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Regulation of protein kinases in steady-state contraction of cat gastric smooth muscle

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Abstract

Cat gastric smooth muscle strips were used to investigate the involvement of protein kinases in the steady-state contraction induced by 1 μM acetylcholine or 20 mM KCl. The steady-state contraction induced by acetylcholine or KCl was inhibited by EGTA dose dependently. Voltage-dependent Ca^{2+} channel antagonists dose dependently inhibited the contractions induced by KCl as well as by acetylcholine. Inhibitory effects of voltage-dependent Ca^{2+} channel antagonists were significantly more prominent on KCl-induced contractions than on acetylcholine-induced contractions. The acetylcholine-induced contraction was dose dependently inhibited by 8-(*N*,*N*-diethylamino)octyl-3,4,5-trimethoxybenzoate (TMB-8, a blocker of intracellular Ca^{2+} release), but the KCl-induced contraction was not inhibited at all. Therefore both intracellular Ca^{2+} release and extracellular Ca^{2+} influx seem to be necessary for the acetylcholine-induced contraction, but intracellular Ca^{2+} release is not necessary for the KCl-induced contraction. Protein kinase C inhibitors, 10 μM 1-(5-isoquinolinylsulfonyl)-2-methyl-piperazine 2HCl (H-7) and 1 μM staurosporine, significantly inhibited the contraction induced by acetylcholine or KCl. Calmodulin antagonists, 30 μM trifluoperazine and 50 μM *N*-(6-aminohexyl)-5-chloro-2-naphthalenesulfonamide HCl (W-7), however, significantly inhibited the contraction induced by acetylcholine but not by KCl. A tyrosine kinase inhibitor, 50 μM genistein, did not affect the acetylcholine-induced contraction but significantly inhibited the KCl-induced contraction. These results strongly suggest that the involvement of protein kinases in regulation of the steady-state contraction may be agonist-dependent.

Keywords: Ca^{2+} channel antagonist; Protein kinase C; Calmodulin; Tyrosine kinase; Smooth muscle contraction

1. Introduction

Agonists that cause a contraction of smooth muscle generally activate phosphatidylinositol specific phospholipase C, which hydrolyzes phosphatidylinositol 4,5-bisphosphate into inositol 1,4,5-trisphosphate (IP_3) and diacylglycerol (Takuwa et al., 1986; Duncan et al., 1987; Sim et al., 1993). Smooth muscle contraction in response to agonist is thought to be mediated by an increase in the concentration of intracellular free Ca^{2+} via a release of Ca^{2+} from intracellular stores by IP_3 and an influx of extracellular Ca^{2+} (Somlyo et al., 1985; Ozaki et al., 1991; Abdel-Latif et al., 1992; Ohta et al., 1995). An increase in intracellular Ca^{2+} concentration results in the activation of a calmodulin-dependent pathway and protein kinase C (Dillon et al., 1981; Kamm and Stull, 1985; Nishizuka, 1984). Agonist-induced contraction of smooth muscle is significantly inhibited by a variety of Ca^{2+} channel antagonists, calmodulin antagonists and protein kinase C inhibitors (Benham et al., 1987; Chijiiwa et al., 1991; Biancani et al., 1994).

Therefore, a calmodulin-dependent pathway and protein kinase C as well as an increase in intracellular Ca^{2+} concentration are involved in the regulation of smooth muscle contraction. But the Ca^{2+} source and the involvement of protein kinase seem to be different during different phases of contraction: the initial phase of contraction is dependent on both intracellular Ca^{2+} release and the activation of a calmodulin-dependent pathway (Washabau et al., 1994), whereas the steady-state phase is dependent on the influx of extracellular Ca^{2+} and the activation of protein kinase C (Rasmussen et al., 1987).

Recently, many studies have reported that tyrosine kinase is involved in vascular smooth muscle contraction

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(Tsuda et al., 1991; Molloy et al., 1993; Laniyonu et al., 1994). Jinsi and Deth (1995) have shown that tyrosine kinase inhibitors, genistein and erbstatin, inhibit α_2-adrenoceptor-mediated contractions in vascular smooth muscle of rabbits but the involvement of tyrosine kinase in gastric smooth muscle has not been studied. In the present study the Ca^{2+} source and the involvement of protein kinases in steady-state contraction induced by acetylcholine or KCl were investigated in cat gastric smooth muscle.

