Activation of SH2-Containing Proteins by Insulin in Proliferating Mouse Parotid Gland Acinar Cells (43822)

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Abstract. **Chronic treatment of mice with insulin results in hypertrophy and hyperplasia of the parotid and submandibular glands (Wang *et al.*: 1994, Proc Soc Exp Biol Med 205:353–361). Hyperplasia of the parotid gland is mediated by the elevation of tyrosine phosphorylation of phospholipase Cγ, p21^{ras}-GTPase activating protein (p21^{ras}-GAP) and phosphatidylinositol 3-kinase. These proteins were found to be associated with the insulin receptor substrate-1 most likely through src homology (SH2) domains of these proteins. There was also a transient increase in intracellular cAMP and protein kinase A during the first day of treatment which declined by Day 3 to near control values. Protein kinase C activity, on the other hand, remained elevated for the 3-day injection regimen. Thus, acinar cell proliferation induced by insulin requires activation of many of the same signaling components as other tyrosine kinase possessing growth factor receptors.** [P.S.E.B.M. 1994, Vol 207]

The hormone insulin is responsible for the control of blood glucose levels in mammals. Insulin's actions are mediated by its ability to stimulate glucose uptake and metabolism primarily in muscle cells and adipocytes (1). In general, insulin affects the physiology and biochemical activities or expression of a variety of enzymes in most cell types (2). The action of insulin is regulated by the specific interaction of the hormone with its receptor (2, 3). The insulin receptor is a transmembrane glycoprotein of 185 kDa containing an intrinsic tyrosine kinase activity (3). Intracellular responses toward insulin are thus dependent upon serum levels of the hormone and the subsequent ability of the bound receptor to stimulate tyrosine phosphorylation on itself and substrates such as the insulin receptor substrate-1 (IRS-1) (2, 3).

Ligand-bound receptors in the plasma membrane regulate cellular processes through activation of cytoplasmic domains containing intrinsic tyrosine kinase activity. Not only the insulin receptor but the receptors for other growth factors, such as members of the epidermal growth factor-like (EGF-like) growth factor family and platelet-derived growth factor (PDGF), function in this fashion (2–5). Subsequent to receptor autophosphorylation, a number of cytoplasmic proteins associate with the membrane-bound receptors through src homology 2 (SH2) domains to propagate second messenger signaling (3, 4, 6). For EGF or PDGF receptors, intracellular signal propagation by the tyrosine kinase second messenger signaling pathway involves the association and activation of phosphatidylinositol (PI) 3-kinase, p21^{ras}-GTPase-activating protein (p21^{ras}-GAP), phospholipase Cγ (PLCγ), GRB-2, and Son of Sevenless (SOS) (6–8). GRB-2, a small SH2 containing peptide, links tyrosine kinase activated receptors to mitogen-activated protein kinase (MAPK) and cell proliferation by mediating the activity of p21^{ras} (9). A stable complex is formed between GRB-2 and the ras-specific nucleotide exchange factor, SOS, through the GRB-2 SH3 domain (9).

By contrast, the activation of SH2-containing proteins by the insulin receptor, as well as insulin-like growth factor 1, takes place through the tyrosine phos-

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phorylation of IRS-1, which in turn is capable of binding the SH2 domains of many of the signal transduction proteins listed above (3).

A previous report from our laboratory presented evidence to suggest that chronic high doses of insulin injected into mice caused a transient increase in parotid and submandibular gland hypertrophy and hyperplasia (10). Along with these observations were reported decreased levels of secretory proteins in saliva while saliva volume remained unchanged. In the present investigation, we have examined the activation of the signaling components of both the cAMP-dependent secretory response and the recruitment and phosphorylation of SH2-containing proteins by IRS-1 and the insulin receptor following the injection of high doses of insulin reported earlier.

