Vol. 179, No. 1, 1991 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
August 30, 1991 Pages 176-182

VASOACTIVE INTESTINAL PEPTIDE RECEPTORS ON AR42J RAT PANCREATIC ACINAR CELLS

Matthew J. Raymond[§] and Steven A. Rosenzweig[§¶‡*]

Departments of [§]Ophthalmology & Visual Science and [¶]Cell Biology
Yale University School of Medicine, New Haven, CT 06510

[‡]Department of Cell and Molecular Pharmacology & Experimental Therapeutics
Medical University of South Carolina, Charleston, SC 29425

Received July 10, 1991

VIP receptors on AR42J rat pancreatic cells were analyzed by competition binding, affinity labeling and by *N*-glycanase digestion analyses. These studies revealed the presence of specific, high affinity (K_d ~1 nM) VIP receptors with a mass of 67 kDa or 59 kDa under reducing or non-reducing conditions, respectively. *N*-glycanase digestion of affinity labeled membranes generated a core receptor protein of ~44 kDa and evidence for at least two *N*-linked glycans on the mature receptor. The receptor lacked *O*-linked oligosaccharides but contained terminal sialic acid residues on its *N*-linked glycan(s) based on digestions with *O*-glycanase and neuraminidase. The similarity of the AR42J VIP receptor to the recently cloned cDNA for human VIP receptors makes this cell line an attractive model for further analysis of VIP receptor signal transduction events.

Vasoactive intestinal peptide (VIP) is a 28 residue neuropeptide having a wide range of effects in diverse tissues. VIP's physiologic effects include: stimulation of pancreatic and intestinal fluid secretion, relaxation of intestinal, bronchial, cerebrovascular, and splanchnic vascular smooth muscle, and stimulation of anterior pituitary cell secretion (1). In the majority of these cell systems, VIP binds to a cell surface receptor thereby activating adenylate cyclase with a resultant increase in cellular cAMP (2-4). Calcium may also play a role in VIP induced signal transduction as calcium-dependent changes have been detected in parotid cells and cerebral cortical membranes upon VIP stimulation (5, 6).

The VIP receptor from a number of tissues has been characterized, including rat liver membranes (7-9), rat intestinal epithelial membranes (10), rat pancreatic acini (11), and rat and bovine retina (9). Affinity crosslinking of VIP receptors on rat liver membranes (7) and guinea pig pancreatic acinar cell membranes (12) revealed species of 80 kDa and 56 kDa (liver) and of 45 kDa and 30 kDa (pancreas), respectively. More recently (13), a 66 kDa protein was affinity labeled on rat and guinea pig pancreatic membranes as were minor components of 80 kDa (rat) and 83 kDa (guinea pig). In contrast, affinity labeling of intact acini labeled components of 80 kDa in the rat and 160 kDa in guinea pig. This variability in size is typical of the VIP receptor crosslinking literature both in different tissues and between species (7-10, 12, 14, 15).

*To whom correspondence should be addressed.

0006-291X/91 $1.50

The AR42J rat pancreatic acinar cell line, originally derived from azaserine-induced hyperplastic nodules of the rat pancreas (16), expresses cholecystokinin (17), somatostatin, (18, 19), insulin (20) and VIP (21) receptors. In the present paper we have extended the analysis of VIP receptors on AR42J cells to include an examination of their glycoprotein nature. The high density of VIP receptors on this cell line and their similarity to VIP receptors on rat pancreatic acinar cells and the structure of human VIP receptors based on their deduced cDNA sequence (22) suggest that this cell line will be useful for analysis of VIP receptor signal transduction events.

