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Protein Kinase C Mediates Increase of Ca^{2+} Sensitivity for Contraction by Cholinoceptor Partial Agonist in Ileal Longitudinal Muscle of Guinea Pig

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ABSTRACT. 1. Experiments were designed to study the roles of protein kinase C in carbachol- and pilocarpine-induced contraction and the increase in cytosolic Ca^{2+} concentration ($[Ca^{2+}]_i$) in guinea pig ileal longitudinal muscle.

2. The protein kinase C inhibitors, GF 109203X (10 μM), calphostin C (10 μM) and H-7 (10 μM), reduced the maximum of the concentration response curve produced by pilocarpine more effectively than that produced by carbachol.

3. The slopes of the regression lines between $[Ca^{2+}]_i$ and tension development for pilocarpine and carbachol in tissues treated with GF 109203X were significantly gentler than those for untreated tissues.

4. The protein kinase C_α- and β_1 selective inhibitor Goe 6976 (1 μM) decreased both $[Ca^{2+}]_i$ and contraction, but did not affect the slopes of the regression lines for pilocarpine and carbachol.

5. These results suggest that protein kinase C (both n- and/or a-type) plays an important role in the increase of Ca^{2+} sensitivity of the contractile element, and that pilocarpine mainly activates the protein kinase C–dependent pathways for contractile mechanisms in guinea pig ileal longitudinal muscle.
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KEY WORDS. Protein kinase C, Ca^{2+} sensitivity, contraction, ileal longitudinal muscle

INTRODUCTION

Hisayama *et al.* (1988) and Takayanagi *et al.* (1989, 1990, 1991b) have reported that, in taenia caecum and ileum longitudinal smooth muscle of guinea pig, there are subtypes of muscarinic receptors, propylbenzilylcholine mustard (PrBCM)-sensitive and -insensitive receptors, which respond differently to PrBCM. The cholinoceptor partial agonist, pilocarpine, induces the contraction of intestinal smooth muscle predominantly through activation of PrBCM-sensitive receptors, whereas full agonists, which induce contraction through interaction with both subtypes, have greater efficacy for PrBCM-insensitive receptors than for PrBCM-sensitive receptors (Takayanagi *et al.*, 1989). Furthermore, PrBCM-sensitive cholinoceptors utilize cytosolic Ca^{2+} for contraction more effectively than PrBCM-insensitive receptors in fura-2-loaded guinea pig intestinal smooth muscle (Takayanagi *et al.*, 1991a). In addition to these findings, the potential involvement of protein kinase C in the regulation of smooth muscle contraction has been suggested by the observation that phorbol esters, which specifically interact with and activate protein kinase C, produce sustained contraction of smooth muscle and increase its Ca^{2+} sensitivity for contraction, especially in the case of vascular smooth muscle (Jiang and Morgan, 1987, 1989; Kokubu *et al.*, 1995; Satoh *et al.*, 1995; Walsh *et al.*, 1994). Recently, several protein kinase C isoforms (c-, n- and a-types) have been identified by differentiating their requirements for Ca^{2+} and lipids. The c-protein kinase C family is divided into α, β_1, β_2 and γ forms, which are synergistically stimulated by diacylglycerol, phosphatidylserine, and Ca^{2+} (Marais and Parker, 1989; Nishizuka, 1988; Parker *et al.*, 1989). On the other hand, the n-protein kinase C family is divided into δ, ϵ, η and θ forms, which are dependent solely on the presence of lipid (Ohno *et al.*, 1988, 1989; Osada *et al.*, 1990; Schaap and Parker, 1990). Finally, the a-protein kinase C family is divided into ζ and λ forms, which have an absolute requirement for phospholipid, but lack Ca^{2+} dependence and are unaffected by diacylglycerol or phorbol ester (Nishizuka, 1992). The potent protein kinase C inhibitor, GF 109203X, inhibits all these isoforms, whereas the novel protein kinase C inhibitor, Goe 6976, inhibits the Ca^{2+}-dependent isoforms α and β_1, but has no effect on the kinase activity of the Ca^{2+}-independent protein kinase C isoforms δ, ϵ and ζ.

