Pertussis Toxin–Treated Dog: A Whole Animal Model of Impaired Inhibitory Regulation of Adenylate Cyclase

John W. Fleming, Timothy D. Hodges, and August M. Watanabe

We have shown previously that stimulation of high-affinity GTP hydrolysis and inhibition of adenylate cyclase activity by muscarinic agonists are mediated by pertussis toxin (IAP) substrates ($G_\alpha$ and $G_\beta$) in canine cardiac sarcolemma. We have now used the pertussis toxin–treated dog as a whole animal model in which $G_\alpha$- and $G_\beta$-mediated biochemical mechanisms can be studied. Mongrel dogs were injected intravenously with IAP 48 hours prior to death and isolation of left ventricular sarcolemma. Treatment of the animal in vivo with the toxin prevented subsequent in vitro IAP-catalyzed $[^{32}P]$ADP-ribosylation of substrates in cardiac, erythrocytic, and renal cortical plasma membranes, suggesting that ADP-ribosylation occurred in vivo from endogenous substrate. Consistent with our previous results obtained by treating sarcolemma in vitro with IAP, muscarinic receptor–mediated stimulation of high-affinity GTP hydrolysis and inhibition of GTP-activated adenylate cyclase activity were attenuated in ventricular membranes from the toxin-treated animals. Proximal to adenylate cyclase, guanine nucleotide regulation of muscarinic receptor affinity for agonists was also abolished in membranes from the toxin-treated animals. In addition, the ability of oxotremorine to attenuate GTP regulation of stimulation of adenylate cyclase activity by magnesium ions was abolished in sarcolemma from the IAP-treated dogs. Thus, cardiac sarcolemma isolated from the IAP-treated animals displayed biochemical characteristics of an adenylate cyclase system in which inhibitory regulatory pathways had been attenuated. The cardiac biochemical studies and the in vivo ADP-ribosylation of noncardiac IAP substrates also suggests considerable potential use of this model in the physiological and biochemical study of regulatory mechanisms mediated by GTP-binding proteins in other systems.

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The mammalian heart is functionally regulated by the opposing influences of the two limbs of the autonomic nervous system.1 Parasympathetic activation of cardiac muscarinic receptors suppresses ventricular function, and this inhibitory effect is greater when $\beta$-adrenergic receptors are activated (e.g., in the presence of sympathetic stimulation).1,2 The biochemical mechanisms that mediate muscarinic effects are varied and include regulation of both cyclic AMP–dependent and cyclic AMP–independent processes.1,3 Because the intracellular effects of catecholamines are largely mediated by increased synthesis of cyclic AMP by adenylate cyclase [ATP pyrophosphate-lyase (cyc- lizing) E.C. 4.6.1.1], considerable experimental effort has been directed toward understanding the mechanism(s) by which muscarinic agonists attenuate the increase in cyclic AMP produced by $\beta$-adrenergic agonists. It is now well established that stimulation and inhibition of adenylate cyclase activity are mediated at the level of two similar yet unique GTP-binding proteins designated $G_\alpha$ and $G_\beta$, respectively.2 Pertussis toxin (islet-activating protein, IAP) has been an important biochemical probe for the selective identification and functional modification of $G_\alpha$ in several tissues.7 Using nicotinamide adenine dinucleotide (NAD) as substrate, IAP catalyzes the covalent ADP-ribosylation of the $\alpha$-subunit of $G_\alpha$ ($G_\alpha^\alpha$), which causes a loss in the ability of $G_\alpha$ to mediate the effects of inhibitory hormones.8 $G_\beta$ and $G_\alpha$ interpose between receptors and adenylate cyclase, and therefore, the functional integrity of the regulatory proteins affects processes both at the level of the autonomic receptors and at the level of adenylate cyclase. We originally reported that muscarinic agonists antagonized the ability of guanine nucleoside 5'-triphosphates to regulate $\beta$-adrenergic receptor affinity for catecholamines in cardiac sarcolemma.9 We have also demonstrated that muscarinic agonists attenuate GTP-activated adenylate cyclase activity by a mechanism that is closely linked with GTP hydrolysis (GTPase activity).9–11 In those studies, muscarinic stimulation of specific GTPase activity was inversely related to muscarinic inhibition of GTP-activated adenylate cyclase activity, and the muscarinic effects on both processes were dependent on functional IAP substrates. We have now further extended these studies using sarcolemma purified from the ventricles of control and IAP-treated dogs. Several inhibitory biochemical regulatory processes mediated by muscarinic receptors were attenuated or abolished in sarcolemma isolated from animals treated in vivo with IAP. In addition to further supporting our original hypotheses,
the present report also suggests that the IAP-treated whole dog is an excellent candidate for studies in noncardiac tissues as a model system in which regulation of biochemical processes by inhibitory receptors linked to IAP substrates has been inactivated.