Materials and Methods

Materials. Male BALB/c mice, 6 months of age, were purchased from the breeding colony of the Department of Pathology, University of Florida. All animals weighed between 15 and 30 g. Insulin, dl-isoproterenol, and propranolol were obtained from Sigma Chemical Co. (St. Louis, MO). Chemicals for SDS-polyacrylamide gels and prestained molecular weight standards were from Bio-Rad (Richmond, CA). Monoclonal antibody to phosphotyrosine, radiolabeled [^{125}I]protein A and the cAMP assay kit were purchased from Amersham (Arlington Heights, IL). The Pep-Tag assay kit for protein kinase A measurements were obtained from Promega (Madison, WI). The reagents for the assay of protein kinase C levels were obtained from GIBCO (Grand Island, NY). Antibodies monospecific for the insulin receptor IRS-1, phospholipase Cγ, p21^{ras}-GAP, and the PI3-kinase were purchased from UBI (Lake Placid, NY). All other reagents were purchased from commercial sources and were of ultrapure quality.

Animal Preparation and Plasma Membrane Isolation. Separate groups of animals (n = 6) were given twice daily intramuscular injections of 0.1 ml containing saline or insulin (50 μM/animal) for 15 min, 1 hr, 24 hr, and 72 hr (10). The animals were sacrificed by cervical dislocation 15 min following the last injection of insulin. The parotid gland was identified by gross morphology, quickly trimmed of connective tissue and lymph nodes, and dispersed in 2.0 ml Tris buffer, pH 8.0, containing 1.0 μM leupeptin, 4 μg/ml phenylmethylsulfonyl fluoride (PMSF), 1 mM Na-orthovanadate and 10 μM Na-pyrophosphate (11). The cells were disrupted by mechanical tissue dispersion followed by lysis using Dounce homogenization. Total membrane separation from cytoplasm was accomplished by centrifugation at 100,000g for 30 min at 4°C. Plasma membranes were isolated by previously reported protocols using sucrose density gradients (12). The protein content of all samples was determined by the method of Bradford (13) using bovine serum albumin (BSA) as a standard.

Immunoprecipitation of PLCγ, PI-3 Kinase, and p21^{ras}-GAP from Parotid Glands. The procedure used for isolation of total soluble cellular material is a modification of the procedure used for isolation of solubilized membrane proteins from plasma membrane preparations (12). Total cell lysates were solubilized in 50 mM HEPES, pH 7.8, containing 100 μM NaF, 10 μM Na-pyrophosphate, 1% Triton X100, 2.0 mM Na-orthovanadate, 4 μg/ml PMSF, and 2.5 mM EDTA (12). The insoluble material was removed by ultracentrifugation at 100,000g for 60 min at 4°C. The resulting supernatant (500 μg protein) was incubated with 10 μg of the antibody specific for insulin receptor or IRS-1 and 20 μg protein A-agarose at 4°C for 18 hr. The immune precipitated complex was collected by centrifugation for 15 min at 2000g. The pellet was washed three times with the above buffer, followed by resuspension in sodium dodecylsulfate (SDS)-polyacrylamide gel sample buffer containing 10 μM dithiothreitol and boiling for 5 min prior to application in polyacrylamide gels for separation of proteins.

Polyacrylamide Gel Electrophoresis and Western Blot Analysis. Immunoprecipitated material was separated on a 1.5-mm thick 8% polyacrylamide gel using the SDS-gel system described by Pugsley and Schnaitman (14). Following electrophoretic separation, the proteins were transferred to nitrocellulose at 17 V for 12 hr (15). The filters were blocked for 1 hr in phosphate buffered saline (PBS) containing 3% nonfat dry milk and 3% BSA. The nitrocellulose blots were incubated at room temperature for 12–15 hr in the same buffer containing a 1:500 dilution of antibody to p21^{ras}-GAP, PI3-kinase, PLCγ, or phosphotyrosine. Following three washings with PBS containing 0.1% Tween-20 to reduce background, the filters were incubated for 1 hr with [^{125}I]-protein A, washed as above and exposed to Kodak XAR-5 film for 72 hr at −80°C. Immunoprecipitation and immunoblotting were repeated on three separate occasions using pooled lysates to verify the consistency of the results.

Determination of cAMP Levels. Parotid gland lysates for each experimental animal were prepared as above using 5% trichloroacetic acid (TCA) in PBS as homogenization buffer. Lysis in TCA results in deproteination and allows for the clarified supernatant to be used as the unknown sample in the quantitation of cAMP as previously detailed (16). The level of cAMP is determined by the competition between the unlabeled cAMP in the sample and a fixed quantity of radiolabeled cAMP for binding to a high-affinity binding protein (17). The concentration of cAMP was determined relative to a standard curve generated from the test kit standards (Amersham). Concentrations of

cAMP were expressed as mean pmole cAMP/mg protein ± standard error (SE).

