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Effects of Arecoline on Colonic Motility in Male Rats

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Arecoline is an important component of the Chinese herbal medicine areca, the purpose of this study is to examine the effects and mechanisms of the action of arecoline on the contractile activity of rat colon. Strips of longitudinal muscle from the distal colon were suspended in organ baths containing Krebs solution. Isometric contraction of colonic smooth muscle in the presence of arecoline was measured. The effects of tetrodotoxin, atropine, 1, 1-dimethyl-4-diphenylacetoxypiperidiniumiodide (4-DAMP), nifedipine and Ca^{2+}-free Krebs solution on arecoline-induced responses were also assessed. The results showed that arecoline (0.1-100 μM) enhanced the contraction of the longitudinal smooth muscle of rat colon in a dose-dependent manner. Pre-treatment with the Na^+ channel blocker tetrodotoxin (1 μM) did not block the effect of arecoline. Muscarinic (M) receptor antagonist atropine (10 μM) and M_3 receptor antagonist 4-DAMP (1 nM-1 μM) blockaded the arecoline-induced contraction. L-type Ca^{2+} channel antagonist nifedipine (1 μM) and Ca^{2+}-free (0.1 mM EGTA) Krebs solution abolished the arecoline-induced contraction. These results suggest that arecoline increases colonic motility *via* the M_3 receptor, which depends on the influx of Ca^{2+}.

Key Words: arecoline, colon, muscarinic receptor, intestinal motility.

Abbreviations: 4-DAMP, 1, 1-dimethyl-4-diphenylacetoxy-piperidiniumiodide, AC, adenylyl cyclase, PLC, phospholipare C, TTX, tetradotoxin, LM, longitndinal muscle.

Introduction

Areca (*Areca catechu* L., a Chinese herbal medicine) had been used to treat abdominal distention and constipation[1]. Our previous study showed that areca stimulates the colonic smooth muscle strips in rats[2]. Arecoline is a major betel-nut alkaloid and one of the most important and effective extractions from areca[3, 4]. The pharmaceutical significance of areca is probably due to the presence of arecoline. Arecoline had been found to enhance the contraction of the guinea pig ileum, the gastrointestinal transit of the mouse and the colonic smooth muscle strips of rabbits[5, 6].

Arecoline has been used as non-selective muscarinic (M) acetylcholine receptor agonist[7, 8]. Smooth-muscle contracting in the gastrointestinal tract is mediated primarily by muscarinic receptors. Muscarinic receptors have been pharmacologically classified into five subtypes, M_1-M_5[9]. All subtypes of muscarinic receptor have been identified in circular smooth muscle cells of guinea pig gastric antrum[10]. Although only several subtypes of muscarinic receptors have been reported to be present in colonic smooth muscle, M_1 muscarinic receptors are reported to be involved in synaptic transmission within intramural plexuses to colonic smooth muscle[11]. Both M_2 and M_3 receptors have been reported to be expressed in rat colon smooth muscle cells[12, 13]. Therefore, arecoline might enhance the colonic contraction *via* M receptors on the smooth muscle cells either directly or indirectly.

It is well known that although the M_2 receptor subtype is abundant in most smooth muscles, the M_3 receptor subtype is the dominant mediator of the contractile response[14, 15]. After binding with an agonist, M_2 and M_3 receptors inhibit adenylyl cyclase (AC) and activate phospholipase C (PLC) [16-18]. Activation of the M_3/PLC/IP_3 system induces the release of stored intracellular Ca^{2+}. M_2 (or M_3) receptor/G_o activation (or M_2-G_i/G_o) opens the muscarinic receptor-gated cationic channel. The influx of cationic ions depolarizes the membrane of smooth muscle and then opens the L-type Ca^{2+} channels. All these process work together to raise the intracellular free Ca^{2+} concentration and then to initiate the contraction of smooth muscle[19-22]. It would be interesting to further investigate the role of Ca^{2+} in arecoline-induced contraction of colonic smooth muscle strips. The purpose of this study was to explore (1) the direct effect of arecoline on the colonic smooth muscle in male rats, (2) the involvement of M receptor and selective M receptor subtype in the action of arecoline on the colon, and (3) the role of Ca^{2+} in the action of arecoline on rat colon.

