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DIFFERENTIAL SENSITIVITY OF α_o AND α_i TO ADP-RIBOSYLATION BY PERTUSSIS TOXIN IN THE INTACT CULTURED EMBRYONIC CHICK VENTRICULAR MYOCYTE

RELATIONSHIP TO THE ROLE OF G PROTEINS IN THE COUPLING OF MUSCARINIC CHOLINERGIC RECEPTORS TO INHIBITION OF ADENYLATE CYCLASE ACTIVITY*

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Abstract—The guanine nucleotide regulatory proteins, α_i and α_o, coexist in a variety of tissues, including heart, brain, and adipose tissues and are ADP-ribosylated by pertussis toxin (Gilman AG, G-proteins and dual control of adenylate cyclase. *Cell* **26**: 577–579, 1984). Previous studies in which purified G proteins were reconstituted with cell membranes and/or phospholipid vesicles have suggested that an α_i-like protein mediates GTP-dependent inhibition of adenylate cyclase activity. However, direct studies comparing the role of α_i and α_o in mediating the inhibition of adenylate cyclase activity in the intact cell have not appeared. In the present study, we demonstrated that, in the intact cell, α_o was more sensitive to ADP-ribosylation in the presence of pertussis toxin than was α_i. The $T_{1/2}$ for pertussis toxin-mediated ADP-ribosylation of α_i was 199 ± 10 min (mean ± SE, N = 10) compared to 157 ± 7 min for α_o. The IC_{50} for pertussis toxin-induced ADP-ribosylation of α_i was 158 ± 40 pg/ml (mean ± SE, N = 11) compared to 35 ± 8 pg/ml for α_o. The differences in both $T_{1/2}$ and IC_{50} for α_i and α_o were statistically significant ($P < 0.001$). Studies were carried out to determine whether α_o was involved in coupling the muscarinic cholinergic receptor to inhibition of adenylate cyclase activity in intact cells. The time course and dose dependence of the pertussis toxin-induced uncoupling of the muscarinic receptor from inhibition of adenylate cyclase closely paralleled the time course and dose dependence for the ADP-ribosylation of α_i but differed significantly ($P < 0.001$) from the time course and dose dependence of the pertussis toxin mediated ADP-ribosylation of α_o. The $T_{1/2}$ and IC_{50} values for the pertussis toxin-induced decrease in the inhibition of adenylate cyclase activity were 210 ± 6 min (mean ± SE, N = 11) and 169 ± 25 pg/ml (mean ± SE, N = 12), respectively, which were not significantly different from the $T_{1/2}$ and IC_{50} for pertussis toxin mediated ADP-ribosylation of α_i. The data are consistent with the hypothesis that, in the intact cell, a pertussis toxin-sensitive α_i-like protein, but not α_o, couples muscarinic receptors to inhibition of adenylate cyclase activity.

Guanine nucleotide binding proteins (G proteins) are a family of plasma membrane proteins which couple receptors to various biologic responses [1]. The stimulatory G protein (G_s) and the inhibitory G protein (G_i) couple receptors to activation and inhibition of adenylate cyclase respectively. Transducin couples light activation of rhodopsin to stimulation of cyclic GMP phosphodiesterase. G_o is another G protein whose function is unknown, but it may be related to inhibition of the neuronal calcium channel [2]. The G proteins are heterotrimers composed of α, β, and γ subunits [3, 4]. The α subunits differ in molecular weight and structure. Recently, multiple forms of the α subunit of putative G_i have been demonstrated and it is unclear whether more than one α_i is present in a particular tissue [5, 6]. While heterogeneity of α_i exists, thus far only one form of α_o has been described. The α subunits of these G proteins also differ in their abilities to serve as substrates for ADP-ribosylation by pertussis or cholera toxins. The α subunits of G_i and G_o are ADP-ribosylated by pertussis toxin, whereas the α subunits of G_s are ADP-ribosylated by cholera toxin [1, 7, 8].

Previous studies using purified α_i demonstrated that α_i is more sensitive to ADP-ribosylation by pertussis toxin than α_o [9]. These studies further demonstrated that the presence of $\beta\gamma$ is necessary for the ADP-ribosylation of the α subunit. Hence, the greater susceptibility of α_i to ADP-ribosylation by pertussis toxin may be related to an increased affinity of α_i for $\beta\gamma$ in comparison with the affinity of α_o for $\beta\gamma$ [9]. In the present study, we investigated whether this differential sensitivity of α_i and α_o to ADP-ribosylation by pertussis toxin could be demonstrated in the intact cell.

