

## Inhibitory Effect of Caffeine on Carbachol-Induced Nonselective Cationic Current in Guinea-Pig Gastric Myocytes

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In gastrointestinal smooth muscle, muscarinic stimulation by carbachol (CCh) activates nonselective cation channel current ( $I_{CCh}$ ) which is facilitated by intracellular  $[Ca^{2+}]$  increase. Caffeine is widely used in experiments to mobilize  $Ca^{2+}$  from intracellular stores. This study shows a strong inhibitory effect of caffeine on  $I_{CCh}$  in guinea-pig gastric myocyte. In this study, the underlying mechanism of the inhibitory effect of caffeine was investigated.  $I_{CCh}$  was completely suppressed by the addition of caffeine (10 mM) to the superfusing solution. Inhibition of  $I_{CCh}$  by caffeine was not related to the intracellular cAMP accumulation which was expected from the phosphodiesterase-inhibiting effect of caffeine. The blockade of  $InsP_3$ -induced  $Ca^{2+}$  release by heparin had no significant effects on the activation of  $I_{CCh}$ . When the same cationic current had been induced by intracellular dialysis of GTP[ $\gamma$ S] in order to bypass the muscarinic receptor, the inhibitory effect of caffeine was significantly attenuated. The results of this study indicate that both intracellular signalling pathways for  $I_{CCh}$ , proximal and distal to G-protein activation, are suppressed by caffeine. A major inhibition was observed at the proximal level.

Key Words: Smooth muscle, Caffeine, Nonselective cationic current, Carbachol

### INTRODUCTION

In mammalian gastrointestinal myocytes, the stimulation of muscarinic receptors by acetylcholine (ACh) or carbachol (CCh) induces a nonselective cationic currents ( $I_{CCh}$ , Benham et al, 1985) as well as the  $Ca^{2+}$ -release mediated by the production of inositol trisphosphate ( $InsP_3$ , Bielkiewicz-Vollrath et al, 1987). The activation of  $I_{CCh}$  is suggested to be responsible for membrane depolarization by muscarinic stimulation in gastrointestinal smooth muscle (Inoue & Isenberg, 1990a, 1990b, 1990c). The gating process of nonselective cation channel is believed to be mediated by a GTP-binding protein (G-protein). Internally-

applied GTP $\gamma$ S, a nonhydrolysable analogue of GTP which permanently activates  $\alpha$ -subunits of GTP-binding proteins, can induce the same cationic currents in intestinal myocytes, while the activation of  $I_{CCh}$  is inhibited by GDP $\beta$ S, a non-hydrolysable GDP analogue which permanently inactivates G-proteins (Inoue & Isenberg, 1990c).

Once  $I_{CCh}$  is activated by CCh, the amplitude of  $I_{CCh}$  is enhanced along with the increase in cytosolic calcium concentration ( $[Ca^{2+}]_c$ ) and such augmentation by  $[Ca^{2+}]_c$  was called 'facilitation of the ACh-gated current' (Inoue & Isenberg, 1990b). Although the mechanism of 'facilitation' is not fully understood yet, it has been suggested that  $Ca^{2+}$ /calmodulin-dependent myosin light chain kinase pathway is involved in such phenomenon (Kim et al, 1995; Kim et al, 1997). Recently, it was also observed that increase of  $[Ca^{2+}]_c$  also induced slow desensitization of  $I_{CCh}$  via activation of  $Ca^{2+}$ -dependent protein

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kinase-C pathway (Ahn et al, 1997; Kim et al, 1998).

