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Effect of Imipramine on Calcium Utilization of Single Cells Isolated from Canine Detrusor

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This study is to investigate the mechanism of inhibitory effect of imipramine on the calcium utilization in single cells isolated from canine detrusor. 2 mm thick smooth muscle chops were incubated in 0.12% collagenase solution at 36°C, and aerated with 95% O_2/5% CO_2, and then cell suspension was examined. Acetylcholine (ACh) evoked a concentration-dependent contraction of the isolated detrusor cells in normal physiologic salt solution (PSS), and the ACh-induced contraction was significantly inhibited by imipramine. In Ca^{2+}-free PSS, ACh-induced contraction was less than those in normal PSS and it was not affected by the pretreatment with imipramine. Ca^{2+}-induced contraction in Ca^{2+}-free PSS was supressed by imipramine, but addition of A 23187, a calcium ionophore, overcomed the inhibitory effect of imipramine. High potassium-depolarization (40 mM KCl) evoked cell contraction, which was inhibited by imipramine. Caffeine, a releasing agent of the stored Ca^{2+} from sarcoplasmic reticulum, evoked a contraction of the cells that was not blocked by the pretreatment with imipramine. These results suggest that imipramine inhibits the influx of calcium in the detrusor cells through both the receptor-operated- and voltage-gated-calcium channels, but does not affect the release of calcium from intracellular storage site.

Key Words: Imipramine, Calcium, Single cells, Canine detrusor

INTRODUCTION

Imipramine, a tricyclic antidepressant, is an old drug widely used to treat detrusor instability (Wall, 1990), nocturnal enuresis of children (Warady et al, 1991), detrusor hyperreflexia (Kato et al, 1991), female urge incontinence (Martan et al, 1993), post-prostatectomy uninary incontinence (Gilja et al, 1997), and overactive bladder (Wein, 1998). In spite of its adverse effects, such as urinary retention, dry mouth, indigestion, drowsyness or nervousness and postural hypotension, imipramine is still considered effective and important in treating vesical instabilities regardless of the etiology (Zubiaur-Libano et al, 1997). If we can elucidate the mechanism of its action, we may find a way to use it more safely and effectively.

Although the mechanism underlying the inhibitory effect of imipramine on the smooth muscle of urinary bladder is not clear, there are several convincing which argue that imipramine inhibited the reuptake of catecholamine at the sympathetic nerve endings, which would enhance the relaxant effect of the smpathetic nerve in detrusor (Axelrode et al, 1961; Sulcer et al, 1978). Imipramine blocked the cholinergic-muscarinic receptor in detrusor muscle in a ligand-binding study, but receptor blocking is not the major mechanism of inhibitory action (Pietra et al, 1990). Imipramine exerts an inhibitory action on the micturition reflex by a central cholinergic mechanism accompanying a stimulation of the muscarinic receptors at the supraspinal level (Sohn & Kim, 1997).

An early study by Malcovicz et al (1978) observed that imipramine had a competitive activity with extracellular calcium in rabbit detrusor muscle strips *in vitro*, suggesting that such a mechanism would play

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a role in its therapeutic effect on hyperreflexia or instability of urinary bladder. Imipramine blocked the influx of extracellular calcium like a calcium channel blocker in isolated detrusor from rat (Lee et al, 1992). Imipramine exerts a muscarinic blockade action in isolated canine detrusor cells (Huh et al, 1994).

In the present study, we employed single cells isolated from canine detrusor to observe the mechanism of imipramine's direct action that affects the calcium utilization of the smooth muscle cells.

METHODS

Detrusor muscle pieces were obtained from anesthetized dogs which were sacrificed after an experimental procedure related to a brain research. Fine chops of detrusor smooth muscle were digested in modified Krebs-Henseleit buffer solution (PSS) containing 120 mM NaCl, 4.6 mM KCl, 1.17 mM KH_2PO_4, 23.8 mM $NaHCO_3$, 1.8 mM $CaCl_2$, 1.2 mM $MgSO_4$, 10 mM Glucose, 0.08 mg/ml soybean trypsin inhibitor (Sigma biochemical), 1.2 mg/ml of collagenase (Worthington Biochemical, Type 2) and 2% bovine serum at 36°C. The solution was aerated by continuous breeze of 95% O_2/5% CO_2 mixed gas on the surface, and the pH was adjusted at 7.4. At the end of the digestion period, the tissue was poured over a 450 μm nylon mesh (Tekto, Elmsford, NY), rinsed with collagenae-free PSS to remove any trace of collagenase, and then incubated in this PSS in shaking (2 cm/sec) water bath at 36°C for 12~15 minutes for the cells dissociate freely.