Determination of Protein Kinase A Activity. Cyclic AMP-dependent protein kinase A (PKA) was measured in acinar cell lysates using the Pep-Tag nonradioactive detection system (16). In brief, the Pep-Tag assay utilizes a colored fluorescent peptide substrate with the amino acid sequence L-R-R-A-S-L-G. Phosphorylation of the substrate by PKA in the sample alters the charge, thereby distinguishing the phosphorylated form from the nonphosphorylated version of the substrate on an agarose gel. The reaction mixture in 25 μl contained the reaction buffer, PKA activator, substrate, and sample. Incubation was carried out for 30 min at 30°C and terminated by boiling in a water bath for 10 min. The samples were separated on a 0.8% agarose gel at 90 V for 1 hr. The negatively charged phosphorylated peptide was visualized on a UV light box and cut from the gel and the product was extracted. The fluorescence associated with the band was measured in a spectrofluorimeter (Perkin-Elmer) with excitation at 570 nm and emission at 593 nm. Protein kinase A activity was expressed as pmole phosphate transferred to the peptide substrate/min/mg protein ± SE.

Determination of Protein Kinase C Activity. The activity of protein kinase C (PKC) in detergent solubilized parotid cell lysates was obtained through the protocol of the GIBCO/BRL assay system (18). The assay is based on the phosphorylation of a peptide from myelin basic protein which is capable of serving as a substrate for PKC. The specificity of the reaction was confirmed by the inclusion of a pseudosubstrate inhibitor peptide. Total cell lysates were prepared by homogenization of the parotid gland in 2 m*M* Tris buffer, pH 7.5, containing 0.5 m*M* EDTA, 0.5 m*M* EGTA, 0.5% Triton X100, 0.5-m*M* PMSF, and 10 μg/ml aprotinin followed by centrifugation at 100,000*g* at 4°C for 30 min to remove detergent-insoluble material. Reaction mixtures in a final volume of 50 μl contained 10 m*M* β-mercaptoethanol, 0.2 *M* NaCl, 5 μl lipid, and 5 μl cell lysate. Separate tubes containing all the above reagents except lipid but containing 10 μl of pseudosubstrate inhibitor were included as controls. The reactions were initiated by the addition of [^{32}P-γ] ATP and substrate peptide, and incubation continued for 5 min at 30°C. The samples were spotted onto phosphocellulose disks and washed with 1% phosphoric acid, and the radioactivity associated with the disk was quantified by liquid scintillation counting. All values of enzyme activity are expressed as pmoles phosphate transferred/min/mg of protein ± SE.

Statistical Analysis. The Bonferroni paired comparisons test was used to determine significant differences among means after a significant analysis of variance (ANOVA) test. $P < 0.05$ was considered to be a significant difference. All values are expressed as mean ± standard error.

Results

Insulin is capable of stimulating cellular responses through interaction with the insulin receptor, which possesses intrinsic tyrosine kinase activity (1–3). Evaluation of parotid gland total cell lysates from animals treated with high doses of insulin show obvious changes in the phosphotyrosine containing proteins (Fig. 1). There were noticeable increases in the phosphorylation beginning at 1 hr after the first intramuscular injection of bovine insulin in the region of the gel at 85, 120, and 140 kDa. Decreased tyrosine phosphorylation appeared in protein above 150 kDa at times subsequent to 1 hr, although it is difficult to resolve whether there are several proteins which are comigrating. By densitometer analysis, there was an increase detected in the phosphorylation at 185 kDa, which presumably includes the insulin receptors (2, 3), during the first 15 min of drug treatment.