Materials and Methods

Animals

Male Sprague-Dawley rats (200-250 g) were housed in a temperature (22 ± 1°C) and light (6 a.m.-8 p.m.)-controlled

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environment. The use and treatment of animals was approved by National Yang-Ming University Animal Care and Use Committee. All animals received care in compliance with the Principles of Laboratory Animal Care and the Guide for the Care and Use of Laboratory Animals, published by the National Science Council, Taiwan, ROC.

Tissue Bath Studies

The rats were fasted for 24-hour and decapitated. A segment of the distal colon (5 cm proximal to the anus) was quickly removed and was opened along the mesentery. Longitudinal muscle (LM) strips were prepared as previously described[23]. In brief, muscle strips (8 × 2 mm) were cut parallel to the longitudinal fibers and the mucosa on each strip was carefully removed. The muscle strip was suspended in a tissue chamber containing 5 ml Krebs solution (37°C) and bubbled continuously with 95% O_2 and 5% CO_2. One end of the strip was fixed to a hook on the bottom of the chamber. The other end was connected to an external isometric force transducer and placed in a 5-ml thermostatically controlled (37°C) organ bath. The composition (in mM) of the Krebs solution was used: NaCl 119, KCl 4.75, KH_2PO_4 1.2, $NaHCO_3$ 25, $MgSO_4$ 1.5, $CaCl_2$ 2.5 and glucose 11.

Spontaneous contractile activity of colonic strips (under an initial tension of 1 g) was simultaneously recorded on ink-writing recorders. All experiments started after a minimal 60-min equilibration period. Dose-response curves of the arecoline on distal colon were obtained by means of application at ascending concentrations spaced by four- or ten-fold. The contraction-response curves were also measured at 10-30 min intervals in the presence of the antagonist.

Effect of Arecoline on Distal Colonic Motility

The distal colonic strips were stabilized for 60 min. Preliminary experiments showed that arecoline produced its maximal effect within 3 min. Arecoline (0.1, 0.4, 1, 10 and 100 μM) dose-response curves were constructed by applying concentrations at 3-min intervals. Three min after the administration of the highest concentration of arecoline, atropine (10 μM) was added to investigate the role of M receptors in mediating to the effects of arecoline on the colonic muscle contraction.

Effect of Tetrodotoxin (TTX) on Arecoline-Induced Colonic Contractions

The effect of TTX (1 μM), a selective Na^+ channel blocker, on the arecoline-induced response was examined. Thirty min after the administration of TTX, arecoline (0.1-100 μM) was added to tissue chamber at ascending concentrations. The effect of TTX (1 μM) on the action of veratridine (a Na^+-channel activator, 10 μM) was also examined.

Effects of Nicotinic Receptor Antagonist and Muscarinic Receptor Antagonist on Arecoline-Induced Contraction

To determine if arecoline acts on the colon *via* nicotinic or muscarinic receptors, the effects of nicotinic receptor antagonist hexamethonium (0.1 mM) and muscarinic receptor antagonist atropine on arecoline-induced response were examined. After a stable level of spontaneous contraction had been recorded, muscle strip motility was observed in the presence of hexamethonium or atropine. Arecoline was added 10 min after addition of antagonists.

To determine if arecoline acts on the colon *via* M_3 receptor, the effects of preferring M_3 receptor antagonist, 4-DAMP (1 nM-1 μM) on arecoline-induced response were examined. After a stable level had been recorded, spontaneous motility was observed in the presence of 4-DAMP. Arecoline (0.1-100 μM) was added 10 min after addition of 4-DAMP.

Effect of Ca^{2+}-Free Krebs Solution and Nifidipine on Arecoline-Induced Colonic Contractions

To determine if the effect of arecoline on colonic contractions depend on extracellular Ca^{2+}, the normal bathing solution was replaced by a Ca^{2+}-free Krebs solution. After stability had been achieved, spontaneous motility was observed in the Ca^{2+}-free Krebs solution. Arecoline (0.1-100 μM) was added 10 min after addition of Ca^{2+}-free Krebs solution.

To determine if the effect of arecoline on colonic contractions depend on L-type Ca^{2+} channel, the effects of L-type Ca^{2+} channel blocker, nifidipine (1 μM) were examined. After stable level had been recorded, spontaneous motility was observed in the presence of nifidipine. Arecoline (0.1-100 μM) was added 10 min after addition of nifidipine.

