IDENTIFICATION OF GUANINE NUCLEOTIDE-BINDING REGULATORY PROTEINS N_i AND N_s IN THE RECTAL GLAND OF THE SHARK *Squalus Acanthias*

Grant G. Kelley, William S. Curtis, and John N. Forrest, Jr., Department of Medicine, Yale University School of Medicine, New Haven, CT

Inhibition and stimulation of adenylate cyclase are now known to occur via two distinct guanine nucleotide-binding regulatory proteins: one (N_i) mediates inhibition and one (N_s) mediates stimulation. Both are heterotrimeric proteins composed of alpha, beta and gamma subunits. The beta and gamma subunits are identical by peptide mapping whereas the alpha subunits differ. The stimulatory alpha subunit is 45-50 kDa while the inhibitory alpha subunit is 39-41 kDa. Binding of hormone promotes dissociation of the N proteins into alpha and beta gamma subunits. For stimulation, the stimulatory alpha subunit directly activates adenylate cyclase. The mechanism for inhibition is less clear but may involve a combination of free beta gamma from N_i binding to free alpha from N_s (Gilman, J. Clin. Invest. 73, 1984) and a direct action of inhibitory alpha subunit on adenylate cyclase (Hildebrandt et al., J. Bio. Chem. 258, 1983).

Pertussis toxin (PT) and cholera toxin (CT) have been instrumental in purifying these regulatory units and determining their mechanism of action. Both are ADP-ribosyltransferases and require specific receptors to be translocated across membranes. CT ADP ribosylates the stimulatory alpha subunit which prevents reassociation with the beta gamma subunit leading to irreversible stimulation of adenylate cyclase. PT ADP ribosylates the inhibitory alpha subunit and stabilizes N_i in its inactive state and irreversibly blocks adenylate cyclase inhibition. PT has also been used in receptor binding studies to determine if certain receptors are coupled to N_i. PT uncouples receptors from N_i and this can be measured by a decrease in binding affinity.

The rectal gland, a model for cAMP-mediated chloride transport, has both stimulatory (adenosine, vasoactive intestine peptide [VIP], rectin) and inhibitory (adenosine, somatostatin) receptors suggesting that both N_i and N_s are present in this tissue. In the present studies, we establish the presence of these regulatory proteins, N_i and N_s, in the rectal gland and demonstrate that inhibitory (A_1) adenosine receptors are functionally coupled to N_i.

Rectal gland membranes were prepared and binding of ^3H NECA was performed as previously described (Poeschla et al., Bull. MDIBL 24, 1984). For the ADP-ribosylation of membranes, PT was preactivated by adding DTE (25 mM) and incubating for 30 min at 37°C. The ADP-ribosylation reaction was started with the addition of membranes to a reaction mixture containing 10 µg/ml PT, 25 mM Tris acetate pH 7.5, 1 mM ATP, 0.05 mM GTP, 10 mM thymidine, and 10 µM NAD, ^{32}P NAD 5000-10000 cpm/pmole cold NAD and incubated for 30 minutes at 30°C. The reaction was stopped by the addition of ice-cold 10% TCA and then centrifuged at 1000 G for 30 min. The supernant was discarded and the pellet was saved for SDS-PAGE. For the binding experiments, membranes were washed twice and incubated with PT in the above reaction mixture except that 1 mM NAD was added and ^{32}NAD was not included. The reaction was stopped with the addition of buffer, pelleted at 40000 G, and washed. A portion of these membranes was exposed to PT a second time as above to quantitate the amount of ADP-ribose incorporated during the first exposure.

