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ATTENUATION OF CHICK HEART ADENYLATE CYCLASE BY MUSCARINIC RECEPTORS AFTER PERTUSSIS TOXIN TREATMENT

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SUMMARY: Pertussis toxin selectively modifies the function of N_i , the inhibitory guanine nucleotide binding protein of the adenylate cyclase complex. In chick heart membranes, guanine nucleotide activation of N_i resulted in a decrease in the apparent affinity of the muscarinic receptor for the agonist oxotremorine, inhibition of basal adenylate cyclase activity, and the attenuation of adenylate cyclase by oxotremorine. Treatment of chicks with pertussis toxin caused the covalent modification of 80-85% of cardiac N_i. After this treatment Gpp(NH)p had no effect on muscarinic receptor affinity and GTP stimulated basal adenylate cyclase activity. In contrast, the GTP-dependent attenuation of adenylate cyclase caused by muscarinic receptors was unaffected.

In cardiac tissue, adenylate cyclase is activated by β -adrenergic receptors and attenuated by muscarinic cholinergic receptors (1). Receptor mediated stimulation or attenuation of adenylate cyclase occurs indirectly via the guanine nucelotide binding proteins N_s[1] and N_i, respectively (2). A Bordetella pertussis toxin, known as islet-activating protein or IAP, perturbs hormonal attenuation of adenylate cyclase in many cell types (3) by causing ADP-ribosylation of N_i. This modification of N_i results in the loss (4-6) of the following measurable functions of N_i: a) receptor mediated attenuation of cyclase; b) GTP-induced attenuation of basal adenylate cyclase activity; and c) control by guanine nucleotides of the apparent affinity of the inhibitory re-

The abbreviations used are: IAP, islet-activating protein; QNB, quinuclidinyl benzilate; Gpp(NH)p, guanylimidodiphosphate; N_i and N_s, guanine nucleotide-binding proteins involved in attenuation and stimulation of adenylate cyclase, respectively; α_i, ADP-ribosylatable subunit of N_i; GN, guanine nucleotides.

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ceptors for agonist. In the present study we measured the effects of IAP on the muscarinic receptor/adenylate cyclase system in chick heart. Under the conditions of this study, IAP had marked effects on two putative functions of N_i but did not diminish the ability of muscarinic receptors to inhibit cyclase.

MATERIALS AND METHODS

IAP injection. Chicks (1-5 days old) were injected i.p. with IAP or saline vehicle and sacrificed 48 hr later. Unless otherwise stated the dose of IAP used was 1 μg. All injected amounts were per 40 g body weight. The 1 μg dose used was the highest amount allowable since 3 or 5 g of IAP were lethal within 36-48 hrs (N = 2) and 24-36 hrs (N = 3) respectively.
Membrane isolation. Crude membranes were prepared from twice-washed pellets (31,000 g x 20 min) in 20 mM Tris-HCl, pH 7.4, 1 mM EDTA, 0.1 mM dithiothreitol. Semi-purified membranes were prepared according to method 1 of (7).
Muscarinic receptor assay. Assays were performed in duplicate at 37°C in 1 ml reactions containing 10 mM Na ,K -phosphate, pH 7.4, 1 mM EDTA, 10 mM MgCl , 5-10 μg crude membrane protein; 100-300 pM [^{3}H]QNB, ± 0.1 mM Gpp(NH)p and varying concentrations of the agonist oxotremorine as in (8).
Adenylate cyclase assay. Assays were conducted at 37°C for 10 min using 2'-dATP as substrate (9) to avoid the production of adenosine since R_i type adenosine receptors are present (22). Reactions, in triplicate, contained 10 mM histidine, pH 7.5; 50 mM NaCl; 9 mM MgCl ; 0.1 mM dcAMP; 5 mM creatine phosphate; 0.4 mg/ml creatine phosphokinase; 2.5 units/ml adenosine deaminase; 0.5 mM RO 20-1724, a phosphodiesterase inhibitor; 0.1% bovine serum albumin; 0.1 mM [α - ^{32}P]dATP (50-100 cpm/pmole); 1-3 μg membrane protein; and GTP as indicated in a volume of 0.1 ml. Basal activities (in the absence of GTP or agonist) in control and IAP-treated membranes were 463 ± 84 (N=9) and 614 ± 54 (N=6) pmols dcAMP formed/mg protein/min, respectively.
In vitro assays of IAP-catalyzed ADP-ribosylation. Membrane protein (60 μg) from control or IAP-treated animals was incubated with [^{32}P]NAD (2 μ Ci), ± 3 μg IAP, ± 0.16% digitonin/0.032% cholate for 30 min at 37°C in 0.1 ml according to (10). Protein was precipitated with trichloroacetic acid, solubilized in electrophoresis sample buffer, heated for 1 min at 100°C, and applied to NaDodSO polyacrylamide (12%) gels prepared as described (11). Radioactivity in the IAP substrate was quantitated by densitometry of radioautograms prepared from the gels or by liquid scintillation counting.
Materials. Fertilized White Leghorn chicken eggs or newborn chicks were obtained from SPAFAS (Roanoke, IL) or Corn Belt Hatcheries (Forrest, IL). IAP was a gift from Dr. Erik Hewlett (Charlottesville, VA). [^{3}H]QNB (34 Ci/mmole) and [α - ^{32}P]dATP were from Amersham. [^{3}H]dcAMP and [^{32}P]NAD were from New England Nuclear. Gpp(NH)p was from P-L Biochemicals. RO 20-1724 was from Dr. H. Sheppard, Hoffman-LaRoche (Nutley, NJ). All other reagents were from Sigma.

