Possible antagonistic effects of the TRPC4 channel blocker ML204 on M2 and M3 muscarinic receptors in mouse ileal and detrusor smooth muscles and atrial myocardium

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ABSTRACT. ML204, a potent transient receptor potential canonical 4 (TRPC4) channel blocker, is often used to elucidate the involvement of TRPC4 channels in receptor-operated signaling processes in visceral smooth muscles. In the present study, we investigated the possible antagonistic actions of ML204 on M2 and M3 muscarinic receptors, which mediate contractions in mouse ileal and detrusor smooth muscles. In ileal and detrusor smooth muscle preparations, ML204 (3 or 10 µM) significantly inhibited electrical field stimulation (EFS)-evoked cholinergic contractions. However, it did not significantly inhibit high K+-induced and EFS-evoked non-cholinergic contractions in the ileal preparations. When the muscarinic agonist, carbachol was cumulatively applied, ML204 (1, 3 and 10 µM) caused a rightward parallel shift of the concentration-response curves of carbachol. Additionally, ML204 (1, 3 and 10 µM) inhibited carbachol-induced negative chronotropic response in atrial preparations, which is mediated by M2 muscarinic receptors. Furthermore, ML204 significantly inhibited the contractions evoked by carbachol-induced intracellular Ca2+ release, which is mediated by M3 muscarinic receptors. These results suggested that ML204 might exhibit antagonistic actions on M2 and M3 muscarinic receptors; in addition, the inhibitory effects of ML204 against EFS-induced cholinergic contractions might be attributed to this receptor antagonism rather than inhibition of TRPC4 channel activity. Therefore, these effects should be considered when ML204 is used as a TRPC4 channel blocker.

KEY WORDS: detrusor smooth muscle, ileal smooth muscle, 4-methyl-2-(1-piperidinyl)-quinoline, muscarinic receptor subtypes, transient receptor potential canonical channel 4
Recently, a potent and selective TRPC4 channel blocker, 4-methyl-2-(1-piperidinyl)-quinoline (ML204) has been developed. The IC₅₀ of ML204 for TRPC4 channels was 0.96 µM, whereas at concentrations of 10–20 µM, it resulted in no appreciable blockade of the transient receptor potential vaniloid 1 and 3 (TRPV1 and 3), transient receptor potential ankyrin 1 (TRPA1), transient receptor potential melastatin 8 (TRPML8), voltage-gated potassium channel subfamily Q member 2 (KCNQ2), and native voltage-gated sodium, potassium, and calcium channels [19]. ML204 (10 µM) reduced TRPC4 channel currents activated by bath application of carbachol by >85%, without affecting TRPC6 channels, in HEK 293 cells heterologously expressing TRPC channels and muscarinic receptors [19]. Griffin et al. [8] reported that ML204 significantly inhibited electrical field stimulation (EFS)-evoked and exogenously applied carbachol-induced cholinergic contractile responses in mouse detrusor smooth muscle cells and suggested that TRPC4 channels might be also involved in muscarinic receptor-mediated contractions. Thus, ML204, a potent and selective TRPC4 channel blocker, is often used to elucidate the physiological role of TRPC4 channels in native tissues. However, radioligand binding assays showed that ML204 (10 µM) also bound to G protein-coupled receptors (GPCRs), including α₁A and α₂A adrenergic receptors, histamine H₁ receptors, and M₂ muscarinic receptors [19]. If ML204 competitively antagonizes the muscarinic receptors at certain concentrations, the inhibition of muscarinic contractile responses in detrusor smooth muscle cells described by Griffin et al. [8] might be attributed to antagonism at the muscarinic receptors, but not blockade of TRPC4 channels.

Thus, in the present study, we aimed to investigate the possible antagonistic actions of ML204 on the muscarinic receptors, which mediate contractions in mouse ileal and detrusor smooth muscles. We also investigated the effects of ML204 on chronotropic responses using an atrial preparation.

