Volume 286, number 1,2, 105–109 FEBS 09925 July 1991
© 1991 Federation of European Biochemical Societies 00145793/91/$3.50
ADONIS 001457939100638X

The effect of glycosyl inositol-phosphate on cAMP production in isolated rat fat-cells is transduced by a pertussis toxin sensitive G-protein

Laurent Martiny[1], Karim Dib[2], Bernard Haye[1], Claude Corrèze[2], Claude Jacquemin[2] and Bernard Lambert[1]

[1] *Laboratoire de Biochimie, UFR de Sciences Exactes et Naturelles, 51062 Reims, France* and [2] *INSERM U96 Unité de Recherches sur la Glande Thyroïde et la Régulation Hormonale, 94275 Le Kremlin-Bicêtre, France*

Received 22 February 1991; revised version received 3 May 1991

In fat-cells, insulin increases synergistically the inhibitory effects of adenosine and prostaglandin E_2 on adenylyl cyclase activity. When the endogenous production of these feedback inhibitors is suppressed, insulin is no more active in conditions where glycosyl inositol-phosphate which is a putative mediator of its action, is always efficient. Moreover, glycosyl inositol-phosphate signal is transduced by a G protein sensitive to IAP intoxication.

Adipocyte; Glycosyl inositol-phosphate; Pertussis toxin; cAMP

1. INTRODUCTION

The anti-lipolytic activity of insulin was reported to affect several steps of the signalling cascade which controls triacylglycerol lipase activity. Among the various potential targets, the most important to transduce insulin message were adenylyl cyclase and cAMP phosphodiesterase which adjust the level of the cyclic nucleotide, the cAMP-dependent protein kinase and the lipase itself. Some years ago, we focused our attention on the control by insulin of adenylyl cyclase activity [1]. Insulin decreased by 40–50% the rate of accumulation of cAMP in experimental conditions where phosphodiesterase activity was completely inhibited. The efficiency of this inhibition was demonstrated by a constant accumulation rate of cyclic AMP for at least 20 min.

We also studied the relationships existing between insulin and adenosine or prostaglandin E_2, in their action at the level of adenylyl cyclase. We concluded that insulin synergized the retroinhibitory effects of the endogenous regulators [2,3]. Indeed, when adenosine was destroyed by adenosine deaminase and when prostaglandin E_2 synthesis was inhibited by indomethacin, insulin became unable to decrease the stimulated cAMP accumulation. Nevertheless, its inhibitory activity was restored when a small concentration of either of the negative regulators, too low to be active by itself, was added to the cell suspension.

Since these results were obtained, the discovery and partial characterization of an insulin modulator were reported [4,5]. This substance, identified to be a glycosyl inositol-phosphate, reproduced some of the effects of insulin on its target-cells, particularly the effects on the adenylyl cyclase activity of fat cells [6] and their consequences on the lipolytic activity [7], as well as the phosphorylation status of target proteins of the hormone [8].

Recently, we described the important production of such a glycosyl inositol-phosphate by cultured pig thyroid reconstituted follicles under the control of thyrotropin [9]. The aim of the present work was to compare the mode of action of glycosyl inositol-phosphate with those of insulin, adenosine and prostaglandin E_2 on the synthesis of cAMP. Unlike insulin, glycosyl inositol-phosphate inhibited adenylyl cyclase even in the absence of adenosine and prostaglandin E_2. This inhibition was relieved by the intoxication of the cells with pertussis toxin, which suggests the participation of G_i-protein in the transduction of the effects of glycosyl inositol-phosphate.