Caffeine is a pharmacological agent used widely for the investigation of ryanodine receptors (RyRs) both in smooth and striated muscles (Endo, 1977; Ganitkevich & Isenberg, 1992). RyRs distribute in the endoplasmic reticulum membrane of various excitable and non-excitable cells. They are known to be responsible for the  $\text{Ca}^{2+}$ -induced  $\text{Ca}^{2+}$ -release (CICR) for excitation-contraction coupling (Berridge, 1997). The threshold of RyRs for cytosolic calcium concentration ( $[\text{Ca}^{2+}]_c$ ) is lowered by caffeine and therefore, RyRs are activated even in the resting state of  $[\text{Ca}^{2+}]_c$  (Endo, 1977). Transient increase of  $[\text{Ca}^{2+}]_c$  and subsequent depletion of sarcoplasmic reticulum (SR)  $\text{Ca}^{2+}$  content are well-described in various muscle cells (Ganitkevich & Isenberg, 1992; Baro & Eisner, 1995).

Since the application of caffeine would induce transient increase of  $[\text{Ca}^{2+}]_c$ , it was expected that such changes may induce corresponding increase of  $I_{\text{CCh}}$  due to the  $\text{Ca}^{2+}$ -dependent facilitation effect. In this experiment, however, we have observed opposite inhibitory effect of caffeine on  $I_{\text{CCh}}$ . The present study was designed to elucidate the underlying mechanisms for the inhibitory effect of caffeine on  $I_{\text{CCh}}$ .

## METHODS

### Cell isolation

Guinea-pigs of either sex weighing 300~350 g were exsanguinated after stunning. The stomach was isolated and cut in the longitudinal direction along the lesser curvature in phosphate-buffered Tyrode solution. The antral part of stomach was cut and the mucosal layer was separated from the muscle layers. The circular muscle layer was dissected from the longitudinal layer using fine scissors and made into small segments ( $2 \times 3$  mm). These segments were incubated in a medium modified from the Kraft-Brühe (K-B) medium (Isenberg & Klöckner, 1982) for 30 min at  $4^\circ\text{C}$ . Then, they were transferred into nominal  $\text{Ca}^{2+}$ -free physiological salt solution (PSS) containing 0.1% collagenase (Wako, Japan), 0.05% dithiothreitol, 0.1% trypsin inhibitor, and 0.2% bovine serum albumin, and incubated for 15~25 min at  $35^\circ\text{C}$ . After digestion, the supernatant was discarded and softened muscle segments were transferred again into the modified K-B medium. Single cells were dispersed

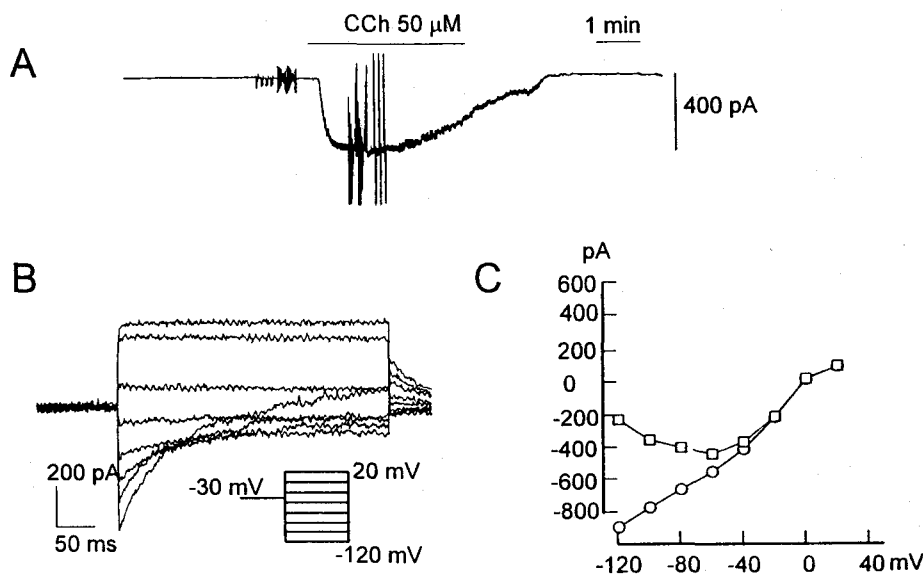
by gentle agitation with a wide-bored glass pipette. Isolated gastric myocytes were kept in the modified K-B medium at  $4^\circ\text{C}$  until used. All experiments were carried out within 12 hours of harvesting cells and performed at room temperature.