## MATERIALS AND METHODS

### Animals

Male mice (ddY mice and hybrid mice of 129S4 and CF-1 strain; aged 9–17 weeks; weighing, 30–50 g) were used. The mice were housed in polycarbonate-ventilated cages in an animal room maintained at 22–25°C with a daily light/dark cycle (07:00–19:00), as previously described [26]. Food (MF; Oriental Yeast Co., Ltd., Japan) and water were supplied ad libitum. All animal procedures were performed according to the guidelines approved by the Animal Ethics Committee at the Faculty of Applied Biological Sciences, Gifu University.

### Tissue preparation

Following cervical dislocation, the whole intestine and urinary bladder were quickly excised and placed in a Petri dish filled with Tyrode’s solution (composition described below). Segments (1.5–2 cm) of the ileum, except those at distances greater than 2 cm from the ileocecal junction, were dissected. The intestinal content was flushed away by Tyrode’s solution, and the adhering tissues were cut off. Then, ileal segments were settled in an organ bath (see below). The urinary bladder was freed of the connective tissues and then cut open by longitudinal incision. The mucosa of the urinary bladder was carefully removed, and detrusor muscle strips were prepared (2–3 × 6–8 mm). The beating heart was also isolated from each animal and immersed in ice-cold bubbling Kreb’s solution (composition described below). Spontaneously beating left and right atria were dissected together from the ventricles, and their lumens were rinsed well to remove blood.

### Isometric tension recording and electrical field stimulation

Ileal segment preparations and bladder strip preparations were mounted in a 10-ml organ bath filled with Tyrode’s solution bubbled with air and kept at 30°C and 37°C, respectively, as previously described [8, 28]. The ileal tissues were subjected to a load of 0.3–0.4 g tension and incubated for 20 min, followed by a further 60 min incubation in fresh Tyrode’s solution containing guanethidine (1 µM), which blocks the adrenergic neurons, and No-nitro-L-arginine methyl ester hydrochloride (L-NAME, 100 µM), which inhibits nitric oxide synthase. These drugs were added to the bathing solution to minimize the possible inhibitory effects because of adrenergic or nitricergic nerves. After the second incubation, hypertonic 70 mM KCl was added for 1 min at intervals of 10–15 min until reproducible contractions were obtained. The detrusor smooth muscle preparations were subjected to a tension of 0.5 g and allowed to equilibrate for 60 min in fresh Tyrode’s solution. Then, they were briefly exposed to hypertonic 70 mM KCl at 10–15 min until reproducible contractions were obtained. To inhibit the purinergic components, α,β-methylene ATP (50 µM) was added to the bathing solution [7, 12]. The atrial preparations were placed vertically in a 10-ml organ bath filled with Kreb’s solution, gassed with 95% O₂ + 5% CO₂ and kept at 37°C, and allowed to equilibrate for 60 min [14].

A force-displacement transducer (T7-30-240, Orientec, Tokyo, Japan) was used to record the changes in tension isometrically, as previously described [26]. The transducer was coupled with a strain DC amplifier (AS1202, NEC, Tokyo, Japan). The output of the amplifier was displayed on an ink-writing chart recorder (U-228, Nippon Denshi Kagaku, Kyoto, Japan) and captured at a sampling rate of 1 KHz using analog-digital converter (PowerLab 8/35, ADInstruments Inc., Nagoya, Japan) interfaced to a computer (CF-S9, Panasonic, Osaka, Japan) running the Chart program version 8.0 (ADInstruments Inc.).

EFS (pulse width, 0.5 msec; strength, 50 V) was generated using a stimulator (SEN-3301, Nihon Kohden Corp., Tokyo, Japan) and delivered using a pair of platinum ring electrodes placed in parallel on either side of the strips. For the ileal preparations, EFS was applied for 5 sec at different frequencies (0.2–50 Hz). The time intervals between successive trials varied from 5–10 min since more time was required for the responses at higher stimulus frequencies to subside. Since the preparations exhibited spontaneous activity throughout the experiments, the mean peak level of the spontaneous contractions ~2 min before each EFS was considered the baseline for measurement of the contractions. After construction of a frequency-response curve as a control, ML204 was added...
for 15 min, and then EFS was applied using the same parameters. For the bladder preparations, EFS was applied for 5 sec at different frequencies (1–100 Hz), and frequency-response curves were constructed before and after application of ML204.