Drugs

The following drugs were used: arecoline, atropine sulfate, dimethyl sulfoxide (DMSO), 4-diphenylacetoxy-N-methylpiperidine-methiodide (4-DAMP), nifedipine, ethylene glycol-bis (β-aminoethyl ether)-N, N, N', N'-tetraacetic acid (EGTA), tetrodotoxin (TTX), hexamethonium, and veratridine. All the chemicals were purchased from Sigma Co. Ltd. (St. Louis, MO, USA). Atropine sulfate was first dissolved in 75% ethanol, and then diluted with deionized water. Nifedipine was first dissolved in DMSO, and then diluted with deionized water. Other chemicals were dissolved in the deionized water. The control experiments performed in the presence of solvent alone demonstrated that the solvent did not affect the contractile response of colonic smooth muscle strips.

Statistical Analysis

Contraction responses were expressed as a percentage of the maximal area under contraction curve produced by arecoline (100 μM). The agonist concentration-response curve was analyzed using statistic software (Prism, Graphpad, La Jolla, CA, USA) that fit the data directly with a logistic function, providing the EC_{50} value (the concentration required for an agonist to produce a half-maximal response), the maximum response (E_{max}), and a slope factor for the curve (Hill slope). Data are presented as mean ± S.E.M., with n indicating the number of rats. Student's unpaired t test was used to determine the statistical significance of differences between two groups and one-way analysis of variance

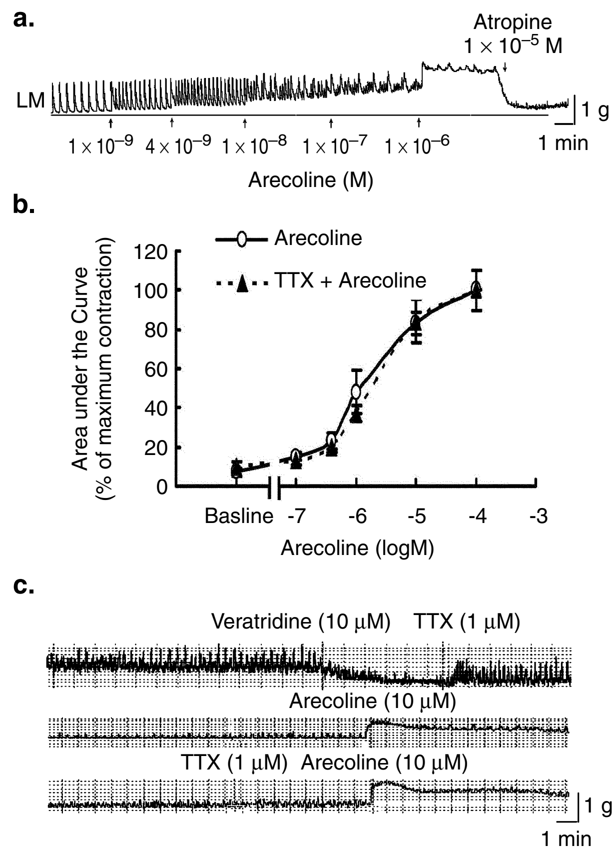

Figure 1. Dose-dependent response effect of arecoline (0.1-100 μM) on the contraction of longitudinal muscle (LM) in rat distal colon. (a) Representative of the recordings of colonic motility. The upward arrows denote the addition of arecoline, and the downward arrow indicates the addition of atropine. (b) Dose-dependent response curves induced by arecoline on colonic contraction and effect of tetrodotoxin (TTX, 1 μM) on arecoline-induced contraction of colonic LM. Each point represents the mean ± S.E.M. as a percentage of maximum contraction. Maximum contraction value is the area under the contraction curve produced by arecoline (100 μM). Baseline equals the area under the contraction curve before drug administration. (c) The effects of veratridine and TTX on arecoline-induced contraction in colonic smooth muscle strips of rats.