2. Materials and methods

2.1. Materials

Verapamil, nifedipine, diltiazem, acetylcholine bromide, 8-(*N*,*N*-diethylamino)octyl-3,4,5-trimethoxybenzoate (TMB-8), EGTA, atropine, 1-(5-isoquinolinylsulfonyl)-2-methyl-piperazine 2HCl (H-7), staurosporine, *N*-(6-aminohexyl)-5-chloro-2-naphthalenesulfonamide HCl (W-7), trifluoperazine and genistein were purchased from Sigma (St. Louis, MO, USA). All other reagents used were analytical grade.

2.2. Preparation of gastric smooth muscle strips

Cats of either sex (2.0–3.4 kg) were anesthetized with 20% urethane (5 ml/kg, intraperitoneal) following 16 h of fasting but with water ad libitum. The whole stomach was removed from each cat, and the mucous membrane was peeled off in ice-cold Krebs bicarbonate solution (mM: 120.8 NaCl, 4.5 KCl, 15.5 $NaHCO_3$, 1.8 $CaCl_2$, 1.2 $MgCl_2$, 1.2 NaH_2PO_4 and 5.6 dextrose). The Krebs bicarbonate solution was aerated with 95% O_2-5% CO_2 until the pH was 7.4. Circular muscle strips (1.0×0.2 cm) were prepared from the fundus, cutting at a right-angle to the greater curvature (Mayer et al., 1982).

2.3. Measurement of contractile response

The circular muscle strips were used to measure the contraction in a cylinder-shaped muscle chamber (10 ml capacity) filled with Krebs bicarbonate solution. The solution of the chamber was kept at 37°C and was bubbled with a mixture of 95% O_2/5% CO_2 at pH 7.4. To record the isometric contraction, the lower end of the muscle preparation was anchored to a steel hook and the upper end to a force transducer (FT.03, Grass Instruments, Quincy, MA, USA) connected to a Grass 7E polygraph. The preparation was loaded with a tension of 2.0 g and allowed to equilibrate with the solution for 30 min. The final concentrations of agonist, antagonists or inhibitors used were achieved by adding 0.01 ml to the chamber. The steady-state contraction induced by 1 μM acetylcholine or 20 mM KCl (an equimolar concentration of NaCl in Krebs bicarbonate solution was replaced by KCl; the solution also contained 1 μM tetrodotoxin to prevent the release of neurotransmitters from myenteric plexus) was maintained for more than 2 h. The steady-state contraction immediately declined to the basal tone after strips were washed with Krebs bicarbonate solution or after addition of 1 μM atropine or 2 mM EGTA.

To investigate the inhibitory effects of Ca^{2+} channel antagonists or protein kinase inhibitors on the steady-state contraction, these agents were added to the muscle chamber after 10 min of steady-state contraction induced by acetylcholine or KCl. The relaxation induced by Ca^{2+} channel antagonists or protein kinase inhibitors was measured for another 10 min and finally complete relaxation was induced by adding 1 μM atropine to the bath during the acetylcholine-induced contraction or 2 mM EGTA during the KCl-induced contraction. The relaxation induced by each agent was expressed as a percentage of the maximum relaxation obtained after treatment with 1 μM atropine or 2 mM EGTA (Fig. 1A).

