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90-kDa S6 kinase is insufficient or not involved in the activation of glycogen synthase induced by insulin

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Abstract

Insulin and growth factors increase glycogen synthesis via complex pathways including protein phosphorylation/dephosphorylation processes. We investigated the involvement of 90-kDa S6 kinase in the control of insulin- or epidermal growth factor (EGF)-stimulated glycogen synthase activation using newly synthesized compounds which selectively inhibit 90-kDa S6 kinase. HH-5709 (1-(5-hydroxy-naphthalenesulfonyl)-1*H*-hexahydro-1,4-diazepine) inhibited 90-kDa S6 kinase at lower concentrations than observed for protein kinases A or C. The inhibition by HH-5709 was competitive with respect to ATP with a K_i value of 1.3 μM. H-7, an inhibitor of protein kinases A and C, and HA-1077 (1-(5-isoquinolinesulfonyl)-homopiperazine), where the naphthalene ring of HH-5709 was replaced with isoquinoline, also inhibited 90-kDa S6 kinase to a similar extent as HH-5709. In 3Y1 fibroblasts, H-7 and HA-1077 attenuated the activation of glycogen synthase. HH-5709, however, failed to affect the glycogen synthase activation by either insulin or EGF. These findings suggest that 90-kDa S6 kinase is unrelated or insufficient to mediate activation of glycogen synthase and that unidentified pathway(s) sensitive to H-7 or HA-1077 would be involved in the activation of glycogen synthase by insulin or EGF in 3Y1 fibroblasts.

Keywords: S6 kinase, 90 kDa; Insulin; EGF (epidermal growth factor); Glycogen synthesis; Protein phosphatase 1G

1. Introduction

Insulin plays important roles in cell growth and nutrient metabolism including glucose transport, glycogen synthesis and lipid synthesis. Insulin signal is mediated by intracellular transduction systems including protein kinases/phosphatases. Binding of insulin to its receptor causes a rapid increase in receptor autophosphorylation and receptor tyrosine kinase activity (White and Kahn, 1994), which also phosphorylates insulin receptor substrate (IRS)-1 (White et al., 1985), IRS-2 (Araki et al., 1994) and Shc (*S*rc *h*omologous *c*ollagen-like protein; Src is the product of *src* proto-oncogene which is a cellular homologue of Raus sarcoma virus) (Pronk et al., 1993; Skolnik et al., 1993). These tyrosine-phosphorylated proteins directly bind proteins which possess Src homology 2 (SH2) domains. IRS-1 interacts with various SH2 containing proteins such as p85 regulatory subunit of phosphatidylinositol (PI) 3-kinase, Syp (SH2 containing phosphotyrosine phosphatase)/SH-PTP2 (SH2 containing protein tyrosine phosphatase 2) and Ash (Abundant *src* homology)/Grb2 (Growth factor-receptor bound protein 2) (Myers and White, 1993). Ash/Grb2 binds both IRS-1 and Shc and forms a complex with son-of-sevenless (Sos), a guanine nucleotide releasing factor of Ras (the product of *ras* proto-oncogene which was originally identified as rat-derived Harvey and Kirsten murine sarcoma virus) (Baltensperger et al., 1993; Li et al., 1993, Simon et al., 1993). The activated Ras · GTP complex triggers sequential activation of downstream protein kinases such as Raf-1 kinase (the product of *raf* proto-oncogene whose virus oncogene induces fibrosarcoma), mitogen-activated protein kinase kinase and mitogen-activated protein kinase (Davis, 1993; Blenis, 1993).

90-kDa S6 kinase (also termed insulin-stimulated protein kinase-1), one of the substrates of mitogen-activated protein kinase, was originally identified as the protein kinase that phosphorylates ribosomal protein S6 in response to insulin or growth factors (Sturgill and Wu, 1991). The kinase has been suggested to participate in activation of glycogen synthase by insulin, since this kinase induced phosphorylation of the regulatory subunit of protein phosphatase 1G on site 1 results in dephosphorylation and activation of glycogen synthase (Dent et al.,

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1990). Moreover, glycogen synthase kinase-3, another substrate of 90-kDa S6 kinase, is inactivated when phosphorylated (Sutherland and Cohen, 1994).

