Muscarinic Cholinergic-Receptor Stimulation of Specific GTP Hydrolysis Related to Adenylate Cyclase Activity in Canine Cardiac Sarcolemma

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One component of muscarinic receptor inhibition of the function of cardiac ventricles is mediated by the inhibition of activated adenylate cyclase activity in sarcolemma. We have shown previously that muscarinic agonists inhibit GTP- but not Gpp(NH)p-activated adenylate cyclase activity, and various studies in other tissues indicate that nonhydrolyzable GTP analogues prevent inactivation of the enzyme. These data have suggested a role for GTP hydrolysis in the mechanism of inhibition of adenylate cyclase. The present study demonstrates that purified canine cardiac sarcolemma displays high-affinity GTPase activity that is reciprocally related to adenylate cyclase activity. The high-affinity GTPase activity was stimulated by muscarinic agonists and blocked by atropine. Furthermore, the one-half maximal effects of oxotremorine for binding to muscarinic receptors, stimulation of high-affinity GTPase activity, and inhibition of adenylate cyclase activity were similar. Muscarinic stimulation of GTPase activity and inhibition of adenylate cyclase activity required functional activity of the pertussis toxin (IAP) substrate(s). Treatment of sarcolemmal membranes with IAP attenuated the ability of oxotremorine to both stimulate high-affinity GTPase activity and inhibit adenylate cyclase activity. These studies indicate that muscarinic receptor stimulation of high-affinity GTPase activity dependent on functional IAP substrate(s) is closely linked to the mechanism of muscarinic inhibition of adenylate cyclase activity. (Circulation Research 1988;64:340-350)
Preparation of Pertussis Toxin

Preparation of Pertussis Toxin was performed as described by Flemming and Watanabe.

Materials and Methods

**Materials**

- Materials for sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS PAGE) were from Bio-Rad, Richmond, California. CNBr-activated Sepharose was purchased from Pharmacia, and fetuin was from Sigma Chemical, St. Louis, Missouri.
- Hydroxylapatite (fast flow) was from Calbiochem, San Diego, California. App(NH)p and Gpp(NH)p were obtained from Boehringer Mannheim, Mannheim, FRG, and forskolin was from Calbiochem-Behring. Other nucleotides, ligands, and reagents were from Sigma or Fisher Scientific.

**Preparation of Pertussis Toxin**

*Bordetella pertussis* strain 165 was grown in modified Stainer-Sholte medium as described by Hewlett et al. Pertussis toxin was purified from the culture supernatants through the use of hydroxyapatite and fetuin-agarose chromatography with a modification of procedures described elsewhere as communicated by Dr. Erik Hewlett of the University of Virginia. Briefly, 71 culture supernatant were passed through a 200-ml column of hydroxylapatate that had been preequilibrated with 10 mM potassium phosphate (pH 9.0). The eluate from the pH 9 column was adjusted to pH 6.0 with HCl and applied to a 200-ml column of hydroxylapatate that had been preequilibrated with 10 mM potassium phosphate (pH 6.0). This column was then washed sequentially with 500 ml of 100 mM potassium phosphate (pH 6.0) and 500 ml of 100 mM potassium phosphate (pH 7.0). Pertussis toxin was eluted from the column with 400 ml of 100 mM potassium phosphate (pH 7.0) containing 0.5 M NaCl. The resulting eluate was applied to a 35-ml column of fetuin-Sepharose. The column was washed with 105 ml of 100 mM potassium phosphate (pH 7.0)-0.5 M NaCl. Pertussis toxin was eluted with 100 ml of 100 mM potassium phosphate (pH 7.0)-0.5 M NaCl containing 3 M KSCN. The purified toxin was dialyzed and concentrated with an Amicon YM-10 ultrafiltration membrane (43 mm) and 100 mM potassium phosphate (pH 7.0)-0.5 M NaCl. Pertussis toxin was stable for at least 1 year when stored in frozen aliquots at −20°C.

