Acetylcholine-Induced Desensitization of Muscarinic Contractile Response in Guinea Pig Ileum Is Inhibited by Pertussis Toxin Treatment

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ABSTRACT

We investigated the effects of pertussis toxin treatment on acetylcholine-induced desensitization of the muscarinic contractile response in guinea pig ileum. Incubation of the isolated ileum with acetylcholine (30 µM) for 20 min caused a decrease in the sensitivity of the ileum to the contractile action of the muscarinic agonist oxotremorine-M. This desensitization was characterized by an increase in the EC50 value of oxotremorine-M without a change in its maximal effect. A maximal 4- to 5-fold increase in the EC50 value of oxotremorine-M was measured at the earliest time investigated after acetylcholine treatment (5 min), and normal sensitivity recovered within approximately 20 min after washout of acetylcholine. Treatment of the ileum with pertussis toxin caused a small increase in the contractile response to oxotremorine-M when measured without prior exposure to acetylcholine. After exposure to acetylcholine, little desensitization was observed in ilea that had been treated with pertussis toxin. Pertussis toxin-treatment caused a small increase in oxotremorine-M-mediated phosphoinositide hydrolysis and a large decrease in oxotremorine-M-mediated inhibition of forskolin-stimulated cAMP accumulation in slices of the longitudinal muscle of the ileum. Exposure of the ileum to acetylcholine had no desensitizing effect on the ability of oxotremorine-M to elicit phosphoinositide hydrolysis, indicating that the mechanism for desensitization of the contractile response occurs at a level downstream from the receptor and phosphoinositide hydrolysis. Our results suggest that activation of muscarinic receptors coupled to pertussis toxin-sensitive Gi and Go is required for most of the desensitization observed in this study.

Desensitization is a common, if not universal, phenomenon occurring at receptors from all major families. The mechanisms for desensitization can involve several loci in the train of events between receptor activation and final tissue response, depending upon the tissue or receptor system and the duration of exposure to the desensitizing agonist. The contractile response of the guinea pig ileum has long been known to undergo desensitization after short-term exposure to muscarinic agonists (Cantoni and Eastman, 1946; Dale, 1958; Paton, 1961). A substantial component of the desensitization can be attributed to mechanisms downstream from the receptor because prior exposure to a muscarinic agonist causes a subsequent desensitization of responses to agonists for other receptors as well as the muscarinic receptor.

When exposed to a muscarinic agonist, the isolated guinea pig ileum exhibits cellular responses that can be attributed to activation of both M2 and M3 muscarinic receptors. These receptor subtypes are expressed abundantly in the longitudinal muscle layer in a ratio of about four to one (for reviews, see Eglen et al., 1996; Ehlert et al., 1997). Activation of the M3 receptor elicits phosphoinositide hydrolysis and contraction, whereas activation of the M2 receptor does not appear to contribute to the highly potent contractile response to muscarinic agonists observed in the absence of other heterologous agents (Lambrecht et al., 1989; Candell et al., 1990). Moreover, genetic studies have revealed that the responsiveness of various smooth muscle types to muscarinic agonists is greatly reduced in mice lacking the M3 gene (Matsui et al., 2000), whereas a much smaller decrement in contractile function occurs in mice lacking the M2 gene (Stengel et al., 2000). Nevertheless, activation of the M2 receptor does inhibit the increase in cAMP elicited by forskolin and agonists acting through Gs-linked receptors, such as isoproterenol (Candell et al., 1990; Griffin and Ehlert, 1992; Ostrom and Ehlert, 1997). Moreover, the M2 receptor has been shown to elicit contraction through a mechanism involving disinhibi-

ABBREVIATIONS: KRB, Krebs-Ringer-bicarbonate; Emax, maximal response; 4-DAMP mustard, N-2-chloroethyl-4-piperidinyldiphenylacetate; AF-DX 116, [2-(diethylamino)methyl]-1-piperidinyldiacetyl]-5,11-dihydro-6H-pyrido[2,3b][1,4]-benzodiazepine-6-1.
tion. That is, M₂ receptors mediate an inhibition of the relaxant effects of isoproterenol and forskolin on histamine-induced contractions (Thomas et al., 1993; Thomas and Ehler, 1994; Reddy et al., 1995). Similarly, M₂ receptors mediate an inhibition of the relaxant effect of isoproterenol and forskolin on contraction elicited via the M₃ receptor (Thomas et al., 1993; Thomas and Ehler, 1994; Ostrom and Ehler, 1997).

