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Actions of Two Main Metabolites of Propiverine (M-1 and M-2) on Voltage-Dependent L-Type Ca^{2+} Currents and Ca^{2+} Transients in Murine Urinary Bladder Myocytes

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Abstract

The anticholinergic propiverine, used for the treatment of overactive bladder (OAB) syndrome, has functionally active metabolites (M-1 and M-2), but the site of actions of these metabolites is uncertain. Propiverine is rapidly absorbed after oral administration and is extensively biotransformed in the liver, giving rise to several active metabolites (M-1, the propiverine N-oxide, and M-2, the N-oxide lacking the aliphatic side chain). This study determines the effect of M-1 and M-2 on voltage-dependent nifedipine-sensitive inward Ca^{2+} currents (I_{Ca}) using patch-clamp techniques and fluorescent Ca^{2+} imaging (following electrical field stimulation (EFS) and acetylcholine (ACh)) in the murine urinary bladder. In conventional whole-cell recording, propiverine and M-1 but not M-2 inhibited the peak amplitude of I_{Ca} in a concentration-dependent manner at a holding potential of -60 mV (propiverine, $K_i = 10$ μM ; M-1, $K_i = 118$ μM). M-1 shifted the steady-state inactivation curve of I_{Ca} to the left at -90 mV by 7 mV. Carbachol (CCh) reversibly inhibited I_{Ca} . This inhibition probably occurred through muscarinic type 3 receptors, coupling with G-proteins, since nanomolar concentrations of 4-DAMP greatly reduced this inhibition, while pirenzepine or AF-DX 116 at concentrations up to 1 μM were almost ineffective. In the presence of M-2, the CCh-induced inhibition of I_{Ca} was blocked. In fluorescent Ca^{2+} imaging, M-2 inhibited EFS-induced and ACh-induced Ca^{2+} transients. These results suggest that M-1 acts, at least in part, as a Ca^{2+} channel antagonist (as it inhibited I_{Ca}), while M-2 has more direct antimuscarinic actions.

Keywords

Gastrointestinal; Hepatic; Pulmonary; Renal

Introduction

It is well known that contraction of the urinary bladder, voluntary or involuntary, is mediated by stimulation of muscarinic receptors by acetylcholine (ACh), released from excited cholinergic parasympathetic nerves. Since muscarinic receptors mediate both normal detrusor contraction and the principal contraction of disturbed urinary bladder, muscarinic receptors are useful drug targets to regulate the function of urinary bladder. Thus, various anticholinergic drugs have been utilized for the treatment of overactive bladder (OAB) syndrome but their usefulness is limited by significant side effects (Andersson et al., 1999; Fry et al., 2002; Herbison et al., 2002; Michel and Hegde, 2006). However, many antimuscarinic drugs act, at least in part, as prodrugs, so their metabolites might provide effective therapies with fewer side effects (Michel and Hegde, 2006).

Propiverine (1-methyl-4-piperidyl diphenylpropoxyacetate) is an anticholinergic agent which is commonly used for the treatment of patients with overactive bladders (Dorschner et al., 2000; Madersbacher et al., 1999). Propiverine is rapidly absorbed after oral administration and is extensively biotransformed in the liver, giving rise to several active metabolites (such as M-1, the propiverine N-oxide, and M-2, the N-oxide lacking the aliphatic side chain; for chemical structures, see Fig. 1). Twenty-four hours after ingestion, these compounds can be recovered among other derivatives from human urine (as a percent of the original dose): propiverine, 3%; M-1, 20%; M-2, 5% (Haustein and Hüller, 1988). Moreover, after five days of treatment with propiverine in a single-dose application, both propiverine and M-1 were detected in the serum whilst M-2 was not detectable in the serum (Siepmann et al., 1998). Despite their high *in vivo* concentrations (M-1 reaches a peak of around 2 μ M, Siepmann et al., 1998), the pharmacological properties of propiverine metabolites remain elusive (Andersson et al., 1999; Madersbacher and Murtz, 2001). Thus, it is essential to investigate whether or not the two main metabolites of propiverine (M-1 and M-2) are related to the therapeutic actions of propiverine (Michel and Hegde, 2006).

It has been reported that propiverine appears to possess spasmolytic effects as a Ca^{2+} channel antagonist in addition to its antimuscarinic actions (Andersson et al., 1999; Madersbacher and Murtz, 2001). The contraction in response to muscarinic receptor activation in the urinary bladder, particularly in the mouse but also in humans, is sensitive to pharmacological blockade of L-type Ca^{2+} channels (Wuest et al., 2007) and depends significantly on the $\text{Ca}_v1.2$ gene (Wegener et al., 2004). The most well-established link between muscarinic receptors and L-type Ca^{2+} channels is an indirect one: intracellular Ca^{2+} stores are released following muscarinic receptor activation, and their filling requires L-type Ca^{2+} channels, at least in guinea-pig (Rivera and Brading, 2006). However, in the rat urinary bladder, contraction due to muscarinic type 3 receptors activation is not inhibited by effective PLC inhibition with U 73,122 (Schneider et al., 2004). Furthermore, PLD and PLA2 pathways play only a minor role in the contraction that follows this muscarinic receptor activation (Schneider et al., 2004), implying that other as yet unidentified pathways functionally link the functionally important muscarinic type 3 receptors with L-type Ca^{2+} channels.

