cytochrome c oxidase was dependent on the extent of enzyme purification (Fig. 2). Cytochrome c oxidase that had been acid-precipitated twice (*13*) was significantly more sensitive to sulfide than, for example, the crude supernatant preparation. This finding suggested that the mechanisms for protecting respiration from sulfide poisoning might involve factors present in the blood or cytosol that render sulfide unable to inhibit cytochrome c oxidase.

To examine whether the relative insensitivity to sulfide of cytochrome c oxidase in crude supernatant preparations was due to a general protein binding of sulfide, we added bovine serum albumin (BSA) to the assay cuvette to a final BSA concentration of 100 mg/ml. No protection against sulfide poisoning occurred. When we tested the vascular blood from *R. pachyptila* for its protective effects, however, we found that blood concentrations as low as 0.45 mg of protein per milliliter in the cuvette could completely reverse the inhibitory effects of sulfide (Fig. 2). In contrast, whole blood from a freshly collected whitetip shark (*Carcharhinus longimanus*) had no disinhibitory effects. This indicates that the factors present in *R. pachyptila* blood are not ubiquitous blood constituents. The protective factors do not appear to be present in the cytosol of *R. pachyptila* tissues. Portions of homogenates of *R. pachyptila* vestimental muscle had no disinhibitory effects. Because vestimental muscle, unlike the plume, is very poorly vascularized, extremely little blood was present in the muscle homogenates in comparison with the plume homogenates, which were bright red in color. We conclude that blood-borne factors are responsible for protecting the respiration of *R. pachyptila* from sulfide poisoning.

Arp and Childress (*11*) provided strong evidence that these blood-borne factors are sulfide-binding proteins. These factors appear not to be previously characterized forms of modified hemoglobin, such as methemoglobin, which bind sulfide in other organisms (*14*). Nor are they enzymes capable of oxidizing sulfide to less toxic sulfur compounds. Although sulfide-oxidizing enzymes are present in the bacteria-containing trophosome of *R. pachyptila*, no capacities for sulfide oxidation were observed in plume or blood (*6, 7*). Thus, the sulfide-binding proteins in the blood of *R. pachyptila* may prevent sulfide poisoning of aerobic metabolism because these proteins have higher binding affinities for sulfide than do sulfide-sensitive enzymes like cytochrome c oxidase. These sulfide-binding proteins may have the dual role of sulfide transport (*11*) and protection against sulfide poisoning.

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Insulin Receptor: Evidence That It Is a Protein Kinase

Abstract. *Highly purified preparations of insulin receptor catalyzed the phosphorylation of the 95,000-dalton subunit of the insulin receptor. This subunit of the insulin receptor was also labeled with [α-^{32}P]8-azidoadenosine 5′-triphosphate, a photoaffinity label for adenosine triphosphate binding sites. The identity of the 95,000-dalton band was confirmed in both cases by precipitation with a monoclonal antibody to the insulin receptor. These results suggest that the insulin receptor is itself a protein kinase.*

Insulin binds to specific membrane receptors on the surface of target cells and then regulates a variety of metabolic processes (*1*). However, it is not known how the interaction of insulin with its receptor elicits changes in cellular functions (*1*). Since insulin can regulate the phosphorylation of several proteins (*2*), one possibility is that insulin exerts its effects via the stimulation of a protein kinase or inhibition of a protein phosphatase. Kasuga *et al.* (*3*) reported that the insulin receptor is itself phosphorylated in intact cells and that the extent of this phosphorylation is regulated by insulin. Recently, the phosphorylation of the insulin receptor was observed in solubilized rat liver plasma membranes and the phosphorylation of the receptor was shown to be on a tyrosine residue (*4*). These studies raise the possibility that the insulin receptor is itself a protein kinase. The present studies were designed to test this hypothesis by using a highly purified insulin receptor preparation and a photoaffinity label for proteins binding adenosine triphosphate (ATP).

Insulin receptors were purified from cultured lymphocytes (IM-9 cells) by using an affinity column containing a monoclonal antibody to the insulin receptor (*5*). Since the binding of this antibody to the receptor is competitive with that of insulin (*5*), the receptor could be specifically eluted from the column with 1 μ*M* insulin. The insulin receptor was then further purified on a wheat germ agglutinin column (*6*). To determine the purity of the insulin receptor preparations, the IM-9 cells were first cultured in the presence of [^{35}S]methionine for 16 hours to label all the proteins in the cell. The labeled receptor preparation could then be visualized by autoradiography of sodium dodecyl sulfate (SDS)–polyacrylamide gels. Under reducing conditions, the purified insulin receptor preparation exhibited two major bands (Fig. 1A) with apparent molecular weights of 135,000 (135K) and 95,000 (95K), values identical to those reported for the α and β subunits, respectively, of the insulin receptor (*1*). Densitometric scans of the autoradiographs showed that more than 90

percent of the labeled protein in the peparation was accounted for by these two bands. Two minor bands of molecular weights 200K and 210K which were also observed may be the precursor form of the insulin receptor that has recently been described by Hedo *et al.* (*7*).