EGF is another peptide hormone which has multiple actions via similar mechanisms to insulin, although tyrosine-phosphorylated proteins such as IRS-1 or Shc seems to be absent in the EGF-dependent pathway (White and Kahn, 1994). 90-kDa S6 kinase has been suggested to participate in EGF activation of glycogen synthase in Swiss 3T3 cells (Chan et al., 1987).

However, a recent report casts doubt on this hypothesis. EGF, which activates mitogen-activated protein kinase to a similar extent to insulin, stimulated glycogen synthesis much less than insulin in 3T3-L1 adipocytes (Robinson et al., 1993).

In this paper, we synthesized 90-kDa S6 kinase inhibitors, HH-5709 (1-(5-hydroxynaphthalenesulfonyl)-1*H*-hexahydro-1,4-diazepine), and HA-1077 (1-(5-isoquinolinesulfonyl)-homopiperazine). Using these compounds and H-7 (1-(5-isoquinolinesulfonyl)-2-methylpiperazine), an isoquinolinesulfonamide which inhibits protein kinases (Hidaka et al., 1984), we investigated the role of 90-kDa S6 kinase in the activation of glycogen synthase by insulin and EGF.

2. Materials and methods

2.1. Materials

Bovine insulin, rat EGF, a peptide inhibitor of protein kinase A (fragment 6–22 amide), UDP-glucose were purchased from Sigma. [γ-^{32}P]ATP was from Amersham Japan, UDP-[U-^{14}C]glucose was from DuPont NEN. P81 phosphocellulose paper was from Whatman. Glucose-6-phosphate, sodium β-glycerophosphate, bovine serum albumin were from Wako. Dulbecco's modified Eagle's medium (DMEM) was from Irvine Scientific. Peptide substrate for 90-kDa S6 kinase was synthesized using 431A peptide synthesizer (Applied Biosystems). All other substances were of the purest grade available.

2.2. Protein kinase assays

90-kDa S6 kinase was purified from rabbit skeletal muscle as described previously (Dent et al., 1990). The activity of 90-kDa S6 kinase was measured by using synthetic peptide (KKPLNRTLSVASLPGL) derived from N-terminal domain of glycogen synthase as described by Dent et al. (1990). The assay was performed at 30°C in 50 μl of reaction mixture (pH 7.0) containing 25 mM 3-(*N*-morpholino) propanesulfonic acid (MOPS), 6 mM sodium β-glycerophosphate, 10 mM $MgCl_2$, 2 mM EGTA, 0.2 mM EDTA, 0.1% Tween 20, 0.5 mg/ml bovine serum albumin, 30 μM peptide substrate and the indicated concentrations of [γ-^{32}P]ATP. In the early steps of 90-kDa S6 kinase purification, the activities were measured in the presence of 10 μM peptide inhibitor of protein kinase A to eliminate the phosphorylating activity of protein kinase A. Following a 10-min incubation, reactions were terminated by spotting 40 μl of the reaction mixture onto small pieces of P81 phosphocellulose paper, followed by immediate immersion in 75 mM phosphoric acid. After washing 3 times, the P81 phosphocellulose paper was dried and residual radioactivity was quantified by scintillation counting. The assays of protein kinases A and C were performed using purified materials as reported by Hidaka et al. (1984).

2.3. 90-kDa S6 kinase assay using 3Y1 cell extracts

3Y1 fibroblasts were cultured in DMEM supplemented with 10% fetal calf serum on 90 mm dishes. After pretreatment with HH-5709 for 30 min, 3Y1 cells were washed twice with PBS and homogenized on ice in 100 μl of lysis buffer containing 25 mM MOPS (pH 7.0), 10 mM NaF, 20 mM β-glycerophosphate, 1 mM sodium orthovanadate, 4 mM EGTA, 0.4 mM EDTA and 1 mM phenylmethylsulfonyl fluoride. The homogenates were spun at 4°C for 20 min at $100\,000 \times g$, and the supernatant was used for 90-kDa S6 kinase assay. The kinase assay was carried out basically as described above (Section 2.2), except the presence of 3Y1 cell extracts (600 μg/ml protein as a final concentration) for purified 90-kDa S6 kinase, and a mixture of phosphatase inhibitors (4 mM NaF, 8 mM β-glycerophosphate and 0.4 mM sodium orthovanadate) in the assay mixture. At the end of the 15 min incubation, trichloric acid (final 25%) was added to terminate the reaction and precipitate proteins in the mixture. After spinning at $10\,000 \times g$ for 5 min, the supernatant was collected, spotted and counted by Cerenkov counting as above. Difference between the counts with and without the peptide substrate under parallel conditions was expressed as the activity of 90-kDa S6 kinase. Usually, the count in the absence of the peptide substrate did not exceed 20% of that with the substrate.