Cell contractions induced by acetylcholine and extracellular calcium

Every 1 ml of the cell suspension was transfered into 5 ml test tubes. Various concentrations of ACh were added into the tubes so that the muscle cells contracted. To fix the contracted cells, diluted acrolein was added as 1% of final concentration in 5 seconds after the administration of ACh. The Ca^{2+}-induced contraction was observed in Ca^{2+}-free PSS, which contained 0.5 mM EGTA instead of $CaCl_2$ in normal PSS. In this case, acrolein was added every 10 seconds for 1 minutes following the administration of $CaCl_2$ solution into the Ca^{2+}-free PSS to determine the time of maximum contractile effect of extracellular Ca^{2+}. Acrolein- fixed cells were transfered onto slide glasses, and the length of cells were measured. Drugs and reagent for these experiments were A 23187, EGTA, calcium chloride and potassium chloride ordered from Sigma Biochemical; caffeine ordered from Fluka; and imipramine, a product of Eisai, donated from Hwan-In pharmaceuticals.

Effect of Imipramine on cell contractions in various conditions

ACh (10^{-11} M and 10^{-9} M) were added to cell suspensions after a 5 minutes preincubation with imipramine (10^{-8} M and 10^{-7} M) in normal PSS, and the same experiments were done in Ca^{2+}-free PSS. To observe the effect of extracellular Ca^{2+} addition, the cell suspension in Ca^{2+}-free PSS were pretreated with imipramine and then added with $CaCl_2$ (0.2 mM and 2 mM). Effect of a concomitant existence of A 23187 and $CaCl_2$ was observed to allow the calcium ions move freely. The cells were fixed 50 seconds after the addition of calcium, and that 50 seconds was the time point when Ca^{2+}-induced contraction reached its maximum. In normal PSS, the effect of pretreated imipramine on 40 mM KCl-induced and 1 mM caffeine-induced contractions were also observed.

Measurement of cell length with microscaler

On a phase-contrast microscope, the cells showing a clear nucleus and a bright halo around the cell body and repelling trypan blue were selected as healthy vital cells. A CCTV camera (Hidacchi VK-C 50) was employed to catch the images of cells on a phase-contrast microscope. The image sent to a microscaler (FOR · A, IV-550) was displayed on a TV screen where the measuring lines produced by the microscaler were also displayed.

Statistical analysis

The average length of 50 cells from one slide was accepted as the value of one case. Values were expressed as mean±SE of experimental cases and were checked for significance ($p<0.05$) by Student's t-test.

RESULTS

In normal PSS, resting cell length of canine de-

tusor smooth muscle was 91.3±2.0 μm. ACh shortend cells in a concentration-dependent manner, from 90.6±0.1 μm by 10^{-14} M to 76.1±0.3 μm by 10^{-9} M. The maximum shortening was 16.0±0.4% (Fig. 1).

In normal PSS, ACh 10^{-11} M and 10^{-9} M contracted cells as much as 13.0±0.4% and 17.5±1.1%, respectively. Imipramine 10^{-8} M reduced the later to 8.0±3.0% ($p<0.05$) and at 10^{-7} M to 5.2±3.7% ($p<0.05$) (Fig. 2A). ACh contracted the smooth muscle cells in Ca^{2+}-free PSS. The extent of contraction was 8.2±1.8% by 10^{-11} M and 12.1±1.5% by 10^{-9} M, and these contractions were tended to be inhibited by imipramine to the extent of 7.9±1.0% and 10.3±1.3% at 10^{-8} M concentration, and 6.8±1.7% and 9.9±1.8% at 10^{-7} M. These data were not statistically significant (Fig. 2B).

