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Intracellular calcium was involved in muscarinic currents increased by hypoosmotic membrane stretch in gastric myocytes of guinea pig[1]

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KEY WORDS muscarinic receptors; pyloric antrum; membrane stretch; hypotonic solusions; calcium; carbachol; patch-clamp techniques

ABSTRACT

AIM: To investigate the role of intracellular calcium in muscarinic currents increased by hypoosmotic membrane stretch in gastric antral circular myocytes of the guinea pig. **METHODS:** The whole cell patch-clamp technique was used, and the myocytes were isolated by collagenase. Cells were swelled by hypoosmotic solution (200 Osmmol/kg). **RESULTS:** The hypoosmotic membrane stretch markedly increased carbachol-induced muscarinic currents (I_{CCh}). The I_{CCh} and the increase of I_{CCh} were completely blocked by quinidine 3 μmol/L, a specific muscarinic current blocker. In external calcium-free solution hypoosmotic membrane stretch could not increase I_{CCh}, but in the presence of nicadipine 5 μmol/L, a L-type calcium channel blocker or gadolinium chloride 100 nmol/L, a stretch-activated cation channel blocker, the I_{CCh} was still increased by hypoosmotic membrane stretch. When both nicardipine and gadolinium chloride were added into external solution, I_{CCh} were not increased by hypoosmotic membrane stretch any more. Ryanodine, a calcium-induced calcium release (CICR) agonist completely blocked hypoosmotic membrane stretch-induced increase of I_{CCh}. **CONCLUSION:** Hypoosmotic membrane stretch increased I_{CCh} and the increment was related to influx of external calcium and CICR.

INTRODUCTION

In smooth muscles of various visceral organs, cell membrane stretch is an important physiological stimulus. It has been reported that stretching smooth muscle caused depolarization of cell membrane, increased the frequency of action potentials, and subsequent contraction[1-5]. The effect of membrane stretch on smooth muscle is mediated by activating or modulating ionic channels. Membrane stretch activates stretch-activated nonselective cation channel and causes sodium and calcium ions influx so that membrane potential depolarized[6]. It is interesting to note that stretch of cell membrane by either direct application of positive pressure through a pipette or osmotic swelling increased the voltage-gated inward calcium current in the rat basilar arterial myocytes under conventional and perforated whole-cell patch clamp condition[7]. Matsuda *et al* also reported that osmotic cell swelling and cell inflation caused by applying positive pressure reversibly increased L-type calcium current in rabbit cardiac myocytes[8].

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Our previous study also demonstrated that hypoosmotic membrane stretch significantly increased L-type calcium current and the increase was mediated by actin microfilament[9,10]. Gastrointestinal motility is regulated by neural, humoral, and myogenic mechanisms[11]. Muscarinic stimulation activates nonselective cation conductance in isolated smooth muscle cells[12,13]. Nowadays the relationship between muscarinic current and membrane stretch is seldom reported. Wanish *et al* reported that reduced extracellular tonicity preferentially increased the muscarinic receptor-activated inward current in guinea pig ileal smooth muscle[14].

In present study, we observed the effect of hypoosmotic membrane stretch on I_{CCh} and investigated the role of intracellular calcium in antral gastric smooth muscle of guinea pig.

MATERIALS AND METHODS

Preparation of cells EWG/B guinea pigs (obtained from the Experimental Animal Department of Norman Bethune University, Certificate No. 10-6004) of either sex, weighing 250-350 g, were euthanized by lethal dose of intravenous pentobarbital sodium (50 mg/kg). The antral part of the stomach was rapidly cut. First the muscosal layer was separated from the muscle layer, dissected from the longitudinal layer using fine scissors, and cut into small segments (1 mm×4 mm). These segments were kept in a modified Kraft-Bruhe (K-B) medium at 4 °C for 15 min. Next they were incubated at 36 °C in 4 mL of digestion medium [Ca-free Physiological Salt Solution (Ca-free PSS)] containing 0.1 % collagenase (II), 0.1 % dithioerythreitol, 0.15 % trypsin inhibitor, and 0.2 % bovine serum albumin for 25-35 min. Then, the softened muscle segments were transferred into the modified K-B medium, and cells were individually dispersed by gentle trituration with a wide-bore fire-polished glass pipette. Isolated gastric myocytes were kept in modified K-B medium at 4 °C until use.

