyridoxal-5-phosphate-6-azophenyl-2′,4′-disulphonic acid (PPADS), acetylcholine, atropine, tetrodotoxin (TTX), carbachol and nifedipine were all obtained from Sigma Chemical Company (St. Louis, MO). Drugs were dissolved in distilled H_2O as stock solutions and added to the organ bath to achieve the stated concentrations. Nifedipine was made up as a stock solution in ethanol that was then diluted in Krebs' solution to its final concentration. Krebs' solution contained the following (mmol·L^{-1}); 120.4 NaCl, 5.9 KCl, 15.5 $NaHCO_3$, 11.5 glucose, 1.2 $MgCl_2$, 1.2 NaH_2PO_4 and 2.5 $CaCl_2$; pH 7.3–7.4 at 36°C when bubbled with 97% O_2/3% CO_2.

Results

Bladders from both wild-type and W/W^v animals were emptied of urine by making a longitudinal incision through the detrusor, blotted dry on absorbent tissue and weighed before being placed immediately into saline solution. The mean mass of bladders from W/W^v mice was significantly higher than bladders from age-matched wild-type animals (mass of wild type 0.022 ± 0.002 g compared with W/W^v 0.03 ± 0.003 g, $P = 0.042$; $n = 8$ for each group). Macroscopic examination of the bladders did not indicate marked differences in appearance between the two groups.

Immunohistochemistry

Preparations of bladder detrusor from C57BL wild-type and W/W^v mutant mice were labelled with an antibody to the *c-Kit* receptor (an established marker of ICC) and imaged with a confocal microscope. Wild-type detrusor preparations had *c-Kit*-positive cells with the typical morphology of ICC reported for guinea-pig bladder (McCloskey and Gurney, 2002; Davidson and McCloskey, 2005) and were spindle-shaped with several lateral branches (Figure 1a,b). They were observed throughout the thickness of the detrusor in all regions of the bladder, located on the boundary of the smooth muscle bundles that were just visible due to their autofluorescence, running in parallel with the bundle orientation. Bladder preparations from W/W^v mice also had *c-Kit*-positive ICC-like cells (Figure 1d,e). These were of similar morphology and distribution to those observed in detrusors from wild-type mice. We did not observe marked differences in the numbers or distribution of *c-Kit*-positive cells between the wild-type ($n = 8$) and the mutant mouse bladders ($n = 8$). These results reveal that the W/W^v mice detrusor, like some regions of the gastrointestinal tract, contains apparently normal ICC. Double-labelling with anti-*c-Kit* and an antibody to vesicular acetylcholinetransferase showed that *c-Kit*-positive cells (red) were located close to cholinergic nerves (green) in both wild-type and W/W^v bladders (Figure 1c,f).

Vimentin antibodies are often used to label ICC as vimentin (intermediate) filaments are found in ICC but are absent from smooth muscle cells. This approach is less specific than the use of *c-Kit* antibodies as vimentin filaments are found in other cell phenotypes including fibroblasts; however, it is an important method to confirm the presence of ICC in tissue preparations. In the present study, both wild-type and W/W^v detrusors contained vimentin-positive cells that had a variety of morphologies: some were elongated and were located on the boundary of the smooth muscle bundles; others were stellate with several branches emanating from a central nuclear region; others had a more rounded shape (Figure 2). The vimentin-positive cell population is likely to contain both ICC and fibroblasts. Double-labelling experiments with anti-*c-Kit* and anti-vimentin were not carried out as each antibody requires a specific fixative (detailed in *Methods*). Clear differences between the pattern of vimentin immunolabelling between wild-type and W/W^v bladders were not detected.

Contractile response to EFS

Strips of bladder from wild-type and W/W^v mice were mounted in organ baths and allowed to equilibrate for up to 1 h. Spontaneous contractile activity typically did not develop during this period whether strips were mounted to an initial tension of 100, 200 or 500 mg, consistent with the findings of others (Petkov *et al.*, 2001). Preparations were initially stimulated at 8 Hz for 10 s (100 V, pulse width 0.3 ms) and responded with a contraction that was maintained for the duration of the stimulation. The stimulation was repeated after 5 min to ensure reproducibility of responses. Figure 3 shows contractile responses of wild-type and W/W^v bladder strips to stimulations of 1, 2, 4, 8 and 16 Hz. These were abolished in the presence of TTX (0.5 μmol·L^{-1}), indicating that the stimulation parameters were directly stimulating nerves. Overall, there was no significant difference in the amplitude of EFS-induced contractions produced by strips from W/W^v bladders ($n = 16$ strips from six animals) compared with those from wild type ($n = 12$ strips from four animals) at all the frequencies tested [amplitude evoked by 16 Hz stimulation; 738 ± 138 mg (wild type), compared with 612 ± 138 mg (W/W^v); $P = 0.53$].