The contribution of both M₂ and M₃ receptors to the overall muscarinic response in the isolated ileum raises the question as to which receptor, if not both, is involved in mediating desensitization. It has been shown that the desensitizing effect of agonist treatment is prevented by coinubcation with the M₃-selective antagonist p-fluorohexahydrosiladifenidol, but not with M₁- or M₂-selective antagonists (Eglen et al., 1992). These results suggest that excessive activation of M₃ receptors causes desensitization in the guinea pig ileum.

In this report, we have investigated the role of pertussis toxin-sensitive Gₛ and Gₛ, in acetylcholine-mediated desensitization of contractions elicited to the muscarinic agonist oxotremorine-M in the guinea pig ileum. We found that treatment of the isolated ileum with acetylcholine (30 μM; 20 min) causes a modest decrease in contractile sensitivity to oxotremorine-M (5-fold increase in EC₅₀ value), while having little or no inhibitory effect on oxotremorine-M-mediated phosphoinositide hydrolysis. These results show that the majority of the desensitization is the result of a change downstream from phosphoinositide hydrolysis. Most of the acetylcholine-induced desensitization was prevented by pertussis toxin-treatment, indicating a role of Gₛ or Gₛ in mediating short-term desensitization by muscarinic agonists.

Materials and Methods

Isolated Ileum. The contractile response of the isolated ileum was measured as described previously (Thomas et al., 1993). Male guinea pigs (Hartley, 300–500 g; Simonsen Labs, Gilroy, CA) were euthanized with CO₂, and segments of the ileum (approximately 2.5 cm) were removed in a rostral direction, starting at a point approximately 10 cm from the caecum. Ileal segments were mounted longitudinally in an organ bath containing Krebs-Ringer bicarbonate buffer (KRB buffer: 124 mM NaCl, 5 mM KCl, 1.3 mM MgSO₄, 26 mM NaHCO₃, 1.2 mM KH₂PO₄, 1.8 mM CaCl₂, and 10 mM glucose) gassed with O₂/CO₂ (19:1). Resting tension was adjusted to a load of 0.5 g, and ilea were allowed to equilibrate for at least 60 min before isometric contractions were measured with a force-displacement transducer and polygraph. Three test doses of the muscarinic agonist oxotremorine-M (40 nM) were added to the bath sequentially to ensure the reproducibility and magnitude of contractile responses. The ileum was washed and allowed to rest 5 min between each test dose. Concentration-response curves to oxotremorine-M were measured using a cumulative technique as described previously (Thomas et al., 1993). Approximately 5 to 7 min were required to measure a complete concentration-response curve. All contractile responses are expressed as mass equivalents (i.e., g) minus resting tension. Control experiments showed that there was no significant difference in the EC₅₀ and maximal response (Eₘₙₐₓ) values of oxotremorine-M for eliciting contraction when these parameters were measured sequentially in the same ileum, with a 20-min rest period between each concentration-response curve.

Phosphoinositide Hydrolysis. Phosphoinositide hydrolysis was measured in strips of the longitudinal muscle of the ileum by using a procedure similar to that described previously by Thomas et al. (1993). Our technique is based on the [³H]inositol-labeling and ion exchange separation method of Berridge et al. (1982), and it incorporates the perchloric acid extraction method of Kendall and Hill (1990). Segments of isolated ileum (approximately 10 cm) were removed from euthanized guinea pigs (see above), washed with KRB buffer, and mounted on a glass pipette. The outer longitudinal muscle layer was obtained by gentle rubbing with a cotton swab as described by Paton and Vizi (1969). This layer was cut into small strips (0.5 cm), and these were placed in an Erlenmeyer flask (50 ml) containing [³H]inositol (200 μCi; PerkinElmer Life Science Products, Boston, MA) and KRB buffer (10 ml) gassed with O₂/CO₂ (19:1) and sealed with a rubber stopper. The tissue was incubated at 37°C for 2 h with gentle shaking. The atmosphere in the flask was flushed with O₂/CO₂ every 30 min. After this labeling phase, the tissue was washed three times with warm KRB buffer and incubated at 37°C for 20 min in 10 ml of KRB buffer containing 10 mM nonradioactive inositol. After this incubation, the tissue was washed twice with warm KRB buffer.

