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RESEARCH PAPER

Distinct effects of CGRP on typical and atypical smooth muscle cells involved in generating spontaneous contractions in the mouse renal pelvis

Hikaru Hashitani[1], Richard J Lang[2], Retsu Mitsui[3], Yoshio Mabuchi[4] and Hikaru Suzuki[1]

[1]*Department of Cell Physiology, Nagoya City University Graduate School of Medical Sciences, Nagoya, Japan,* [2]*Department of Physiology, School of Biomedical Sciences, Monash University, Clayton, Victoria, Australia,* [3]*Department of Health Science and Social Welfare, Faculty of Human Sciences, Waseda University, Tokorozawa, Japan, and* [4]*Department of Functional Morphology, Nagoya City University Graduate School of Medical Sciences, Nagoya, Japan*

Background and purpose: We investigated the cellular mechanisms underlying spontaneous contractions in the mouse renal pelvis, regulated by calcitonin gene-related peptide (CGRP).
Experimental approach: Spontaneous contractions, action potentials and Ca^{2+} transients in typical and atypical smooth muscle cells (TSMCs and ATSMCs) within the renal pelvis wall were recorded separately using tension and intracellular microelectrode recording techniques and Fluo-4 Ca^{2+} imaging. Immunohistochemical and electron microscopic studies were also carried out.
Key results: Bundles of CGRP containing transient receptor potential cation channel, subfamily V, member 1-positive sensory nerves were situated near both TSMCs and ATSMCs. Nerve stimulation reduced the frequency but augmented the amplitude and duration of spontaneous phasic contractions, action potentials and Ca^{2+} transients in TSMCs. CGRP and agents increasing internal cyclic adenosine monophosphate (cAMP) mimicked the nerve-mediated modulation of TSMC activity and suppressed ATSMCs Ca^{2+} transients. Membrane hyperpolarization induced by CGRP or cAMP stimulators was blocked by glibenclamide, while their negative chronotropic effects were less affected. Glibenclamide enhanced TSMC Ca^{2+} transients but inhibited ATSMC Ca^{2+} transients, while both 5-hydroxydecanoate and diazoxide, a blocker and opener of mitochondrial ATP-sensitive K^+ channels, respectively, reduced the Ca^{2+} transient frequency in both TSMCs and ATSMCs. Inhibition of mitochondrial function blocked ATSMCs Ca^{2+} transients and inhibited spontaneous excitation of TSMCs.
Conclusions and implications: The negative chronotropic effects of CGRP result primarily from suppression of ATSMC Ca^{2+} transients rather than opening of plasmalemmal ATP-sensitive K^+ channels in TSMCs. The positive inotropic effects of CGRP may derive from activation of TSMC L-type Ca^{2+} channels. Mitochondrial Ca^{2+} handling in ATSMCs also plays a critical role in generating Ca^{2+} transients.
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Keywords: renal pelvis; CGRP; mitochondria; sensory nerve; intracellular calcium; ATP-sensitive K^+ (K_{ATP}) channels; smooth muscle

Abbreviations: 5-HD, 5-hydroxydecanoate; ATSMC, atypical smooth muscle cell; BK channel, large-conductance Ca^{2+} activated K^+ channel; CCCP, carbonyl cyanide 3-chlorophenylhydrazone; CGRP, calcitonin gene-related peptide; CPA, cyclopiazonic acid; ER, endoplasmic reticulum; ICC, interstitial cells of Cajal; K_{ATP} channels, ATP-sensitive K^+ channels; PBS, phosphate-buffered saline; PCJ, pelvicalyceal junction; PSS, physiological salt solution; STDs, spontaneous transient depolarizations; TRPV1, transient receptor potential cation channel, subfamily V, member 1; TSMC, typical smooth muscle cell; TTX, tetrodotoxin

Introduction

Spontaneous contractions of smooth muscle in the renal pelvis are the basis of peristaltic contractions that transport urine from the kidney to the bladder. These contractions are considered to originate in atypical smooth muscle cells (ATSMCs), which are preferentially distributed in the most

Correspondence: Hikaru Hashitani, Department of Cell Physiology, Nagoya City University Graduate School of Medical Sciences, Nagoya 467-8601, Japan. E-mail: hasitani@med.nagoya-cu.ac.jp
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proximal renal pelvis (Klemm *et al.*, 1999). In the mouse renal pelvis, ATSMCs exhibit spontaneous Ca^{2+} transients. These transients arise from Ca^{2+} release from intracellular stores and are thought to open Ca^{2+}-activated cation channels to generate spontaneous transient depolarizations (STDs) (Lang *et al.*, 2007a,b). ATSMCs may act as 'point sources' of excitation sending an electrical input, that is, STDs, into adjacent typical smooth muscle cells (TSMCs) to drive bundles of TSMCs, that increase in number from the point of attachment between the papilla and calyx (pelvicalyceal junction, PCJ) to the pelviureteric junction until only TSMCs are present within the ureter. Thus propagation of action potentials and associated intercellular Ca^{2+} waves via gap junctions within and between TSMC bundles may be the fundamental mechanism underlying peristaltic contractions (Lang *et al.*, 2007a).

In spite of an extensive plexus of cholinergic and noradrenergic nerves in the renal pelvis (Rolle *et al.*, 2008), local motor responses produced by transmural nerve stimulation are unaffected by either atropine or guanethidine, although cholinergic or adrenergic agonists are able to modulate spontaneous contractions (see Santicioli and Maggi, 1998). Instead, the stimulation of sensory nerves with electrical stimuli or capsaicin, modulates spontaneous contractility by releasing neuropeptides, including calcitonin gene-related peptide (CGRP), substance P and neurokinin A. Thus, sensory nerves distributed in the renal pelvis may uniquely have an 'efferent' as well as 'afferent' signalling role and play a fundamental role in regulating or sustaining the intrinsic contractile properties of the renal pelvis (Lang *et al.*, 2002). However, it is not yet known whether sensory nerves target ATSMCs or TSMCs to modulate the spontaneous phasic contractions of the renal pelvis.

CGRP is the main mediator involved in the local inhibition of motility in the renal pelvis. CGRP, either endogenous and released from sensory nerves or exogenous and added *in vitro*, causes a negative inotropic effect on spontaneous contractions (Maggi *et al.*, 1992; Teele and Lang, 1998), and also reduces the frequency of spontaneous action potentials in the distal renal pelvis (Maggi *et al.*, 1995a; Exintaris and Lang, 1999). In the ureter, the mechanisms underlying the negative inotropic effect of CGRP are thought to involve the opening of plasmalemmal ATP-sensitive K^+ (K_{ATP}) channels via a cyclic adenosine monophosphate (cAMP)-dependent pathway to hyperpolarize the membrane. However, the role of plasmalemmal ATP-sensitive K^+ channels (K_{ATP} channels) is more controversial in the renal pelvis (Maggi *et al.*, 1995a; Teele and Lang, 1998; Exintaris and Lang, 1999).

The notion that membrane hyperpolarization upon the opening of plasmalemmal K_{ATP} channels reduces cell excitability is well established in smooth muscle. However K_{ATP} channels are also located on the inner membrane of mitochondria (Garlid *et al.*, 1996) and are thought to play an important role in regulating their function. The opening of mitochondrial K_{ATP} channels has been reported to increase matrix volume, although their direct effect on respiration, membrane potential or Ca^{2+} uptake has been rather conflicting (Holmuhamedov *et al.*, 1998; Kowaltowski *et al.*, 2001). Accumulating evidence now indicates that mitochondria also play a fundamental role in the initiation of the spontaneous activity in interstitial cells of Cajal (ICC) that act as pacemaker cells (Ward *et al.*, 2000; Fukuta *et al.*, 2002; Balemba *et al.*, 2008; Sergeant *et al.*, 2008) or in smooth muscles themselves (Kubota *et al.*, 2003). As ATSMCs, the putative pacemaker cells of the renal pelvis are richly endowed with mitochondria and endoplasmic reticulum (ER) (Klemm *et al.*, 1999), thus it is pertinent to ascertain whether mitochondrial function plays a role in the initiation of the spontaneous activity in a manner that can be modulated by CGRP.

In the present study, we investigated the effects of sensory nerve stimulation and bath-applied CGRP on the spontaneous contractions, action potentials and Ca^{2+} transients in TSMCs as well as Ca^{2+} dynamics in ATSMCs of the mouse renal pelvis. The effects of agents known to increase cAMP production, to modulate plasmalemmal and mitochondrial K_{ATP} channels or to inhibit mitochondrial function on the spontaneous excitations in TSMCs and ATSMCs were also examined. Immunohistochemistry and electron microscopy were employed to identify the distribution of CGRP-containing sensory nerves in relation to ATSMCs and TSMCs.

Methods

Tissue preparation

All animal care and experimental procedures were approved by the animal experimentation ethics committee of the Physiological Society of Japan. Male BALB/c mice, aged 6–8 weeks, were killed by stunning followed by cervical dislocation and exsanguinations, the kidneys and attached ureters removed through an abdominal incision and the kidney was bathed in a bicarbonate-buffered physiological salt solution (PSS; see below for composition). The upper urinary tract, from its point of attachment to the papilla and calyx, the PCJ, to the pelviureteric junction, was dissected free of the kidney, opened along its longitudinal axis and pinned out in a dissecting dish.

Isometric tension recordings

Under a dissecting microscope, spontaneous peristaltic contractions of the whole renal pelvis that propagated longitudinally from the proximal to mid renal pelvis were carefully observed to find a region that exhibited the most vigorous contractions. Nylon threads were tied around both ends of the most contractile transverse region (approximately 0.5 mm wide) of whole renal pelvis preparations. Preparations were transferred to 2 mL organ baths and were superfused with warmed (36°C) PSS at a constant flow rate (2 $mL \cdot min^{-1}$). One thread was fixed to the bottom of the organ bath, while the other was connected to an isometric force transducer connected to a bridge amplifier. Isometric tension changes were digitized using a Digidata 1200 interface (Axon Instruments, Inc., Foster City, CA, USA) and stored on a personal computer for later analysis. Preparations almost instantly started to contract, and were stretched appropriately to exhibit spontaneous phasic contractions without detectable increases in basal tension. Preparations were then left to equilibrate for about 30 min until spontaneous phasic contractions, which were stable in both amplitude and frequency, were generated.

The preparations were placed between a pair of silver plates in the organ bath and were stimulated by passing brief pulses of constant current (duration 50 μs at supramaximal voltage).

Intracellular Ca^{2+} imaging

To image intracellular Ca^{2+} dynamics in ATSMCs and TSMCs, longitudinal preparations were pinned out with the serosal surface uppermost on a block of Sylgard plate (silicone elastomer, Dow Corning Corporation, Midland, MI, USA). To minimize tissue distortion due to muscle contractions, preparations were stretched using 15–20 tungsten wires (20 μm in diameter). After 30 min incubation with warmed (36°C) PSS, and the visual detection of spontaneous muscle contractions that travelled from the proximal to distal (with respect to the kidney) renal pelvis, preparations were then incubated in low Ca^{2+} PSS [$(Ca^{2+})_o$ = 0.5 mM] containing 3 μM Fluo-4 AM (special packaging, Dojindo, Tokyo, Japan) and cremphor EL (0.01%, Sigma, St. Louis, MO, USA) for 90 min at room temperature to visualize TSMCs Ca signals. To visualize Ca^{2+} signals in ATSMCs, preparations were incubated in low Ca^{2+} PSS [$(Ca^{2+})_o$ = 0.1 mM] containing 1 μM Fluo-4 AM and cremphor EL for 30 min at 36°C, and then were left for another 30 min at room temperature. Following incubation, the preparations were superfused with dye-free, warmed (36°C) PSS at a constant flow rate (about 2 mL·min^{-1}) for 30 min.