**Preparation of Membranes**

Cardiac membrane vesicles were prepared exactly as described by Jones et al. Cardiac sarcolemma was prepared according to Jones et al. from Procedure II membrane vesicles with the exception that 0.75 M NaCl-10 mM l-histidine was substituted for 0.75 M KCl-5 mM l-histidine in the starting buffer. The total ouabain-sensitive Na+,K+-ATPase activity of the purified sarcolemma was routinely 70-90 μmol P/hr/mg protein; the maximum specific binding of [3H]QNB to muscarinic receptors was 5.5 pmol/mg protein.

**Adenylate Cyclase Assay**

Adenylate cyclase activity was assayed by measuring the conversion of [α-32P]ATP to cyclic [32P]AMP, essentially as described earlier. Unless otherwise indicated, the 0.1-ml assays contained 40 mM Tris-HCl (pH 7.5), 1 mM (20 cpm/pmol) [α-32P]ATP, 10 mM MgCl₂, 0.5 mM cyclic AMP (cAMP), 5 mM phosphocreatine, and 5 units/ml creatine phosphokinase. Reactions were initiated by the addition of 4-6 μg membrane protein and were allowed to incubate for 10 minutes at 37°C. Reactions were terminated by the addition of 0.8 ml of a stopping solution containing 5 mM ATP, 0.175 mM cyclic AMP, and 0.25% sodium dodecyl sulfate (pH 7.5). Cyclic [8-3H]AMP was added (0.1 ml, 10,000 cpm) to allow quantitation of cyclic AMP recovery (70-80%). Cyclic AMP formed during the reactions was isolated by the method of Salomon et al.
Results are expressed as mean±SEM of triplicate determinations as shown in the figures. Single experiments representative of three to five similar studies are depicted.

GTPase Assay

GTP hydrolysis was determined by measuring the release of $^{32}$P, from $[^{32P}]$GTP. Unless otherwise indicated, the 0.1 ml triplicate assays contained 40 mM Tris-HCl (pH 7.5), 0.25 uM (3,000 cpm/pmol) $[^{32P}]$GTP, 5 mM creatine phosphate, 5 U/ml creatine phosphokinase, 1 mM ATP, 1 mM App(NH)P, 10 mM MgCl$_2$, and 1 mM EDTA. Reactions were initiated by the addition of 4–6 uM membrane protein and were allowed to incubate for 10 minutes at 37° C. Reactions were terminated by the addition of 0.9 ml activated charcoal suspension (Norit A in 20 mM phosphoric acid, 5% wt/vol) at 0° C. After 5 minutes on ice, the mixtures were centrifuged for 10 minutes at 4° C in a Beckman TJ-6 centrifuge (Fullerton, California) at 2,700 rpm (TH-4 rotor, 1,520^G^,). Radioactivity remaining in the supernatant was identified as $^{32}$P, by thin-layer chromatography as described in Materials and Methods. The tubes were placed on ice, and 0.5 ml of the clear supernatant was removed for liquid scintillation spectrometry. Nonspecific $^{32}$P$^-$ release and $^{32}$P$^-$ contamination of the substrate were determined in parallel assays containing 100 uM unlabeled GTP for each incubation condition. Specific GTPase activity was expressed as the difference between the mean total and nonspecific activities with the SEM shown in the figures. Single experiments representative of three to five similar studies are depicted.

Atomic Absorption

The concentration of sodium ion was determined in the indicated experiments with an Instrumentation Laboratories model 951 spectrophotometer (Dayton, Ohio).

Muscarinic Receptor Assays

The affinity of oxotremorine binding to sarcolemmal muscarinic receptors was determined at equilibrium by competition with the agonist ligand [H]$^-$QNB, essentially as we have described previously.17,21

Preparation of ADP-Ribosylation Factor

Cholera toxin-catalyzed ADP-ribosylation of G$_{ma}$ from certain species requires a protein cofactor called “ADP-ribosylation factor” (ARF), and a functionally similar protein is required for the modification of cardiac sarcolemmal G$_{ma}$, For the present study, ARF was the soluble fraction from canine cardiac left ventricles. During preparation of Procedure I membrane vesicles according to Jones et al., the soluble fraction from the 45,000g$_{max}$ centrifugation was saved and further centrifuged at 35,000 rpm for 60 minutes in a Beckman 35 rotor. This cytosolic fraction was frozen in aliquots at −20° C and used in the $[^{32P}]$ADP-ribosylation of G$_{ma}$ as described below. The use of more highly purified preparations of ARF did not alter the effects of cholera toxin on adenylate cyclase or GTPase activities (data not shown).