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The physiogic role of α_i and α_o in mediating the hormone-sensitive inhibition of adenylate cyclase activity was based on reconstitution studies using purified α_i or α_o with cell membranes from cyc-S49 lymphoma cells or with phospholipid vesicles containing the catalytic unit of adenylate cyclase [10–12]. These studies demonstrated that a GTPγS-activated α_i, but not a GTPγS-activated α_o, was able to inhibit adenylate cyclase activity. No evidence has been presented to rule out the possibility that α_o may couple the muscarinic receptor to inhibition of adenylate cyclase activity in the intact cell. Since pertussis toxin uncouples muscarinic receptors from inhibition of adenylate cyclase, a differential sensitivity of α_i and α_o to pertussis toxin would help determine whether ADP ribosylation of α_o or an α_i-like protein is responsible for uncoupling the muscarinic receptor from inhibition of adenylate cyclase. In the present study, we found that, in the intact cell, α_o and α_i have different susceptibilities to ADP-ribosylation in the presence of pertussis toxin. We used this differential sensitivity to pertussis toxin to study whether α_o or an α_i-like protein is involved in coupling the muscarinic cholinergic receptor to inhibition of adenylate cyclase activity in the intact cell by determining whether the pertussis toxin-mediated ADP-ribosylation of α_o or an α_i-like protein correlates with the pertussis toxin-mediated decrease in muscarinic inhibition of adenylate cyclase activity.

MATERIALS AND METHODS

ATP, GTP, creatine phosphate, creatine kinase, lima bean trypsin inhibitor, soybean trypsin inhibitor, leupeptin, *l*-isoproterenol, alamethicin, carbamylcholine and dithiothreitol were all purchased from Sigma; [^{3}H]ATP was from ICN. ^{14}C-labeled cyclic AMP ([^{14}C]cAMP) and [^{32}P]NAD were from New England Nuclear. Pertussis toxin was purchased from List Biologicals and bovine albumin was from Miles Scientific. Embryonated chicken eggs were purchased from Spafas Inc. Embryonic ages were determined according to the description of Hamburger and Hamilton [13].

Adenylate cyclase assay. Adenylate cyclase activity was determined as described previously [14]. ATP prepared by phosphorylation of adenine, and hence low in contaminating GTP, was used as the substrate. The reaction mixture contained 0.4 mM ATP, [^{3}H]ATP (80 mCi/mmol), 10 mM magnesium chloride, 10 mM phosphocreatine, 0.84 units of creatine phosphokinase, 1 mM cyclic AMP, 1 mM dithiothreitol, 75 mM sodium chloride, 100 μM GTP, 50 mM Tris-hydrochloride (pH 7.6), 1 mM EDTA, 75 mM sucrose, and protease inhibitors at the concentrations indicated. Unless otherwise indicated, the concentrations of (−)-isoproterenol and carbamylcholine were 10^{-5} M and 10^{-3} M respectively. Following incubation for 10 min at 37° diluting solution containing unlabeled cAMP, ATP and [^{14}C]cAMP was added, and the reaction mixture was boiled for 2 min; it was then loaded onto a Dowex AG50W X2 column and the cyclic AMP peaks were collected. Further separation of the remaining adenine and guanine nucleotides was accomplished by barium hydroxide and zinc sulfate precipitation. Following the precipitation, recovery of [^{3}H]cAMP was determined by measurement of the ratio of ^{3}H to ^{14}C in aliquots of the supernatant fraction in the Beckman liquid scintillation counter. Recoveries of cAMP in these procedures were usually about 40–50%.

Preparation of tissues. Ventricular myocytes from chick embryos 14 days *in ovo* were grown in culture for 3 days according to methods previously described [15], and experiments were performed on day 3. Cells were broken and homogenized in a Dounce homogenizer in buffer containing the following components: 50 mM Tris hydrochloride (pH 7.4), 2 mM $MgCl_2$, 1 mM ethyleneglycolbis(aminoethylether)tetra acetate (EGTA), 1 mM EDTA, 20 μg/ml of leupeptin, 32 μg/ml of lima bean trypsin inhibitors, 32 μg/ml of soybean trypsin inhibitors, as well as 0.01% of the detergent 3-(3-cholamidopropyl)-dimethylammonio)-1-propane sulfonate (CHAPS). Cell homogenates were then pretreated with 20 μg/ml of an antibiotic detergent, alamethicin, at room temperature for 5 min prior to adenylate cyclase assay. We found that homogenates prepared this way rarely contained unbroken cells and consisted mostly of vesicles in which determination of the inhibition of adenylate cyclase activity was reproducible from experiment to experiment. All experiments were carried out in the presence of protease inhibitors, alamethicin and CHAPS unless otherwise indicated.