2.4. Cell preparation for glycogen synthase assay

After 3Y1 cells reached subconfluence, the medium was replaced with DMEM containing 0.25% fetal calf serum, and the cells were further incubated for 3 days. After washing once with phosphate-buffered saline (PBS), the cells were incubated with DMEM containing 0.25% fetal calf serum for 2 h. The cells were incubated with or without inhibitors as indicated for 30 min before addition of insulin (1 μg/ml) or EGF (100 ng/ml). After an incubation period indicated in the figure captions, the cells were rinsed twice with ice-cold PBS and scraped into 1 ml of extraction mixture containing 50 mM Tris, pH 7.8, 10 mM EDTA, 100 mM NaF, 1 mM dithiothreitol, 1 mM phenylmethylsulfonyl fluoride. The cell suspensions were transferred to 1.5 ml Eppendorf tubes and centrifuged

briefly. The resulting pellets were frozen in liquid nitrogen and stored at −80°C until assayed. Frozen pellets were sonicated in 100 μl of extraction mixture, and centrifuged at 8000 × *g* for 20 min. The supernatant was used for glycogen synthase assay.

2.5. Glycogen synthase assay

Glycogen synthase was assayed by a modification of the method described previously (Sodering et al., 1970). Briefly, 35 μl of cell extract was mixed with 17.5 μl of reaction mixture (final composition: 65 mM Tris, pH 7.8, 10 mM EDTA, 6.3 mg/ml glycogen, 0.5 mM UDP-[U-^{14}C]glucose, 66.7 mM NaF, 0.67 mM dithiothreitol, 0.67 mM phenylmethylsulfonyl fluoride and 10 mM glucose-6-phosphate. The mixture was incubated for 30 min at 30°C. The reaction was terminated by the addition of 60% KOH and boiled for 15 min. Glycogen was precipitated in ethanol containing 1% (w/v) NaCl, and the pellet was dissolved in 1 ml of H_2O, and counted by scintillation spectrometry. The total activity of glycogen synthase was measured in the presence of glucose-6-phosphate, an allosteric activator of the enzyme. The activity of the dephosphorylated form of glycogen synthase is measured without the addition of glucose-6-phosphate. Thus the glycogen synthase ratio (activity without the addition of glucose-6-phosphate/activity in the presence of glucose-6-phosphate) indicates the phosphorylation state of glycogen synthase. This ratio is a useful index to determine the activation state of glycogen synthase in vivo (Chan et al., 1987).

2.6. Statistical analysis of the data

Statistical significance of the data was assessed by unpaired Student's *t*-test.

3. Results

3.1. Effects of H-7, HA-1077 and HH-5709 on protein kinases A and C, and 90-kDa S6 kinase in vitro

The chemical structures of H-7, HA-1077 and HH-5709 are shown in Fig. 1. We investigated the effects of these

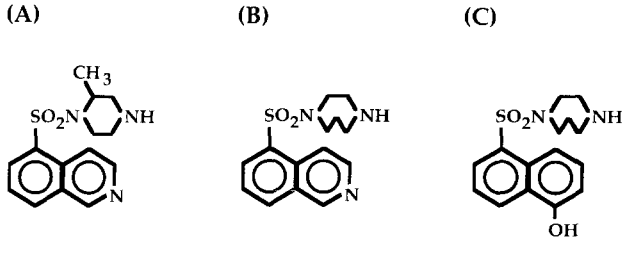

Fig. 1. Chemical structures of H-7 (A), HA-1077 (B) and HH-5709 (C).