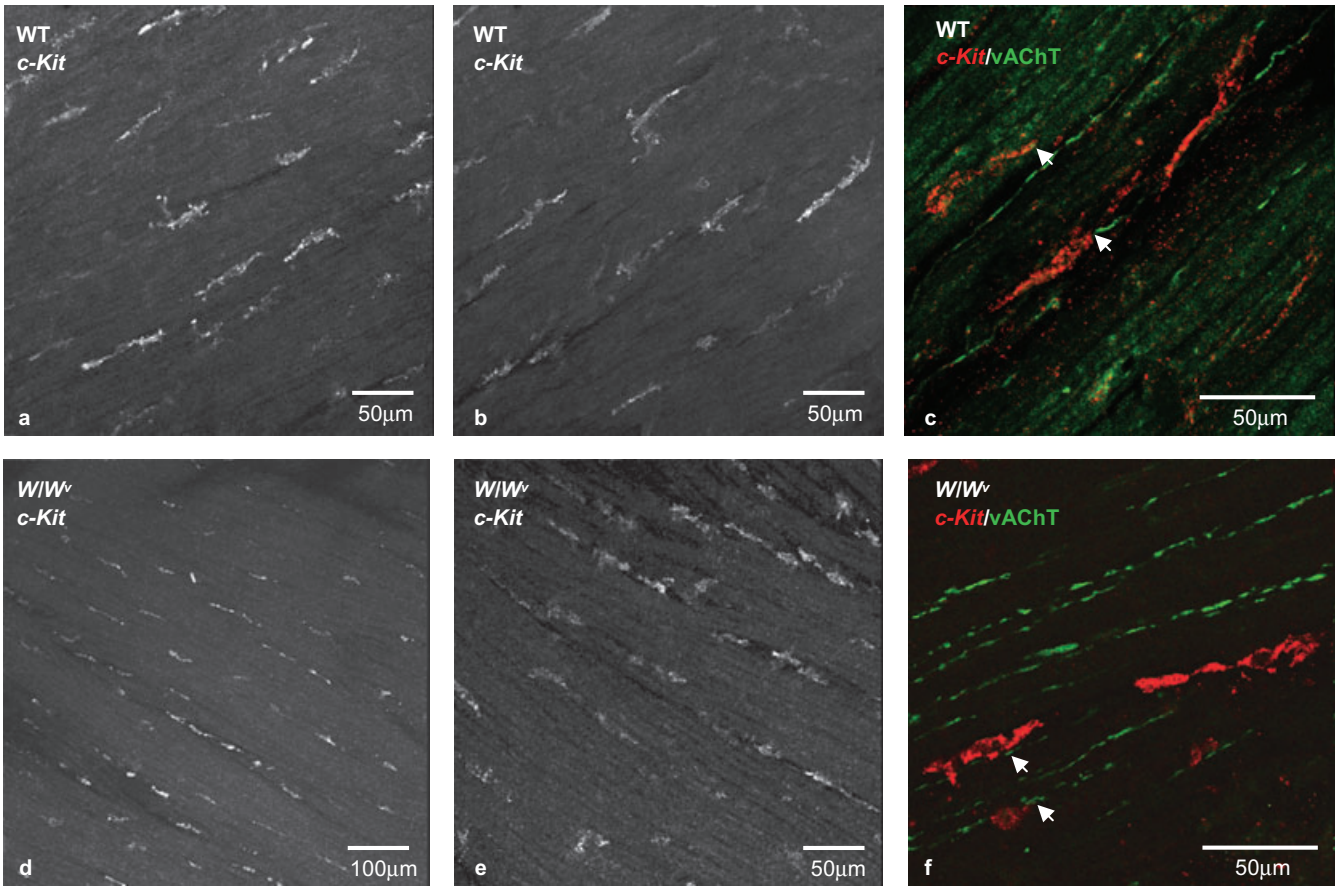

Figure 1 *c-Kit*-positive cells in wild-type and *W/W*^{v} bladder. *c-Kit*-positive cells in whole-mount preparations of wild-type (a,b) and *W/W*^{v} (d,e) mouse detrusor. Immunoreactive cells were elongated with several small lateral branches and were orientated parallel to the detrusor muscle fibres. Double-labelling with an antibody to vesicular acetylcholine transferase (vAChT, green) and anti-*c-Kit* (red) revealed close proximity between interstitial cells of Cajal and cholinergic neurons in both tissue types (c,f). All images are projections of a stack of optical sections captured with a confocal microscope.

Cholinergic and purinergic mediated contractile responses to EFS

Tissues were perfused with the muscarinic antagonist atropine (1 μmol·L^{-1}) after responses to the range of stimulations had been obtained in the absence of drugs. In strips from wild-type bladders, atropine significantly reduced the contractions at all frequencies (except 1 Hz). Subsequent addition of the purinergic receptor antagonist, PPADS (30 μmol·L^{-1}), abolished the atropine-resistant contractions. A typical experiment is shown in Figure 4a and data for $n = 9$ strips from three animals are summarized in Figure 4c. Atropine also caused a significant reduction in the EFS contractions in *W/W*^{v} animals at all frequencies and the residual component was abolished by the subsequent addition of PPADS ($n = 9$ strips from three animals, Figure 4b). While both wild-type and *W/W*^{v} bladder strips had both a cholinergic and a purinergic component of neurogenic contractions, it is interesting to note that the relative proportions were different. The amplitude of the contraction to 16 Hz in wild-type detrusor strips was 52% cholinergic and 48% purinergic (control 571 ± 185 mg reduced to 272 ± 97 mg in atropine and further reduced to 24 ± 8 mg by PPADS), whereas responses in *W/W*^{v} detrusor strips were comprised of 27% cholinergic and 73% purinergic components (control 1054 ± 115 mg, atropine 774 ± 115 mg and PPADS 76 ± 15 mg).

