Carbachol but Not Acetylcholine Inhibits Contraction by the Protein Kinase C-Dependent and -Independent Pathways in the Smooth Muscle of Guinea Pig Taenia Caeci

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ABSTRACT—In the intestinal smooth muscle of guinea pig taenia caeci, acetylcholine and carbachol induced a transient contraction followed by a sustained contraction. The magnitudes of the transient and sustained contractions were similar when muscle was stimulated with acetylcholine (0.1 μM−1 mM) or a lower concentration (0.1 μM) of carbachol. However, higher concentrations of carbachol (1−100 μM) induced significantly smaller sustained contraction than the transient contraction. In the 45 mM KCl-stimulated strips, addition of 100 μM carbachol induced a transient increase followed by a sustained decrease in the contractile tension. In contrast, acetylcholine (0.1 μM−1 mM) showed only weak inhibitory effects on the high K^+-induced contraction either in the absence or presence of a cholinesterase inhibitor, 0.5 μM diisopropylfluorophosphate. The same concentration of diisopropylfluorophosphate shifted the concentration-response curve for acetylcholine to lower concentrations. In the muscles pretreated with 3 μM phorbol 12-myristate 13-acetate for 24 hr to desensitize protein kinase C, sustained contractions induced by higher concentrations of carbachol (1−100 μM) were significantly greater than those in the strips without the treatment with phorbol ester. However, the transient contraction and the contraction induced by a lower concentration (0.1 μM) of carbachol were not changed by the treatment with phorbol ester. Pretreatment with phorbol ester attenuated the inhibitory effect of carbachol on the high K^+-induced contraction. These results suggest that the inhibitory effects of carbachol is composed of two phases: protein kinase C-independent transient inhibition and protein kinase C-dependent sustained inhibition.

Keywords: Smooth muscle (intestinal), Acetylcholine, Carbachol, Phorbol ester, Protein kinase C

It has been shown that acetylcholine and carbachol induce contraction by increasing the Ca^{2+} influx in intestinal smooth muscle (1). Higher concentrations of acetylcholine and carbachol also stimulate phosphoinositide turnover (2−4), and one of the hydrolysis products, inositol 1,4,5-trisphosphate, releases Ca^{2+} to induce transient contraction (5).

We have previously reported that high concentrations of carbachol inhibit the sustained contraction induced by carbachol itself by a decrease of cytosolic Ca^{2+} in the intestinal smooth muscle of guinea pig taenia caeci. We have also reported that high concentrations of carbachol inhibit high K^+ induced contraction (6). Phorbol ester, 12-deoxyphorbol 13-isobutyrate, also inhibited high K^+-induced contraction, and this effect was abolished when the protein kinase C was desensitized (7). These results suggest that the inhibitory effect of a high concentration of carbachol on high K^+-induced contraction may be mediated by the activation of protein kinase C.

To further clarify the physiological role of protein kinase C in intestinal smooth muscle contraction, we compared the effects of carbachol and acetylcholine on the contractile response in guinea pig taenia caeci. We also examined the contractile and relaxant effects of carbachol in muscle strips in which the protein kinase C activity had been desensitized.

MATERIALS AND METHODS

Male guinea pigs, weighing 250−300 g, were killed by a blow on the neck and bled. A section of taenia (5−10 mm in length) was dissected from the caecum. For the desensitization of protein kinase C, the muscle strips were incubated in Dulbecco's modified Eagle medium with 10% fetal calf serum for 24 hr at 37℃ in a CO_2 incubator in the presence of 3 μM phorbol 12-myristate 13-acetate or its

vehicle, 0.3% dimethylsulfoxide (7).

Normal physiological salt solution contained: 136.9 mM NaCl, 5.4 mM KCl, 5.5 mM glucose, 23.8 mM $NaHCO_3$, 1.5 mM $CaCl_2$, 1.0 mM $MgCl_2$ and 0.01 mM ethylenediamine-tetraacetic acid. High K^+ solution was made by adding KCl to the normal solution. These solutions were aerated with a 95% O_2 and 5% CO_2 mixture at 37℃ (pH 7.4). Muscle tension was recorded isometrically with a force-displacement transducer. A passive tension of 2 mN was initially applied, and then tissues were allowed to equilibrate for 60–90 min until the contractile response to 45 mM K^+ solution became stable. The magnitude of the high K^+-induced contraction in the muscle pretreated with phorbol ester (5.5±1.2 mN, n=11) was not significantly different from that of the vehicle-treated muscle (4.3±0.9 mN, n=11). At the end of an experiment, 12-deoxyphorbol 13-isobutyrate (1 μM) was added to the high K^+-induced contraction in the strips pretreated with phorbol ester to determine if the contractile effect of phorbol ester had disapeared.