Addition of 2 mM Ca^{2+} ($CaCl_2$) in the Ca^{2+}-free PSS contracted cells. The average length of cells in Ca^{2+}-free PSS was 107.7±2.0 μm, which was shortened to 106.8±2.1 μm in 10 seconds, 101.6±2.5 μm in 20 seconds, 97.2±2.1 μm in 30 seconds, 93.5±

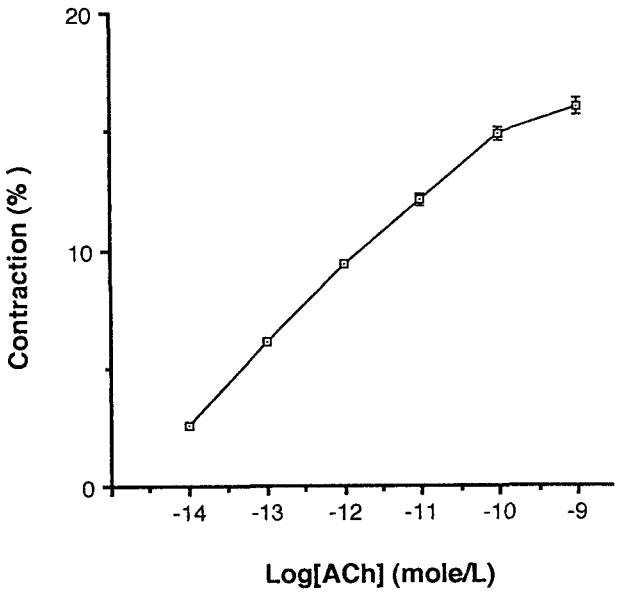

Fig. 1. Concentration-response to acetylcholine of the single cells isolated from canine detrusor. Bars on data points express the SEM (n=11).

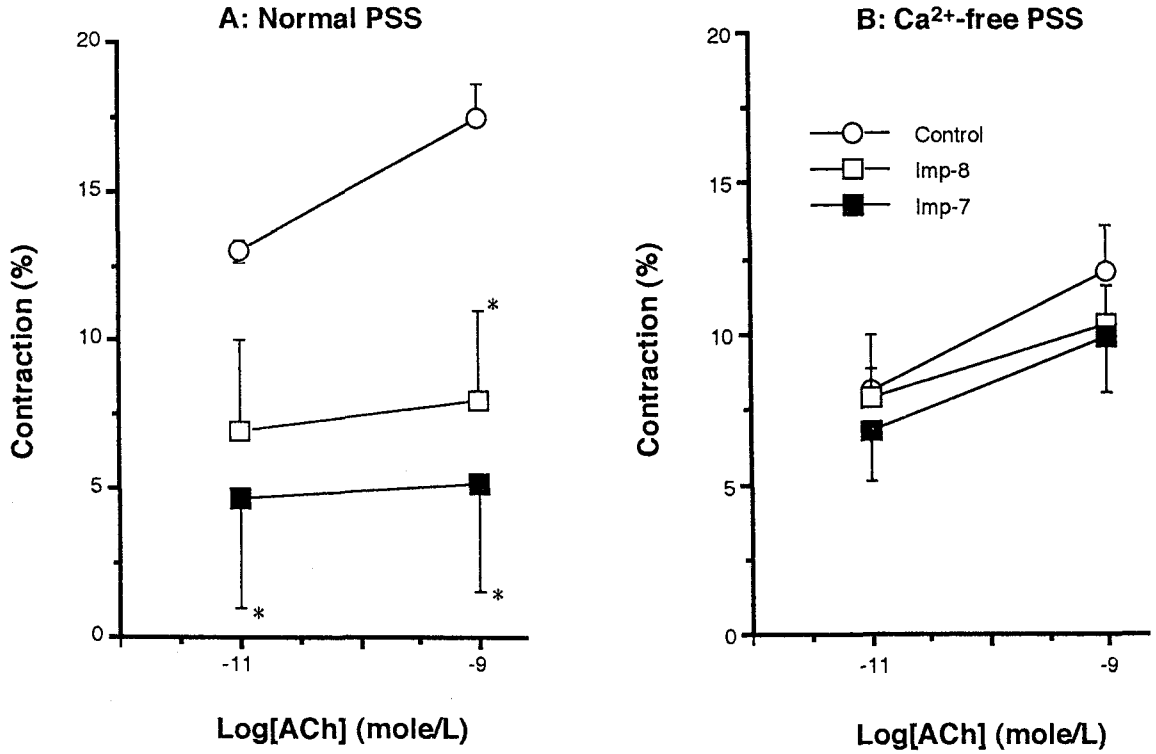

Fig. 2. Effect of imipramine on the acetylcholine-induced contraction of single cells isolated from canine detrusor in normal PSS (A) and Ca^{2+}-free PSS (B). Bars on data points express S.E.M. (n=7 for each group). Imp-8 and Imp-7 mean the concentrations of pretreated imipramine 10^{-8} M and 10^{-7} M, respectively. *$p<0.05$; significantly different from control.

