Pertussis Toxin Treatment Blocks Hyperpolarization by Muscarinic Agonists in Chick Atrium

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SUMMARY. Atrial and ventricular adenylate cyclase activity and atrial membrane potentials were measured in hearts from hatched chicks at 2-3 days after intravenous administration of pertussis toxin (0.5-1.0 μg, total) or saline. Both in atrium and ventricle, treatment with pertussis toxin antagonized inhibition by carbachol of basal and isoproterenol-stimulated adenylate cyclase activity without changing either basal or isoproterenol-stimulated adenylate cyclase. In atria from pertussis toxin-treated animals (5.4 ± 0.3 mV, n = 9) and did not increase resting potassium conductance. In contrast, carbachol hyperpolarized the resting membrane by 4.5 ± 0.8 mV (n = 11) and increased resting potassium conductance more than 4-fold in saline-treated animals. Carbachol did not significantly affect the atrial action potential peak or duration at 50% repolarization of pertussis toxin-treated animals. This muscarinic agonist reduced action potential peak by 7.8 ± 1.2 mV and the duration at 50% repolarization by 22.1 ± 3.0 msec in atria from saline-treated animals. Pertussis toxin treatment also prevented the negative inotropic effect and the inhibition of calcium-dependent action potentials caused by carbachol in atrial muscle. Neither the affinity nor the maximal specific binding of \(^{3}\text{H}\)quinuclidinyl benzilate in ventricular homogenates was changed by pertussis toxin treatment. The inhibition of carbachol-induced hyperpolarization by pertussis toxin treatment implicates a guanosine 5'-triphosphate-dependent protein (N, or a similar protein) as an essential link that permits muscarinic receptor to regulate atrial potassium channels. (Circ Res 57: 748-758, 1985)

MUSCARINIC agonists decrease the slow inward conductance (\(g_{s,i}\)) and increase background K⁺ conductance (\(g_{K_s}\)) in chick atrium (Inoue et al., 1983) as in the atria of other vertebrates (reviewed in Brown, 1982). The reduction of \(g_{s,i}\) by muscarinic agonists has been attributed to inhibition of adenylate cyclase activity with an attendant inhibition of cyclic adenosine monophosphate (cAMP)-dependent phosphorylation of the slow inward current channel (reviewed in Reuter, 1983; Tsien, 1983; Watanabe, 1983). Less well understood is the mechanism by which muscarinic agonists increase \(g_{K_s}\).

The relationship between muscarinic receptors (mAChR) and atrial K⁺ channels has several interesting features. There is an irreducible delay (tens of milliseconds) between agonist release (iontophoretically or from nerves) and the increase of \(g_{K_s}\) (Purves, 1976; Glitsch and Pott, 1978; Hartzell, 1980; Osterrieder et al., 1982). From experiments with a light-activated muscarinic antagonist, it has been concluded that the rate-limiting step in muscarinic agonist activation of \(g_{K_s}\) is not the agonist occupancy of mAChR, but a subsequent stage in the reaction (Nargeot et al., 1982). Some investigators have attributed this delay to a requirement for a metabolic link between mAChR and K⁺ channels (Pott and Pusch, 1979; Pott, 1979; Hartzell, 1980). However, possible intermediates including cyclic guanosine monophosphate (cGMP) (Nawrath, 1977; Trautwein et al., 1982; Nargeot et al., 1983), CAMP (Trautwein et al., 1982; Nargeot et al., 1983), and \(Ca^{++}\) (Trautwein et al., 1982) have no effect on K⁺ flux, resting membrane potential, or the ability of carbachol to hyperpolarize the resting membrane in atria or the sinoatrial node. The plausibility of a metabolic intermediate has also been questioned by the results of Osterrieder et al. (1982). The time-course of acetylcholine (ACh)-induced hyperpolarization could be adequately described by a kinetic scheme, adapted from the model of the nicotinic receptor-end plate conductance, simply by adjusting the rate constants in the reaction (see also Pott, 1983). Additional evidence against a metabolic intermediate is found in the experiments of Soejima and Noma (1984) who reported that in rabbit atrial cells, ACh increased K⁺ current when applied to the membrane under a patch clamp electrode, but not when ACh was applied to the membrane outside the patch.