$[^{32P}]$ADP-Ribosylation of Pertussis Toxin Substrates

Covalent labeling of G$_{ma}$ and G$_{m}$ was performed exactly as for G$_{ma}$, except that activated IAP was substituted for cholera toxin, and ARF was omitted.

SDS PAGE of Toxin-Treated Sarcolemma

After intoxication with either cholera toxin or IAP, membranes were subjected to SDS PAGE as described by Laemmi.42 Samples containing 60 ug protein were loaded onto each gel (8% resolving gel). The gels were run at 20 mA per gel until the bromphenol blue dye front exited from the bottom of the gel. The gels were stained with Coomassie Blue, destained, dried, and subjected to autoradiography with Dupont Cronex Pellicula X-ray Film and Lightning Plus intensifying screens.

Results

Isotopic dilution experiments identified a specific, high-affinity GTPase activity in cardiac sarcolemma (Figure 1). Approximately 10% of 100 nM $[^{32P}]$GTP was hydrolyzed by the membranes during incubation at 37° C for 10 minutes. Increasing concentrations of unlabeled GTP reduced the hydro-
lysis of \([\gamma^{32}\text{P}]\text{GTP}\) until a plateau was reached at concentrations greater than 10 \(\mu\text{M}\), indicating the presence of low-affinity GTPase activity. The muscarinic agonist oxotremorine stimulated GTP hydrolysis at all concentrations of GTP below 10 \(\mu\text{M}\), indicating that the muscarinic agonist stimulated the activity of a high-affinity GTPase in the sarcolemmal membranes. In contrast, oxotremorine had no effect on the low-affinity (nonspecific) enzyme(s).

After correction for nonspecific hydrolysis (see "Materials and Methods") and specific activity of the \([\gamma^{32}\text{P}]\text{GTP}\), saturation of the specific, high-affinity GTPase was observed (Figure 1, inset). In the absence of muscarinic ligands, the \(V_{\text{max}}\) of the high-affinity GTPase was 22.8 pmol \(\text{P}_i/\text{min/mg protein}\) (21.0–24.8), and the \(K_m\) for GTP was 0.18 \(\mu\text{M}\) (0.14–0.23); the Hill coefficient was 1.01. (The asymmetric confidence intervals were calculated according to Johnson and Frasier.\(^4^3\)) Oxotremorine stimulated the \(V_{\text{max}}\) of the enzyme over 100% to 51.0 pmol \(\text{P}_i/\text{min/mg protein}\) (47.3–54.9) and had essentially no effect on the \(K_m\) (0.2–0.3 \(\mu\text{M}\)). Another muscarinic receptor agonist, methacholine, had qualitatively and quantitatively similar effects on high-affinity GTPase activity (data not shown). Specific GTPase activity was linear with respect to time (2–15 minutes) and protein (3–18 \(\mu\text{g}\)) in the absence or presence of oxotremorine.\(^3^0\)

The \(K_{\text{oxo}}\) for oxotremorine stimulation of high-affinity GTPase activity was 0.14 \(\mu\text{M}\) (0.11–0.17) (Figure 2). The muscarinic receptor antagonist atropine completely abolished the effects of oxotremorine, except at millimolar concentrations of oxotremorine. The addition of atropine (1 \(\mu\text{M}\)) resulted in a parallel 716-fold rightward shift in the concentration-effect curve, the \(K_{\text{oxo}}\) for oxotremorine being increased to 100.2 \(\mu\text{M}\) (73.2–127.0).