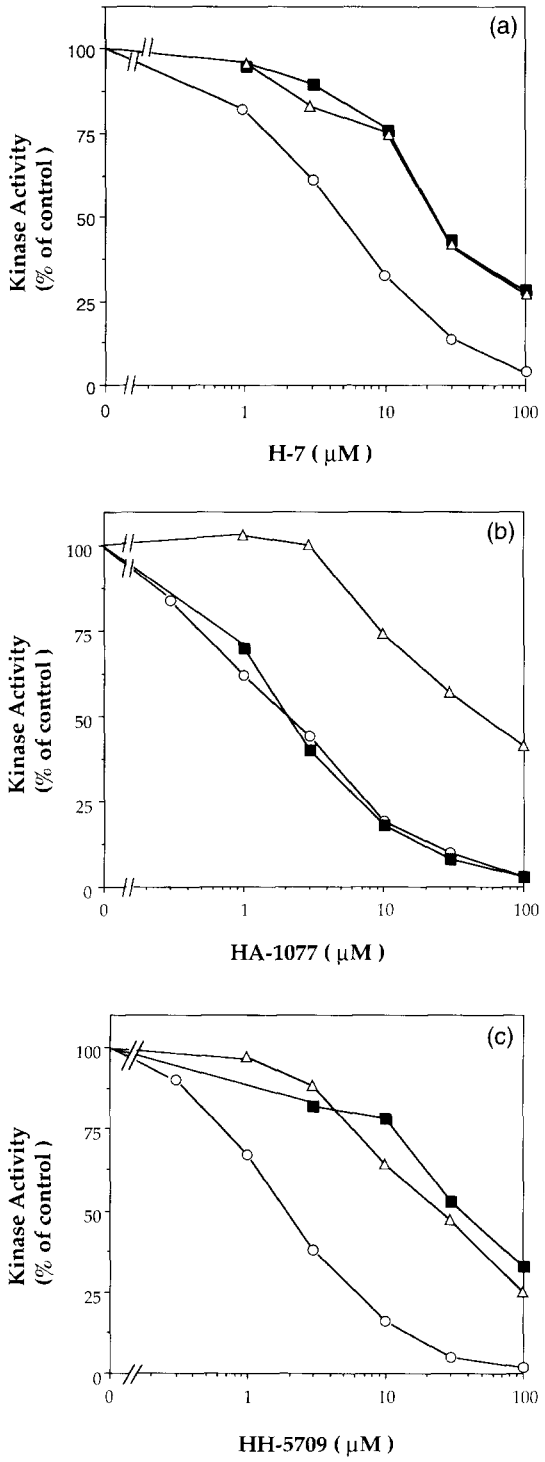

Fig. 2. Effects of H-7, HA-1077 and HH-5709 on 90-kDa S6 kinase (○), protein kinase A (■) and protein kinase C (△). Activities of these protein kinases were measured in vitro in the presence of various concentrations of H-7 (panel a), HA-1077 (panel b) or HH-5709 (panel c) as described in Materials and methods. Results are shown as the mean of duplicate determinations.

three compounds on 90-kDa S6 kinase, and protein kinases A and C which have also been suggested to modulate the activity of glycogen synthase. As shown in Fig. 2a, H-7,

an inhibitor of protein kinases A and C, also inhibited 90-kDa S6 kinase, and the inhibitory effect on 90-kDa S6 kinase was observed at slightly lower concentrations than on the other protein kinases. The IC_{50} values of H-7 with 90-kDa S6 kinase, protein kinases A and C were 4.2 μM, 22 μM and 22 μM, respectively. HA-1077, another isoquinolinesulfonamide derivative, potently inhibited 90-kDa S6 kinase. This compound inhibited the activity of protein kinase A, but not C, as potently as 90-kDa S6 kinase. The IC_{50} values of HA-1077 with 90-kDa S6 kinase, protein kinase A and protein kinase C were 2 μM, 2 μM and 40 μM respectively (Fig. 2b).

We have newly synthesized HH-5709, a novel naphthalenesulfonamide derivative which shares the homopiperazine group with HA-1077. The inhibitory effects of HH-5709 on the three protein kinases are demonstrated in Fig. 2c. HH-5709 inhibited 90-kDa S6 kinase as potently as HA-1077, but the inhibition of protein kinase A was smaller than that for HA-1077. The IC_{50} values of HH-5709 with 90-kDa S6 kinase, protein kinase A and protein kinase C were 1.8 μM, 38 μM and 18 μM respectively. To assess the mode of inhibitory action of HA-1077 and HH-5709, 90-kDa S6 kinase activity was measured with varying concentrations of ATP and the inhibitors. The results analyzed by Dixon plot indicated that both HA-1077 and HH-5709 inhibited 90-kDa S6 kinase in an ATP-competitive manner and the K_i values were 1.1 μM and 1.3 μM, respectively (Fig. 3a and b).