In view of these divergent conclusions, we conducted a series of experiments to examine the effect of islet-activating protein (IAP, pertussis toxin) on the relationship between mAChR and K⁺ channel in atrial muscle. Islet-activating protein blocks muscarinic inhibition of adenylate cyclase by inhibiting N, the guanine nucleotide-binding protein that cou-
mM) were used to estimate polarization resistance (Rpd). Hyperpolarizing electronic potentials (5 mV) were used to prepare homogenates for adenylate cyclase assays. Ventricular homogenate was used in adenylate cyclase and binding assays. Cardiac muscle strips were superfused with gassed (95% O2, 5% CO2) modified Tyrode's solution (mm concentrations: Na+, 149; K+, 5.4; Ca++, 1.8; Mg++, 1.0; Cl-, 148; HCO3-; 11.9; HPO42-; 0.4; and glucose, 5.5) at 37°C. Elevated K+-saline solutions (25 mm) were prepared by substitution of KCl for NaCl in Tyrode's solution; the sum of K+ and Na+ concentrations was 154.4 mm. Membrane potentials were recorded with standard microelectrode techniques. Cells were impaled with glass microelectrodes filled with 3 M KCl, with tip resistances of 15–30 MΩ. The membrane potential was led through a high input impedance preamplifier (WPI type M701) with capacitance neutralization for optimum recording of potential transients, and was displayed on an oscilloscope. The signal also was led through a differentiator (Tektronix, type 0) with a response linear from 0–500 V/sec in order to record the maximum rate of rise (Vmax) of the action potential.Rectangular constant-voltage stimuli were applied to the muscle through a Teflon-insulated Ag wire (tip diameter, 100 μm) at a voltage 1.5 times diastolic threshold and at a frequency of either 0.67 Hz (2.7 and 5.4 mm K+-Tyrode's solution) or 0.1 Hz (25 mm K+-Tyrode's solution). A sucrose gap method was used to elicit electrotonic potentials and to obtain an estimate of changes in membrane resistance produced by carbachol. The experimental arrangement and its limitations have been described in a report from this laboratory (Inoue et al., 1983). In brief, the voltage changes (ΔV) produced by constant current pulses were measured at fixed distances (±400 μm) from the source of current. Provided that internal longitudinal resistance is constant, the change in electrotonic potential produced by carbachol in response to a given constant current pulse yields an estimate of the ratio of membrane resistance (ΔR) by the relation (Rm_inj/Rm_control) = k (ΔV_morph/ΔV_morph). Hyperpolarizing electronic potentials (5 mV) were used to estimate polarization resistance (Rp). The error involved in estimating the change in membrane resistance by this method is 29% at the end of the 0.5–mm length of muscle in the test compartment and will be considerably less than this at the distances used for recording electronic potentials. Data were taken only from those cells in which a stable impalement was maintained throughout the experiment. Muscle contractions were recorded with a force-displacement transducer (Grass FT03C) using the technique described previously (Higgins and Pappano, 1981) at a rest length that provided maximum twitch tension.

Adenylate cyclase was measured by the method of Salomon (1979). The assay mixture contained the following: NaCl, 100 mM; Tris-HCl, pH 7.8, 50 mM; MgCl2, 5 mM; dithiothreitol, 1 mM; EGTA, 0.1 mM; ATP, 0.3 mM; cAMP, 1.0 mM; GTP, 10 μM; phosphocreatine, 1.7 mg/ml; and creatine phosphokinase, 0.4 mg/ml. l-Isoproterenol bitartrate, carbamyl chloride (carbachol), and atropine were added as indicated. The reaction volume, 0.1 ml, contained approximately 1.5 X 105 counts/min of α-[32P]ATP (New England Nuclear). Reactions were initiated with the addition of 30–50 μg of homogenate protein and were terminated after 10 minutes at 30°C with the addition of 0.1 ml of 2% sodium dodecyl sulfate, 1.4 mM cAMP, and 4 mM ATP. [3H]cAMP (approximately 5000 counts/min per tube) was added as a recovery standard. Recovery of CAMP ranged from 60–80%. Samples were boiled and cAMP was isolated by chromatography over Dowex and alumina by the method described by Salomon (1979).

A filtration assay was used to study 1-[3H]quinuclidinyl benzilate ([3H]QNB) (Amersham, 56 Cl/mmol) binding to ventricular homogenate (Yamamura and Snyder, 1974). For direct [3H]QNB binding, 15-25 μg of homogenate protein were added to a total volume of 4.0 ml of Tyrode's solution (no added glucose) containing increasing concentrations of [3H]QNB. In displacement experiments, 0.1 nm [3H]QNB, 30–50 μg of homogenate protein, and varying concentrations of carbachol were incubated in 4.0 ml of Tyrode's solution. Reactions were initiated with the addition of homogenate. After 60 minutes at 37°C, tubes were filtered over Schleicher and Schuell no. 32 glass fiber filters and washed with 3 × 5 ml of ice-cold Tyrode's solution. Filters were placed in minivials and counted on a Tractor analytical model 6892 liquid scintillation counter at 45% efficiency at least 10 hours after the addition of Liquiscint (National Diagnostics). Nonspecific binding, defined as binding in the presence of 1 μM atropine, was subtracted from all tubes to determine specific [3H]QNB binding.