Electrophysiologic recording Isolated cells were transferred to a small chamber (0.1 mL) on the stage of an inverted microscope (IX-70 Olympus, Japan) for 10-15 min to settle down. The cells were continuously superfused with isosmotic PSS by gravity (0.9-1.0 mL/min). An 8-channel perfusion system (L/M-sps-8, List Electronics, Germany) was used to change solution. Experiments were performed at 20-25 °C and the whole-cell configuration of the patch-clamp technique was used. Patch-clamp pipettes were manufactured from borosilicate glass capillaries (GC 150T-7.5, Clark Electromedical Instruments, UK) using a two-stage puller (PP-83, Narishige, Japan). The resistance of the patch pipette was 3-5 MΩ, when filled with pipette solution. Liquid junction potentials were canceled prior to seal formation. Whole-cell currents were recorded with an Axopatch 1-D patch-clamp amplifier (Axon Instrument, USA) and command pulses were applied by using the IBM-compatible 486-grade computer and pCLAMP software (Version 6.02).

Drugs and solutions All drugs were purchased from Sigma Chemical Co USA. Tyrode's solution contained NaCl 147, KCl 4, $MgCl_2 \cdot 6H_2O$ 1.05, $CaCl_2 \cdot 2H_2O$ 0.42, $Na_2HPO_4 \cdot 2H_2O$ 1.81, glucose 5.5 mmol/L, and its pH was adjusted to 7.35 with NaOH. The pH of Ca-free solution containing NaCl 134.8, KCl 4.5, glucose 5 and HEPES 10 mmol/L, was adjusted to 7.4 with TRIZMA BASE (Tris). The isosmotic solution (290 Osmmol/kg) contained NaCl 80, KCl 4.5, $MgCl_2 \cdot 6H_2O$ 1, $CaCl_2 \cdot 2H_2O$ 2, glucose 5, HEPES 10, sucrose 110 mmol/L, and its pH was adjusted to 7.4 with Tris. The isosmotic solution for recording carbachol current contained CsCl 85, $MgCl_2 \cdot 6H_2O$ 1, $CaCl_2 \cdot 2H_2O$ 2, glucose 5, HEPES 10, sucrose 110 mmol/L, and its pH was adjusted to 7.4 with Tris. Hypoosmotic solution (200 Osmmol/kg) contained sucrose 30 mmol/L, and the others are the same as the isoosmotic solution. Modified K-B solution containing *L*-glutamate 50, KCl 50, taurine 20, KH_2PO_4 20, $MgCl_2 \cdot 6H_2O$ 3, glucose 10, HEPES 10, and egtazic acid 0.5 mmol/L, had its pH adjusted to 7.4 with KOH. The pipette solution contained K-aspartic acid 110, Mg-ATP 5, $MgCl_2 \cdot 6H_2O$ 1, KCl 20, egtazic acid 0.1 or 10, di-*tris*-creatine phosphate 2.5, disodium-creatine phosphate 2.5 mmol/L, and its pH was adjusted to 7.3 with KOH. The pipette solution for recording mucarinic current containing CsCl 135, Na_2ATP 3, $MgCl_2$ 3, di-*tris*-creatine phosphate 2.5, disodium-creatine phosphate 2.5. HEPES 5, egtazic acid 0.5, and pH was adjusted to 7.3 with Tris. Carbachol (Sigma) was prepared as aqueous stock solutions (10 mmol/L) and niflumic acid and nicardipine (Sigma) were prepared as Me_2SO stock solutions (20 mmol/L and 10 mmol/L, respectively).

Data analysis Data were expressed as mean±SD. Statistical significance was evaluated by *t*-test.

RESULTS

Effect of hypoosmotic membrane stretch on I_{CCh} in guinea-pig gastric myocytes Under the whole-cell configuration, the membrane potential was clamped at -20 mV. When sodium chloride in external solution and potassium chloride in the pipette solution were replaced by CsCl, the mucarinic current was elicited by carbachol 50 μmol/L (Fig 1A). The mean amplitude of carbachol-induced muscarinic current (I_{CCh}) was (546±99) pA at -20 mV (n=50). Quinidine 3 μmol/L, a muscarinic receptor gated channel blocker, completely blocked I_{CCh} (Fig 1B, n=6). When the cells were superfused with hypoosmotic solution (200 Osmmol/kg and containing carbachol 50 μmol/L) I_{CCh} was enhanced and increased by 126 %±13 % at -20 mV (Fig 2A and 2B, n=14). Hypoosmotic membrane stretch-induced increase of I_{CCh} was completely blocked by quinidine (3 μmol/L), a muscarinic receptor gated channel blocker (Fig 2C, n=6).

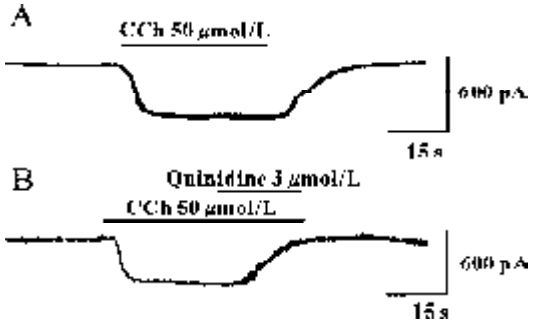

Fig 1. Effect of quinidine, a specific muscarinic current blocker on carbachol-induced muscarinic current. A: raw trace of muscarinic current induced by carbachol 50 μmol/L. B: the inhibitory effect of quinidine 3 μmol/L on carbachol current.

