Role of Extracellular Electrolytes in the Activation of Ribosomal Protein S6 Kinase by Epidermal Growth Factor, Insulin-like Growth Factor 1, and Insulin in ZR-75-1 Cells

Ilse Novak-Hofer, Willy Küng, and Urs Eppenberger

Laboratory of Biochemistry-Endocrinology, Department of Research, and Department of Gynecology and Obstetrics, University Clinic Medical School, Basel, Switzerland

Abstract. Activation of ribosomal protein S6 kinase by epidermal growth factor (EGF), insulin, and insulin-like growth factor 1 (IGF1) was studied in the human mammary tumor cell line ZR-75-1 in isotonic buffers. In contrast to growth factor-dependent S6 phosphorylation which is strongly dependent on extracellular pH (Chambard, J. C., and J. Pouyssegur. 1986. *Exp. Cell Res.* 164:282–294.) preincubation of cells in buffers with different pH values ranging from 7.5 to 6.5 had no effect on basal or EGF-stimulated S6 kinase activity. Replacement of extracellular Na^+ with choline or replacement of extracellular Ca^{++} with EGTA also did not inhibit stimulation of S6 kinase by EGF. When intracellular Ca^{++} was buffered with the permeable Ca^{++} chelator quin2, EGF stimulation was reduced 50%. A similar inhibition of the EGF response was observed when cells were incubated in buffers with high K^+ concentrations or in the presence of the K^+ ionophore valinomycin. Insulin and IGF1 stimulation of S6 kinase were also inhibited by high K^+ concentrations and by buffering intracellular Ca^{++}. In contrast to the responses to EGF, insulin- and IGF1-activation of S6 kinase was enhanced when glucose was present and depended on the presence of bicarbonate in the medium.

The results indicate that ionic signals generated by growth factors and insulin, such as increases in intracellular pH or Na^+, do not seem to be involved in the activation of S6 kinase. However, effects of growth factors or insulin on membrane potential and/or K^+ fluxes and redistribution of intracellular Ca^{++} may play a role in the activation process. Furthermore, the mechanism of insulin activation of S6 kinase is distinct from the growth factors by its dependency on extracellular bicarbonate.

GROWTH of the human mammary tumor cell line ZR-75-1 is stimulated by 17 β-estradiol (E_2) and growth factors such as epidermal growth factor (EGF)[1] and insulin like growth factor 1 (IGF1) (Allegra and Lippman, 1980; Darbre et al., 1983; Küng et al., 1986). An early prereplicative response of ZR-75-1 cell to stimulation with EGF, but not with E_2, is the phosphorylation of the 40S ribosomal protein S6 (Novak-Hofer et al., 1987). In ZR-75 cells as well as in other cell systems S6 phosphorylation is temporally linked to an increase in protein synthesis (Novak-Hofer and Thomas, 1985; Novak-Hofer et al., 1987; Thomas et al., 1982). In vivo as well as in vitro studies suggest that phosphorylation of S6 may facilitate initiation of protein synthesis (Gressner and Van de Leur, 1980; Duncan and McConkey, 1982; Palen and Traugh, 1987).

EGF-induced S6 phosphorylation is preceded by the activation of a specific S6 kinase (Novak-Hofer and Thomas, 1985) which can be recovered in its active form in cell free extracts (Novak-Hofer and Thomas, 1984). Activation of S6 kinase is a rapid and transient response to EGF with kinetics similar to the growth factor–mediated induction of several genes, including c-fos (Müller et al., 1984; Morgan and Curran, 1986). Similar to the induction of this class of "early" growth related genes (Lau and Nathans, 1987), activation of S6 kinase is also potentiated in the presence of protein synthesis inhibitors (Tabarini et al., 1985; Blenis and Erikson, 1986). Furthermore, it was found by chromatographic fractionation of extracts from stimulated cells that the action of a number of different growth factors and mitogens converges in activating the same S6 kinase (Novak-Hofer et al., 1986; Blenis and Erikson, 1986).

The mechanism by which EGF causes activation of S6 kinase is unknown, but there are indications that phosphorylation of the kinase may be involved (Novak-Hofer and Thomas, 1985). Because activation takes place within minutes, rapid ionic changes induced by EGF may mediate S6 kinase activation. Effects of growth factors on ion fluxes across the plasma membrane cannot be reproduced in a cell-free system, and this may explain why to date no such system

1. *Abbreviations used in this paper*: EGF, epidermal growth factor, IGF1, insulin-like growth factor; pH_i, intracellular pH; pH_o, extracellular pH.

exists for activating S6 kinase in vitro. The experimental approach used by us to assess the importance of ionic signals was to investigate how extracellular electrolytes influence the activation of S6 kinase by EGF.

In analogy to other growth factors EGF activates a Na^+/H^+ antiporter, thereby shifting the intracellular pH to slightly (~0.2 U) more alkaline values (Rothenberg et al., 1983; Hesketh et al., 1985; Moolenaar et al., 1986). It has been demonstrated that the manipulation of intracellular pH (pH_i) by changing extracellular pH (pH_o) modifies growth factor-induced DNA synthesis and S6 phosphorylation and that both of these events can only take place above a certain pH_o value (Pouyssegur et al., 1985; Chambard and Pouyssegur, 1986). To investigate if EGF-induced changes of the pH_i are responsible for the activation of S6 kinase, the effect of imposing different pH_o values in the medium on the response was studied.