Carbachol response
To construct concentration-response curves, increasing concentrations of carbachol, differing by 3- or 10-fold, were applied in a cumulative manner before and after application of ML204. In some experiments, carbachol (10 µM) was applied in Ca²⁺ free Tyrode’s solution containing 140 mM KCl to induce intracellularly released Ca²⁺-mediated contractions but not Ca²⁺ influx from the extracellular matrix [26] before and after application of cyclopiazonic acid (CPA), 2-aminoethoxydiphenyl borate (2-APB) and ML204.

Data analysis
EFS- and carbachol-evoked contractile amplitudes in the ileal and detrusor smooth muscle preparations were expressed as the percentage of the reproducible 70 mM KCl-induced contractions measured in the same preparation. Data are expressed as the mean ± standard error of the mean (S.E.M.; n=the number of tissue preparations used). The Student paired t-test was performed to determine the statistical significance of differences between two groups or one-way analysis of variance (ANOVA) followed by Bonferroni’s post-hoc comparison test were used when more than two groups were compared. Differences were considered statistically significant when P<0.05. Averaged curves representing the relationships between stimulus frequencies or carbachol concentrations and contraction size were constructed by sigmoid nonlinear regression fit using GraphPad Prism version 6 (Tokyo, Japan). EC₅₀ values of carbachol were calculated from the conventional sigmoid concentration-response-curve using GraphPad Prism version 6. The pA₂ value of ML204 was obtained using Schild plot analysis. To analyze the chronotropic activity in the atrial preparations, the beating frequency was counted by LabChart 8.0 (ADInstruments Inc.).

Solutions
The solutions used had the following composition (mM): Tyrode’s solution, NaCl 136.9, KCl 2.68, CaCl₂ 1.8, MgCl₂ 2.1, NaH₂PO₄ 0.41, NaHCO₃ 11.9, and glucose 5.55; and Kreb’s solution, NaCl 118, KCl 4.75, MgSO₄ 1.2, KH₂PO₄ 1.2, CaCl₂ 2.5, NaHCO₃ 25, and glucose 11.5. To record high K⁺-induced contractions, 3.5 M KCl stock solution was prepared (without any other components) and applied hyperosmotically at the desired concentration (70 or 140 mM).

Drugs
ML204 was purchased from Focus Biomolecules (Plymouth Meeting, Pennsylvania, PA, U.S.A.), whereas atropine and carbachol were obtained from Wako (Osaka, Japan). L-NAME, guanethidine, and α,β-methylene ATP were purchased from Sigma Chemical Co. (St. Louis, MO, U.S.A.). CPA was purchased from LKT Laboratories, Inc. (St. Paul, MN, U.S.A.) and 2-APB was purchased from StressMarq Biosciences Inc. (Victoria, Canada). ML204 was dissolved in dimethyl sulfoxide (DMSO), where the final concentration of DMSO (up to 0.1%) had no effect on EFS- or carbachol-evoked contractions.

RESULTS

Effects of ML204 on EFS-induced cholinergic contractions in detrusor smooth muscle preparations
Griffin et al. [8] showed that ML204 inhibited EFS-evoked cholinergic contractions in detrusor muscle preparations and suggested that TRPC4 channels were involved in the cholinergic contractile responses. First, we confirmed these inhibitory effects of ML204 (3 µM) on cholinergic contractions in mouse detrusor smooth muscle preparations. Detrusor smooth muscle preparations were bathed in Tyrode’s solution containing α,β-methylene ATP (50 µM) to suppress purinergic contractions.