In patch-clamp experiments, propiverine inhibited the peak amplitude of voltage-dependent Ca^{2+} currents in detrusor myocytes (murine, Wuest et al., 2005; rat, Tokuno et al., 1993; guinea-pig, Tokuno et al., 1993; human, Wuest et al., 2005). However, surprisingly, neither M-1 nor M-2 caused inhibitory effects on voltage-dependent Ca^{2+} currents in murine detrusor myocytes (Wuest et al., 2005). Furthermore, although functional interactions between muscarinic receptors and voltage-dependent Ca^{2+} currents were reported in urinary bladder smooth muscle cells (Fry et al., 2002; Schneider et al., 2004), which muscarinic receptors may regulate voltage-dependent Ca^{2+} currents remains to be elucidated. Moreover,

the effects of two main propiverine metabolites on Ca^{2+} transients in intact smooth muscle cells in intact urinary bladder have not yet been directly measured.

In the present experiments, therefore, we initially studied the effects of propiverine and two of its main metabolites (M-1 and M-2) on voltage-dependent nifedipine-sensitive macroscopic Ca^{2+} currents (I_{Ca} , i.e., caused by Ca^{2+} entry through L-type Ca^{2+} channels) in single freshly-dispersed smooth muscle cells from murine urinary bladder using patch-clamp techniques. Secondly, we have further investigated whether or not M-2 prevented the CCh-induced inhibition of I_{Ca} through muscarinic receptors in whole-cell recordings. Finally, we have studied the actions of M-2 on the EFS-induced and ACh-induced Ca^{2+} transients in murine detrusor muscles with fluorescent Ca^{2+} imaging.

Materials and Methods

Cell Dispersion

Eight- to twelve-week-old male BALB/c mice (Charles River Laboratories INC., Shiga, Japan; Harlan, Bicester, U.K.) were killed by cervical fracture and the urinary bladders removed. A segment of detrusor was excised and quickly transferred into nominally Ca^{2+} -free solution (mM): Na^+ 140, K^+ 5, Mg^{2+} 0.5, Cl^- 146, HEPES 10, titrated to pH 7.35-7.40 with Tris base. Murine detrusor myocytes were freshly isolated under a microscope by gently tapping after treatment with collagenase (Type IA, 1-2 mg/mL; Sigma Chemical K.K., Tokyo, Japan), as described previously (Teramoto and Brading, 1996). Relaxed spindle-shaped cells were isolated and stored at 4°C. The dispersed cells were used within 4-5 h for experiments.

Recording Procedure

Patch-clamp experiments were performed at room temperature (21-23°C), as described previously (Teramoto et al., 2005). Junction potentials between bath and pipette solutions were measured with a 3 M KCl reference electrode and were < 2 mV, so that correction of these potentials was not necessary. Capacitance noise was kept to a minimum by maintaining the bath solution in the electrode as low as possible. After establishing a conventional whole-cell configuration, voltage-dependent Ca^{2+} inward currents (I_{Ca}) evoked by a depolarizing pulse to 0 mV from a holding potential of -60 mV increased slightly in amplitude until the peak currents reached steady state approximately 4 min after the rupture of the membrane patch. This peak value was then maintained for at least 30 min (the peak amplitude of I_{Ca} at 30 min being $98 \pm 1\%$ to control, $n = 15$ cells, 10 different animals) when test depolarization pulses (500 ms duration) were applied at 20 s intervals in conventional whole-cell recording. Consequently, all experiments were performed within 30 min after the peak amplitude of I_{Ca} became stable in a conventional whole-cell configuration at -60 mV.

Drugs and Solutions

To record voltage-dependent Ca^{2+} currents (I_{Ca}) in whole-cell configuration, pipettes containing a high concentration of caesium were used; the composition of the pipette solutions was (mM): Cs^+ 130, tetraethyl ammonium (TEA^+) 10, Mg^{2+} 2, Cl^- 144, glucose 5, EGTA 5, ATP (ATP-Tris) 5, HEPES 10, titrated with Tris base (pH 7.35-7.40). The bath solution contained (mM): Na^+ 140, K^+ 5, Mg^{2+} 1.2, Ca^{2+} 2, glucose 5, Cl^- 151.4, HEPES 10/Tris (pH 7.35-7.40). The bath solution was superfused by gravity throughout the experiments at a rate of 2 ml min⁻¹. Propiverine (1-methyl-4-piperidyl diphenylpropoxyacetate) and its active metabolites (M-1, 1-methyl-4-piperidyl diphenylpropoxyacetate N-oxide; M-2, 1-methyl-4-piperidyl benzilate N-oxide) were kindly provided by Taiho Pharmaceutical Co. Ltd. (Tokushima, Japan; see Fig. 1). The rest of the

drugs were obtained from Sigma-Aldrich (Tokyo, Japan). M-1 and M-2 were prepared as 100 mM stock solutions in dimethylsulfoxide (DMSO). The final concentration of DMSO was less than 0.3%, and this concentration was shown not to affect I_{Ca} in murine urinary bladder.

Data Analysis

The whole-cell current data were low-pass filtered at 1 kHz with an 8 pole Bessel filter, sampled at 1 kHz and analysed on a computer (PowerMac G4, Tokyo, Japan) by the commercial software 'Powerlab' (ADInstruments Pty Ltd., Castle Hill, Australia). Data are expressed as mean with the standard deviation (s.e.m.). To account for leak and capacitive currents, I_{Ca} in the presence of 100 μM Cd^{2+} was subtracted from the raw current data (Teramoto et al., 2005).