Classic competitive inhibition by atropine indicated that stimulation of the high-affinity GTPase was mediated by muscarinic receptors.

Both basal and oxotremorine-stimulated high-affinity GTPase activities required \(\text{Mg}^{2+}\). The \(K_{\text{Mg}}\) for \(\text{MgCl}_2\) (0.8 \(\text{mM}\)) was unchanged by oxotremorine, and concentrations of \(\text{MgCl}_2\) >5 \(\text{mM}\) had no further effect on the activity. High-affinity GTPase activity was stable at pH 6.5–8.2, with oxotremorine-stimulated activity decreasing at pH >8.0. Because of the pronounced stimulatory effects of the plant diterpene derivative forskolin on adenylate cyclase activity,\(^1^3\)\(^1^7\)\(^4^4\) it was of interest to examine its effects on the high-affinity GTPase activity. Whereas 50 \(\mu\text{M}\) forskolin typically stimulated cardiac adenylate cyclase activity eightfold,\(^1^7\) forskolin had no effect on the high-affinity GTPase activity in the absence or presence of oxotremorine (Table 1).

Because hydrolysis of GTP by the sarcolemmal membranes would not necessarily bear any relation to adenylate cyclase activity per se, such a relation was evaluated using several approaches. First, it is well established that sodium ions have modulatory effects in dually regulated adenylate cyclase systems, affecting muscarinic receptor binding affinity.\(^4^5\)

### Table 1. Effects of Forskolin on High-Affinity GTPase Activity

<table>
<thead>
<tr>
<th></th>
<th>GTPase activity (pmol P/min/mg protein)</th>
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<tbody>
<tr>
<td></td>
<td>Basal</td>
</tr>
<tr>
<td>Control</td>
<td>17.9±2.0</td>
</tr>
<tr>
<td>Forskolin (50 (\mu\text{M}))</td>
<td>17.5±1.3</td>
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Cardiac sarcolemma was assayed for basal and oxotremorine-stimulated high-affinity GTPase activity in the absence (Control) and presence of 50 \(\mu\text{M}\) forskolin as described in "Materials and Methods."
and the effects of inhibitory receptors on adenylate cyclase activity.46,47 Accordingly, we used sodium chloride as a probe of GTPase and adenylate cyclase activities. In general, GTPase and adenylate cyclase activities were inversely related (Figure 3), consistent with the hypothesis that inhibition of GTP hydrolysis would increase the availability of $G_{o}\cdot GTP$ and thereby increase adenylate cyclase activity. Increasing concentrations of sodium chloride progressively reduced both basal and oxotremorine-stimulated high-affinity GTPase activities (Figure 3, right). In contrast, sodium chloride stimulated adenylate cyclase activity over the same range of concentrations that inhibited GTPase activity (Figure 3, left). Moreover, the ability of oxotremorine to stimulate high-affinity GTPase activity declined with increasing concentrations of sodium chloride, from 54.5% stimulation in the absence of NaCl to 39.0% in the presence of 150 mM NaCl (Figure 3, right). In parallel, sodium chloride reduced the ability of oxotremorine to inhibit GTP-activated adenylate cyclase activity (Figure 3, left). In the absence of sodium chloride, 10 $\mu$M GTP stimulated adenylate cyclase activity 86.4%, and 10 $\mu$M oxotremorine almost completely abolished the increase in activity due to GTP. However, at 50, 100, and 150 mM NaCl, oxotremorine inhibition of GTP-activated adenylate cyclase activity was reduced to 96.6%, 71.0%, and 33.7%, respectively. Thus, both basal and hormone-regulated GTPase and adenylate cyclase activities appear linked, both to each other and to modulation by sodium chloride. In addition to demonstrating a reciprocal relation between GTPase and adenylate cyclase activities, these data posed new questions regarding the specificity of the sodium chloride effect and the apparent lack of a sodium ion requirement for muscarinic regulation of the activities.