The kinase activity of this purified insulin receptor preparation was then tested by the addition of 5 μM [γ-^{32}P]adenosine 5′-triphosphate. When the receptor preparation was subsequently analyzed on SDS gels, the 95K band was found to be labeled with the ^{32}P (Fig. 1B, lane a). This labeling was greatly increased by the addition of 2 mM $MnCl_2$ and was still linear after 30 minutes at 24°C (data not shown). The labeling of the 95K band was blocked by the addition of 10 mM 5′adenylylimidodiphosphate (AMP-PNP), an ATP analog that can bind to kinases and antagonize their action (Fig. 1B, lane b) (*8*). To verify that the phosphorylated 95K band was a real constituent of the insulin receptor and not a contaminating protein, we precipitated the phosphorylated receptor preparation with either a monoclonal antibody to the receptor or a control antibody and then analyzed the bound proteins by SDS gel electrophoresis. The 95K phosphoprotein was precipitated with only the monoclonal antibody to the insulin receptor (Fig. 1B, lanes c and e). Furthermore, the precipitation of the 95K phosphoprotein by the monoclonal antibody was blocked by the presence of 10 μM insulin (Fig. 1B, lane d).

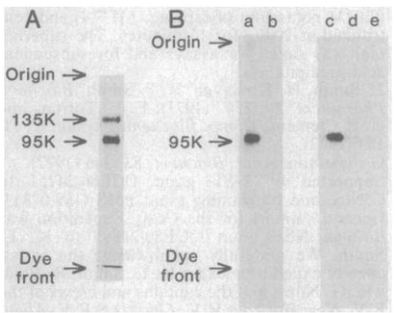

Fig. 1. (A) Autoradiogram showing the purity of the insulin receptor preparation. IM-9 lymphocytes (2×10^7 cells) were grown for 16 hours at 37°C in methionine-free RPMI-1640 medium with 10 percent dialyzed fetal calf serum and 2.5 mCi of [^{35}S]methionine. Cells were washed twice with unlabeled medium, lysed by addition of 5 ml of 1 percent Triton X-100 in 50 mM Hepes, pH 7.6, containing 1 mM phenylmethylsulfonyl fluoride (PMSF) and 100 units of bacitracin per milliliter, and were centrifuged at 100,000g for 1 hour. The supernatant was passed over an 8-ml column of monoclonal antibody to receptor coupled (*15*) to Affi-Gel 15 (2 mg of antibody per milliliter of gel). The column was first washed with 100 ml of buffer A (50 mM Hepes, pH 7.6, 150 mM NaCl, 0.1 percent Triton X-100, 1 mM PMSF, and 100 units of bacitracin per milliliter) and then with 20 ml of 1M NaCl. The insulin receptor was then eluted with 1 μM insulin in buffer A and further purified on a 5-ml column containing wheat germ agglutinin coupled to agarose (*6*). The receptor was then eluted off this column with 300 mM *N*-acetyl-D-glucosamine in buffer A. Twenty microliters of the purified insulin receptor preparation were electrophoresed on a 7.5 percent polyacrylamide SDS gel as described (*13*). After electrophoresis, the slab gel was stained, destained, treated with Enhance (New England Nuclear), and autoradiographed. (B) Autoradiogram showing the phosphorylation of the β subunit of the insulin receptor by purified insulin receptor. The purified insulin receptor was made 2 mM in $MnCl_2$, and then (a) 5 μM [γ-^{32}P]ATP or (b) 5 μM [γ-^{32}P]ATP plus 10 mM AMP-PNP was added and the reaction was allowed to proceed for 1 hour at 24°C. For the immunoprecipitation, the reaction mixture was incubated with (c) monoclonal antibody to the insulin receptor, (d) monoclonal antibody plus 1 μM insulin, or (e) normal immunoglobulin G for 14 hours at 4°C. The antibodies were precipitated by the addition of *Staphylococcus aureus*, coated with rabbit antiserum to mouse immunoglobulin, washed three times, and solubilized as described (*5*). The gels were processed as described above except that they were not treated with Enhance but instead the autoradiographs were developed with the use of Dupont Cronex Lightning-Plus intensifying screen to increase the detection of ^{32}P.