### The measurement of membrane currents

Isolated cells were transferred to a small chamber (400  $\mu\text{l}$ ) on the stage of an inverted microscope (IMT-2, Olympus, Japan). The chamber was perfused with physiological salt solution (PSS, 2~3 ml/min). Glass pipettes with a resistance of 2~4  $\text{M}\Omega$  were used to make a gigaseal of 5~10 giga  $\Omega$ . Standard whole-cell patch clamp techniques were used (Hamill et al, 1981). An Axopatch-1C patch-clamp amplifier (Axon instruments, Burlingame, USA) was used to record membrane currents and command pulses were applied using an 486-grade PC and pCLAMP software v.5.51 (Axon Instrument, Burlingame, USA). The data were filtered at 5 kHz and displayed on a digital oscilloscope (PM 3350, Phillips, Netherlands), a computer monitor, and a pen recorder (Recorder 220, Gould, Cleveland, USA).

### Solutions

Phosphate-buffered Tyrode solution contained (in mM) NaCl 147, KCl 4,  $\text{MgCl}_2$  1,  $\text{CaCl}_2$  1.8,  $\text{NaH}_2\text{PO}_4$  0.42,  $\text{Na}_2\text{HPO}_4$  1.81, glucose 5.5, pH 7.3.  $\text{Ca}^{2+}$ -free PSS contained (in mM) NaCl 135, KCl 5,  $\text{MgCl}_2$  1, glucose 5, HEPES (N-[2-hydroxyethyl]piperazine-N'-[2-ethanesulfonic acid]) 5, and its pH was adjusted to 7.3 by Tris. PSS contained 1.8 mM  $\text{CaCl}_2$  in the  $\text{Ca}^{2+}$ -free PSS.  $\text{Na}^+$  and  $\text{K}^+$  of extracellular solution were replaced by  $\text{Cs}^+$  in the experiments recording CCh-activated inward current except one experiment (Fig. 3B). Modified K-B solution contained (in mM) L-glutamate 50, KCl 50, taurine 20,  $\text{KH}_2\text{PO}_4$  20,  $\text{MgCl}_2$  3, glucose 10, HEPES 10, EGTA (ethylene-glycol-bis(8-aminoethyl ether)-N,N,N',N'-tetraacetic acid) 0.5 and its pH was adjusted to 7.3 by KOH. Pipette solution consisted of (in mM) CsCl 135, MgATP 5, tris-GTP 0.5, di-tris-creatine phosphate 2.5,  $\text{Na}_2$  creatine phosphate 2.5,  $\text{MgCl}_2$  1, HEPES 5, EGTA 0.5; the pH was adjusted to 7.3 using Tris.



**Fig. 1.** Carbachol-activated cationic current ( $I_{CCh}$ ) in guinea-pig gastric myocytes. **A.** In symmetrical CsCl solution,  $I_{CCh}$  was induced by CCh application as shown in the trace. **B.** From a holding potential of  $-30$  mV, various step pulses (from  $-120$  mV to  $+20$  mV, 350 msec) were applied. The current traces are difference currents where control currents are obtained in the absence of CCh. **C.** I/V relationships of  $I_{CCh}$  where open circles indicate peak values of each traces and open squares indicate values obtained at the end of each step pulse (lower right panel).