Addition of the muscarinic agonist, carbachol, to wild-type and *W/W*^{v} detrusor strips evoked contractions in a concentration-dependent fashion. Typical traces are shown in Figure 5. Concentrations of carbachol (0.5, 1, 5, 10 μmol·L^{-1}) were applied to the tissue for 2 min and then washed out for at least 20 min before the next concentration was added. Experiments showed that this time was sufficient to allow reproducible responses. The area under the curve for the first 5 min of the carbachol-induced contraction at each concentration was measured and normalized to that of the maximum concentration. Mean data were plotted against concentration and fitted with a Hill equation so that the EC_{50} could be calculated ($E/E_{max} = E_{min} + (E_{max} - E_{min})[xn/(kn + xn)]$ where E/E_{max} was the relative response to carbachol, kn was the EC_{50} and n was the Hill coefficient). Wild-type strips had an EC_{50} of 1.2 ± 0.05 μmol·L^{-1} whereas *W/W*^{v} strips had an EC_{50} of 2.6 ± 0.5 μmol·L^{-1}, indicating that the *W/W*^{v} tissues were perhaps less sensitive to carbachol. The shape of the carbachol-induced contractions did not differ between wild-type and *W/W*^{v} strips.

Intracellular recordings

Wild-type detrusor preparations had mean resting membrane potentials of –47.5 ± 1.1 mV ($n = 18$ impalements of tissues

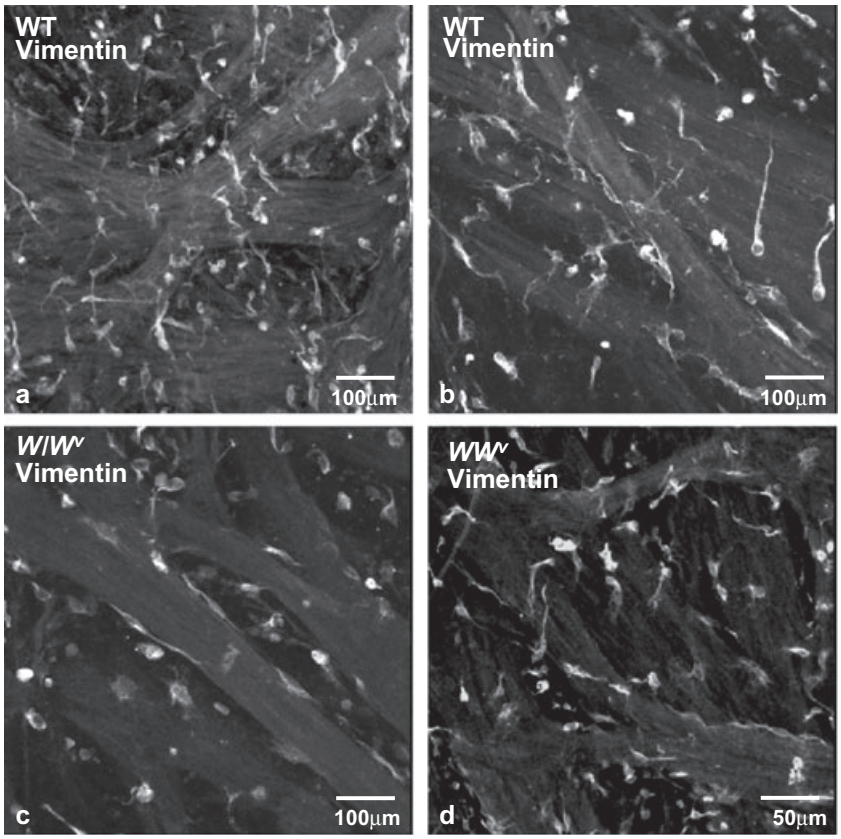

Figure 2 Vimentin-positive cells in wild-type and W/W^v bladder. Vimentin-positive cells in whole-mount preparations of wild-type (a,b) and W/W^v (c,d) mouse detrusor. Vimentin (intermediate) filaments are found in interstitial cells of Cajal (ICC) and fibroblasts but not in smooth muscle cells and vimentin antibodies are often used to label ICC. Vimentin-positive cells had several morphologies including rounded, stellate or elongated cell bodies with branched processes and are likely to include both ICC and fibroblasts. All images are projections of a stack of optical sections captured with a confocal microscope.

from six animals) and those from W/W^v detrusors had mean resting membrane potentials of -47.7 ± 1.0 mV ($n = 12$ impalements of tissues from three animals). There was no significant difference between the two groups ($P = 0.8966$). Spontaneous electrical activity was recorded in both wild-type and W/W^v preparations. This activity typically comprised spontaneous unitary potentials and action potentials. Both wild-type and W/W^v tissues exhibited similar electrical activity and typical recordings are illustrated in Figure 6. Action potentials were fired at a frequency of 3.3 ± 1.6 min^{-1} (range: 0–14 min^{-1}, $n = 11$ from four animals) in wild-type tissues and 11.3 ± 3.4 min^{-1} in W/W^v (range: 2–26 min^{-1}, $n = 8$ from two animals). This apparent difference in frequency was not tested statistically as tissues from only two mutant animals were available. The mean amplitude of action potentials in wild-type and W/W^v tissues were 30 ± 3.9 and 36.0 ± 4.04 mV respectively. Unitary potentials were fired at a frequency (and amplitude) of 7.7 ± 1.2 min^{-1} (6.3 ± 1.0 mV) in wild-type ($n = 11$ from six animals) and 10.9 ± 2.5 min^{-1} (6.2 ± 0.5 mV) in W/W^v tissues. Action potentials were inhibited by 1 μmol·L^{-1} nifedipine. However, unitary potentials remained and continued to fire regularly (9.4 ± 2.6 min^{-1} in wild type and 7.0 ± 2.2 min^{-1} in W/W^v ($n = 5$ and $n = 4$ respectively, $P > 0.05$ in both wild type and W/W^v). Addition of atropine (1 μmol·L^{-1}) did not affect the frequency or amplitude of unitary potentials nor the resting membrane potential, but addition of PPADS (100 μmol·L^{-1}) caused a small depolarization (in the region of 5 mV) and abolished unitary potentials in both wild-type ($n = 2$) and W/W^v ($n = 3$) tissues.