All preparations showed little spontaneous activity. EFS (50 V in strength and 0.5 msec in duration) was applied at ascending series of frequencies of 1, 2, 5, 10, 20, 50, and 100 Hz (each for 5 sec). As shown in Fig. 1A, EFS evoked rapid, phasic contractions (data not shown). EFS-induced cholinergic contractile responses were reproducible, and DMSO (0.1%), used as a solvent of ML204, itself had little effect on the mean peak amplitude in the 2nd trial of EFS. For example, the mean peak amplitudes evoked by 50 Hz EFS in the absence and presence of DMSO were 87.5 ± 11.2 and 82.5 ± 6.5% (n=6), respectively.

ML204 did not affect the amplitude of 70 mM KCl-induced contractions. The mean amplitudes of KCl-induced contractions before (control) and after ML204 treatment (3 µM) were 0.61 ± 0.14 and 0.59 ± 0.13 g (n=6), respectively, which was not a significant difference (P>0.05). However, as shown in Fig. 1A, ML204 treatment (3 µM) inhibited EFS-evoked cholinergic contractions. The peak amplitude of the cholinergic contractions evoked by 10 and 20 Hz EFS were 24.6 ± 5.1 and 82.3 ± 13.7% (n=6), respectively, which were estimated to be relative value of 70 mM KCl-induced contractions in the same preparation. ML204 significantly decreased these contractile responses to 12.5 ± 2.6 and 42.3 ± 7% (n=6), respectively. Thus, in line with the findings of Griffin et al. [8], ML204 suppressed the cholinergic contractions in detrusor smooth muscle preparations.

Effects of ML204 on EFS-induced cholinergic and non-cholinergic contractions in ileal preparations
We next examined whether ML204 could affect the cholinergic contractile responses in ileal preparations, where cholinergic
Constrictions are believed to be mediated mainly by TRPC4 channels [24]. Ileal segment preparations were bathed in Tyrode’s solution containing guanethidine (1 µM) and L-NAME (100 µM), and tension changes in their longitudinal directions were recorded.

All preparations showed spontaneous contractions, and application of ML204 did not apparently affect the spontaneous activity (Fig. 2A). The mean amplitudes of 70 mM KCl-evoked contractions before and after ML204 application (10 µM) were 0.89 ± 0.05 and 0.88 ± 0.06 g (n=9), respectively, which was not statistically significant (P>0.05).

EFS (50 V in strength and 0.5 msec in pulse duration) was applied at ascending series of frequencies of 0.2, 0.5, 1, 2, 5, 10, 20, and 50 Hz (each for 5 sec). As shown in Fig. 2A, EFS evoked fast, brief contractions followed by slower, sustained ones, where spontaneous contractions were superimposed [28]. Both the initial fast and later slow contractions increased as the stimulus frequency increased, and the maximal response was attained at 10–50 Hz for the initial contractions and at 20 or 50 Hz for the later ones. The initial fast contractions appeared immediately after the beginning of EFS, reached the peak within ~5 sec, and then rapidly declined. The later slow contractions showed not only variability in amplitude but also in duration with different stimulus frequencies or among different preparations. For instance, at 20 Hz, it reached the peak after 30–90 sec and then disappeared within 2–8 min. Application of atropine (2 µM) inhibited only the initial fast contractions (Fig. 3A). Thus, the initial fast...
ML204 ANTAGONIZES MUSCARINIC RECEPTORS

Effects of ML204 on carbachol-induced contractions in ileal preparations

ML204 was shown to exhibit binding affinities not only to TRPC4 channels but also to certain types of receptors [19]. To evaluate the antagonistic actions of ML204 at the muscarinic receptors, the effects of ML204 on the concentration-response curves of carbachol were investigated.

Cumulative application of carbachol (3 nM–10 µM) produced a concentration-dependent increase in contractions (Fig. 4A). As shown in Fig. 4B, ML204 treatment (1, 3 and 10 µM) caused a rightward parallel shift of the concentration-response curve of carbachol. The pA2 value of ML204 estimated using Schild plot analysis was 6.25. These results suggested that ML204 might not only inhibit TRPC4 channels but also competitively bind to the muscarinic receptors.