Protein was determined by the method of Lowry et al. (1951) with delipidated bovine serum albumin as standard. The independent t-test was used for statistical comparisons, except for adenylate cyclase data, for which statistical significance was determined by the analysis of variance (ANOVA).

**Results**

**Effect of Pertussis Toxin on Adenylate Cyclase Activity**

Since pertussis toxin exerts at least some of its biological effects by blocking the action of certain agonists to inhibit adenylate cyclase (reviewed by Ui, 1984), we first examined the effect of toxin treatment upon adenylate cyclase activity in ventricular homogenates. Carbachol can directly reduce

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adenylate cyclase activity in the avian ventricle (Pappano, 1984). In saline-treated animals, basal adenylate cyclase activity (44.4 ± 6.4 pmol cAMP/min per mg protein), defined as activity in the presence of 10^{-5} M GTP, was inhibited by 10.0 ± 3.8 pmol cAMP/min per mg protein (n = 8) at 10^{-4} M carbachol with an EC_{50} of 3 × 10^{-7} M (Fig. 1). This degree of inhibition is similar to that reported by others who have studied ventricular membranes from dog (Murad et al., 1962, Watanabe et al., 1978), rabbit atrial membranes (Jakobs et al., 1979), and embryonic chick atrial membranes (Halvorsen and Nathanson, 1984). Pertussis toxin treatment abolished the inhibitory effect of carbachol on ventricular adenylate cyclase activity, even with the highest concentration (10^{-4} M) tested (Fig. 1). Toxin treatment had no effect on basal adenylate cyclase (44.8 ± 4.7 pmol cAMP/min per mg protein) activity.

In a separate group of experiments, the effects of pertussis toxin treatment on drug-induced changes of homogenate adenylate cyclase activity in ventricle (part A) and atrium (part B). The concentrations of drugs when present are: isoproterenol, 10^{-6} M carbachol, 10^{-6} M atropine, 10^{-4} M. All tubes contained 10^{-5} M GTP. Experiments labeled as "basal" were done in the absence of isoproterenol. Statistical comparisons were made with ANOVA. The significance of values in saline-treated animals compared with toxin-treated animals is given above each pair of column bars (NS = no significant difference). *P < 0.05 when effects of carbachol on basal activity are compared to carbachol plus atropine. **P < 0.05 when effects of carbachol on isoproterenol-stimulated adenylate cyclase activity are compared to carbachol plus atropine. Values plotted are the mean ± SEM (ventricle, n = 10; atrium, n = 5).

pertussis toxin treatment on drug-induced changes of adenylate cyclase activity in atrium and ventricle were compared. Basal adenylate cyclase activities (pmol cAMP/min per mg protein) in ventricular homogenates were 39.4 ± 5.2 (saline-treated, n = 10) and 39.5 ± 3.1 (toxin-treated, n = 10). As shown in Figure 2A, carbachol (10^{-4} M) reduced basal adenylate cyclase activity by 10.2 ± 2.4 pmol cAMP/min per mg protein in saline-treated animals. Pertussis toxin treatment significantly (P < 0.05) reduced inhibition by this concentration of carbachol. Atropine (10^{-6} M) prevented inhibition of adenylate cyclase activity by carbachol in saline-treated animals (P < 0.05; Fig. 2A). Essentially the same results were obtained under basal conditions in atrial preparations (Fig. 2B). Under basal conditions, there was no difference between adenylate cyclase activity...
(pmol cAMP/min per mg protein) in atrial preparations from saline-treated (48.1 ± 5.5; n = 5) and pertussis toxin-treated (51.3 ± 5.5; n = 5) animals. Isoproterenol stimulation of ventricular adenylate cyclase activity was not significantly affected by pertussis toxin treatment. The EC₅₀ levels were 1.7 × 10⁻⁶ M and 1.9 × 10⁻⁶ M in saline-treated and toxin-treated animals, respectively. Activity was stimulated 1.97 ± 0.18-fold and 1.67 ± 0.11-fold in saline and toxin-treated animals, respectively, by 10⁻⁴ M isoproterenol (not shown). In the experiments dealing with carbachol-induced inhibition of isoproterenol-stimulated adenylate cyclase activity, the ventricular levels of adenylate cyclase activity (pmol cAMP/min per mg protein) in isoproterenol (10⁻⁵ M) alone were 54.8 ± 7.2 (saline-treated, n = 10) and 52.9 ± 4.9 (toxin-treated, n = 10). In atrial homogenates, adenylate cyclase activities in the presence of isoproterenol were 60.7 ± 6.9 (n = 5) and 66.9 ± 6.1 (n = 5) in saline-treated and pertussis toxin-treated animals, respectively.