RESULTS

Effect of IAP on agonist interaction with chick heart muscarinic receptors. The muscarinic receptor agonist oxotremorine bound with high affinity to receptors in control membranes (Fig. 1). Gpp(NH)p caused an apparent decrease in receptor affinity, as evidenced by an increase in the IC_{50} value for the agonist from 0.1 to 1.0 μM (Fig. 1). In membranes prepared from chicks injected

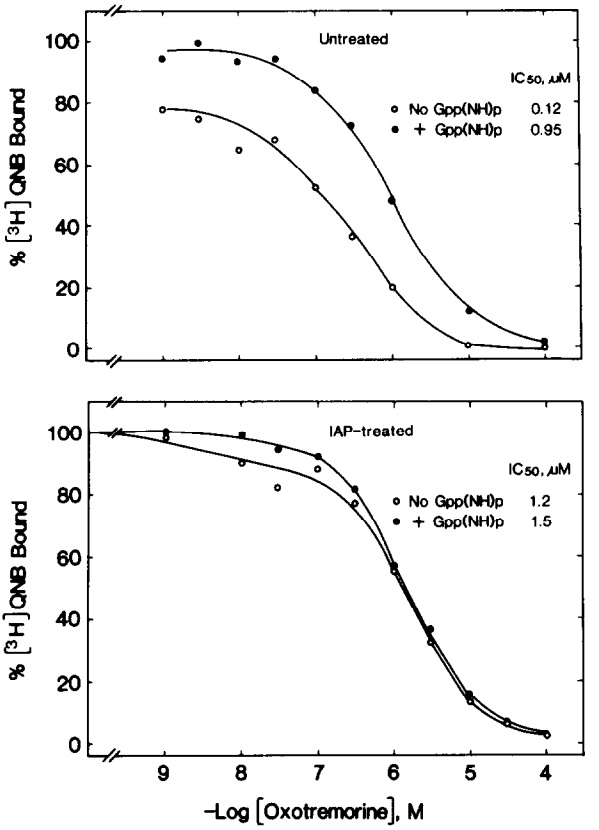
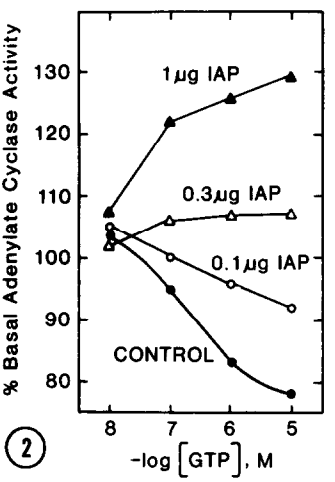


Figure 1. Effects of IAP treatment on the apparent affinity of the cardiac muscarinic receptor for oxotremorine. Chicks were treated + IAP, crude cardiac membranes were prepared, and oxotremorine/[^{3}H]QNB competition experiments were performed. The protein concentration was 25-50 μg/ml and [^{3}H]QNB was 250-300 pM. Gpp(NH)p was 0.1 mM which was found to be a saturating dose. The data shown are from a representative experiment. The IC (μM) and n_H values from a series of experiments were: Control, 0.12 ± 0.03 (n_H = 0.56, N = 6); control plus Gpp(NH)p, 0.98 ± 0.17 (n_H = 0.79, N = 3); IAP, 1.45 ± 0.4 (n_H = 0.86, N = 4); IAP plus Gpp(NH)p, 1.65 (n_H = 0.89, N = 2).

with IAP, the apparent affinity for oxotremorine decreased and the effect of Gpp(NH)p was abolished (Fig. 1). The effect of IAP was dose dependent, i.e., lower doses of IAP caused smaller increases in the IC_{50} values (data not shown). Both Gpp(NH)p and the IAP treatment steepened the competition curves compared to the controls (Fig. 1) and the n_H values approached 1.0.