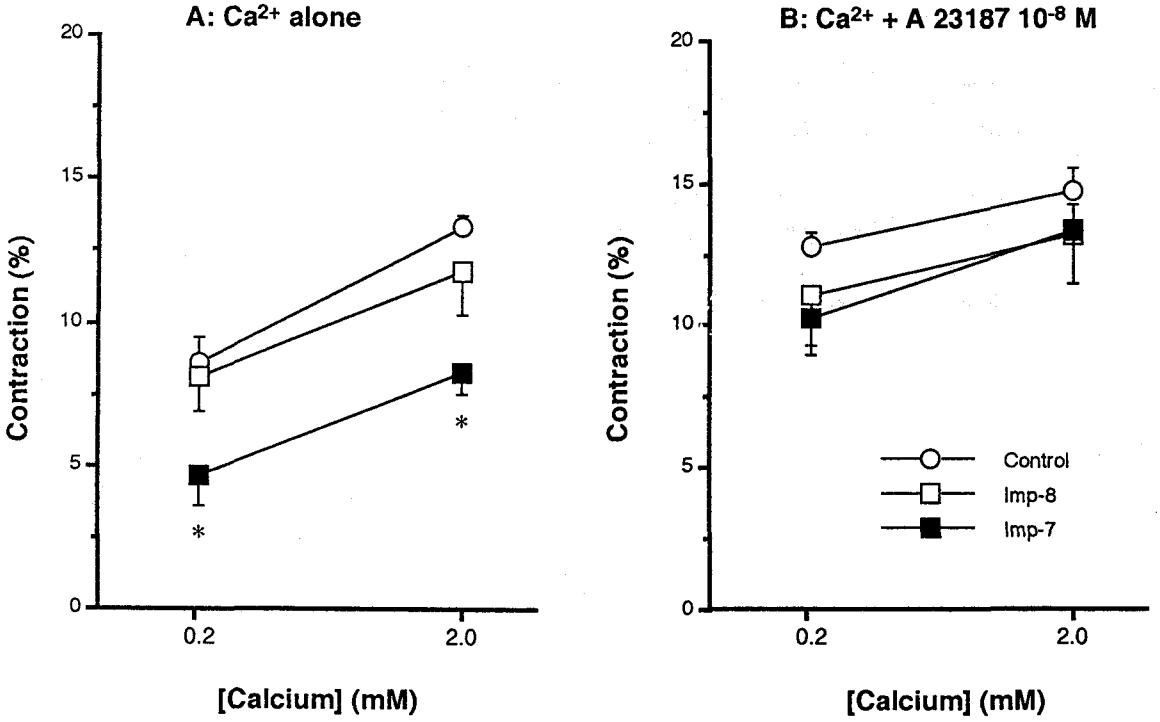

Fig. 3. Effect of imipramine on the Ca^{2+}-induced and Ca^{2+}-A 23187 10^{-8} M induced contractions of single cells isolated from canine detrusor in Ca^{2+}-free PSS. Bars on data points express S.E.M. (n=7 for each group) Imp-8 and Imp-7 mean the concentrations of pretreated imipramine 10^{-8} M and 10^{-7} M, respectively. *$p<0.05$: Significantly different from control.

1.4 μm in 50 seconds and 92.5±1.3 μm in 60 seconds. The maximum rate of contraction was 14.1 ±0.8% (data not shown on figure).

In Ca^{2+}-free PSS with 10^{-8} M imipramine, the addition of 0.2 mM Ca^{2+} contracted cells upto 8.1± 1.2%, which was not significantly different from control (8.6±0.9%). In the presence of 10^{-7} M imipramine, the contraction caused by the addition of 0.2 mM Ca^{2+} was 4.6±1.0%, significantly ($p<0.05$) lower than that of the control. The addition of 2 mM Ca^{2+} in the presence of imipramine 10^{-8} M contracted the cells upto 11.8±1.5%, which was not significantly different from the contraction level of the control (13.3±0.4%), while the contraction by 2 mM Ca^{2+} in the presence of 10^{-7} M imipramine was 8.3±0.8%, significantly ($p<0.05$) lower than that of the control (Fig. 3A). Effects of imipramine on cell contraction by concomitant administration of Ca^{2+} and A 23187 were measured in Ca^{2+}-free PSS. With A 23187 10^{-8} M, 0.2 mM calcium ion contracted cells by 12.8±0.5%, and 2 mM calcium, by 14.8± 0.8%. In the presence of 10^{-8} M or 10^{-7} M imipramine, those contractions were not reduced signi-