The recording chamber was mounted on the stage of an upright epifluorescence microscope (BX51WI, Olympus, Tokyo, Japan) equipped with an electron multiplier CCD camera (C9100, Hamamatsu Photonics) and a high-speed scanning polychromatic light source (C7773, Hamamatsu Photonics, Hamamatsu, Japan). Preparations were viewed with a water-immersion objective (UMPlanFI x20 or LUMPlanFI x60, Olympus) and illuminated at 495 nm. The fluorescence emission in a rectangular window was measured through a barrier filter above 515 nm, and images were obtained every 100 or 200 ms (frame interval) with an exposure time of 17.4–58.7 ms using a micro photoluminescence measurement system (AQUACOSMOS, Hamamatsu Photonics). Relative amplitude of Ca^{2+} transients was expressed as the ratio F/F_0 of the fluorescence generated by an event (F) against baseline (F_0).

Intracellular recordings

For intracellular recordings, longitudinal full-length strips of the renal pelvis with part of the renal calyx attached were dissected free and firmly pinned, with the urothelial layer uppermost, on a Sylgard plate (silicone elastomer, Dow Corning Corporation) at the bottom of the recording chamber (volume approximately 1 mL). The recording chamber was mounted on the stage of either an inverted or upright microscope, and the preparations were superfused with warmed (36°C) PSS at a constant flow rate (2 mL·min^{-1}). Preparations were allowed to equilibrate for some 30 min. Individual TSMCs in the muscle bundles were impaled with glass capillary microelectrodes, filled with 0.5 M KCl (tip resistance, 150–250 MΩ). Membrane potential changes were recorded using a high input impedance amplifier (Axoclamp-2B, Axon Instruments, Inc.), and displayed on a cathode-ray oscilloscope (SS-5702, Iwatsu, Tokyo, Japan). After low-pass filtering (cut-off frequency, 10 kHz), membrane potential changes were digitized using a Digidata 1440A interface (Axon Instruments, Inc.) and stored on a personal computer for later analysis.

Immunohistochemistry

Samples of renal pelvis were stretched until they were flat and secured with tungsten wires on a Sylgard plate (Dow Corning Corporation). The whole mount preparations were immersed in phosphate-buffered saline (PBS) containing 4% formaldehyde at 4°C overnight, and then were rinsed in PBS several times. All antibodies used in this study were diluted in PBS containing 2% bovine serum albumin. Preparations were incubated with 0.3% Triton X-100 in PBS for 10 min, incubated with Block Ace (Dainippon Seiyaku, Osaka, Japan) for 20 min at room temperature.

To examine the distribution of CGRP-containing nerve fibres, whole mounts were incubated with rabbit anti-PGP 9.5 antibody (1: 500, RA95101, Ultraclone, Isle of Wight, UK) and guinea pig anti-CGRP antibody (1:1000, T-5027, Peninsula Laboratories, San Carlos, CA, USA) for 2 days at 4°C. The primary antibodies were washed out in PBS. Preparations were incubated with tetramethylrhodamine isothiocyanate-conjugated anti-rabbit IgG antibody (1:20, R0156, Dako, Glostrup, Denmark) and Alexa 488-conjugated anti-guinea pig IgG antibody (10 μg·mL^{-1}, S11223, Molecular Probes, Eugene, OR, USA) for 2 h at room temperature.

To examine colocalization of CGRP and transient receptor potential cation channels (TRPV1; vanilloid receptors), whole mounts were incubated with rabbit anti-CGRP antibody (1:50, 11189, Progen Biotechnik, Heidelberg, Germany) and guinea pig anti-TRPV1 antibody (1:1000, GP14100, Neuromics, Edina, MN, USA) for 2 days at 4°C. After washing with PBS, preparations were incubated with pig biotinylated anti-rabbit IgG antibody (1:300, E0353, Dako) for 30 min at room temperature and washed again with PBS. Preparations were then incubated with Cy-3-conjugated streptavidin (Jackson ImmunoResearch, West Grove, PA, USA) and the Alexa 488-conjugated anti-guinea pig IgG antibody for 2 h at room temperature. After washing with PBS or distilled water, preparations were mounted on glass slides and coverslipped with Vectorshield (Vector Laboratories, Burlingame, CA, USA). Preparations were examined on a confocal laser scanning microscope (TCS SP2, Leica, Tokyo, Japan).

Electron microscopy

To identify the ultrastructural characteristics of the renal pelvis wall, preparations from 3 animals were fixed with glutaraldehyde (2.5% v·v^{-1}) made up in 0.1 M phosphate buffer (pH 7.4) for 1 h at room temperature. Tissues were rinsed with the same buffer and postfixed with osmium tetroxide in the same buffer (2% v·v^{-1}) for 2 h at 4°C. Tissues were subsequently rinsed in the same buffer, dehydrated through a graded series of ethanol solutions,and embedded in Epon epoxy resin. Ultrathin sections of 90 nm thickness were prepared from each block, and double-stained with uranyl acetate and lead citrate and observed using transmission electron microscopy.

Data analysis

The following parameters of spontaneous action potentials and Ca^{2+} transients were measured: peak amplitude, measured as the value from the resting level to the peak of events; half-duration, measured as the time between 50% peak amplitude on the rising and falling phases; and frequency, which was defined as an average of 3 or 5 min recordings.

Measured values are expressed as mean ± SD. Statistical significance was tested using a paired Student's *t*-test, and accepted if $P < 0.05$.

Materials

The composition of PSS was (in mM): Na^+ 137.5, K^+ 5.9, Ca^{2+} 2.5, Mg^{2+} 1.2, HCO_3^- 15.5, $H_2PO_4^-$ 1.2, Cl^- 134 and glucose 11.5. The pH of PSS was 7.2 when bubbled with 95% O_2 and 5% CO_2, and the measured pH of the recording bath was approximately 7.4.

The human CGRP (hCGRP) and hCGRP fragment (8–37) were from the Peptide Institute, Osaka, Japan; the atropine, antimycin-A, 8-bromo-cyclic AMP, carbonyl cyanide 3-chlorophenylhydrazone (CCCP), diazoxide, forskolin, glibenclamide, guanethidine, 5-hydroxydecanoate (5-HD), isoprenaline, L-nitro arginine, oligomycin, rotenone and SQ22536 were from Sigma; and the capsaicin, oligomycin and tetrodotoxin (TTX) from Wako Pure Chemical Institute, Osaka, Japan. Drugs were dissolved in distilled water, except for CCCP, diazoxide, forskolin and glibenclamide, which were dissolved in dimethyl sulphoxide; and capsaicin and oligomycin, which were dissolved in absolute ethanol. The final concentration of these solvents in the PSS did not exceed 1:1000. Drug/molecular target nomenclature conforms to the *British Journal of Pharmacology*'s Guide to Receptors and Channels (Alexander *et al.*, 2008).

Results

Effects of sensory nerve stimulation and of bath-applied CGRP on spontaneous contractions

Strips of the mouse renal pelvis generated about 10 spontaneous phasic contractions every minute, which were relatively stable in amplitude (0.23 ± 0.08 mN, $n = 25$), half-duration (1115 ± 331 ms) and frequency (12.0 ± 2.4 min^{-1}) for several hours (Figure 1Aa,B,C). The frequency of spontaneous contractions was significantly higher than that of circumferential strips without renal calyx (mid renal pelvis about 6 min^{-1}, Lang *et al.*, 2007a), suggesting that the

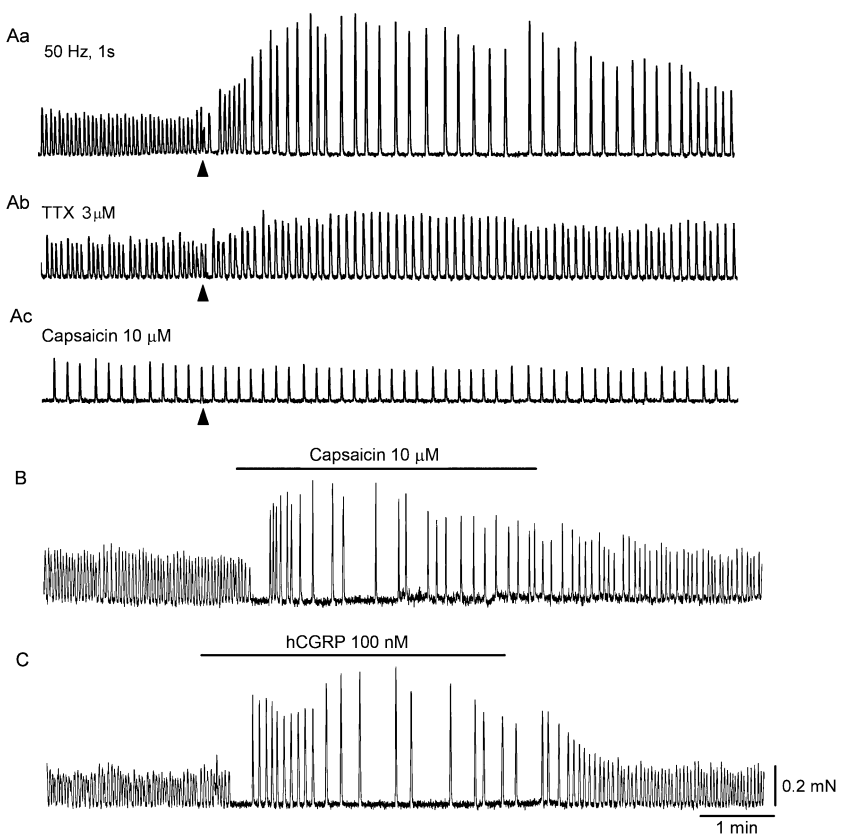

Figure 1 Effects of sensory nerve stimulation and bath-applied calcitonin gene-related peptide (CGRP) on spontaneous contractions of the mouse renal pelvis. Trains of stimuli (50 Hz, 1 s; triangles) reduced the frequency of the spontaneous contractions but increased their amplitude (Aa). In preparations that had been exposed to tetrodotoxin (TTX) (3 μM), the effects of nerve stimulation on spontaneous contractions were diminished (Ab). In capsaicin (10 μM)-treated preparations, nerve stimulation failed to modulate spontaneous contraction (Ac). Aa–c were recorded from the same preparation. In another preparation, capsaicin (10 μM) reduced the frequency of spontaneous contractions and increased their amplitude (B). In a different preparation, human CGRP (hCGRP) (100 nM) also caused negative chronotropic and positive inotropic effects on the spontaneous contractions (C). Scale bars for C refer to all traces.

dominant pacemaker cells may be distributed in the renal calyx or most proximal region of the renal pelvis.