Chemicals used were 12-deoxyphorbol 13-isobutyrate, phorbol 12-myristate 13-acetate (Funakoshi, Tokyo), acetylcholine, carbachol and diisopropylfluorophosphate (Sigma Chemicals, St. Louis, MO, USA).

Results of the experiments are expressed as the mean±S.E.M. Student's *t*-test was used for statistical analysis of the results, and $P<0.05$ was considered to be significant.

RESULTS

Magnitude of the transient and the sustained contractions

In the guinea pig taenia caeci, acetylcholine induced transient contraction followed by sustained contraction. The magnitude of the transient and sustained contractions induced by acetylcholine increased in a concentration-dependent manner, and the magnitude of the sustained contraction measured 20 min after the addition of acetylcholine was not significantly different from that of the transient contraction (Fig. 1). Carbachol also induced a transient contraction followed by a sustained contraction. The magnitude of the sustained contraction induced by 100 nM carbachol was not significantly different from that of the transient contraction (Fig. 2A). However, the magnitude of the sustained contraction induced by 1 μM or higher concentrations of carbachol was significantly smaller than that of the transient contraction (Fig. 2: B–D).

In the presence of cholinesterase inhibitor, 0.5 μM diisopropylfluorophosphate, contractions induced by lower concentrations of acetylcholine were augmented, shifting the concentration-response curve for acetylcholine to the left (Fig. 1). The magnitude of the sustained contraction induced by 1 μM acetylcholine was smaller than that of the transient contraction ($P<0.05$). However, 0.5 μM diisopropylfluorophosphate did not change the contractions induced by higher concentrations of acetylcholine. Furthermore, 0.5 μM diisopropylfluorophosphate did not modify the carbachol-induced contractions (data not shown).

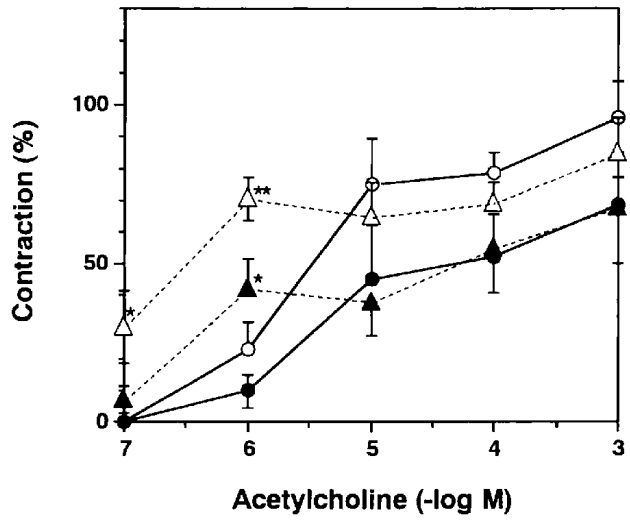

Fig. 1. Concentration-response curves for acetylcholine in the absence and presence of diisopropylfluorophosphate. ○ and △: Acetylcholine-induced transient contraction, ● and ▲: acetylcholine-induced sustained contraction. Diisopropylfluorophosphate (0.5 μM) was added 10 min before the addition of acetylcholine (△ and ▲). Each point represents the mean±S.E.M. of 4–6 experiments. 100% represents the magnitude of sustained contraction induced by 45 mM K^+. * and **: Significantly different from the value in the absence of diisopropylfluorophosphate with $P<0.05$ and $P<0.01$, respectively.

Effects of acetylcholine and carbachol on high K^+-induced contraction

During high K^+-induced sustained contraction, addition of 100 nM carbachol induced a slight increase of contraction. Higher concentrations of carbachol (1–100 μM) transiently increased and then inhibited the high K^+-induced contraction. The inhibition became greater when the concentration of carbachol was increased (Fig. 3).

Acetylcholine also transiently enhanced the high K^+-induced contraction. However, acetylcholine showed only a small inhibitory effect on high K^+-induced contraction at 100 μM–1 mM in the presence or absence of diisopropylfluorophosphate (0.5 μM, Fig. 3). Diisopropylfluorophosphate (0.5 μM) did not modify high K^+-induced contractions (data not shown).