Two experimental approaches further defined the relation of ionic strength and sodium ion to GTPase and adenylate cyclase activities. In these experiments, the Tris salts of reagents (e.g., ATP, GTP, and phosphocreatine) were used where appropriate to create a "sodium-free" medium; the $[Na^+]$ contributed by the sodium-free medium and membranes was measured to be <600 $\mu$M. The sodium...
chloride–induced decrease in GTPase activity and parallel increase in GTP-stimulated adenylate cyclase activity described above apparently resulted from relatively specific effects of the monovalent cation rather than from nonspecific effects of ionic strength on the enzymic activities. NaCl, KCl, and LiCl (150 mM) each decreased GTPase activity and increased GTP-stimulated adenylate cyclase activity (Figure 4). In contrast, 150 mM ChCl had the opposite effect: GTPase activity was stimulated, and GTP-stimulated adenylate cyclase activity was decreased (Figure 4). The effects on the enzymic activities were thus due to the monovalent cation rather than to chloride ion or ionic strength. Furthermore, Na⁺ was apparently not required for the observation of muscarinic stimulation of GTPase activity and inhibition of GTP-stimulated adenylate cyclase activity. In the sodium-free medium ([Na⁺] = 585 μM), 10 μM oxotremorine resulted in a characteristic 70% stimulation of GTPase activity (Figure 5). In parallel assays in sodium-free medium ([Na⁺] = 537 μM), GTP stimulated basal adenylate cyclase activity from 508 to 923 pmol cAMP/min/mg protein (81.7%), and the increment in enzymic activity due to GTP was inhibited 55.3% by 10 μM oxotremorine (Figure 5).

Another assessment of the muscarinic receptor–GTPase–adenylate cyclase relation used was the comparison of the concentration-effect curves for oxotremorine binding to the muscarinic receptor, oxotremorine stimulation of the high-affinity GTPase activity, and oxotremorine inhibition of GTP-activated adenylate cyclase activity (Figure 6). In these experiments, the effects of oxotremorine on [3H]QNB binding, stimulation of the high-affinity GTPase activity, and inhibition of GTP-stimulated adenylate cyclase activity were determined simultaneously in the same incubation tubes. Cardiac sarcolemma was incubated in the presence of [3H]QNB, [γ-32P]GTP, [α-32P]ATP, and various concentrations of oxotremorine. At the end of the incubation period, aliquots of the reaction were removed for filtration ([3H]QNB binding), determination of [32P] liberation (GTPase activity), and determination of cyclic [32P]AMP formation (adenylate cyclase activity). The effects of oxotremorine on the three parameters were compared with respect to the maximum effect of the muscarinic agonist in each case (Figure 6). The affinities of oxotremorine for each process were 16 μM (inhibition of [3H]QNB binding), 7 μM (stimulation of GTPase), and 5 μM (inhibition of GTP-stimulated adenylate cyclase activity. Correcting for the concentration of [3H]QNB and its affinity for the receptors (5.9 nM; Kd = 15.3 pM), the Kd for oxotremorine binding to muscarinic receptors (42 nM) was similar to the Kd for oxotremorine stimulation of high-affinity GTPase activity (110–170 nM, Figure 2; the higher Kd for oxotremorine stimulation of GTPase in Figure 6 relative to that in Figure 2 is also due to the presence of QNB). That the concentration effect of oxotremorine was essentially identical for each process suggested a close relation between oxotremorine binding to muscarinic receptors and reciprocal regulation of GTPase and adenylate cyclase activities.