Transmural nerve stimulation with a single impulse or trains of stimuli at frequencies less than 10 Hz failed to alter either amplitude or frequency of the spontaneous contractions. Trains of stimuli at higher frequency (20 or 50 Hz, 1–5 s, n = 25) reduced the frequency of the spontaneous contractions (negative chronotropic effect) but increased their amplitude (positive inotropic effect; Figure 1Aa). These nerve-mediated changes in muscle contractility were not prevented by atropine (1 μM, n = 5), guanethidine (10 μM, n = 5) or L-nitro arginine (100 μM, n = 4), and thus did not result from the activation of cholinergic, adrenergic or nitrergic innervations. TTX (3 μM) diminished the effects of nerve stimulation (Figure 1Ab, n = 6), while capsaicin (10 μM) completely inhibited these responses (Figure 1Ac, n = 8), confirming that both the negative chronotropic and positive inotropic effects resulted from the release of neurotransmitters from capsaicin-sensitive sensory nerves.

Capsaicin itself (10 μM, n = 10, Figure 1B) and hCGRP (100 nM, n = 8, Figure 1C) both had negative chronotropic and positive inotropic effects on the spontaneous contractions very similar to the effects of transmural nerve stimulation. In preparations that had been exposed to hCGRP 8–37 (1 μM), a CGRP receptor antagonist, for 10 min (n = 5), transmural nerve stimulations reduced the frequency of spontaneous phasic contractions to 74.5 ± 8.5% of normal unstimulated values, less than the reduction in the absence of the antagonist (57.5 ± 7.6%; $P < 0.05$) and increased their amplitude to 182 ± 73% of normal, again less than that in the absence of the antagonist (265 ± 117; $P < 0.05$), suggesting that CGRP may be the major neurotransmitter involved during the sensory nerve-mediated modulation of spontaneous contractions of the renal pelvis.

Distribution of CGRP immunoreactive sensory nerves in the mouse renal pelvis

A plexus of PGP9.5-immunoreactive nerve fibres were seen throughout the renal pelvis including the proximal renal calyx down to the distal region near the pelviureteric junction (Figure 2Aa). Thick nerve fibres ran along the outer longitudinal muscle bundles and connected to smaller nerve bundles running transversely in the suburothelial layers or inner circular smooth muscles, and thus created a three-dimensional network. PGP9.5-positive nerve bundles predominantly contained CGRP-immunoreactive fibres (Figure 2Ac), and thus CGRP-positive nerve fibres also form a three-dimensional plexus (Figure 2Ab). TRPV1 immunoreactive nerve fibres were also widely distributed throughout the renal pelvis (Figure 2Ba) and a majority of these TRPV1-positive nerve fibres also expressed CGRP-immunoreactivity (Figure 2Bb), suggesting that the dominant nerve population in the mouse renal pelvis is CGRP-containing TRPV1-positive sensory nerves (Figure 2Bc).

Figure 3A and 3B shows the typical electron micrographs of the renal pelvis wall that demonstrate the presence of unmyelinated nerves bundles within the muscle wall of the renal pelvis wall (Figure 3A,B). Although these nerves bundles were often in near apposition with, or surrounded by neighbouring TSMCs and ATSMCs, close contacting synapses were never observed, suggesting that only global ('volume') neurotransmission may be occurring.

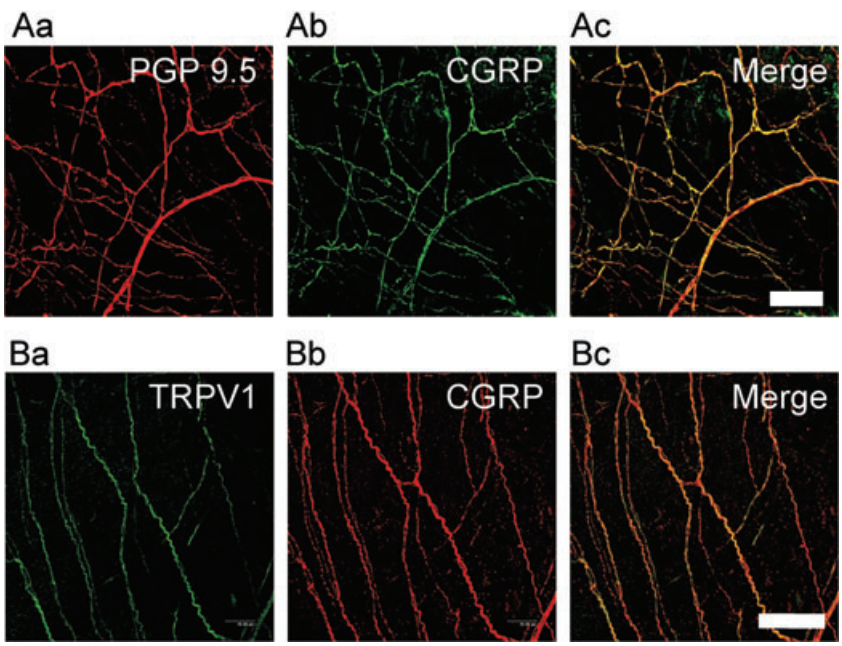

Figure 2 Distribution of PGP9.5-, calcitonin gene-related peptide (CGRP)- and transient receptor potential cation channel, subfamily V, member 1 (TRPV1) immunoreactive nerves in the mouse renal pelvis. In the proximal renal pelvis, nerve fibres were stained with antibodies against the neural marker PGP 9.5 (Aa). CGRP-immunoreactive nerve fibres were abundant in the same preparation (Ab). A merged image indicates that most of thick and thin nerve bundles contain CGRP-immunoreactive fibres (Ac). Double staining of the mid renal pelvis with TRPV1 (Ba) and CGRP (Bb) antibodies showed that many CGRP-positive fibres also expressed TRPV1-immnoreactivity (Bc). Scale bars: 80 μm.

Role of cAMP in CGRP-mediated modulations of spontaneous Ca^{2+} waves in TSMCs

To elucidate the cellular mechanism underlying the CGRP-induced negative chronotropic and positive inotropic effects on the spontaneous contractions, we examined the effects of sensory nerve stimulation and bath-applied CGRP on the Ca^{2+} waves in TSMCs that originated in the most proximal regions of the renal pelvis and propagated distally. At higher magnifications (×60), individual TSMCs could be clearly identified by their gross morphology to be the cells generating these Ca^{2+} transients (Lang *et al*., 2007a).

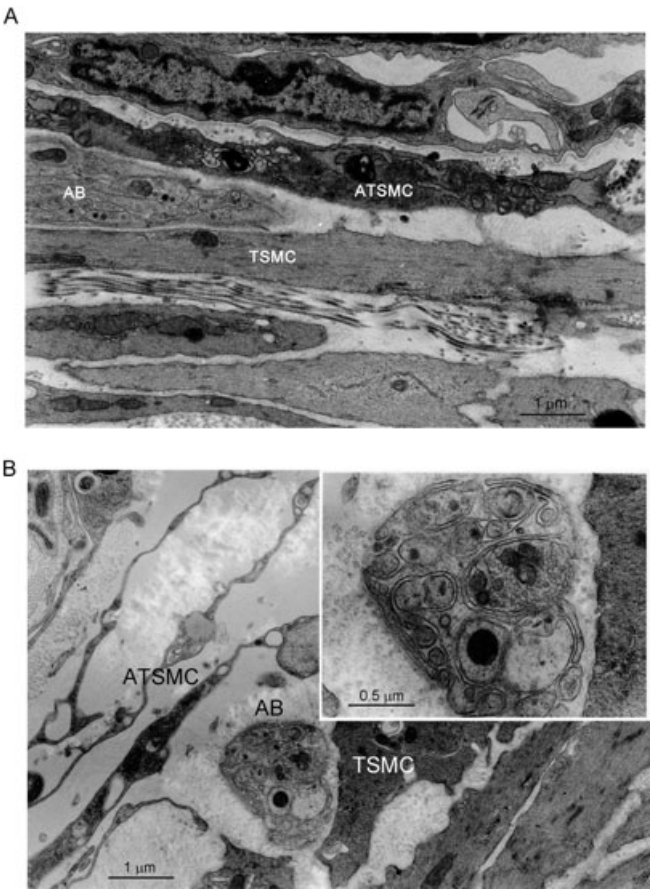

Figure 3 Electron microscopy of unmyelinated sensory nerves in association with typical smooth muscle cells (TSMCs) and atypical smooth muscle cells (ATSMCs). In the suburothelial muscle layer, axonal bundles (AB) of unmyelinated nerves were surrounded by TSMCs and ATSMCs (A). Another axonal bundle of unmyelinated nerves was in near apposition with TSMC (B). Atypical smooth muscle cells (ATSMCs) in close association with each other were also seen nearby. The AB is shown at a higher magnification in the inset.

Stimulation of TRPV1 receptors on sensory nerves with capsaicin (10 μM; Figure 4Aa) reduced the frequency of TSMC Ca^{2+} transients, but increased their amplitude and duration (Figure 4Ab). Note that capsaicin did not alter the direction (from proximal to distal) of Ca^{2+} wave propagation (Figure 4Ab). Similarly, bath-applied CGRP (100 nM) slowed spontaneous Ca^{2+} transient discharge, and increased their amplitude and duration (Figure 4Ba,b). CGRP did not affect the direction of Ca^{2+} wave propagation either (Figure 4Bb), suggesting that negative chronotropic effects of CGRP released from sensory nerves may result from its action on ATSMCs, while the positive inotropic effects may be, at least partly, attributed to enhancement of the TSMC Ca^{2+} transients. These results are summarized in Table 1.

In other species, the effects of CGRP in the renal pelvis have been considered to be associated with an increase in cAMP production. Thus the effects of agents that are known to increase cAMP (cAMP stimulators) on TSMC Ca^{2+} transients were compared to those of CGRP.

Forskolin, an activator of adenylate cyclase (1 μM; Figure 4Ca,b) and isoprenaline (10 μM; Figure 4Da,b), known to increase cAMP production through β-adrenoceptor stimulation, reduced the frequency of spontaneous TSMC Ca^{2+} transients but increased both the amplitude and duration of individual Ca^{2+} transients. 8-bromo-cAMP (8Br-cAMP 1 mM), a membrane-permeable cAMP analogue, also reduced the frequency of spontaneous Ca^{2+} transients and increased their amplitude and duration (Figure 4Ea, b). These effects of cAMP stimulators on the parameters of TSMC Ca^{2+} transients are also summarized in Table 1.

SQ22536, a cell-permeable adenylate cyclase inhibitor, antagonized the inhibitory effects of CGRP on TSMCs Ca^{2+} transients (Figure 4Fa, b). In preparations that had been exposed to SQ22536 (300 μM) for 10 min, CGRP (100 nM) reduced the frequency of Ca^{2+} transients to 53 ± 9% of control (a lesser effect of CGRP than in the absence of SQ22536 (reduced to 28 ± 5%: $P < 0.05$, $n = 3$) but did not significantly change their amplitude or duration.

Table 1 Effects of capsaicin, hCGRP and cAMP stimulators on the parameters of spontaneous Ca^{2+} transients in TSMCs

Chemicals	*Frequency (min^{-1})*	*Amplitude (F_t/F_0)*	*Half-duration (ms)*
Control ($n = 8$)	14.5 ± 1.4	0.79 ± 0.24	652 ± 65
Capsaicin (10 μM)	5.4 ± 1.3*	0.99 ± 0.25*	904 ± 92*
Control ($n = 8$)	15.6 ± 5.1	0.61 ± 0.15	653 ± 68
hCGRP (100 nM)	3.6 ± 1.3*	0.81 ± 0.31*	994 ± 160*
Control ($n = 6$)	16.3 ± 4.3	0.71 ± 0.23	654 ± 125
Forskolin (1 μM)	5.0 ± 2.6*	0.87 ± 0.27*	955 ± 162*
Control ($n = 4$)	14.6 ± 1.8	0.83 ± 0.09*	697 ± 72
Isoprenaline (10 μM)	5.7 ± 1.3*	1.1 ± 0.14*	1074 ± 183*
Control ($n = 6$)	14.7 ± 2.2	0.69 ± 0.22	701 ± 51
8Br-cAMP (1 mM)	6.7 ± 1.6*	0.82 ± 0.26*	912 ± 103*

Data shown are mean ± SD.
*Significantly different from control values ($P < 0.05$).
hCGRP, human calcitonin gene-related peptide; TSMC, typical smooth muscle cell.

