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Essential role of phosphatidylinositol 3-kinase in insulin-induced activation and phosphorylation of the cGMP-inhibited cAMP phosphodiesterase in rat adipocytes

Studies using the selective inhibitor wortmannin

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Abstract

Incubation of rat adipocytes with wortmannin, a potent and selective phosphatidylinositol 3-kinase (PI 3-kinase) inhibitor, completely blocked the antilipolytic action of insulin ($IC_{50} \approx 100$ nM), the insulin-induced activation and phosphorylation of cGMP-inhibited cAMP phosphodiesterase (cGI-PDE) as well as the activation of the insulin-stimulated cGI-PDE kinase ($IC_{50} \approx 10$–30 nM). No direct effects of the inhibitor on the insulin-stimulated cGI-PDE kinase, the cGI-PDE and the hormone-sensitive lipase were observed. These data suggest that activation of PI 3-kinase upstream of the insulin-stimulated cGI-PDE kinase in the antilipolytic insulin signalchain has an essential role for insulin-induced cGI-PDE activation/phosphorylation and anti-lipolysis.

Key words: Insulin; Phosphatidylinositol 3-kinase; Inhibitor; Adipocyte; Lipolysis; cGMP-inhibited cAMP phosphodiesterase

1. Introduction

The mechanisms whereby insulin regulates metabolic pathways and cell proliferation and are not fully understood even though substantial progress during recent years has been achieved [1–3]. Activation of the intrinsic tyrosine kinase of the insulin receptor is a critical event that initiates the diverse effects of insulin. The activated receptor phosphorylates specific target proteins triggering a series of events resulting in activation of serine/threonine protein kinases and phosphatases, that alter the phosphorylation state/activities of key proteins involved in the regulation of cell metabolism and proliferation. One of the most important metabolic actions of insulin is the inhibition of catecholamine-induced lipolysis in adipose tissue. Activation of the adipocyte cGMP-inhibited cAMP phosphodiesterase (cGI-PDE) by insulin (reviewed in [4]) is believed to be the major mechanism whereby insulin reduces cellular cAMP leading to inactivation of cAMP-dependent protein kinase (cAMP-PK), net dephosphorylation of hormone- sensitive lipase (HSL) and antilipolysis [5–8]. This activation of cGI-PDE is the result of serine phosphorylation [9,10]. A more than additive activation and phosphorylation of cGI-PDE is seen in response to insulin in the presence of catecholamines, i.e. the conditions during which insulin normally exerts its antilipolytic effect [10,11]. The insulin-induced phosphorylation/activation of the cGI-PDE is catalyzed by an insulin-stimulated cGI-PDE serine kinase (cGI-PDE IK) [12–14]. The identity of this protein kinase and the mechanism of its regulation by insulin are still unknown.

Phosphatidylinositol 3-kinase (PI 3-kinase) has been suggested to be important in mediating metabolic effects of insulin [15] and other non-mitogenic and mitogenic stimuli that act through tyrosine kinases [16]. Wortmannin, a potent and selective PI 3-kinase inhibitor [15,17], has recently been reported to block the antilipolytic action of insulin and the insulin-stimulated hexose uptake in adipocytes [15]. PI 3-kinase, a dual specificity [18] lipid- and protein serine kinase, consists of a 110 kDa catalytic subunit and an 85-kDa regulatory subunit which contains *src* homology-2 (SH2) domains [19–22]. Activation of PI 3-kinase by insulin seems to involve

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Abbreviations: PI 3-kinase, phosphatidylinositol 3-kinase; cGI-PDE, cGMP-inhibited cAMP phosphodiesterase; cAMP-PK, cAMP-dependent protein kinase; HSL, hormone-sensitive lipase; cGI-PDE IK, insulin-stimulated cGMP-inhibited cAMP phosphodiesterase kinase; IRS-1, insulin receptor substrate 1; SH2, src homology-2; PAGE, polyacrylamide gelelectrophoresis; KRH, modified Krebs-Ringer buffer with HEPES.