Finally, bacterial toxins with selectivity for Gs or Gi were used to further probe the relation between the high-affinity GTPase and adenylate cyclase activities. Cholera and pertussis toxins selectively catalyze the ADP-ribosylation of Gs and Gi, respectively. In canine cardiac sarcolemma, cholera toxin catalyzed the [32P]ADP-ribosylation of Gs, and pertussis toxin (IAP) catalyzed the [32P]ADP-ribosylation of Gi (Figure 7). An additional substrate for IAP, presumably analogous to Gs previously identified in bovine brain, chick heart,
and rabbit heart.54 was also identified in the cardiac membranes (Figure 7). Cassel and Selinger55 have shown that the stimulatory effects of cholera toxin on adenylate cyclase activity are mediated by inhibition of GTPase activity resulting from ADP-ribosylation of $G_{o}$. Adenylate cyclase activity is greater in the presence of Gpp(NH)p than in the presence of GTP due to the resistance of the analogue to hydrolysis by GTPases.6 The cholera toxin-induced decrease in GTPase activity allows GTP stimulation of adenylate cyclase activity to be comparable with stimulation by Gpp(NH)p.655 In the sarcolemmal membranes, cholera toxin-catalyzed $^{32}$P-ADP-ribosylation of $G_{o}$ resulted in a 3.4-fold increase in adenylate cyclase activity stimulated by GTP plus isoproterenol (Figure 8). However, basal and oxotremorine-stimulated GTPase activities were similar regardless of whether $G_{o}$ was modified by cholera toxin (Figure 8).

In contrast to cholera toxin, pertussis toxin had little effect on GTP-activated adenylate cyclase activity but reduced the ability of oxotremorine to inhibit the enzyme. Adenylate cyclase activity in the presence of isoproterenol and GTP was 34% of the activity in the presence of isoproterenol and Gpp(NH)p (data not shown). ADP-ribosylation of the membranes with cholera toxin increased GTP-stimulated adenylate cyclase activity to 83% of that achieved in the presence of Gpp(NH)p. However, adenylate cyclase activity in the presence of isoproterenol and GTP was unaffected by pertussis toxin treatment of the membranes. In contrast, oxotremorine stimulation of the high-affinity GTPase activity and oxotremorine inhibition of GTP-stimulated adenylate cyclase activity were both attenuated by pertussis toxin treatment of the membranes. In control sarcolemma, adenylate cyclase activity due to isoproterenol and GTP was inhibited 79% by oxotremorine (Figure 9, left). In contrast, inhibition by oxotremorine was reduced to 35% in the membranes that had been treated with pertussis toxin (Figure 9, right). IAP treatment of the membranes also reduced the ability of oxotremorine to stimulate the high-affinity GTPase activity (Figure 10). Whereas oxotremorine-stimulated GTPase activity 87% in the control (untreated) membranes (Figure 10, left), stimulation by the muscarinic agonist was reduced to 29% in the membranes that had been treated with pertussis toxin (Figure 10, right). Thus, two GTPases were functionally distinguished by these assays: 1) the GTPase activity that turns off activation of adenylate cyclase and is inhibited by cholera toxin–catalyzed $^{32}$P-ADP-ribosylation of $G_{o}$ and 2) the GTPase activity that is stimulated by muscarinic agonists and inactivated by IAP.

**Discussion**

Purified canine cardiac sarcolemma displayed high-affinity GTP hydrolysis that was stimulated by oxotremorine acting at muscarinic receptors (Figures 1 and 2). This high-affinity GTPase activity and its stimulation by oxotremorine closely paralleled adenylate cyclase activity as evaluated with three different approaches. First, inhibition of basal GTPase activity by Na$^{+}$ resulted in stimulation of GTP-activated adenylate cyclase activity (Figures 3 and 4). Furthermore, inhibition of oxotremorine stimulation of GTPase activity was reflected in decreased ability of the muscarinic agonist to inhibit GTP-activated adenylate cyclase activity (Figure 3). Second, the $E_{C_{0}}$ values for oxotremorine binding to muscarinic receptors, oxotremorine stimulation of high-affinity GTPase activity, and oxotremorine inhibition of GTP-activated adenylate cyclase activity were similar (5–16 µM, Figure 6). Third, preincubation of the sarcolemmal membranes with IAP resulted in attenuation of the ability of oxotremorine to both stimulate high-affinity GTPase activity and inhibit GTP-activated adenylate cyclase activity (Figures 9 and 10). These results indicate that
the high-affinity GTPase activity measured in these experiments is reciprocally related to adenylate cyclase activity and that muscarinic stimulation of this GTPase is closely linked to muscarinic inhibition of adenylate cyclase.