ADP ribosylation with pertussis toxin. The *in vitro* ADP-ribosylation reaction was carried out at 37° for 40 min. The assay mixture contained 10 μM NAD^+, 2 μCi [^{32}P]NAD^+, 2.5 mM ATP, 2 mM GTP, 10 mM isoniazid, 10 mM thymidine, 5 mM magnesium chloride, 1 μg pertussis toxin, 10 mM phosphocreatine, 0.84 units of creatine phosphokinase and tissue (50 μg protein/assay) in a total volume of 50 μl. All of the protease inhibitors were present from the time of initial homogenization of the tissue. The reaction was stopped by the addition of 2% sodium dodecyl sulfate (SDS) Laemmli sample buffer followed by boiling for 1 min. Analysis of the protein bands following ADP-ribosylation was performed on 11% polyacrylamide gels prepared according to Laemmli [16]. Dry gels were exposed to Kodak XAR film with or without an enhancing screen for 1–2 days at −70°. The autoradiograms from the dried gels were scanned on an LKB laser densitometer, and the two peaks representing the α_i and α_o bands were cut out and weighed. The weights obtained represented the relative levels of α_i and α_o and were normalized as a percentage of the weight of peaks from the control sample (percent maximum). In previous studies of changes in levels of α_o during embryonic development of the chick heart, we demonstrated that quantification of the relative levels of α_o by immunoblotting and by pertussis toxin-mediated [^{32}P]ADP-ribosylation gives results that are not significantly different [14].

In the intact cell treated with pertussis toxin, the extent of ADP-ribosylation of G proteins by endogenous NAD was determined by quantifying the amount of pertussis toxin-catalyzed [^{32}P]ADP ribose incorporated into the unreacted G proteins in

homogenates of these cells. The amount of [^{32}P]ADP-ribosylation in these cell homogenates, reflecting the extent of α_i and α_o that was not ADP-ribosylated in the intact cell, was inversely related to the time course and dose dependence of pertussis toxin-mediated ADP-ribosylation of α_i and α_o in intact cells by endogenous NAD^+. These data were plotted as the ratio (×100%) of the level of [^{32}P]ADP-ribosylation in homogenates of cells pretreated with pertussis toxin to the level of [^{32}P]ADP-ribosylation in homogenates of control cells (% maximum). Thus, 100% represented zero ADP-ribosylation in the intact cell and 0% represented 100% ADP-ribosylation in the intact cell.

Protein assay. Proteins were determined according to the method of Lowry *et al.* [17] using bovine serum albumin as the standard.

RESULTS

Differential sensitivity of α_i and α_o to pertussis toxin. To determine whether α_i and α_o have differential susceptibility to ADP-ribosylation by pertussis toxin, we compared the dose dependence of the pertussis toxin-induced ADP-ribosylation of α_i with that of the ADP-ribosylation of α_o. After incubation of chick ventricular cells from hearts 14 days *in ovo* with various concentrations of pertussis toxin for 8.5 hr, the level of pertussis toxin-catalyzed ADP-ribosylation of α_o and α_i by endogenous NAD was quantified by measuring the amount of [^{32}P]ADP ribose incorporation into the unreacted α_i and α_o in homogenates of these cells. A typical experiment is illustrated in the autoradiogram in Fig. 1. In control cells, the band corresponding to α_o was darker and broader than the band corresponding to α_i. With increasing time of exposure of cells to pertussis toxin, the intensity of the bands decreased progressively, reflecting reduced incorporation of [^{32}P]ADP ribose into the unreacted G proteins in cell homogenates. Relative to its initial control level, the α_o band decreased faster than the α_i band at each of the pertussis toxin concentrations tested.

To quantify the changes in the level of [^{32}P]ADP-ribosylation, autoradiograms were scanned with a laser densitometer. Initially, the peak corresponding to the α_o band in tho densitometry tracing was broader than the peak corresponding to the α_i band (Fig. 2). Relative to controls, the height and width of the α_o peak decreased more rapidly than that of α_i, suggesting a more rapid rate of ADP-ribosylation of α_o by the endogenous NAD in intact myocytes. The relative levels of α_o and α_i are summarized in Fig. 3. The data were plotted as ratios of α_i and α_o that were not ADP-ribosylated in intact cells (represented by the level of [^{32}P]ADP-ribosylation in homogenates of cells pretreated with pertussis toxin) to the total (represented by the level of [^{32}P]ADP-ribosylation in homogenates of control). These data demonstrated that α_o was more sensitive to ADP-ribosylation by pertussis toxin than was α_i at each of the pertussis toxin concentrations studied ($P < 0.01$), and the IC_{50} for the pertussis toxin-mediated ADP-ribosylation of α_o was less than the IC_{50} for the ADP-ribosylation of α_i (Table 1).