The muscle strips were carefully transferred withforceps to small plastic cylindrical inserts having a nylon mesh bottom (Netwell; Costar, Cambridge, MA) through which the incubation media rose and bathed the tissue when the insert was placed in an incubation bath with gentle shaking. Use of these plastic vessels enabled the rapid transfer of muscle strips to different incubation environments, all at 37°C in KRB buffer in an enclosed chamber with an atmosphere of O₂/CO₂ (19:1). After the final washing (see above), the strips undergoing acetylcholine treatment were equilibrated for 5 min and then transferred to a bath containing 500 ml of KRB buffer and acetylcholine (30 μM) and were incubated for 20 min. After exposure to acetylcholine, the strips were washed with KRB buffer by using a squeeze bottle and then transferred to an incubation bath containing KRB buffer for 5 min. After this incubation, the strips were transferred to wells containing 2 ml of KRB buffer, LiCl (10 mM), and various concentrations of oxotremorine-M for 5 min. The incubation with oxotremorine-M was terminated by the addition of atropine at a final concentration of 10 μM. The muscle strips were quickly transferred to plastic tubes containing perchloric acid (0.1 ml of 10% w/v) and KRB buffer (0.3 ml) for extraction of [³H]inositol-phosphates as described previously (Shehbaz et al., 2001). Control tissue was handled in a similar manner except for exposure to acetylcholine.

The incorporation of [³H]inositol into phosphoinositides was determined by extracting the muscle strips with chloroform/methanol/ HCl (100:200:1) and subsequently separating the extract into aqueous and organic layers by the addition of water and chloroform, essentially as described previously (Thomas et al., 1993). An aliquot of the chloroform layer was removed and placed in a scintillation vial for estimation of [³H]inositol-labeled phospholipid after evaporation of the chloroform. Subsequently, all residual solvents were removed from the tube containing the tissue with a Speed Vac concentrator connected to a waterjet aspirator (Savant Instruments, Farmingdale, NY). The residual tissue was dissolved in 1 ml of NaOH (0.5 M), and protein was estimated using the Bradford reagent (Bio-Rad, San Diego, CA). Estimates of phosphoinositide hydrolysis are expressed as the percentage of conversion of labeled phosphoinositides into [³H]inositol-phosphates. Incorporation of [³H]inositol into phosphoinositides is expressed as cpm per milligram of protein.

Cyclic AMP Accumulation. CAMP accumulation was measured in slices of the longitudinal muscle of the ileum by using a procedure similar to that described previously by Thomas et al. (1993). Our technique is based on the [³H]adenine-labeling method of Daly et al. (1981), and it incorporates the separation method of Salomon et al. (1974). Briefly, the longitudinal muscle of the ileum was isolated as described above, and cross-chopped (0.35 mm) using a McIlwaine tissue chopper. The slices were washed three times and placed in gassed (O₂/CO₂; 19:1) KRB buffer containing [³H]adenine (1 μM; 50 μCi) in a final volume of 10 ml. The slices were incubated with [³H]adenine for 40 min at 37°C and then washed three times. Aliquots (100–50 μl) of gently packed tissue slices were pipetted into
small plastic conical tubes containing freshly gassed KRB buffer, 0.5 mM isobutylmethylxanthine, and various drugs in a final volume of 0.7 ml. The tubes were capped and incubated at 37°C for 10 min. The reaction was stopped by the addition of 0.2 ml of 30% trichloroacetic acid (w/v). The tubes were centrifuged at 2000g for 10 min, and most of the supernatant was removed and applied to a column containing 1.5 ml of Dowex AG 50W-X4 (200–400 mesh). The eluate together with that from two additional washes with water (1.5 ml each) was collected into scintillation vials and saved for estimation of [3H]-cAMP content. The Dowex column was placed on top of a column of neutral alumina (0.6 g), and [3H]-cAMP was eluted onto the alumina with 5 ml of water. [3H]-cAMP was eluted from the alumina and into scintillation vials with 4 ml of imidazole, pH 7.5. Estimates of [3H]-cAMP are expressed as the percentage of total labeled nucleotides converted into [3H]-cAMP.

Calculations. $E_{\text{max}}$, concentration of agonist eliciting a half-maximal response (EC50 value), and the Hill coefficient of oxotremorine-M for eliciting contraction, stimulation of phosphoinositide hydrolysis, and inhibition of cAMP accumulation were estimated by nonlinear regression analysis of the concentration-response curves according to logistic equations as described previously (Candell et al., 1990). The significance of the desensitizing effect of acetylcholine on the EC50 value of oxotremorine-M was determined by measuring the log shift in the EC50 value of oxotremorine-M caused by acetylcholine treatment. This shift was calculated as the difference between the log EC50 value estimated after acetylcholine treatment minus that measured in the same tissue before acetylcholine treatment. The effect of acetylcholine treatment was considered significant if the log shift value was significantly different from zero (paired Student’s t test, two-tailed; minimum level of significance = 0.05). To determine whether the log shift in control tissue was significantly different from that observed in pertussis toxin-treated tissue, an unpaired Student’s t test (two-tailed) was used.