Effect of extracellular calcium on hypoosmotic membrane stretch-induced increase of I_{CCh} I_{CCh} was elicited by carbachol 50 μmol/L at -20 mV of membrane potential. When the cells were superfused with calcium-free isoosmotic solution, the I_{CCh} was completely inhibited and calcium-free hypoosmotic solution did not increase I_{CCh} any more (Fig 3A, n=6). However, the hypoosmotic membrane stretch-induced increase of I_{CCh} was not blocked by nicardipine 5 μmol/L, a L-type calcium channel blocker (Fig 3B, n=6), and by gadolinium chloride 100 nmol/L, a stretch-activated non-selective cation channel blocker (Fig 3C, n=5), but I_{CCh} was completely blocked by both nicardipine and gadolinium chloride (Fig 3D, n=6).

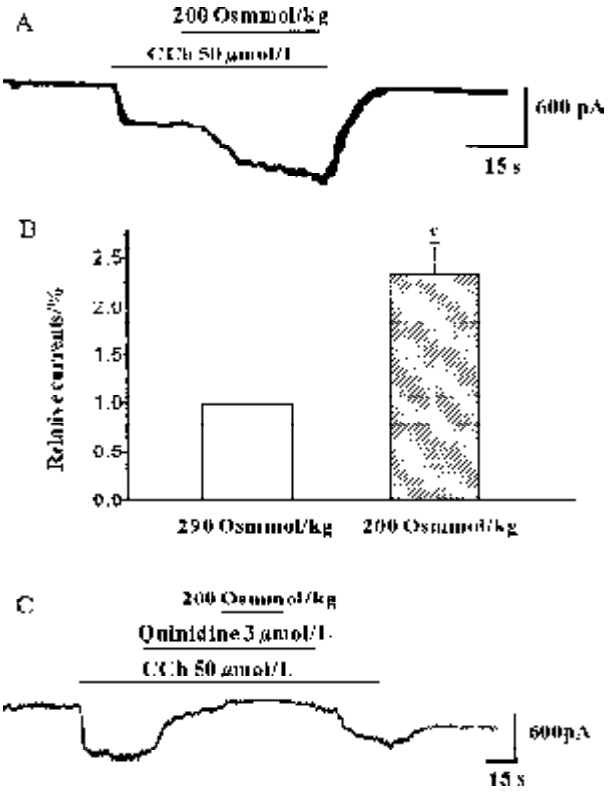

Fig 2. Effect of hypoosmotic membrane stretch on carbachol current. A: raw trace of hypoosmotic membrane stretch-induced increase of carbachol current. B: statistical summary of hypoosmotic membrane stretch-induced increase of carbachol current. C: hypoosmotic membrane stretch-induced increase of carbachol current was completely blocked by quinidine, a specific carbachol current blocker. ^cP<0.01 *vs* control.

Effect of calcium mobilization on hypoosmotic membrane stretch-induced increase of I_{CCh} I_{CCh} increased by hypoosmotic membrane stretch was mediated by external calcium influx. In order to investigate whether the calcium come from external solution triggered calcium-induced calcium release, the effect of ryanodine , a CICR agonist, on hypoosmotic membrane stretch-induced increase of I_{CCh} was observed. Ryanodine 10 mol/L firstly increased I_{CCh} and subsequently blocked I_{CCh} completely. In the present of ryanodine, hypoosmotic membrane stretch did not increase I_{CCh} again (Fig 4, n=6).

DISCUSSION

In gastric antral circular myocytes of the guinea-pig, the I_{CCh} was increased by hypoosmotic membrane