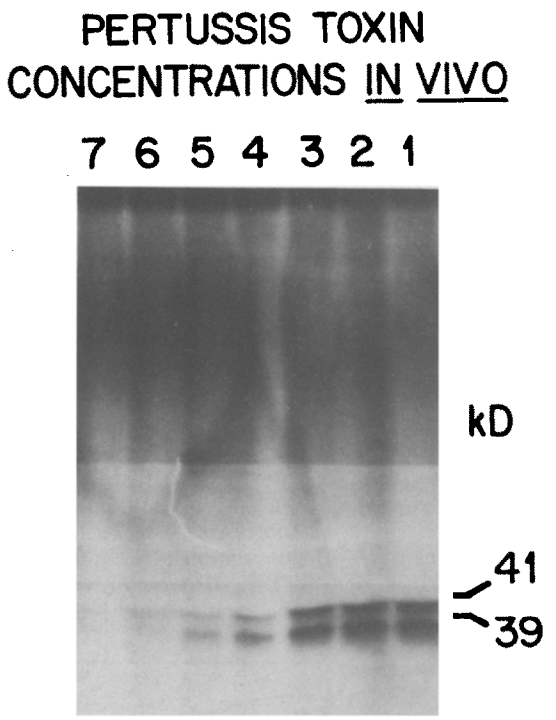

Fig. 1. Typical autoradiogram showing the extent of [^{32}P]ADP-ribosylation of α_i and α_o in homogenates of cells treated with various concentrations of pertussis toxin. Cells incubated with the indicated concentrations of pertussis toxin were washed, harvested, and dounce homogenized. Cell homogenates were then incubated in the presence of [^{32}P]NAD^+ and pertussis toxin, and loaded onto a polyacrylamide gel as described in Materials and Methods. Samples loaded onto each lane contained the same amount of protein (50 μg). Lane 1: control cells; lanes 2–7 pertussis toxin-catalyzed [^{32}P]ADP-ribosylation of the α_o and α_i in homogenates of cells exposed to 10 pg/ml, 30 pg/ml, 100 pg/ml, 1 ng/ml, 10 ng/ml and 100 ng/ml of pertussis toxin, respectively, for 8.5 hr.

To further characterize the differential sensitivity of α_o and α_i to ADP-ribosylation by pertussis toxin, we compared the time course of pertussis toxin-induced ADP-ribosylation of α_i with that of the ADP-ribosylation of α_o. The extent of ADP-ribosylation of α_o and α_i in the intact cell was determined

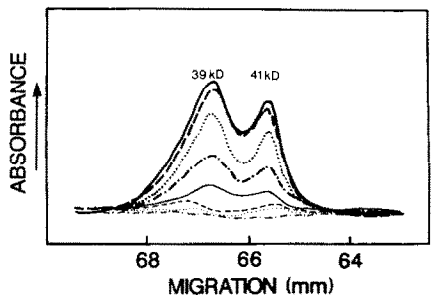

Fig. 2. Densitometry tracings of an autoradiogram of α_o and α_i. An autoradiogram from Fig. 1 was scanned on an LKB densitometer, and the baselines for the scan at each concentration were superimposed. Data was typical of scans of at least ten different determinations. Key: (——) control; (— —) 10 pg/ml; (●●●) 30 pg/ml; (—●—) 100 pg/ml; (——) 1 ng/ml; (– – –) 10 ng/ml; (· · · ·) 100 ng/ml; and (—·—) blank.

Table 1. Effect of pertussis toxin on the ADP-ribosylation of α_i and α_o

	$T_{1/2}$ (min)	IC_{50} (pg/ml)
α_o	157 ± 7	35 ± 8
α_i	199 ± 10*	158 ± 40*

The means ± SE were derived from individual $T_{1/2}$ (N = 10) and IC_{50} (N = 11) values. It should be noted that the IC_{50} value for α_i in Table 1 is slightly different from that in Fig. 3. This difference may be explained by the fact that each point in Fig. 3 was derived from the curve defined by points representing the means of ten determinations, whereas the IC_{50} value in Table 1 is the mean of eleven IC_{50} values.