Materials and Methods

Reagents. VIP, secretin and glucagon were purchased from Bachem (Philadelphia, PA). $Na^{125}I$ was from Amersham (Arlington Heights, IL). Aprotinin, bacitracin, dexamethasone, leupeptin, *N*-acetyl D-glucosamine (GlcNAc), neuraminidase type X, phenyl methyl sulfonyl fluoride (PMSF) were from Sigma (St. Louis, MO). Proteins for molecular weight standards were obtained from Bio-Rad Laboratories (Richmond, CA). Soybean trypsin inhibitor was purchased from Worthington Diagnostics (Freehold, NJ). Recombinant *N*-glycanase and *O*-Glycanase were obtained from Genzyme (Boston, MA). Cross-linking reagents were from Pierce Chemical Co. (Rockford, NJ). All other reagents were of reagent grade.

Cell Culture. AR42J Cells (ATCC, Rockville, MD) were maintained as continuous subconfluent cultures in DMEM containing Penicillin, Streptomycin, and 10% fetal calf serum. Cells were plated at 5 x 10^5 cells/ml in 75 cm^2 or 150 cm^2 flasks and grown in 5% CO_2 atmosphere at 37° C (23). After five days they were fed media +/– 10 nM dexamethasone and allowed to grow for 48 hours.

Radioiodination of VIP. VIP was iodinated and purified as previously described (24, 9). Fractions were collected into 250 µl of HMS (25 mM HEPES, pH 7.4, 5 mM $MgCl_2$ and 104 mM NaCl) containing 0.2% gelatin and were scanned for radioactive content. Peak fractions were pooled and the volume was reduced *in vacuo*. The specific activity of the peptide was ≥ 2000 Ci/mmol, based on the specific activity of the ^{125}I.

AR42J Cell Membrane Preparation. Cells from subconfluent flasks were mechanically removed, washed in PBS containing 1 mM EDTA and harvested in HMS containing 0.1% STI, 1 mM PMSF, 1 mM bacitracin, 0.2 µM aprotinin, 5 µM leupeptin and 5 mM EDTA. Following centrifugation, the cells were resuspended in 25 mM HEPES, pH 7.4, containing the above indicated protease inhibitors. The cells were incubated for five minutes at 4°C, passed through a 25 gauge needle ten times to disrupt the cell membranes and the lysate was centrifuged at 800 x g for 8 min at 4° C. The supernatant was pelleted in a microcentrifuge, washed twice in HMS containing protease inhibitors and stored in aliquots at -80°C until needed (no more than two months).

Receptor Binding. AR42J membranes (30-50 µg) were washed, resuspended in HMS containing protease inhibitors, and added to start the reactions. After incubating for 45 min at 25°C the tubes were placed on ice, diluted with ice cold HMS buffer and centrifuged at 12,000 x g for 8 min at 4°C. The supernatants were removed and the membranes were washed twice and the final pellet was scanned in a gamma counter. Nonspecific binding was determined in the presence of 1 µM unlabeled VIP and total binding was determined in the absence of any competing ligand.

Affinity Labeling. ^{125}I-VIP was crosslinked to AR42J membranes using DSS, BSOCOES or MBS essentially as described by Pilch and Czech (25). The pellets were resuspended in HMS containing protease inhibitors and 0.2% gelatin and were incubated with 0.1 ml ^{125}I-VIP in the presence or absence of 1 µM VIP for 60 minutes at 4°C. The incubation was stopped by dilution with cold HMS buffer and the tubes were centrifuged to remove unbound counts. The pellets were resuspended in 98 µl of ice cold HMS and 2 µl of a 25 mM solution of DSS, BSOCOES or MBS freshly dissolved in DMSO was added (final concentration of 0.5 mM). After ten min on ice, the incubation was quenched by the addition of 20 mM Tris, pH 7.4. The cells or membranes were washed several times and SDS sample buffer was added to the pellet for gel electrophoresis.

Gel electrophoresis and Autoradiography. Affinity labeled membrane pellets were solubilized in SDS sample buffer (0.125 M Tris, pH 6.95 containing 4% SDS, 10 mM EDTA, 15% sucrose, 0.01% bromophenol blue in the presence or absence of 0.1M DTT), boiled for three min and pelleted. The supernatants were resolved on 7.5% acrylamide SDS gels, according to the method of Laemmli (26). After electrophoresis the gels were fixed, stained, dried and exposed to Kodak XAR 5 X-ray film using a Dupont cronex lightning plus intensifying screen for 3-21 days.