Drugs and Chemicals. The reagents used in this study were obtained from the following sources: pertussis toxin (List Biochemicals, Campbell, CA); [3H]adenine and [3H]inositol (PerkinElmer Life Science Products); oxotremorine-M (Sigma/RBI, Natick, MA); forskolin (Calbiochem, San Diego, CA); atropine, isobutylmethylxanthine, indomethacin, and tetrodotoxin (Sigma Chemical Co., St. Louis, MO); and 4-DAMP mustard was synthesized in our laboratory as described previously (Thomas et al., 1993).

Results

Isolated Ileum. To optimize our conditions for detecting desensitization, we initially measured the time course for recovery of the contractile response to oxotremorine-M after exposure of the ileum to acetylcholine. In each experiment, a total of four ilea from the same guinea pig was used. A control concentration-response curve to oxotremorine-M was measured first in each ileal segment. The ilea were washed thoroughly and allowed to rest for 20 min. Acetylcholine (30 μM) was applied to each organ bath for 20 min, and the ilea were washed quickly and effectively, so that the contractile response was reduced to resting levels or slightly below within 2 min. After this wash, a single concentration-response curve to oxotremorine-M was measured in each of the four ilea at 5, 10, 20, or 40 min. After this measurement, another concentration-response curve was measured in each ileum 70 min after washout of acetylcholine. Thus, in one experiment, a total of four ileal segments was used from a single guinea pig, each corresponding to a different time point (5, 10, 20, and 40 min) for the second EC50 determination. Because measurement of a concentration-response curve to oxotremorine-M had no effect on the same measurement 20 min later (see Materials and Methods), it was assumed that measurement of the second concentration-response curve (i.e., at 5, 10, 20, or 40 min) was without effect on the third concentration-response curve (70-min curve). Acetylcholine treatment (30 μM; 20 min) elicited a time-dependent contractile response. A maximal contraction was elicited within a few seconds of application. This contraction began to wane in about 10 s and reached a low value of approximately 50% of the maximal response in about 2 min. This level of contraction was maintained throughout the remainder of the incubation with acetylcholine.

The effects of acetylcholine treatment (30 μM; 20 min) on the sensitivity of the ileum to oxotremorine-M at various times after washout are shown in Fig. 1. Exposure to acetylcholine caused a 4.2-fold increase in the EC50 value of oxotremorine-M when estimated 5 min after washout (Fig. 1a). Under the present desensitizing conditions, recovery from desensitization was nearly complete within 20 min, and acetylcholine-treatment had no significant effect on the maximal response to oxotremorine-M (Fig. 1b). Inclusion of tetrodotoxin (0.1 μM) had no effect on the magnitude of the desensitization elicited by acetylcholine (30 μM; 20 min) when the sensitivity of the ileum to oxotremorine-M was measured 5 and 10 min after washout of acetylcholine (data not shown).

To investigate the role of G proteins of the Gi family in mediating desensitization, we measured the effects of pertussis toxin treatment on acetylcholine-induced desensitization. In these experiments, guinea pigs were injected in vivo with pertussis toxin (75 μg/kg i.p.) 3 days before being euthanized for the subsequent in vitro experiments. The effects of pertussis toxin treatment on acetylcholine-induced desensitization are shown in Fig. 2 together with a new set of control experiments different from those shown in Fig. 1. Pertussis toxin treatment caused a small increase in the contractile response to oxotremorine-M. This effect was manifest as a significant increase in the maximal response from 4.04 ± 0.304 g in control ileum to 5.26 ± 0.382 g in ilea from pertussis toxin-treated guinea pigs. This effect was associated with a small, nonsignificant increase in the EC50 value from 24.5 nM in control ileum to 29.5 nM in ilea from pertussis toxin-treated guinea pigs (Fig. 2a). Acetylcholine treatment caused a significant 4.9-fold increase in the EC50 value.