Carbachol (10⁻⁵ M) significantly inhibited isoproterenol-stimulated adenylate cyclase activity (P < 0.05) in atrial and ventricular homogenates from saline-treated animals (Fig. 2, A and B). Treatment with pertussis toxin significantly reduced (P < 0.05) the inhibitory effect of carbachol on isoproterenol-stimulated adenylate cyclase activity in both atrium and ventricle (Fig. 2, A and B). Inhibition by carbachol of isoproterenol-stimulated adenylate cyclase activity in atrium and ventricle from saline-treated animals was atropine sensitive (P < 0.05; Fig. 2, A and B). In all comparisons of the effects of atropine on carbachol-induced inhibition of adenylate cyclase activity, there was no significant difference between the values in saline- and pertussis toxin-treated preparations, except in the atrium in the presence of isoproterenol. We have no explanation for the latter observation.

### Effect of Pertussis Toxin on mAChR Binding

<table>
<thead>
<tr>
<th></th>
<th>Saline-treated</th>
<th>Pertussis toxin</th>
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<tr>
<td>[³H]QNB</td>
<td></td>
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<tr>
<td>Kᵦ (pM)</td>
<td>12.5 ± 1.8</td>
<td>11.5 ± 1.5</td>
</tr>
<tr>
<td>Bₘₐₓ (fmol/mg protein)</td>
<td>368 ± 20</td>
<td>362 ± 37</td>
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Carbachol

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<tr>
<td>IC₅₀ (µM)</td>
<td>37 ± 6</td>
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<tr>
<td>Hill slope</td>
<td>0.69 ± 0.04</td>
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</table>

The binding of [³H]QNB to ventricular homogenate was assayed as described in Methods. The Kᵦ and Bₘₐₓ values for [³H]QNB were determined by Scatchard analysis. Carbachol IC₅₀ values are the concentrations of carbachol needed to displace 50% of the specific binding of 0.1 nM [³H]QNB. All values presented are the mean ± SEM of four experiments. The IC₅₀ for carbachol and the Hill slope for carbachol were significantly altered by pertussis toxin treatment (P < 0.01).

### Effect of Pertussis Toxin on Membrane Potentials and on Responses to Carbachol

Membrane potentials were measured in left atrial fibers of hearts whose ventricles were used in adenylate cyclase assays. The initial experiments were done in Tyrode's solution with 5.4 mM K⁺. As shown in Figure 3, carbachol (10⁻⁶ M) evoked a 5.5-mV hyperpolarization that reached steady state within 50 seconds and persisted for the duration of exposure to this agonist. In contrast, the same concentration of carbachol had no effect on Vᵢ in any atrial cell from a pertussis toxin-treated animal (Fig. 3). Treatment with pertussis toxin did not significantly

### FIGURE 3. Effect of pertussis toxin (IAP) treatment on carbachol-induced hyperpolarization.

Experiments were done in atrial cells from a saline-treated (upper panel) and a pertussis toxin-treated (lower panel) animal. Tyrode's solution contained 5.4 mM K⁺. Carbachol (10⁻⁶ M) was introduced (at arrows) and was present for the remainder of recording. Horizontal calibration is 1 minute; vertical calibration is 10 mV. The Vᵢ values were -79 and -80 mV in the cells from saline-treated and pertussis toxin-treated animals, respectively. The "spike" in the upper panel is a spontaneously occurring action potential.
Experiments were done with the single sucrose gap atrial cell from a saline-treated animal, the \( V_m \) was negative, an action of carbachol on \( g_K \), can be ascertained from these experiments. If the driving force is zero or from a failure of the drug to increase \( g_K \), or if \( V_m < 0 \) in 5.4 mM K+-Tyrode’s solution (Fig. 5A). The \( I-V \) relationship displayed inward-going rectification for depolarizing current; polarization resistance (\( R_{pol} \)), measured from hyperpolarizing voltage changes, was 12.1 K\( \Omega \) (Fig. 4A). In the presence of carbachol \( (10^{-6} \text{ M}) \), \( V_m \) increased to \(-88 \text{ mV} \) and \( R_{pol} \) decreased to 4.3 K\( \Omega \). These results are essentially the same as those previously reported for ACh (Inoue et al., 1983). In a series of five such experiments done in 5.4 mM K+-Tyrode’s solution, \( R_{pol} \) averaged 6.7 \( \pm \) 2.0 K\( \Omega \) in the absence and 1.7 \( \pm \) 0.8 K\( \Omega \) in the presence of \( 10^{-4} \text{ M} \) carbachol. When the ratio, \( R_{carb}/R_{CTR} \), was determined for each experiment and averaged, membrane resistance decreased to 0.23 \( \pm \) 0.06 of control values in the presence of carbachol. The results in Fig. 4B indicate that the \( I-V \) relationship displayed inward-going rectification in the cell from a pertussis toxin-treated animal, and \( R_{pol} \) was 8.3 K\( \Omega \). The addition of carbachol did not change \( V_m \) or \( R_{pol} \) in this cell (Fig. 5B). In a series of seven experiments, \( R_{pol} \) averaged 7.1 \( \pm \) 2.2 K\( \Omega \) in atrial cells from pertussis toxin-treated animals. This value was not significantly different from that in saline-treated animals. Moreover, \( R_{pol} \) averaged 6.9 \( \pm \) 1.8 K\( \Omega \) in the presence of carbachol and the averaged value of \( R_{carb}/R_{CTR} \) from these experiments was 1.01 \( \pm \) 0.09. Therefore, carbachol did not significantly change \( g_K \) in cells from pertussis toxin-treated animals superfused with 5.4 mM K+-Tyrode’s solution.