Effect of IAP on the modulation of basal adenylate cyclase activity by GTP. In control membranes, GTP inhibited basal adenylate cyclase activity by 20-35% (Fig. 2). Inhibition was observed with GTP greater than 10^{-8} M and was maximal at 10 μM. This inhibitory effect of GTP was abolished and reversed to a



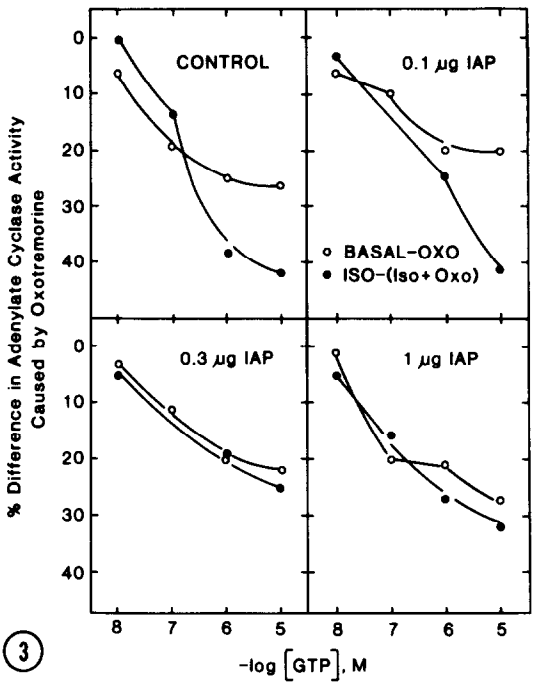


Figure 2. Effect of varying GTP on basal adenylate cyclase in cardiac membranes from control and IAP treated chicks. Adenylate cyclase activity was determined in purified cardiac membranes prepared from control (●) or IAP-treated chicks (○, 0.1 µg; △, 0.3 µg; ▲, 1 µg) in the presence of varying concentrations of GTP. The data shown are the means from one experiment done in triplicate. Similar results were obtained with membranes prepared from another group of chicks injected with varying concentrations of IAP and with 3-5 preparations from control and 1 g IAP-treated chicks.

Figure 3. Comparison of the attenuation of adenylate cyclase by oxotremorine in cardiac membranes from control and IAP-treated chicks. The data shown were derived by determining the percent change in basal or isoproterenol-stimulated activity caused by the addition of oxotremorine, i.e., the data shown by (-○-) represent the percent decrease in basel activity caused by oxotremorine, while (-●-) shows the percent decrease in isoproterenol stimulated activity caused by oxotremorine. The data are from a representative experiment that was repeated 3 times.

stimulation by IAP treatment (Fig. 2). The degree of the reversal of the GTP inhibition was dependent on the dose of IAP used (Fig. 2).

Effect of IAP treatment on muscarinic receptor mediated attenuation of chick heart adenylate cyclase. In control membranes, oxotremorine inhibited basal and β-adrenergic receptor-stimulated adenylate cyclase activity in a GTP-dependent manner (Fig. 3). Unexpectedly, IAP did not alter these effects of oxotremorine (Fig. 3). The net decrease in basal or isoproterenol-stimulated cyclase activity caused by oxotremorine was unchanged by any IAP treatment (Fig. 3. The only significant effect of any dose of IAP on adenylate cyclase was on

the GTP-dependent modulation of basal activity Fig. 2). Similar results were obtained with the muscarinic agonist carbachol, and the effects of both agonists were appropriately antagonized by atropine (data not shown).

Effect of IAP treatment on the potency of oxotremorine to attenuate adenylate cyclase. Oxotremorine attenuated basal adenylate cyclase with IC_{50} values of 0.17 and 0.21 μM in membranes from control and IAP-treated chicks, respectively (Fig. 3). The maximum degree of inhibition of basal adenylate cyclase was 35 ± 3% in control (N = 3) and 25 ± 4% (N = 3) in IAP treated membranes (Fig. 3). IC_{50} values for oxotremorine to attenuate isoproterenol-stimulated adenylate cyclase were 0.85 μM for membranes from both control and IAP-treated chicks (data not shown).