Correspondence address: L. Martiny, Laboratoire de Biochimie, UFR de Sciences Exactes et Naturelles, 51062 Reims, France

2. MATERIALS AND METHODS

[^{32}P]NAD$^+$ (30 Ci/mmol) was obtained from New England Nuclear (UK). D,L-Isoproterenol-HCl (IPNE) (log 1-5627), pertussis toxin (IAP), prostaglandin E_2 (PGE_2), crystalline bovine B-grade insulin (INS) (25.6 IU/mg) were purchased from Sigma. Collagenase (CLS; 207 IU/mg) was obtained from Worthington Biochemicals, N^6(*R*-phenyl-isopropyl)-adenosine (PIA) and adenosine-deaminase (ADA) were from Boehringer. 3-(3,4-Dimethoxybenzyl)-2-imidazolidinone (RO-7-2956) was kindly donated by Hoffmann-La-Roche (Basel). Indomethacin (Indo) was provided by Merck, Sharp and Dohme. Bovine serum albumin fraction V, fatty acid-free was obtained from Armour. Pertussis toxin B-oligomer was obtained from List Biological Lab. (CA, USA). Male Wistar rats (180 g) from Depré (Saint Doulchard, France) were kept at room temperature and given free access to food and water.

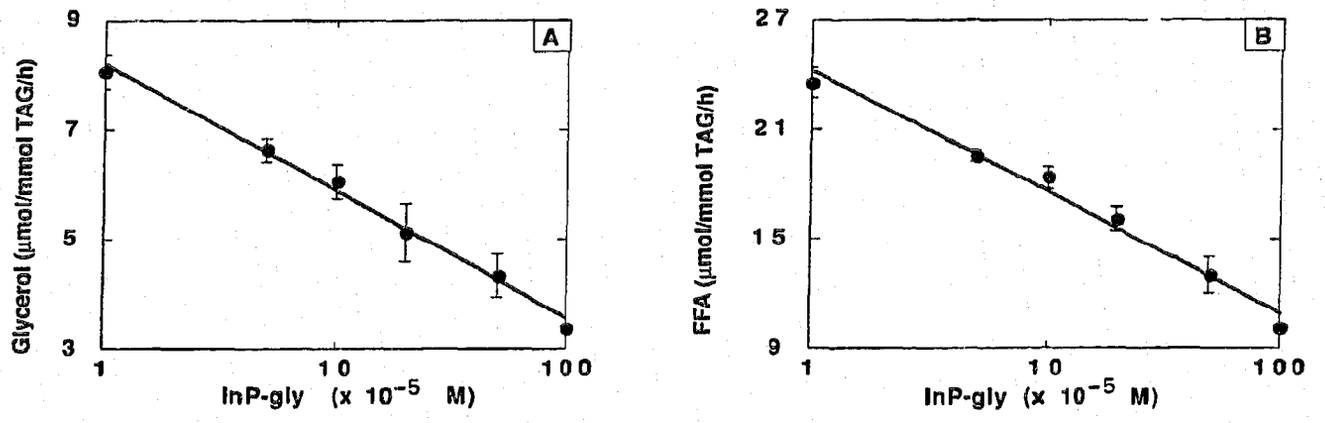


Fig. 1. Concentration-dependent inhibition of lipolysis by glycosyl inositol-phosphate. Fat cells were incubated at 37°C for 1 h in the presence of 10^{-6} M IPNE supplemented or not with 100 µU insulin or various concentrations of InP-gly. (A) Glycerol release – basal: 1.31 ± 0.01, IPNE: 8.07 ± 0.38; IPNE+insulin: 3.14 ± 0.07. (B) Fatty acid release – basal: 3.79 ± 0.10, IPNE: 23.15 ± 0.77; IPNE+insulin: 9.01 ± 0.09. Values (µmol/mmol TAG/h) are the mean ± SE of 4 experiments.

In order to prepare glycosyl inositol-phosphate (InP-gly) according to [9], pig thyroid cells were cultured for 1 day with TSH (0.1 mU/ml) before adding [1,6-^{3}H]glucosamine (20 µCi/20 × 10^6 cells) to the medium for 48 h. The cells were then washed and resuspended. Glucosamine-labelled cells (2 × 10^6 cells) were incubated for 20 min in 3 ml of Earle's-HEPES solution. The reaction was stopped by adding $HClO_4$ (final concentration 5% (v/v)) and centrifuging at 1200 × g. The supernatant was neutralized with 9 M KOH, centrifuged at 3000 × g and percolated on a column (8 × 0.5 cm) of Dowex AG_1X_8 (200–400 mesh). Column was washed with 9 ml of H_2O and 10 ml of 0.1 M ammonium formate, and InP-Gly eluted with 0.1 M formic acid. Formic acid was removed by lyophilization and the InP-Gly concentration was determined by reacting the non-*N*-acetylated amino group of its glucosamine moiety with fluorescamine. A molecular weight of 1000 was assumed.