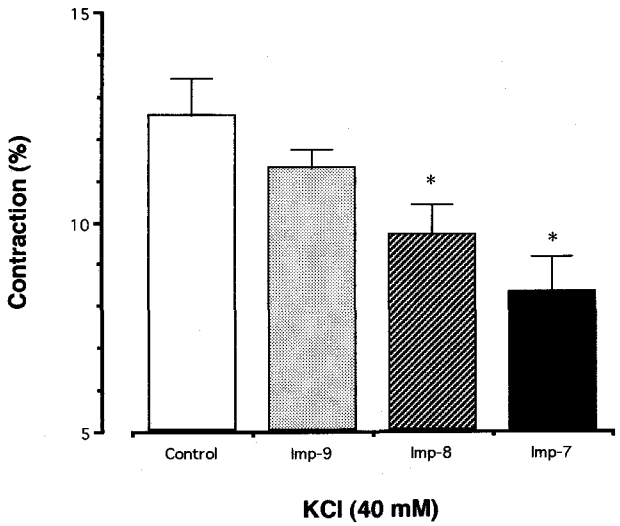

Fig. 4. Effect of imipramine on the high potassium-induced contraction of single cells isolated from canine detrusor. Error bars indicate S.E.M. (n=7 for each group) Imp-9, Imp-8 and Imp-7 mean the concentrations of imipramine 10^{-9} M, 10^{-8} M and 10^{-7} M, respectively. *$p<0.05$; significantly different from control.

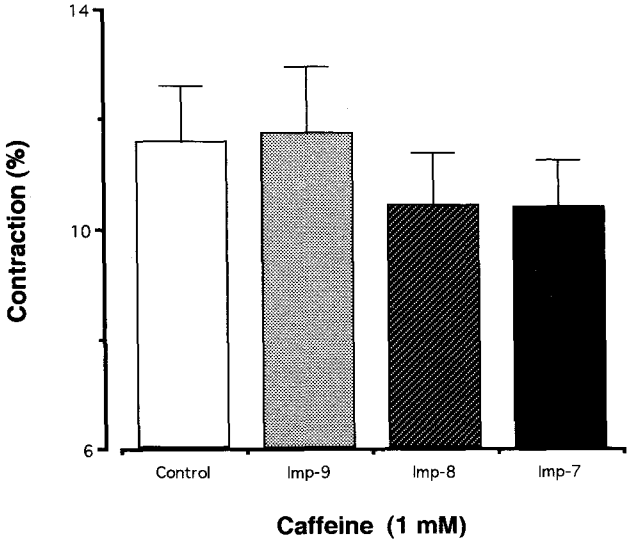

Fig. 5. Effect of imipramine on the caffeine-induced contraction of single cells isolated from canine detrusor. Bars on data points express the SEM (n=7 for each group) Imp-9, Imp-8 and Imp-7 mean the concentrations of imipramine 10^{-9} M, 10^{-8} M and 10^{-7} M, respectively.

ficantly (Fig. 3B).

High potassium (40 mM)-induced contraction of the detrusor cells in normal PSS was 12.5±0.9% (91.3±1.1 μm, control). Imipramine 10^{-9} M appeared to reduce this contraction upto 11.3±0.5%, which was not significant. Pretreated imipramine 10^{-8} M allowed the cells to contract only upto 9.7± 0.7% (94.2±0.8 mm) by 40 mM KCl, and this contraction was significantly different ($p<0.05$) from that of the control. Imipramine 10^{-7} M inhibited the KCl-induced contraction further, to 8.3±0.9% (95.6 ±0.5 mm) (Fig. 4).

In normal PSS, 1 mM caffeine contracted the cells upto 11.6±1.0%. When imipramine concentrations of 10^{-9} M, 10^{-8} M, and 10^{-7} M were pretreated, caffeine contracted the cells upto 11.7±1.2%, 10.4± 1.0% and 10.4±0.9%, respectively. These contractions were not significantly different from that of the control (Fig. 5).

DISCUSSION

Individual cells digested from detrusor are free from sympathetic, parasympathetic or any other innervation. In this way we excluded the influence of catecholamine inhibition (Axelrode et al, 1961; Sulcer et al, 1978) or central cholinergic activity (Sohn & Kim, 1997).