In this study, we used protein kinase C isoform-specific inhibitors to examine the possibility that activation of protein kinase C is involved in the Ca^{2+} sensitization mediated by PrBCM-sensitive cholinoceptors, and to look for differences in the intracellular mechanisms of the contractile responses involving novel subtypes (PrBCM-sensitive and -insensitive) with partial and full agonists in guinea pig ileal longitudinal muscle strips.

MATERIALS AND METHODS

Tissue preparation and physiological solution

Male guinea pigs, weighing 250–350 g, were killed by a blow on the head. A longitudinal muscle strip was isolated by carefully slipping an ileal segment over a tapered glass rod. A piece (about 3 cm) of strip was suspended in a 20-ml organ bath filled with a physiological solution of the following composition: 118 mM NaCl, 1.2 $MgCl_2$, 2.5 $CaCl_2$, 1.2 KH_2PO_4, 25.0 $NaHCO_3$ and 11.0 glucose dissolved in distilled water (pH 7.4 at 37°C). Responses to agonists were isometrically recorded under a tension of 0.7 g. Concentration–response

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curves of agonists were obtained cumulatively. After determination of control concentration–response curves, the strips were equilibrated with a protein kinase C inhibitor for 30 min, and then concentration–response curves were obtained in the presence of the inhibitor.

Measurement of cytosolic Ca^{2+} in fura-2-loaded preparation

The strips were incubated with 5 μM fura-2/AM in normal PSS for 4 hr at room temperature in the presence of 0.2% Cremophor EL, then rinsed with the same medium for 15 min thereafter, experiments were performed with a double wavelength excitation fluorimeter (CAF-100, Japan Spectroscopic, Tokyo). The mechanical activity was monitored isometrically and, simultaneously, the ratio of 500-nm fluorescence emitted by 340-nm excitation (F340) to that by 380-nm excitation (F380) was calculated automatically from successive illumination periods (48 Hz) and referred to as $R_{340/380}$. In the muscle strips successfully loaded with fura-2, the increase in cytosolic Ca^{2+} level resulted in an increase in F340, a decrease in F380 and an increase in $R_{340/380}$. The $R_{340/380}$ was used to monitor the relative cytosolic Ca^{2+} level, $[Ca^{2+}]_i$.

Statistics

Numerical results are expressed as means±SE, and statistical significance was calculated by Student's *t*-test or Duncan's new multiple range test. $P<0.05$ was considered to indicate a significant difference.

Drugs

The following drugs and chemicals were used: carbachol chloride, pilocarpine hydrochloride (Sigma Chemical Co., St. Louis, MO), 1-(5-isoquinolinesulfonyl)-2-methylpiperazine dihydrochloride (H-7) (Seikagaku Co, Tokyo), 2-(1-(3-dimethylaminopropyl)-indol-3-yl)-3-(-indol-3-yl)-maleimide (GF 109203X; Wako Pure Chemical, Osaka, Japan), Goe 6976 (Calbiochem), fura-2 pentaacetoxymethylester (fura-2/AM), ethyleneglycolbis(β-aminoethylether)*N*,*N*′-tetraacetic acid (EGTA), *N*-2-hydroxyethylpiperazine-*N*′-ethanesulphonic acid (HEPES), *N*,*N*,*N*′,*N*′-tetrakis(2-pyridylmethyl) ethylenediamine (TPEN) (Dojindo Laboratories, Kumamoto, Japan) and Cremophor EL (Nacalai Tesque, Kyoto, Japan). Other chemicals used were of analytical grade.

RESULTS

Effects of GF 109203X, calphostin C and H-7 on the contraction induced by carbachol and pilocarpine

In guinea pig ileal longitudinal muscle, carbachol and pilocarpine elicited concentration-dependent contraction. pD_2 values estimated from the concentration–response curves for these agonists were 7.26±0.03 and 6.24±0.02, respectively, and the intrinsic activity (i.a.) of pilocarpine was 0.91±0.21. The inhibitory effects of protein kinase C inhibitors on the contraction produced by carbachol and pilocarpine are shown in Figure 1. GF 109203X, calphostin C and H-7 shifted the concentration–response curves for carbachol and pilocarpine slightly to the right and reduced the maximum contraction. The magnitudes of inhibition of pilocarpine-induced contraction by these inhibitors were significantly greater than those of carbachol-induced contractions (Table 1).