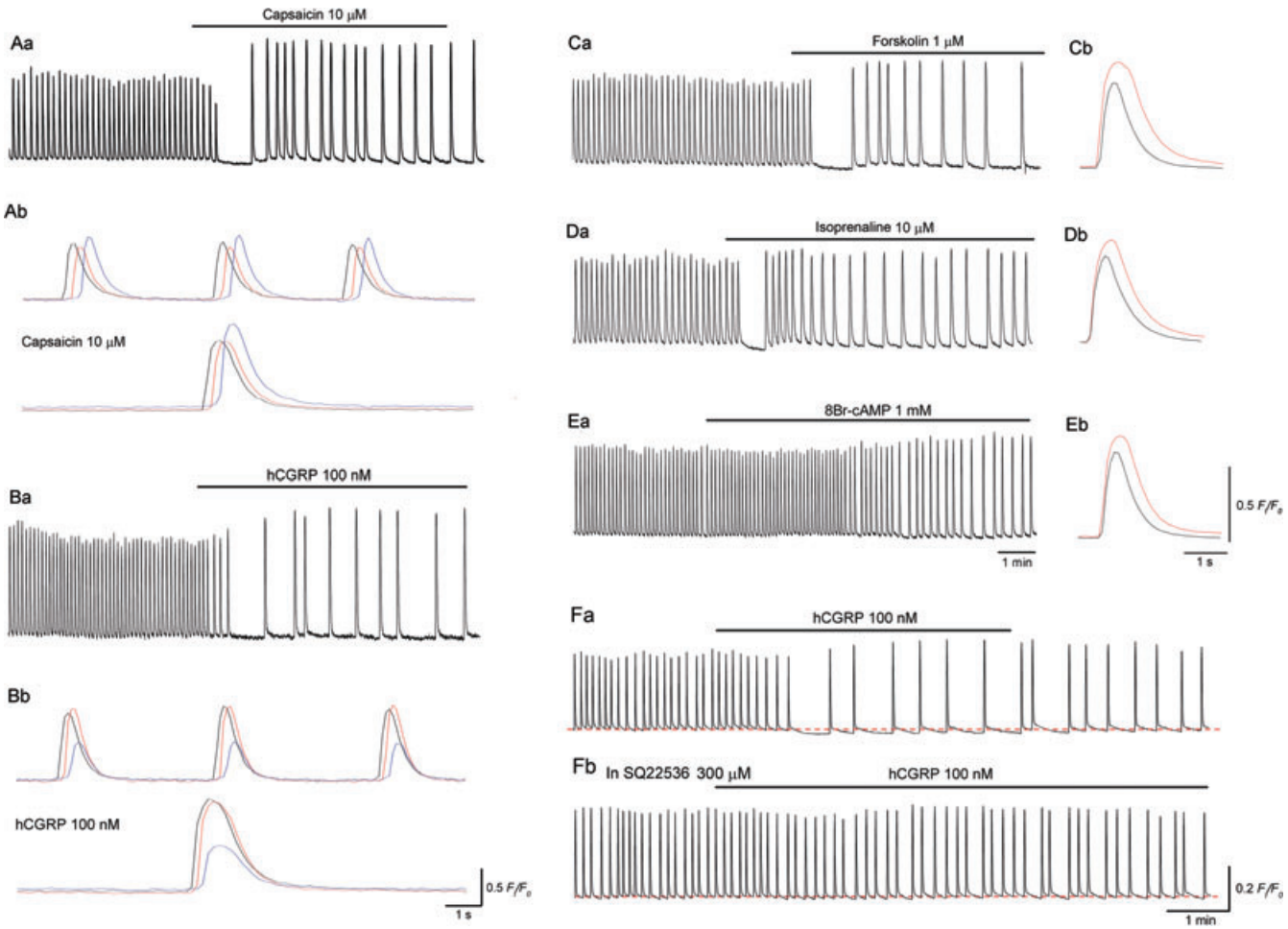

Figure 4 Role of cAMP in calcitonin gene-related peptide (CGRP)-mediated modulations of spontaneous Ca waves in typical smooth muscle cell (TSMC). Capsaicin (10 μM) reduced the frequency of spontaneous Ca^{2+} transient (Aa) and increased their amplitude (Bb) in TSMCs. Capsaicin did not affect the direction of Ca^{2+} wave propagation (black, red and blue traces from proximal to distal in order, Ab). In a different preparation, bath-applied human CGRP (hCGRP) (100 nM) slowed spontaneous Ca^{2+} transient discharge (Ba) and increased the amplitude and duration of individual Ca^{2+} transient (Bb). Again, hCGRP did not alter intercellular Ca^{2+} waves (black, red and blue traces from proximal to distal in order). Forskolin (1 μM, C), isoprenaline (10 μM, D) and 8Br-cAMP (1 mM, E) all reduced the frequency of spontaneous Ca^{2+} transients (Ca, Da, Ea) and increased both their amplitude and duration (Cb, Db, Eb). F compares the effects of hCGRP (100 nM) on the frequency and time course of TSMC Ca^{2+} transients in the absence (Fa) and presence of SQ22536 (300 μM). In the same preparation, which had been treated with SQ22536, hCGRP caused weaker inhibitory effects (Fb). The scale bars for Bb also refer to Ab. The scale bars for E refer to all corresponding traces.

These results suggested that increases in cAMP may be underlying CGRP-mediated regulation of TSMC Ca^{2+} transients.

Effects of CGRP and cAMP stimulators on ATSMCs Ca^{2+} transients

In preparations that had been exposed to nifedipine (1 μM) for over 30 min, high-frequency spontaneous Ca^{2+} transients that propagated over distances <50 μm were recorded in cells that were preferentially located in the renal calyx or most proximal regions of the renal pelvis. At higher magnifications (×60), short, spindle-shaped ATSMCs could be clearly identified by their gross morphology to be the cells generating these Ca^{2+} transients (Lang *et al*., 2007a).

Capsaicin (10 μM, Figure 5A) and CGRP (100 nM, Figure 5B) both reduced the frequency and amplitude of ATSMC Ca^{2+} transient discharge as well as their basal Ca^{2+} level. These effects were mimicked by forskolin (1 μM, Figure 5C) and 8Br-cAMP (1 mM) as summarized in Table 2.

In preparations that had been pretreated with SQ22536 (300 μM for 10 min), CGRP (100 nM) reduced the frequency of Ca^{2+} transients to 68 ± 8% of control, a lesser effect of CGRP than in the absence of SQ22536 (reduction to 19 ± 8%; $P < 0.05$, $n = 3$) but did not significantly change their amplitude or duration. Thus, SQ22536 again antagonized the inhibitory action of CGRP on ATSMC Ca^{2+} transients (Figure 5Da,b; $n = 3$), suggesting that CGRP-mediated inhibition of ATSMC Ca^{2+} transients may also result from an increase in cAMP production.

Effects of CGRP and cAMP stimulators on spontaneous action potentials

Membrane hyperpolarization consequent to the opening of K_{ATP} channels has been shown to underlie CGRP-mediated

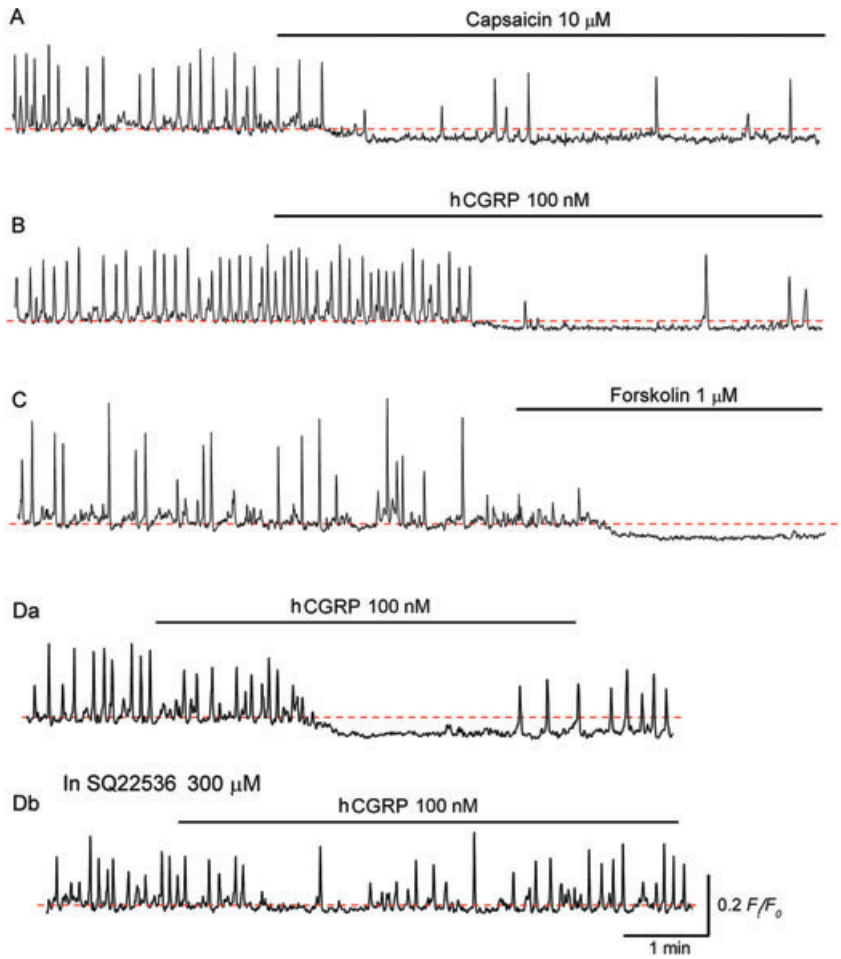

Figure 5 Role of cAMP in calcitonin gene-related peptide (CGRP)-mediated modulations of spontaneous Ca^{2+} transients in atypical smooth muscle cells (ATSMC)s. Capsaicin (10 μM) reduced the frequency of spontaneous Ca^{2+} transients in ATSMCs and reduced basal Ca^{2+} (A). In another preparation, human CGRP (hCGRP) (100 nM) slowed spontaneous Ca^{2+} transients in ATSMCs and reduced basal Ca^{2+} level (B). Forskolin (1 μM) abolished Ca^{2+} transients with a fall in basal Ca^{2+} (C). In a different preparation (Da,b), SQ22536 (300 μM) partially prevented the CGRP (100 nM)-induced reduction in the frequency of spontaneous Ca^{2+} transients and fall in basal Ca^{2+}.All traces were recorded from preparations pretreated with nifedipine (1 μM) for 30 min. The scale bars for Db refer to all traces.