The antiserum AS/7 directed against a synthetic decapeptide KENLKDCGLF, representing the carboxyl-terminus of α-transducin was kindly provided by Dr A. Spiegel (NIH Bethesda). This antiserum recognized the αG_i1 and αG_i2 isoforms [10].

White fat cells were isolated by collagenase digestion and incubated in Krebs-Ringer bicarbonate buffer 0.1 M (pH 7.4) with Ca^{2+} (1.3 mM) and 4% (w/v) fatty acid-free albumin. Free fatty acids were extracted as in [11] and determined according to [12]. Triacylglycerols (TAG) were extracted according to [13]. Aliquots of total lipid extracts were saponified. Glycerol was assayed as in [14]. Cyclic AMP was measured by a radioimmunological method described in [15] except that bound ligand was separated from the free ligand by polyethylene glycol precipitation. Free fatty acids, glycerol and cAMP were expressed in function of TAG content of the cells. In some experiments, 1 h preincubation was performed in the presence of pertussis toxin (10 µg/ml) and indomethacin (20 µg/ml) before addition of drug or hormone. Adipocytes preincubated with or without pertussis toxin were washed twice with Krebs-Ringer bicarbonate buffer, 0.1 M, pH 7.4, and disrupted in 40 mM Tris-HCl, pH 7.4, containing 250 mM sucrose, 40 µg/ml leupeptin and aprotinin, 0.1 mM PMSF and 0.1 mM DTT. The homogenate was centrifuged for 30 min at 30000 × g. The resulting pellet was resuspended in the homogenization buffer at a concentration of 1–2 mg/ml. Aliquots of membrane (100 µg protein) were ADP-ribosylated with 3 × 10^{-6} M [^{32}P]NAD and 3 µg of activated pertussis toxin as described in [16]. ADP-ribosylated membrane proteins were solubilized and separated by SDS-PAGE. Membrane proteins were then electrotransferred onto nitrocellulose sheets (BA 83, Schleicher and Schüll). The nitrocellulose sheets were then reacted with AS/7 antipeptide (1/300) followed by radioiodinated protein A as described [17].

The statistical differences were calculated by the Student's *t*-test, the mean values ± SE are given.

3. RESULTS

Glycosyl inositol-phosphate (InP-gly), purified from culture media of pig reconstituted thyroid follicles [9] was assayed on isolated fat cells. In these cells, lipolysis was stimulated 6 times in 1 h by 10^{-6} M isoproterenol (IPNE); the addition of 100 µU/ml insulin inhibited by 60% the stimulated release of glycerol and fatty acids. Fig. 1 shows the concentration-dependent inhibition of the lipolytic rate evaluated either in glycerol or in free fatty acid between 10^{-5} M and 10^{-3} M InP-gly. A similar inhibition was obtained with 100 µU/ml insulin or 10^{-3} M InP-gly.

The accumulation of cAMP in 6 min was stimulated 14 times by 10^{-6} M IPNE; the addition of 10^{-3} M RO-7-2956 (RO), which inhibited completely the phosphodiesterase activity in fat cells [3], multiplied the effect of IPNE by 2.5 times. The relief of the tonic inhibition of adenylyl cyclase mediated by G_i, resulting from the addition of indomethacin (Indo) and adenosine deaminase (ADA), increased further by 5.5 times the joint effect of IPNE and RO (Fig. 2). Insulin (100 µU/ml) or InP-gly (3.3 × 10^{-4} M) inhibited cAMP accumulation whether phosphodiesterase was active or not. On the other hand, in the presence of Indo and ADA, insulin was no more antilipolytic as previously reported [2,3], whereas InP-gly kept its inhibitory effect. The functional character of G_i pathway of adenylyl cyclase was assessed by the very efficient inhibition obtained with phenyl-isopropyl-adenosine (PIA) or prostaglandin E_2 (PGE_2).