Wheat Germ Agglutinin-Agarose Chromatography. Affinity labeled AR42J membranes (~1 mg) were solubilized in 2% Nonidet-P40 (NP-40) in HMS containing protease inhibitors for 1 h at 4°C. Insoluble material was removed by centrifugation and the soluble fraction was diluted to 0.2% NP40 with HMS. WGA bound agarose (1 ml; Vector Laboratories, Burlingame, CA) in 25 mM HEPES, pH 7.5 containing 0.1 M NaCl and 0.1% NP40 was added to the sample and the suspension was incubated for 15 min at 4°C with rotation. The sample was packed into a 0.8 x 4 cm column and the flow through recycled three times. The gel was then sequentially washed with: 5 ml of 25 mM HEPES, pH 7.5 containing 0.1 M NaCl and 0.1% NP40; 5 ml of HEPES/ NP40 containing 0.5 M NaCl, and 2.5 ml of 0.3 M GlcNAc in the high salt HEPES buffer. Fractions (0.25 ml) were collected from each step and counted in a gamma counter. 0.1 mg STI was added to each fraction and proteins were precipitated with ice cold 10% TCA. After centrifugation the pellets were washed twice with ice cold acetone and solubilized in SDS sample buffer in preparation for SDS gel electrophoresis.

Glycosidase Digestion of the Affinity Labeled Receptor. Affinity labeled membranes were washed and resuspended in *N*-glycanase buffer (0.1M $NaPO_4$ pH 7.4 containing 50 mM EDTA, 1% NP40, 0.1% SDS and 1% b-mercaptoethanol) or *O*-Glycanase buffer (20 mM Tris-maleate, pH 6.0 containing 1mM Calcium acetate, 1% NP40, 0.1% SDS, 2 mM DTT and 1 mM PMSF). Recombinant *N*-glycanase (0.3 units), crude endo ß-*N*-acetylglucosaminidase F/peptide: *N*-glycosidase F (10-20 units, prepared as described previously, 27, 28) or buffer was then added to the samples and the reaction was allowed to proceed for 18-24 hours at 37°C. For *O*-Glycanase digestions, neuraminidase (0.1 U) or buffer was added to the samples for 1h at 37°C followed by 4.0 mU of *O*-Glycanase. The reaction was allowed to continue for 4-6 hours at 37° C. Incubations were terminated by addition of 25 µl of 5X SDS sample buffer. The samples were boiled and run on fresh SDS gels for analysis by autoradiography.

Results

VIP Binding and Receptor Identification. The affinity and specificity of AR42J membrane VIP receptors was examined in competition binding experiments. As shown in Fig. 1, AR42J membranes possessed a high affinity receptor for VIP (K_d ~1 nM). Neither secretin or