ADP-ribosylation of cardiac membranes from control and IAP-injected chicks. In vitro ADP-ribosylation of membranes from control and IAP-treated chicks was performed in order to determine the extent of modification of N_i caused by the the in vivo IAP treatment. Analysis of such experiments by gel electrophoresis and autoradiography indicated that IAP incorporated [^{32}P]ADP-ribose into a peptide of 39,000 daltons. This is presumably α_i (12-14). In both the absence and presence of digitonin-cholate, the in vitro ADP-ribosylation of this peptide was reduced in membranes from IAP-treated chicks to 15%-19% (N=3) of control membranes.

DISCUSSION

The injection of IAP into chicks caused the covalent modification of 80-85% of a 39,000 dalton peptide, presumably the α-subunit of N_i (12-14). After the IAP treatment the affinity of the muscarinic receptor for agonist was decreased and was unaffected by GN. In addition, GTP stimulated, rather than inhibited, basal adenylate cyclase activity. Surprisingly, under these conditions, the ability of oxotremorine to inhibit adenylate cyclase activity was not affected by IAP.

Muscarinic receptors can exist in high and low affinity states in vitro (15). The interpretation of the present results differs depending on what one

assumes physically constitutes the individual affinity states of the receptor. The highest affinity state of the receptor may be an RN_i complex that can be converted to a lower affinity state by the binding of a guanine nucleotide to N_i (16-18). The lower affinity state may be free R created by the dissociation of N_i (17), or it may be a complex of RN_i liganded with a GN as suggested by Rodbell (16). One explanation of the present findings is that the low affinity state is RN_i liganded with GN (16). N_i is believed to be activated by GTP and inactivated by GDP (2). The present results can be explained by postulating that GTP can readily exchange with GDP bound to the unmodified N_i, but after covalent modification of N_i, this exchange only occurs in the presence of an agonist. GDP may be become bound to N_i *in vivo* and may be released from the unmodified N_i during the preparation or assay of control membranes. This would allow the *in vitro* detection of high affinity binding. In contrast, GDP may not be released from ADP-ribosylated N_i under the same conditions, and *in vitro* high affinity binding would not be detected. According to this formulation high affinity binding is largely an *in vitro* phenomenon created by emptying N_i of GN.

The effect of GTP on basal adenylate cyclase activity is probably a consequence of opposing effects of GTP on N_s and N_i. The proposed inability of GTP to exchange for GDP bound to ADP-ribosylated N_i would result in the activation of more N_s than N_i by GTP and thus explain the IAP-induced reversal of the effects of GTP on basal cyclase activity. If oxotremorine can promote the exchange of GTP for GDP bound to the ADP-ribosylated N_i, this would explain why oxotremorine mediated inhibition of adenylate cyclase activity after IAP treatment. This reasoning is supported by the work of Jakobs *et al.* who demonstrated that the effect of IAP is to *slow* the activation of N_i (19). Accordingly, ADP-ribosylated N_i may contain GDP that is more tightly bound and exchanges more slowly than that bound to native N_i.

Alternative explanations of the present results are found if one assumes that the low affinity state of the muscarinic receptor is free R. In that case, then either: 1) there are pools of N_i with different accessibility to

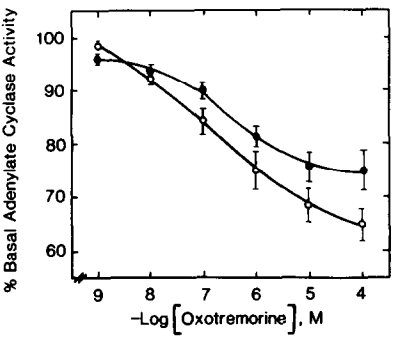


Figure 4. Dose-response curves for oxotremorine-mediated attenuation of basal adenylate cyclase in cardiac membranes from control and IAP-treated chicks. Adenylate cyclase activity was determined in the presence of 10 μM GTP and varying concentrations of oxotremorine as shown. The results are the mean of 3 experiments. Control (○); IAP-treated (●).

IAP, or 2) more than one GTP-binding protein participates in hormonal attenuation of chick heart cyclase, or 3) there may be "spareness" in the N_i and/or receptor population. With regard to the last possibility, it has been suggested that only 10% of the muscarinic receptors in the chick heart may be necessary for full effects on cyclic AMP metabolism (20). However, the dose-response curves for oxotremorine to inhibit cyclase were not shifted to the right after IAP treatment (Fig. 4), arguing against involvement of "spare" components in this response.

Pertussis toxin usually blocks all known functions of N_i, although others reported that IAP blocked dopamine receptor-N_i interactions but not N_i-cyclase interactions (21). It is not clear why the chick heart muscarinic receptor system is different from other cyclase attenuating systems in its response to IAP, but it may be useful for probing receptor:cyclase interactions.

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