Electrophoresis was carried out as per the procedure of Lamelli (1970). Radiolabelled samples were dissolved in sample buffer containing 1% SDS, 5% 2-mercaptoethanol, 10% glycerol, 50 mM tris-HCl pH 6.8, and 0.02% bromophenol blue, and heated for 3 minutes at 100°C. An aliquot (10 µg protein) was loaded onto a minislab 3½ x 4 inch discontinuous SDS polyacrylamide gel (separating gel 12.5% acrylamide, stacking gel 3% acrylamide) and electrophoresed at 20 mA constant current for approximately 60 minutes. Gels were stained with Coomassie Brilliant Blue R-250, 50% MeOH, and 10% AcOH for 10 min; destained with 30% MeOH and 7% AcOH for 1½ hours; and swelled in 10% glycerol for 10 min. Gels were dried overnight between nitrocellulose and subjected to autoradiography at -80°C using Kodak X-Omat film for 1-2 days. Amount of ^{32}P ADP-ribose incorporation was determined by scanning densitometry. Molecular weight standards used were phosphorylase a (94 kDa), bovine serum albumin (18 Kda), ovalbumin (45 kDa), carbonic anhydrase (32 kDa) and lactoglobin (18 kDa).

Rectal gland membranes were incubated with activated PT and CT and subjected to SDS-PAGE and autoradiography. PT labelled a 41 kd protein and CT labelled a 45 kDa and a 50 kDa proteins shown in Figure 1. These molecular weights correspond to the inhibitory alpha and stimulatory alpha subunits previously described and characterized by others.

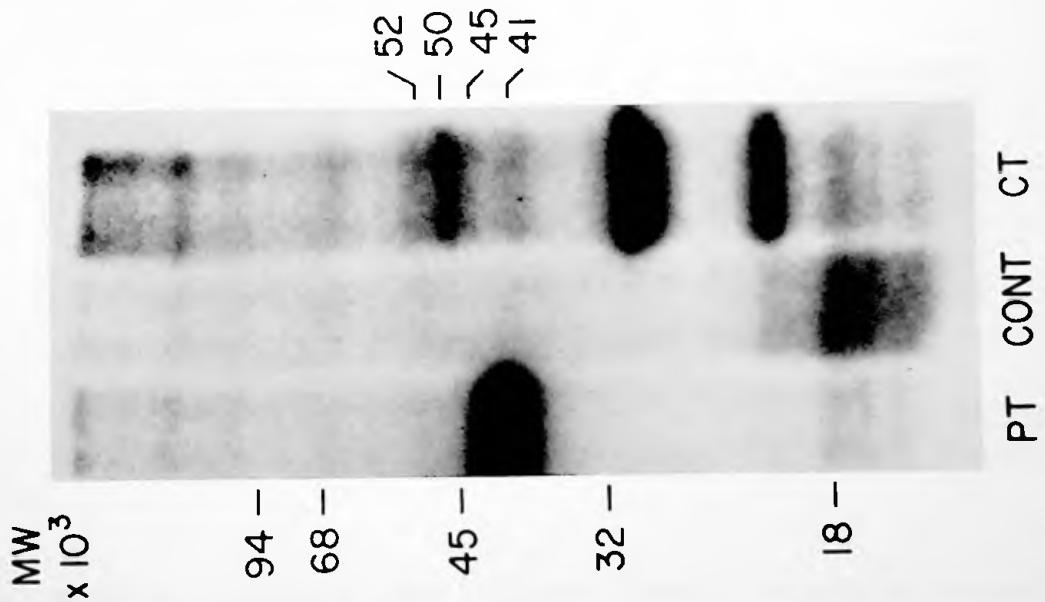

Figure 1: Autoradiograph of SDS-gel showing a 41 kDa protein labelled with ^{32}P ADP-ribose by PT and a 45 and 50 kDa proteins labelled by CT. C=control, PT=Pertussis toxin, CT=cholera toxin, PT media, CT media=plus magnesium and phosphate.

In separate experiments, we showed that CT and PT catalyzed ADP-ribosylation was dependent on the reaction media. CT catalyzed ADP-ribosylation required the presence of magnesium and phosphate while these reagents slightly decreased PT catalyzed ADP-ribosylation. Both ATP and GTP enhanced PT calalyzed ADP ribosylation in an additive manner. No ADP-ribosylation occurred at 0°C. The amount of ADP-ribose incorporated was dependent on the time, amount of toxin and the amount of protein present.