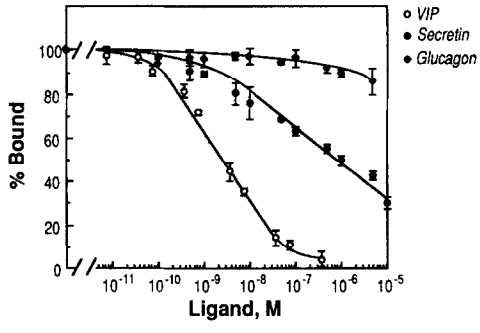
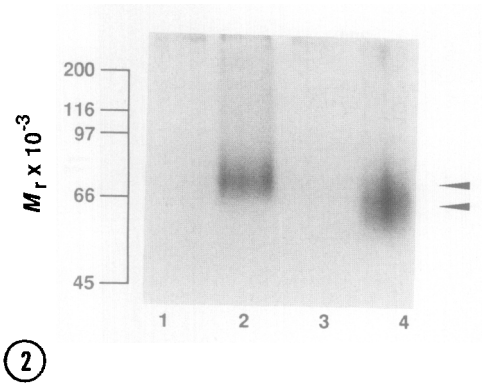
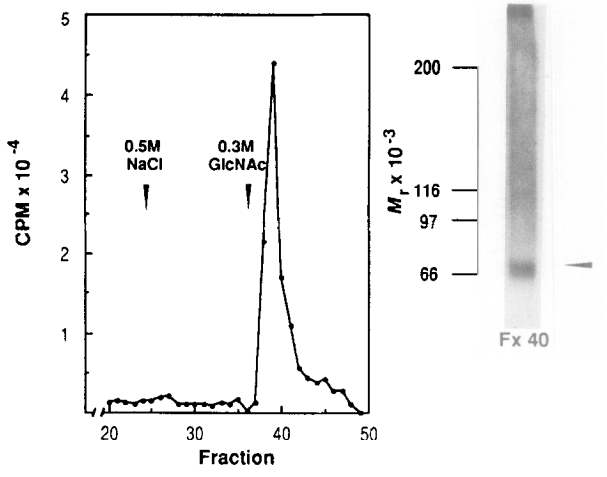


Figure 1. Binding of VIP to AR42J membranes. ^{125}I-VIP and the indicated dilutions of unlabeled ligands were added to polypropylene tubes. Membranes (30-50 µg) were added to start the incubation (45 min, 25°C). The samples were then placed on ice, diluted with ice-cold buffer and the membranes were pelleted by centrifugation. This was repeated twice before scanning the final pellet for radioactivity in the gamma counter. Nonspecific binding was determined in the presence of 1 µM VIP and total binding was determined in the absence of competing ligand. Each experiment was performed in duplicate with the data shown being representative of the means of three experiments +/– one standard deviation. VIP: O. Secretin: ●. Glucagon: ◆.



(2)



(3)

Figure 2. Affinity labeling of AR42J membranes. AR42J membranes were incubated with ^{125}I-VIP +/– 1 μM cold VIP. Unbound peptide was removed by centrifugation and the pellets were resuspended in protein-free buffer. DSS (500 μM final concentration) was added and allowed to react for five minutes on ice to covalently cross-link the bound ^{125}I-VIP. Membranes were washed, solubilized in sample buffer under reducing or nonreducing conditions and resolved on 7.5% acrylamide, SDS gels. Gels were fixed, stained, dried and autoradiographed for 5-7 days. **Lane 1**: Reduced plus cold VIP, **Lane 2**: Reduced minus cold VIP, **Lane 3**: Unreduced plus cold VIP, **Lane 4**: Unreduced minus cold VIP. The data shown is representative of three separate experiments.

Figure 3. Wheat germ agglutinin-agarose chromatography of solubilized, affinity labeled membranes. AR42J membranes were affinity labeled with ^{125}I-VIP, solubilized and chromatographed on WGA-agarose as described in Methods. Arrows designate changes in the elution buffers. Inset: autoradiograph of labeled proteins eluting in fraction 40 and resolved on a 7.5% acrylamide SDS gel. These results are representative of two experiments.

glucagon were able to compete with ^{125}I-VIP for binding to the same degree as cold VIP. At higher concentrations however, secretin (K_d ~1μM) was capable of limited competition.

VIP binding sites on crude AR42J membranes were analyzed biochemically using the technique of affinity labeling. As shown in Fig. 2, ^{125}I-VIP labeled a 67 kDa component (64 kDa when corrected for the molecular mass of ^{125}I-VIP) under reducing conditions (lane 2). Under non-reducing conditions this component migrated with a mass of 59 kDa (56 kDa, corrected, lane 4). When 1 μM VIP was added during binding, the labeling of these components was abolished (lanes 1 and 3). Other chemical crosslinking reagents (MBS, sulfo-DSS, and BSOCOES) generated identical results, with sulfo-DSS and MBS producing weaker autoradiographic signals (not shown). The signal obtained with MBS is presumably the result of reaction with a free SH group (cysteine residue) in the receptor and not the purported cleavage of an intramolecular disulfide bond (4); in good agreement with the deduced cDNA sequence indicating an odd number of cysteines in the extracellular domain (22).