Preparations were stimulated with single pulses having a range of durations (0.1, 0.2, 0.3, 0.4, 0.5, 0.75 ms), which typically evoked excitatory junction potentials (EJPs). In the absence of drugs, similar responses were elicited in both wild-type and W/W^v tissues (Figure 7). Application of atropine (1 μmol·L^{-1}) caused a small reduction in the amplitude of the EJPs in wild-type preparations but did not inhibit those from W/W^v detrusors (see expanded traces). In contrast, PPADS (100 μmol·L^{-1}) abolished the atropine-resistant component of EJPs in all tissues.

Discussion

The study of ICC in the urinary bladder has been aided by the use of the specific marker anti-*c-Kit* that has enabled the cells to be identified in fixed tissues, in preparations *'in situ'* and in enzymically dispersed cells. While progress has been encouraging on investigations of the morphological characteristics of ICC, their structural interactions with nerves and smooth muscle cells, their Ca^{2+}-signalling response to cholinergic

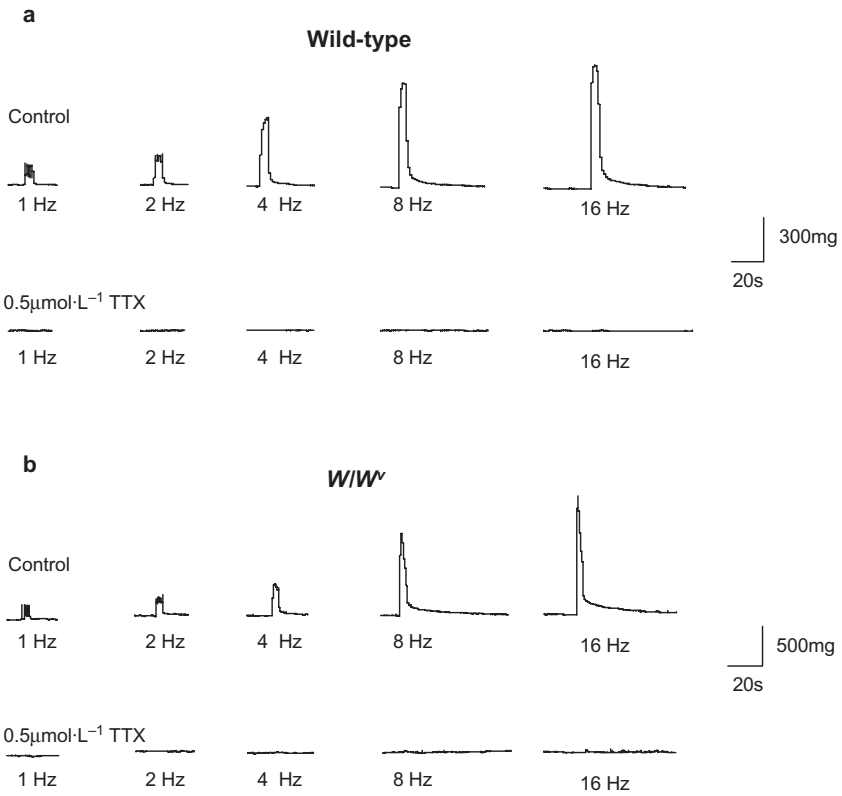

Figure 3 Contractile responses to electrical field stimulation. Detrusor strips from wild-type (a) and *W/W*^{v} (b) animals contracted in response to electrical field stimulation applied for 10 s at frequencies of 1, 2, 4, 8 and 16 Hz; pulse width durations of 0.3 ms. All contractions were abolished in the presence of tetrodotoxin (TTX) (0.5 μmol·L^{-1}).

stimulation and their electrophysiological properties, the study of mice having mutations in Kit-signalling pathways could potentially provide the opportunity of studying bladders devoid of ICC and therefore help determine the functional role of ICC in the bladder. In the present study, the bladders of *W/W*^{v} mice were investigated and compared with wild-type bladders to ascertain whether these mutants are a useful tool in the assessment of the role of ICC in the urinary bladder.

Identification of ICC in W/W^{v} *bladder*

Our immunohistochemistry experiments demonstrated that *c-Kit*-positive ICC and vimentin-positive ICC-like cells were present in both wild-type and *W/W*^{v} murine bladders. ICC in the guinea-pig bladder are immunopositive for both *c-Kit* and vimentin antibodies (Davidson and McCloskey, 2005), comparable with the ICC in the gastrointestinal tract. Vimentin (intermediate) filaments are found in ICC and fibroblasts but not smooth muscle cells and vimentin-labelling is therefore a useful tool in the identification of ICC in smooth muscle tissues. The vimentin-positive cells in the present study will include both ICC and fibroblasts and therefore represents a larger, heterogeneous population compared with the *c-Kit*-positive cells. The *c-Kit*-positive ICC had the typical morphology and arrangement of ICC in the guinea-pig bladder; moreover, there was no evidence of differences in quantities or arrangement of ICC in wild-type or *W/W*^{v} bladders. This finding was rather disappointing as it indicates that the *W/W*^{v} strain is not the model of choice for study of the role of ICC in the bladder. However, it is consistent with findings in gastrointestinal tissues where several classes of ICC in the gut are apparently unaffected by *W/W*^{v} mutations. ICC-MY are present in the *W/W*^{v} stomach comparable with those in wild-type animals (Ordög *et al.*, 2002; Hirst *et al.*, 2002; Sanders *et al.*, 2006 for review) and it has been reported that while ICC are reduced in the colons of *W/W*^{v} mice, no class of ICC are entirely lost in this region (Sanders and Ward, 2007).