The effect of pretreatment of membranes with PT on ^{3}H NECA binding was determined. Figure 2 shows a typical experiment and correlates the amount of PT catalyzed ADP-ribose incorporation with specific binding of ^{3}H NECA. The autoradiograph in Figure 2 shows the amount of ^{32}P ADP ribose incorporated during a second exposure to PT. The amount of label incorporated is inversely proportional to the amount of cold ADP-ribose that was incorporated during the first exposure to PT. It is apparent that the amount of inhibitory alpha subunit ribosylated during the first exposure to PT increases with time to a maximum of approximately 90% of control membranes. Total binding of ^{3}H NECA decreased proportionally with the amount of alpha subunit ribosylated. The average inhibition was 32% (n=3 experiments, $p<0.01$, paired t). These results suggest that a subpopulation of adenosine receptors are functionally coupled to N_i.

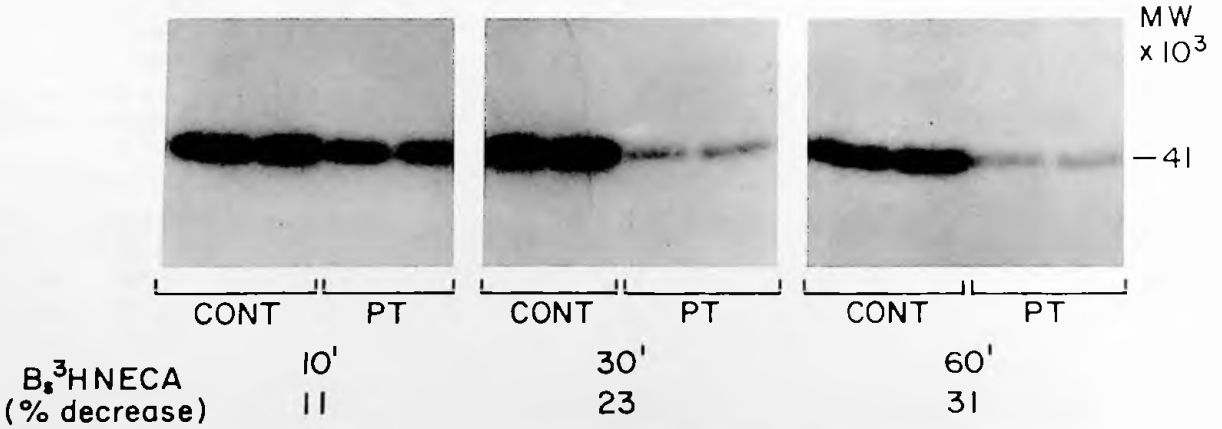

Figure 2: Autoradiograph of SDS-gel showing time dependent decrease in PT catalyzed ADP-ribosylation. Cont=control membranes, PT=membranes previously exposed to pertussis toxin.

In two sharks innoculated with PT at approximately 10 µg/kg and in two glands perfused with 0.1 µg/ml for 6 hours there was no difference in the amount of ^{32}P label incorporated compared to controls. This suggests that the rectal gland lacks the receptor necessary for PT recognition and translocation across to membrane.

In summary, we have determined that the rectal gland has a 41 kd protein labelled by PT and two proteins 45 and 50 kd labelled by CT which correspond to inhibitory alpha and stimulatory alpha subunits. Adenosine and 2 Chloroadenosine in high concentrations decrease PT catalyzed ADP-ribosylation. PT preincubation decreases total binding of ^{3}H NECA in a time dependent manner. The rectal gland probably lacks the PT recognition and translocation receptor. Taken together, these studies establish the presence of N_i proteins in the rectal gland that are functionally coupled to an inhibitory adenosine receptor.