The increase in phosphorylation of proteins in the region of 80–150 kDa was further analyzed by immunoprecipitation to evaluate their potential as members of the proteins previously identified as signal transduction substrates for kinase activity following stimulation of the insulin receptor (for review, see Ref. 3). Detergent soluble cell lysates were immunoprecipitated with antibody monospecific to IRS-1. Subsequent to transfer to nitrocellulose the immunoprecipitated material was reacted with antibody to PLCγ,

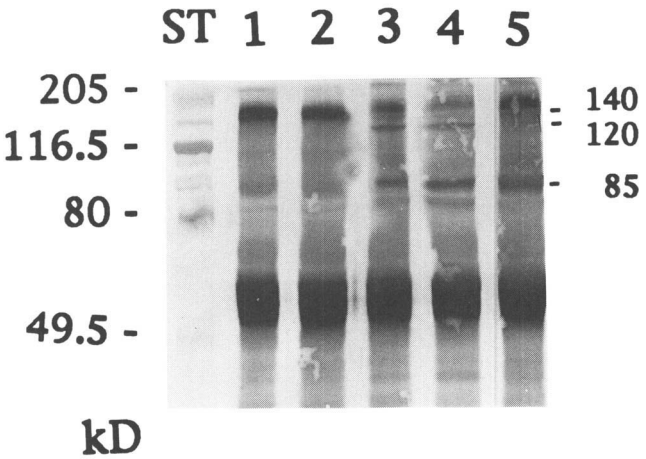

Figure 1. Time course in the detection of phosphotyrosyl-containing proteins in mouse parotid gland lysates following administration of insulin. Thirty-five micrograms of protein were transferred to nitrocellulose and probed with a monospecific antibody to phosphotyrosine. Lane 1, control, saline-injected animals; Lane 2, cell lysates from animals injected with insulin 15 min prior to sacrifice; Lane 3, cell lysates 1 hr after insulin injection; Lane 4, cell lysates 24 hr after insulin injections; Lane 5, cell lysates after 72 hr of chronic insulin treatment. Prestained molecular weight standards are myosin, 205,000 kDa; β-galactosidase, 116,500 kDa; bovine serum albumin, 80,000 kDa; ovalbumin, 49,500 kDa.

p21[ras]-GAP, and PI3-kinase. As shown in Figure 2, there was an increase in the association of all three enzymes with IRS-1 following insulin treatment. This increase appeared to begin by 15 min after the initiation of insulin treatment and continued for 24 hr. By Day 3 of chronic treatment, there was a decrease in PLCγ and 21[ras]-GAP, while PI3-kinase levels in the immunoprecipitation remained constant (Table I). The PI3-kinase consists of two subunits of 110 and 85 kDa, of which the antibody specifically recognizes the phosphorylated p85 subunit (9). For both PLCγ and GAP, a doublet appears on the nitrocellulose blots. Using rat salivary tissue, we have previously reported only a single protein species for both proteins (11, 12). The appearance of these doublets has been consistently observed in the mouse salivary gland lysates using antibodies to GAP and PLCγ. In the case of GAP (Fig. 2, Panel B), it is possible that the lower molecular weight species represents a degradation product, although we have no explanation for the minor higher molecular weight species observed in Panel A for the PLCγ immunoblot.