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insulin receptor substrate 1 (IRS-1) (reviewed in [23,24]). The activated insulin receptor tyrosine kinase phosphorylates IRS-1 on a number of tyrosines in YXXM/YMXM motifs [25,26]. The SH2 domains of the p85 regulatory subunit of PI 3-kinase are believed to bind to phosphorylated YXXM/YMXM motifs on IRS-1 [25,26]. This insulin-induced association of PI 3-kinase with tyrosine phosphorylated IRS-1 increases the kinase activity of the 110-kDa catalytic subunit towards PI and/or its phosphorylated derivatives PI(4)P and PI(4,5)P_2 [27,28]. The PI 3-kinase also acts as a serine protein kinase by phosphorylating the p85 subunit causing a feed-back auto-inhibition [18]. No other exogenous protein substrate has been identified.

The observation that wortmannin blocked the antilipolytic effect of insulin [15] prompted us to evaluate the effect of this PI 3-kinase inhibitor on several links believed to be important in the antilipolytic signalling chain activated by insulin. In this report we demonstrate that wortmannin inhibited the insulin-induced activation of cGI-PDE IK as well as the activation/phosphorylation of cGI-PDE in isolated adipocytes.

2. Materials and methods

2.1. Preparation and fractionation of adipocytes

Adipocytes were prepared according to [30] with modifications [31] from 36- to 38-day-old male Sprague–Dawley rats (B&K Universal, Stockholm) and resuspended in Krebs-Ringer medium, pH 7.4, containing 25 mM HEPES, 200 nM adenosine, 119 mM NaCl, 4.95 mM KCl, 2.54 mM $CaCl_2$, 1.19 mM KH_2PO_4, 1.19 mM $MgSO_4$, 2 mM glucose and 3.5% bovine serum albumin (KRH). Adipocytes (1.5–2 ml of an 8–10% cell suspension) were incubated with wortmannin (0–100 nM) (Sigma, St. Louis, USA) for 10 min and then with hormones as indicated. The cells were quickly washed once and homogenized at 21°C (10 strokes) in 0.8 ml of 50 mM *N*-tris(hydroxymethyl)-methyl-2-aminoethanesulfonic acid (TES), pH 7.5, containing 40 mM *p*-nitrophenyl-phosphate, 0.2 mM EGTA, 2 mM EDTA, 250 mM sucrose, 1 mM dithioerythriol, 0.1 mM vanadate and 10 μg/ml each of antipain, leupeptin and pepstatin A, immediately cooled to 4°C and centrifuged (50.000 × *g*, 60 min, 4°C). The crude membrane fraction containing cGI-PDE [32] was either resuspended in 50 mM Tris buffer, pH 7.5, containing 5 mM $MgCl_2$, 1 mM EDTA, 3 mM benzamidine and 10 μg/ml each of antipain, leupeptin and pepstain A or solubilized in the same buffer with 1% $C_{13}E_{12}$ (non-ionic alkyl polyoxyethylene glycol detergent from Berol Kemi AB, Stenungsund, Sweden) as described [32]. Solubilized cGI-PDE from control cells was used as substrate for detection of cGI-PDE IK activity. The fat free cytosolic fraction containing cGI-PDE IK activity was assayed as described below.

2.2. Isolation of cGI-PDE from ^{32}P-labelled adipocytes

Adipocytes (10% cell suspension in KRH containing 300 μM KH_2PO_4) were labelled with ^{32}P (1 mCi/ml) (Amersham, Little Chalfont, UK) for 2 h. Wortmannin (0–1000 nM) was added to the incubations (1.5 ml of a 10% cells suspension) 10 min prior to stimulation by 1 nM insulin. After 10 min with insulin, the cells were washed twice with 50 mM Tris, pH 7.4, containing 250 mM sucrose, 1 mM EDTA and 10 μg/ml each of antipain, leupeptin and pepstain A and homogenized. cGI-PDE was immunoisolated from the solubilized crude membrane fraction [9,10]. Antiserum raised against a peptide (corresponding to amino acid 423–440 based on the deduced sequence of the recently cloned rat adipose tissue cGI-PDE [33,34]) was used to immunoprecipitate the cGI-PDE. [^{32}P]cGI-PDE was visualized after SDS-PAGE [35] by digital imaging of ^{32}P (Fujix BAS 2000).