Table of Abbreviations

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<th>Abbreviation</th>
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<tr>
<td>G&lt;sub&gt;i&lt;/sub&gt;, G&lt;sub&gt;x&lt;/sub&gt;, G&lt;sub&gt;q&lt;/sub&gt;</td>
<td>Stimulatory and inhibitory guanine nucleotide binding and regulatory proteins of adenylate cyclase, respectively</td>
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<tr>
<td>ARF</td>
<td>ADP-ribosylation factor</td>
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<tr>
<td>QNB</td>
<td>(±)quinuclidinylbenzilate</td>
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Materials and Methods

-[α-<sup>32</sup>P]ATP, [γ-<sup>32</sup>P]GTP, [adenylate-<sup>32</sup>P]NAD, and [3H]QNB were obtained from New England Nuclear, Boston, Massachusetts. The purity of the radionucloides was verified using ascending thin-layer chromatography on polyethyleneimine cellulose (Brinkmann, Westbury, New York) in 0.75 M potassium phosphate (pH 3.4), followed by autoradiography. Cyanogen bromide–activated Sepharose was purchased from Pharmacia, Piscataway, New Jersey, and fetuin was from Sigma Chemical, St. Louis, Missouri. Hydroxylapatite (fast flow) was from Calbiochem, San Diego, California. All other reagents were from Sigma or Fisher Scientific, Pittsburgh, Pennsylvania.

Preparation of IAP

*Bordetella pertussis* strain 165 was grown in modified Stainer-Scholte medium as described by Hewlett et al. Pertussis toxin was purified from the culture supernatants using hydroxylapatite and fetuin-Sepharose chromatography using a modification of procedures described elsewhere as communicated by Dr. Erik Hewlett of the University of Virginia, Charlottesville. Briefly, 71 culture supernatant were passed through a 200-ml column of hydroxylapatite 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 hydroxylapatite 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) and 0.5 M NaCl. Pertussis toxin was eluted with 100 ml of 100 mM potassium phosphate (pH 7.0) and 0.5 M NaCl containing 3 M KSCN. The purified toxin was dialyzed and concentrated on an Amicon YM-10 ultrafiltration membrane (43 mm) (Danvers, Massachusetts) using 100 mM sodium phosphate (pH 7.0) and 0.5 M NaCl. Pertussis toxin was stable for at least 1 year when stored in frozen aliquots at −20° C.

Experimental Design

Conditioned mongrel dogs (22–30 kg) were injected with either 100 mM sodium phosphate (pH 7.0) and 0.5 M NaCl (control) or pertussis toxin (30 μg/kg i.v.) in the same buffer. The animals showed no obvious signs of discomfort or disease. Left ventricular sarcolemma was purified 48 hours postinjection of toxin and subjected to the indicated biochemical assays.

Preparation of Membranes

Purified cardiac left ventricular sarcolemma was prepared according to Jones et al. and Jones from Procedure II membrane vesicles with the exception that 0.75 M NaCl and 10 mM l-histidine were substituted for 0.75 M KCl and 5 mM l-histidine in the starting buffer. The total ouabain-sensitive Na<sup>+</sup>,K<sup>+</sup>-ATPase activity of the purified sarcolemma was routinely 70–90 μmol P<sub>1</sub>/hr/mg protein.