There are other strains of mice with *W* mutations that might be potentially useful in the study of bladder ICC. *W/W* is a homozygotic lethal mutation arising from a *c-Kit*^{W} allele that encodes a shortened protein lacking the transmembrane portion, leading to non-expression of the Kit receptor on the cell membrane. The majority of *W/W* animals die *in utero* or before postnatal day 10 due to anaemia, significantly limiting their ability to be used in ICC-related research. Waskow *et al.* (2004) generated viable transgenic erythropoietin-overexpressing *W/W* mice, termed *WEPO*, which are potentially useful to analyse the role of *c-Kit*-positive ICC in mature animals. Although these animals do not have surface expression of *c-Kit*, transgenic expression of erythropoietin appears to overcome the lethality caused by the *W/W* mutation and the typical macrocytic anaemia is reduced to at a level that enables survival.

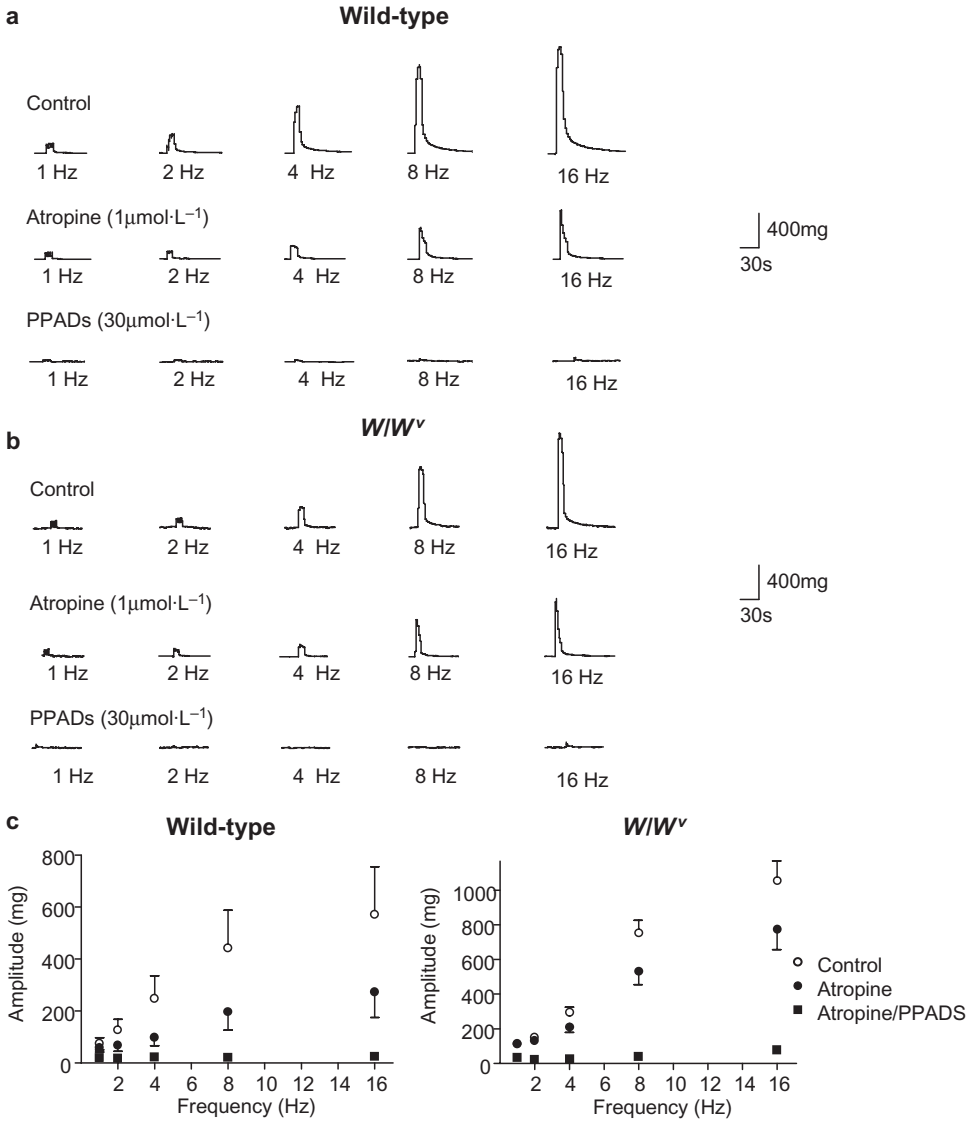

Figure 4 Cholinergic and purinergic components of neurogenic-evoked contractions. Detrusor strips from wild-type (a) and *W/W*^{v} (b) animals contracted in response to electrical field stimulation as before. In both tissue types, atropine (1 μmol·L^{-1}) reduced the amplitude of the contractions with the remaining responses inhibited by pyridoxal-5-phosphate-6-azophenyl-2′,4′-disulphonic acid (PPADS) (30 μmol·L^{-1}). Summary graphs (c) reveal that atropine blocked approximately 50% of the contraction in wild-type strips, whereas in *W/W*^{v} strips, atropine reduced the contractions by only about 25%. Contraction amplitude at 16 Hz in wild-type strips was 52% cholinergic and 48% purinergic (control 571 ± 185 mg reduced to 272 ± 97 mg in atropine and further reduced to 24 ± 8 mg by PPADS), whereas responses in *W/W*^{v} strips were comprised of 27% cholinergic and 73% purinergic components (control 1054 ± 115 mg, atropine 774 ± 115 mg and PPADS 76 ± 15 mg).