2.3. Detection of insulin-stimulated cGI-PDE kinase activity

Aliquots (70 μl) from fat free cytosolic fractions from control and wortmannin/insulin treated adipocytes were incubated with solubilized cGI-PDE (30 μl) and a phosphorylation buffer (25 μl) containing 200 μM [^{32}P]ATP (10,000–50,000 cpm/pmol), 50 mM $MgSO_4$, 5 mM dithioerythritol, 250 mM sucrose and 25 μM cAMP PK inhibitor (Sigma, St. Louis, USA) for 10 min at 30°C. Reactions were terminated by the addition of 1 ml of 10 mM Tris, pH 7.5, containing 250 mM sucrose, 10 mM NaF, 2 mM sodium-pyrophosphate, 1 mM EDTA, 0.1 mM EGTA and 10 μg/ml each of antipain, leupeptin and pepstatin A. cGI-PDE was isolated and analyzed as described above.

2.4. Lipolysis

Adipocytes, (2% cell suspension) were incubated with wortmannin or vehicle (DMSO) in KRH for 10 min. Samples (1 ml) were added to vials containing either buffer, 100 nM noradrenaline alone or together with 1 nM insulin. Glycerol released to the medium during 30 min was then determined [36].

2.5. Assay of cGI-PDE and HSL activities

Crude membrane fractions were assayed for cAMP-PDE activity [37] for 8 min at 30°C in a total volume of 0.3 ml containing 50 mM HEPES, pH 7.5, 0.1 mM EDTA, 8.3 mM $MgCl_2$, 0.5 μM [^{3}H]cAMP (200–500 cpm/pmol) (New England Nuclear, Dreieich, Germany). Under these conditions cGI-PDE represented > 90% of total cAMP-PDE activity in the membrane fraction [9,32]. HSL was assayed as described [38].

3. Results and discussion

To evaluate the effects of PI 3-kinase inhibition on the regulatory mechanisms of lipolysis, isolated adipocytes were incubated with wortmannin (0–1000 nM) for 10 min and the effects of insulin on cGI-PDE IK activity, cGI-PDE phosphorylation/activity and antilipolysis were determined. As expected 1 nM insulin almost com-

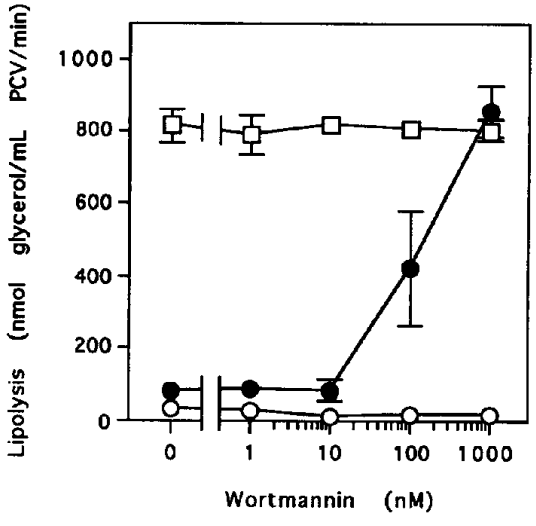

Fig. 1. Effect of wortmannin on the antilipolytic action of insulin. Adipocytes were incubated for 10 min with vehicle (DMSO) or wortmannin at the indicated concentrations before the addition of 100 nM noradrenaline alone (□) or together with 1 nM insulin (●) or no hormone addition (○). Lipolysis was measured as glycerol released to the medium during 30 min. Each point represents mean values ± S.E.M. (n = 3). Basal lipolysis was 82 ± 15 nmol glycerol/ml packed cell volume (PCV)/min and lipolysis stimulated by 100 nM noradrenaline was 815 ± 47 nmol glycerol/ml PCV/min.

