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Inhibition of oxotremorine-induced desensitization of guinea-pig ileal longitudinal muscle in Ca^{2+}-free conditions

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Abstract

The aim of this study was to investigate the differences between oxotremorine-induced and acetylcholine (ACh)-induced desensitization, particularly under Ca^{2+}-free conditions, in guinea-pig ileal longitudinal muscle, and to elucidate the different mechanisms of desensitization that might exist between these two muscarinic agonists. Pretreatment of the tissue with $10^{-7}-10^{-5}$ M oxotremorine (desensitizing treatment) in normal Tyrode solution caused desensitization of the responses to ACh, as did the desensitizing treatment with ACh. However, Ca^{2+}-free conditions significantly reduced oxotremorine-induced desensitization, contrary to the previous findings that Ca^{2+}-free conditions enhanced ACh-induced desensitization. The desensitizing treatment with oxotremorine caused suppression of the responses to high K^+ (tonic phase), as did the ACh treatment. Ca^{2+}-free conditions removed this suppression, whereas this condition enhanced ACh-induced suppression of the K^+ response. A protein kinase C inhibitor, 1-(5-isoquinolinesulfonyl)-2-methylpiperazine (10^{-4} M) had no effect on oxotremorine-induced desensitization of the ACh response. The results suggest that a voltage-gated Ca^{2+} channel was involved in oxotremorine-induced desensitization, as in ACh-induced desensitization, but that the process of inactivation of Ca^{2+} channels was different between oxotremorine and ACh, and that oxotremorine-induced desensitization was due not only to Ca^{2+} channel, but also to other unknown factors. Protein kinase C did not participate in oxotremorine-induced desensitization.

Introduction

Activation of muscarinic receptors in intestinal smooth muscles causes an increase in cellular Ca^{2+} through two pathways. One is mediated by a pertussis toxin (PTX)-sensitive G protein and activates cationic channels, leading to the membrane depolarization (Benham et al 1985; Inoue & Isenberg 1990; Komori et al 1992; Zholos & Bolton 1994). The other is mediated by a PTX-insensitive G protein and activates phosphoinositide hydrolysis to result in the formation of inositol 1,4,5-triphosphate (IP_3) and diacylglycerol (Komori et al 1992). The former pathway increases Ca^{2+} entry through voltage-gated Ca^{2+} channels (Pacaud & Bolton 1991), and the latter pathway releases Ca^{2+} from internal stores (Komori & Bolton 1991; Pacaud & Bolton 1991), both leading to smooth muscle contraction.

Muscarinic agonist-induced desensitization in intestinal smooth muscle has been shown to be heterologous, suggesting that changes in the effector system rather than in the receptor level are responsible for desensitization (Siegel et al 1984;

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Horio et al 1990). Thus, inactivation of voltage-gated Ca^{2+} channel currents is probably one cause of this desensitization (Russell & Aaronson 1990; Himpens et al 1991; Unno et al 1995). Desensitization of cationic currents could be another possible cause of this desensitization (Zholos & Bolton 1996).

It has been shown that the muscarinic agonist oxotremorine has a somewhat different profile in terms of cellular signalling pathways compared with other agonists, such as acetylcholine (ACh) and carbachol. That is, oxotremorine shows comparable effects with ACh and carbachol in the inhibition of cyclic AMP formation (Olianas et al 1983; Brown & Brown 1984), but it only partially stimulates phosphoinositide hydrolysis (Fisher et al 1983; Evans et al 1985; Ek & Nahorski 1988; Konno & Takayanagi 1989). In guinea-pig ileum, carbachol induces both Ca^{2+} influx and the release of Ca^{2+} from internal stores, but oxotremorine induces only Ca^{2+} influx (Wang et al 1992). Binding studies suggest that carbachol and ACh bind to both high- and low-affinity sites of the receptor, but that oxotremorine only binds to the high-affinity site (Fisher et al 1983; Evans et al 1985; Ek & Nahorski 1988; Safrany & Nahorski 1994). Therefore oxotremorine may be useful for clarifying the signalling pathway that participates in the muscarinic receptor-mediated desensitization process, particularly with respect to the role of phosphoinositide hydrolysis and protein kinase C in the desensitization process. In this study, we investigated the differences between oxotremorine-induced and ACh-induced desensitization of guinea-pig ileal muscle, particularly under Ca^{2+}-free conditions.