Effects of ML204 on carbachol-induced chronotropic responses in atrial preparations

To further characterize the competitive binding ability of ML204 to the muscarinic receptors, we used atrial preparations and recorded the spontaneous beating activity. In mouse atrial preparation, M2 muscarinic receptors are responsible for the negative chronotropic responses of carbachol [14], whereas there is no report describing about the involvement of TRPC4 channels in this cholinergic signal transduction.

Atria exhibited spontaneous beating activities (352 ± 32.1 beats/min; n=3). Cumulative application of carbachol at approximately 3 min intervals (10 nM–10 µM) inhibited the spontaneous beating activities in a concentration-dependent manner (Fig. 5). ML204 (1, 3 and 10 µM) significantly inhibited the negative chronotropic effects of carbachol and induced a parallel shift of the concentration-response curve of carbachol. The pA2 value of ML204 estimated using Schild plot analysis was 6.79. These results suggested that ML204 could bind to the muscarinic receptors (probably M2) to suppress muscarinic chronotropic responses.

Effects of ML204 on intracellularly released Ca2+-induced contractions in ileal and detrusor smooth muscle preparations

Activation of M3 muscarinic receptors is known to induce Ca2+ release from the intracellular stores through the Gq protein/phospholipase C/inositol 1,3,5,trisphosphate (IP3) pathway [20, 22, 23]. To examine whether ML204 could bind to M3 muscarinic receptors, we investigated the effects of ML204 on carbachol-induced contractile responses evoked by intracellularly released Ca2+ in ileal and detrusor smooth muscle preparations. The preparations were depolarized with 140 mM KCl and extracellular Ca2+ was removed. Under these conditions, carbachol (10 µM) induced phasic contractions (Fig. 6A) owing to the release of stored Ca2+ [26]. We confirmed the involvement of stored Ca2+ in the carbachol-induced phasic contraction using CPA and 2-APB, which inhibits Ca2+ uptake by sarcoplasmic reticulum resulting in its depletion of stored Ca2+ [29] and Ca2+ release by inhibiting IP3 receptors [17], respectively. In the ileal preparations, CPA (30 µM) and 2-APB (100 µM) strongly inhibited phasic contractile responses to carbachol (10 µM) by 91.1 ± 4.6% and 51.5 ± 3.5% (each n=3), respectively. Similarly, in detrusor smooth muscle preparations, CPA (30 µM) and 2-APB (100 µM) showed 90.0% and 57.5% (each n=2) of inhibition of carbachol (10 µM)-induced contractile responses. These results indicated that contractile responses to carbachol (10 µM) in the Ca2+-free, 140 mM KCl containing Tyrode’s solution were mainly mediated due to release of stored Ca2+ in the ileal and detrusor smooth muscles.

ML204 (3 µM) significantly inhibited carbachol-induced contractions in the ileal preparations (Fig. 6A). The mean peak amplitude of carbachol-induced phasic contractions was reduced from 18.2 ± 3.6 to 1.2 ± 0.4% (n=5) after ML204 (3 µM) treatment (Fig. 6B). Similar results were obtained in the detrusor muscle preparations. The mean peak amplitude of carbachol-induced phasic contractions was reduced from 17.2 ± 7.5 to 4.2 ± 2% (n=4) after ML204 (3 µM) treatment in the detrusor muscle preparations. These results suggested that ML204 could bind to the M3 subtype of muscarinic receptors to suppress Ca2+ release.

DISCUSSION

ML204 has been used as an excellent tool for the investigation of TRPC4 channels to reveal their physiological and pathophysiological roles underlying the receptor-operated signal transduction, including muscarinic receptor-related transduction. In detrusor smooth muscle tissues, involvement of TRPC4 channels in muscarinic contractions has been suggested in mechanical studies using ML204 [8]. However, a radioligand binding assay of 68 GPCRs, ion channels, and transporters showed that ML204 (10 µM) significantly interacted with seven of the 68 assays (with the criterion of no inhibition of radioligand binding greater than 50% at 10 µM), namely adrenergic (α1A and α2A), histamine (H1), imidazole I2, muscarinic M2, nicotinic, and sigma (σ1) receptors.
These results prompted us to explore the effects of ML204 on muscarinic receptor subtypes by functional assays. Therefore, we examined the effects of ML204 on the contractile responses mediated via stimulation of M2 and M3 muscarinic receptors in ileal, detrusor muscle, and atrial preparations.