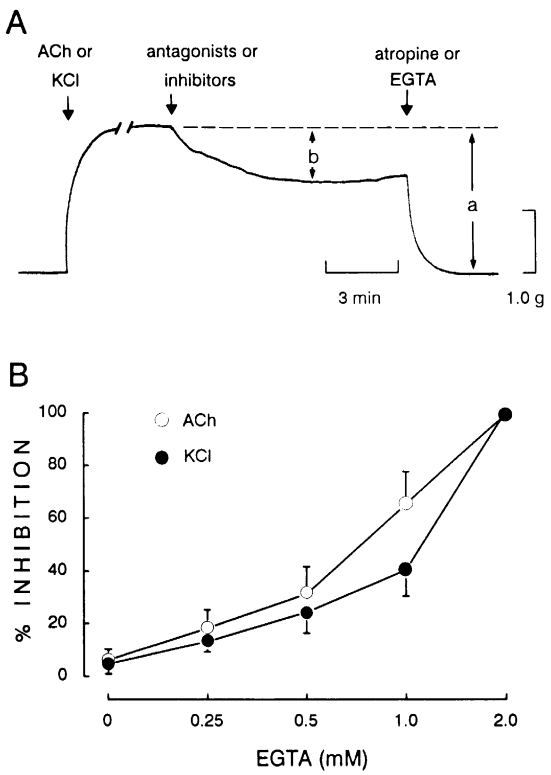

Fig. 1. Isometric contraction of cat gastric smooth muscle strips. (A) Tonic contraction was induced by acetylcholine (ACh, 1 μM) or 20 mM KCl. The relaxation (*b*) induced by Ca^{2+} channel antagonists or protein kinase inhibitors is expressed as percentage inhibition ($b/a \times 100$) of the maximum relaxation (*a*) obtained after treatment with 1 μM atropine or 2 mM EGTA. (B) EGTA dose-response curves of steady state contraction induced by 1 μM acetylcholine or 20 mM KCl. The results indicate that steady-state contraction may depend on the extracellular Ca^{2+} concentration. Results are means ± S.D. of eight experiments. Responses were recorded 10 min after addition of EGTA.

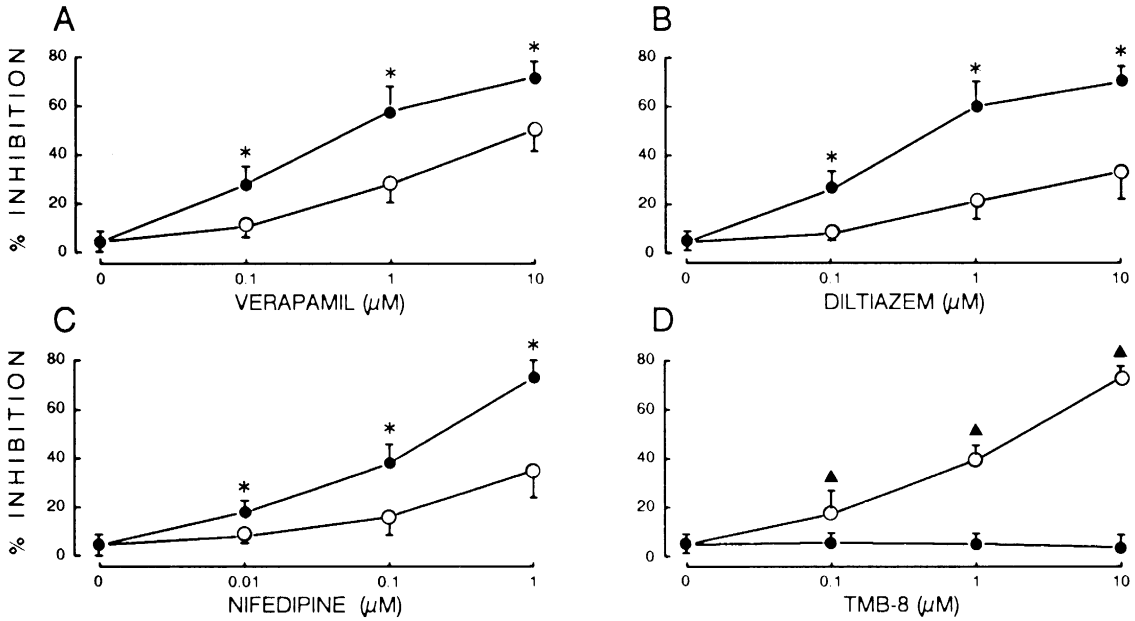

Fig. 2. The effects of Ca^{2+} channel antagonists on steady-state contraction induced by 1 μM acetylcholine (○) or 20 mM KCl (●). Voltage-dependent Ca^{2+} channel antagonists, verapamil, diltiazem and nifedipine, dose dependently inhibited acetylcholine- and KCl-induced contractions. Such an inhibitory effect of voltage-dependent Ca^{2+} channel antagonist was significantly more prominent on KCl-induced contraction than on ACh-induced contraction. TMB-8, a blocker of intracellular Ca^{2+} release, dose dependently inhibited acetylcholine-induced contraction but not KCl-induced contraction. Results are means ± S.D. of eight experiments. Responses were recorded 10 min after addition of Ca^{2+} channel antagonists. * $P < 0.05$ vs. ACh-induced contraction. ▲ $P < 0.05$ vs. KCl-induced contraction.

2.4. Statistical analysis

The results are presented as means ± S.D. and analyzed statistically by analysis of variance (ANOVA), and differences between groups were determined with Newman-Keuls test. The level of significance was set at 5%.