The peak amplitude of I_{Ca} elicited by a step pulse to 0 mV from the holding potential just before application of a drug was normalized as one. Curves were drawn by fitting the following equation using the least-squares method:

$$\text{Relative amplitude of } I_{Ca} = \frac{1}{1 + \left(\frac{D}{K_i}\right)^{n_H}}$$

where K_i , D and n_H are the inhibitory dissociation constant, concentration of drug (μM) and Hill's coefficient, respectively.

For steady-state inactivation curves, the peak amplitude of I_{Ca} in the absence and presence of drugs without application of any conditioning pulse was normalized to one. The lines were drawn by fitting the data to the following equation using the least-squares method,

$$I = \frac{I_{\max} - C}{1 + e^{\frac{(V - V_{\text{half}})}{k}}} + C$$

including the following parameters: the relative amplitude of I_{Ca} observed at various amplitudes of conditioning pulse (I), that observed with a -100 mV conditioning pulse ($I_{\max}$), the amplitude of the conditioning pulse (V), the conditioning pulse amplitude (V_{half}) which evokes I_{Ca} of amplitude half $I_{\max}$, a slope factor (k) and the fraction of the non-inactivating component of I_{Ca} (C).

Activation curves were derived from the current-voltage relationships. Conductance (G) was calculated from the equation $G = I_{Ca} / (E_m - E_{Ca})$, where I_{Ca} is the peak current elicited by depolarizing test pulses of -50 to $+40$ mV from a holding membrane potential of -60 mV and E_{Ca} is the equilibrium potential for Ca^{2+} . $G_{\max}$ is the maximal Ca^{2+} conductance (calculated at potentials above 0 mV). Values for $G/G_{\max}$ (a relative amplitude) were plotted against membrane potential.

Ca^{2+} Transients

The urinary bladder was cut from base to dome and exposed to 10 μM Oregon Green 488 BAPTA-1 AM (Invitrogen, U.K.) in 1% DMSO/0.2% pluronic F-127 (Sigma-Aldrich, U.K.) in physiological salt solution (PSS) for 90 minutes at 36°C . Each urinary bladder was then pinned flat, serosal side up, in a Sylgard-lined organ bath and rinsed in PSS containing: Na^+ 144.53, K^+ 4.7, Mg^{2+} 1.3, Ca^{2+} 1.8, Cl^- 129.3, HCO_3^- 25, H_2PO_4^- 1.13 and glucose 11.1

which was bubbled with 95% O₂ and 5% CO₂. This preparation was transferred to the stage of an upright Olympus microscope with a 40x 0.8 NA water immersion objective and Hamamatsu ORCA-AG camera. The urinary bladders were continuously superfused with PSS (bath temperature 33–34°C). A series of 1000 frames were captured using Wasabi software (Hamamatsu Photonics Deutschland, Herrsching, Germany) at 20 ± 1 Hz with 4×4 on-chip binning to generate one image set. Such sets were acquired once every 3 minutes. Three such sets were acquired at each drug concentration (or protocol step). Image analysis was performed with Image SXM (<http://www.liv.ac.uk/~sdb/ImageSXM/>); to correct for lateral movement (particularly that generated by contraction), all images were automatically aligned to a template image using the 'Autoregister' function of Image SXM and custom-written macros. A region of interest was established which encompassed the portion of a SMC that was consistently within the field of view. The fluorescent signal in this region was measured over time throughout the image set. Data were exported to Spike 2 (Cambridge Electronic Design Ltd, Cambridge, U.K.) for automated detection and measurement of spikes in the Ca²⁺ signal. The threshold for spike detection (based on the amplitude of the first derivative of the fluorescent signal) was manually chosen to match the sensitivity of manual detection for that cell. The Ca²⁺ wave frequency was calculated by counting the number of waves crossing a small, randomly chosen region in each of three smooth muscle cells from each preparation. The smooth muscle cells were chosen to be well-separated (wherever possible) and clearly in focus throughout at least 50% of the recording period. Some portions of the recordings could not be analysed because of preparation movement (including contraction).

Immunohistochemical Studies

Tissue samples from murine urinary bladder were embedded in OCT compound (Tissues-Tek, SAKURA, Tokyo, Japan) in disposable plastic tubes and rapidly frozen in hexane surrounded by liquid nitrogen. Sections were cut in a cryostat (Leica, CM3050S, Tokyo, Japan) at a thickness of 6 µm and mounted on silane-precoated glass slides, then allowed to air dry at room temperature for approximately 30 min. Sections were fixed in cold acetone and washed thoroughly in phosphate-buffered saline (PBS) before staining. The tissue sections were treated in 2% normal goat serum (Santa Cruz Biotechnology, CA, U.S.A.), 1% bovine serum albumin (GIBCO, NY, U.S.A.) and 0.1% Triton-X 100 (Amersham Biosciences, Uppsala, Sweden) in PBS for 1 h to avoid non-specific staining, and then reacted with the primary antibody, polyclonal rabbit anti-muscarinic type 3 receptor (Santa Cruz Biotechnology, CA, U.S.A.) antibody (diluted at 1: 200) at 4°C overnight. After washing three times (5 min duration) in PBS, sections were incubated with phycoerythrin-conjugated goat anti-rabbit IgG (Santa Cruz Biotechnology, CA, U.S.A.) diluted to 1:50 in PBS for 60 min at room temperature under dark conditions. Sections were then washed three times (5 min duration) in PBS. Coverslips were mounted onto slides with a fluorescence mounting medium and the slides were viewed with a fluorescence microscope (Olympus BX51, Olympus Optical Co. Ltd., Tokyo, Japan). Non-immunized rabbit IgG was also used instead of the primary antibody as a negative control.