Canine renal cortical plasma membranes were prepared using differential centrifugation essentially according to the method of Marx et al. exactly as described by Queener et al. Canine erythrocrytic plasma membranes were prepared by hypotonic lysis of erythrocytes obtained at the time of death and removal of heart and kidneys. Blood collection, lysis, and washing of membranes by centrifugation were exactly as described by Fleming and Ross. All membranes were quick-frozen using a dry ice–acetone bath and stored at −80° C.

Adenylate Cyclase Assay

Adenylate cyclase activity was assessed by measuring the conversion of [α-<sup>32</sup>P]ATP to cyclic [<sup>32</sup>P]AMP, exactly as described previously. Cyclic AMP formed during the reactions was isolated by the method of Salomon et al. Results are expressed as the mean adenylate cyclase activity ± SEM of several different determinations, each using preparations of sarcolemma from individual hearts.

GTPase Assay

GTP hydrolysis was determined by measuring the release of <sup>32</sup>P from [γ-<sup>32</sup>P]GTP, exactly as recently described by us. Specific GTPase activity of each membrane preparation was calculated as the difference between the mean total and nonspecific activities in duplicate determinations. Results are expressed as the mean specific GTPase activity ± SEM of several different determinations, each using preparations of sarcolemma from individual hearts.
\[^{125}P\]ADP-Ribosylation of \(G_{ia}\) and \(G_{io}\)

Covalent modification of the \(\alpha\)-subunits of the IAP substrates was by modification of well-established procedures using \[^{125}P\]NAD as substrate.\(^{24,25}\) Purified sarcolemmal membranes (60 \(\mu\)g) were incubated in 1.44 ml containing potassium phosphate (pH 8.0) 61 mM, NaCl 54 mM, dithiothreitol (DTT) 2.7 mM, MgCl\(_2\), 5 mM, thymidine 10 mM, GTP 0.1 mM, ATP 1 mM, phosphocreatine 3.3 mM, creatine phosphokinase 0.3 units/ml, \[^{125}P\]NAD (\(-10,000\) cpnm/pmol) 50 \(\mu\)M, and 14.4 \(\mu\)g IAP (preactivated with 20 mM DTT, 30 minutes at 30\(^\circ\)C). The reactions were initiated by the addition of \[^{125}P\]NAD, and intoxication proceeded for 30 minutes at 30\(^\circ\)C. Intoxication was terminated by the addition of 4 ml ice-cold 0.25 M sucrose and 10 mM L-histidine, and the sarcolemmal membranes were sedimented at 40,000 rpm for 20 minutes in a Beckman 40 rotor (145,000g\(_{max}\)). The pellets were resuspended to a final concentration of 0.6 mg/ml and boiled for 5 minutes in gel dissociation medium\(^{26}\) [Tris (pH 6.8) 62.5 mM, sodium dodecyl sulfate (SDS) 1.25\% (vol/vol), \(\beta\)-mercaptoethanol 0.25\% (vol/vol); glycerol 10\% (vol/vol), and bromphenol blue 0.05\% (vol/vol)].

SDS Polyacrylamide Gel Electrophoresis of IAP-Treated Sarcolemma

After intoxication with IAP, membranes were subjected to SDS polyacrylamide gel electrophoresis as described by Laemmli.\(^{28}\) Samples containing 60 \(\mu\)g protein were loaded onto each lane of a 12\% resolving gel. The gels were run at 20 ma per gel for 30 minutes after 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 using Du Pont Cronex Pellicula X-ray film and Du Pont Lightning Plus intensifying screens (Wilmington, Delaware).

Muscarinic Receptor Assays

The ability of guanine nucleotides to regulate muscarinic receptor affinity for agonists was determined in sarcolemmal membranes purified from control and toxin-treated animals in equilibrium binding assays using the antagonist ligand \([^{3}H]\)quinuclidinylbenzilate (\([^{3}H]\)QNB) \(^{29}\) essentially as we have previously reported.\(^{30}\) Briefly, the 0.1-mI assays contained 60 \(\mu\)g protein were loaded onto each lane of a 12\% resolving gel. The gels were run at 30 ma per gel for 30 minutes after 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 using Du Pont Cronex Pellicula X-ray film and Du Pont Lightning Plus intensifying screens (Wilmington, Delaware).