The possibility that treatment with pertussis toxin somehow modifies the \( K^+ \) channel directly is not supported by the several lines of evidence presented below. One such piece of evidence is that \( 0.1 \mu \text{M} \) carbachol was used. The \( Ca^{2+} \)-dependent action potential was completely blocked in five of six \( Ca^{2+} \)-dependent action potentials can be examined in the presence of isoproterenol. Carbachol concentration was 1 \( \mu \text{M} \) in all cases except for the saline-treated \( Ca^{2+} \)-dependent action potentials where only 0.1 \( \mu \text{M} \) carbachol was used. The \( Ca^{2+} \)-dependent action potential was completely blocked in five of six saline-treated preparations by 0.1 \( \mu \text{M} \) carbachol. Values of zero were used for \( APD_{50} \), where there was no action potential. All abbreviations used are: \( V_m \) resting membrane potential; \( V_p \), the peak of the action potential; \( APD_{50} \), action potential duration at 50% repolarization. The \( P \) values indicate significance of the effect of carbachol within each group using Student’s paired \( t \)-test.

### Table 2: Effects of Pertussis Toxin on Atrial Membrane Potential

<table>
<thead>
<tr>
<th>Condition</th>
<th>( V_m ) (mV)</th>
<th>( V_p ) (mV)</th>
<th>( APD_{50} ) (msec)</th>
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<tbody>
<tr>
<td>Control</td>
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<tr>
<td>Carbachol</td>
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<td>Difference</td>
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<td>( P )</td>
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**Table**: Membrane potential was measured in paced (0.67 Hz) atrial fibers from chicks treated 2-3 days earlier with pertussis toxin. In 25 mM external \( K^+ \), voltage-activated \( Na^+ \) channels are inactivated and \( Ca^{2+} \)-dependent action potentials can be examined in the presence of isoproterenol. Carbachol concentration was 1 \( \mu \text{M} \) in all cases except for the saline-treated \( Ca^{2+} \)-dependent action potentials where only 0.1 \( \mu \text{M} \) carbachol was used. The \( Ca^{2+} \)-dependent action potential was completely blocked in five of six saline-treated preparations by 0.1 \( \mu \text{M} \) carbachol. Values of zero were used for \( APD_{50} \), where there was no action potential. All abbreviations used are: \( V_m \) resting membrane potential; \( V_p \), the peak of the action potential; \( APD_{50} \), action potential duration at 50% repolarization. The \( P \) values indicate significance of the effect of carbachol within each group using Student’s paired \( t \)-test.
HYPERPOLARIZATION BY 10^{-6}M CARBACHOL

![Graph showing hyperpolarization by 10^{-6}M carbachol]

**Figure 4.** Effect of $[K^+]_o$ on carbachol-induced hyperpolarization in atria from saline-treated (O) and pertussis toxin-treated (•) animals. Ordinate: hyperpolarization (mV). Abscissa: $[K^+]_o$ (mM) on logarithmic scale. Number of cells indicated in parentheses; carbachol concentration was 10^{-6}M in all experiments.