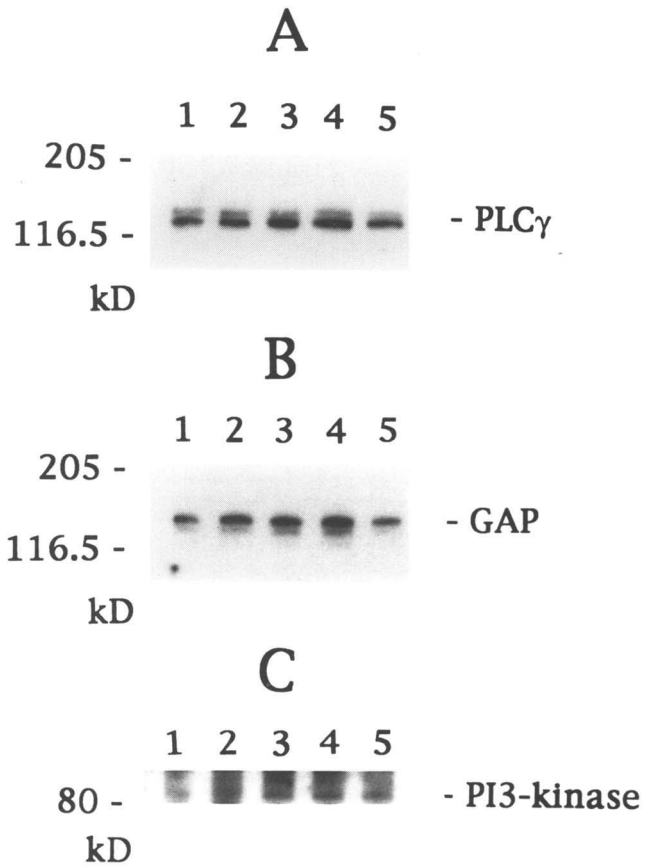

Figure 2. Autoradiograph of the distribution of PLCγ (Panel A), p21[ras]-GAP (Panel B), and PI3-kinase (Panel C) associated with IRS-1 following the same time course of insulin treatment as in Figure 1. Immunoprecipitation of the complex was accomplished with a polyclonal antibody monospecific for IRS-1 as described in Materials and Methods, using 50 μg of protein in each sample.

Table I. Densitometer Values* for PLCγ-, PI3-kinase-, or GAP Associated Acinar Cell Membranes Following Western Blot Analysis[a]

Treatment/blot	Time				
	CT	15 min	1 hr	1 day	3 day
PLCγ	1	1.2[e]	1.5[c]	2.0[c]	1.8[c]
GAP	1	1.5[e]	1.5[c]	1.8[c]	1.3[e]
PI3-kinase	1	2.0[c]	2.0[c]	2.0[c]	2.0[c]
PI3-kinase[b]	1	3.0[d]	3.0[d]	3.0[d]	2.5[c]

[a] Densitometer analysis (Hoeffer GS300 gel scanner) was performed on determinations of PLCγ, GAP, or PI3-kinase.
[b] Following immunoprecipitation using antibody to anti-insulin receptor and subsequent detection by Western blotting.
[c] $P < 0.05$.
[d] $P < 0.01$.
[e] Not significant, $P > 0.05$.

It has been reported that the PI3-kinase can be directly immunoprecipitated with antibody to the insulin receptor (19). Therefore, using purified plasma membrane fractions from insulin-stimulated parotid glands, we attempted to detect the coprecipitation of the PI3-kinase in receptor complexes. Figure 3 presents the results of this experiment. As can be seen, immunoprecipitation of the PI3-kinase takes place with the antibody to the insulin receptor. Densitometer analysis revealed a 3-fold increase ($P < 0.01$) in kinase associated with the receptor from 15 min to 24 hr after insulin treatment (Table I). Only by Day 3 was there a detectable decline in PI3-kinase found in the receptor complex, which was still significantly greater than control levels ($P < 0.05$).

The activation of PLCγ typically increases the formation of diacylglycerol and inositol 3,4,5-trisphosphate (IP_3) in growth factor stimulated cells (20, 21). Diacylglycerol causes an increase in the activity of PKC, while IP_3 causes the release of intracellular calcium (21). Subsequent to the injection of insulin into mice, there was a trend towards increased PKC activity in parotid gland acinar cells (Fig. 4). By 1 hr following the initial hormone treatment, there was a statistically significant change in kinase activity over control levels ($P < 0.02$; Fig. 4). The maximum increase was achieved 24 hr after initiation of the drug regimen which then declined on Day 3 of treatment. This decline did not, however, result in a return to control values (i.e., 13.2 pmoles phosphate transferred/min/

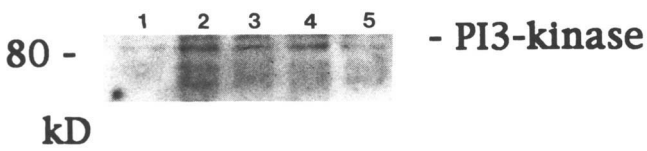

Figure 3. Autoradiograph of PI3-kinase associated with the insulin receptor in plasma membrane preparations following the insulin injection time course *in vivo*. Immunoprecipitation was performed with an antibody monospecific for the insulin receptor.