Fig. 1. Recovery of the contractile response to oxotremorine-M after exposure of the guinea pig ileum to acetylcholine (30 μM) for 20 min. Concentration-response curves for oxotremorine-M-stimulated contractions were measured before and at various times after exposure of the guinea pig ileum to acetylcholine for 20 min. a, ordinate (Shift) represents the EC50 value of oxotremorine-M divided by that measured before acetylcholine treatment. b, $E_{\text{max}}$ value of oxotremorine-M is expressed as a percentage of that measured before acetylcholine treatment. The data represent the mean values ± S.E.M. from four experiments, each done on a different guinea pig. For each experiment, a total of four ilea from a single guinea pig was used as described under Results.
of oxotremorine-M when measured 5 min after washout of acetylcholine (Fig. 2b). This shift is very similar to what we observed in previous experiments under identical conditions (Fig. 1a). The shift in the oxotremorine-M EC$_{50}$ value was reduced to 2.2-fold by pertussis toxin treatment (Fig. 2c). Similar behavior was observed 10 min after washout of acetylcholine, although the desensitizing effects of acetylcholine were less at 10 min (Fig. 1). A summary of the effects of pertussis toxin treatment on acetylcholine-induced desensitization of contractions to oxotremorine-M is summarized in Table 1.

A conspicuous effect of acetylcholine treatment was an increase in the steepness of the concentration-response curve to oxotremorine-M (Fig. 2, Table 1). The Hill coefficient increased from a value of 1.50 in control ilea to a value of 3.28 at 5 min after acetylcholine treatment. This effect was reduced by pertussis toxin treatment. Some possible explanations for this effect are described under Discussion.

**cAMP Accumulation and Phosphoinositide Hydrolysis.** Pertussis toxin is known to catalyze the ADP ribosylation of G$_i$, thereby preventing receptor-mediated inhibition of adenyl cyclase (Kurose et al., 1983). To verify the effectiveness of pertussis toxin in the experiments described in Fig. 2, we measured the effects of pertussis toxin treatment on oxotremorine-M-mediated inhibition of forskolin-stimulated cAMP accumulation (Fig. 3a). In control ilea, forskolin (10 $\mu$M) caused an 11.1-fold increase in cAMP levels relative to basal. Oxotremorine-M caused a concentration-dependent inhibition of cAMP with the EC$_{50}$ and $E_{\text{max}}$ values for this effect being 0.17 $\mu$M and 82.5% inhibition, respectively. In pertussis toxin-treated ilea, the effects of oxotremorine-M were substantially reduced; the EC$_{50}$ value increased 5.9-fold and the $E_{\text{max}}$ decreased to only 31.8% inhibition. Thus, pertussis toxin treatment was effective in producing effects characteristic of an ADP ribosylation of G$_i$.

In contrast, pertussis toxin treatment had no inhibitory effect on oxotremorine-M-stimulated phosphoinositide hydrolysis, but rather, caused a modest potentiation in this response (Fig. 3b). This effect corresponded to 4.0-fold increase in the EC$_{50}$ value of oxotremorine-M (control EC$_{50}$ value = 57 $\mu$M) without a significant effect on the maximal response (control $E_{\text{max}} = 13.4\%$ conversion of labeled phospholipids into $[^3H]$inositolphosphates). This pertussis toxin-mediated enhancement in phosphoinositide hydrolysis correlates with the small increase in the contractile response noted in pertussis toxin-treated ilea (Fig. 2a).

M$_3$ muscarinic receptors are known to mediate the contractile response to muscarinic agonists on the isolated guinea pig ileum (Lambrecht et al., 1989). This receptor subtype signals through G$_i$ to trigger phosphoinositide hydrolysis in ileal smooth muscle (Candell et al., 1990) as well as in cell lines transfected with recombinant M$_3$ receptors (Peralta et al., 1991).

### Table 1

<table>
<thead>
<tr>
<th>Condition</th>
<th>$E_{\text{max}}$</th>
<th>EC$_{50}$</th>
<th>Hill Coefficient</th>
<th>Shift$^d$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control tissue</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control (13)</td>
<td>4.04 ± 0.30</td>
<td>0.025 (7.61 ± 0.089)</td>
<td>1.50 ± 0.13</td>
<td></td>
</tr>
<tr>
<td>5 min after ACh-treatment (7)</td>
<td>4.09 ± 0.46</td>
<td>0.113 (6.95 ± 0.041)</td>
<td>3.28 ± 0.37$^e$</td>
<td>4.85 (0.69 ± 0.073)</td>
</tr>
<tr>
<td>10 min after ACh-treatment (6)</td>
<td>4.35 ± 0.58</td>
<td>0.061 (7.22 ± 0.10)</td>
<td>2.70 ± 0.32$^e$</td>
<td>2.33 (0.37 ± 0.089)</td>
</tr>
<tr>
<td>Pertussis toxin-treated tissue</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control (13)</td>
<td>5.26 ± 0.38$^f$</td>
<td>0.030 (7.53 ± 0.076)</td>
<td>1.32 ± 0.051</td>
<td></td>
</tr>
<tr>
<td>5 min after ACh-treatment (7)</td>
<td>5.64 ± 0.42</td>
<td>0.079 (7.16 ± 0.10)</td>
<td>1.41 ± 0.21</td>
<td>2.20 (0.34 ± 0.065)</td>
</tr>
<tr>
<td>10 min after ACh-treatment (6)</td>
<td>5.47 ± 0.62</td>
<td>0.052 (7.28 ± 0.093)</td>
<td>1.46 ± 0.27</td>
<td>1.94 (0.29 ± 0.061)</td>
</tr>
</tbody>
</table>