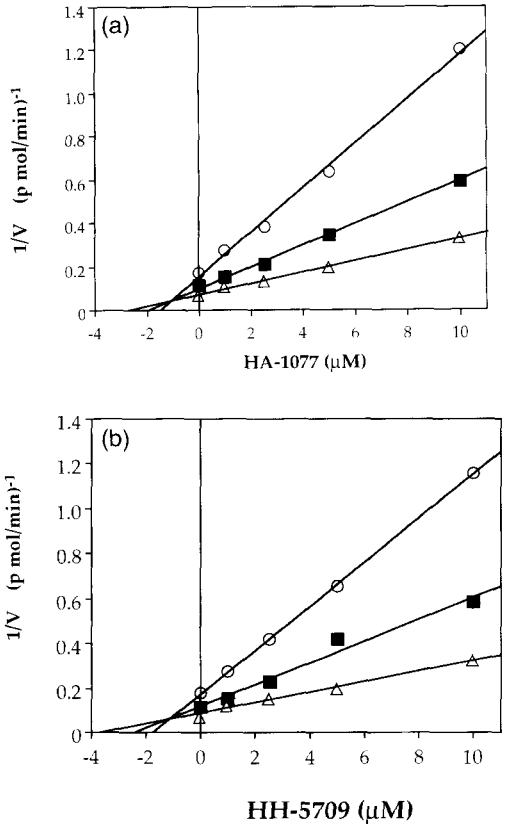

Fig. 3. Kinetic analysis of inhibition of 90-kDa S6 kinase by HA-1077 and HH-5709. Reciprocal velocity versus [inhibitor] for 90-kDa S6 kinase activity with 12.5 (○), 25 (■) and 50 (△) μM ATP in the presence of the indicated concentrations of HA-1077 (panel a) and HH-5709 (panel b). Each symbol is the value from a single determination.

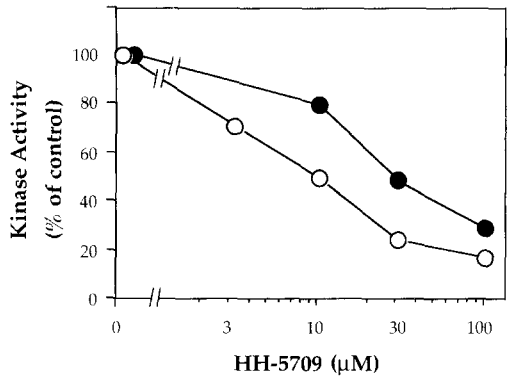

Fig. 4. Effects of HH-5709 on 90-kDa S6 kinase activity in 3Y1 cell extracts. 3Y1 cell extracts were incubated with various concentrations of HH-5709 and [γ-^{32}P]ATP (final concentration 30 μM) in the presence or absence of the peptide substrate for 15 min (open circle). In another set of experiments, 3Y1 cells were pretreated with HH-5709 for 30 min under the culture condition and extracted (closed circle). The kinase assay was terminated by 25% TCA. Radioactivity incorporated into the peptide was counted by Cerenkov counting, and the activity of 90-kDa S6 kinase was determined as the difference in the counts between with and without the peptide substrate. Data are the mean of duplicate determination.

3.2. Inhibition of 90-kDa S6 kinase by pretreatment of 3Y1 cells with HH-5709

As shown in Fig. 4, 30 min pretreatment of 3Y1 cells with HH 5709 dose-dependently decreased the activity of 90-kDa S6 kinase. 50% inhibition was achieved at 30 μM

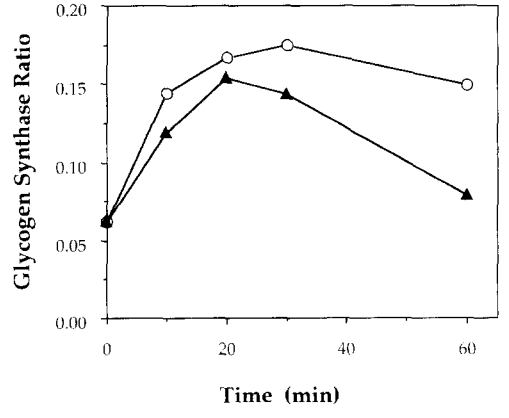

Fig. 5. Time course of glycogen synthase activation by insulin or EGF in 3Y1 cells. 3Y1 fibroblasts were stimulated for 0, 10, 20, 30 and 60 min by 1 μg/ml bovine insulin (○) or 100 ng/ml rat EGF (▲). Cells were extracted and the glycogen synthase assay was performed as described in Materials and methods. The glycogen synthase ratio was calculated as glucose-6-phosphate-independent activity/total activity. Each value is the mean of duplicate determinations.