Statistical Analysis

Unless otherwise indicated, reported probability (p) values were derived from repeated measures analysis of variance. In some experiments, statistical significance was tested using Student’s t test.

Results

Pertussis toxin treatment of the animals in vivo altered the ability of the toxin to catalyze the \[^{125}P\]ADP-ribosylation of \(G_{ia}\), during subsequent in vitro exposure to the toxin and \[^{125}P\]NAD. IAP catalyzed the \[^{125}P\]ADP-ribosylation of \(G_{ia}\) in plasma membranes purified from canine cardiac left ventricles, erythrocytes, and renal cortex of the control animals (Figure 1). As we have recently reported,\(^{11}\) two substrates for IAP were identified in the cardiac tissue: \(G_{ia}\) (\(M_r = 41,000\)) and another protein corresponding to \(G_{ia}\) described in brain\(^{20}\) and heart\(^{31}\) (\(M_r = 59,000\)). In contrast, the ability of IAP to label \(G_{io}\) was nearly abolished in all three membrane types examined from the animals treated with the toxin in vivo (Figure 1). \(G_{io}\) was not identified in the erythrocytic and renal cortical plasma membranes (Figure 1). \[^{125}P\]ADP-ribosylated IAP substrates in cardiac sarcolemma were isolated from polyacrylamide gels and quantitated using liquid scintillation spectrometry. Whereas IAP catalyzed the incorporation of 212.8 \(\pm\) 38 fmol ADP-ribose/mg sarcolemmal protein in membranes from the hearts (\(n = 5\)) of control animals, only 23.8 \(\pm\) 8.8 fmol ADP-ribose/mg sarcolemmal protein could be incorporated in membranes from the hearts (\(n = 7\)) of the animals that received IAP in vivo. Thus, intravenous injection of IAP effectively catalyzed the endogenous ADP-ribosylation of cardiac \(G_{ia}\) and \(G_{io}\) (p < 0.001).

We have recently shown that the ability of muscarinic receptor agonists to inhibit adenylate cyclase activity is closely linked to their ability to stimulate specific,
high-affinity GTP hydrolysis displayed by canine cardiac sarcolemma. In the present study, in vivo pretreatment of dogs with IAP markedly reduced oxotremorine stimulation of specific GTPase activity in subsequently purified sarcolemma, and adenylate cyclase activity was refractory to inhibition by the muscarinic agonist. Concentration-effect curves for oxotremorine stimulation of specific GTPase activity are shown in Figure 2. Oxotremorine stimulated specific GTPase activity in a concentration-dependent manner in sarcolemma purified from both control and IAP-treated animals (p < 0.001). However, maximal muscarinic stimulation of specific GTPase activity was markedly reduced in membranes from the hearts of the toxin-treated animals (17.1%) compared with that seen in the control membranes (70.2%). Although oxotremorine stimulated GTPase activity in sarcolemma from the IAP-treated animals, muscarinic stimulation was significantly reduced in membranes from the IAP-treated animals at essentially all concentrations tested (p < 0.001). In vivo ADP-ribosylation of the LAP substrates thus attenuated the ability of oxotremorine to stimulate the specific GTPase activity of subsequently isolated cardiac sarcolemma.

That muscarinic stimulation of GTPase activity was attenuated in sarcolemma from the IAP-treated dogs suggested that inhibition of GTP-stimulated adenylate cyclase activity might be reduced in membranes from the toxin-treated animals. Concentration-effect curves for oxotremorine inhibition of GTP-stimulated adenylate cyclase activity are shown in Figure 3. Oxotremorine inhibited GTP-stimulated adenylate cyclase activity in a concentration-dependent manner in sarcolemma purified from both control and IAP-treated animals

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**Figure 2.** Concentration-effect curves for oxotremorine stimulation of specific GTPase activity in sarcolemma (SL) purified from control and islet-activating protein (IAP)-treated dogs. Oxotremorine-stimulated GTPase activity was assessed in membranes purified from control and toxin-treated animals as described in "Materials and Methods." Basal specific GTPase activity was 21.9 ± 3.1 pmol P/min/mg protein (control) and 10.7 ± 2.2 pmol P/min/mg protein (IAP treated). (n = 3, control; n = 3, IAP treated.)