Effects of GF 109203X and Goe 6976 on $[Ca^{2+}]_i$–tension relationship in fura-2-loaded strips

Carbachol and pilocarpine caused a rapid increase in $[Ca^{2+}]_i$ ($R_{340/380}$) and tension in fura-2-loaded longitudinal muscle. Both these responses were increased by both agonists in a concentration-dependent manner. High K^+ PSS at 70 mM also caused a rapid increase in $[Ca^{2+}]_i$ ($R_{340/380}$) and muscle tension, expressed as a percentage of that induced by 70 mM K^+. As shown in Figure 2, there was a positive correlation between $[Ca^{2+}]_i$ ($R_{340/380}$) and tension. A regression line between $[Ca^{2+}]_i$ ($R_{340/380}$) and tension was obtained from all the points and the zero point for each agonist. The slopes of the regression lines were 1.32±0.048 (r^2=0.979, n=6) for carbachol and 2.88±0.216 (r^2=0.984, n=5) for pilocarpine. The slope for pilocarpine was significantly steeper than that for carbachol, indicating that $[Ca^{2+}]_i$ utilization during pilocarpine-induced contraction was greater than that during carbachol-induced contraction. The slopes of the regression lines in the presence of GF 109203X (10 μM) were 0.858±0.090 (r^2=0.968, n=5) for carbachol and 0.746±0.098 (r^2=0.951, n=5) for pilocarpine. The slopes obtained in the presence of protein kinase C inhibitors were significantly gentler than those obtained in their absence. Although the carbachol-induced increase of $[Ca^{2+}]_i$ tended to be reduced by the presence of Goe 6976, as shown in Figure 2B, the regression lines obtained for carbachol and pilocarpine were not affected by Goe 6976.

DISCUSSION

Takayanagi *et al.* (1989), using the irreversible cholinoceptor blocking agent, PrBCM, characterized two distinct M3-cholinoceptor subtypes (PrBCM-sensitive and -insensitive) in guinea pig intestinal smooth muscle. They demonstrated that the full agonists, acetylcholine and carbachol, induce contraction through both subtypes, whereas the partial agonist, pilocarpine, acts only through the PrBCM-sensitive subtype. In the present experiment, the magnitudes of inhibition of pilocarpine-induced contraction by the potent protein kinase C inhibitor, GF 109203X, which has an IC_{50} value of 0.07 μM (Toullec *et al.*, 1991), and calphostin C, which has an IC_{50} value of 0.05 μM (Kobayashi *et al.*, 1989), were significantly greater than the inhibition of carbachol-induced contraction. These observations indicate that pilocarpine produces a contractile response predominantly through activation of protein kinase Cs, and that the PrBCM-sensitive cholinoceptor subtype activates the protein kinase C–dependent pathway in ileal longitudinal muscle. It is suggested that the PrBCM-sensitive cholinoceptor activates mainly the n- or a-type protein kinase C isoform-dependent pathway in muscle contraction. In smooth muscle cells, expression of various protein kinase C isoforms has been reported (Assender *et al.*, 1994; Ohanian *et al.*, 1996; Ohno *et al.*, 1988), and these may have distinct functions related to the regulation of smooth muscle contraction. In vascular smooth muscle, Ca^{2+}-dependent isoenzymes (α, β_1 and β_2) are involved in Ca^{2+}-dependent contraction (Singer *et al.*, 1992), and a Ca^{2+}-independent isoenzyme (ε) is involved in the Ca^{2+}-independent translocation of protein kinase Cε from the cytosol to the sarcolemma (Khalil *et al.*, 1992), suggesting that receptor stimulation induces Ca^{2+} sensitization of the contractile apparatus through activation of protein kinase C isoforms in smooth muscle.