* $P < 0.001$, compared to α_o values.

by studying the amount of pertussis toxin-catalyzed [^{32}P]ADP-ribosylation of α_o and α_i in homogenates of cells pretreated with pertussis toxin. The data for the kinetics of ADP-ribosylation of α_i and α_o are summarized in Fig. 4. These data demonstrate that the ADP-ribosylation of α_o preceded the ADP-ribosylation of α_i in intact cells at each time point studied ($P < 0.01$ by t-test) and that the $T_{1/2}$ for the ADP-ribosylation of α_o was significantly different from the $T_{1/2}$ for the ADP-ribosylation of α_i (Table 1).

It should be noted that, although only one form of α_o has been described, recent studies have demonstrated the presence of multiple isoforms of α_i in rat and human brains [5, 6]. Since little is known about the expression of these forms of α_i in cultured chick ventricular cells, it is possible that the pertussis toxin substrate migrating at 41 kD on sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS–PAGE) shown in Fig. 1 represents several forms of α_i with identical electrophoretic mobilities. However, in the present study, the pertussis toxin substrate(s) which migrates at a molecular weight of 41 kD was referred to as α_i.

Dose dependence of pertussis toxin effects on adenylate cyclase activity. Since pertussis toxin uncouples the muscarinic receptor from inhibition of adenylate cyclase, the differential sensitivity of α_i and α_o to ADP-ribosylation by pertussis toxin suggested that it may be possible to determine whether ADP-

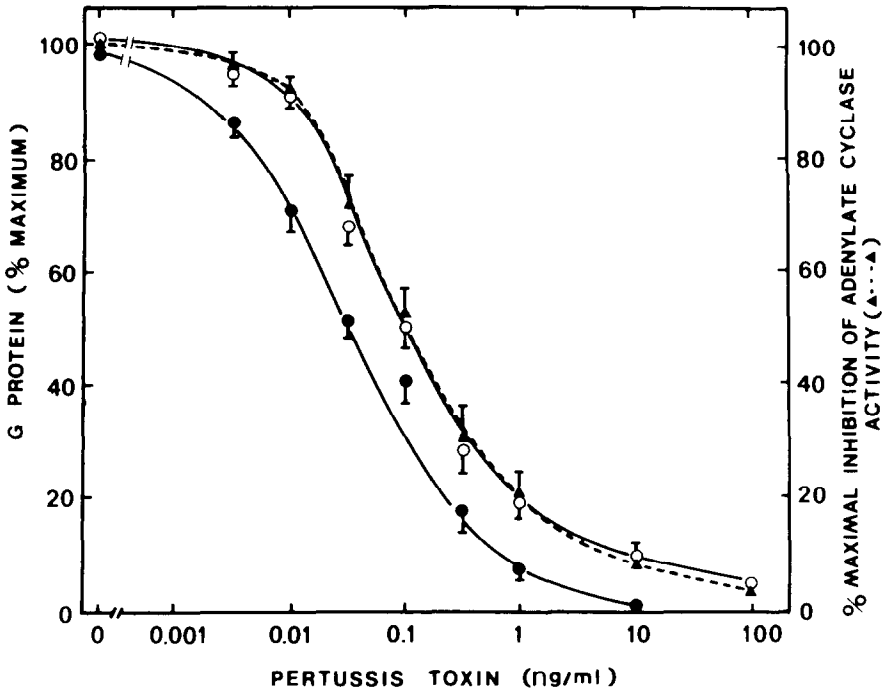

Fig. 3. Concentration dependence of the effect of pertussis toxin on the ADP-ribosylation of α_i and α_o and on the muscarinic inhibition of adenylate cyclase activity. The fraction of α_i or α_o which was not ADP-ribosylated by endogenous NAD^+ in the intact cell at various concentrations of pertussis toxin was determined as the ratio (×100%) of the level of [^{32}P]ADP-ribosylation in homogenates of cells pretreated with pertussis toxin to the level of [^{32}P]ADP-ribosylation in homogenates of control cells (% maximum) (●—●, α_o; ○—○, α_i). Data were determined by quantifying the size of the peaks in scans of autoradiograms as described in Materials and Methods. Each point is the mean ± SE of eleven determinations. In parallel experiments, cultured ventricular cells were incubated with various concentrations of pertussis toxin for 8.5 hr. Membranes were prepared for determination of carbamylcholine inhibition of isoproterenol-stimulated adenylate cyclase activity. Inhibition by carbamylcholine was obtained by determining the difference between the level of adenylate cyclase activity in the presence of 10 μM isoproterenol and in the presence of 10 μM isoproterenol plus 1 mM carbamylcholine. Data are expressed as the ratio of percent inhibition of isoproterenol-stimulated adenylate cyclase activity in homogenates of cells exposed to pertussis toxin to the percent inhibition in homogenates of control cells (as percent maximal inhibition) (▲– – –▲). The actual value for the maximum inhibition of adenylate cyclase activity was 99 ± 8 pmol cAMP/mg protein/10 min. Data are the means ± SE of eleven independent determinations carried out in triplicate.