Effects of pretreatment with phorbol ester on acetylcholine- and carbachol-induced contractions

Figure 2 shows the time-courses of the contractions induced by different concentrations of carbachol in the muscle pretreated with phorbol ester. Pretreatment with phorbol 12-myristate 13-acetate did not change the contractions induced by 100 nM carbachol (Fig. 2A). In contrast, pretreatment with phorbol ester changed the contractions induced by higher concentrations (10 and 100 μM) of carbachol; carbachol induced a transient contraction followed by an increase of the contraction. Thus, the contraction measured 0.4 to 0.8 min after the addition of carbachol was smaller, whereas the contraction measured 4 to 10 min after the addition of carbachol was greater in the muscles pretreated with phorbol ester than the vehicle-treated muscles (Fig. 2: C and D).

Pretreatment with phorbol ester did not change the transient and sustained contractions induced by 100 μM acetylcholine (vehicle control: 104.9±4.9% and 48.4±8.2% of the contraction induced by 45 mM K^+, respectively, n=4 each; phorbol ester: 110.3±28.5% and 57.0±5.1%, respectively, n=4 each).

Effects of pretreatment with phorbol esters on carbachol-induced inhibition of high K^+-induced contraction

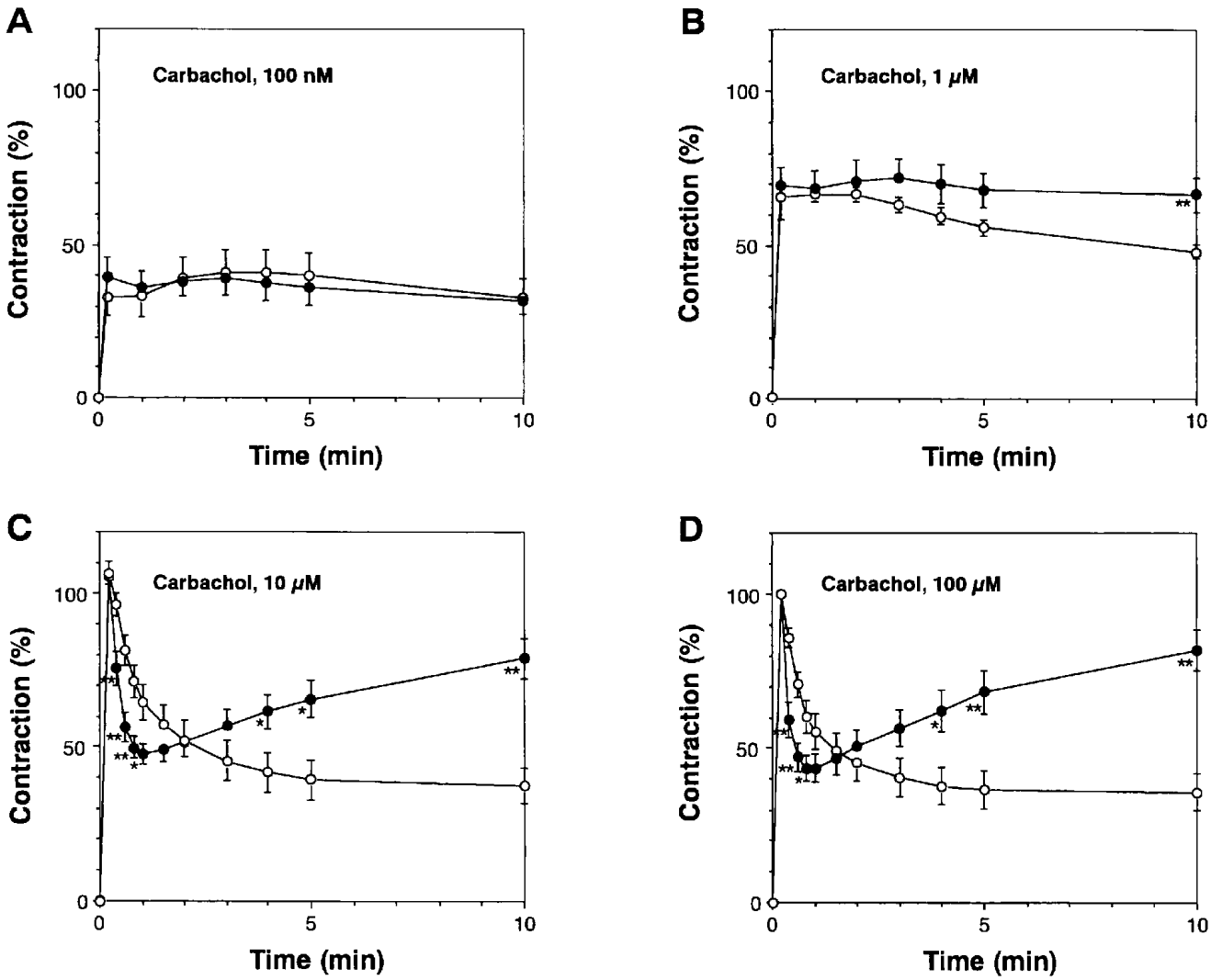

Fig. 2. Time-course of the contraction induced by various concentration of carbachol (100 nM−100 μM) in the muscle pretreated with 3 μM phorbol 12-myristate 13-acetate (●, n=6) or its vehicle (○, n=8) for 24 hr at 37°C. Each point represents a mean±S.E.M. 100% represents the magnitude of the transient contraction induced by 100 μM carbachol. Pretreatment with phorbol ester did not change the magnitude of the transient contraction induced by 100 μM carbachol (vehicle control: 147.8±9.7% of the contraction induced by 45 mM K^+, n=8; phorbol ester: 145.3±9.9%, n=6). * and **: Significantly different from the vehicle value with $P<0.05$ and $P<0.01$, respectively.

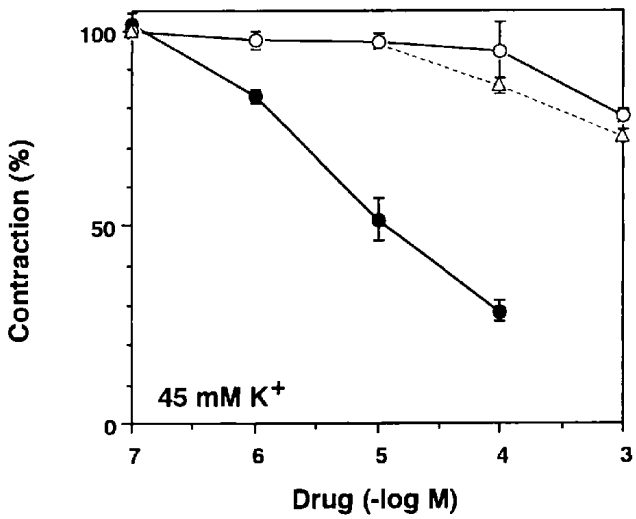

Fig. 3. Concentration-response curves for the inhibitory effects of acetylcholine and carbachol. Acetylcholine (○) or carbachol (●) was added during the sustained contraction induced by 45 mM K^+. Diisopropylfluorophosphate (0.5 μM) was added 10 min before the addition of acetylcholine (△). Each point represents the mean±S.E.M. of 4 experiments. 100% represents the muscle tension before the addition of acetylcholine or carbachol.