## RESULTS

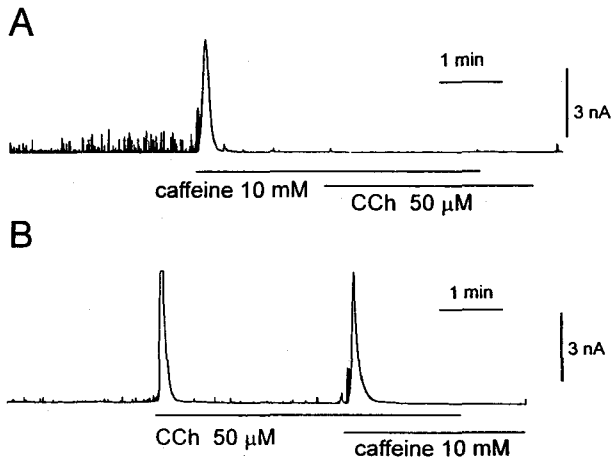
### *CCh-activated inward current*

The membrane potential was held at  $-20$  mV, and  $50 \mu\text{M}$  CCh was applied to the externally perfusing bath solution. In CsCl-bath solution ( $140$  mM CsCl), CCh application induced an inward current ( $I_{CCh}$ , Fig. 1A). In Fig. 1A, various levels of step pulses were applied in the presence or absence of CCh.  $I_{CCh}$  at various membrane potentials were obtained by subtracting the control currents from the currents recorded in the presence of CCh. The time-dependent relaxation of inward currents (Fig. 1B) was observed in the negative membrane potential range, and therefore, the steady-state I-V relationship (open square, Fig. 1C) of  $I_{CCh}$  was U-shape. The response to depolarizing pulse was, however, instantaneous and showed no significant time-dependent relaxation. The reversal potential of  $I_{CCh}$  was about  $0$  mV and was not significantly changed when extracellular  $\text{Cs}^+$  was replaced by  $\text{Na}^+$  or  $\text{Li}^+$  (data not shown). However, substitution with N-methyl-D-glucamate (NMDG), a non-permeable cation, could shift the reversal potential to more negative values (data not shown). This

indicates that the CCh-activated channel is non-selectively permeable to small monovalent cations (Inoue & Isenberg, 1990a).

### *Effects of caffeine on $I_{CCh}$*

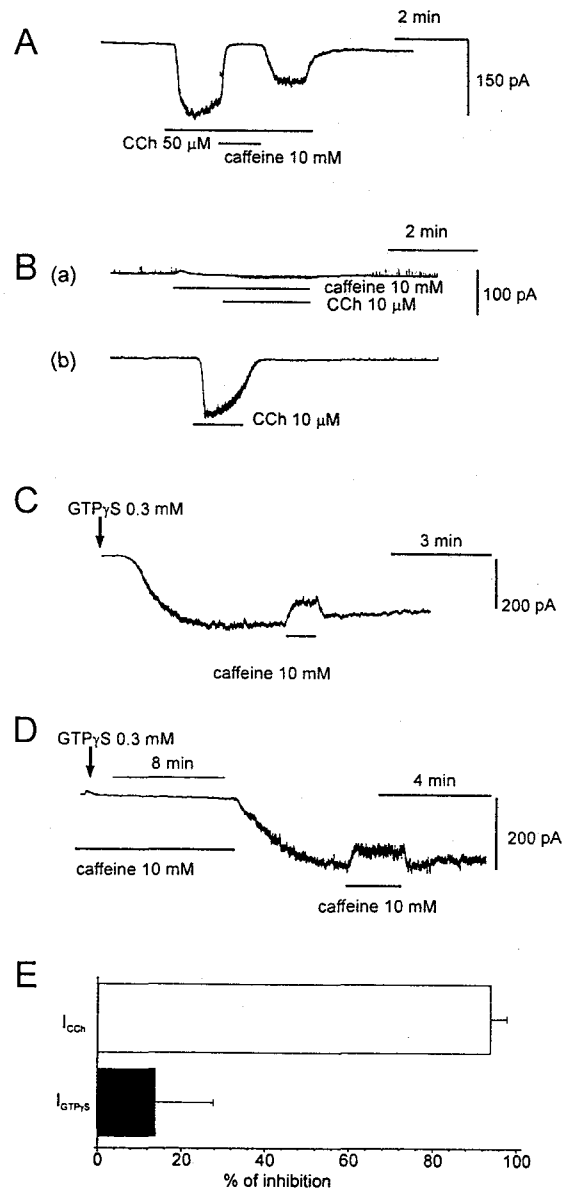
In Fig. 2, the caffeine-induced  $\text{Ca}^{2+}$ -release was indirectly observed from the  $\text{Ca}^{2+}$ -activated  $\text{K}^+$  current. The gastric myocyte was clamped at  $0$  mV and KCl-pipette solution was adopted to record potassium current. At this membrane potential, activations of spontaneous transient outward currents (STOCs), which indicate a spontaneously localized  $\text{Ca}^{2+}$  release from SR, were observed. Bath applied caffeine ( $10$  mM) induced a large transient outward current (the  $\text{Ca}^{2+}$ -activated  $\text{K}^+$  current) which was considered to reflect simultaneous activation of RyR and  $\text{Ca}^{2+}$  release from SR (Bolton & Imaizumi, 1996). In the continued presence of caffeine, additional application of CCh had no further effect (Fig. 2A). In Fig. 2B, the sequence of application was reversed with CCh being applied first and then caffeine coming next. CCh also induced a large transient increase of outward current, which might have been caused by the simultaneous  $\text{Ca}^{2+}$ -release via