Statistics

Statistical analyses were performed with a paired *t* test (two-factor with replication). Changes were considered significant at *P* < 0.05.

Results

Electrophysiological Properties of Voltage-Dependent Ca^{2+} Currents in Murine Urinary Bladder Myocytes

When depolarizing step pulses (10 mV increments from -50 mV to $+40$ mV for 500 ms duration) were applied from a holding potential of -60 mV in whole-cell recordings, voltage-dependent Ca^{2+} inward currents (I_{Ca}) were evoked in murine urinary bladder myocytes. As shown in Fig. 2A, at potentials more positive than -40 mV, I_{Ca} was evoked which reached a peak and then gradually decayed. Fig. 2C shows the current-voltage relationships of I_{Ca} obtained from a holding potential of -60 mV. The maximum peak amplitude was obtained at approximately 0 mV and the amplitude was reduced at more positive potentials. M-1 ($100 \mu\text{M}$) inhibited the peak amplitude of I_{Ca} evoked by depolarizing pulses (500 ms duration) from a holding potential of -60 mV to potentials more positive than -30 mV, Fig. 2A, B. When the peak amplitude of I_{Ca} in the absence (i.e. control) and presence of M-1 was superimposed, the time course of current decay is shown to be accelerated in the presence of $100 \mu\text{M}$ M-1 (Fig. 2B). Fig. 2C shows the current-voltage relationships of I_{Ca} in the absence and presence of $100 \mu\text{M}$ M-1 ($n = 5$ cells, 3 different animals).

Effects of M-1 and M-2 on I_{Ca} in Murine Urinary Bladder Myocytes

Fig. 3A shows the time course of the effect of M-1 on I_{Ca} evoked by a depolarizing pulse to 0 mV from -60 mV after M-1 (10 and $100 \mu\text{M}$) had been cumulatively applied. The depolarizing pulses were applied every 20 s. M-1 (10 and $100 \mu\text{M}$) reduced the peak amplitude of I_{Ca} . On removal of M-1, the peak amplitude of I_{Ca} gradually recovered, but not to the control level. Subsequently, application of $10 \mu\text{M}$ nifedipine suppressed I_{Ca} to zero. Fig. 3B shows the time course of the effects of M-2 (10 and $100 \mu\text{M}$) on I_{Ca} evoked by a depolarizing pulse to 0 mV from a holding potential of -60 mV. Although M-2 ($10 \mu\text{M}$) caused no reduction of the peak amplitude of I_{Ca} , M-2 ($100 \mu\text{M}$) caused a small inhibition ($100 \mu\text{M}$, 0.87 ± 0.07 , $n = 5$ cells, 3 different animals; 1 mM , 0.84 ± 0.09 , $n = 4$ cells, 3 different animals; Fig. 3C). After removal of $100 \mu\text{M}$ M-2, the peak amplitude of I_{Ca} recovered. Subsequent application of nifedipine ($10 \mu\text{M}$) suppressed I_{Ca} . Fig. 3C shows the relative peak amplitude of I_{Ca} evoked by depolarizing pulses to 0 mV from two different holding potentials (-60 and -90 mV) applied every 20 s, plotted against concentration of M-1, M-2 or propiverine. M-1 inhibited the peak amplitude of I_{Ca} in a concentration-dependent manner (-60 mV, $K_i = 118 \mu\text{M}$; -90 mV, $K_i = 505 \mu\text{M}$).

The voltage-dependence was investigated before and after application of M-1 using the experimental protocol shown in Fig. 4 (conditioning pulse duration, 10 s; test pulse duration, 1 s; holding membrane potential, -100 mV). In the absence of M-1 (control), inactivation of I_{Ca} occurred with depolarizing pulses more positive than -50 mV. After application of M-1 (approximately 5 min later), the voltage-dependent inactivation curve (the 50% inactivation potentials) in the same cells was significantly shifted to the left (by approximately 7 mV; control, -36.0 ± 1.5 mV, $n = 6$ cells, 3 different animals; M-1, -42.8 ± 2.5 mV, $n = 6$ cells, 3 different animals, $P < 0.05$).

Fig. 4 shows the activation curves obtained from the current-voltage relationships in Fig. 2, fitted to the Boltzmann equation. M-1 ($100 \mu\text{M}$) caused little shift of the activation curve (the 50% activation potentials; -14 mV (control; -14.0 ± 0.3 mV, $n = 5$) versus -13 mV (M-1; -13.4 ± 0.3 mV, $n = 5$ cells, 3 different animals, $P > 0.05$).