Carbohydrate Analysis of the AR42J VIP Receptor. To examine the presence of oligosaccharides on the VIP receptor, AR42J membranes were affinity labeled, solubilized and chromatographed on a WGA-agarose column (Fig. 3). The peak fraction from the GlcNAc eluate of this column was further analyzed by SDS gel electrophoresis under reducing conditions (Fig.

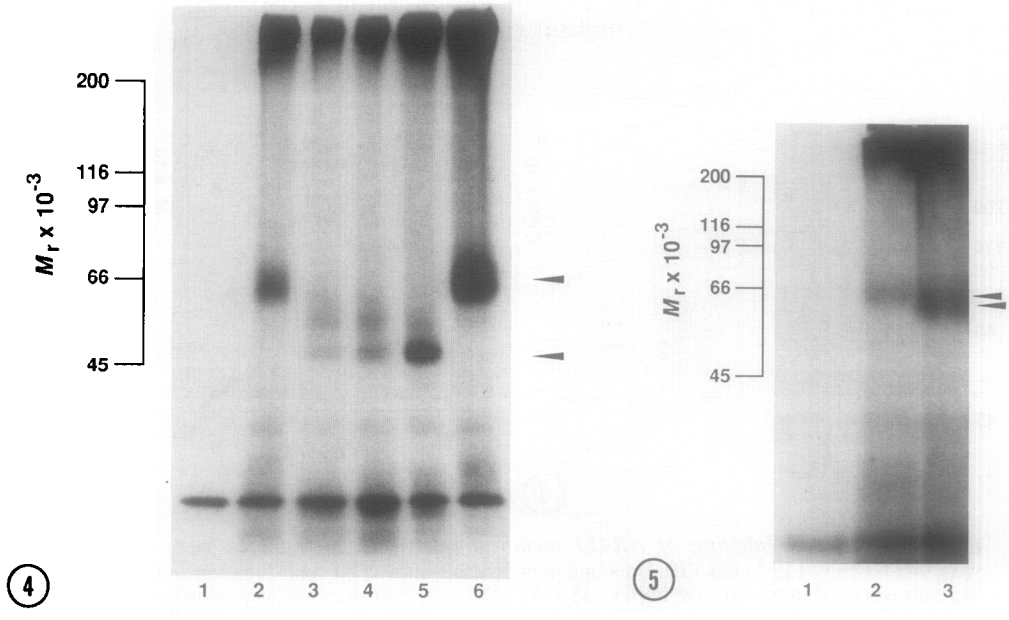


Figure 4. ***Endoglycosidase digestion of affinity labeled membranes.*** Affinity labeled AR42J membranes were resuspended in *N*-glycanase or endo F digestion buffer, boiled, equilibrated to 23°C and digested with the indicated enzyme for 18 h at 37°C. Digested proteins were resolved on a 7.5% acrylamide SDS gel. **Lane 1**: Control membranes, **Lane 2**: Affinity labeled membranes – enzyme, **Lane 3**: Affinity labeled membranes + 10 U crude endo F/PNGase F, **Lane 4**: Affinity labeled membranes + 20 U crude endo F/ PNGase F, **Lane 5**: Affinity labeled membranes + 0.3 U *N*-glycanase, **Lane 6**: Affinity labeled receptor – enzyme. The results shown are representative of three experiments.

Figure 5. ***Neuraminidase digestion of affinity labeled membranes.*** Affinity labeled AR42J membranes were resuspended in *O*-glycanase digestion buffer and digested with neuraminidase for 18 hours at 37°C. Sample buffer was added and the proteins were resolved on fresh 7.5% acrylamide SDS gels as described. **Lane 1**: Control competed with 1 mM VIP during binding, **Lane 2**: Affinity labeled receptor minus neuraminidase, **Lane 3**: Affinity labeled receptor plus neuraminidase. The results shown are representative of three separate experiments.