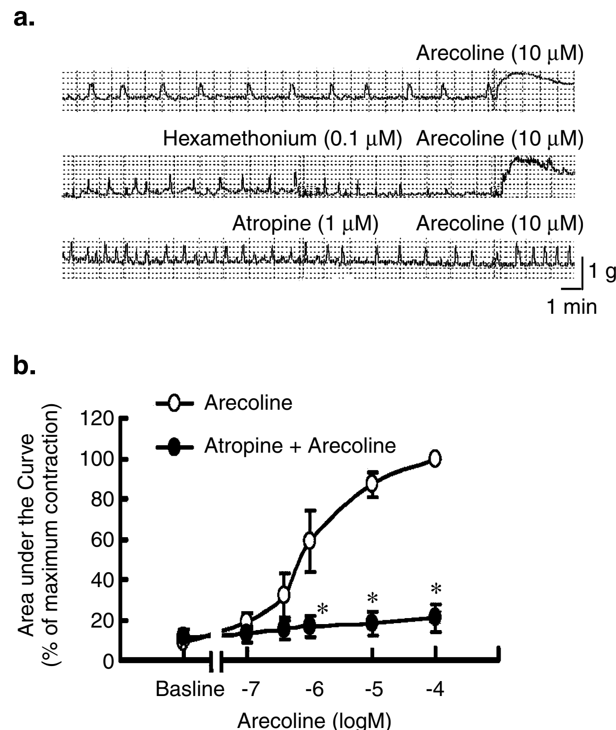

Figure 2. (a) The effects of nicotinic receptor antagonist hexamethonium and muscarinic receptor antagonist atropine on arecoline-induced contraction in colonic smooth muscle strips of rats. (b) The effects of nonselective muscarinic antagonist atropine (1 μM) on arecoline-induced contraction of the longitudinal muscle (LM) of distal colon. Each point indicates the mean ± S.E.M. of percentage of maximum contraction ($n = 6$). Baseline equals the area under contraction curve before drug administration. *$P < 0.05$ as compared to the arecoline treated group.

(ANOVA) followed by Dunnett's multiple range test was used to compare multiple groups. A probability level of $P < 0.05$ was considered to be significant.

Results

Effects of Arecoline on Colonic Motility in Strips

Arecoline (1 nM-1 μM) dose-dependently stimulated the contraction of LM of distal colonic smooth muscle strip (Fig. 1a). Maximum contraction of distal colon was achieved at an arecoline dose of 100 μM (Fig. 1b). The EC_{50} value of arecoline was 1.60 ± 0.25 μM, the 95% confidence interval of the EC_{50} value was 0.4 - 6 μM, and the E_{max} value for arecoline was 101.8 ± 6.9%. After arecoline induced the contraction, atropine (10 μM) was added, E_{max} value for arecoline decreased from 101.8 ± 6.9% to 6 ± 1% ($P < 0.05$). The dose-dependent response of the arecoline on colonic strips is shown in Fig. 1b.

Effects of TTX on Arecoline-Induced Contraction

TTX (1 μM) partially reversed the inhibitory response of veratridine (10 μM) (Fig. 1c). But TTX (1 μM) had no effect on rat distal colonic spontaneous contraction. The E_{max} and Hill slope values of arecoline in the presence and absence of TTX were 101.2 ± 4.6% and 1.0 ± 0.2%, 101.8 ± 6.9% and 0.9 ± 0.2%, respectively, which had no difference (also see Fig. 1b, $P > 0.05$).

Effects of Nicotinic Receptor Antagonist and Muscarinic Receptor Antagonist on Arecoline-Induced Contraction

The results showed that atropine but not hexamethonium blocked the arecoline-induced contraction in colonic smooth muscle strips of rats (Fig. 2a). Atropine (1 μM) alone had no effect on the spontaneous contraction of rat distal colon. The effect of arecoline (0.1-100 μM) on colonic contraction was blocked when atropine was given 10 min before the