Materials and Methods

Drugs

ACh chloride was obtained from Daiichi Pharmaceutical Co. (Tokyo, Japan). Oxotremorine sesquifumarate was from Sigma (St Louis, MO). 1- (5-Isoquinolinesulfonyl)-2-methylpiperazine (H-7) and *N*- (2-guanidinoethyl)-5-isoquinolinesulfonamide hydrochloride (HA1004) were from Seikagaku Co. (Tokyo, Japan).

Solutions

The Tyrode solution had the following composition (mM): NaCl 136.9, KCl 2.7, $CaCl_2$ 1.8, $MgCl_2$ 1.05, NaH_2PO_4 0.42, $NaHCO_3$ 11.9, and glucose 5.6. High-K^+ (40 mM K^+) solution was prepared by replacing NaCl in the Tyrode solution with equivalent amounts of KCl. Ca^{2+}-free solution was prepared by removing $CaCl_2$ from the Tyrode solution.

Preparations and contractile measurements

Male guinea-pigs, 250–450 g, were killed by a blow on the head and their throats cut. The ileum was removed and strips of longitudinal muscle were obtained according to the method of Rang (1964). The strips were suspended in Tyrode solution at 31°C and bubbled with air under a resting tension of approximately 0.5 g. Isotonic contractions were recorded with a lever on a smoked drum.

Measurement of desensitization

Desensitization of the responses to ACh or high-K^+ was induced by pretreatment of the muscle strips with appropriate concentrations of oxotremorine or ACh. For the measurement of desensitization of the ACh response, cumulative dose–response curves for ACh were measured on a muscle strip at intervals of approximately 1 h. The muscle strip was then treated with a desensitizing agent (3×10^{-8}–10^{-5} M oxotremorine or 10^{-8}–10^{-3} M ACh) for 30 min. This treatment was done either in normal Tyrode solution or in Ca^{2+}-free solution (exchanged 5 min before the treatment). The muscle strip was then washed with Tyrode solution for 10 min after the treatment with ACh, and for 10–23 min after the treatment with oxotremorine (10 min for 3×10^{-8}–10^{-7} M, 13 min for 10^{-6} M, 20 min for 3×10^{-6} M, and 23 min for 10^{-5} M). After the washing periods, the muscle tone recovered to the baseline. We did not use concentrations of oxotremorine higher than 10^{-5} M, because such treatment required a long washing period (more than 30 min) for the muscle tone to recover to the baseline. The dose–response curve for ACh was then re-examined in Tyrode solution. The curve shifted almost in parallel to the right after treatment with ACh or oxotremorine. The dose ratio for shifted curves was determined at 50% response, to assess the extent of desensitization (Paton 1961; Horio et al 1990). Here, the dose ratio is the ratio of the concentration of ACh required to elicit 50% of the maximum response in the desensitized state to the concentration needed to elicit the same response in the control experiment.

For the measurement of desensitization of high-K^+-induced contraction, the response to 40 mM K^+ solution was measured at intervals of approximately 1 h until it became stable. The muscle strip was then treated with 3×10^{-7} M oxotremorine for 30 min in Tyrode solution or in Ca^{2+}-free solution, washed for 10 min, and the

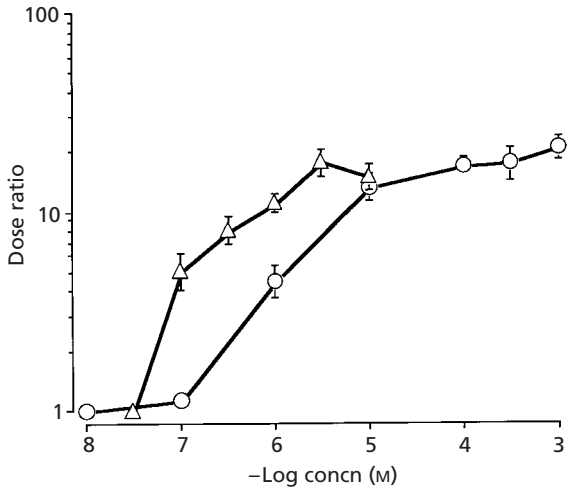

Figure 1 Desensitization of ACh responses induced by muscarinic agonists, ACh (○) and oxotremorine (△) in guinea-pig ileal longitudinal muscle. The desensitizing treatments were done for 30 min with 10^{-8}–10^{-3} M ACh or 3×10^{-7}–10^{-5} M oxotremorine. Dose ratios were determined from the shifted dose–response curves to express the extent of desensitization. Each point represents the mean ± s.e.m., n = 3–6.

response to 40 mM K^+ was re-examined. High-K^+-induced contraction is composed of two components, phasic and tonic (Bolton 1979), and the desensitized response of the tonic component was examined as described previously (Horio et al 1999). The treatment of the muscle strips with Ca^{2+}-free solution for 35 min followed by a 10-min washout had no significant effect on the response to either ACh or 40 mM K^+.