3. Results

3.1. Effect of extracellular Ca^{2+} concentration

As shown in Fig. 1A, when gastric smooth muscle strips were exposed to 1 μM acetylcholine or 20 mM KCl, a prompt contractile response (2.4 ± 0.3 g) was observed and then a steady-state contraction was maintained for more than 2 h. EGTA dose dependently relaxed both acetylcholine- and KCl-induced contractions (Fig. 1B), suggesting that the maintenance of steady-state contraction is dependent on the concentration of extracellular Ca^{2+}.

3.2. Effects of Ca^{2+} channel antagonists

Since the maintenance of steady-state contraction required the influx of extracellular Ca^{2+}, we used three structurally different Ca^{2+} channel antagonists, verapamil, nifedipine and diltiazem (Catterall and Striessnig, 1992), to investigate whether this influx was mediated via a voltage-dependent Ca^{2+} channel (VDCC). Verapamil, diltiazem and nifedipine dose dependently inhibited both acetylcholine- and KCl-induced contractions. The inhibitory effects of VDCC antagonists were significantly greater on the KCl-induced contraction than on the acetylcholine-induced contraction (Fig. 2A,B,C). TMB-8, a blocker of intracellular Ca^{2+} release, also dose dependently inhibited the acetylcholine-induced contraction but TMB-8 did not inhibit the KCl-induced contraction at all (Fig. 2D). The combined inhibitory effect of TMB-8 and verapamil was significantly greater than that of verapamil or TMB-8 alone on the acetylcholine-induced contraction (Fig. 3).

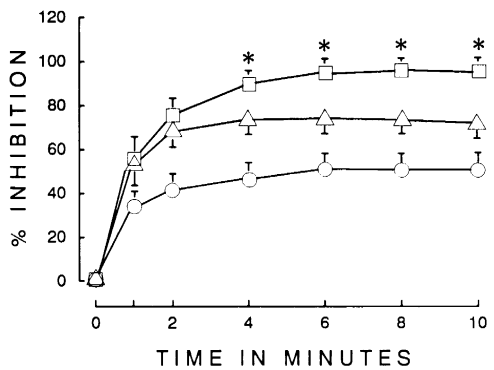

Fig. 3. The combined effect of verapamil and TMB-8 on steady state contraction induced by 1 μM acetylcholine. The combined inhibitory effect of VP and TMB-8 (□) was significantly greater than that of verapamil (○, 10 μM) or TMB-8 (△, 10 μM) alone. Results are means ± S.D. of eight experiments. * $P < 0.05$ vs. VP or TMB-8 alone.

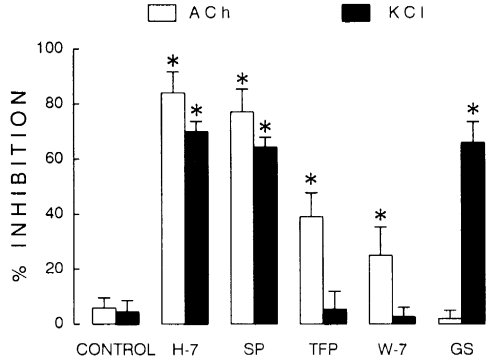

Fig. 4. The effects of protein kinase inhibitors on steady-state contraction induced by 1 μM acetylcholine or 20 mM KCl. Control was obtained after adding 0.1% dimethyl sulfoxide (DMSO). Protein kinase C inhibitors, H-7 (10 μM) and staurosporine (SP, 1 μM), significantly inhibited acetylcholine- or KCl-induced contraction, while the calmodulin antagonists, W-7 (50 μM) and trifluoperazine (TFP, 30 μM), partly inhibited acetylcholine-induced contraction but did not inhibit KCl-induced contraction. Tyrosine kinase inhibitor, genistein (GS, 50 μM) did not have any effect on acetylcholine-induced contraction but significantly inhibited KCl-induced contraction. Results are means ± S.D. of eight experiments. Responses were recorded 10 min after addition of protein kinase inhibitors. * $P < 0.05$ vs. control.