**Figure 3.** Concentration-effect curves for oxotremorine inhibition of GTP-activated adenylate cyclase activity in sarcolemma (SL) purified from control and islet-activating protein (IAP)-treated dogs. Adenylate cyclase activity was assessed in sarcolemma purified from control and toxin-treated animals as described in "Materials and Methods." Basal adenylate cyclase activity was 685.1 ± 172.3 pmol/min/mg protein (control) and 679.0 ± 7.5 pmol/min/mg protein (IAP treated). Percent oxotremorine inhibition of that increment in adenylate cyclase activity due to the presence of 10 μM GTP is shown. (n = 3, control; n = 2, IAP treated.)
(p < 0.001). Maximal muscarinic inhibition of GTP-
stimulated adenylate cyclase activity of 84.3% in the
control membranes was reduced to 20.4% in mem-
branes from the hearts of the toxin-treated animals.
Although oxotremorine somewhat inhibited GTP-
stimulated adenylate cyclase activity in sarcolemma
from the IAP-treated animals, muscarinic inhibition
was significantly reduced in membranes from the
IAP-treated animals at all concentrations tested
(p < 0.001). In summary, IAP-catalyzed ADP-ribo-
sylation of sarcolemmal substrates in vivo attenuated
the ability of GTP-7S to regulate muscarinic receptor
affinity for agonist (Figure 4). In sarcolemma from
the control animals, specific oxotremorine binding to
the cardiac muscarinic receptors was evaluated
further with nonlinear least-squares curve-fitting anal-
ysis of the data using either a two-site (control) or
one-site (IAP) mass action binding model according to
the equation:

\[
\text{BOUND} = [R] \sum_{i=1}^{n} \frac{n_i [\text{free}]}{K_{di} + [\text{free}]}
\]

**Table 1. Effect of In Vivo IAP Treatment on Guanine Nucleotide Regulation of Muscarinic Receptor Affinity in Purified Sarcolemma**

<table>
<thead>
<tr>
<th>Sarcolemma from normal dog</th>
<th>100 µM GTP7S</th>
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<tr>
<td>14 (1.2–1.7) 61%</td>
<td>0.8 (0.6–1.2) 33%</td>
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<tr>
<td>14 (0.8–1.9)</td>
<td>20.3 (11.9–40.9)</td>
</tr>
<tr>
<td>0.9 (0.7–1.1) 39%</td>
<td>1.6 (1.4–1.2) 67%</td>
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<tr>
<td>76.0 (38.0–152.9)</td>
<td>135.9 (66.9–206.7)</td>
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<tr>
<th>Sarcolemma from IAP dog</th>
<th>100 µM GTP7S</th>
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<tbody>
<tr>
<td>2.0 (1.8–2.1) 100%</td>
<td>2.1 (2.0–2.1) 100%</td>
</tr>
<tr>
<td>117.9 (89.1–157.6)</td>
<td>140.6 (118.5–166.7)</td>
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Muscarinic receptors in purified sarcolemma were assayed in the absence and presence of 100 µM GTP7S as described in "Materials and Methods." The results of nonlinear least-squares curve-fitting analysis of the oxotremorine binding isotherms depicted in Figure 4 are summarized for two representative experiments. The constants above indicate the dissociation constants for the high- (14Kd) and low-(14Kd) affinity states of the receptors and the corresponding receptor density of each affinity class (14 and 14). The Kd values were calculated from the binding data by the method of Cheng and Prusoff.44 (The Kd for [1H]QNB was 15.3 pM, and the concentration of [1H]QNB in the assays was 5.5 nM.) Asymmetric confidence intervals shown in parentheses were calculated according to Johnson and Frasier.45 IAP, islet-activating protein.
was totally absent in sarcolemma from the hearts of control animals. In contrast, guanine nucleotide regulation of muscarinic receptor affinity for oxotremorine (Table 1). Least-squares analysis revealed that 100% of the receptors remaining in a relatively high-affinity binding state (HKd = 1.4 nM), while 39% were in the low-affinity state (LKd = 76.0 nM). The presence of GTP-7S shifted the majority (67%) of the receptors into the low-affinity state (LKd = 117.9 nM), and the addition of 100 µM GTP-7S resulted in no significant change in affinity (LKd = 140.6 nM). Furthermore, the pseudo-Hill coefficient for oxotremorine binding to muscarinic receptors in sarcolemma from the IAP-treated animals was approximately 1.0, indicating simple binding to a homogeneous population of receptors. ADP-ribosylation of the cardiac IAP substrates thus totally abolished GTP-7S regulation of muscarinic receptor affinity for oxotremorine.