To determine the effects of two protein kinase inhibitors, H-7 and HA1004, on oxotremorine-induced desensitization, the muscle strips were pretreated with each inhibitor for 30 min, and then treated with 3×10^{-7} M oxotremorine in the continued presence of the drug for 30 min. After a 10-min washout, the dose–response curve for ACh was examined in the absence of the drug. The treatment of muscle strips with each inhibitor for 60 min followed by the 10-min washout had no significant effect on the dose–response curve for ACh.

Statistical evaluation of significant differences was performed using the Student's *t*-test.

Results

Dose–response curves for oxotremorine and ACh were determined on guinea-pig ileal longitudinal muscle. Both drugs developed maximum contractile responses to a similar extent with no significant difference. The pD_2 values for oxotremorine and ACh were 7.47 ± 0.09 (n = 3) and 7.23 ± 0.05 (n = 5), respectively; the sensitivity to oxotremorine was somewhat greater than that to ACh, but this difference was not significant.

Pretreatment of the tissue with oxotremorine or ACh (desensitizing treatment) for 30 min, followed by a 10-min washout, caused desensitization of the response to ACh. Desensitization was observed as a shift to the right in the dose–response curve for ACh. To quantitatively assess the extent of desensitization, we calculated the dose ratio from the shifted curves as described previously (Paton 1961; Horio et al 1990). As shown in Figure 1, both oxotremorine and ACh induced concentration-dependent desensitization. Oxotremorine was approximately 10-fold more effective than ACh in inducing desensitization. It should be noted that a longer washing time was required after the desensitizing treatment with oxotremorine. During these washing periods, desensitization might have recovered to some extent, as reported previously (Horio et al 1990). Thus, there remained the possibility that the extent of actual oxotremorine desensitization might have been even greater than shown in Figure 1.

To determine the effect of external Ca^{2+} concentrations on oxotremorine-induced desensitization of the ACh responses, we desensitized the tissue with oxotremorine under Ca^{2+}-free conditions. As shown in Figure 2A, this desensitizing treatment suppressed the desensitization. The dose ratios for control and Ca^{2+}-free desensitization were 7.60 ± 0.61 and 3.08 ± 0.24, respectively, and were significantly different ($P < 0.01$; n = 4). The treatment with Ca^{2+}-free solution alone (without oxotremorine) had no significant effect on the response to ACh, as described previously (Horio et al 1999).

We studied the effect of the desensitizing treatment with oxotremorine on high-K^+-induced contractions. High-K^+-induced contraction was composed of two components, the phasic and the tonic components. Pretreatment with oxotremorine suppressed both the phasic and the tonic components of high- K^+-responses. However, there was a marked difference in the degree of desensitization between the two components; the tonic component was far more sensitive to the desensitizing treatment. The phasic component was reduced to 83.3 ± 3.2% of the control ($P < 0.01$ compared with control response, n = 7), and the tonic component was reduced to 46.5 ± 3.7% of the control ($P < 0.01$ compared with control response, n = 7). This was probably due to the difference in the types of Ca^{2+} channels involved in the