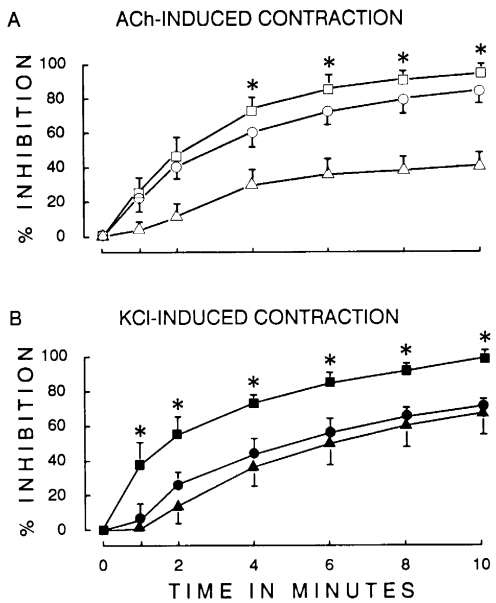

Fig. 5. The combined effects of protein kinase inhibitors on steady-state contraction induced by 1 μM acetylcholine or 20 mM KCl. (A) For the ACh-induced contraction, the combined inhibitory effect of H-7 and trifluoperazine (□) was significantly greater than that of H-7 (○, 10 μM) or trifluoperazine (△, 30 μM) alone and completely inhibited the contraction, suggesting that both protein kinase C and the calmodulin-dependent pathway may be involved in the steady-state contraction. (B) For the KCl-induced contraction, the combined inhibitory effect of H-7 and genistein (■) was significantly greater than that of H-7 (●, 10 μM) or genistein (▲, 50 μM) alone and completely inhibited the contraction, suggesting that both protein kinase C and tyrosine kinase may be involved in the steady-state contraction. Results are means ± S.D. of eight experiments. * $P < 0.05$ vs. H-7, trifluoperazine or genistein alone, respectively.

3.3. Effects of protein kinase inhibitors

To investigate what kind of protein kinase is involved in the maintenance of the steady-state contraction of smooth muscle, we used a variety of protein kinase inhibitors (a protein kinase C inhibitor, a calmodulin antagonist and a tyrosine kinase inhibitor). Unfortunately, we rarely observed the significant effects of H-7 and staurosporine at a concentration of 1 μM and 100 nM, respectively (data not shown). However, the protein kinase C inhibitors (10 μM H-7 and 1 μM staurosporine) significantly inhibited the acetylcholine-induced contraction by 70–80% and the calmodulin antagonists (50 μM W-7 and 30 μM trifluoperazine) inhibited it by 30–40%. But a tyrosine kinase inhibitor (50 μM genistein) did not inhibit the contraction at all (Fig. 4). The combined inhibitory effect of H-7 and trifluoperazine on acetylcholine-induced contraction was greater than that of H-7 and trifluoperazine alone (Fig. 5A). Both protein kinase C inhibitors and tyrosine kinase inhibitor significantly inhibited the KCl-induced contraction by 70% and 66%, respectively, but calmodulin antagonists did not inhibit it at all (Fig. 4). The combined inhibitory effect of H-7 and genistein on the KCl-induced contraction was greater than that of H-7 and genistein alone (Fig. 5B).

4. Discussion

Both acetylcholine and KCl caused contraction and maintained the steady-state contraction for more than 2 h. EGTA dose dependently inhibited the contraction, with complete inhibition occurring at a concentration of 2 mM. These results indicate that the steady-state contraction induced by acetylcholine or KCl is dependent on the influx of extracellular Ca^{2+}.

To determine the involvement of the VDCC in the influx of extracellular Ca^{2+}, three structurally different VDCC antagonists (verapamil, diltiazem and nifedipine) were used. All of the VDCC antagonists dose dependently inhibited acetylcholine-induced contractions, but the inhibitory effects of VDCC antagonists at high concentrations of 1–10 μM did not exceed 50%. Therefore some kind of Ca^{2+} channel, other than VDCC, may be involved in the influx of extracellular Ca^{2+}. TMB-8, a blocker of intracellular Ca^{2+} release, dose dependently inhibited acetylcholine-induced contraction; the inhibitory effect of TMB-8 was greater than that of the VDCC antagonists. The combined inhibitory effect of TMB-8 and verapamil was greater than that of TMB-8 or verapamil alone and was similar to that of 2 mM EGTA. These results indicate that both intracellular Ca^{2+} release and the influx of extracellular Ca^{2+} are necessary for the maintenance of the steady-state contraction induced by acetylcholine, which substantiates that acetylcholine- and cholecystokinin-induced contractions rely on both extracellular

Ca^{2+} and intracellular Ca^{2+} stores in guinea pig gall bladder smooth muscle (Renzetti et al., 1990; Shaffer et al., 1992). Nevertheless, the steady-state contraction induced by acetylcholine was completely inhibited in the presence of 2 mM EGTA. The inhibitory effect of TMB-8 was more potent than that of VDCC antagonists.