Muscarinic Suppression of I_{Ca} in Smooth Muscle Cells of Murine Urinary Bladder

Fig. 5A shows a time course of the effects of CCh (10 and 100 μ M) on I_{Ca} evoked by a depolarizing pulse to 0 mV from a holding potential of -60 mV. CCh inhibited the peak amplitude of I_{Ca} in a concentration-dependent manner, Fig. 5B. On removal of CCh, the peak amplitude of I_{Ca} did not recover to the control level. When the pipette solution contained 1 mM GDP- β -S, which completely inhibits the dissociation of α and $\beta\gamma$ complexes of GTP-binding proteins, the application of CCh (100 μ M) had little effect on the peak amplitude of I_{Ca} . In the presence of 1 μ M cyclopentolate, CCh (100 μ M) changed the peak amplitude of I_{Ca} negligibly, Fig. 5B. To determine further the type of muscarinic receptors involved in the inhibition of I_{Ca} , we tested the effects of three relatively subtype-specific muscarinic antagonists: pirenzepine, AF-DX116 and 4-DAMP (Eglen et al., 1996). As shown in Fig. 5B, 4 min pretreatment with 1 μ M pirenzepine or AF-DX 116 (concentrations 10-100 times higher than the K_d values for muscarinic type 1 and type 2 receptors, Eglen et al., 1996) greatly reduced I_{Ca} induced by 100 μ M CCh. In contrast, in the presence of 100 nM 4-DAMP, a muscarinic type 3 receptor-specific antagonist, the same concentration of CCh failed to inhibit the amplitude of I_{Ca} , Fig. 5B. Although thorough inspection of antagonism between CCh and the three muscarinic antagonists over the full concentration range was not technically feasible, these results are consistent with the muscarinic receptor involved in I_{Ca} inhibition being of the muscarinic type 3 receptors.

Immunohistochemical Localization of Muscarinic Type 3 Receptor in Murine Detrusor

Immunohistochemistry was performed to determine whether muscarinic type 3 receptors were expressed, Fig. 5C, D. As shown in Fig. 5D, muscarinic type 3 receptor immunoreactivity is clearly visible in the membranes of the smooth muscle cells.

Immunohistochemistry using non-immune rabbit IgG instead of primary antibody (as a control) gave a negative result, Fig. 5E. Similarly, no specific immuno-reactive signal was seen when primary antibody was preabsorbed with the immunizing muscarinic type 3 receptor antigen (data not shown).

Effect of M-2 on CCh-Induced Suppression of I_{Ca} in Detrusor Smooth Muscle Cells

Fig. 6A shows a time course of the effects of CCh (10 and 100 μ M) on I_{Ca} evoked by a depolarizing pulse to 0 mV from a holding potential of -60 mV in the absence and presence of 3 μ M M-2. M-2 had little effect on the peak amplitude of I_{Ca} . Additional application of CCh (10 and 100 μ M) failed to inhibit I_{Ca} . On removal of M-2, CCh (100 μ M) caused an inhibition of I_{Ca} . Fig. 6B and 6C summarize the CCh-induced suppression of I_{Ca} in the presence of M-2 (1 and 3 μ M) when the peak amplitude of I_{Ca} was normalized as one just before application of several drugs (i.e. control).

Effect of M-2 on Smooth Muscle Cells in Intact Urinary Bladder

Evoked Ca^{2+} transients in urinary bladder smooth muscle cells were characterised as present when the fluorescent signal rose rapidly within 0.5 s of the field stimulus (while stimulating at 0.33 Hz) to stimulate nerves. As shown in Fig. 7A, such evoked Ca^{2+} transients fell into two groups. Firstly, a 'fast' group defined on the basis of a very fast rising phase and characterised by a Ca^{2+} response over the whole cell. Secondly, a 'slow' group defined on the basis of a slower rising phase and characterised by the presence of Ca^{2+} waves. Under control conditions (DMSO or saline only), most field stimuli induced an increase in fluorescence within the smooth muscle cells (fast, $P = 0.82 \pm 0.06$; slow, $P = 0.09 \pm 0.04$; total, $P = 0.91 \pm 0.02$; Fig. 7B). In response to a cumulative concentration-response protocol ($n = 4$ urinary bladders; 12 cells), more than half of the fast Ca^{2+} transients were abolished at 30 nM M-2 and only slow transients remained. These slow Ca^{2+} transients were present until concentrations exceeded 1 μ M. Upon exposure of the intact mice urinary bladder to

ACh (100 μM), Ca^{2+} waves could be induced in Fig. 8A. These waves were often periodic rather than occurring randomly (Fig. 8B). After washing out the ACh and leaving the preparation for 25 min, a repeat application of ACh could again generate Ca^{2+} waves with a similar frequency (Fig. 8C; $n = 9$ cells from 3 urinary bladders). M-2 (3 μM) was then added and had no significant effect on the wave frequency at rest. However, when ACh was applied in the presence of M-2 (3 μM), ACh no longer induced Ca^{2+} waves, Fig. 8.

Discussion

We have been able to demonstrate that M-1 but not M-2 inhibited I_{Ca} in dispersed myocytes from murine detrusor, that M-2 antagonized the CCh-induced suppression of I_{Ca} through muscarinic type 3 receptors and that M-2 suppressed ACh-induced Ca^{2+} transients in intact murine urinary bladder.

Effects of Propiverine and Two of its Main Metabolites (M-1 and M-2) on I_{Ca}

It is well known that propiverine suppresses I_{Ca} in dispersed detrusor myocytes (rat, $K_i = 7$ μM , Tokuno et al., 1993; guinea-pig, $K_i = 21$ μM , Tokuno et al., 1993; human, $K_i = 4$ μM , Wuest et al., 2007). Our data confirm the inhibitory effects of propiverine on I_{Ca} (i.e. L-type Ca^{2+} currents) in murine detrusor myocytes with a similar potency (murine, $K_i = 10$ μM). Recently, it has been reported that propiverine and M-1, but not M-2, have significant binding affinity to L-type Ca^{2+} channels (competing with a known antagonist) in rat urinary bladder (propiverine, $K_d = 18$ μM ; M-1 $K_d = 154$ μM ; Uchida et al., 2007), similar to the inhibitory potency in the present experiments (murine detrusor myocytes; propiverine, $K_i = 10$ μM ; M-1, $K_i = 118$ μM).