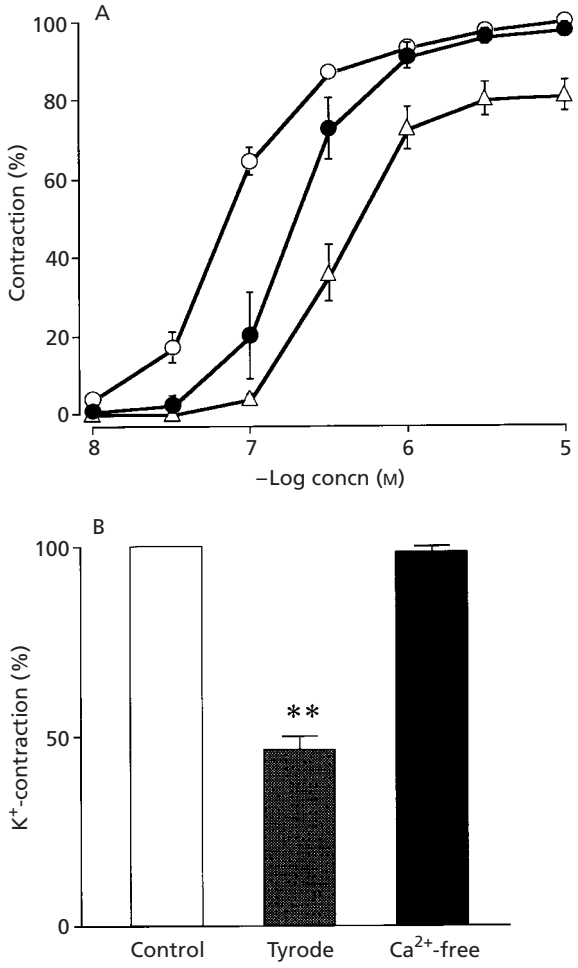

Figure 2 A. Effect of the desensitizing treatment with oxotremorine under normal and Ca^{2+}-free conditions, on the dose–response curve for ACh in guinea-pig ileal longitudinal muscle. After a control dose–response curve (○) was obtained on a muscle strip, the strip was treated with 3×10^{-7} M oxotremorine for 30 min either in normal Tyrode solution (△) or in Ca^{2+}-free solution (●), washed for 10 min, and the dose–response curve was re-examined. Maximum contraction in the control dose–response curve was taken as 100%. Each point represents the mean ± s.e.m., n = 4. B. Effect of the desensitizing treatment with oxotremorine under normal and Ca^{2+}-free conditions on the response to 40 mM K^+. Muscle strips were pretreated with 3×10^{-7} M oxotremorine for 30 min in normal Tyrode solution or in Ca^{2+}-free solution. Each tonic component of the response to 40 mM K^+ after the treatment is expressed as a percentage of its control response. Each bar represents the mean ± s.e.m., n = 7. $^{**}P < 0.01$, significantly different compared with control.

phasic and tonic components of the K^+-induced responses. The tonic component of the K^+-response seems to represent L-type Ca^{2+} channels, since it is sensitive to Ca^{2+}-channel blockers. The phasic component seems to come from another type of Ca^{2+} channel, since it is far less sensitive to the blockers (Grbovic & Radmanovic 1987; Karaki & Mitsui 1988; Usune et al 1995). Since the tonic component of the K^+-responses underwent profound desensitization, this component, rather than the phasic component, was probably related to desensitization of the ACh responses. Therefore, in this study, we mainly focussed on the tonic component (Figure 2B).

We studied the effect of Ca^{2+}-free conditions on oxotremorine-induced suppression of the K^+-responses. As shown in Figure 2B, Ca^{2+}-free conditions completely removed the suppression. Thus Ca^{2+}-free conditions inhibited oxotremorine-induced suppression of Ca^{2+}-channel currents, in addition to inhibiting oxotremorine-induced desensitization. It should be noted that the removal of the suppression of Ca^{2+} currents was complete but that the inhibition of desensitization was only partial. It is of interest that the absence of Ca^{2+} had opposite effects on oxotremorine-induced and ACh-induced desensitization processes, that is, it inhibited the former but facilitated the latter (Horio et al 1999). Therefore, there must be some differences in the mechanism of desensitization between the two muscarinic agonists.

As oxotremorine differs from ACh in the stimulation of phosphoinositide hydrolysis and activation of protein kinase C, we examined whether protein kinase C was involved in the oxotremorine-induced desensitization process. The effects of two protein kinase inhibitors, H-7 and HA1004, on oxotremorine-induced desensitization in guinea-pig ileal longitudinal muscle were studied. After the desensitizing treatment with 3×10^{-7} M oxotremorine for 30 min in the presence or absence of 10^{-4} M H-7 or 10^{-4} M HA1004, dose ratios were determined from the shifted dose–response curves to express the extent of desensitization. Dose ratios for desensitizations in the control, with H-7, and with HA1004 were 7.60 ± 0.61, 6.73 ± 0.45, and 7.10 ± 0.29, respectively, and were not significantly different (n = 4). Thus, a protein kinase C specific inhibitor, H-7, and a non-specific kinase inhibitor, HA1004, had no effect on oxotremorine-induced desensitization, indicating that protein kinase C was not involved in oxotremorine-induced desensitization.

Discussion

This study showed that oxotremorine-induced desensitization in guinea-pig ileal muscle had quite different

profiles to ACh-induced desensitization. Oxotremorine was more effective than ACh in inducing desensitization, and oxotremorine-induced desensitization was inhibited under Ca^{2+}-free conditions, whereas ACh-induced desensitization was enhanced under the same conditions (Horio et al 1999). Although both oxotremorine and ACh suppressed Ca^{2+}-channel currents, oxotremorine-induced suppression was completely blocked under Ca^{2+}-free conditions, whereas ACh-induced suppression was enhanced under this condition (Horio et al 1999). The protein kinase C inhibitor (H-7) had no effect on oxotremorine-induced desensitization, but was previously shown to enhance ACh-induced desensitization (Horio et al 1999). These results suggest that the mechanism of desensitization differs between oxotremorine and ACh.