In the present study, the slope of the regression line between $[Ca^{2+}]_i$ and tension development for pilocarpine was significantly steeper than that for carbachol (Fig. 2), in accord with previous findings reported by Takayanagi *et al.* (1991a). Furthermore, in the presence of GF 109203X, the slopes of the regression lines were gentler than those in the absence of protein kinase C inhibitors. These observations suggest that the contractile pathway operated through the PrBCM-sensitive cholinoceptor, which is activated by pilocarpine, more effectively utilizes cytosolic Ca^{2+} for contraction than the pathway operated through the PrBCM-insensitive cholinocep-

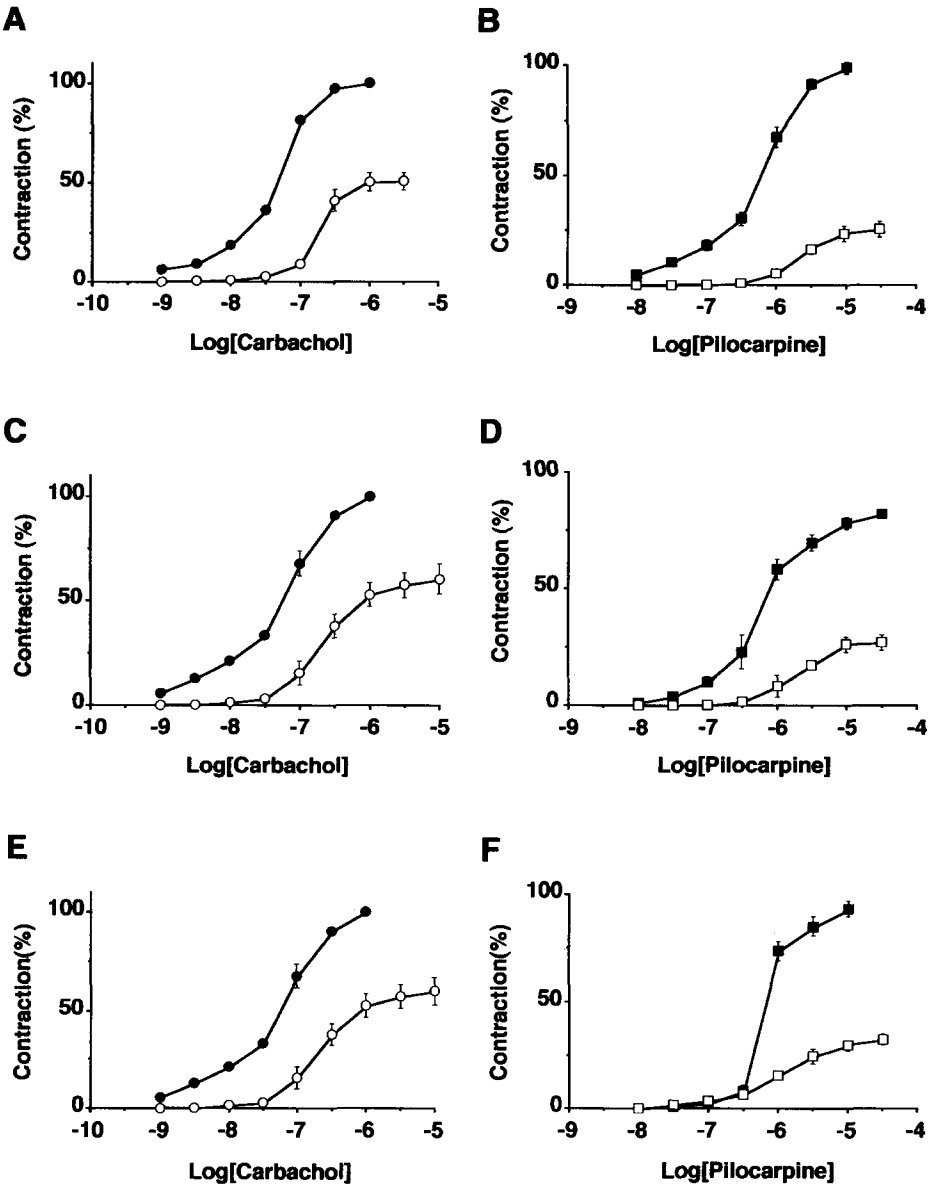

FIGURE 1. Inhibitory effects of GF 109203X (A, B), calphostin C (C, D) and H-7 (E, F) on concentration–response curves of carbachol and pilocarpine in guinea pig ileal longitudinal muscle. Ordinate: contraction (%) which is expressed as a percentage of the contractile response to carbachol (10^{-6} M); abscissa: logarithm of drug concentration (M). (●) Agonist alone; (○) calphostin C (10 μM), GF 109203X (10 μM) or H-7 (10 μM). Each point represents the mean±SE (bar) of four experiments.