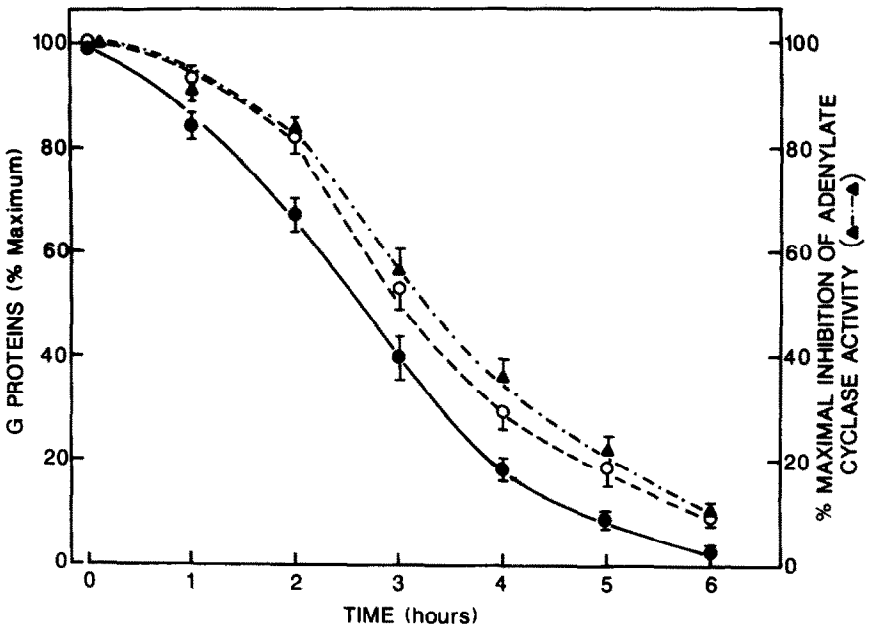

Fig. 4. Time course of the pertussis toxin effect on the ADP-ribosylation of α_i and α_o and on the inhibition of adenylate cyclase activity. The fraction of α_i or α_o which was not ADP-ribosylated by endogenous NAD^+ in the intact cell at various times was determined as the ratio (×100%) of the level of [^{32}P]ADP-ribosylation in homogenates of cells pretreated with pertussis toxin to the level of [^{32}P]ADP-ribosylation in homogenates of control cells (as percent maximum) (●—●, α_o; ○--○, α_i). Data were derived by quantifying the relative size of the peaks in scans of autoradiograms such as that shown in Figs. 1 and 2 by the procedure described in Materials and Methods. Each point is the mean ± SE of ten determinations. In parallel experiments, myocytes were incubated with 2 ng/ml of pertussis toxin for the same time periods. Membranes were prepared for assay of adenylate cyclase activity as described in Materials and Methods. Inhibition by carbamylcholine was obtained by determining the difference between the level of adenylate cyclase activity in the presence of 10^{-5} M isoproterenol and in the presence of 10^{-5} M isoproterenol plus 10^{-3} M carbamylcholine. Data are expressed as the ratio of percent inhibition of isoproterenol-stimulated adenylate cyclase activity in homogenates of cells exposed to pertussis toxin to the percent inhibition in homogenates of control cells (as percent maximal inhibition) (▲--▲). The actual value for the maximum inhibition of adenylate cyclase activity was 95 ± 9 pmol cAMP/mg protein/10 min. Data are the means ± SE of ten determinations carried out in triplicate.

ribosylation of α_o or α_i is responsible for uncoupling the muscarinic receptor from inhibition of adenylate cyclase. To study the correlation between pertussis toxin-induced attenuation of muscarinic inhibition of adenylate cyclase activity and pertussis toxin-mediated ADP-ribosylation of α_i and α_o in the intact cell, the ability of carbamylcholine to inhibit adenylate cyclase activity was determined after treatment of cultured myocytes with pertussis toxin. Incubation of ventricular myocytes with 100 ng/ml pertussis toxin for 8.5 hr abolished nearly all the inhibition caused by 1 mM carbamylcholine (the inhibition was 84 pmol cAMP per mg protein per 10 min for the control and 14 for the pertussis-intoxicated cultures) (Table 2). Treatment with pertussis toxin also caused an increase in the GTP- and isoproterenol-stimulated adenylate cyclase activity. This finding is consistent with the notion reported by others [1] that inac-