The main difference between the two drugs is that oxotremorine stimulates phosphoinositide hydrolysis only slightly compared with ACh, probably due to its binding only to the high affinity site of the muscarinic receptor (Fisher et al 1983; Evans et al 1985; Ek & Nahorski 1988; Konno & Takayanagi 1989; Safrany & Nahorski 1994). Previous results showing that oxotremorine stimulated Ca^{2+} influx, but did not release Ca^{2+} from internal stores in guinea-pig ileal smooth muscle (Wang et al 1992), indicate that oxotremorine couples only to a PTX-sensitive G protein, whereas ACh couples to both a PTX-sensitive G protein and a PTX-insensitive G protein (Komori et al 1992).

The result that prolonged treatment with oxotremorine caused suppression of Ca^{2+}-channel currents is in good agreement with previous data demonstrating that muscarinic treatment suppressed Ca^{2+}-channel currents (Mitsui & Karaki 1990; Russell & Aaronson 1990; Unno et al 1995; Horio et al 1999), and suggests that voltage-gated Ca^{2+} channels were involved in oxotremorine-induced desensitization. However, there was a marked difference between oxotremorine-induced and ACh-induced suppression of Ca^{2+}-channel currents. The former was fully removed, whereas the latter was rather potentiated under Ca^{2+}-free conditions. If cellular Ca^{2+} were required in this inactivation process, Ca^{2+}-free conditions would depress this process. Therefore, it was suggested that the suppression of Ca^{2+} currents by oxotremorine required cellular Ca^{2+}, in contrast to the previous finding indicating that the suppression of Ca^{2+} currents by ACh required little or no cellular Ca^{2+} (Russell & Aaronson 1990; Unno et al 1995; Horio et al 1999). These data indicated that there might be two mechanisms to suppress voltage-gated Ca^{2+}-channel currents by muscarinic agonists, one mediated by a PTX-sensitive G protein (Himpens et al 1991) and requiring cellular Ca^{2+}, and the other mediated by a PTX-insensitive G protein (Unno et al 1995) and requiring little or no cellular Ca^{2+}. Oxotremorine might stimulate the former process only, whereas ACh might stimulate both processes.

Oxotremorine-induced desensitization was only partially inhibited (Figure 2A), whereas the suppression of Ca^{2+}-channel currents by oxotremorine was completely inhibited (Figure 2B) under Ca^{2+}-free conditions. This indicates that oxotremorine-induced desensitization consisted of at least two mechanisms, that is a Ca^{2+}-dependent (i.e. inactivation of Ca^{2+} channels) and Ca^{2+}-independent mechanism. At present, however, we can not confirm the Ca^{2+}-independent mechanism. Desensitization of non-selective cationic currents could have been a possible cause of this desensitization, since this process is insensitive to Ca^{2+} (Zholos & Bolton 1996).

We previously found that ACh-induced desensitization was enhanced under Ca^{2+}-free conditions, and that this enhancement was due to the inhibition of protein kinase C, which may activate or protect Ca^{2+} channels during desensitization under normal conditions (Horio et al 1999). In contrast, this study showed that oxotremorine-induced desensitization was rather inhibited under Ca^{2+}-free conditions. This difference probably resulted from the different mechanisms that inhibited Ca^{2+} channels between oxotremorine and ACh, and from the differences between these drugs in stimulating protein kinase C. Protein kinase C was not involved in oxotremorine-induced desensitization, probably because oxotremorine did not stimulate this kinase. Thus, oxotremorine-induced desensitization was inhibited under Ca^{2+}-free conditions, because the inhibition of Ca^{2+} current by oxotremorine was Ca^{2+}-dependent and because Ca^{2+}-free conditions did not enhance desensitization through the inhibition of protein kinase C. Oxotremorine was more effective than ACh in inducing desensitization (Figure 1), probably because protein kinase C, which prevents Ca^{2+} channel desensitization, did not participate in oxotremorine-induced desensitization, but participated in ACh-induced desensitization.

In conclusion, this study showed several differences between oxotremorine-induced and ACh-induced desensitizations in guinea-pig ileal smooth muscle. These differences probably resulted from the different properties of these agonists in stimulating the signalling pathway. Further investigation of the differences between the desensitizations induced by these two muscarinic agonists should provide valuable information for the elucidation of the mechanism of muscarinic receptor-mediated desensitization.

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