excluded by measuring the effects of carbachol on membrane potential and resistance. That $V_m$ was not significantly different at 5.4 mM $K^+$ in cells from saline- and pertussis toxin-treated animals indicates that the regulation of $V_m$ by the transmembrane distribution of $K^+$ is not importantly affected by pertussis toxin treatment. Another indication that pertussis toxin had no effect on $K^+$ channels directly was the observation that reduction of $[K^+]_o$ to 2.7 mM made the $V_m$ more negative in cells from pertussis toxin-treated animals (Table 2). In 2.7 mM $[K^+]_o$, the $V_m$ values of atrial cells from saline-treated and toxin-treated animals were not significantly different. When $[K^+]_o$ is reduced, the $V_m$ hyperpolarizes because the driving force on $K^+$ increases. However, a reduction of $[K^+]_o$ also diminishes $g_K$, so that the difference between $V_m$ and $V_r$ becomes greater (reviewed in Sperelakis, 1979). Therefore, carbachol-induced increases of $g_K$ should evoke a larger hyperpolarization when $[K^+]_o$ is reduced, as reported for atrial cells (Trautwein and Dudel, 1958; Garnier et al., 1978) and Purkinje fibers (Mubagwa and Carmeliet, 1983). This phenomenon was observed in chick atrium for carbachol (10^{-6}M) hyperpolarized the membrane by 8.5 ± 0.7 mV in atrial cells from saline-treated animals (Fig. 4; Table 2). Carbachol also hyperpolarized the membrane (1.6 ± 0.7 mV) in cells from pertussis toxin-treated animals, but significantly less than in the saline-treated animals (Fig. 5). This result indicates that the effect of pertussis toxin is not as complete in 2.7 mM $[K^+]_o$ as in 5.4 mM $[K^+]_o$. Resting membrane I-V relationships indicated that $R_{in}$ was 15.2 KΩ in a saline-treated animal (n = 1) and 13.3 ± 5.2 KΩ in pertussis toxin-treated animals (n = 4). These values of membrane resistance are greater than in 5.4 mM $[K^+]_o$, and are consistent with the observations of others that $g_K$ is directly related to $[K^+]_o$ (see above). When the increased driving force for $K^+$ measurement is considered, along with the increased membrane resistance in 2.7 mM $[K^+]_o$, as compared to 5.4 mM $[K^+]_o$, a small increase of $g_K$ by carbachol in cells from pertussis toxin-treated animals can be expected to hyperpolarize the membrane.

In 25 mM $[K^+]_o$, $V_m$ was −43 ± 0.5 mV in saline-treated animals (Table 2) and carbachol (10^{-6}M) elicited a small but significant hyperpolarization (1.0 ± 0.3 mV; Fig. 5). Treatment with pertussis toxin had no significant effect on $V_m$ in 25 mM $K^+$ (Table 2); however, carbachol did not hyperpolarize the atrial membrane in these preparations (Fig. 5).

**Action Potentials**

In atrial cells from pertussis toxin-treated animals, the ability of carbachol to reduce action potential
peak ($V_p$) and duration at 50% repolarization ($APD_{50}$) was suppressed, as was the carbachol-induced hyperpolarization. As shown in Figure 6 (A–C), in a paced (0.67-Hz) atrial fiber from a saline-treated animal, carbachol ($10^{-6}$ M) hyperpolarized the resting membrane ($V_m$) ($-82$ mV) by $4$ mV and reduced the peak of the action potential ($V_p$) and the action potential duration at 50% repolarization ($APD_{50}$) by $3$ mV and $23$ msec, respectively. These effects of carbachol were largely prevented in an atrial fiber from a pertussis toxin-treated animal (Fig. 6, D–F). The resting membrane potential ($-83$ mV) did not change while $V_p$ and the $APD_{50}$ decreased by $2$ mV and $1$ msec, respectively (Fig. 6, D–F). The results of all such experiments, done in Tyrode’s solution with $5.4$ mM K+, are given in Table 2.

Carbachol had no significant effect on $V_p$ and $APD_{50}$ when atrial cells from pertussis toxin-treated animals were superfused with Tyrode’s solution containing $2.7$ mM K+. Pertussis toxin treatment alone had no significant effect on $V_p$ at $2.7$ and $5.4$ mM K+. In Tyrode’s solution with $5.4$ mM K+, but not with $2.7$ mM K+, the $APD_{50}$ was decreased significantly ($P < 0.05$) by pertussis toxin treatment alone ($33.1 \pm 2.3$ msec in saline-treated chicks vs. $24.1 \pm 1.8$ msec in toxin-treated chicks (Table 2)). The mechanism of this effect is not known. Nevertheless, the $APD_{50}$ in the presence of $10^{-7}$ M carbachol was still greater in pertussis toxin-treated animals ($22.1 \pm 1.9$ msec) than in saline-treated animals ($11.3 \pm 1.2$ msec) (Table 2).