The kinetic and concentration-dependent effects of the treatment of the cells with pertussis toxin (IAP) on lipolytic rate and cAMP accumulation were studied in [18]. After a 1 h incubation period, the level of cAMP in cells treated with IAP alone, raised to 285.48 ± 20.42 pmol/mmol TAG. The effect of IAP treatment on the ability of InP-gly to inhibit cAMP accumulation

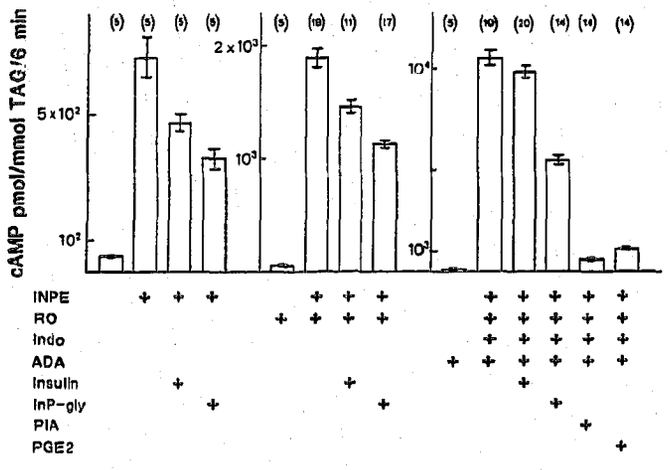


Fig. 2. Effect of various inhibitors on cAMP accumulation by fat cells. Fat cells were incubated at 37°C for 6 min in the presence of 10^{-6} M IPNE and various agonists and antagonists: 10^{-3} M RO-7-2956 (RO), 20 μg/ml indomethacin (Indo), adenosine deaminase, 2 μg/ml (ADA), 100 μU/ml insulin, 3.3×10^{-4} M glycosyl inositol-phosphate (InP-gly), 10^{-6} M phenyl-isopropyl-adenosine (PIA), 10^{-6} M prostaglandin E_2 (PGE_2). Results are mean ± SE for the number of experiments indicated above each bar in the graph.

was determined in the presence of 10^{-6} M IPNE, 10^{-3} M RO, 2 μg/ml ADA and 20 μg/ml Indo. One hour preincubation of fat cells with 10 μg/ml IAP, which enhanced by 35% the accumulation of cAMP promoted by IPNE + RO + Indo + ADA, completely prevented the inhibition by 10^{-6} M PIA, 10^{-6} M PGE_2 and 10^{-3} M InP-gly (Fig. 3). The replacement of the holotoxin by B oligomer during the preincubation of