Various effects of Na⁺ on inhibitory receptors and adenylate cyclase activity have been described by others.45-47,56-58 The literature indicates considerable species and experimental variation in Na⁺ effects on inhibitory receptor-coupled GTPase and adenylate cyclase activities. The exact locus and even direction of effect of this monovalent cation on GTPase and adenylate cyclase activities remain obscure. Milligan and Klee56 have reported that Na⁺ inhibited the high-affinity GTPase activity of NG108-15 hybrid membranes but stimulated the GTPase activity of purified G₁ from bovine brain. Koski et al57 demonstrated that Na⁺ stimulated adenylate cyclase activity in crude membrane preparations but inhibited activity in more purified preparations. The specific mechanisms underlying the effects of Na⁺ on the coupling of inhibitory receptors with their effectors were not the focus of the present study. Rather, Na⁺ was used as a general probe to evaluate the relation between GTPase and adenylate cyclase activities. However, it was surprising to note that muscarinic stimulation of GTPase and reciprocal inhibition of GTP-activated adenylate cyclase activities in the cardiac membranes did not require millimolar concentrations of Na⁺ as reported for other inhibitory receptor-regulated systems. Because the [Na⁺] was ~600 μM in the sodium-free medium used in the present study, it cannot be stated unequivocally that muscarinic regulation does not require the presence of Na⁺. However, the canine cardiac sarcolemma appears to be significantly different from other systems in this regard. For example, others have reported that 100-150 mM [Na⁺] is necessary for maximal facilitation of inhibitory hormone effects (e.g., Koski et al57), and Blume and coworkers46 have reported that the one-half maximally effective concentration of Na⁺ regulation of opiate receptors in NG108-15 membranes was 20 mM.46 Blume’s group has also

![Figure 6](image_url)

**Figure 6.** Plots of concentration-effect curves for oxotremorine inhibition of [³H]QNB binding, stimulation of high-affinity GTPase activity, and inhibition of GTP-activated adenylate cyclase activity. Incubation medium (100 μl) contained 40 mM Tris-HCl (pH 7.5), 1 mM (20 cpm/pmol) [α-³²P] ATP+APP(NH)P, 10 μM GTP, 10 μM isoproterenol, 10 mM MgCl₂, 1 mM EDTA, 100 μM ascorbic acid, 5 mM phosphocreatine, and 5 U/ml creatine phosphokinase. Sarcolemmal membranes were assayed for the indicated activities in the presence of various concentrations of oxotremorine. Maximal specific [³H]QNB binding, maximal stimulation of high-affinity GTPase activity, and maximal inhibition of GTP-activated adenylate cyclase activity were set equal to 100%.

![Figure 7](image_url)

**Figure 7.** [¹²⁵I]ADP-ribosylation of cardiac sarcolemma by cholera toxin and IAP. Sarcolemmal membranes were incubated with [¹²⁵I]NAD and cholera toxin or IAP and subjected to SDS PAGE and autoradiography as described in "Materials and Methods." Two substrates were identified for IAP: Mᵢ=41,000 (G₆₅) and Mᵢ=39,000 (G₄₅). A single substrate for cholera toxin was identified: Mᵢ=45,000 (G₄₅).
reported that 20–40 mM Na⁺ (or Li⁺) must be present in the extracellular medium for the observation of enkephalin regulation of intracellular cyclic AMP concentration in NG108-15 cells. In contrast, muscarinic stimulation of GTPase and reciprocal inhibition of adenylate cyclase activities in canine cardiac sarcolemma was similar, whether in the presence of 150 mM Na⁺ or in the presence of sodium-free concentrations far below the \( K_m \) for previously reported facilitative effects of the monovalent cation in other systems.