Nonhydrolyzable GTP analogues characteristically inhibit forskolin-stimulated adenylyl cyclase activity 15-30% in membranes that display functional Gi, and we have shown previously that Gpp(NH)p inhibits the forskolin-stimulated canine cardiac enzyme. In sarcolemma from the control animals, 0.1 µM GTP-7S inhibited adenylyl cyclase activity in the presence of 10 µM forskolin by 14.5% (Table 2). In contrast, GTP-7S inhibited the forskolin-stimulated enzyme by only 4.3%. The ability of GTP-7S to inhibit the forskolin-stimulated enzyme was significantly reduced (p < 0.05) in membranes from the toxin-treated animals (Table 2).

Finally, muscarinic effects on the ability of GTP and magnesium ions to regulate adenylyl cyclase activity were examined in sarcolemma from the hearts of control and IAP-treated animals. In sarcolemma from both control and IAP-treated animals, MgCl2 augmented GTP-stimulated adenylyl cyclase activity in a concentration-dependent manner (p < 0.001, Figure 5). Although 10 µM oxotremorine markedly inhibited GTP-stimulated adenylyl cyclase activity at all concentrations of MgCl2 tested in the control sarcolemma (p < 0.001), muscarinic inhibition of the enzyme was essentially abolished in sarcolemma from the IAP-treated animals (Figure 5).

Discussion

Treatment of intact dogs with IAP resulted in alterations in the interactions of the components of the hormone-regulated adenylyl cyclase system in cardiac sarcolemma from control and IAP-treated dogs. Adenylyl cyclase activity was assessed as described in “Materials and Methods.” The assays contained the indicated concentrations of oxotremorine (OXO) and MgCl2. The increment in adenylyl cyclase activity due to the presence of 10 µM GTP is shown. (n = 3, control; n = 2, IAP treated.)

<table>
<thead>
<tr>
<th>Table 2. Effect of In Vivo IAP Treatment on Guanine Nucleotide Inhibition of Forskolin-Stimulated Adenylyl Cyclase Activity</th>
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<tr>
<td>Additions</td>
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<tr>
<td>None</td>
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<td>0.1 µM GTP-7S</td>
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where R is the concentration of receptor molecules, i is an index referring to each of the m different classes (affinities) of receptors, n is the number of receptors in each of the i affinity states, and Kdi is the corresponding dissociation constant for each of the affinity states of the receptors. Sarcolemma purified from the hearts of control animals displayed two populations of muscarinic receptors with high- and low-affinity states differing approximately 100-fold in relative affinity for oxotremorine (Table 1). In the absence of regulation by guanine nucleotide, 61% of the receptors displayed high-affinity binding (Kd = 0.4 nM), while 39% were in the low-affinity state (Kd = 76.0 nM). The presence of GTP-7S shifted the majority (67%) of the receptors into the low-affinity state (Kd = 135.9 nM), with 33% of the receptors remaining in a relatively high-affinity state (Kd = 20.3 nM). Therefore, GTP-7S converted the majority of the high-affinity receptors to the low-affinity state in sarcolemma from the hearts of control animals. In contrast, guanine nucleotide regulation of muscarinic receptor affinity for oxotremorine was totally absent in sarcolemma from the hearts of IAP-treated animals (Figure 4 and Table 1). Least-squares analysis revealed that 100% of the receptors (~2 pmol/mg protein) were in the low-affinity state (Kd = 117.9 nM), and the addition of 100 µM GTP-7S resulted in no significant change in affinity (Kd = 140.6 nM). Furthermore, the pseudo-Hill coefficient for oxotremorine binding to muscarinic receptors in sarcolemma from the IAP-treated animals was approximately 1.0, indicating simple binding to a homogeneous population of receptors. ADP-ribosylation of the cardiac IAP substrates thus totally abolished GTP-7S regulation of muscarinic receptor affinity for oxotremorine.