3). As expected, the 67 kDa component was present in this fraction indicating that the VIP receptor binds to wheat germ agglutinin.

To further examine the extent of *N*-linked glycosylation of the VIP receptor, glycosidase digestions of the affinity labeled component were carried out. As shown in Fig. 4, digestion of crude affinity labeled membranes with crude endo F/PNGase F or recombinant *N*-glycanase generated two distinct products. In each case the 67 kDa component was digested to a 47 kDa core protein with a single intermediate appearing between the mature and fully deglycosylated bands. These results indicate the presence of at least two *N*-linked oligosaccharide chains on the core protein accounting for ~20 kDa of the mass of the mature receptor. These results were confirmed by digestion of the affinity labeled bands isolated from gels (data not shown).

That at least one of the *N*-linked glycans was a complex oligosaccharide was confirmed by digestion with neuraminidase (Fig. 5). Upon digestion, the 67 kDa VIP receptor exhibited an increased mobility to approximately 59 kDa. This suggested that one or more of the *N*-linked glycans was terminally sialylated. This was corroborated by *O*-glycanase digestions to test for the presence of *O*-linked carbohydrates. Treatment of labeled receptor from individual gel bands or of

affinity labeled membranes with *O*-glycanase did not affect the electrophoretic mobility of the receptor protein suggesting the absence of *O*-linked oligosaccharides (data not shown).

Discussion

The VIP receptor on AR42J cell membranes was found to be a single chain glycoprotein with a mass of 67 kDa (64 kDa corrected) containing an intramolecular disulfide bond(s), corroborating a previous report (21). The receptor contains at least two *N*-linked glycans, one or both terminating with sialic acid. This was demonstrated by the sensitivity of the receptor to neuraminidase digestion. Removal of the *N*-linked oligosaccharides with *N*-glycanase generated a 47 kDa (44 kDa, corrected) core protein and an intermediate digestion product. This is in good agreement with the deduced sequence of human VIP receptor cDNA which encodes for a 362 residue polypeptide with a predicted mass of ~42 kDa and having three potential *N*-linked glycosylation sites (22). No *O*-linked sugars were detected on the receptor based on digestions with *O*-glycanase. The receptor bound VIP with high affinity (K_d ~1nM) with secretin (K_d ~1μM) having a modest and glucagon a negligible ability to compete for binding sites.

Svoboda et al. (13) reported that the VIP receptor on rat pancreatic membranes was a 66 kDa component specific for VIP. On intact pancreatic acini, the principle affinity labeled component appeared as an 80 kDa protein with an additional complex at M_r 160,000 (13). These data, obtained by using whole cells, was similar to what had been observed for the VIP receptor on rat liver plasma membranes by Nguyen et al. (7). A more recent analysis of the rat hepatic and retinal VIP receptors revealed that ~14 kDa (liver) and ~11 kDa (retina) of the apparent mass of these receptors was contributed by *N*-linked carbohydrate (9). In both cases, the core size of deglycosylated receptor was ~45 kDa. Our present data indicate that the AR42J receptor is structurally similar to the VIP receptor on rat liver plasma membranes (9). However, pharmacologically, their properties differ as VIP binding to the retinal receptor, unlike the rat liver and AR42J receptors, could not be effectively inhibited by high concentrations of secretin. The reason for this discrepancy and the strong inhibition of VIP binding by glucagon in COS cells transfected with the human VIP receptor cDNA (22) remain to be determined.

Acknowledgments

We wish to thank Linda Hodgdon-Anandan and Gloria Tressler for their assistance in cell culture. We also thank Drs. Norman Law and Barry Oemar for critical review of this manuscript. Finally, we thank Dr. Blake Horio for help in radiolabeling VIP. This work was supported by NIH grants DK 34389 and EY 06581 awarded to S. A. Rosenzweig.