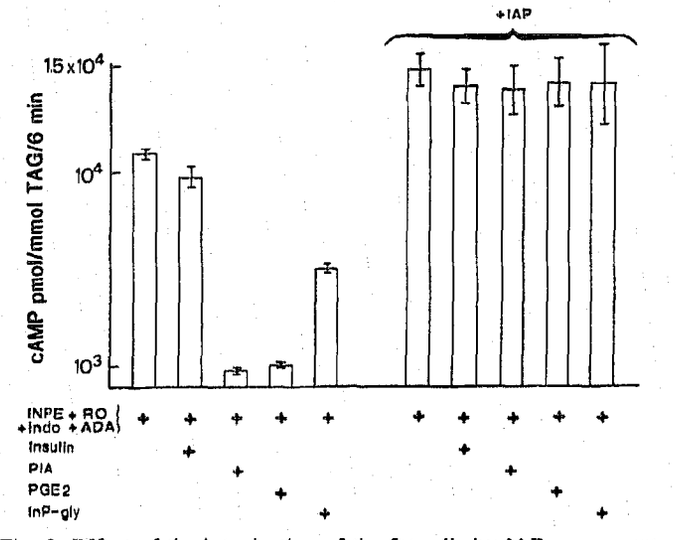


Fig. 3. Effect of the intoxication of the fat cells by IAP component of pertussis toxin on cAMP accumulation. Cells were stimulated at 37°C for 6 min by 10^{-6} M IPNE in the presence of 10^{-3} M RO, 2 μg/ml ADA and 20 μg/ml Indo, supplemented or not with 100 μU/ml insulin, 10^{-6} M PIA, 10^{-6} M PGE_2 or 10^{-3} M InP-gly. When present, 10 μg/ml IAP was preincubated for 1 h with the cells. IAP alone, after 1 h incubation raised the level of cAMP to 285.48 ± 20.42 pmol/mmol TAG. Values are the mean ± SE of 3 experiments.

Table I

Failure of pertussis toxin B oligomer treatment to suppress the PIA- or InP-gly-induced inhibition of cAMP accumulation stimulated by IPNE

Additions	cAMP accumulation (pmol/mmol TAG)	
	Control	B oligomer
IPNE-RO-ADA	8352 ± 645	7420 ± 609
IPNE-RO-ADA-PIA	1288 ± 140	1132 ± 147
IPNE-RO-ADA-InP-gly	4043 ± 497	4196 ± 61

Fat cells were preincubated for 1 h in the absence or in the presence of 6.6 μg/ml of B oligomer, then 1 mM RO-7-2956 and 2 μg/ml ADA were added to each sample, supplemented or not with 10^{-6} M PIA or 3.3×10^{-4} M InP-gly. The incubation was pursued a further 6 min. cAMP was assayed as described in Fig. 2. Values were expressed as the mean value ± SE of quadruplicate assays.

the cells, did affect neither the stimulatory effect of IPNE, nor the inhibitory effect of PIA or InP-gly (Table I).

To determine if IAP treatment resulted in ADP-ribosylation of G-proteins in fat cells, crude plasma membranes were ADP-ribosylated in vitro using [^{32}P]NAD and activated IAP. As shown in Fig. 4, in vitro ADP-ribosylation of membranes from cells incubated without IAP resulted in the specific transfer of ADP-ribose to a doublet of proteins of approximately

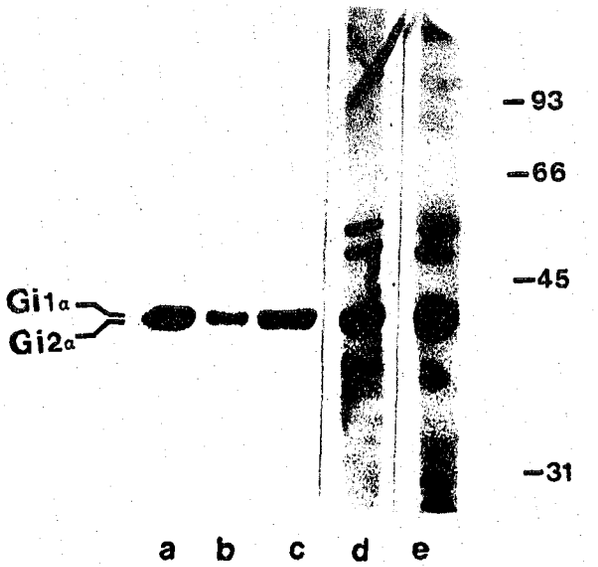


Fig. 4. Effect of intoxication of fat cells with IAP on the ADP-ribosylation and immunoreactivity of G_i protein. Crude plasma membranes were prepared from cells preincubated with 10 μg/ml IAP or with 6.66 μg/ml B oligomer for 1 h and from cells preincubated for 1 h in control conditions. Then they were ADP-ribosylated by 3 μg of activated IAP in the presence of 3×10^{-6} M [^{32}P]NAD. Mobilities of molecular mass standard proteins are shown on the right (values in kDa). (Lane a) Untreated cells; (lane b) IAP-treated cells; (lane c) B-oligomer-treated cells. (Lane d) Immunoreactivity of untreated cells. (Lane e) Immunoreactivity of IAP treated cells.