Although a previous study showed that ML204 bound to M2 receptor using a radioligand binding assay [19], whether ML204 could functionally inhibit cholinergic responses by receptor antagonism has not been investigated yet. In the present study, ML204 inhibited carbachol-induced contractions that resulted in a shift of its concentration-response curve to the right direction. The pA2 value of ML204 estimated from the parallel shift of carbachol-induced concentration-response curves was 6.25 in...
ML204 antagonizes muscarinic receptors

The ileal preparations. ML204 also inhibited the negative chronotropic effects of carbachol and produced a parallel shift of the concentration-response curve of carbachol in the atrial preparations. The estimated pA₂ value of ML204 was 6.79, which was comparable to that estimated in ileal preparations. The negative chronotropic response has been shown to be mediated by the M₂ receptor using M₂ receptor knockout mice [14]. Therefore, ML204 might competitively bind to the M₂ receptor to inhibit the muscarinic negative chronotropic responses. Thus, the pA₂ values estimated in the present study might be overlapped with a range of the inhibitory concentrations of ML204 (IC₅₀ for TRPC4 channels, 0.96 µM; [19]). It has been proposed that binding of ML204 to the muscarinic receptors is unlikely to account for its inhibitory effects against muscarinic receptor-activated cationic current in smooth muscle myocytes freshly isolated from guinea pig ileum [19] and cholinergic contractions in mouse ileum [8]. However, we could not exclude the possibility that the inhibitory effects of ML204 against the muscarinic cationic channel opening and

Fig. 5. Effects of ML204 on carbachol-induced chronotropic responses in atrial preparations. (A) Representative recording showing the effects of carbachol on spontaneous atrial beating before (upper trace) and after (lower trace) the application of ML204 (3 µM). Carbachol was applied in a cumulative manner (10⁻⁷–10⁻³ M) as indicated by closed triangles. ML204 suppressed the inhibitory effects of carbachol against spontaneous heart beating, in terms of frequency and amplitude. (B) Summarized data showing the inhibitory effects of ML204 (1, 3 and 10 µM) against carbachol effects on spontaneous heart beating frequency. Each point represents the mean ± S.E.M. (n=8 for control, 5 for ML204 (1 µM), 6 for ML204 (3 µM) and 3 for ML204 (10 µM)). *Significantly different (P<0.05) between group means evaluated by one-way ANOVA followed by a post-hoc Bonferroni test.

Fig. 6. Effects of ML204 (3 µM) on carbachol-induced intracellularly released Ca²⁺-evoked contractions in ileal preparations. (A) Representative recording of carbachol-induced contractions before (left trace) and after (right trace) the application of ML204 (3 µM) in ileal preparations bathed in 140 mM KCl with removal of extracellular Ca²⁺. (B) Summarized data showing the inhibitory effects of ML204 (3 µM) against intracellularly released Ca²⁺-evoked contractile responses. The amplitude of the contractions was expressed as percentage of the 70 mM KCl-evoked contraction in the same preparation. Each column represents the mean ± S.E.M. (n=5). *Significantly different (P<0.05) from the corresponding control values.
cholinergic contractions might be, at least partly, attributed to its competitive binding to the muscarinic receptors.