**Fig. 2.** CCh- and caffeine-induced transient outward currents. The pipette and bath solutions were high-K solution with low (0.1 mM) EGTA and normal Tyrode solution, respectively. Extracellular application of caffeine (10 mM) or CCh (50  $\mu$ M) induced a large, transient outward current. In the presence of caffeine, CCh had no further effect (A), while caffeine applied after CCh could induce a large transient outward current (B).

InsP<sub>3</sub> receptor channel in SR membrane (IICR). At this membrane potential (0 mV, reversal potential for nonselective cation channel), no inward current was observed. Even in the presence of CCh, caffeine could induce an additional transient outward current (Fig. 2B). Similar response was observed in two other cells. Above results matched the Ca<sup>2+</sup>-releasing action of caffeine and also indicate that pretreatment with caffeine inhibit subsequent Ca<sup>2+</sup> release by CCh.

Since the concentration of caffeine used in the experiments is 10 mM, the effect of 10 mM caffeine on I<sub>CCh</sub> was also tested. Again, the membrane potential was held at -20 mV and 50  $\mu$ M CCh was applied. I<sub>CCh</sub> was almost completely suppressed during the presence of caffeine and the suppression was reversed when CCh was washed out (Fig. 3A). Same inhibitory effect on I<sub>CCh</sub> was observed by the pretreatment with caffeine (Fig. 3B). In this cell, the internal pipette solution was composed of KCl instead of CsCl and as a result, spontaneous transient outward currents (STOCs) were observed. Even though the caffeine application did not induce large transient increase of I<sub>K(Ca)</sub> in this myocyte, it still could abolish STOCs, which indicated the depletion of SR Ca<sup>2+</sup> content by caffeine. In the same myocyte, second application of CCh induced usual amplitude of I<sub>CCh</sub>



**Fig. 3.** Comparison of the effects of caffeine on I<sub>CCh</sub> and on GTP $\gamma$ S-induced current. A. I<sub>CCh</sub> was completely suppressed during the application of 10 mM caffeine. B. The pretreatment with caffeine could also block the activation of I<sub>CCh</sub> (Ba, upper trace). In the same myocyte, usual activation of I<sub>CCh</sub> was observed after washout of caffeine (Bb, lower trace). Note that K<sup>+</sup>-rich pipette solution was used in B. C. Caffeine (10 mM) was applied after the full activation of GTP $\gamma$ S-induced current. Caffeine showed weaker inhibitory effect on I<sub>GTP $\gamma$ S</sub> than on I<sub>CCh</sub>. D. Caffeine was applied before the intracellular dialysis of GTP $\gamma$ S. The pretreatment with caffeine blocked the activation of I<sub>GTP $\gamma$ S</sub> while the usual inward current (I<sub>GTP $\gamma$ S</sub>) was induced after the washout of caffeine. Addition of caffeine after full activation of I<sub>GTP $\gamma$ S</sub> did not completely block the inward current (E).