The plant diterpene forskolin stimulates adenylate cyclase activity markedly in many tissues. Although purified \( G_o \) and \( G_o \) each display GTPase activity, the muscarinic-stimulated high-affinity GTPase activity of the sarcolemma was predominantly displayed by the IAP substrate(s). Cholera toxin-catalyzed \([^{32}P]ADP-ribosylation of \( G_o \) increased isoproterenol plus GTP-activation of adenylate cyclase activity as expected but had no effect on basal or muscarinic-stimulated GTPase activities (Figure 8). In contrast, inactivation of \( G_o \) and \( G_o \) by IAP resulted in only a slight increase in (isoproterenol plus GTP)–activated adenylate cyclase activity (Figure 9), perhaps due to attenuation of the inhibitory influence of \( G_o \). Moreover, inactivation of \( G_o \) attenuated the effects of oxotremorine on both GTPase and adenylate cyclase activities (Figures 9 and 10). Therefore, functional IAP substrate(s) was required for both muscarinic stimulation of GTPase activity and muscarinic inhibition of GTP-activated adenylate cyclase activity.

Analogous to the inactivation of \( G_o \cdot GTP \), the hydrolysis of GTP is thought to be the mechanism for inactivation of \( G_o \cdot GTP \). However, direct inhibition of adenylate cyclase by \( G_o \cdot GTP \) has only been observed under nonphysiological conditions. Kanaho et al demonstrated that \( G_p \) increased the hydrolysis of GTP by \( G_o \). Thus, muscarinic stimulation of GTP hydrolysis related to adenylate cyclase inactivation may reflect inactivation of \( G_o \cdot GTP \) by \( G_p \), released from \( G_o \) in the presence of GTP and muscarinic receptor agonists. IAP treatment of the sarcolemma attenuated the ability of oxotremorine to stimulate GTPase activity and inhibit adenylate cyclase activity, and IAP-
Becky Wilson.

tinue to appreciate the skillful artwork of Phil and their patient advice

Bordetella pertussis

gative roles of G\(_i\) and Go in regulation of adenylate

associated with inhibition of adenylate cyclase activ-

rinic receptor affinity in brain, 3263 only Gj has been

Figure 9  legend.  Pretreated membranes were subse-

stimulation of high-affinity GTPase activity. Sarco-

catalyzed ADP-ribosylation of G\(_\omega\) inhibits the dis-

Therefore, IAP may attenuate the inhibitory effects of muscarinic agonists by decreasing their ability to increase the pool of inhibitory G\(_p\). In

summary, purified cardiac sarcolemma displayed muscarinic-stimulated high-affinity GTPase activity that required functional IAP substrate(s) for inhibitory receptor coupling to adenylate cyclase activity. Although two substrates for IAP were identified, and both G\(_i\) and G\(_o\) have been shown to regulate musca-

receptor affinity in brain,\(^2,63\) only G\(_j\) has been

associated with inhibition of adenylate cyclase activ-

ity. Future studies will further explore the exact locus of muscarinic-stimulated GTP hydrolysis and the relative roles of G\(_i\) and G\(_o\) in regulation of adenylate cyclase activity.

Acknowledgments

We would like to thank Paul Cantrell, Elizabeth Price, and Debra Klingberg for their excellent technical assistance; Dr. Erik Hewlett and Gwendolyn Myers of the University of Virginia for the gift of Bordetella pertussis and their patient advice that enabled us to produce purified IAP; and Dr. L. R. Willis and M. Christine Broughton for determinations of sodium ion concentration. We continue to appreciate the skillful artwork of Phil and Becky Wilson.

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FIGURE 10. Bar charts of effects of IAP on muscarinic stimulation of high-affinity GTPase activity. Sarco-

lemmal membranes were the same as those described in

Figure 9 legend. Pretreated membranes were subse-

quently assayed for high-affinity GTPase activity in the absence and presence of 10 \(\mu\)M oxotremorine (OXO).

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KEY WORDS • muscarinic receptor • GTP hydrolysis
Muscarinic cholinergic-receptor stimulation of specific GTP hydrolysis related to adenylate cyclase activity in canine cardiac sarcolemma.

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doi: 10.1161/01.RES.63.2.340

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Print ISSN: 0009-7330. Online ISSN: 1524-4371

The online version of this article, along with updated information and services, is located on the World Wide Web at:
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