tor, which is activated by carbachol. Protein kinase C causes an increase of $[Ca^{2+}]_i$ or Ca^{2+} influx through activation of Ca^{2+} channels (Xuan *et al.*, 1994). These mechanisms are important for the agonist-induced sustained and high K^+-induced contraction of smooth muscle such as that in the ileum and arteries. In the present study, Goe 6976, which has an IC_{50} value of 6.2 nM (Martiny-Baron *et al.*, 1993), reduced the elevation of sustained $[Ca^{2+}]_i$ and the sustained contraction, suggesting that the activation of protein kinase C may be inhibited by Goe 6976 and that intracellular Ca^{2+} concentration is regulated by Goe 6976–sensitive protein kinase C isoforms. In addition to these Ca^{2+}-related mechanisms, agonist-induced activation of protein kinase C can induce strong and long-lasting contraction at the low Ca^{2+} concentration. Satoh *et al.* (1992a, b, 1994) and Kokubu *et al.* (1995) reported that, in rabbit thoracic aorta, α_{1A}-

TABLE 1. Effects of GF 109203X, calphostin C and H-7 on pEC_{50} and maximum response of carbachol or pilocarpine

	Carbachol		Pilocarpine	
	pEC_{50}	Contraction (%)	pEC_{50}	Contraction (%)
Untreated	7.26 ± 0.03 (12)	100	6.24 ± 0.02 (17)	100
GF 109203X	6.74 ± 0.03 (4)	47.9 ± 4.59[a]	5.74 ± 0.04 (6)	26.2 ± 3.42[a']
Calphostin C	6.63 ± 0.07 (4)	60.2 ± 7.07[b]	5.56 ± 0.20 (4)	32.5 ± 3.90[b']
H-7	6.97 ± 0.10 (4)	41.5 ± 2.34[c]	6.00 ± 0.09 (4)	33.5 ± 2.28[c']

[a-a', b-b', c-c']Significantly different from each other ($P < 0.05$).