40 kDa. Treatment of the fat cells with IAP prior to preparing the plasma membranes decreased significantly the in vitro ADP-ribosylation, indicating that the IAP substrate in vitro is also an in vivo toxin substrate. When cells were treated with an equivalent amount of B-subunits of the toxin, devoid of ADP-ribosyltransferase activity, the corresponding membranes were not different from membranes of untreated cells in their capacity to be ADP-ribosylated in vitro. The ADP-ribosylated proteins were identified as $\alpha G_i1/\alpha G_i2$ by their reaction with the antiserum AS/7. The amount of αG_i was not modified by the intoxication of the cells by IAP.

4. DISCUSSION

The identification of the various steps implied in the signalling of insulin effects is the object of intense research. The first element consists in the heterotetrameric receptor which upon stimulation is activated and autophosphorylated by its tyrosine protein kinase activity [19]. At this stage, two relatively independent processes occur: a cascade of phosphorylation-dephosphorylation steps leading for example to the stimulation of glycogen synthesis [20] and an interaction with a G-protein, sensitive to pertussis toxin, responsible for the stimulation of the glycosyl phosphatidylinositol specific phospholipase C releasing the glycosyl inositol-phosphate mediator into the medium [21]. To date, there is no information about the mode of action of this mediator. The control of pyruvate dehydrogenase phosphatase activity by glycosyl inositol-phosphate added to intact cells or isolated mitochondria [22], suggests the existence of a transmembrane and intracellular transport system. On the other hand, the inhibition of adenylyl cyclase is more controversial: previous results by Saltiel [6] and Malchoff et al. [2] support an action by inhibiting the catalytic subunit of the enzyme, whereas our present results are in favour of the participation of membrane receptors. These two kinds of pathways are not mutually exclusive.

We have previously described the modulation by insulin of the activity of endogenous retroinhibitors: adenosine and prostaglandin E_2, on cAMP accumulation. In the case of the latter, we observed that the hormone seemed to increase the binding affinity of the receptors for prostaglandin E_2, thus enhancing the sensitivity of the cells to its inhibitory effect [2]. In the case of the former, the nature of the modulation was somewhat different: insulin also enhanced the inhibitory action of adenosine on cAMP accumulation, but likely by an increase of the binding capacity of the receptors [3]. A similar situation was recently reported in human blood platelet where insulin treatment increased the binding capacity of prostanoid receptors [24].

Our present results bring information on the negative control of adenylyl cyclase by glycosyl inositol-phosphate. Unlike insulin which needs the presence of minimal concentrations of adenosine [3] or prostaglandin E_2 [2] to become active, glycosyl inositol-phosphate alone, inhibited in a concentration-dependent manner the stimulated adenylyl cyclase. The consequential effects of this primary action can be observed on the release of glycerol and fatty acids. The other important observation was the prevention by pertussis toxin treatment of the cells, of the inhibition of stimulated adenylate cyclase by glycosyl inositol-phosphate, phenyl-isopropyl-adenosine and prostaglandin E_2. As the two latter substances exert their action under the mediation of G_i-protein, it is likely that the same mechanism is operating for glycosyl inositol-phosphate. This interpretation is supported by a good correlation between the ADP-ribosyl-transferase activity of the toxin and its inhibitory potency: the pretreatment of the cells with B-subunits, which exert in rat adipocytes an insulin-like action enhancing glucose oxidation [25], do not suppress in vivo the inhibitory character of PIA, PGE_2 and InP-gly and do not affect in vitro the subsequent ADP-ribosylating potency of activated A-subunits on their G-protein substrates.

Moreover, our results suggest that in the pathway of insulin effects mediated by glycosyl inositol-phosphate, G-proteins sensitive to pertussis toxin ADP-ribosylation take action successively at two levels. The first one is the scene of the interaction between liganded insulin receptor and a G-protein activated by adenosine and/or prostaglandin E_2, which controls the activity of glycosyl phosphatidylinositol phospholipase C. The second level corresponds probably to G_i as its ADP-ribosylation prevents the inhibition of adenylyl cyclase by glycosyl inositol-phosphate but also by adenosine or prostaglandin E_2.