Table 2 Effects of capsaicin, hCGRP and cAMP stimulators on the parameters of spontaneous Ca^{2+} transients in ATSMCs

Chemicals	*Frequency (min^{-1})*	*Amplitude (F_t/F_0)*	*Half-duration (ms)*	*Basal Ca level (F_t/F_0)*
Control (n = 8)	7.2 ± 1.5	0.59 ± 0.2	698 ± 94	–
Capsaicin (10 μM)	1.3 ± 0.44*	0.49 ± 0.23*	683 ± 79	–0.09 ± 0.03
Control (n = 8)	8.7 ± 2.7	0.51 ± 0.15	629 ± 83	–
hCGRP (100 nM)	2.5 ± 1.3*	0.38 ± 0.11*	648 ± 69	–0.09 ± 0.06
Control (n = 4)	8.2 ± 1.1	0.54 ± 0.23	682 ± 50	–
Forskolin (1 μM)	1.4 ± 0.5*	0.34 ± 0.15*	637 ± 68	–0.11 ± 0.06
Control (n = 5)	8.2 ± 1.8	0.53 ± 0.21	688 ± 61	–
8-Br-cAMP (1 mM)	4.0 ± 1.6*	0.39 ± 0.16*	715 ± 67	–0.03 ± 0.01

Data shown are mean ± SD.
*Significantly different from control values ($P < 0.05$).
hCGRP, human calcitonin gene-related peptide; ATSMC, atypical smooth muscle cell.

inhibition of spontaneous activity in a number of smooth muscles. We therefore examined the effects of CGRP and cAMP stimulators on spontaneous action potentials recorded in TSMCs.

Capsaicin (10 μM) caused a transient membrane depolarization (for 1–2 min) that was followed by a prolonged hyperpolarization and reduction in the frequency of spontaneous action potential discharge (Figure 6A). It should be noted that the capsaicin-induced reduction in discharge frequency occurred before the membrane hyperpolarization, even during the initial transient depolarization, suggesting that action potential frequency was not directly related to the

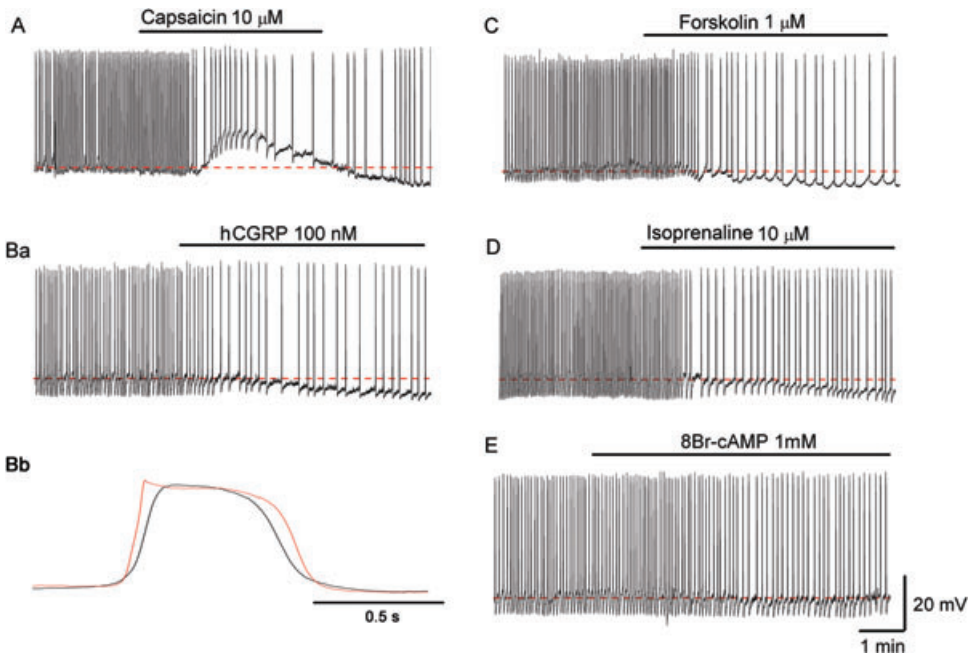

Figure 6 Effects of calcitonin gene-related peptide (CGRP) and cAMP stimulators on spontaneous action potentials. Capsaicin (10 μM) caused a transient membrane depolarization and subsequent hyperpolarization, both associated with a reduction of action potential frequency (A). Human CGRP (hCGRP) (100 nM) evoked a membrane hyperpolarization (Ba), the frequency of action potential discharge was reduced but action potential peak amplitudes and durations were increased (Bb). Forskolin (1 μM; C) and isoprenaline (10 μM; D) both hyperpolarized the membrane and reduced action potential frequency. 8-Br-cAMP (1 mM) reduced action potential frequency without hyperpolarizing the membrane (E). A–E were recorded from different preparations. The scale bars for E refer to all traces.

Table 3 Effects of capsaicin, hCGRP and cAMP stimulators on the parameters of spontaneous action potentials

Blockers	*RMP (mV)*	*Frequency (min^{-1})*	*Amplitude (mV)*	*Half-duration (ms)*
Control (n = 8)	−62.0 ± 3.2	11.9 ± 1.9	43.4 ± 3.2	537 ± 132
Capsaicin (10 μM)	−63.9 ± 3.2	3.9 ± 1.0*	45.3 ± 3.5*	632 ± 157*
Control (n = 7)	−66.7 ± 4.2	13.4 ± 1.3	47.1 ± 3.6	533 ± 103
hCGRP (100 nM)	−68.9 ± 3.2	3.9 ± 0.9*	48.4 ± 3.3*	636 ± 138*
Control (n = 4)	−60.9 ± 1.9	11.3 ± 2.2	44.8 ± 2.8	493 ± 108
Forskolin (1 μM)	−63.5 ± 1.8	3.1 ± 1.5*	46.8 ± 1.8*	691 ± 157
Control (n = 3)	−62.7 ± 4.6	12.0 ± 1.0	45.3 ± 2.9	497 ± 63
Isoprenaline (10 μM)	−64.9 ± 5.3	4.1 ± 0.5*	49 ± 1.8*	656 ± 91*
Control (n = 6)	−66.3 ± 2.5	13.0 ± 1.6	47.9 ± 3.1	565 ± 114
8Br-cAMP (1 mM)	−66.7 ± 3.1	7.9 ± 0.8*	48.7 ± 3.1*	618 ± 132*

Data shown are mean ± SD.
*Significantly different from control values (P < 0.05).
RMP, resting membrane potential; hCGRP, human calcitonin gene-related peptide.

membrane potential. hCGRP (100 nM) also reduced the frequency of action potential firing before there was a substantial membrane hyperpolarization (Figure 6Ba). During the application of either capsaicin or hCGRP (Figure 6Bb), the peak amplitude and duration of spontaneous action potentials were increased. Figure 6Bb shows the effects of hCGRP on action potential configuration. These results were summarized in Table 3.

Forskolin (1 μM; Figure 6C) and isoprenaline (10 μM; Figure 6D) both first reduced the frequency of spontaneous action potentials before hyperpolarizing the membrane. 8-Br-cAMP (1 mM) did not significantly hyperpolarize the membrane, but reduced action potential frequency (Figure 6E). These agents that raise internal cAMP also mimicked the action of CGRP on all action potential parameters (Table 3).

Role of plasmalemmal K_{ATP} channels in CGRP/cAMP-mediated reduction of action potential frequency

Glibenclamide (10 μM) increased the frequency of spontaneous action potentials (from 12.1 ± 2.5 min^{-1} to 21.3 ± 3.2 min^{-1}, n = 7, P < 0.05) with a small depolarization of the membrane (from −65.9 ± 2.3 mV to −64.3 ± 1.6 mV, P < 0.05). In preparations that had been pretreated with glibenclamide for over 20 min, hCGRP failed to hyperpolarize the membrane (from −65.5 ± 1.8 mV to −63.9 ± 1.5 mV, P > 0.05), but still reduced action potential frequency (from

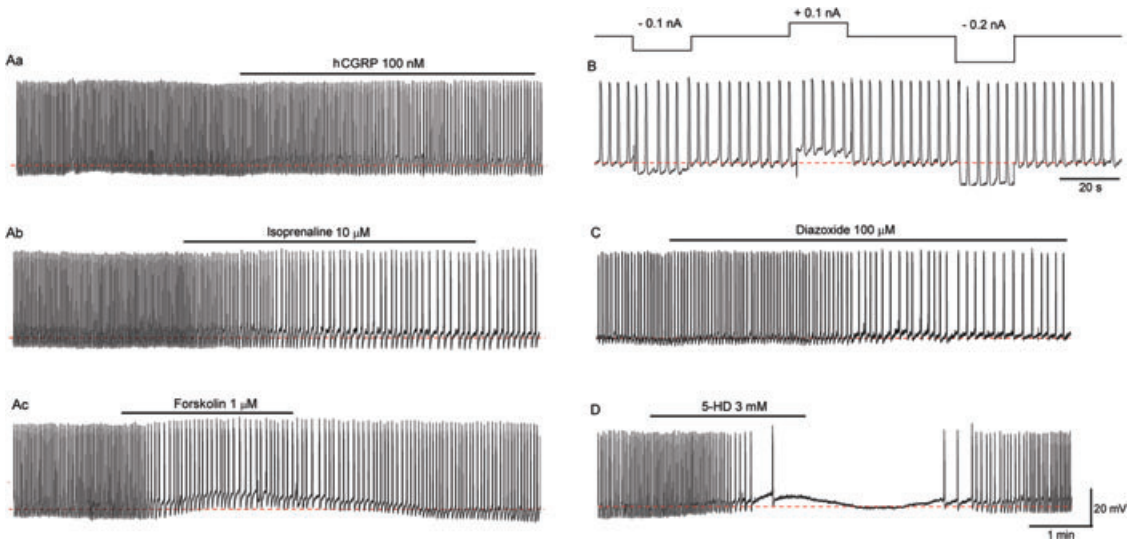

Figure 7 Role of K_{ATP} channel opening and membrane hyperpolarization in calcitonin gene-related peptide (CGRP)-/cAMP-mediated reductions of action potential frequency. In a preparation pretreated with glibenclamide (10 μM), human CGRP (hCGRP) (100 nM) failed to hyperpolarize the membrane but still reduced action potential frequency (Aa). In different preparations that had been exposed to glibenclamide (10 μM), isoprenaline (10 μM, Ab) and forskolin (1 μM, Ac) caused a small depolarization but reduced the action potential frequency. Inward (–0.1 and –0.2 nA) or outward (+0.1 nA) currents injected through the recording electrode, respectively, hyperpolarized or depolarized the membrane, but failed to alter the frequency of spontaneous action potentials (B). In a different preparation, diazoxide (100 μM) reduced action potential frequency but depolarized the membrane potential (C). 5-hydroxydecanoate (5-HD) (3 mM) prevented the generation of spontaneous action potentials in a manner associated with a small membrane depolarization (D). The scale bars for D refer to all traces.

22.2 ± 1.8 min^{-1} to 10.9 ± 2.1 min^{-1}, $P < 0.05$, $n = 3$, Figure 7Aa). Similarly, in glibenclamide-treated preparations, neither isoprenaline ($n = 2$, Figure 7Ab) nor forskolin ($n = 3$, Figure 7Ac) hyperpolarized the membrane. Rather, both agents depolarized the membrane <10 mV and reduced the frequency of action potential firing (isoprenaline, from 23.1 ± 1.3 min^{-1} to 11.1 ± 1.1 min^{-1}, $P < 0.05$; forskolin, from 21.8 ± 1.8 min^{-1} to 9.8 ± 0.9 min^{-1}, $P < 0.05$). These results suggested that the reductions in action potential frequency with CGRP/cAMP do not result from the membrane hyperpolarizations evoked upon the opening of plasmalemmal K_{ATP} channels.