As shown in Fig. 4A, addition of 100 μM carbachol induced a transient increase (to 179.7±16.5%, n=5) followed by a sustained decrease (to 45.1±4.5%, at 30 min) of the 45 mM K^+-induced contraction. In the muscle pretreated with phorbol ester, carbachol also augmented the high K^+-induced contraction (to 161.9±6.2%, n=5). However, the inhibitory effect of carbachol was transient in the muscle pretreated with phorbol ester. The maximum inhibition was reached within 5 min of carbachol application (to 54.6±5.5%), and muscle tension gradually increased thereafter (to 83.9±6.4%, at 30 min, Fig. 4B).

DISCUSSION

Different effects of acetylcholine and carbachol

It has been shown that the contractions induced by acetylcholine and carbachol are mediated by the muscarinic M_3-receptor followed by stimulation of the phosphatidilinositol turnover in intestinal smooth muscle (2, 4). We have also reported that both the contractile and relaxant effects of carbachol are due to the activation of M_3-receptors sensitive to 4-diphenylacetoxy-*N*-methylpiperidine methiodide (6). Carbachol was more potent than acetylcholine in inducing contraction in guinea pig taenia caeci (present experiment) and rat airway smooth muscles (8). In contrast, acetylcholine was stronger than carbachol in inducing contraction in guinea pig ileum (9) and bovine coronary artery (10). The difference may be due to the difference in the cholinesterase activity in these tissues. In guinea pig taenia caeci, however, Mitchelson and Ziegler (11) suggested that acetylcholine and carbachol interact with different muscarinic receptors since these agonists exhibited different sensitivities towards muscarinic antagonist or Ca^{2+} channel blockers in the presence of a cholinesterase inhibitor. Takayanagi et al. (12) also showed that there are two types of M_3-receptors in the taenia caeci. In the present experiments, we showed that carbachol but not acetylcholine inhibited high K^+-induced contraction, and the inhibitory effect of acetylcholine was not augmented in the presence of cholinesterase inhibitor. Carbachol but not acetylcholine may stimulate one of the M_3-receptor subtypes that induces muscle relaxation.