General properties of W/W^{v} *smooth muscle*

The electrical and mechanical properties of *W/W*^{v} detrusor smooth muscle in our study did not appear to be different from wild-type smooth muscle. There was no significant difference between resting membrane potentials, patterns of spontaneous electrical activity or the maximal amplitude of contraction in response to nerve stimulation in the two strains of animals. This is first consistent with the *c-Kit* immunohistochemistry, which indicated that ICC were present in both tissues and second indicates that *W/W*^{v} bladder smooth muscles are not affected by Kit mutations. Studies of *W/W*^{v} gastrointestinal smooth muscles in which ICC-IM were absent showed that nerve processes were present as in the controls; there was no reduction in transmitter release; there was no loss of response to exogenous transmitter (showing that integrity of smooth muscle contraction had not been compromised) and that there was no loss of nerve cell bodies or fibres (Ward *et al.*, 1994; 2000; Burns *et al.*, 1996; Beckett *et al.*, 2002).

Up-regulation of purinergic responses

In spite of the presence of ICC in *W/W*^{v} detrusors and the apparent normality of the smooth muscle, the contractile response to electrical nerve stimulation had a different pharmacology from that recorded in strips from wild-type

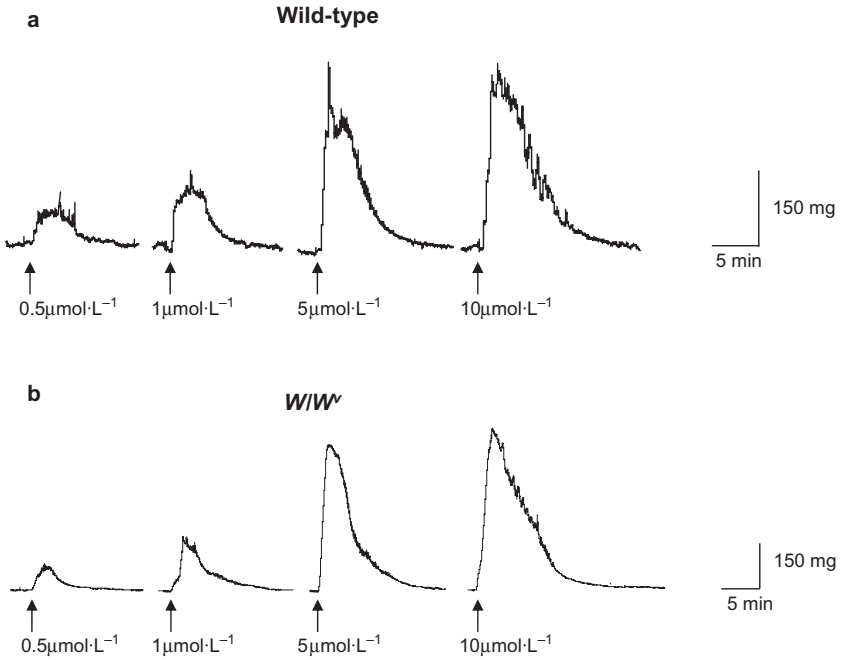

Figure 5 Exogenously applied carbachol. Carbachol was applied to detrusor strips from wild-type (a) and W/W^v (b) bladders as indicated on the traces. Agonist was applied for 2 min and tissues were subsequently washed for at least 20 min before the next concentration was added.

bladders. While both wild-type and W/W^v strips responded to nerve stimulation with TTX-sensitive contractions of comparable amplitude, the proportion of cholinergic and purinergic components were notably different. Overall, wild-type bladder strips exhibited an approximate 50 : 50 ratio of cholinergic versus purinergic components of contractions, whereas this was about 25 : 75 in W/W^v strips. Our control data are consistent with published results for EFS-induced contractions in mouse bladder in which atropine causes a 50% reduction (Werner *et al.*, 2007). The reason for the difference in W/W^v strips is unclear. However, it is reminiscent of several pathological bladder conditions in which purinergic-evoked contractions either emerge for the first time or, alternatively, are up-regulated.