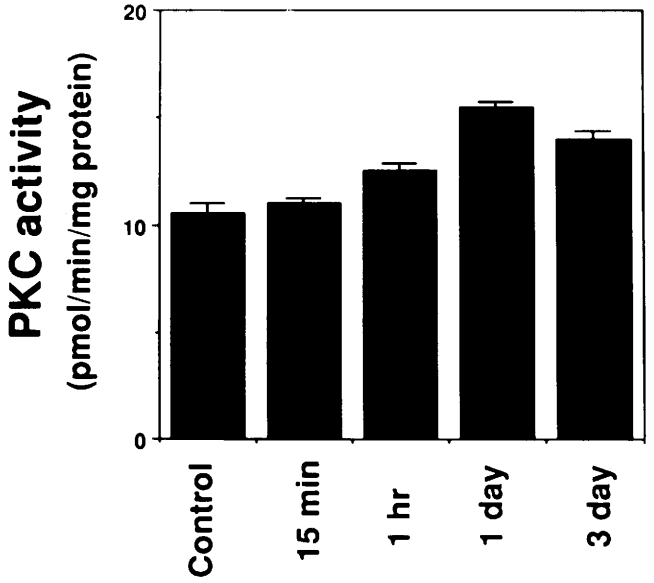

Figure 4. Histogram of Protein Kinase C activity following the time course of insulin treatment of BALB/c mice. All values are expressed as mean pmoles phosphate transferred to substrate/min/mg protein ± SE. Each value was determined from two separate preparations of $n = 6$ animals per time point. The assays were performed in duplicate on three separate occasions.

mg protein vs 10.8 pmoles phosphate transferred/min/mg protein, respectively).

In rat acinar cells treated with EGF, we have previously observed the activation of adenylate cyclase through a β-adrenergic receptor independent mechanism (12). Thus, it was of interest to examine whether increased cAMP in salivary glands was a general phenomenon of growth factor application. Protein kinase A activity, the down-stream substrate of the cAMP second messenger pathway, is dependent on the levels of cAMP in the cell to modulate its actions. In detailing cAMP levels, we observed a significant increase in cAMP levels with the time of the drug regimen that peaked at 24 hr (Fig. 5A). Again, the level of cAMP declined at 72 hr of treatment, as had been observed for the PKC activity shown above.

The level of a second serine/threonine kinase, namely PKA, was examined following a single or multiple injection of insulin. As indicated in Figure 5B, there was a significant increase in PKA activity 15 min after the initial treatment with insulin ($P < 0.05$). PKA activity increased in the acinar cell lysates until 24 hr. By 72 hr, PKA levels had fallen to a third of their peak level. This level of activity at 3 days was 2-fold higher than the basal unstimulated levels of kinase activity assayed in control cells ($P < 0.05$).

Discussion

Signal transduction by growth factors has been shown to involve receptor autophosphorylation and changes in the cytosolic localization of signal transduction components mediated by SH2 domains (4, 5).

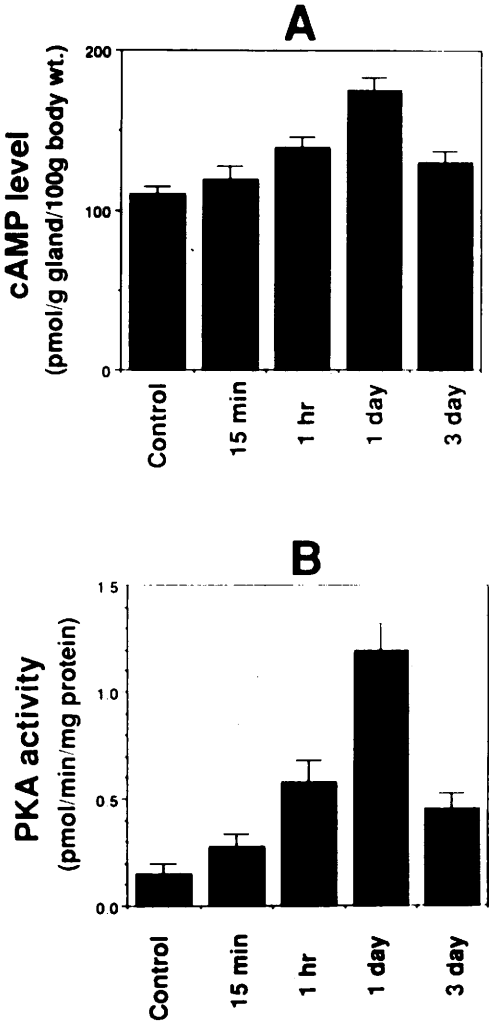

Figure 5. Histogram of time course changes in the level of cAMP (Panel A) and protein kinase A activity (Panel B) following insulin injection into BALB/c mice. Values for cAMP concentration are expressed as pmoles cAMP/g parotid gland/100 g body wt ± SE. For PKA, all values are expressed as pmoles phosphate transferred to substrate/min/mg protein. Each assay was performed on two separate occasions in duplicate with $n = 6$ animals per time point.