Inhibitory effects of carbachol

In the present experiments, desensitization of protein kinase C did not modify the effects of a low concentration of carbachol (100 nM), suggesting that protein kinase C is not involved in this contraction. On the other hand, desensitization of protein kinase C increased the magnitude of sustained contraction induced by 1−100 μM carbachol (Fig. 2). These results suggest that high concentrations of carbachol inhibit its own contraction by a protein kinase C-dependent pathway. This suggestion is consistent with the finding that high concentrations of carbachol are necessary to stimulate phosphatidylinositol turnover (2). Sasaguri and Watson (13) have also reported that desensitization of the protein kinase C activity potentiated the sustained contraction induced by 1 μM carbachol in guinea pig ileum. Since phorbol ester activates the voltage-dependent Ca^{2+} channel in this muscle (14), activation of the membrane Ca^{2+} extrusion pump (15) may contribute to the carbachol-induced inhibition of its own contraction.

In the presence of 45 mM K^+, addition of carbachol (100 μM) induced a transient increase followed by a sustained decrease of the contraction (Fig. 4A). In the muscle in which protein kinase C was desensitized, 100 μM carbachol only transiently inhibited 45 mM K^+-induced contraction (Fig. 4B). These results suggest that high concentrations of carbachol induced transient inhibition of high K^+-induced contraction by the protein kinase C-independent pathway and sustained inhibition by the protein kinase C-dependent pathway.

We have previously reported that higher concentrations of carbachol inhibited the increase in cytosolic Ca^{2+} induced by high K^+ in the taenia caeci (6). Therefore, the inhibitory effects of carbachol may be due to inhibition of voltage-dependent Ca^{2+} channels or activation of the membrane Ca^{2+} extrusion pump. It has been reported that carbachol inhibited the voltage-dependent Ca^{2+} cur-