Normal detrusors of many species of mammal including mouse, rat, guinea pig (Brading and Williams 1990; Kennedy *et al.*, 2007) and rabbit (Chancellor *et al.*, 1992) have both cholinergic and purinergic components of the contractile response to neurogenic stimulation (for review see Burnstock 2001). In human detrusor strips, EFS-mediated contractions are normally exclusively atropine-sensitive (see Fry *et al.*, 2005 for review); however, in pathological conditions, an atropine-insensitive component emerges. Strips of detrusor from patients suffering from interstitial cystitis had an atropine-resistant portion of EFS-induced contraction, which was abolished after desensitization with α,β-methylene ATP, in contrast to control detrusor strips where an atropine-insensitive component did not exist (Palea *et al.*, 1993). A purinergic component of EFS-induced contractions has also been reported for idiopathic unstable female human detrusor strips, which comprised 50% of the total response. This finding was explained by an up-regulation of $P2X_2$ receptors in the idiopathic unstable samples and interestingly, other P2X receptors decreased (O'Reilly *et al.*, 2002). A study of human detrusor strips from stable, unstable and obstructed bladders demonstrated that unstable and obstructed strips had atropine-resistant contractions that were sensitive to TTX or α,β-methylene ATP (Bayliss *et al.*, 1999). The emergence of a purinergic component was apparently not due to differences in smooth muscle contractility as the response to direct muscle stimulation was similar in all three patient groups. Unstable bladders arising from a neuropathic instability conversely did not have atropine-resistant contractions.

In a rabbit model of detrusor instability secondary to bladder outlet obstruction, the purinergic component increased and cholinergic component decreased in the early hypertrophic stage (Calvert *et al.*, 2001). However, in a model of partial bladder outlet obstruction in the same species, up-regulation of the P2X component of bladder contraction did not occur 3 weeks after the obstruction, leading the authors to suggest that it perhaps occurred later in bladder hypertrophy/pathology. They also noted no difference in P2X receptor subtypes expressed on detrusor smooth muscle between control and partially obstructed groups (Banks *et al.*, 2006).

It is possible that the W/W^v bladder has undergone changes in the expression of cell types other than ICC, related to the W/W^v *c-Kit* mutation. For example, it has been reported that mast cells are absent in W/W^v bladder (Saban *et al.*, 2001). D'Andrea *et al.* (2005) demonstrated spatial relationships between mast cells and nerves in the mucosa and detrusor regions of murine bladder. There is considerable evidence that purinergic mechanisms play key roles in inflammatory responses of many tissues (Kolachala *et al.*, 2007) and it is likely that purinergic neurons contact mast cells in the bladder. It seems feasible that in the mast cell-deficient W/W^v detrusor, there may therefore be a richer innervation of smooth muscle cells leading to a larger neurogenic-evoked