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sarcolemma purified from the hearts taken from these dogs. We have previously shown that muscarinic agonists modulate both β-adrenergic receptor affinity for catecholamines and GTP-activation of adenylate cyclase. It has been shown that the biochemical effect of IAP is the ADP-ribosylation of Gα, (and possibly Gβγ), this modification by the toxin results in the inactivation of the ability of the IAP substrates to mediate inhibition of adenylate cyclase activity. The present report describes the attenuation of biochemical mechanisms mediated by IAP substrates in cardiac sarcolemma isolated from the hearts of animals treated in vivo with the toxin.

In vivo ADP-ribosylation of Gαi and Gαq from endogenous NAD was confirmed by the inability of the substrates to be [32P]ADP-ribosylated on subsequent exposure to the toxin and [32P]NAD in vitro (Figure 1). The ability of oxotremorine to stimulate high-affinity GTPase activity and inhibit GTP-activated adenylate cyclase activity was markedly attenuated or abolished in membranes from the toxin-treated animals (Figures 2 and 3). In addition, the ability of GTPγS to regulate muscarinic receptor affinity for agonists was abolished in membranes from the toxin-treated animals (Figure 4 and Table 1). Thus, several biochemical regulatory systems related to adenylate cyclase were interrupted by in vivo ADP-ribosylation of cardiac sarcolemma with IAP: muscarinic stimulation of GTPase activity, muscarinic inhibition of adenylate cyclase activity, inhibition of forskolin-stimulated adenylate cyclase activity by GTPγS, and, proximal to adenylate cyclase, guanine nucleotide regulation of muscarinic receptor affinity for agonists.

Apart from the effects of in vivo IAP administration to uncouple guanine nucleotide regulation of oxotremorine binding to subsequently isolated cardiac muscarinic receptors, it was of interest to us that GTPγS did not shift 100% of the receptors from the high-affinity state to the low-affinity state in the sarcolemma from the control animals (Figure 4 and Table 1). Rather, 33% of the receptors remained in a relatively high, intermediate affinity state (Kd = 20.3 nM) in the presence of GTPγS. Because the data were obtained in the presence of MgCl2 and a high concentration of GTPγS (100 μM), it seemed unlikely that the intermediate state of receptor affinity resulted simply from an incomplete effect of GTPγS. We have consistently observed that approximately one third of the receptors do not shift completely to the low-affinity state but rather shift to an affinity intermediate between the affinities displayed in the absence of guanine nucleotide. Furthermore, this observation was true regardless of whether the guanine nucleotide was GTPγS or Gpp(NH)p (data not shown).

Although there is substantial precedent in the literature for muscarinic receptors existing in three affinity states, the present data did not allow strict differentiation between the possibilities of two or three affinity states of the canine cardiac receptors. Birdsell et al first demonstrated mammalian neuronal muscarinic receptors existing in three affinity states, and Hoyer et al have observed three affinity states of the chick cardiac muscarinic receptor. In addition, treatment of chicks with IAP 48 hours before preparation of cardiac membranes resulted in elimination of the highest affinity state of the muscarinic receptors and increases in the proportions of receptors in the intermediate- and low-affinity states. The Kd of the high-affinity state of the canine cardiac receptor (1.4 nM) was similar to that reported in other cardiac tissues, but lower-affinity states did not differ to the degree reported in other systems. For example, the highest and lowest affinity states described in the mammalian neuronal and chick cardiac membranes differed by two to three orders of magnitude and required fitting of the data to a three-site binding model. In contrast, the high- and low-affinity states of the muscarinic receptors in the present study differed by two orders of magnitude or less and did not require the hypothesis of a third affinity state for adequate fit of the data. It can be seen in Table 1 that the confidence interval for the intermediate affinity state of the receptor in the presence of GTPγS (Kd = 11.9-40.9 nM) overlapped with that of the low-affinity state seen in the absence of guanine nucleotide (Kd = 38.0-152.9 nM). Thus, it cannot be stated unequivocally whether the canine receptors actually exist in three affinity states that differ by a factor of 10 or less or whether the receptors exist in only two affinity states. Regardless of the strict interpretation of the oxotremorine displacement curves, the important conclusion remains that the highest affinity state of the receptors was eliminated by either the presence of GTPγS or by complete uncoupling of the receptors from IAP substrate(s) by administration of the toxin to the animals in vivo.