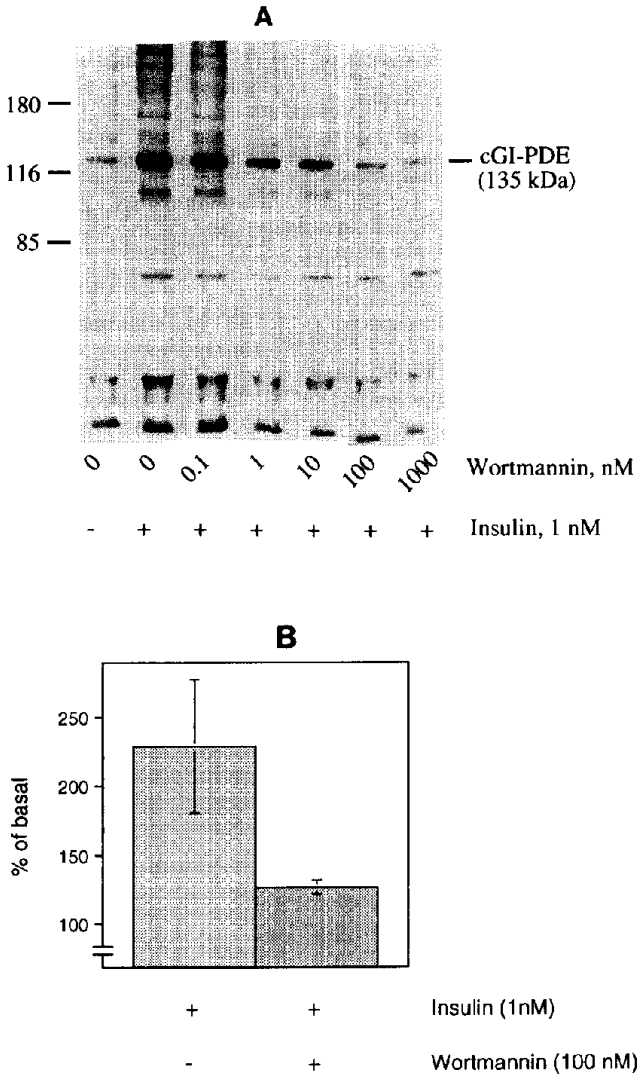

Fig. 2. Effect of wortmannin on the insulin-stimulated phosphorylation and activation of cGI-PDE. (A) ^{32}P-labelled adipocytes were incubated for 10 min with vehicle (DMSO) or wortmannin at the indicated concentrations before addition of 1 nM insulin. cGI-PDE was immunoisolated, subjected to SDS-PAGE and [^{32}P]cGI-PDE visualized by digital imaging of ^{32}P. The same result was obtained in 4 experiments (B). In parallel, using unlabelled adipocytes from the same cell preparation, the effect of 100 nM wortmannin on activation of cGI-PDE by 1 nM insulin was measured in the membrane fraction. Results are expressed as% of basal (mean ± S.E.M., $n = 3$). Basal cGI-PDE activity was 200 pmol/min/ml PCV.

pletely inhibited lipolysis stimulated by 100 nM noradrenaline (Fig. 1). Wortmannin completely blocked this antilipolytic effect with an $IC_{50} \approx 100$ nM, confirming and extending a recent report [15]. Wortmannin did not effect noradrenaline-stimulated or basal lipolysis, indicating that adenylate cyclase-induced increase in cAMP, cAMP PK- and HSL activities per se were not affected. In an in vitro assay, wortmannin did not inhibit HSL activity (data not shown).

Wortmannin completely blocked ($IC_{50} \approx 10$ nM) the five-fold increase in the phosphorylation of the cGI-PDE (Fig. 2A) as well as the activation of the enzyme (Fig. 2B) induced by insulin stimulation. Wortmannin added to the in vitro assay had no effect on cGI-PDE activity (data not shown).