Table 2. Effect of pertussis toxin on adenylate cyclase activity

	Adenylate cyclase activity (pmol cAMP/mg protein/10 min)	
	Control	Treated*
GTP (10^{-4} M)	211 ± 18	296 ± 16†
GTP + isoproterenol (10^{-4} M)	394 ± 30	513 ± 27†
GTP + isoproterenol + carbamylcholine (10^{-3} M)	310 ± 19‡	499 ± 20

Data are the means ± SE of six experiments carried out in triplicate.
* Cells were treated with 100 ng/ml pertussis toxin for 8.5 hr.
† $P < 0.01$, compared to control values.
‡ $P < 0.01$ (by Student's paired t-test), comparing data for GTP plus isoproterenol with GTP plus isoproterenol plus carbamylcholine for each of the six experiments.

tivation of the inhibitory G protein by pertussis toxin results in the release of adenylate cyclase from a tonic inhibition.

To correlate the pertussis toxin-induced decrease in the ability of carbamylcholine to inhibit adenylate cyclase activity with the pertussis toxin-mediated ADP-ribosylation of α_i and α_o, we determined the dose dependence of the pertussis toxin-mediated decrease in the ability of carbamylcholine to inhibit adenylate cyclase activity. The percent carbamylcholine-mediated inhibition of isoproterenol-stimulated adenylate cyclase activity in pertussis toxin-treated cells was normalized to the percent carbamylcholine inhibition of isoproterenol-stimulated adenylate cyclase activity in control cells (maximal inhibition) and was plotted as percent maximal inhibition. Inhibition of isoproterenol-stimulated adenylate cyclase activity by carbamylcholine represented 40 ± 3% of the increase in adenylate cyclase activity stimulated by isoproterenol. Data summarized in Fig. 3 demonstrated that pertusis toxin caused a dose-dependent decrease in the ability of carbamylcholine to inhibit adenylate cyclase activity. At each of the pertussis toxin concentrations tested, the pertussis toxin-induced decrease in muscarinic inhibition of adenylate cyclase activity was similar to the pertussis toxin-induced ADP-ribosylation of α_i ($P > 0.1$) but differed significantly from the pertussis toxin-induced ADP-ribosylation of α_o ($P < 0.001$). The IC_{50} for the pertussis toxin-induced decrease in muscarinic inhibition of adenylate cyclase activity (169 ± 25 pg/ml, mean ± SE, N = 12) was similar to the IC_{50} for pertussis toxin-mediated ADP-ribosylation of α_i (158 ± 40 pg/ml, mean ± SE, N = 11, Table 1) but was significantly different from the IC_{50} for α_o (35 ± 8 pg/ml, mean ± SE, N = 11, Table 1).

Kinetics of pertussis toxin effect on adenylate cyclase activity. To further study the correlation between the pertussis toxin-mediated ADP-ribosylation of α_i and α_o and the pertussis toxin-induced loss of muscarinic inhibition of adenylate cyclase activity, we compared the time course of the pertussis toxin-mediated decrease in the ability of carbamylcholine to inhibit adenylate cyclase activity with that of the ADP-ribosylation of α_i and α_o (Fig. 4). At each time point studied, the pertussis toxin-induced decrease in muscarinic inhibition of adenylate cyclase activity paralleled the pertussis toxin-induced ADP-ribosylation of α_i ($P > 0.1$) but differed from the pertussis toxin-induced ADP ribosylation of α_o ($P < 0.01$). Hence, the $T_{1/2}$ for pertussis toxin-induced attenuation of muscarinic inhibition of adenylate cyclase activity (210 ± 6 min, mean ± SE, N = 11) was similar to the $T_{1/2}$ of pertussis toxin-induced ADP-ribosylation of α_i (199 ± 10 min, mean ± SE, N = 10; Table 1) but was significantly different from the $T_{1/2}$ of pertussis toxin-mediated ADP-ribosylation of α_o (157 ± 7 min, mean ± SE, N = 10, Table 1).