A voltage-independency of action potential discharge was further confirmed by altering the membrane potential with the injection of inward or outward currents through the intracellular microelectrode. The frequency of action potentials was not changed by either membrane hyperpolarization or depolarization within a range of –55 mV to –75 mV (Figure 7B, $n = 5$), suggesting that the generation of action potential in TSMCs may have less voltage dependency than other L-type Ca^{2+} channel dependent action potentials in smooth muscles such as the bladder (Hashitani and Brading, 2003).

Even though CGRP and cAMP stimulators readily opened plasmalemmal K_{ATP} channels to cause a membrane hyperpolarization of TSMCs, this phenomenon may not be the fundamental mechanism underlying the CGRP/cAMP-mediated negative chronotropic effects in the renal pelvis. Therefore, we have examined whether the opening of mitochondrial K_{ATP} channels might be involved in the negative chronotropic effects observed.

Diazoxide (100 μM), a mitochondrial K_{ATP} channel opener, reduced the frequency of spontaneous action potentials (from 13.3 ± 4.2 min^{-1} to 3.8 ± 1.7 min^{-1}, $P < 0.05$, $n = 7$), associated with a small depolarization of the membrane (from –64.9 ± 3.0 mV to –60.1 ± 2.3 mV, $P < 0.05$; Figure 7C).

Unexpectedly, 5-HD, a blocker for mitochondrial K_{ATP} channels, either reduced the frequency of TSMC action potentials firing (1 mM, from 12.3 ± 3.3 min^{-1} to 4.0 ± 1.6 min^{-1}, $n = 4$, $P < 0.05$) without changing the membrane potential (–62.1 ± 2.9 mV in control, –63.8 ± 3.5 in 5-HD, $P > 0.05$), or abolished their discharge associated with a small depolarization of the membrane (3 mM, $n = 3$, Figure 7D).

Comparison of the effects of glibenclamide, 5-HD and diazoxide on spontaneous Ca^{2+} transients in TSMCs and ATSMCs

As the effects of 5-HD on action potential frequency were paradoxically similar to those of diazoxide, but opposite to the actions of glibenclamide, the effects of these K_{ATP} channel modulators on spontaneous Ca^{2+} transients in TSMCs and ATSMCs were examined to explore their site of action.

Consistent with the results of electrophysiological studies above, glibenclamide (10 μM) increased the frequency of spontaneous Ca^{2+} transients in TSMCs (Figure 8Aa), while 5-HD (1 mM Figure 8Ba) and diazoxide (100 μM Figure 8Ca) reduced their frequency. These results are summarized in Table 4A.

In preparations exposed to nifedipine (1 μM) for >30 min, both glibenclamide (10 μM Figure 8Ab) and 5-HD (1 mM Figure 8Bb) reduced the frequency of spontaneous Ca^{2+} transients as well as the basal Ca^{2+} level in ATSMCs. In contrast, diazoxide (100 μM) also reduced ATSMC Ca^{2+} transient frequency but increased the basal Ca^{2+} level (Figure 8Cb). These results are summarized in Table 4B.

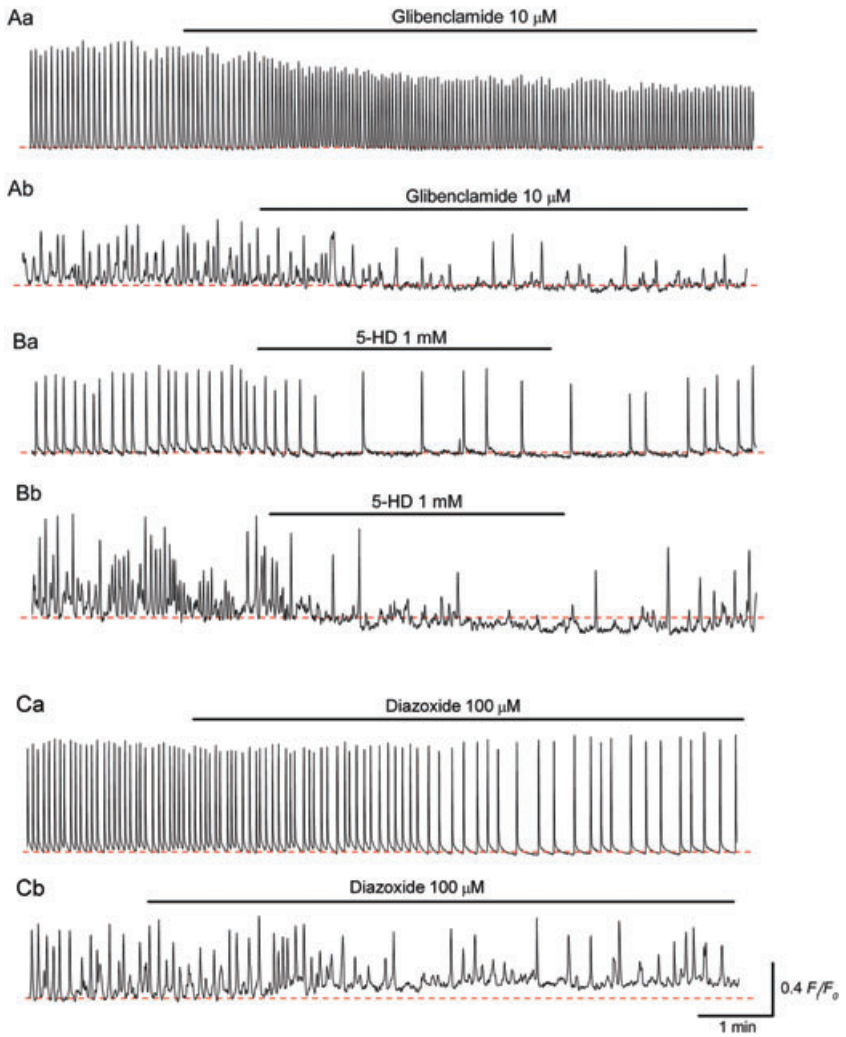

Figure 8 Comparison of the effects of glibenclamide, 5-hydroxydecanoate (5-HD) and diazoxide on spontaneous Ca^{2+} transients in typical smooth muscle cells (TSMCs) and atypical smooth muscle cells (ATSMC)s. Glibenclamide (10 μM) increased the frequency and reduced the amplitude of spontaneous Ca^{2+} transients in TSMCs (Aa). In another preparation, exposed to nifedipine (1 μM), glibenclamide (10 μM) reduced the frequency of spontaneous Ca^{2+} transients in ATSMCs and the basal Ca^{2+} level (Ab). In different preparations, 5-HD (1 mM B) or diazoxide (100 μM C) reduced the frequency of spontaneous Ca^{2+} transients in TSMCs (Ba, Ca) in control solutions and the frequency of ATSMC Ca^{2+} transients when bathed in nifedipine (1 μM) (Bb, Cb), Recordings in B and C are in different preparations. The scale bars for Cb refer to all traces.

Role of mitochondrial function in the generation on spontaneous action potentials in TSMCs

The above experiments indicate that CGRP/cAMP-mediated negative chronotropic effects may be attributed to the suppression of ATSMC Ca^{2+} transients rather than the opening of plasmalemmal K_{ATP} channels in TSMCs. As ATSMCs, pacemaker cells in the renal pelvis, are characterized by numerous mitochondria and mitochondrial K_{ATP} channel modulators inhibited ATSMC Ca^{2+} transients, the possible involvement of mitochondrial function in generating spontaneous excitation was further examined.

CCCP (1 μM, $n = 8$), a mitochondrial uncoupler, caused a transient membrane depolarization followed by a prolonged hyperpolarization (from –63.8 ± 3.0 mV to –68.9 ± 3.6 mV, $P < 0.05$). CCCP blocked the generation of all action potentials (Figure 9Aa), this blockade of action potential discharge preceded the membrane hyperpolarization (Figure 9Ab). In preparations that had been pretreated with glibenclamide (10 μM, $n = 5$) for 20 min, CCCP depolarized the membrane by approximately 10 mV (from –63.8 ± 1.4 mV to –53.1 ± 2.1 mV, $P < 0.05$) but still prevented action potential generation (Figure 9Ab).

Antimycin-A (10 μM, $n = 7$), an inhibitor for complex III in the electron transport chain, also prevented or reduced the frequency of spontaneous action potentials with little change in the membrane potential (from –64.4 ± 2.1 mV to –62.9 ± 4.4 mV, Figure 9B).

Oligomycin (5 μM), a blocker for mitochondrial ATP synthesis, reduced the frequency of spontaneous action potentials (from 10.5 ± 2.1 min^{-1} to 4.8 ± 1.4 min^{-1}, $P < 0.05$, $n = 6$) without changing either the membrane potential (from –66.5 ± 3.3 mV to –65.4 ± 3.6 mV) or action potential amplitude (from 44.4 ± 4.23 mV to 44.1 ± 3.6 mV, Figure 9C).

Table 4 Effects of K_{ATP} channel modulators on spontaneous Ca transients in ATSMCs

A. TSMC Chemicals	*Frequency (min^{-1})*	*Amplitude (F_t/F_0)*	*Half-duration (ms)*	
Control (n = 8)	11.5 ± 1.6	0.84 ± 0.16	672 ± 43	
Glibenclamide (10 μM)	18.4 ± 3.2*	069 ± 0.13*	661 ± 37	
Control (n = 6)	9.2 ± 1.0	0.61 ± 0.12	702 ± 131	
5-HD (1 mM)	2.6 ± 0.9*	0.65 ± 0.12*	678 ± 124	
Control (n = 5)	9.4 ± 1.1	0.89 ± 0.2	707 ± 43	
Diazoxide (100 μM)	2.9 ± 0.72*	0.91 ± 0.2	720 ± 42	
B. ATSMC Chemicals	*Frequency (min^{-1})*	*Amplitude (F_t/F_0)*	*Half-duration (ms)*	*Basal Ca level (F_t/F_0)*
Control (n = 7)	9.1 ± 1.6	0.47 ± 0.13	697 ± 51	–
Glibenclamide (10 μM)	2.2 ± 0.3*	0.36 ± 0.11*	707 ± 54	–0.08 ± 0.02
Control (n = 6)	6.9 ± 0.78	0.49 ± 0.15	714 ± 37	–
5-HD (1 mM)	1.8 ± 0.67*	0.41 ± 0.15*	727 ± 59	–0.1 ± 0.03
Control (n = 6)	8.7 ± 2.2	0.49 ± 0.18	673 ± 55	–
Diazoxide (100 μM)	3.6 ± 0.92*	0.42 ± 0.09*	692 ± 42	+0.06 ± 0.04

Data shown are mean ± SD.
*Significantly different from control values ($P < 0.05$).
5-HD, 5-hydroxydecanoate; ATSMC, atypical smooth muscle cell; TSMC, typical smooth muscle cell.