These results demonstrated that a protein kinase activity had copurified with the insulin receptor. The kinase, however, could still be a minor contaminating protein in the preparation. To test this possibility, we used [α-^{32}P]8-azidoadenosine 5′-triphosphate (*9*) as a photoaffinity ligand to identify the ATP binding proteins present in a partially purified receptor preparation. Although 8-azido-ATP can be utilized by the kinase, this does not result in the labeling of the protein substrate since the label is in the α position. Thus, as expected, no labeling of the 95K band was observed in the dark (data not shown). However, when a partially purified insulin receptor preparation (*10*) containing the [α-^{32}P]8-azido-ATP was irradiated with light at 360 nm to cross-link the reagent to ATP binding proteins, the 95K band was labeled. The amount of radioactivity associated with the 95K band increased with the length of time of exposure to light (Fig. 2A). When the cross-linked receptor preparation was precipitated, the 95K phosphoprotein was found to be bound by the monoclonal antibody to the insulin receptor but not by control antibody (Fig. 2B, lanes a and b). Furthermore, 10 mM AMP-PNP inhibited the labeling of the 95K band (Fig. 2B, lane c).

Two other bands of molecular weights 55K and 45K were also observed after cross-linking [α-^{32}P]-8-azido-ATP to the insulin receptor preparation. These bands did not appear to be adenosinetriphosphatases since very little ATP hydrolysis was observed during the phosphorylation reactions. Also, phosphoamino acid analyses of the phosphorylated β subunit revealed only phosphotyrosine. This last result indicates that the insulin receptor is a tyrosine-specific kinase and is in agreement with the finding of phosphotyrosine in the β subunit of the insulin receptor after phosphorylation by solubilized plasma membranes (*4*). Since tyrosine-specific kinases are relatively rare, these results make it unlikely that the 55K and 45K components are also kinases. Another possibility is that these two components are proteolytic fragments of the β subunit of the insulin receptor as has previously been described (*1*). This hypothesis was supported by the finding that both components were precipitated by antibody to the receptor (Fig. 2B, lane b). However, both bands also were precipitated by control antibody (Fig. 2B, lane a). Since a large amount of protein also electrophoresed in this area of the gel (the protein arose from albumin and immunoglobulin heavy chain), it is possible that these bands result from radioactivity trapped by these proteins.

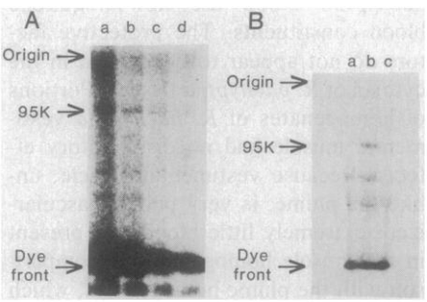

Fig. 2. (A) Autoradiogram showing the labeling of the β subunit of the insulin receptor with [α-^{32}P]8-azido-ATP. [α-^{32}P]8-azido-ATP (Schwarz/Mann, Inc., Spring Valley, New York) was dried, resuspended in one-tenth the volume of 50 mM Hepes, pH 7.6, and added to an insulin receptor preparation at a final concentration of 10 μM. The preparation at a distance of 8 cm was exposed to ultraviolet light with a peak wavelength of 364 nm (UVSL-25, Ultra Violet Products, Inc., San Gabriel, California) for (a) 40, (b) 20, (c) 10, or (d) 5 minutes. The reaction mixtures were made 1 percent in SDS and 2 percent in 2-mercaptoethanol, put in boiling water for 1 minute, and electrophoresed on 7.5 percent polyacrylamide gels as described above. (B) Immunoprecipitation of insulin receptor labeled with [α-^{32}P]8-azido-ATP. Insulin receptor preparation was incubated with 10 μM 8-azido-ATP for 30 minutes with ultraviolet light and then precipitated with (a) control antibody or (b) monoclonal antibody to the insulin receptor. (c) The receptor preparation was incubated with the [α-^{32}P]8-azido-ATP and 10 mM AMP-PNP for 30 minutes with ultraviolet light and then precipitated with the monoclonal antibody to the insulin receptor.