To explain the difference in the inhibitory effects, the capacitative model proposed by Putney (1993) was used. The resulting IP_3, produced by acetylcholine, released Ca^{2+} from intracellular stores and resulted in the depletion of intracellular Ca^{2+} stores. The depletion of intracellular stores induced the influx of extracellular Ca^{2+}. The inhibitory effect of TMB-8 may be due to a block of both intracellular Ca^{2+} release and the influx of extracellular Ca^{2+} via some Ca^{2+} channel other than VDCC, thereby preventing the depletion of intracellular Ca^{2+} stores. VDCC antagonists also dose dependently inhibited the KCl-induced contraction, whereas TMB-8 did not. This finding further supports the above results of the inhibitory action of TMB-8 on acetylcholine-induced contraction.

A variety of protein kinases including those of the calmodulin-dependent pathway and protein kinase C play an important role in the regulation of smooth muscle contraction. Recently, tyrosine kinase has been found to be related to the contraction of vascular smooth muscle (Laniyonu et al., 1994; Jinsi and Deth, 1995). It has been reported that H-7 and staurosporine inhibited other protein kinases including cAMP- and cGMP-dependent protein kinases or tyrosine kinase as well as protein kinase C, and that IC_{50} values for H-7 and staurosporine are 4.5 μM and 30 nM, respectively (Hidaka et al., 1984; Rüegg and Burgess, 1989; Herbert et al., 1990). Since a high concentration of H-7 (10 μM) or staurosporine (1 μM) was used in the present study, we can not exclude the possible actions of H-7 and staurosporine on other protein kinases. Nevertheless, other investigators have used such high concentrations of H-7 and staurosporine to inhibit protein kinase C activity (Sasaki et al., 1991; Biancani et al., 1994; Sohn et al., 1995; Yang and Black, 1995).

Protein kinase C inhibitors (H-7 and staurosporine) significantly inhibited the acetylcholine-induced contraction by 70% and the calmodulin antagonists (W-7 and trifluoperazine) slightly inhibited it by 30–40%, but the tyrosine kinase inhibitor (genistein) did not inhibit the contraction at all. The combined inhibitory effects of two agents (H-7 and trifluoperazine) were greater than the effect of each one. These results suggest that both protein kinase C and calmodulin-dependent pathway appear to be involved in the maintenance of acetylcholine-induced contraction. Both protein kinase C inhibitors and tyrosine kinase inhibitor significantly inhibited the KCl-induced contraction whereas calmodulin antagonists did not. The combined inhibitory effects of two agents (H-7 and genistein) were greater than the effect of each one, which suggests that both protein kinase C and tyrosine kinase are involved in the maintenance of KCl-induced contraction. There is much evidence that protein kinase C plays an important role in the maintenance of steady-state contraction (Rasmussen et al., 1987; Sato et al., 1992; Yang and Black, 1995). Our results also showed that protein kinase C is mainly involved in the maintenance of steady-state contraction induced by acetylcholine or KCl. In addition to the involvement of protein kinase C, a calmodulin-dependent pathway and tyrosine kinase may be partly involved in the maintenance of the steady-state contraction induced by acetylcholine and KCl, respectively. These data suggest that the participation of protein kinase in maintaining steady-state contraction may be agonist-dependent.

It is of interest that both TMB-8 and calmodulin antagonists had a significant influence on acetylcholine-induced contraction but had no effect on KCl-induced contraction. Hillemeier et al. (1991) have reported that calmodulin antagonists block contraction mediated by the release of intracellular Ca^{2+} induced by acetylcholine or IP_3. Also Hill et al. (1988) have shown that calmodulin antagonists inhibit IP_3-induced Ca^{2+} release. The above results, together, suggest that there is a close interaction between intracellular Ca^{2+} release and a calmodulin-dependent pathway in cat gastric smooth muscle contraction.

In summary, both protein kinase C and a calmodulin-dependent pathway regulate the maintenance of the steady-state contraction induced by acetylcholine, for which both intracellular Ca^{2+} release and extracellular Ca^{2+} influx may be necessary. However, both protein kinase C and tyrosine kinase regulate the maintenance of the KCl-induced steady-state contraction under the influence of extracellular Ca^{2+} influx, rather than intracellular Ca^{2+} release.

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