$^a$ Estimates were calculated from the data shown in Fig. 2.
$^b$ Number of experiments is denoted in parentheses.
$^c$ The EC$_{50}$ value (concentration of oxotremorine-M eliciting a half-maximal contraction) is indicated in micromolar units. The values in parentheses for each EC$_{50}$ estimate represent the mean negative log molar EC$_{50}$ value ± S.E.M.
$^d$ Denotes the EC$_{50}$ value divided by that for the control (i.e., that measured without prior exposure to acetylcholine). The values in parentheses for each shift estimate represent the mean log shift ± S.E.M. Each shift value in the table was estimated from the corresponding paired control value and not from the overall control value listed in the table, which represents the average of all of the 5- and 10-min control values.
$^e$ Significantly different from the paired control value, $P < 0.0001$.
$^f$ Significantly different from the paired control value, $P < 0.02$.
$^g$ Significantly different from the corresponding $E_{\text{max}}$ observed in untreated tissue, $P < 0.02$.
$^h$ Significantly different from the corresponding EC$_{50}$ observed in untreated tissue, $P < 0.05$.
$^i$ Significantly different from the corresponding shift observed in untreated tissue, $P < 0.005$.  

**Fig. 2.** Effects of pertussis toxin treatment on the contractile response to oxotremorine-M, before and after exposure to acetylcholine (30 $\mu$M; 20 min). a, contractile response to oxotremorine-M was measured in ilea from control and pertussis toxin-treated (PTX) guinea pigs. b, contractile response to oxotremorine-M was measured before and 5 min after exposure to acetylcholine (Post ACh) in ilea from untreated guinea pigs. c, contractile response to oxotremorine-M was measured before and 5 min after exposure to acetylcholine in ilea from pertussis toxin-guinea pigs. The results of these experiments are summarized in Table 1.
Thus, to investigate the extent of desensitization of M₃ receptors in the ileum, we measured the effects of acetylcholine treatment on oxotremorine-M-stimulated phosphoinositide hydrolysis in control and pertussis toxin-treated tissue (Fig. 4). In these experiments, phosphoinositide hydrolysis was measured 5 min after washout of acetylcholine by using a 5-min incubation with oxotremorine-M. This incubation time was chosen because it is nearly the same as that required to measure a concentration-response curve for the contractile response to oxotremorine-M (5 to 7 min). Thus, both the contractile assay at 5 min after acetylcholine treatment and the phosphoinositide assay were measured during the same time interval after acetylcholine washout. In control ilea, the mean values ± S.E.M. for basal and agonist-stimulated [³H]inositolphosphate accumulation at an oxotremorine-M concentration of 0.25 mM were 611 ± 115 and 10,800 ± 2080 cpm/mg of protein, respectively. Prior exposure to acetylcholine had little influence on oxotremorine-M-stimulated phosphoinositide hydrolysis (Fig. 4a). Similarly, acetylcholine treatment had no significant effect on oxotremorine-M-stimulated phosphoinositide hydrolysis in pertussis toxin-treated ilea (Fig. 4b).

Acetylcholine treatment had little effect on the labeling of phospholipids with [³H]inositol. When expressed as cpm per milligram of protein, the labeling of phospholipids in control tissue was 55,800 ± 13,600 and 68,000 ± 24,300 for untreated and acetylcholine-treated ilea, respectively. The corresponding values in pertussis toxin-treated ilea were 24,600 ± 3970 and 29,600 ± 5420, respectively.

**Discussion**

The desensitization observed in this study is consistent with that observed by numerous other investigators who have shown that incubation of the isolated guinea pig ileum with acetylcholine or muscarinic agonists causes a subsequent decrease in the contractile response to muscarinic agonists (Cantoni and Eastman, 1946; Dale, 1958; Paton, 1961). It seems likely that the desensitization is mediated through the direct activation of muscarinic receptors on the sarclemma, because the desensitization is prevented by muscarinic antagonists (Himpens et al., 1991; Eglen et al., 1992) and is unaffected by tetrodotoxin. The desensitization observed in this study does not require the production of cyclooxygenase products, because our experiments were carried out in the presence of indomethacin.