The present study demonstrates that a highly purified insulin receptor preparation retains kinase activity and that the β-subunit of the insulin receptor is phosphorylated by this kinase activity and has an ATP binding site. The results suggest, therefore, that the insulin receptor is itself a kinase as well as a substrate for phosphorylation. Prior studies have indicated that the α subunit of the insulin receptor (the 135K band) is predominantly labeled when [^{125}I]insulin is cross-linked to the receptor (*1*). These results, together with the data in the present study, suggest that the insulin receptor is composed of two subunits with separate functions: an α subunit that binds insulin and a β subunit with kinase activity.

The addition of insulin to either whole cells or solubilized plasma membranes increases the extent of phosphorylation of the β subunit of the insulin receptor (*3, 4*). The simplest explanation for these results would be for insulin to stimulate the kinase activity of the receptor which, in turn, phosphorylates itself as well as other proteins. These phosphorylations could then initiate a cascade of reactions resulting in the various effects of insulin. The increased kinase activity of the insulin receptor may not, however, be sufficient to induce all the effects of insulin on cells. In analogy, the epidermal growth factor (EGF) receptor has also been shown to be a protein kinase (*11*). Most interesting was the finding that although cyanogen bromide–cleaved EGF was capable of inducing the phosphorylation of the EGF receptor and some of the short-term effects of EGF, it was not capable of stimulating cell division (*12*). Thus, it remains to be determined whether the kinase activity of the insulin receptor is both necessary and sufficient to generate all of the multiple effects of insulin.

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Prostaglandins Mediate Inhibition of Gastric Acid Secretion by Somatostatin in the Rat

Abstract. *Somatostatin, a tetradecapeptide with potent inhibitory actions on gastric acid secretion, potentiated carbamylcholine-induced synthesis and release of prostaglandin E_2 from isolated perfused rat stomachs. The ability of somatostatin to inhibit acid secretion was blocked by indomethacin, an inhibitor of prostaglandin synthesis. These results suggest that prostaglandins mediate gastric acid inhibition by somatostatin in the rat.*

Somatostatin, a tetradecapeptide initially isolated from ovine hypothalamus, occurs in virtually all areas of the mammalian gut (*1*). One of its many actions is inhibition of gastric acid secretion (*1–3*). This action of somatostatin may be physiological since it has been observed with doses comparable to those released into plasma after meal ingestion (*3*). Since the acid-inhibitory activity is independent of the peptide's ability to inhibit release of the gastric secretagogue gastrin (*3*), somatostatin is thought to inhibit gastric parietal cells directly. However, attempts to demonstrate a direct inhibitory action of somatostatin on isolated parietal cell preparations have met with little success (*4*). Because somatostatin inhibits adenosine 3′,5′-monophosphate (cyclic AMP) production in gastric glands (*5*) and because prostaglandins are thought to inhibit gastric acid secretion by inhibiting parietal cell adenylate cyclase (*6*), we examined the possibility that somatostatin may inhibit acid secretion by promoting the synthesis and release of prostaglandins.

Isolated stomachs from anesthetized male Sprague-Dawley rats (150 to 250 g) were perfused through the aortic remnant with Krebs-Ringer buffer containing 0.2 percent human serum albumin and 3 percent dextran T-40 (Pharmacia Fine Chemicals) as described (*7*). The perfusate was oxygenated with 95 percent O_2 and 5 percent CO_2 throughout the experiments and the temperature was constantly monitored and maintained at 37°C. Portal vein effluent was collected in ice-chilled containers at 2-minute intervals for subsequent measurement of prostaglandin E_2 by radioimmunoassay (*8*). As shown in Fig. 1A, the procedures used for perfusion were as follows: after a 30-minute stabilization period a 10-minute baseline was obtained, then 10-minute pulses of stimulants were administered followed in each instance by a 10-minute washout with unmodified perfusate.

Initial studies indicated that somatostatin (somatostatin-14, Peninsula Laboratories) by itself had no ability to stimulate prostaglandin release (Fig. 1B). Because the preparation was extrinsically denervated and because of preliminary evidence that cholinergic agonists stimulate prostaglandin synthesis, we reasoned that resting vagal tone might be required for somatostatin to influence prostaglandin production and release. When we applied increasing concentrations of somatostatin (10^{-10} to 10^{-8} *M*) to our preparations, we observed a dose-dependent potentiation of the effect of carbamylcholine (10^{-6} *M*) to release prostaglandins (Fig. 1, A and B). This biphasic stimulatory effect was inhibited completely by atropine (10^{-7} *M*), indicating that muscarinic receptors must be activated for somatostatin to act on prostaglandin production. Inhibition of pros-