Recently, it has been reported that M-1 and M-2 (which Wuest *et al.* abbreviate as M-5 and M-6, respectively) have little inhibitory effect on I_{Ca} in human detrusor myocytes, with M-2 slightly inhibiting the peak current with an EC_{50} of just over 10 μM and no significant effect of M-1 up to 100 μM (Wuest et al., 2007). In the present experiments, M-1, but not M-2, inhibited the peak amplitude of I_{Ca} in a concentration- and voltage-dependent manner. We are not certain whether these differences between the present work and that of Wuest et al. (2005) are due to different experimental conditions (such as different charge carriers for Ca^{2+} channels, i.e. Ca^{2+} vs. Ba^{2+}), or species differences (humans compared with mice).

When the holding potential was elevated from -90 mV to -60 mV, the concentration response curve for M-1 was shifted to the left. The voltage-dependent inactivation curve was also significantly shifted to the left after application of 100 μM M-1, without altering the activation kinetics of I_{Ca} . These results strongly suggest the voltage-dependent inhibitory actions of M-1 occur at the inactivated state of the L-type Ca^{2+} channels in murine urinary bladder smooth muscle cells (i.e. voltage-dependent block). On removal of M-1, the peak amplitude of I_{Ca} did not recover to the control level. We suggest that M-1 may tightly bind L-type Ca^{2+} channels, producing a long lasting effect on L-type Ca^{2+} channels. However, the high concentrations of M-1 at which this occurs are unlikely to be reached during *in vivo* administration of propiverine (where M-1 reaches a peak of around 2 μM ; Siepmann et al., 1998).

Cholinergic Suppression of I_{Ca} Through Muscarinic Type 3 Receptor

Application of CCh gradually inhibited the peak amplitude of I_{Ca} . After washout of CCh, the peak amplitude of I_{Ca} slowly recovered. These results suggest that CCh inhibits I_{Ca} through indirect pathways, such as G-protein-coupled regulatory mechanisms.

In the present experiments, CCh caused a concentration-dependent inhibition of the peak amplitude of I_{Ca} in murine detrusor myocytes when EGTA (5 mM) was included in the

pipette solution. Furthermore, there was no suppression induced by CCh when GDP- β -S was present in the pipette solution. A similar observation was obtained in human and pig detrusor myocytes (Kajioka et al., 2002). Thus, we suggest that intracellular Ca^{2+} -insensitive and G-protein-coupled mechanisms seem to be involved in the inhibitory mechanisms. While both muscarinic type 2 and muscarinic type 3 receptors are expressed in the urinary bladder (mRNA transcripts, Yamaguchi et al., 1996; specific immunoprecipitation, Wang et al., 1995), it is generally believed that the muscarinic type 3 receptor is dominant in the urinary bladder from several species (Wang et al., 1995). Of the three subtype-specific antagonists for muscarinic receptors, only 4-DAMP, the muscarinic type 3 receptor antagonist, potently prevented the CCh-induced inhibition of I_{Ca} in murine detrusor myocytes. Moreover, immunohistochemical studies also indicated the presence of muscarinic type 3 receptor proteins in detrusor smooth muscle bundles. Taken together, these results indicate that G-protein activation *via* muscarinic stimulation, probably through muscarinic type 3 receptors, inhibits I_{Ca} in murine detrusor myocytes.

So, muscarinic type 3 receptor activation in the intact preparation has two opposing effects on Ca^{2+} influx. Firstly, it causes depolarization, probably activating SKF-96365-sensitive receptor operated cation channels (Kajioka et al., 2005). The depolarization opens voltage-gated Ca^{2+} channels. Secondly, activating muscarinic type 3 receptors changes voltage-gated Ca^{2+} channel kinetics as described in the present work; this action reduces or limits the Ca^{2+} current activated by depolarization.

Recently, using binding techniques, it has been reported that propiverine and its main metabolites (M-1 and M-2) bind to all five subtypes of human muscarinic receptors (i.e. muscarinic type 1-5 receptors) which were expressed in CHO cells and that the order of affinity for all subtypes of muscarinic receptors is mostly M-2 > propiverine > M-1 (Wuest et al., 2006; Maruyama et al., 2006). In the present experiments, pretreatment with 3 μM M-2 completely antagonized the CCh-induced suppression of I_{Ca} in murine detrusor myocytes. These results suggest that M-2 may functionally antagonize muscarinic type 3 receptors.

M-2 Modulates Ca^{2+} Transients in Intact Urinary Bladder Smooth Muscle

Recently, it has been reported that propiverine and M-2 suppressed CCh (1 μM)-induced $[\text{Ca}^{2+}]_{\text{i}}$ elevation in HEK-293 cells stably transfected with human muscarinic type 3 receptors, with very similar potency (M-2, $\text{IC}_{50} = 5 \mu\text{M}$; propiverine, $\text{IC}_{50} = 3 \mu\text{M}$, Wuest et al., 2006). Thus, they concluded that the antimuscarinic actions of propiverine *in vivo* are mainly due to the actions of M-2 (Wuest et al., 2006).