For EGF and PDGF, signal transduction components such as PLCγ, $p21^{ras}$-GAP, GRB-2, and PI3-kinase bind directly to sites of tyrosine phosphorylation on the receptor (2, 6). In the case of insulin, the receptor, although containing intrinsic tyrosine kinase activity, mediates the tyrosine second messenger signaling cascade through a protein intermediate IRS-1 (3). As presented here for parotid gland acinar cells, and by others for different cell types (2, 3, 9), the signaling intermediates consisting of PLCγ, $p21^{ras}$-GAP, and PI3-kinase bind to the IRS-1 peptide upon stimulation with insulin. The changes in cytosolic distribution of these proteins begin within 15 min after the initial injection *in vivo*.

Uncontrolled changes in cellular tyrosine kinase activity have been implicated in the aberrant cell growth of transformed cells (6, 7). Insulin treatment

has been shown to lead to the activation of signal transduction components distal to $p21^{ras}$, SOS, and GAP (9, 22). The activation of PLCγ leads directly to an increase in the activity of downstream components of phosphatidylinositol metabolism, namely the alteration in PKC enzyme activity. The activity of PKC has been shown to down-regulate the intrinsic tyrosine kinase activity of the insulin receptor by phosphorylation of serine/threonine residues (23) and may thus play a role in controlling acinar cell response to chronic insulin by 72 hr of treatment (10).

Immunoprecipitation of the insulin receptor has indicated that, at least in some cells, PI3-kinase may associate directly with the receptor (19). In acinar cells, while the majority of PI3-kinase was immunoprecipitated with the IRS-1 complex, a fraction of kinase protein was coprecipitated with the activated insulin receptor. The mechanism by which the PI3-kinase is detected in association with the insulin receptor is not clear. Since we did not detect any of the kinase associated with the unstimulated receptor, it is possible that autophosphorylation of receptor tyrosine residues creates a conformation change which is recognized by the SH2 domain of PI3-kinase.

In acinar cells, insulin also had a positive effect on intracellular concentration of cAMP and the activity of the cAMP-dependent protein kinase A. Our laboratory and others (24, 25) have been able to show that, in at least some cell types, adenylate cyclase can be activated by EGF treatment through a β-adrenergic receptor independent mechanism involving signaling pathway crosstalk (24). The parallel increases in cAMP and PKA suggest that for insulin action as well there may be an increase in the cAMP second messenger signaling pathway by a β-adrenergic receptor independent mechanism, since pretreatment of animals with the antagonist propranolol failed to block insulin-associated changes in cAMP levels (data not shown). Insulin has been shown to effect cAMP-dependent phosphorylation of cellular proteins and thus alter enzyme activity (1). In rat adipocytes, adenylate cyclase activity appears to be modulated by a phosphoprotein phosphatase activity regulated by insulin (26). In other cell types, insulin has been implicated in controlling specific dephosphorylation of phosphatases through the activation of PKA activity (27). The increase in intracellular cAMP in response to insulin in acinar cells was not as dramatic as that observed for β-adrenergic agonist stimulation but similar to that observed for rat parotid glands treated with EGF (24). In addition, by 72 hr following chronic administration of insulin, both cAMP levels and PKA activity were observed to decrease. Cyclic AMP and PKA play a role in secretion of protein into saliva (28). The previous observation of decreased salivary protein content in saliva following chronic insulin (10) may therefore be related to the down regulation of cAMP accumulation and subsequent PKA activity with continued use of the drug regimen.

In conclusion, the present investigation has shown that chronic insulin treatment of mice activates components of the tyrosine kinase signaling pathway in parotid gland acinar cells. The specific association of these proteins with membrane tyrosine kinases leads to increased enzymatic activity through subsequent tyrosine phosphorylation. Activating these proteins has been shown to be involved in regulating signal transduction leading to cell proliferation. Thus, the observed acinar cell growth in response to high doses of insulin *in vivo* suggests that the proliferation of cells in the parotid gland is mediated by the tyrosine phosphorylation cascade.

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