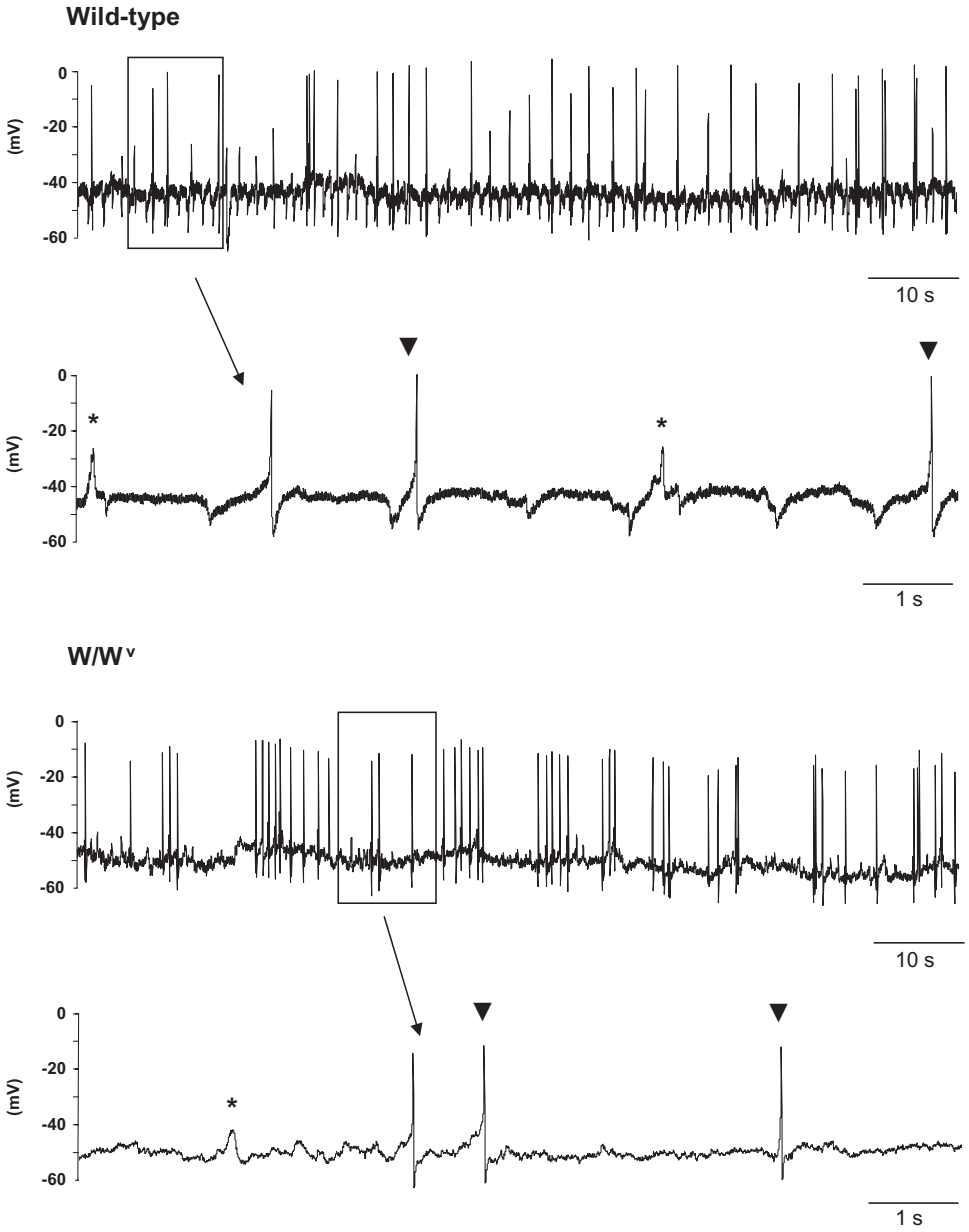

Figure 6 Intracellular recordings of spontaneous electrical activity. Wild-type and *W/W*^{v} detrusor preparations exhibited electrical activity comprising spontaneous unitary potentials (denoted by asterisks) and larger events resembling action potentials (indicated by arrowheads). Nifedipine (1 μmol·L^{-1}) inhibited action potentials in both tissue types; however, unitary potentials were unaffected (trace not shown).

purinergic component. Alternatively, our findings might be explained by the *W/W*^{v} mutation causing an up-regulation of purinergic nerve fibre expression or indeed an increase in purinoceptor expression. An up-regulation of purinoceptor expression in *W/W*^{v} gastric fundus has been shown by Sergeant *et al*. (2002) who used cDNA microarrays to demonstrate that P2Y receptors were up-regulated in *W/W*^{v} muscles lacking ICC-IM.

In summary, the present study has shown that *c-Kit*-positive ICC and vimentin-positive ICC-like cells are present in the *W/W*^{v} bladder. There was little difference in spontaneous electrical activity or contractile properties between wild-type and *W/W*^{v} detrusor strips. The neurogenic contractile response had a greater proportion from purinergic nerves than cholinergic nerves in *W/W*^{v} tissues compared with wild type. Other strains of mutant mice may offer an advantage over *W/W*^{v} in the study of ICC in the urinary bladder.

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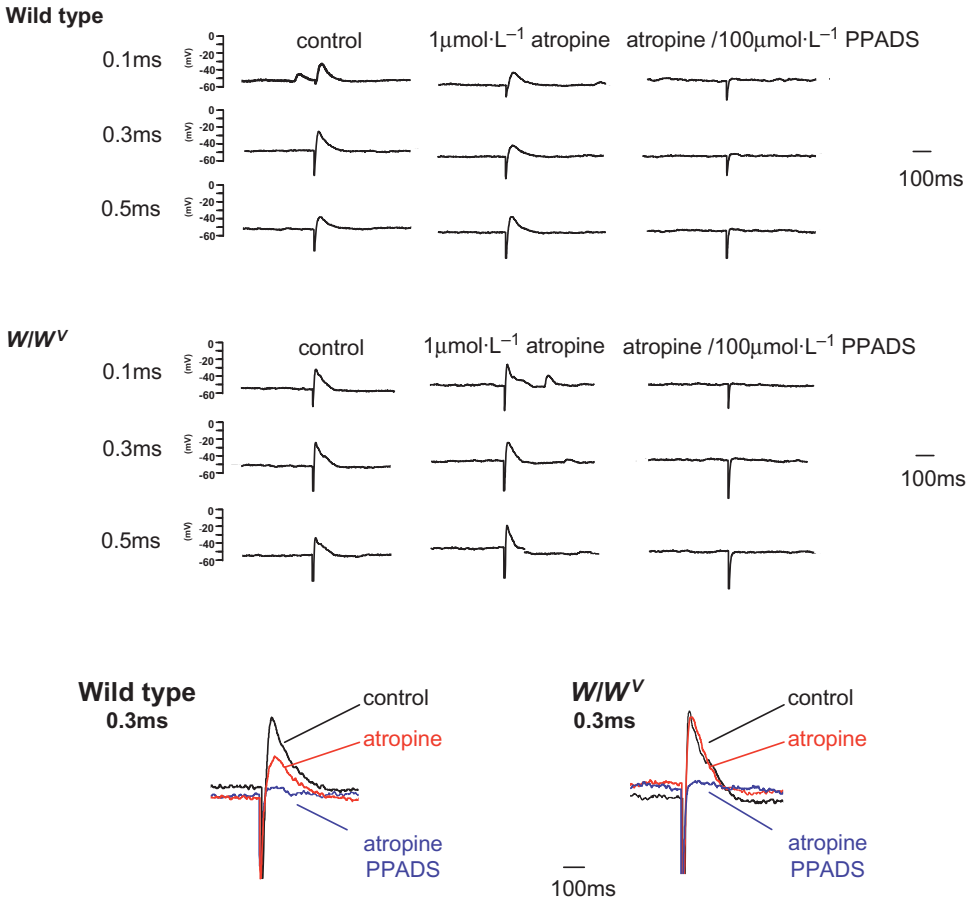

Figure 7 Neurogenic-evoked electrical responses. Wild-type and *W/W*^{v} detrusor preparations were stimulated with single pulses having durations of 0.1, 0.3 and 0.5 ms, which typically evoked excitatory junction potentials (EJPs). In the absence of drugs, similar responses were elicited in both wild-type and *W/W*^{v} tissues. Application of atropine (1 μmol·L^{-1}) caused a small reduction in the amplitude of the EJPs in wild-type preparations but did not inhibit those from *W/W*^{v} detrusors (see expanded traces of the responses to a pulse of 0.3 ms). In contrast, PPADS (100 μmol·L^{-1}) abolished the atropine-resistant component of EJPs in all tissues.

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Conflicts of interest

None.

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