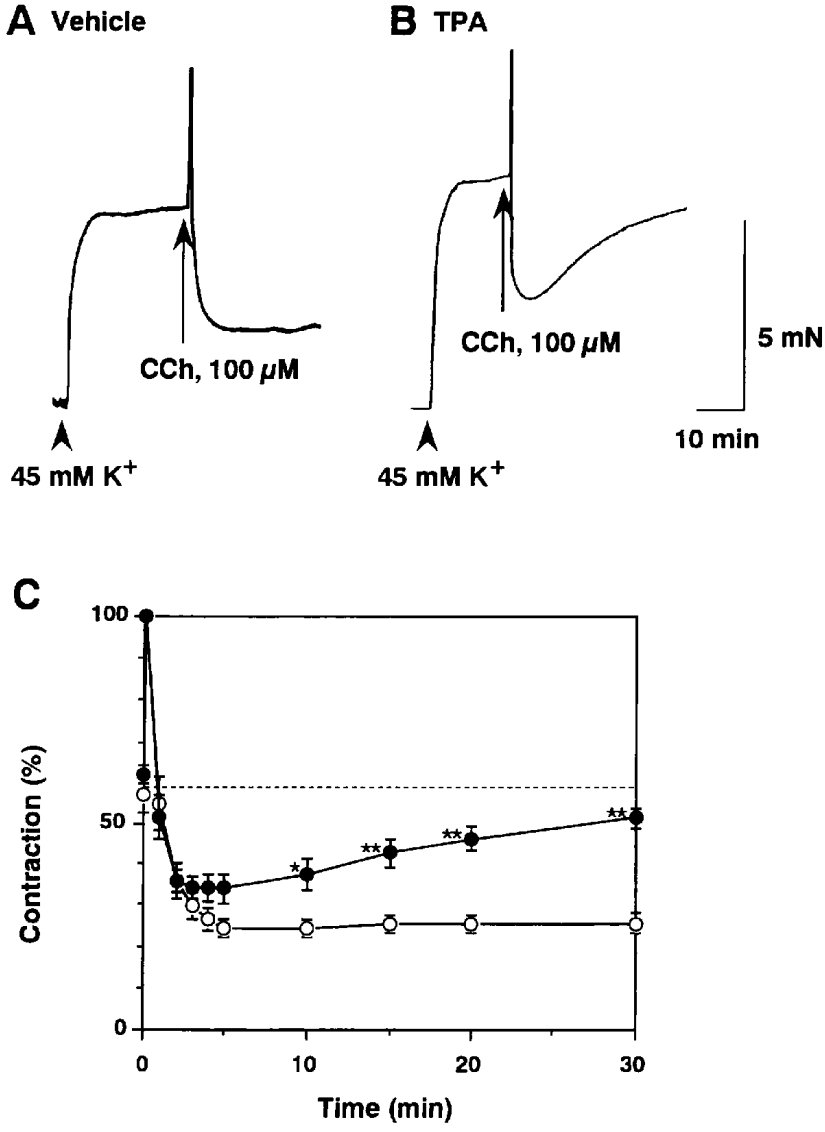

Fig. 4. Effects of 100 μM carbachol (CCh) on the 45 mM K^+-induced contraction in the muscle pretreated with 3 μM phorbol 12-myristate 13-acetate (TPA) (B) or its vehicle (A). C: Time-course of the effects of 100 μM carbachol on the high K^+-induced contraction in the muscle pretreatment with 3 μM phorbol 12-myristate 13-acetate (●) or its vehicle (○). 100% represents the magnitude of the transient contraction induced by 100 μM carbachol in the presence of high K^+. The magnitude of the contraction induced by 45 mM KCl before the addition of carbachol was 59.7±2.6% (n=10)) as shown by the dotted lines. Each point represents the mean±S.E.M. of 5 experiments. * and **: Significantly different from the vehicle value with $P<0.05$ and $P<0.01$, respectively.

rent in rabbit jejunum (16) and guinea pig ileum (17). Komori and Bolton (18) showed that inositol 1,4,5-trisphosphate-induced increase of Ca^{2+} inactivated the voltage-dependent Ca^{2+} channel in rabbit small intestine, which might at least partly be responsible for the carbachol-induced decrease of the Ca^{2+} current. We have previously reported that phorbol ester decreased cytosolic Ca^{2+} stimulated by high K^+ in the taenia caeci (7). However, phorbol ester had an excitatory effect on the voltage-dependent Ca^{2+} current in guinea pig taenia caeci (14). In addition, phorbol ester had little or no inhibitory effect on the Ca^{2+} current in the ileum (17). These results suggest that high concentrations of carbachol decrease the voltage-gated Ca^{2+} current by the protein kinase C-independent pathway, and this may be the reason why carbachol transiently inhibited high K^+-induced contraction.

In the muscle in which protein kinase C was desensitized, addition of 10–100 μM carbachol induced a transient increase followed by an immediate decrease of the

contraction (Fig. 2). When the protein kinase C activity was desensitized, the decrease of contraction became faster than that in the vehicle-treated muscles (Fig. 2: C and D). This result suggests that protein kinase C attenuates the transient inhibition induced by carbachol. We have previously reported that activation of protein kinase C by phorbol ester transiently augmented the contraction induced by high K^+ and carbachol in guinea pig taenia caeci (7). Augmentation of contraction mediated by protein kinase C may overcome the protein kinase C-independent relaxation induced by carbachol. Another possibility is that activation of protein kinase C may increase the voltage-dependent Ca^{2+} current as has been reported (14). However, these mechanisms do not explain why the decrease of 45 mM K^+-induced contraction induced by 100 μM carbachol in the muscle pretreated with phorbol ester (Fig. 4C) was not faster than that in vehicle-treated strips. Further experiments are necessary to examine these possibilities.

In the present experiments, acetylcholine showed little inhibitory effect, although it may also stimulate the phosphatidylinositol turnover. The subtype of the M_3-receptor that is stimulated by acetylcholine may not activate protein kinase C strongly enough to relax the high K^+-induced contraction.

In conclusion, it is suggested that high concentrations (10–100 μM) of carbachol have dual effects to induce contraction and to inhibit contraction. The inhibitory effect of carbachol is composed of two phases. The sustained relaxation, but not the transient relaxation, may be mediated by the activation of protein kinase C.

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