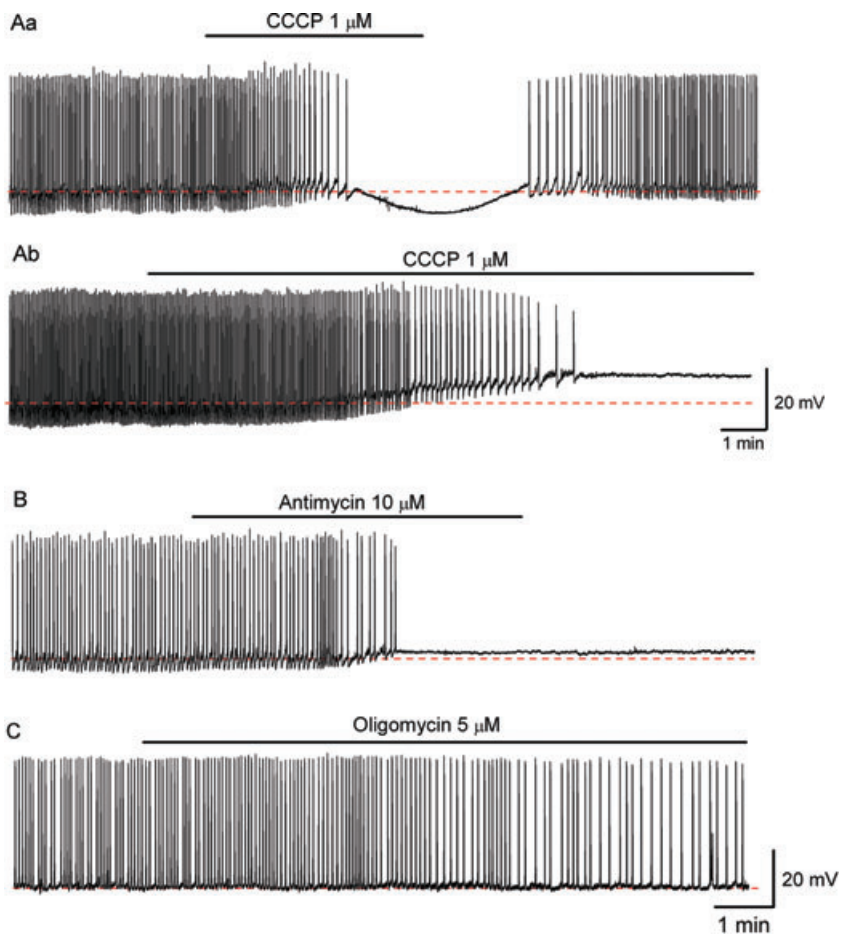

Figure 9 Effects of mitochondrial inhibitors on spontaneous action potentials. Carbonyl cyanide 3-chlorophenylhydrazone (CCCP) (1 μM) caused a transient membrane depolarization followed by a prolonged hyperpolarization and abolished action potential discharge (Aa). When the same preparation was pretreated with glibenclamide (10 μM for 30 min), CCCP again depolarized the membrane and prevented action potential generation (Ab). In another preparation, antimycin-A (10 μM) prevented the generation of spontaneous action potentials in a manner associated with a small membrane depolarization (B). Oligomycin (5 μM) reduced the frequency of action potential without changing the membrane potential (C). The scale bars for Ab also refer to Aa. The scale bars for C refer to B.

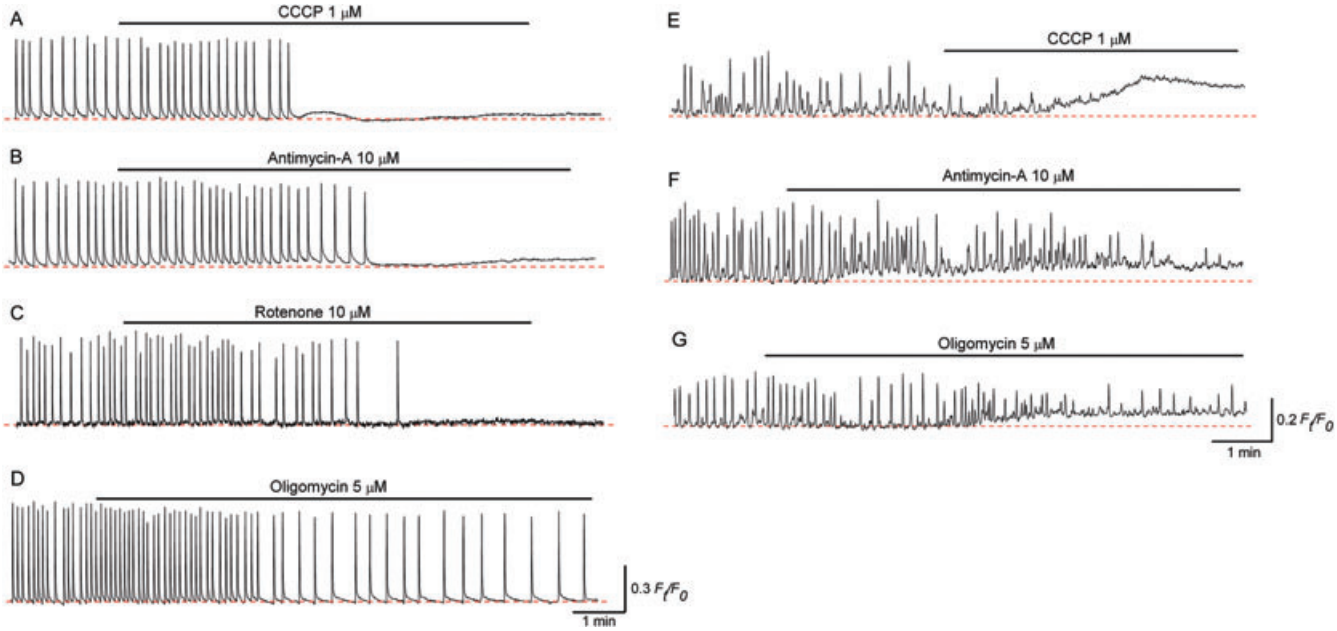

Figure 10 Role of mitochondrial function in the generation of spontaneous Ca^{2+} transients in typical smooth muscle cells (TSMCs) and atypical smooth muscle cells (ATSMCs). Carbonyl cyanide 3-chlorophenylhydrazone (CCCP) (1 μM) prevented the generation of spontaneous Ca^{2+} transients in TSMCs with a small increase in basal Ca^{2+} level (A). Antimycin-A (10 μM) also abolished TSMC Ca^{2+} transients with a small increase in basal Ca^{2+} level (B). In a different preparation, rotenone (10 μM) abolished Ca^{2+} transient generation (C). Oligomycin (5 μM) reduced the frequency of TSMC Ca^{2+} transients (D). The scale bars for D refer to all traces. CCCP (1 μM) caused a large increase in basal Ca^{2+} level and prevented Ca^{2+} transient discharge in ATSMCs (E). Antimycin-A (10 μMF) and oligomycin (5 μM) (E) both reduced ATSMC Ca^{2+} transients with a rise in basal Ca^{2+} level (F). All traces were recorded from different preparations pretreated with nifedipine (1 μM) for 30 min. The scale bars for G refer to all traces.

Table 5 Effects of mitochondrial inhibitors on the parameters of Ca transients in TSMC

Chemicals	*Frequency (min^{-1})*	*Amplitude (F_t/F_0)*	*Half-duration (ms)*
Control (n = 5)	12.3 ± 3.2	0.61 ± 0.17	684 ± 70
Antimycin-A (10 μM)	3.5 ± 0.7*	0.63 ± 0.13	662 ± 74
Control (n = 6)	9.7 ± 2.2	0.75 ± 0.16	662 ± 123
Rotenone (10 μM)	3.9 ± 1.1*	0.78 ± 0.13	679 ± 109
Control (n = 6)	9.6 ± 1.5	0.71 ± 0.11	686 ± 85
Oligomycin (5 μM)	4.8 ± 1.2*	0.70 ± 0.12	663 ± 64

Data shown are mean ± SD.
*Significantly different from control values ($P < 0.05$).
TSMC, typical smooth muscle cell.

Role of mitochondrial function in the generation on spontaneous Ca transients in TSMC

CCCP (1 μM, n = 8) prevented the generation of spontaneous Ca^{2+} transients in TSMCs (Figure 10A). The effects of CCCP on basal Ca^{2+} level were complex. Typically, CCCP caused a transient increase in basal Ca^{2+} level that was followed by a transient reduction and then a slow increase in Ca^{2+}.

Antimycin-A (10 μM) reduced the frequency of spontaneous Ca^{2+} transients and eventually prevented their generation within 10 min (n = 8, Figure 10B). It also caused a small, slow rise in the basal Ca^{2+} level. In five out of eight preparations, the effects of antimycin-A on the parameters of Ca^{2+} transients before their complete cessation were able to be analysed as illustrated in Table 5.

Rotenone (10 μM), an inhibitor for complex I, also reduced the frequency of spontaneous Ca^{2+} transients (n = 6) or abolished their generation (n = 2, Figure 10C) with a marginal effect on the basal Ca^{2+} level (Table 5). Both mitochondrial inhibitors abolished or reduced the frequency of spontaneous Ca^{2+} transients, but failed to increase either their amplitude or duration, suggesting that the positive inotropic effects of CGRP may not be causally related with the suppression of mitochondrial function.

Oligomycin (5 μM, n = 7) also reduced the frequency of spontaneous Ca^{2+} transients, although its action was weaker than the other mitochondrial inhibitors (Figure 10D, Table 5).

Role of mitochondrial function in the generation of spontaneous Ca transients in ATSMC

CCCP (1 μM n = 7, Figure 10E) and antimycin-A (10 μM n = 6, Figure 10F) caused a large increase in basal Ca^{2+} level (0.33 ± 0.07 F_t/F_0 and 0.15 ± 0.04 F_t/F_0 respectively) and blocked the spontaneous generation of Ca^{2+} transients in ATSMCs.

Oligomycin (5 μM) reduced both the frequency (from 7.4 ± 1.3 min^{-1} to 3.3 ± 0.98 min^{-1}, $P < 0.05$, $n = 6$) and amplitude (from 0.62 ± 0.09 F_t/F_0 to 0.38 ± 0.11 F_t/F_0, $P < 0.05$) of spontaneous ATSMC Ca^{2+} transients, which was associated with a rise in basal Ca^{2+} (0.12 ± 0.04 F_t/F_0, Figure 10G).

Discussion

In the mouse renal pelvis, stimulation of sensory nerves with electrical stimuli or capsaicin caused the release of substances that induced negative chronotropic and positive inotropic effects on the spontaneous contractions. Our observations suggest that these negative chronotropic effects primarily result from the inhibition of ATSMC Ca^{2+} transients that reduced the triggering of action potential discharge in the TSMC layer. On the other hand, the positive inotropic effects may be attributed to a direct effect on TSMCs to increase the amplitude and duration of action potentials and associated Ca^{2+} transients. Thus sensory nerves may project dual innervations to ATSMCs and TSMCs as is evident by electron microscopy.

The major transmitter for these neuronal modulations of spontaneous contractions is likely to be CGRP as the effects of sensory nerve stimulation were well mimicked by bath-applied hCGRP and the renal pelvis is richly innervated with CGRP-containing TRPV1-positive sensory nerves. The CGRP receptor antagonist, CGRP 8–37 (1 μM) partly antagonized the sensory nerve-mediated modulation of spontaneous contractions, suggesting that high frequency stimuli may be releasing relatively high concentrations of CGRP locally (Maggi *et al.*, 1992).