(closed circle). This value was higher than the IC_{50} value when the inhibitor was directly added to the assay mixture (10 μM, see Fig. 4, open circle), presumably because of a dissociation of the inhibitor from the kinase during extraction (~ 1.5 h).

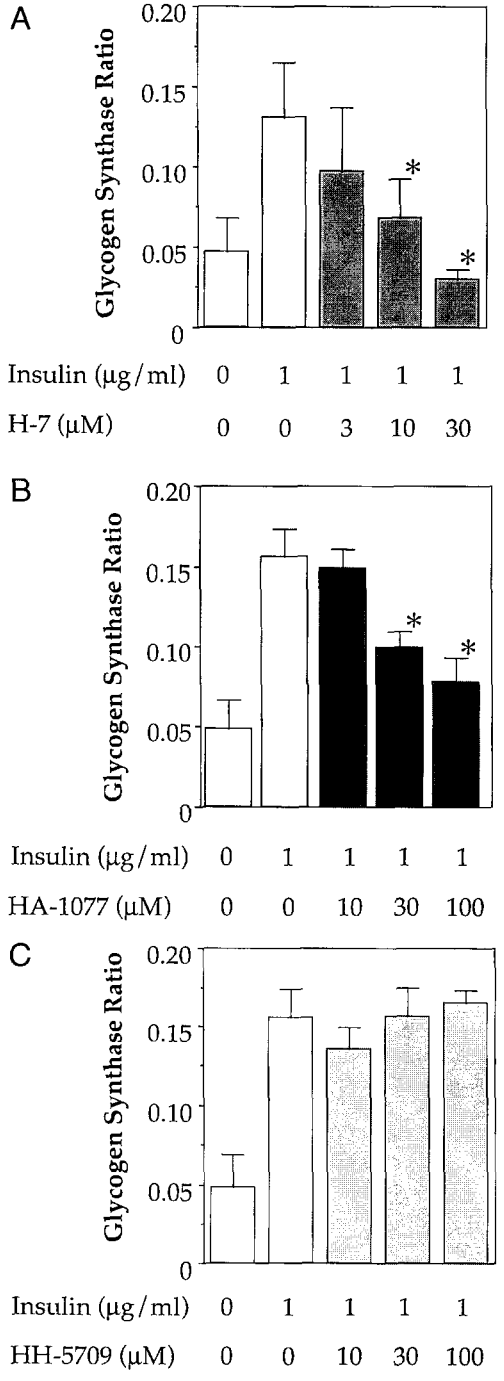

Fig. 6. Effects of H-7 (A), HA-1077 (B) and HH-5709 (C) on the insulin activation of glycogen synthase in 3Y1 fibroblasts. 3Y1 fibroblasts pretreated with increasing concentrations of the inhibitors for 30 min were stimulated with 1 μg/ml insulin for 20 min. Cell extracts by sonication were used for the glycogen synthase assay as described in Materials and methods. The results are expressed as the mean ± S.E. of 3–5 observations from three independent experiments. * $P < 0.05$ compared to controls (without inhibitors).

3.3. Activation of glycogen synthesis by insulin and EGF in 3Y1 fibroblasts

Treatment of the quiescent 3Y1 fibroblasts with insulin or EGF resulted in a 3-fold increase in the activity of glycogen synthase (Fig. 5). The activation was transient and the maximal activation was achieved at 30 min after insulin stimulation and 20 min after EGF stimulation. The activity was then gradually decreased. The decline was more rapid in EGF-treated cells than that observed for insulin stimulation.