Our results show that a pertussis toxin-sensitive G protein (Gᵢ or Gₒ) is involved in muscarinic receptor-mediated desensitization of the guinea pig ileum, because pertussis toxin treatment inhibited a substantial amount of the desensitization caused by acetylcholine. This observation seems surprising because pertussis toxin treatment had no inhibitory effect on contraction, indicating that Gᵢ or Gₒ is not involved in the highly potent contractile response of the guinea pig ileum.
Muscarnic receptors can be divided into two groups, depending upon whether they signal through \( G_i \) (M2 and M4) or \( G_o \) (M1, M3, and M5) (Peralta et al., 1988; Lai et al., 1991; Dell’Acqua et al., 1993). In the longitudinal muscle of the ileum, muscarinic receptor-mediated inhibition of adenyl cyclase is sensitive to pertussis toxin, and it exhibits a pharmacological profile consistent with an \( M_2 \) mechanism (Candell et al., 1990; Thomas and Ehler, 1994). These results are consistent with the high expression of \( M_2 \) receptors in the ileum, and they provide no evidence for a functional role of the other \( G_i \)-linked muscarinic receptor (i.e., the \( M_4 \)). With regard to \( G_o \)-mediated responses, the muscarinic phosphoinositide response in the ileum is insensitive to pertussis toxin, and it exhibits a pharmacological profile consistent with that of the \( M_3 \) receptor (Candell et al., 1990; Thomas and Ehler, 1994). Thus, studies on second messenger responses in the ileum indicate that the predominant muscarinic receptors are the \( M_3 \) and \( M_2 \) and that these receptors mediate responses through pertussis toxin-sensitive and -insensitive G proteins, respectively.

The effects of pertussis toxin on the contractile response of the guinea pig ileum are also consistent with the hypothesis that pertussis toxin interrupts \( M_2 \) receptor-mediated responses, but not those of the \( M_3 \) receptor. It is well known that the \( M_3 \) receptor mediates the contractile response to muscarinic agonists in the ileum and that this response is insensitive to pertussis toxin (Eglen et al., 1988; Lambrecht et al., 1989; Candell et al., 1990; Thomas and Ehler, 1994). In contrast, \( M_2 \) muscarinic receptors are known to mediate contraction through a mechanism of disinhibition. That is, \( M_2 \) receptors mediate an inhibition of the relaxant effects of isoproterenol and forskolin on \( H_1 \) histamine and \( M_2 \) muscarinic receptor-mediated contractions (Thomas et al., 1993; Reddy et al., 1995). These contractile effects of the \( M_2 \) receptor are pertussis toxin-sensitive (Thomas and Ehler, 1994; Ostrom and Ehler, 1999; Sawyer and Ehler, 1999).

Collectively, the results summarized above suggest that the \( M_2 \) muscarinic receptor is involved in mediating desensitization because the desensitization is largely prevented by pertussis toxin treatment, and pertussis toxin is useful for discriminating between responses elicited by the two main muscarinic receptors of ileal smooth muscle, \( M_3 \) and \( M_2 \). Part of the increase in \([\text{35S}]\text{guanosine-5'-O-(3-thio)triphosphate} \) binding observed in homogenates of cell lines expressing \( M_3 \) receptors is pertussis toxin-sensitive, indicating that \( M_2 \) receptors do couple with \( G_i \) and \( G_o \) in cell lines when expressed in high abundance (Lazareno et al., 1993; Burford et al., 1995). However, there is no evidence for such a mechanism in smooth muscle. Moreover, it is difficult to imagine how the \( M_3 \) receptor could activate \( G_o \) substantially in the ileum because the \( M_3 \) receptor couples more effectively to \( G_o \), and the \( M_2 \) receptor outnumbers the \( M_3 \) by a factor of approximately four. Thus, any contribution of the \( M_3 \) receptor to \( G_o \) signaling in the ileum would probably be insignificant relative to the large activation through \( M_2 \) receptors. Moreover, any potential \( M_2 \)-\( G_o \) signaling might possibly be inhibited by competition with \( M_2 \) receptors for \( G_o \).