References

1. Said, S.I. (1984) Peptides **5**, 143-150.
2. Fahrenkrug, J. (1979) Digestion **19**,149-169.
3. Inoue, Y., Kaku, K., Kaneko, T., Yanaihara, N. and Kanno, T. (1985) Endocrinology **11**, 686-692.
4. Robberecht, P., Waelbroeck, M., Camus, J.C., de Neef, P. and Christophe, J. (1984) Biochim. Biophys. Acta. **73**, 271-278.
5. Scott, J. and Baum,B.J. (1985) Biochem. Biophys. Acta **847**, 255-262.
6. Magistretti, P.J. and Schorderet, M. (1984) Nature **308**, 280-282.
7. Nguyen, T.D., Williams, J.A. and Gray, G.M. (1986) Biochemistry **25**, 3361-3368.
8. Couvineau, A. and Laburthe, M. (1985) Biochem. J. **225**, 473-479.

9. Swedlund, A.P. and Rosenzweig, S.A. (1990) Exp. Eye Res. **51**, 317-323.
10. Laburthe, M., Breant, B. and Rouyer-Fessard, C. (1984) Eur. J. Biochem. **139**, 181-187.
11. Bissonette, B.M., Collen, M.J., Adachi, H., Jensen, R.T. and Gardner, J.D. (1984) Am J. Physiology **246**, G710-G717.
12. Mcarthur, K.E., Wood, C.L., O'Dorisio, M.S., Zhou, Z., Gardner, J.D and Jensen, R.T. (1987) Am. J. Physiology **252**, G404-G412.
13. Svoboda, M., Poloczek, P., Winand, J., Robberecht, P. and Christophe, J. (1988) Eur. J. Biochem. **174**, 59-66.
14. Wood, C.L. and O'Dorisio, M.S. (1985) J. Biol. Chem. **260**, 1243-1247.
15. Muller, J.-M., Luis, J., Fantini, J., Abadie, B., Giannellinni, F., Marvaldi, J. and Pichon, J. (1985) Eur J. Biochem. **151**, 411-417.
16. Jessop, N.W. and Hay, R.J. (1980) In Vitro **16**, 212.
17. Logsdon, C.D. (1986) J. Biol. Chem. **261**, 2096-2101.
18. Viguerie, N., Estève, J.-P., Susini, C., Logsdon, C.D., Vaysse, N. and Ribet, A. (1987) Biochem. Biophys. Res. Comm. **147**, 942-948.
19. Brown, P.J., Lee, A.B., Norman, M.G., Prasky, D.H. and Schonbrunn, A. (1990) J. Biol. Chem. **29**, 17995-18004.
20. Moessner, J., Logsdon, C.D., Williams, J.A. and Goldfine, I.D. (1985) Diabetes **34**, 891-897.
21. Svoboda, M., DeNeef, P., Tastenoy, M. and Christophe, J. (1988) Eur. J. Biocem. **176**, 707-713.
22. Sreedharan, S.P., Robichon, A., Peterson, K.E. and Goetzl, E.J. (1991) Proc. Natl. Acad. Sci. USA **88**, 4986-4990.
23. Logsdon, C.D., Moessner, J., Williams, J.A. and Goldfine, I.D. (1985) J. Cell Biol. **100**, 1200-1208.
24. Martin,J.L., Rose, K., Hughes, G.J. and Magistretti, P.J. (1986) J. Biol. Chem. **261**, 5320-5327.
25. Pilch, P.F. and Czech, M.P. (1979) J. Biol. Chem. **254**, 3375-3380.
26. Laemmli, U.K. (1979) Nature **227**, 680-685.
27. Rosenzweig, S.A., Madison, L.D. and Jamieson, J.D. (1984) J. Cell Biol. **99**, 1110-1116.
28. Tarentino, A.L., Gómez, C.M. and Plummer, T.H., Jr. (1985) Biochemistry **24**, 4665-4671.