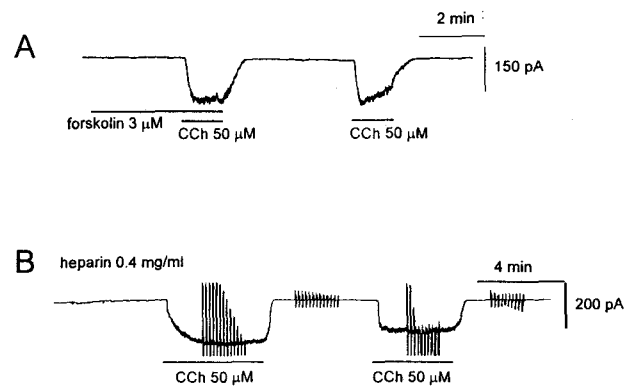
(Fig. 3B-(b)). Effects of both agents (caffeine and CCh) on STOCs were reversed by washout. Effects of caffeine on  $I_{CCh}$  were tested in 9 cells and the mean inhibition was  $94 \pm 3.9\%$  (Fig. 3E).

As reported elsewhere, the same cationic current with  $I_{CCh}$  was induced by the application of internal  $GTP\gamma S$ , non-hydrolyzable GTP analogue.  $GTP\gamma S$  (0.3 mM) was added to the pipette solution and the whole-cell configuration was made. With the onset of dialysis, an inward current ( $I_{GTP\gamma S}$ ) was slowly induced at a holding potential of  $-20$  mV (Fig. 3C, see also Inoue & Isenberg, 1990c). The current increased with the passage of time and before saturated within 10 min.  $I_{GTP\gamma S}$  showed the same  $I/V$  relationship with  $I_{CCh}$  (data not shown here).

The inhibitory effect of caffeine on  $I_{GTP\gamma S}$  was far less potent than the effect on  $I_{CCh}$ . In 13 cells examined, less than 20% ( $14 \pm 13.8\%$ ) of maximum amplitude was lost by 10 mM caffeine (Fig. 3C & E). The weak inhibitory effect was also reversed by washout. In Fig. 3D, caffeine was applied before the diffusion of  $GTP\gamma S$  into the cytosol. Quite interestingly, this kind of pretreatment with caffeine blocked the slow activation of  $I_{GTP\gamma S}$  for about 10 min. Normal activation of  $I_{GTP\gamma S}$  was confirmed by the washout of caffeine and once activated,  $I_{GTP\gamma S}$  was only weakly suppressed by caffeine (Fig. 3D). Similar effect of caffeine pretreatment was observed in 5 out of 6 cells tested.

Like many methylxanthines, caffeine inhibits cyclic nucleotide phosphodiesterase activity (Butcher & Sutherland, 1962), which would lead to the accumulation of cAMP. A test was performed to verify whether an accumulation of cAMP by caffeine might have inhibited  $I_{CCh}$ . Forskolin ( $3 \mu M$ ), an activator of adenylate cyclase, was applied before the activation of  $I_{CCh}$ . As shown in Fig. 4A, forskolin had no significant effect on  $I_{CCh}$ . Slight change in the contour of  $I_{CCh}$  was within usual range of experimental variation.