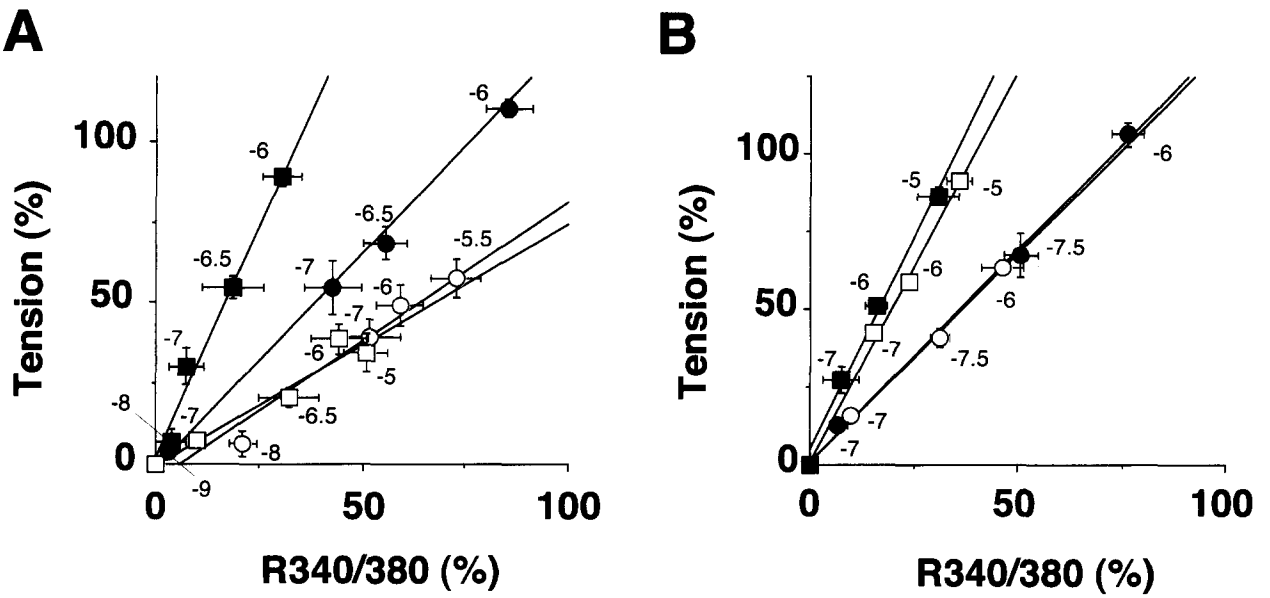

FIGURE 2. Effects of GF 109203X (A) and Goe 6976 (B) on the relationship between $R_{340/380}$ and tension development in the presence of various concentrations of carbachol and pilocarpine. (●) Carbachol; (○) carbachol with GF 109203X (10 μM) or Goe 6976 (1 μM); (■) pilocarpine; (□) pilocarpine with GF 109203X (10 μM) or Goe 6976 (10 μM). Ordinate and abscissa: tension (%) and $[Ca^{2+}]_i$ ($R_{340/380}$), of which 100% represents a 70 mM K^+-induced increase. Each number shown is the negative logarithm of the concentration used. Each point represents the mean±SE of four experiments.

and α_{1B}-adrenoceptor subtypes were activated by norepinephrine and that the Ca^{2+} sensitization produced by α_{1A} subtypes was mediated through G-protein and protein kinase C. Nishimura and Van Breemen (1989) provided evidence that receptor mediated GTP-binding protein-coupled activation of protein kinase C increases the Ca^{2+} sensitivity of contractile elements. Karaki (1989) also stated that the contractility of smooth muscle induced by receptor stimulants is regulated not only by the increase in $[Ca^{2+}]_i$ but also by increases in the Ca^{2+} sensitivity of contractile elements in the ileum and arteries, and this hypothesis was supported by Takayanagi *et al.* (1997) and other investigators (Hirata *et al.*, 1992; Hori *et al.*, 1992; Kitazawa *et al.*, 1991). As shown in Figure 2, the decrease in the slopes of the $[Ca^{2+}]_i$–tension relationship with the use of protein kinase C inhibitors is due to the decrease in Ca^{2+} sensitivity involving the inhibition of protein kinase C upon contraction. According to our finding that Goe 6976 selectively inhibits the Ca^{2+}-dependent isoforms α and β_1, whereas Goe 6976 at micromolar concentration has no effect on the kinase activity of the Ca^{2+}-independent protein kinase C isoforms δ, ε and ζ, the protein kinase C isoforms activated by stimulation through PrBCM-insensitive cholinoceptors are Goe 6976 sensitive (α and β_1 isoforms). Calphostin C binds the cystein-rich regulatory domain of protein kinase C, and inhibits c- and n-protein kinase C isoforms. Also, H-7, an inhibitor of the catalytic domain of protein kinase C, inhibits all isoforms (Fabbri *et al.*, 1994). The concentration–response curves for carbachol and pilocarpine were inhibited by the application of GF 109203X, calphostin C and H-7, and Ca^{2+} utilization in agonist-induced contraction was also inhibited by GF 109203X. These findings lead to the suggestion that Goe 6976–sensitive (α and β_1 isoforms) may control Ca^{2+} concentration in smooth muscle cells, whereas other isoforms may regulate Ca^{2+} sensitivity of muscle contraction induced through cholinoceptor. Determination of the protein kinase C isoforms for the regulation of intracellular Ca^{2+} concentration and Ca^{2+} sensitivity in agonist-induced contraction of ileum smooth muscle will require further experiments using more selective tools and/or α-toxin-permeabilized muscle preparations.

In conclusion, the present results suggest that activation (induced by pilocarpine) of PrBCM-sensitive cholinoceptor subtypes predominantly increases the Ca^{2+} sensitivity of contractile elements involving protein kinase Cs, and that intracellular Ca^{2+} concentration is regulated by Goe 6976–sensitive protein kinase C isoforms, which are activated through PrBCM-insensitive subtypes.

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