Our hypothesis that \( M_2 \) receptors are involved in mediating desensitization might first appear to conflict with a report by Eglen et al. (1992). These investigators found that the \( M_3 \)-selective antagonist \( p \)-fluorohexahydrodilafenedil prevented desensitization to short-term treatment of the guinea pig ileum with carbachol. In contrast, \( M_1 \) and \( M_3 \) selective muscarinic antagonists were without effect on desensitization. In these experiments, the authors were careful to assess the effects of each antagonist at essentially the same relative concentration. In this context, “relative concentration” denotes the concentration of the antagonist divided by its \( K_D \) at the receptor for which it is selective. Thus, the work of Eglen et al. (1992) indicates that \( M_2 \) receptors are involved in desensitization, whereas the present results implicate a role for the \( M_2 \) receptor. An explanation for these apparently conflicting results may be that activation of both \( M_2 \) and \( M_3 \) receptors is required for desensitization.

This interpretation seems plausible in the light of a related phenomenon that we have previously observed in the guinea pig colon. The muscarinic contractile response of the guinea pig colon, like other smooth muscles, is mediated via activation of the \( M_3 \) receptor (Sawyer and Ehler, 1998). Nevertheless, after a majority of the \( M_2 \) receptors in the colon are selectively inactivated with the irreversible antagonist 4-DAMP mustard, it is still possible to elicit a contractile response to oxotremorine-M although the potency is greatly reduced (Sawyer and Ehler, 1998; Sawyer and Ehler, 1999). Under this condition, the contractile response is moderately sensitive to pertussis toxin, which suggests that the \( M_2 \) receptor is involved in mediating contraction. Nevertheless, the contractile response is relatively insensitive to the \( M_3 \)-selective antagonist AF-DX 116. Thus, the contractile response under this condition is enigmatic; it is \( M_3 \)-like in its sensitivity to pertussis toxin yet is \( M_2 \)-like in its profile for pharmacological antagonism. We have previously shown that a model based on an interaction between \( M_2 \) and \( M_3 \) receptors can rationalize this behavior (Sawyer and Ehler, 1999). According to the model, activation of the \( M_2 \) receptor by itself does not cause contraction; nevertheless, \( M_2 \) receptor activation does potentiate the contractile response elicited through the \( M_2 \) receptor. Analysis of the model shows that the competitive antagonism of the interactive response resembles the pharmacology of the directly acting receptor (i.e., the \( M_3 \)). The model also shows that the interactive response can be moderately sensitive to pertussis toxin. This behavior is similar to the desensitization phenomenon, which is pertussis toxin-sensitive and preferentially blocked by \( M_2 \)-selective antagonists. If both \( M_2 \) and \( M_3 \) receptors interact with each other to elicit contraction in the presence of high concentrations of acetylcholine then it is possible that the downstream signaling mechanisms desensitize in a manner that reflects this interaction.

The desensitization observed in this study is most likely...
caused by a mechanism downstream from the M₂ muscarinic receptor and phosphoinositide hydrolysis because we observed no desensitization of the phosphoinositide response. Such a locus would be expected to cause heterologous desensitization of the contractile effects of agonists acting on other receptors in the ileum. Accordingly, it has been shown that treatment of the isolated guinea pig ileum with muscarinic agonists causes desensitization in the contractile response to histamine (Ishii and Kato, 1987; Leurs et al., 1991; Shehnaz et al., 2001).

As described above, we suggest that activation of both M₂ and M₃ muscarinic receptors is required for the desensitization observed in this study, and that the nature of the desensitization should result in a heterologous desensitization to other contractile agents, such as histamine. We have recently observed that acetylcholine-mediated desensitization of histamine induced contractions is prevented by either pertussis toxin treatment or inactivation of M₃ receptors with 4-DAMP mustard (Shehnaz et al., 2001). These results are consistent with the idea that desensitization requires activation of both M₂ and M₃ receptors and that activation of either receptor by itself is insufficient to cause desensitization.

We noted that the Hill coefficient of the concentration-response curve to oxotremorine-M increased with acetylcholine-induced desensitization, and that this effect was prevented by pertussis toxin-treatment. These results may indicate at least two distinct components to the contractile mechanism characterized by a difference in their potency, and that the higher potency component undergoes greater desensitization. Alternatively, it may be that the increase in the Hill coefficient is simply related to recovery from desensitization during measurement of the cumulative concentration-response curve to oxotremorine-M (Shehnaz et al., 2001).

Although the present results together with those of Eglen et al. (1992) and Shehnaz et al. (2001) suggest that activation of both M₂ and M₃ receptors is required for short-term desensitization, it is possible that different receptor requirements come into play under different conditions of desensitization (e.g., long-term agonist exposure).

References

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