Acknowledgements: We thank O. Legué for her excellent technical assistance and A. Lefèvre, M. Sorgue and M. Bahloul for preparing the manuscript. We are grateful to Dr. A. Spiegel for the gift of antiserum AS/7. This work was supported, in Reims, by the Fondation pour la Recherche Médicale and by a grant from INSERM (CRE 874009).

REFERENCES

[1] Lambert, B. and Jacquemin, C. (1979) FEBS Lett. 105, 19–22.
[2] Lambert, B. and Jacquemin, C. (1980) Prostaglandins and Medicine 5, 375–382.
[3] Lambert, B. and Jacquemin, C. (1983) FEBS Lett. 155, 31–34.
[4] Saltiel, A.R., Fox, J.A., Sherline, P. and Cuatrecasas, P. (1986) Science 233, 967–972.
[5] Mato, J.M., Kelly, K.L., Abler, A. and Jarett, L. (1987) J. Biol. Chem. 262, 2131–2137.
[6] Saltiel, A.R. (1987) Endocrinology 120, 967–972.
[7] Kelly, K.L., Mato, J.M., Merida, I. and Jarett, L. (1987) Proc. Natl. Acad. Sci. USA 84, 6404–6407.
[8] Alemany, S., Mato, J.M. and Stralfors, P. (1987) Nature 330, 77–79.

[9] Martiny, L., Antonicelli, F., Thuilliez, B., Lambert, B., Jacquemin, C. and Haye, B. (1990) Cellular Signalling 2, 21–27.
[10] Goldsmith, P., Rossiter, K., Carter, A., Simonds, W., Unson, C.G., Vinitsky, R. and Spiegel, A.M. (1988) J. Biol. Chem. 263, 6476–6479.
[11] Dole, V.P. and Meinertz, M. (1960) J. Biol. Chem. 235, 2595–2599.
[12] Novak, M. (1965) J. Lipid Res. 6, 431–433.
[13] Folch, J., Less, M. and Stanley, G.H.S. (1957) J. Biol. Chem. 226, 497–509.
[14] Tixier, M. and Claude, J. (1974) Ann. Biol. Clin. 32, 53–57.
[15] Cailla, H., Racine-Weisbuch, M. and Delaage, M. (1973) Anal. Biochem. 56, 396–407.
[16] Jacquemin, C., Thibout, H., Lambert, B. and Corrèze, C. (1986) Nature 233, 967–972.
[17] Saunier, B., Dib, K., Delemer, B., Jacquemin, C. and Corrèze, C. (1990) J. Biol. Chem. 265, 19942–19946.
[18] Lambert, B., Aublin, J.L., Champion, S., Haye, B. and Jacquemin, C. (1985) FEBS Lett. 181, 390–396.
[19] Shoelson, E. and Kahn, C.R. (1989) in: Insulin Action, pp. 23–33, A.R. Liss.
[20] Dent, P., Lavoinne, A., Nakielny, S., Barry Caudwell, F., Watt, P. and Cohen, P. (1990) Nature 348, 302–308.
[21] Luttrell, L., Kilgour, E., Larner, J. and Romero, G. (1990) J. Biol. Chem. 265, 16873–16879.
[22] Jarett, L., Wong, E.H.A., Lance Macaulay, S. and Smith, J.A. (1985) Science 227, 533–535.
[23] Malchoff, C.D., Huang, L., Gillespie, N., Villar Palasi, C., Schwartz, C.F.W., Cheng, K., Hewlett, E.L. and Larner, J. (1987) Endocrinology 120, 1327–1337.
[24] Kahn, N.N. and Sinha, A.K. (1990) J. Biol. Chem. 265, 4976–4981.
[25] Tamura, M., Nogimori, K., Yajima, M., Ase, K. and Ui, M. (1983) J. Biol. Chem. 258, 6756–6761.