Strikingly, in the experiments using ileal preparations, depolarized using 140 mM KCl with removal of extracellular Ca^{2+}, carbachol-induced contractions were inhibited by ML204 (3 \mu M). Depolarization with 140 mM KCl is so extreme that the membrane potential could reach approximately 0 mV, which is close to the equilibrium potential of the cationic channels and is sufficient for depolarization block to cease spike discharge [1, 25]. Because of the absence of extracellular Ca^{2+}, carbachol-induced contractions might not involve Ca^{2+} influx from the extracellular stores into the cytosol of smooth muscle cells. Thus, carbachol-induced contractions in the presence of 140 mM KCl and absence of extracellular Ca^{2+} might be attributed to Ca^{2+} release from intracellular Ca^{2+} stores [15, 16]. Therefore, TRPC4 channels did not mediate the inhibitory effects of ML204 (3 \mu M) against intracellularly released Ca^{2+}-induced contractions induced by carbachol. We previously showed that intracellularly released Ca^{2+}-induced contractions were mediated by only M_{3} receptors in the mouse ileum [20, 22]. In the detrusor smooth muscles, it was reported that ML204 did not affect L-type Ca^{2+} or big conductance Ca^{2+}-activated potassium (BK) channel current amplitudes, caffeine-induced Ca^{2+} transients, and KCl-induced contractions [8]. These results suggested that binding of ML204 to M_{3} receptors might be involved in its inhibitory effects against muscle contractions induced by the release of stored Ca^{2+}.

It has been shown that cholinergic contractions and muscarinic cationic channel opening are strongly inhibited in TRPC4-deficient mice [24]. In ileal smooth muscle cells, guanosine 5′-O-(gamma-thio)triphosphate (GTPγS)-induced cation currents, which do not involve muscarinic receptors, were largely reduced by ML204 [19]. Therefore, TRPC4 channels are believed to essentially contribute to cholinergic contractions of the ileal smooth muscles, and their knockout and knockdown approaches [6, 31] are powerful tools to investigate the roles of TRPC4 channels. However, it has also been reported that gene-deficient approach is not necessarily a suitable model [18]. For instance, the phenotype of caveolin-1-deficient mice is attributed to a developmental defect, rather than to a direct role of caveolae in signaling cascades [30]. Therefore, more selective blockers of TRPC4 channels are still needed to reveal their physiological roles.

Recently, angiotensin II-dependent stimulation of Ca^{2+} flux in the podocytes has been shown to be inhibited by non-specific TRPC channel blockers (SAR7334, SKF96365, clemizole hydrochloride, and La^{3+}), but not by 20 \mu M ML204 [11]. These results suggested that ML204 might not block angiotensin II receptor-mediated responses, and that TRPC4 channels might not contribute to these responses. We also showed that non-cholinergic contractions in ileal preparations were not inhibited by ML204. Moreover, Miller et al. [19] reported that ML204 inhibited current responses mediated by GTPγS-activated TRPC4 channels, which bypass receptor stimulation, in a single ileal smooth muscle cell. In the detrusor smooth muscles, several potential nonselective actions of ML204, such as inhibition of L-type Ca^{2+} channels, BK channels, and Ca^{2+} release pathways, were ruled out [8]. Taken together, ML204 could be used as a potent TRPC4 channel blocker, but not in the case of cholinergic responses.

ML204 was shown to bind to not only the cholinergic receptors (M_{2}) but also adrenergic (\alpha_{1A} and \alpha_{2A}) and histamine (H_{1}) receptors with the criterion of no inhibition of radioligand binding greater than 50% at 10 \mu M [19]. Miller et al. [19] proposed that the functional selectivity of ML204 might differ from the results of the binding study. Thus, functional profiling of ML204 binding to other receptors other than the muscarinic receptors (M_{2} and M_{3}) is not fully understood. Thus, further studies are needed to investigate the selectivity of ML204 to GPCRs, including adrenergic (\alpha_{1A} and \alpha_{2A}) and histamine (H_{1}) receptors, in mediating biological responses.

In conclusion, our findings provided evidence that the inhibitory effects of ML204 against cholinergic contractions were likely to be mediated by its binding to not only TRPC4 channels but also to muscarinic receptors. Therefore, the possible antagonistic effects of ML204 on receptors should be taken into consideration when used as a TRPC4 channel blocker.

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