Effect of Pertussis Toxin on Contraction

The negative inotropic effect of carbachol in atrial muscle strips was greatly reduced by pertussis toxin treatment. Carbachol ($10^{-6}$ M) reduced tension in paced atrial strips to $8 \pm 4\%$ of control tension in saline-treated vs. $91 \pm 2\%$ of control tension in toxin-treated (Fig. 8). The $EC_{50}$ for this effect was $2 \times 10^{-7}$ M in saline-injected chicks, whereas $10^{-4}$ M isoproterenol was always required in toxin-treated chicks. The reason for the difference in isoproterenol sensitivity to restore Ca++-dependent action potentials is not known. Ca++-dependent action potentials were completely eliminated by $10^{-7}$ M carbachol in five of six saline-treated preparations tested. An example of such an experiment is shown in Figure 7 (A–C). In the one preparation where a Ca++-dependent action potential remained at $10^{-7}$ M carbachol, it was of much lower amplitude ($12$ mV decrease in $V_p$) and duration ($13$-msec decrease in $APD_{50}$). In pertussis toxin-treated animals, a 10-fold higher concentration of carbachol ($10^{-6}$ M) never abolished the Ca++-dependent action potential (Fig. 7, D–F). The results of all experiments in $25$ mM K+-depolarized atria appear in Table 2.
FIGURE 7. Calcium-dependent action potentials in paced (0.1 Hz) atrial muscle in 25 mm external potassium in the presence of isoproterenol. The top tracing in each panel is the membrane potential; vertical calibration is 20 mV. The lower tracing in each panel is the first derivative (dV/dt); vertical calibration is 5 V/sec. Horizontal calibration bar is 20 msec and marks zero potential. Panels A, B, and C are from a saline-injected animal. Panels D, E, and F are from a pertussis toxin-injected animal. Panels A and D are with isoproterenol alone. Panels B and E are with isoproterenol and the indicated concentrations of carbachol (10^{-7} M in panel B; 10^{-6} M in panel E). In panels C and F, the action potentials in the absence and presence of carbachol are superimposed.

Discussion
We have demonstrated that 2-3 days after pertussis toxin is injected in hatched chicks, the ability of carbachol to inhibit adenylate cyclase (basal and isoproterenol-stimulated) in atrial and ventricular homogenates is greatly diminished. In atrial muscle from these same animals, the effects of carbachol on membrane potentials are also reduced. After pertussis toxin treatment, carbachol did not reduce action potential duration, suppress Ca^{++}-dependent action potentials, or hyperpolarize the resting membrane to the same extent as in saline-treated animals. Pertussis toxin treatment also blocked the negative inotropic effect of carbachol in atrial muscle. The effects of carbachol on APD, the slow action potential, and contractility probably are related to effects on $g_M$ that are mediated by adenylate cyclase (and intracellular free Ca^{++}) (Inoue et al., 1983). Hyperpolarization of the resting membrane is attributed to an effect of carbachol on $g_K$ (Inoue et al., 1983).

[Atrial membrane potentials from pertussis toxin-treated animals did not differ significantly from those in saline-treated animals with one exception. The APD_{50} tended to be briefer, significantly so in 5.4 mm K+-Tyrode's solution, in cells from pertussis toxin-treated animals. The reason for this is unknown. This effect could result from an increase of a voltage- and time-dependent conductance to K^{+} ($i_K$) which was not measured in our experiments. It is unlikely that the diminished APD_{50} is due to increased $g_K$, because neither the resting membrane potential nor membrane resistance was significantly affected by treatment with pertussis toxin.]

Adenylate cyclase, the enzyme responsible for production of the second messenger cAMP, is regulated by a variety of stimulatory and inhibitory
hormones in different tissues. Membrane receptors for hormones are coupled to the catalytic subunit of adenylate cyclase by either a stimulatory guanine nucleotide regulatory protein (N_s) for receptors that activate adenylate cyclase or by an inhibitory guanine nucleotide regulatory protein (N_i) for receptors that inhibit adenylate cyclase (reviewed in Rodbell, 1980). These guanine nucleotide regulatory proteins have been shown to be at least dimeric proteins composed of an alpha-subunit and a beta-subunit (Northrup et al., 1980; Bokoch et al., 1984). Pertussis toxin, isolated from the supernatant of Bordetella pertussis cultures, has been shown to catalyze the ADP-ribosylation of the alpha-subunit of N_i, and to block the effects of inhibitory agonists on adenylate cyclase [Uj review (1984)].