We have demonstrated two distinct types of Ca^{2+} transients evoked by electrical stimulus in murine urinary smooth muscle cells, namely a 'fast' type and a 'slow' type. In control, the fast Ca^{2+} transients were dominant when field stimuli were applied. These were similar to the nifedipine-sensitive whole cell Ca^{2+} elevations present in guinea-pig urinary bladder that are associated with spontaneous action potentials (Hashitani et al., 2001). When 30 nM M-2 was applied, more than half of the fast Ca^{2+} transients evoked by electrical stimulation were abolished. The slow Ca^{2+} transients were suppressed at 1 μM M-2, showing a different potency of M-2 between the two types of Ca^{2+} transients. The cellular mechanisms generating fast or slow Ca^{2+} transients following field stimulation were not further characterized. However, it has recently been reported that there are two types of nifedipine-sensitive spontaneous action potentials in the mouse urinary bladder, with median half rise times that differ by about 45 ms (8 ms compared with 53 ms); CCh increased the frequency of both types of action potential (Meng et al., 2007). If these two types of action potentials also arise following field stimulation, then perhaps they could be associated with the fast and slow Ca^{2+} transients reported in the present study. Meng et al. (2007) noted that spontaneous

Ca^{2+} transients occurred with a frequency similar to that of spontaneous action potentials, although the recording rate used (5 Hz) would not have been sufficient to distinguish between differences in Ca^{2+} rise times that matched the rise times of the two types of action potentials (Meng et al., 2007).

The present results suggest that M-2 suppressed Ca^{2+} transients in intact urinary bladder smooth muscle with higher potency (lower IC_{50} value) than for overexpressed muscarinic type 3 receptors in HEK-293 cells. Since we detected Ca^{2+} transients evoked by electrical stimulus in intact smooth muscle cells, the Ca^{2+} transients are related to the physiological release of neurotransmitter (ACh and ATP). Moreover, ACh-induced Ca^{2+} transients in intact urinary bladder were almost abolished by pretreatment with M-2 (3 μM), demonstrating that M-2 might also act downstream of the muscarinic receptor to inhibit the Ca^{2+} transients. We cannot rule out an additional effect on purinergic transmission. Thus, the present work supports the view that the propiverine metabolite M-2 functionally modified urinary bladder contraction predominantly through muscarinic receptors. Given the effectiveness of M-2 (at 1 μM) in inhibiting CCh-induced and field-stimulation induced contraction (Wuest et al., 2005), and its lack of action on Ca^{2+} channels, M-2 might prove to be more clinically useful than propiverine.

In conclusion, high concentrations of M-1 but not M-2 inhibited the peak amplitude of I_{Ca} in dispersed smooth muscle cells of murine urinary bladder. M-2 antagonized the CCh-induced suppression of I_{Ca} through muscarinic type 3 receptors. M-2 also suppressed electric field stimulation-induced and ACh-induced Ca^{2+} transients in intact murine urinary bladder.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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A list of non-standard abbreviations used in the paper

ACh	acetylcholine
CCh	carbachol
DMSO	dimethylsulfoxide
EFS	electrical field stimulation
I_{Ca}	voltage-dependent nifedipine-sensitive inward Ca^{2+} currents
OAB	overactive bladder
PBS	phosphate-buffered saline
PSS	physiological salt solution

TEA tetraethylammonium

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Fig. 1. Chemical structures of propiverine and two metabolites, M-1 and M-2. Propiverine (1-methyl-4-piperidyl diphenylpropoxyacetate), M-1 (1-methyl-4-piperidyl diphenylpropoxy acetate N-oxide) and M-2 (1-methyl-4-piperidyl benzilate N-oxide) are shown.

Fig. 2.

Effects of M-1 on I_{Ca} from a holding potential of -60 mV in murine urinary bladder myocytes. Whole-cell recording, pipette solution Cs^+-TEA^+ solution containing 5 mM EGTA and bath solution containing 2 mM Ca^{2+} . **A**, Original current traces before (control) and after application of 100 μ M M-1 at the indicated pulse potentials. **B**, I_{Ca} from **A** scaled to match their peak amplitudes and superimposed. **C**, Current-voltage relationships obtained in the absence (control) or presence of 100 μ M M-1. The curves were drawn by eye. **D**, Relationship between the test potential and relative value of I_{Ca} inhibited by 100 μ M M-1, expressed as a fraction of the peak amplitude of I_{Ca} evoked by a range of depolarizing pulses in the absence of M-1. Each symbol indicates the mean of 5 observations (3 different animals) with $\pm$ s.e.m. shown by vertical lines. The line was drawn by eye.

Fig. 3.

Effects of propiverine metabolites (M-1 and M-2) and nifedipine on I_{Ca} in murine detrusor myocytes. **A**, The time course of the effect of M-1 and nifedipine on the peak amplitude of I_{Ca} evoked by repetitive depolarizing pulses to 0 mV from a holding potential of -60 mV. Time 0 indicates the time when 10 μ M M-1 was applied to the bath. **B**, The time course of the effect of M-2 and nifedipine on the peak amplitude of I_{Ca} evoked by repetitive depolarizing pulses to 0 mV from -60 mV. Time 0 indicates the time when 10 μ M M-2 was applied to the bath. **C**, Relationships between relative inhibition of the peak amplitude of I_{Ca} and the concentration of propiverine and its metabolites (M-1 and M-2) in murine urinary bladder smooth muscle cells. The following parameters were obtained for each curve fit: M-1 from $V_h = -60$ mV, $K_i = 118$ μ M, $n_H = 0.9$; M-1 from $V_h = -90$ mV, $K_i = 505$ μ M, $n_H = 0.9$; propiverine from $V_h = -60$ mV, $K_i = 10$ μ M, $n_H = 1.4$. Each symbol indicates the mean of 4-10 observation with $\pm$ s.e.m. shown by vertical lines. Some of the s.e.m. bars are smaller than the symbol.