In addition to Gαi, we have shown that IAP catalyzes the [32P]ADP-ribosylation of an additional substrate in canine cardiac sarcolemma (Figure 1 and J. W. Fleming and A. M. Watanabe). The additional IAP substrate is analogous to Gαs described previously in bovine brain, chick heart, and rabbit heart. Although Gαs has not been directly demonstrated to affect adenylate cyclase activity, Gαi has been shown to regulate muscarinic receptor affinity for agonists. We have recently shown that the most significant mechanism for muscarinic effects on adenylate cyclase activity in cardiac sarcolemma is mediated indirectly by inhibition of activated Gαi(Gαi-GTP). The mechanism of inhibition is closely linked to the hydrolysis of GTP and may be related to the inhibited receptor-catalyzed release of functionally similar, if not identical, Gαi-GTP subunits from Gαi and Gαs in the presence of GTP. Katada and coworkers have shown that ADP-ribosylation of IAP substrates increases the affinity of the α-subunits for the βγ-subunits, thus reducing the ability of inhibitory βγ-subunits to dissociate from the G proteins in response to inhibitory receptor agonists. It appears likely that the biochemical alterations seen in sarcolemma purified from the hearts of IAP-treated animals result from ADP-ribosylation of the α-subunits of both Gαi and Gαs. Present experiments are directed toward selective definition of the relative roles of Gαi and Gαs in the mechanism of inhibitory regulation of...
receptor affinity and inhibition of adenylate cyclase activity.

It is important to note that studies of inhibitory regulation are not limited to in vitro study or to particular tissues in the model used for the present studies. The IAP-treated dog appears to be an excellent whole animal model in which biochemical mechanisms mediated by IAP substrates can be studied. The IAP-treated dog model is limited only by the accessibility of the toxin to its substrates. The present study indicates that $G_\alpha$ was ADP-ribosylated in vivo in three tissues examined: cardiac, erythrocytic, and renal cortical. Previous use of IAP in whole animals has been limited to small animals of the rodent family. However, the dog is a preferred model for many physiological and biochemical investigations, and the IAP-treated animal is an excellent candidate for such studies. Our preliminary results have indicated complete loss of vagal control of chronotropy in the IAP-treated dogs.* Electrical stimulation of either the right or left vagus nerve produced none of the characteristic electrocardiographic alterations observed in the control animals (e.g., cessation of sinus node activity).

Although our data to date indicate that essentially all of the toxin substrates were labeled in vivo, it is possible that a small fraction of IAP substrates remained unmodified and resulted in the small residual effects of oxotremorine seen on GTPase and adenylate cyclase activities. However, it seems unlikely that a small unlabeled pool of inhibitory $G$ proteins will significantly complicate further physiological studies using this model. Rather, the IAP treatment rendered enzymatic response to oxotremorine highly significantly attenuated when compared with control sarcolemma. Furthermore, guanine nucleotide regulation of muscarinic receptor affinity was completely abolished in membranes from the toxin-treated animals, as was responsiveness of the hearts to vagal stimulation in intact animals. It is, therefore, expected that use of the IAP-treated dog will contribute substantially to the understanding of processes mediated by $G_\alpha$ and $G_\beta$ and also provide a model for answering new questions likely to arise regarding the effects of chronic inactivation of mechanisms mediated by these proteins.

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