Insulin-stimulated kinases that activate cGI-PDE in vitro (cGI-PDE IK) have been detected in adipocytes and in liver after insulin administration to non-diabetic rats [13,14]. A human platelet cGI-PDE IK that phosphorylates and activates cGI-PDE in vitro has been suggested to be regulated by serine/threonine phosphorylation in response to insulin [12]. As shown in Fig. 3, incubation of adipocytes with insulin resulted in a time-dependent activation of cGI-PDE IK measured as its ability to phosphorylate cGI-PDE in vitro. Activation of this kinase correlated with activation of cGI-PDE in adipocytes (Fig. 3). Half-maximal activation of cGI-PDE IK was obtained with 1 pM insulin (data not shown), in the same range as that causing cGI-PDE activation/phosphorylation [9]. Incubation of adipocytes with wortmannin inhibited insulin-induced activation of cGI-PDE IK with an IC_{50} value of about 30 nM (Fig. 4). The 46-kDa ^{32}P-labelled phosphoprotein also observed in Fig. 4 is likely to be a proteolytic fragment of the cGI-PDE [9] consistent with the inhibition of its phosphorylation by wortmannin with the same IC_{50}. Addition of wortmannin to cytosolic fractions from insulin-stimulated cells containing activated cGI-PDE IK did not inhibit phosphorylation of cGI-PDE, indicating that PI 3-kinase is not a cGI-PDE IK.

Wortmannin at nanomolar concentrations inhibits PI 3-kinase as a result of direct association with the 110 kDa

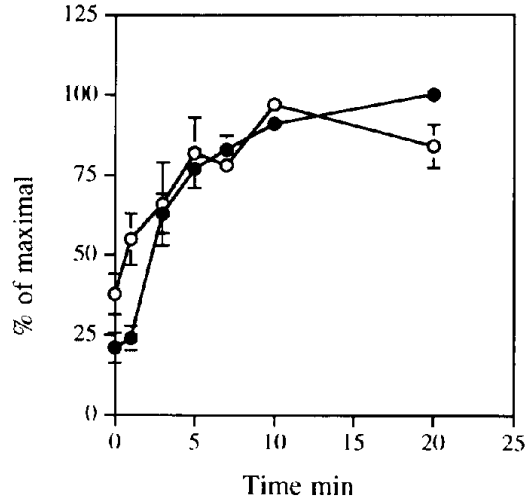

Fig. 3. Time course of insulin stimulation of cGI-PDE IK and cGI-PDE activities in adipocytes. Adipocytes were incubated without or with 1 nM insulin for the times indicated. Cytosolic fractions were obtained and assayed for cGI-PDE IK activity (●), i.e. extent phosphorylation of exogenous added cGI-PDE (see section 2). Crude membrane fractions were obtained and assayed for cGI-PDE activity (○). The results are in each case expressed as% of maximal activity (mean ± S.E.M., $n = 3$). In the absence of insulin, the cGI-PDE activity was 200 pmol/min/ml PCV.

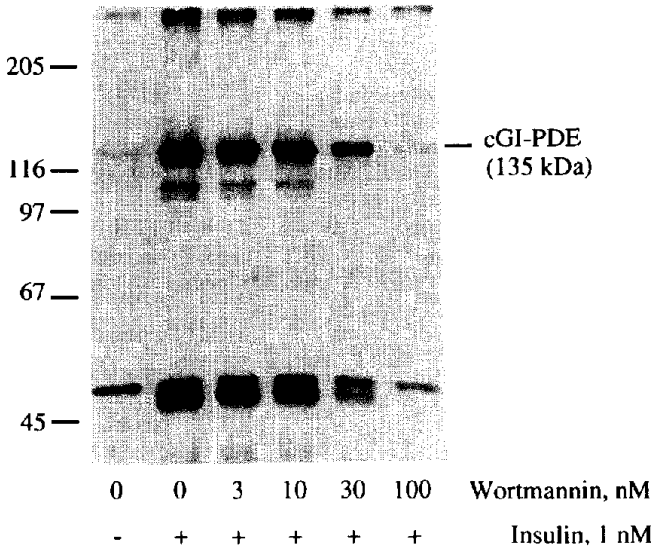

Fig. 4. Effect of wortmannin on insulin stimulation of cGI-PDE IK activity in adipocytes. Adipocytes were incubated for 10 min with vehicle (DMSO) or wortmannin at the indicated concentrations before the addition of 1 nM insulin. Cytosolic fractions were prepared and assayed for cGI-PDE IK activity, i.e. extent phosphorylation of exogenous added cGI-PDE (see section 2). [^{32}P]cGI-PDE was visualized by digital imaging of ^{32}P. The same result was obtained in 3 experiments.