Our results are consistent with previous reports in the upper urinary tract of other species, where CGRP and cAMP stimulators caused membrane hyperpolarization, through the opening of plasmalemmal K_{ATP} channels (Santicioli and Maggi, 1994; Maggi *et al.*, 1995a,b), and reduced the frequency of the spontaneous action potentials in the ureter attached to the renal pelvis (Exintaris and Lang, 1999). It has also been reported that CGRP hyperpolarized the smooth muscle membrane of gall bladder through the opening of plasmalemmal K_{ATP} channels via cAMP-dependent mechanisms (Zhang *et al.*, 1994).

Glibenclamide blocked the membrane hyperpolarization of TSMCs to CGRP or cAMP stimulators but did not prevent their negative chronotropic effects, suggesting that the inhibitory actions of CGRP may not be directly attributed to the opening of TSMC plasmalemmal K_{ATP} channels. This was further supported by the lack of voltage dependency of action potential generation in TSMCs. Similar effects of K_{ATP} channel openers, reducing action potential frequency without membrane hyperpolarization, have been previously reported in detrusor smooth muscle of the guinea pig (Seki *et al.*, 1992; Hashitani *et al.*, 1996), in which action potential frequency is highly voltage sensitive (Hashitani and Brading, 2003). Interestingly, the possible contribution of mitochondria in generating action potentials in detrusor smooth muscles has been suggested because of their sensitivity to CCCP (Kubota *et al.*, 2003).

Interpretation of the effects of K_{ATP} channels modulators on ATSMC Ca^{2+} transients is quite complicated and obscure. Diazoxide, a mitochondrial K_{ATP} channels opener that is expected to have a mild uncoupling effect, indeed reduced the frequency of ATSMC Ca^{2+} transients and raised basal Ca^{2+}, as well as slowing TSMC action potential discharge associated with a membrane depolarization. This is consistent with the idea that mitochondrial K_{ATP} channel opening in ATSMCs reduces their Ca^{2+} buffering capacity. This was further supported by the fact that Y-26763, a K_{ATP} channel opener (Hashitani *et al.*, 1996) that hyperpolarized the membrane and abolished all spontaneous action potential discharge in TSMCs, was still capable of reducing action potential frequency in the presence of glibenclamide without hyperpolarizing the membrane (H. Hashitani, unpubl. obs.). However, 5-HD and glibenclamide that are considered to close mitochondrial K_{ATP} channels, suppressed Ca^{2+} transients in ATSMCs, in a manner associated with a slight reduction in basal Ca^{2+}. How can both openers and blockers of mitochondrial K_{ATP} channels reduce the frequency of ATSMC Ca^{2+} transients? It seems likely that other K_{ATP} channel-independent actions of these K_{ATP} channels modulators may be involved. Diazoxide and glibenclamide have been reported to increase flavoprotein fluorescence, suggesting that both agents have mild uncoupling effects that stimulate oxidation (Hu *et al.*, 1999). On the other hand, diazoxide and 5-HD have been reported to increase mitochondrial matrix volume upon inhibition of respiration (Lim *et al.*, 2002). The common consequence of uncoupling and respiration inhibition may be a diminished ATP production. Such an action is consistent with our observed reduction of ATSMC Ca^{2+} transient frequency with oligomycin.

The opposing actions of 5-HD and glibenclamide on action potentials and Ca^{2+} transient discharge in TSMCs could be explained by a 'selective' inhibition of plasmalemmal K_{ATP} channels in TSMCs with glibenclamide. Thus, even though glibenclamide is likely to be reducing spontaneous Ca^{2+} transients and subsequent STD discharges that are generated by the opening of Ca^{2+}-activated cation-selective channels in ATSMCs, this inhibition could well be more than compensated for by the decrease in the resting conductance in TSMC associated with the blockade of plasmalemmal K_{ATP} channels. Thus, STDs firing in the presence of glibenclamide would have a greater probability of triggering action potentials in TSMCs resulting in the larger Ca^{2+} transients observed. In circular smooth muscle of the gastric antrum of the guinea pig, where intramuscular ICC electrically drive neighbouring smooth muscle bundles, both glibenclamide and 5-HD abolished spontaneous Ca^{2+} transients (Fukuta *et al.*, 2002), suggesting a role of ICC mitochondria in generating their pacemaking activity.

In the present study, disruption of the mitochondrial inner membrane electrical gradient with CCCP, or inhibition of complexes I and III in the mitochondrial respiratory chain with rotenone and antimycin-A, respectively, abolished or reduced the frequency of spontaneous action potentials and Ca^{2+} transients in TSMCs. CCCP and antimycin-A also prevented the generation of ATSMCs Ca^{2+} transients and increased their basal Ca^{2+}. These results suggest that mitochondrial function, presumably its Ca^{2+} buffering capacity in ATSMCs, is critical for the generation of spontaneous activity in the renal pelvis, as is the case for ICC in other smooth muscles (Ward *et al.*, 2000; Fukuta *et al.*, 2002; Balemba *et al.*,

2008; Sergeant *et al*., 2008). As all of these chemicals may subsequently suppress ATP synthesis, such inhibition may result from the reduction of mitochondrial ATP production. This seems particularly consistent with the CCCP-evoked hyperpolarization in TSMCs upon the opening of plasmalemmal K_{ATP} channels. Unlike ICC in the mouse ileum or rabbit urethra, which generate oligomycin-insensitive spontaneous electrical or Ca^{2+} activity, oligomycin inhibited all spontaneous activity in both ATSMC and TSMC, although its action was less potent than the other mitochondrial inhibitors. Oligomycin has been reported to hyperpolarize the mitochondrial membrane that would be expected to facilitate Ca^{2+} buffering (Chalmers and McCarron, 2008). However, oligomycin in fact increased basal Ca^{2+} levels in ATSMCs. As mitochondrial are situated in close apposition to sarcoplasmic reticulum, produced ATP may be transported to maintain CaATPase function. We believe that it is likely that the hyperpolarizations to CGRP results from their direct action on plasmalemmal K_{ATP} channels in TSMCs rather than as a consequence of mitochondrial inhibition. This was supported by our findings that caffeine, a non-specific phosphodiesterase inhibitor, and forskolin increase K_{ATP} currents in enzymatically dispersed TSMCs (M.A. Tonta and R.J. Lang, unpubl. obs.).

In the mouse renal pelvis preparations where L-type Ca^{2+} channels have been blocked, cyclopiazonic acid (CPA) readily prevents the generation of STDs and Ca^{2+} transients in ATSMCs in a manner associated with a large increase in basal Ca^{2+} (Lang *et al*., 2007b). In the present study, CCCP, at concentrations that evokes large increases in the basal Ca^{2+} in ICC (Sergeant *et al*., 2008) or smooth muscle (Fukuta *et al*., 2002; Kubota *et al*., 2003), mimicked the effects of CPA in ATSMCs, suggesting that both organelles may work concurrently to regulate spontaneous Ca^{2+} transient discharge. A recent study employing the simultaneous monitoring of cytoplasmic Ca^{2+} concentration and Ca^{2+} concentration in either ER or mitochondria clearly demonstrates that Ca^{2+} shuttles between ER and mitochondria in phase with the Ca^{2+} oscillations, thus revealing an essential requirement for mitochondria in generating Ca^{2+} transients (Ishii *et al*., 2006). As STDs may result from the opening of Ca^{2+}-dependent cation-selective channels in ATSMCs (Lang *et al*., 2007b), alterations in Ca^{2+} homeostasis as a consequence of mitochondrial Ca^{2+} handling may be crucial in regulating STD frequency.

CGRP and stimulators of cAMP were capable of reducing the frequency of TSMC action potentials in glibenclamide-treated preparations independently of the membrane potential, and thus may have other targets to reduce the frequency of spontaneous activity. As ATSMCs are richly endowed with mitochondria (Klemm *et al*., 1999), it is reasonable to assume that the modulation of ATSMC mitochondria Ca^{2+} handing may be the site of action of CGRP and cAMP stimulators to inhibit spontaneous Ca^{2+} transients. The influx of Ca^{2+} into matrix space relies on the electrical gradient across the inner mitochondrial membrane, and thus the mitochondrial membrane potential plays an important role in regulating mitochondria Ca^{2+} handing (Duchen, 1999). CGRP and cAMP reduced the basal Ca^{2+} levels in ATSMCs, in contrast to the mitochondrial inhibitors that increased Ca^{2+} levels. Therefore, CGRP and cAMP stimulators may enhance Ca^{2+} buffering by mitochondria rather than open mitochondrial K_{ATP} channels that would theoretically reduce mitochondrial Ca^{2+} buffering capacity. It has been reported that the inhibition of mitochondrial Ca^{2+} uptake accelerates intracellular Ca^{2+} waves (Duchen, 1999), and thus CGRP-stimulated Ca^{2+} buffering may suppress not only Ca^{2+} release from individual mitochondria but also their function as part of a coupled intracellular oscillator that is fundamental to STD generation.

Increases in action potential duration may be attributed to the direct action of CGRP and cAMP stimulators on L-type Ca^{2+} channels in TSMCs to enhance associated Ca^{2+} transients. As the duration of action potentials in TSMCs is likely to be predominantly determined by the opening of large conductance potassium (BK) channels (Lang *et al*., 2007c), reduced BK channel activation as a consequence of a reduction in Ca^{2+} concentration in the subplasmalemmal space by mitochondrial Ca^{2+} uptake would prolong action potential duration. In rat cerebral arteries, mitochondrial inhibition results in an increased activation of Ca^{2+} sparks and associated opening of BK channels (Cheranov and Jaggar, 2004). In addition, the accelerated Ca^{2+} uptake into either the sarcoplasmic reticulum or mitochondria by CGRP/cAMP may well be enhanced by the longer intervals between action potentials and reinforce Ca^{2+} release upon subsequent action potential discharge. Finally, the positive inotropic effects of CGRP may involve the activation of excitatory substance P receptors by reducing the metabolism of tonically released substance P from sensory nerves (Gontijo *et al*., 1999).

In conclusion, spontaneous action potential discharge and muscle contraction of the mouse renal pelvis critically depends on mitochondrial function in ATSMCs. The negative chronotropic effects on pyeloureteric contractility, mediated by CGRP released from sensory nerves, may result from the suppression of ATSMC Ca^{2+} transients upon stimulation of mitochondrial Ca^{2+} buffering and, less importantly, upon the opening of plasmalemmal K_{ATP} channels in TSMCs. In contrast, the positive inotropic effects of CGRP may be related to a prolongation in the duration of action potentials and Ca^{2+} transients in TSMCs. Thus, sensory nerves appear to have distinct interactions with ATSMCs and TSMCs to modulate pyeloureteric contractility.

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Conflict of interest

The authors state no conflict of interest.

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Supporting Information

Additional Supporting Information may be found in the online version of this article:

Figure S1 Effects of K channel opener on spontaneous action potentials in typical smooth muscle of the mouse renal pelvis. Y-26763 (1 μM), a K_{ATP} channel opener, hyperpolarized the membrane and prevented spontaneous action potential discharge (Aa). In the same preparation, which had been exposed to glibenclamide (10 μM), Y-26763 (1 μM) did not hyperpolarize the membrane but was still capable of reducing the action potential frequency (Ab). A higher concentration of Y-26763 (10 μM) depolarized the membrane and prevented action potential generation (Ac). The scale bars for Ac refer to all traces.

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