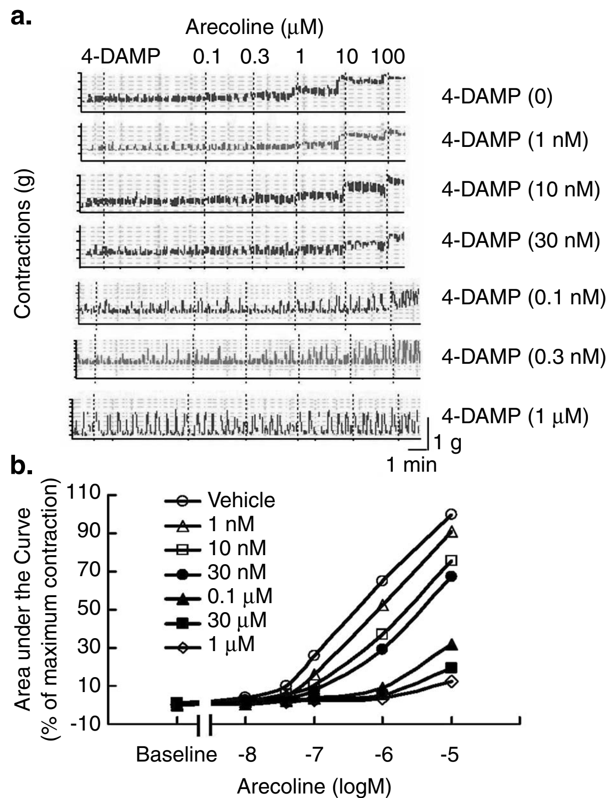

Figure 3. Dose-dependent effect of M_3 receptor antagonist 4-diphenylacetoxy-N-methylpiperidine-methiodide (4-DAMP, 1 nM - 1 μM) on arecoline-induced contraction of longitudinal muscle (LM) of rat distal colon.

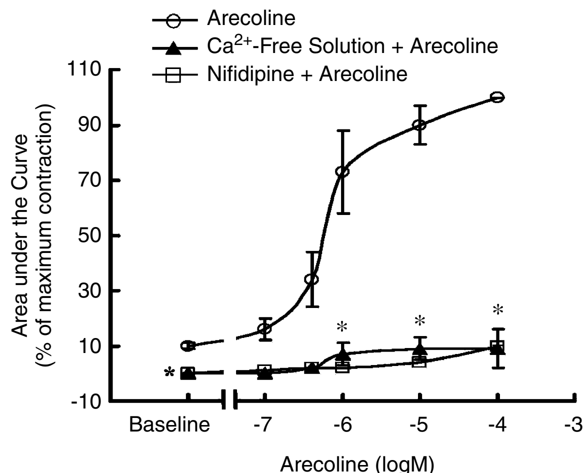

Figure 4. Effects of Ca^{2+}-free Krebs solution and nifedipine (1 μM) on arecoline-induced contractions of longitudinal muscle (LM) of distal colon in rats. Each point indicates the mean ± S.E.M. of percentage of control (n = 6). Baseline means the area under contraction curve before drug administration. $^*P < 0.05$ as compared to the arecoline-treated group.

administration of arecoline (Fig. 2, a and b, the E_{max} value decreased from 99.3 ± 5.6% to 24.8 ± 3.9%, $P < 0.05$).

In the presence of 1 μM 4-DAMP, a preferring M_3 receptor antagonist, arecoline (0.1-100 μM) contraction curve shifted to the right and the E_{max} value decreased from 99.3 ± 5.6% to 47.3 ± 9.8% (Fig. 3b, $P < 0.05$).

The dose-response curves for arecoline (0.1-100 μM) in the presence of several concentrations (1 nM-1 μM) of 4-DAMP showed that 4-DAMP served as a competitive antagonist within the concentration range of 1-30 nM (Fig. 3, a and b). 4-DAMP produced a rightward shift of the arecoline's curve in a concentration-dependent manner without a significant change in E_{max} or Hill slope (At 1, 10, 30 nM, the value of E_{max} and Hill slope are 105.6 ± 2.8%, 111.3 ± 6.1%, 111.4 ± 4.8% and 0.78 ± 0.09, 0.76 ± 0.05, 0.75 ± 0.05, respectively).

Effects of Ca^{2+}-Free Krebs Solution and Nifedipine on Arecoline-Induced Contraction

When Ca^{2+}-free Krebs solution with EGTA was employed, the spontaneous contraction (*i.e.*, in the absence of arecoline) decreased from 12.8 ± 3.0% to 0.8 ± 0.3% ($P < 0.05$). After the strip was equilibrated with Ca^{2+}-free Krebs solution for 10 min, arecoline was applied at ascending concentrations. When 1 μM of arecoline was employed, the contraction of the distal colon increased slightly from 0.8 ± 0.3% to 2.0 ± 0.3% ($P < 0.05$). At dose of 100 μM, arecoline in Ca^{2+}-free Krebs solution only increased contraction to 9 ± 7%, which was similar to the contraction in normal Krebs solution (12.8 ± 3.0%, $P > 0.05$). The E_{max} for arecoline in Ca^{2+}-free Krebs solution decreased greatly than that in normal Krebs solution (Fig. 4, E_{max} decreased from 95.4 ± 5.9% to 9.01 ± 0.02%, $P < 0.05$).