ACh, a cholinergic muscarinic M_3 receptor agonist in smooth muscle, splits phosphatidylinositol biphosphate to liberate inositol 1,4,5-triphosphate (IP_3) which releases calcium from intracellular store and diacylglycerol (DAG) that activates protein kinase C (PKC) to paticipate in the later functional state (Lefkowitz et al, 1996). Biancani et al (1997) observed that in cat lower esophageal sphinctor muscle, ACh-induced contraction required both intracellular calcium release and extracellular calcium influx linked to phosphoinositide metabolism. In the present study, ACh contracted cells in Ca^{2+}-free PSS, although the contraction was much less than that occured in normal PSS. This may suggest that ACh in canine detrusor cells also utilizes both the extracellular and intracellular calciums. This is in agreement with many reports as followings. ACh evokes both the intracellular calcium release and extracellular calcium influx in guinea pig gallbladder (Ryan, 1985), cat gallbladder (Lee et al, 1989), and kitten gallbladder (Hillemeier et al, 1991), respectively. Sato et al (1994) reported that ACh contracts the smooth muscle of canine colon by the influx of calcium through a voltage-depedent calcium channel and the subsequent release of intracellular stored calcium (calcium-induced release of calcium). In this study, ACh-induced contraction mostly relied on intracellular calcium storage in Ca^{2+}-free PSS. Imipramine inhibited the ACh-induced contraction in normal PSS effectively, but not in Ca^{2+}-free PSS. This suggests that imipramine does not affect the release of intracellular calcium storage but selectively blocks the influx of extracellular calcium. In such a condition, the inhibitory action of imipramine does not seem to originate from its muscarinic receptor blockade action but from a blockade of either calcium channel or from an inhibition of DAG-PKC activation mechanism.

Malkovicz et al (1987) & Lee et al (1992) observed that imipramine possesed competitive calcium antagonism in the smooth muscle strips of urinary bladder isolated from rabbit and rat, respectively. The direct action of imipramine on cells were studied on neuronal cells. Ogata et al (1989) observed that imipramine directly blocked calcium channel current in neuroblastoma cells by patch electrode voltage-clamp techniques. Choi et al (1992) reported that imipramine suppressed the calcium current through L-type calcium channel in cultured murine dorsal root ganglia

cells.

In the present study, we used single cells to evaluate direct action mechanism of imipramine on urinary bladder smooth muscle. In Ca^{2+}-free PSS, imipramine suppressed Ca^{2+}-induced contraction by additional extracellular calcium. This finding suggested that imipramine might block some of the calcium channels on cell membrane, but there are still a couple of assumptions to be ruled out. Those are: 1. If imipramine bound Ca^{2+} extracellularly to reduce the concentration in PSS; and 2. If imipramine inhibited the calcium-induced release of calcium from the intracellular store.

A 23187, a calcium ionophore, binds Ca^{2+} temporarily to reduce its polarity and thereby make it more permeable. A 23187 added concomitantly with Ca^{2+} in Ca^{2+}-free PSS significantly enhanced the calcium-induced contraction, and this contraction was not inhibited by imipramine. This result rules out the possibility of imipramine simply binding Ca^{2+}. Imipramine may block the receptor-operated calcium channel related to cholinergic muscarinic receptors.

Voltage-gated calcium channels are excited by a high concentration of potassium ion (Wei & Chiang, 1986), which depolarizes the cell membrane of smooth muscle (Bhattacharya & Bhattakarya, 1992). In the present study, 40 mM KCl evoked a strong contraction, which was effectively suppressed by pretreatments of imipramine. This result may suggest that imipramine blocks voltage-gated calcium channels.

Imipramine could not prevent the muscle contraction responding to ACh in Ca^{2+}-free PSS where the contraction mostly depended on the release of intracellular stored calcium. It suggests that imipramine could not prevent the calcium release evoked by IP_3 liberated by the stimulation of muscarinic receptor. Beside the IP_3 receptor, a ryanodine sensitive receptor that contributes to the release of the stored calcium (Morel et al, 1997; Oh et al, 1997). Caffeine, an agonist of the ryanodine receptor channel (Kuemmerle et al, 1994), contracted the detrusor cells strongly, but imipramine could not inhibit this contraction.

These results suggest that in canine detrusor muscle, imipramine blocks both the receptor-operated- and voltage-gated-calcium channels on cell membrane to prevent the muscle contraction but does not inhibit the release of calcium from intracellular storage sites.

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