Since the ultimate action of caffeine is the depletion of cellular  $Ca^{2+}$  stores, the effect of heparin on  $I_{CCh}$  was compared with the depletive effects of cellular  $Ca^{2+}$  stores. Heparin is known as a competitive antagonist of  $InsP_3$ -receptor in SR membrane and blocks the  $Ca^{2+}$  release by muscarinic stimulation in smooth muscle (Bolton & Lim, 1989). Low-molecular weight heparin was added to the pipette solution (0.4 mg/ml) and waited for more than 10 min after making whole-cell configuration.  $I_{CCh}$



**Fig. 4.** Inhibitory effects of caffeine on  $I_{CCh}$  were not mimicked by forskolin or by intracellular application of heparin. A. The pretreatment with forskolin ( $3 \mu M$ ) had no significant effect on the activation of  $I_{CCh}$ . B. Heparin (0.4 mg/ml) was added in the pipette solution. 12 minutes after making a whole-cell configuration, usual activation of  $I_{CCh}$  was observed.

could be induced even with heparin in the pipette solution (Fig. 4B). Similar activation of  $I_{CCh}$  was obtained in seven cells tested ( $250 \pm 57$  pA).

## DISCUSSION

In this study, prominent inhibitory effect of caffeine on the activation process of  $I_{CCh}$  was observed. In guinea-pig ileal myocyte, a weak inhibitory effect of caffeine on  $I_{CCh}$  has been briefly mentioned (Chen et al, 1993). In the previous report, nystatin-perforated whole-cell patch clamp technique was used and 10 mM caffeine inhibited  $I_{CCh}$  by about 40% which was far less potent than the results observed on  $I_{CCh}$ . This study explained that no effects of caffeine on cellular conditions, e.g. increase of cAMP content and  $Ca^{2+}$ -pool depletion, were involved.

This study suggests the inhibitory effect of caffeine was not prominent on the same cationic current activated by direct stimulation of G-protein. As  $I_{CCh}$  is elicited through G-protein coupled receptor activation, muscarinic receptor, G-protein, and cation channel itself could all be the targets of caffeine. If the effect of caffeine on  $I_{GTP\gamma S}$  was weak, it implies that caffeine may have affected mostly the signalling process proximal to the cation channel. A simple speculation, though not supported by any conclusive evidences, based upon the inhibitory effect of caffeine pretreatment on  $I_{GTP\gamma S}$  (Fig. 3D) may suggest

that caffeine could inhibit the dissociation of trimeric G-protein subunits.

A portion of the decrease in  $I_{GTP\gamma S}$  by the after-treatment with caffeine (Fig. 3C) may have reflected the nonspecific inhibition of distal signalling pathway by caffeine (e.g. direct inhibition of nonselective cation channel). Therefore, even the nonspecific inhibition of  $I_{CCh}$  by caffeine could be broken down into two categories depending upon the acting mechanism 1) major inhibitory effect on proximal signaling pathway (e.g. G-protein); and 2) minor inhibitory effect on distal signaling pathway (e.g. nonselective cation channel). At present, it is not certain whether the effect on distal pathway is due to a channel blocking mechanism or not.

Nonspecific actions of caffeine have been reported in various tissues related with various mechanisms including: effect on ion channels (Islam et al, 1995; Yoshino et al, 1996), inhibition of  $InsP_3$  production (Toescu et al, 1992), inhibitory effect on  $InsP_3$  receptor (Sanchez-Bueno et al, 1994), and nicotinic receptor channel blocking (Xu & Forsberg, 1992). The results of this study suggest another nonspecific effect of caffeine, an inhibitory effect on the muscarinic signalling for cation channel activation.

In conclusion, a strong inhibitory effect of caffeine was observed on the activation of nonselective cationic current in guinea-pig gastric smooth muscle. As mentioned in the *Introduction*, caffeine is frequently used as an experimental tool to mobilize  $Ca^{2+}$  from SR. Effective concentration of caffeine for this purpose (5–20 mM) considerably overlaps the concentration tested here. Therefore, special care is needed in designing the experiment using caffeine and in the interpretation of experiment results, especially in relation to muscarinic action.

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