Fig. 4.

Effects of M-1 on the voltage-dependent activation and inactivation of I_{Ca} in dispersed cells from murine urinary bladder. Steady-state inactivation curves, obtained in the absence (control) and presence of M-1, were fitted to the Boltzmann equation. Peak current values were used. The steady-state inactivation curve was obtained using the double-pulse protocol (see inset). The current measured during the test pulse is plotted against membrane potential and expressed as relative amplitude. The steady-state inactivation curves in the absence or presence of M-1 were drawn using the following values: control, $I_{max} = 1.0$, $V_{half} = -36$ mV, $k = 7$ and $C = 0.05$; M-1, $100 \mu\text{M}$, $I_{max} = 1.0$, $V_{half} = -43$ mV, $k = 9$ and $C = 0.02$. Each symbol indicates the mean of 6 observations (3 different animals) with $\pm$ s.e.m. shown by vertical lines. Activation curves were obtained from the current-voltage relationships of Figure 2, fitting to the Boltzmann equation (see Methods). The activation curves in the absence or presence of M-1 were drawn using the following values: control, $I_{max} = 1.0$, $V_{half} = -14$ mV, $k = 6$ and $C = 0.01$; M-1, $100 \mu\text{M}$, $I_{max} = 1.0$, $V_{half} = -13$ mV, $k = 6$ and $C = 0.01$. Each symbol indicates the mean of 5 observations (3 different animals) with $\pm$ s.e.m. shown by vertical lines. Some of the s.e.m. bars are smaller than the symbol.

Fig. 5.

Effects of CCh on I_{Ca} in murine urinary bladder smooth muscle cells. **A**, The time course of the effects of application of CCh (10 and 100 μ M) on the peak amplitude of I_{Ca} evoked by repetitive depolarizing pulses to 0 mV from a holding potential of -60 mV. Time 0 indicates the time at which 10 μ M CCh was applied to the bath. **B**, Concentration-dependent inhibitory effects of CCh (1 μ M - 1 mM) on I_{Ca} . The effects on the CCh-induced suppression of I_{Ca} in the presence of several types of antimuscarinic agents (cyclopentolate, pirenzepine, AF-DX116 and 4-DAMP). Occasionally, GDP- β -S (1 mM) was included in the pipette solution. * Significantly different from the control ($P < 0.05$). Each column shows the mean of 5-9 observations with + s.e.m. shown by vertical lines. The peak amplitude of I_{Ca} was normalized as one. **C**, Fluorescent image of muscarinic type 3 receptor immunoactivity in murine detrusor bundles. White bar represents 100 μ m. **D**, Expanded image of the dotted white square in **C**. Clear membranous staining was observed. White bar represents 25 μ m. **E**, Negative-control: use of muscarinic type 3 receptor antibody preabsorbed with the immunizing antigen never yielded only a tissue-edge artifact. White bar represents 100 μ m. just before application of several drugs (i.e. control).

Fig. 6.

Effects of CCh on the peak amplitude of I_{Ca} in the absence and presence of M-2. Whole-cell recording, pipette solution Cs^+ -TEA $^+$ solution containing 5 mM EGTA and bath solution. **A**, The time course of the effect of CCh on the peak amplitude of I_{Ca} evoked by repetitive depolarizing pulses to 0 mV from a holding potential of -60 mV. Time 0 indicates the time when 3 μ M M-2 was applied to the bath. **B**, Original current traces before (control, (a)) and after application of M-2 (3 μ M, (b)), CCh (100 μ M (c)) as indicated in **A**. (d) indicates a current trace after removal of M-2. **C**, The effects on the CCh (10 and 100 μ M)-induced suppression of I_{Ca} in the presence of M-2 (1 and 3 μ M). Each column shows the mean of 5-6 observations with + s.e.m. shown by vertical lines. * Significantly different from the control ($P < 0.05$).

Fig. 7. Ca^{2+} transients in murine urinary bladder smooth muscle. **A**, A sample from a recording showing the response of Ca^{2+} fluorescence in one smooth muscle cell during field stimulation at 0.33 Hz. On each stimulus during this control recording the fluorescence increased, but to different amplitudes and at different rates. For the 10 responses shown these were categorized as either fast (F) or slow (S) on the basis of the amplitude of the first derivative. The fall in the fluorescent signal following some large increases may be caused by rapid movement of the smooth muscle cells out of the plane of focus following such events. **B**, Concentration-response curve to M-2.

Fig. 8.

Effects of M-2 on Ca^{2+} transients in murine urinary bladder smooth muscle. **A**, The first trace shows the fluorescent signal from a small area of a smooth muscle cell under control conditions. **B**, The second trace shows an oscillation in the fluorescent signal in the presence of ACh ($100 \mu\text{M}$); the fluorescence oscillates as waves of Ca^{2+} pass through this small region. In both cases the fluorescence traces have been high-pass filtered (0.1 Hz cutoff) to correct for a slow baseline drift. **C**, The frequency of Ca^{2+} waves under control conditions and then in the presence of ACh ($100 \mu\text{M}$). Each control and ACh exposure was separated by 5 minutes. Each test was separated by 25 minutes, with Test 2 demonstrating that ACh can still initiate waves upon re-exposure. Exposure to M-2 ($3 \mu\text{M}$) prevents this action of ACh. * Significantly different from the control ($P < 0.05$).