DISCUSSION

Previous studies [9] using purified components of the G protein heterotrimer suggested that $\alpha_i\beta\gamma$ was more easily ADP-ribosylated in the presence of pertussis toxin than was $\alpha_o\beta\gamma$. Since $\beta\gamma$ was required for ADP-ribosylation of α_i, this finding may be explained by a greater affinity of the α_i for $\beta\gamma$ in the cell-free system where the level of $\beta\gamma$ may be limiting [9]. In the present study in the intact cell, data from studies of both the kinetics and dose dependence of pertussis toxin-mediated ADP-ribosylation suggest that α_o was more easily ADP-ribosylated by pertussis toxin. Potential explanations for the enhanced susceptibility of α_o in the intact cell to ADP ribosylation by pertussis toxin include: (1) α_o is a better substrate for pertussis toxin intrinsically; (2) $\beta\gamma$ is more tightly associated with α_o in the native membranes, thus rendering the complex more easily ADP-ribosylated; (3) α_i is a better substrate for a potential enzyme which removes the ADP ribose moiety in these cells. The possibility that the differential sensitivity of α_i and α_o to ADP-ribosylation by pertussis toxin did not reflect a difference in sensitivity of α_o and α_i to ADP-ribosylation by endogenous NAD in the intact cell but was due to a differential susceptibility of α_i and α_o to [^{32}P]ADP-ribosylation in cell homogenates is unlikely since we have demonstrated previously [14] that the level of [^{32}P]ADP-ribosylation of α_i and α_o in membrane homogenates of embryonic chick hearts does not increase further in the presence of increased concentrations of pertussis toxin (up to 60 μg/ml assay mixture) or increased time of incubation (up to 90 min). Furthermore, adding the detergent cholate (0.1 to 0.01%) to unmask latent pertussis toxin substrate activity does not influence the extent of [^{32}P]ADP-ribosylation of α_o and/or α_i in cell homogenates. Finally, the level of [^{32}P]ADP-ribosylation increases linearly as a function of the amount of cell homogenate added to the reaction mixture up to a limit of 70 μg protein; the amount of protein used in the ADP-ribosylation reaction in the present study was in the linear range of the curve relating the labeling of α_i and α_o to the amount of protein [14].

Since previous studies demonstrating the role of α_i in mediating the inhibition of adenylate cyclase activity were carried out using purified proteins in phospholipid vesicles and/or in membranes in a cell-free system [10–12], the possibility that α_o might couple the muscarinic receptor to inhibition of adenylate cyclase activity in the intact cell has not been ruled out. Since pertussis toxin uncouples the muscarinic receptor from inhibition of adenylate cyclase activity, we used the differential sensitivity of α_o and α_i to ADP-ribosylation by pertussis toxin in the intact cell to determine whether ADP-ribosylation of either α_i or α_o correlated with uncoupling of muscarinic receptor from inhibition of adenylate cyclase activity. The results demonstrated that both the time course and dose dependence of the pertussis toxin-induced decrease in the ability of carbamylcholine to inhibit adenylate cyclase activity were correlated only with the time course and dose dependence of pertussis toxin-mediated ADP-ribosylation of α_i but differed from those of pertussis toxin-mediated ADP-ribosylation of α_o. These data support the notion that an α_i-like protein, but not α_o, couples the muscarinic receptor to inhibition of adenylate cyclase activity in the intact cell.

Recent findings have suggested that an additional G protein (mol. wt 40 kD) other than α_i, α_o, and

transducin is also a substrate for pertussis toxin-catalyzed ADP-ribosylation [18, 19]. In our previous studies and in Figs. 1 and 2 presented here, SDS–PAGE of homogenates of embryonic chick hearts [14] and cultured chick ventricular cells ADP-ribosylated with [^{32}P]NAD in the presence of pertussis toxin demonstrated clear separation of the 41 kD and the 39 kD peptides without the appearance of an intermediate, 40 kD peptide. Thus, it is unlikely that a 40 kD pertussis toxin-sensitive protein is present and mediates the inhibition of adenylate cyclase activity in these cultured heart cells. Recent studies have demonstrated the presence of multiple forms of α_i: α_{i1}, α_{i2}, and α_{i3} [6]. It is unclear whether the expression of a particular α_i is tissue- or species-specific. Rat C6 glioma, mouse macrophage, and human monocyte appear to express α_{i2}, while human and bovine brain contain predominantly α_{i1} [5, 6]. While both α_{i1} and α_{i2} have been found in the whole rat heart, it is possible that one is specific for the ventricles while the other is expressed only in the atria. It is not known which α_i or whether both α_{i1} and α_{i2} are expressed in the cultured chick ventricular cells. Nevertheless, it is possible that the 41 kD band seen on SDS–PAGE represents more than one α_i species with similar electrophoretic mobility. The present results should be interpreted in the context of this possibility. The data, however, help provide evidence in support of the notion that a pertussis toxin-sensitive α_i-like protein, but not α_o, is involved in coupling the muscarinic receptor to inhibition of adenylate cyclase activity in the *intact cell*.

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