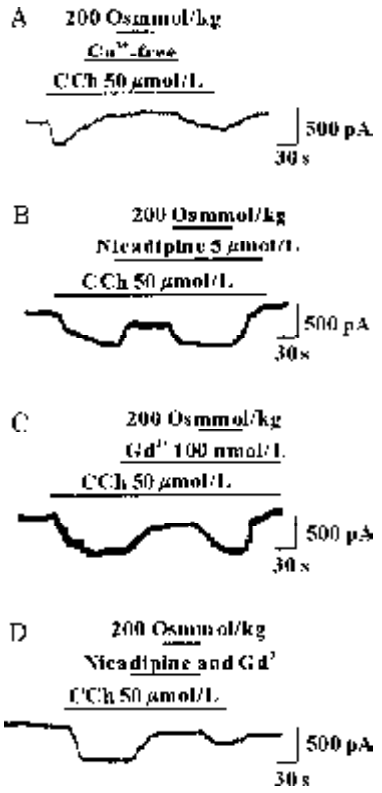

Fig 3. Effect of external calcium on hypoosmotic membrane stretch-induced increase of carbachol current. A: hypoosmotic membrane stretch-induced increase of carbachol current was completely blocked by external calcium-free solusion. B: nicardipine, a specific L-type calcium channel blocker, markedly inhibited carbachol current but could not blocked hypoosmotic membrane stretch-induced increase of carbachol current. C: gadolinium, a specific stretch-activated cation channel blocker, markedly inhibited carbachol current but could not block hypoosmotic membrane stretch-induced increase of carbachol current. D: in presence of both nicardipine and gadolinium condition hypoosmotic membrane stretch did not increase carbachol current any more.

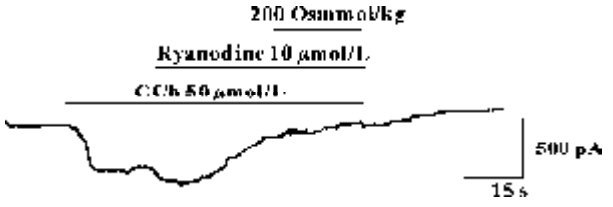

Fig 4. Effect of calcium-induced calcium release on hypoosmotic membrane stretch-induced increase of carbachol current. Ryanodine, a specific calcium induced calcium release inhibitor, completely blocked hypoosmotic membrane stretch-induced increase of carbachol current.

stretch and this effect was completely blocked by quinidine, a specific muscarinic current blocker. In external calcium free condition, I_{CCh} was not increased by hypoosmotic membrane stretch. However, gadolinium, a stretch-activated nonselective cation channel blocker or nicardipine, a L-type calcium channel blocker inhibited I_{CCh} but did not block hypoosmotic membrane stretch-induced increase of I_{CCh}. After pretreatment with gadolinium and nicardipine, the hypoosmotic membrane stretch-induced increase of I_{CCh} was completely blocked. Ryanodine, an inhibitor of calcium-induced calcium release (CICR), completely blocked hypoosmotic membrane stretch-induced increase of I_{CCh}.

Ca^{2+} and G-protein play important roles in the activation of cation channels in smooth muscle cells[15,16] as well as in other cell types[17]. In the absence of acetylcholine (ACh), elevation of the intracellular free calcium concentration to 1 μmol/L itself did not induce a cation current, but once the current was activated by ACh, it was augmented by intracellular calcium[14]. Such augmentation by intracellular calcium was called "facilitation of the ACh-gated current" and the dependence of the ACh or carbachol (CCh)-gated current on intracellular calcium has been demonstrated by others[16,18]. The main point of the present study is to determine the relationship between calcium signaling and hypoosmotic membrane stretch-induced increase of I_{CCh} in gastric antral circular myocytes of guinea pig. Our previous study demonstrated that hypoosmotic membrane stretch increased L-type calcium current in guinea-pig gastric myocytes[9,10]. In the present study, we confirmed that I_{CCh} was increased by hypoosmotic membrane stretch in gastric antral circular myocytes, and the increment was completely blocked by external calcium-free solution. However, neither nicardipine, a L-type calcium channel blocker nor gadolinium, a stretch-activated channel blocker, did not block the membrane stretch-induced increase of I_{CCh}, but administration of both nicardipine and gadolinium together completely blocked the increment. In our previous study, we found that hypoosmotic swelling activated $I_{K(Ca)}$, and the activation was associated with CICR which was triggered by calcium influx through stretch-activated cation channel[19]. Recently, Kirber *et al*[20] found that extracellular calcium influx by membrane stretch via activating stretch-sensitive channels and caused membrane depolarization, which activated voltage-gated calcium channels in toad gastric myocyte. The entry of calcium through stretch-activated chan-

nels was also amplified by calcium release from internal stores. In the present study membrane stretch-induced increase of I_{CCh} was completely blocked when CICR was inhibited by ryanodine. So that the hypoosmotic membrane stretch-induced increase of I_{CCh} may be related with CICR triggered by calcium influx through L-type calcium channel and stretch-activated cation channel.

In summary, hypoosmotic membrane stretch increased I_{CCh} in gastric antral circular myocytes of guinea pig. The increased currents are modulated by intracellular calcium which is released from calcium store via CICR triggered by hypoosmotic membrane stretch-induced calcium influx. In gastric smooth muscle hypoosmotic membrane stretch-induced increase of I_{CCh} is a mechanism of stretch-induced smooth muscle contraction.

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