Nifedipine (1 μM) decreased the spontaneous contraction of LM in rat distal colon from 7 ± 1% to 0.8 ± 0.4% ($P < 0.05$). After addition of nifedipine for 10 min, arecoline was applied at ascending concentrations. Administration of arecoline increased the contraction of the distal colon significantly (0.8 ± 0.4% to 10 ± 2%, $P < 0.05$). The response of arecoline in the presence of nifedipine (100 μM, 10 ± 2%) was lower than that in the absence of nifedipine (Fig. 4, $P < 0.05$).

Discussion

We have demonstrated that arecoline dose-dependently stimulated the contraction of colonic smooth muscle in rats. Since arecoline is one of the most important and effective extractions from areca, it is likely that the stimulation of areca on rat colonic smooth muscle is primarily attributable to the action of arecoline. These results also indicated that the relief of abdominal distention and the constipation after the treatments of areca is likely caused by arecoline.

A variety of neurons in gastrointestinal smooth muscle layers express presynaptic muscarinic receptors which serve to modulate the neuronal firing rate and the neurotransmitters releasing. Muscarinic agonists cause a TTX-sensitive increase in spontaneous acetylcholine release from myenteric neurons[24]. To testify the activity of TTX, veratridine, a Na^+ channel activator was used. Veratridine decreased the

spontaneous contraction of colonic smooth muscle and TTX partially reversed the inhibitory response of veratridine. The results proved that TTX was indeed active. In our studies, TTX did not block the arecoline-induced contractions. The results suggested that the action of arecoline is TTX-independent and arecoline probably could act on the rat colonic smooth muscle directly.

The nonselective muscarinic receptor antagonist atropine completely blocked the arecoline-induced contractions suggesting that arecoline might act on rat colonic smooth muscle *via* a muscarinic receptor. The muscarinic M_3 receptor antagonist (4-DAMP) blocked the arecoline-induced contraction suggesting that arecoline stimulated the contraction of distal colonic smooth muscle in rats *via* muscarinic M_3 receptor.

The involvement of extracellular Ca^{2+} influx in the arecoline-induced contraction had been explored. When the normal Krebs solution is replaced by substituted with the Ca^{2+}-free Krebs solution, the arecoline-induced contraction decreased completely. We found that the L type Ca^{2+} channel blocker nifedipine significantly reduced the arecoline-induced contraction. The results have shown that the arecoline-induced colonic contraction depends on influx of extracellular Ca^{2+}.

It has been well known that muscarinic M_3 receptor causes the contraction of smooth muscles *via* Gq type of GTP-binding protein-phospholipase C-IP_3-Ca^{2+} stores release pathway[8]. Muscarinic M_3 receptor antagonists have been reported to suppress the muscarinic cationic current response of guinea-pig ileal cells[25]. M_3 receptor generates or modulates cationic channel opening through Ca^{2+} store release pathways, including Ca^{2+}/calmodulin/myosin light-chain kinase cascade[26, 27], protein kinase C[28, 29] and tyrosine kinases[30]. Opening of cationic channels activates L type Ca^{2+} channels, which causes the copious influx of Ca^{2+}. M_3 receptors might mediate the arecoline-induced contractions *via* Ca^{2+} stores release-cationic channels activation-extracellular Ca^{2+} influx pathway.

Both M_2 and M_3 muscarinic receptors subtypes are co-expressed in gastrointestinal smooth muscles with the preponderance of the M_2 subtype. Cationic channel opening is mediated by muscarinic M_2 receptors[31] and potentiated by muscarinic M_3 receptors through Ca^{2+} store release[32] or by direct interaction with muscarinic M_2 receptors[33, 34].

In conclusion, the present study demonstrates that arecoline stimulates the distal colonic spontaneous contraction in rats *via* M_3 receptor, and the M_3 receptors mediate the arecoline-induced colonic contraction *via* extracellular Ca^{2+} influx.

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