catalytic subunit [39]. Other kinases such as myosin light chain kinase [40] and phosphatidylinositol 4-kinase [15] have been reported to be inhibited by wortmannin at at least 100 fold higher concentrations than those used in the present investigation. Wortmannin has been reported not to inhibit cyclic nucleotide protein kinases, calmodulin-dependent protein kinase II, protein kinase C [40] nor insulin-stimulated tyrosine autophosphorylation of the insulin receptor β-subunit [15]. We have therefore assumed that our results reflect an inhibition of the PI 3-kinase with wortmannin.

In conclusion, our data confirm that wortmannin, a selective PI 3-kinase inhibitor, acts as an inhibitor of the antilipolytic effect of insulin [15]. In addition we show that this inhibition blocks the insulin-induced activation of cytosolic cGI-PDE IK and phosphorylation/activation of cGI-PDE. The activities of these two enzymes were not inhibited by wortmannin in in vitro assays. Taken together these results strongly indicate that PI 3-kinase is involved in mediating the antilipolytic effect at a step(s) upstream of the activation of cytosolic cGI-PDE IK. The identity of the cGI-PDE IK and the mechanism whereby PI 3-kinase activates cGI-PDE IK is presently investigated in our laboratories.

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References

[1] Rosen, O. (1987) Science 237, 1452–1458.
[2] White, M.F. and Kahn, C.R. (1994) J. Biol. Chem. 269, 1–4.
[3] Skolnik, E.Y., Batzer, A., Li, N., Lee, C.-H., Lowenstein, E., Mohammadi, M., Margolis, B. and Schlessinger, J. (1993) Science 260, 1953–1955.
[4] Manganiello, V., Degerman, E., Smith, C. J., Vasta, V., Tornqvist, H. and Belfrage, P. (1992) In Adv. Cycl. Nucl. Res. (Strada, J., Hidaka, H. eds.) Raven Press, New York, pp. 147–164.
[5] Elks, M.L. and Manganiello, V.C. (1985) Endocrinology 116, 2119–2121.
[6] Nilsson, N.Ö., Strålfors, P., Fredrikson, G. and Belfrage, P. (1986) FEBS Lett. 111, 125–130.
[7] Beebe, S.J., Redmon, J.B., Blackmore, P.F. and Corbin, J.D. (1985) J. Biol. Chem. 260, 15781–15788.
[8] Londos, C., Honner, R.S. and Dhillon, G.S. (1985) J. Biol. Chem. 260, 15139–15145.
[9] Degerman, E., Smith, C.J., Tornquist, H., Vasta, V., Manganiello, V. and Belfrage, P. (1990). Proc. Natl. Acad. Sci. USA 87, 533–537.
[10] Smith, C.J., Vasta, V., Degerman, E., Belfrage, P. and Manganiello, V. (1991) J. Biol. Chem. 266 13385–13390.
[11] Smith, C.J. and Manganiello, V.C. (1988) Mol. Pharmacol. 35, 381–386.
[12] Lopez-Aparicio, P., Belfrage, P., Manganiello, V., Kono, T. and Degerman, E. (1993) Biochem. Biophys. Res. Commun. 193, 1137–1144.
[13] Shibata, H. and Kono, T. (1990) Biochem. Biophys. Res. Commun.167, 614–620.
[14] Shibata, H. and Kono, T. (1990) Biochem. Biophys. Res. Commun. 170, 533–539.
[15] Okada, T., Sakuma, L., Fukui, Y., Hazeki, O. and Ui, M. (1994) J. Biol. Chem. 269, 3563–3567.
[16] Panayotou, G. and Waterfield, M.D. (1993) BioEssays 3, 171–177.
[17] Okada, T., Kawano, Y., Sakakibara, T., Hazeki, O. and Ui, M. (1994) J. Biol Chem. 269, 3568–3573.
[18] Dhand, R., Hiles, I., Panayotou, G., Roche, S., Fry, M. J., Gout, I., Totty, N., F., Truong, O., Vicendo, P., Yonezawa, K., Kasuga, M., Courtneidge, S.A. and Waterfield, M.D. (1994) EMBO J. 13, 522–533.
[19] Carpenter, C.L., Duckworth, B.C., Auger, K.R., Cohen, B., Schaffhausen, B.S. and Canley, L.C. (1990) J. Biol. Chem. 265, 19704–19711.
[20] Morgan, S.J., Smith, A.D. and Parker, P.J. (1990) Eur. J. Biochem. 191, 761–767.
[21] Schibasaki, F., Homma, Y. and Takenawa, Y. (1991) J. Biol. Chem. 266, 8108–8114.
[22] Fry, M.J., Panayotou, G., Dhand, R., Ruiz-Larrea, F., Gout, I., Nguyen, O., Courtneidge, S.A. and Waterfield, M.D. (1992) Biochem. J. 288, 383–393.
[23] Keller, S.R. and Lienhard, G.E. (1994) Trends Cell Biol. 4, 115–119.
[24] Myers Jr., M.G. and White, M.F.(1993) Diabetes 42, 643–650.
[25] Sun, X.J., Rothenberg, C.R., Kahn, J.M., Backer, E., Araki, P.A., Wilden, D.A., Cahill, A., Goldstein, B.J. and White, M.F. (1991) Nature 352, 73–77.
[26] Cantley, L.C., Auger, K.R., Carpenter, C., Duckworth, B., Graziani, A., Kapeller, R. and Soltoff, S. (1991) Cell 64, 381–302.
[27] Backer, J.M., Myers Jr., M.G., Shoelson, S.E., Chin, D.J., Sun, X.-J., Miralpeix, M., Hu, P., Margolis, B., Skolnik, E. Y., Schlessinger, J. and White, M.F. (1992) EMBO J. 11, 3469–3479.