3.4. Effects of protein kinase inhibitors on glycogen synthase activation by insulin or EGF in 3Y1 fibroblasts

We examined the effects of the 90-kDa S6 kinase inhibitors on glycogen synthase activity stimulated by insulin in 3Y1 fibroblasts. Insulin at 1 μg/ml increased the activity of glycogen synthase during the 20 min incubation. H-7 completely inhibited glycogen synthase activation by insulin at 30 μM and the IC_{50} value was 5.2 μM (Fig. 6A). HA-1077 also inhibited insulin-stimulated activation of glycogen synthase by 53% at 30 μM and by 73% at 100 μM (Fig. 6B). Although HH-5709 inhibited 90-kDa S6 kinase most potently among the inhibitors (see Fig. 2), neither the basal (0.036 ± 0.006 without HH-5709 vs. 0.029 ± 0.008 with HH-5709, $n = 4–6$ from two independent experiments, not significant) nor insulin-stimulated activity (Fig. 6C) of this enzyme was altered by the addition of 100 μM HH-5709. HH-5709 did not affect glycogen synthase activity stimulated by EGF either (0.131 ± 0.013 without HH-5709 vs. 0.126 ± 0.017 with HH-5709, $n = 3–5$ from two independent experiments, not significant).

4. Discussion

Naphthalenesulfonamides and isoquinolinesulfonamides, which are membrane-permeable and possess inhibitory actions on various serine/threonine protein kinases, have been widely used for pharmacological studies as specific inhibitors to examine biological functions of protein kinases in living cells (for a review, see Hidaka and Kobayashi, 1993). The isoquinolinesulfonamide derivatives, H-7 and HA-1077, inhibit 90-kDa S6 kinase (Stokoe et al., 1992). Here we raised a novel synthetic naphthalenesulfonamide, HH-5709, which also inhibits 90-kDa S6 kinase more potently than protein kinases A and C. As shown in Fig. 1, the difference in the structures of

HH-5709 and HA-1077 is the naphthalene and the isoquinoline rings, which suggests that the homopiperazine group might be related to their interaction with 90-kDa S6 kinase. HH-5709, a more specific inhibitor of 90-kDa S6 kinase than HA-1077, failed to influence glycogen synthase activation in vivo. These findings suggest that 90-kDa S6 kinase may not be involved in or insufficient for the activation of glycogen synthase by insulin.

There is evidence against the involvement of the classical Ras-dependent mitogen-activated protein kinase/90-kDa S6 kinase pathway in the control of glycogen synthesis by insulin. Overexpression of catalytically inactive SH-PTP 2 protein phosphatase attenuated insulin-induced formation of Ras-GTP complex and subsequent activation of mitogen-activated protein kinase cascade, while insulin activated glycogen synthase in the cell line to a similar extent to that in the parental cell line (Sakaue et al., 1995). Moreover, the activation of 90-kDa S6 kinase by insulin was inhibited by a mitogen-activated protein kinase inhibitor in 3T3-L1 adipocytes or L6 myocytes, while glycogen synthase activation was not affected by this inhibitor (Lazar et al., 1995). HH-5709 exhibited an inhibitory effect on 90-kDa S6 kinase when 3Y1 cells were pretreated with the inhibitor before extraction. It is, therefore, unlikely that HH-5709 had no effect on glycogen synthesis simply because this inhibitor failed to penetrate the plasma membrane.

PI 3-kinase may be involved in the Ras/mitogen-activated protein kinase-independent pathway, because the PI 3-kinase inhibitor, wortmannin, inhibits the activation of glycogen synthase (Sakaue et al., 1995) and glycogen synthase kinase-3 (Moule et al., 1995) by insulin. Protein kinase B has recently been suggested to lie between PI 3-kinase and glycogen synthase kinase-3 (Cross et al., 1995). In the present study, we demonstrated that H-7 and HA-1077 attenuated the activation of glycogen synthase by insulin in 3Y1 fibroblasts. This is, to our knowledge, the first report that glycogen synthesis in vivo is inhibited by protein kinase inhibitors. The target proteins of these protein kinase inhibitors are still missing. Although HA-1077 inhibited protein kinase A as potently as 90-kDa S6 kinase, it is unlikely that suppression of glycogen synthase by this compound results from its inhibitory effect on protein kinase A, since phosphorylation of site-2 of glycogen-targeting subunit (G subunit) of protein phosphatase 1G by protein kinase A causes dissociation of the catalytic subunit from G-subunit, which inactivates its dephosphorylating activity (Nakielny et al., 1991). This occurs when epinephrine inhibits glycogen synthesis via binding to the β-adrenergic receptor.

In conclusion, the inhibition of 90-kDa S6 kinase by protein kinase inhibitors is insufficient or does not participate in the activation of glycogen synthase by insulin or EGF in 3Y1 fibroblasts. Pharmacological tools we described here would be helpful to find the protein kinases involved in the activation of glycogen synthase by insulin.

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