Muscarinic agonists and beta-adrenoceptor agonists have opposing effects on N_i and contractility in myocardium. There is considerable evidence that the effects of beta-adrenoceptor agonists are mediated by an increase in adenylate cyclase activity and cAMP-dependent processes (Reuter, 1983; Tsien, 1983; Watanabe, 1983) and that the opposing effects of muscarinic agonists are due to adenylate cyclase inhibition (Murad et al., 1962; Jakobs et al., 1979; Biegon and Pappano, 1980; Iwasa and Hosey, 1983). Our observation that pertussis toxin blocks the inhibition of adenylate cyclase, APD, and contractility by muscarinic agonists is consistent with the idea that the negative inotropic effect and the decrease in N_i caused by muscarinic agonists are due to adenylate cyclase inhibition.

Treatment with pertussis toxin had no effect on either the K_3 or B_max of [3H]QNB binding to ventricular mACHR indicating that pertussis toxin probably does not bind to mACHR. There is a small (2-fold) decrease in apparent affinity of carbachol for mACHR in pertussis toxin-treated animals, a result qualitatively similar to those reported by McMahon et al. (1985). This finding cannot readily explain why blockade of carbachol-induced effects on adenylate cyclase, membrane potentials, and contractions is not overcome by higher concentrations of muscarinic agonist. The simplest explanation is the postulated action of toxin on N_i which regulates agonist affinity for mACHR (reviewed in Uj, 1984).

The link between muscarinic agonists and increases in N_i is not as well defined. It is not clear whether there is a metabolic intermediate or a receptor-channel complex with slow kinetics (Hartzell, 1982) (see introduction). The lag phase between application of muscarinic agonist and opening of K_+ channels argues for a metabolic intermediate (Hartzell, 1982). There is, however, no evidence supporting a role for any of the known second messengers (cyclic nucleotides and Ca^{++}; see introduction) to link mACHR activation to changes in N_i. The possibility that increased phosphatidyl inositol (PI) hydrolysis is somehow involved as a link between mACHR and K_+ channels has been considered (Brown and Masters, 1984). Oxotremorine, unlike carbachol, has only a small effect on PI turnover in chick atrium (Brown and Brown, 1984), yet oxotremorine is a more potent agonist than carbachol for hyperpolarizing atrial cells (Hartzell, 1980; Subers and Pappano, unpublished observation). However, the possibility that the effect of a small increase of PI turnover may be sufficient to achieve a large increase of N_i, mitigates against this view. Most important, pertussis toxin treatment does not block carbachol-induced increased PI turnover in cultured chick heart cells (Masters et al., 1985). This result, taken together with our observation that pertussis toxin treatment blocks carbachol-induced increase of N_i and hyperpolarization, makes unlikely a role of increased PI turnover as a link between mACHR and atrial K_+ channels. Treatment with pertussis toxin did not affect the membrane hyperpolarization and increased membrane resistance produced by reducing [K_+], from 5.4-2.7 mM. This result indicates that pertussis toxin had no important effect on N_i, by itself at a concentration that prevented muscarinic agonist-induced activation of these K_+ channels.

Our data show that pertussis toxin blocks carbachol-induced increase of N_i and hyperpolarization of chick atrium. These data cannot readily be reconciled with the idea that muscarinic-induced hyperpolarization is due to inhibition of adenylate cyclase because of the evidence against cyclic nucleotides as a link between mACHR and N_i (see introduction). We favor an alternative explanation, namely, that muscarinic-induced hyperpolarization is mediated by a pertussis toxin-sensitive substrate which functions independently of adenylate cyclase. This pertussis toxin substrate could be N_i coupling mACHR to more than one effector or a separate coupling protein. There is increasing evidence that there are as yet uncharacterized guanine nucleotide regulatory protein(s) which may couple receptors to their effectors (Rodbell, 1980; Gomperts, 1983; Heyworth et al., 1983; Uj, 1984; Okajima and Uj, 1984). Some of these protein(s) may be pertussis toxin substrates, since it appears that there are multiple pertussis toxin substrates in bovine cerebral cortex (Neer et al., 1984), rat fat cells (Malbon et al., 1984), rabbit heart (Malbon et al., 1984), and chick atrium (Halvorsen and Nathanson, 1984).

In summary, there are at least three components involved in the regulation of N_i by muscarinic agonists in atrium: a muscarinic receptor, a pertussis toxin-sensitive substrate, and an inwardly rectifying K_+ channel. Although our data do not permit a resolution of the problem of whether a metabolic intermediate links mACHR to atrial K_+ channels, it is reasonable to conclude that a pertussis toxin-sensitive substrate is an essential component for regulation of inwardly rectifying N_i by agonists acting on mACHR.
Note Added in Proof

After this paper had been accepted for publication, we learned that two other laboratories had reached essentially the same conclusion from experiments done in rat (Endoh et al., 1985) and chick (Pfaffinger et al., in press) atrial cells.

A preliminary report of these results has been presented (Sorota et al., 1985).

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