[28] Myers Jr., M.G., Becker, J.M., Sun, X.-J., Shoelson, S., Hu, P., Schlessinger, J., Yoakim, M., Schaffhausen, B. and White, M.F. (1992) Proc. Natl. Acad. Sci. USA 89, 10350–10354.
[30] Rodbell, M. (1964) J. Biol. Chem. 239, 375–380.
[31] Honnor, R.C., Dhillon, G.S. and Londos, C. (1985) J. Biol. Chem. 260, 15122 15129.
[32] Degerman, E., Belfrage, P., Hauck Newman, A., Rice, K.C. and Manganiello, V.C. (1987) J. Biol. Chem. 162, 5797–5807.
[33] Taira, M., Hockman, S.C., Calvo, J.C., Taira, M., Belfrage, P. and Manganiello V.C. (1993) J. Biol. Chem. 268, 18573–18579.
[34] Rascon, A., Degerman, E., Taira, M., Meacci, E., Smith, C.J., Manganiello, V., Belfrage, P. and Tornqvist, H. (1994) J. Biol. Chem. 269, 11962–11966.
[35] Laemmli, U.K. (1970) Nature 227, 680–685.
[36] Garland, P.B. and Randle, P.J. (1962) Nature 196, 987–988.
[37] Manganiello, V.C., Murad, F. and Vaughan, M. (1971) J. Biol. Chem. 246, 2195–2202.
[38] Fredrikson, G., Strålfors, P., Nilsson, N.Ö. and Belfrage, P (1981) J. Biol. Chem. 256, 6311 6320.
[39] Yano, H., Nakanishi, S., Kimura, K., Hanai, N., Saitoh, Y., Fukui, Y., Nonomura, Y., Matsuda, Y. (1993) J. Biol. Chem. 268, 25846–25853.
[40] Nakanishi, S., Kakata, S., Takahashi, I., Kawahara, K., Tsukuda, E., Sano, T., Yamada, K., Yoshida, M., Kase, H., Matsuda, Y., Hashimoto, Y and Nonomura, Y. (1992) J. Biol. Chem. 267, 2157–2163.