In several cultured cell lines EGF leads to mobilization of Ca^{++}, possibly both from extracellular (Hesketh et al., 1985; Moolenaar et al., 1986) as well as intracellular (Pandiella et al., 1987; Hepler et al., 1987) stores. Therefore, the effect of depleting extracellular Ca^{++} with EGTA as well as of buffering intracellular Ca^{++} with the permeable Ca^{++} chelator quin2 was studied next. Since growth factor-stimulated DNA synthesis strongly depends on Na^+_o (Schuldiner and Rozengurt, 1982; Rozengurt, 1986) the effect of replacing NaCl with choline chloride on the activation of S6 kinase by EGF was also investigated. Finally the effects of removing K^+ from the extracellular medium as well as of raising the K^+ concentration in the medium and thereby reversing the normal K^+ gradient across the membrane was studied. To test the hypothesis that the membrane potential maintained by the K^+ gradient regulates activation of S6 kinase by EGF, the effect of the K^+ ionophore valinomycin was also tested.

Attempting to find out if common ionic requirements exist for S6 kinase activation by different growth factors, the effects of IGF1 and insulin in different isotonic buffers was studied. Because of recent reports on the effect of extracellular bicarbonate (Shechter and Ron, 1986) as well as of intracellular Ca^{++} (Pershadsingh et al., 1987) on insulin action in the adipocyte, special attention was directed to these ions. In the course of this study we found a common regulation of the EGF, IGF1, and insulin activation of S6 kinase by extracellular K^+ and by intracellular Ca^{++}, whereas insulin activation of S6 kinase showed a distinct requirement for extracellular bicarbonate.

Materials and Methods

Cell Culture and Stimulation Protocol

Cells (ZR-75-1; American Type Culture Collection, Rockville, MD) were grown in improved minimal essential medium, zinc option ("Richter's" medium) as described previously (Novak-Hofer et al., 1987) in 100-mm^2 dishes up to a density of 5×10^6 cells. Cultures were kept one night in serum-free Richter's medium and were then preincubated for 40 min at 37°C in the buffers indicated in the figure legends. Then cultures were treated either for 15 min with 100 ng/ml EGF (Collaborative Research, Lexington, MA) or for 30 min with 100 ng/ml IGF1 (Amersham, U.K.) or for 30 min with 100 ng/ml insulin (Sigma Chemical Co., St. Louis, MO). Quin2 (acetoxymethyl)ester (quin2/AM) was from Calbiochem and valinomycin was from Boehringer Mannheim (FRG).

Measurement of S6 Kinase Activity

Cells were extracted on ice and soluble extracts were prepared as described previously (Novak-Hofer et al., 1987). 40S ribosomal subunits used as substrate in the S6 kinase assay were prepared from Artemia salina cysts as described (Zasloff and Ochoa, 1974). Phosphorylation assays, SDS-electrophoresis, and autoradiography were performed as described earlier (Novak-Hofer and Thomas, 1984). S6 kinase activity was quantified by cutting out the S6 band from stained and dried SDS gels and its radioactivity was determined by liquid scintillation counting.

Results

Effect of pH_o on the Stimulation of S6 Kinase by EGF

Addition of EGF to A431 cells leads to the activation of a Na^+/H^+ antiporter, resulting in a slight (~0.2 U) intracellular alkalinization (Rothenberg et al., 1982; Moolenaar et al., 1986). It has been demonstrated that growth factor-induced DNA synthesis and phosphorylation of ribosomal protein S6 are very sensitive to pH_o (Chambard and Pouyssegur, 1986; Pouyssegur et al., 1985), a linear relationship existing between pH_o and pH_i (L'Allemain et al., 1984). When cells are exposed to different pH_o values no reinitiation of DNA synthesis and S6 phosphorylation is observed below pH 6.9 (Chambard and Pouyssegur, 1986). To find out if the strict pH dependency observed for S6 phosphorylation is due to a pH sensitivity of S6 kinase activation, cells were stimulated with EGF in bicarbonate-free media buffered to a range of pH values between 7.5 and 6.5. It was observed (Fig. 1) that EGF activation of S6 kinase (5–10-fold increase in activity above control levels) is largely independent of pH_o and takes place at pH_o below 6.8 which is not permissive for S6 phosphorylation. As a control the effect of different pH values on S6 kinase activity in cell-free extracts from controls (Fig. 1, solid triangles) and from EGF-treated (Fig. 1, open triangles) cells was measured. It was found that S6 kinase activity in vitro remains the same over a rather broad range of pH values. In contrast to the unimpaired activation of S6 kinase we have observed, similar to Chambard and Pouyssegur (1986), that no S6 phosphorylation occurred below pH_o 7.1 in ZR-75 cells (data not shown). The tight control which pH exerts on growth factor stimulation of S6 phosphorylation thus appears to be at the level of substrate phosphorylation rather than enzyme activation.

Effect of Extracellular (Ca^{++}_o) and Intracellular (Ca^{++}_i) on the Stimulation of S6 Kinase by EGF

Binding of EGF to its cell surface receptors triggers an increase in Ca^{++}_i, apparently both by increasing Ca^{++} influx from extracellular medium (Hesketh et al., 1985; Moolenaar et al., 1986) as well as by redistribution of intracellular Ca^{++} (Pandiella et al., 1987; Hepler et al., 1987). Because changes in Ca^{++}_i could regulate the response of S6 kinase to EGF, we investigated how buffering of Ca^{++}_o and of Ca^{++}_i affects the stimulation of S6 kinase by EGF. ZR-75-1 cells were serum-deprived overnight and then preincubated in a simple isotonic buffer (modified Krebs–Ringer buffer without bicarbonate and buffered with Hepes to pH 7.3), where overall activity of S6 kinase was about three times lower than in the complex Richter's medium, but where stimulation by EGF was similarly 5–10-fold (Fig. 2, bars 1 and 2). When Ca^{++}_o was replaced by 0.5 mM EGTA, activation of S6 ki-

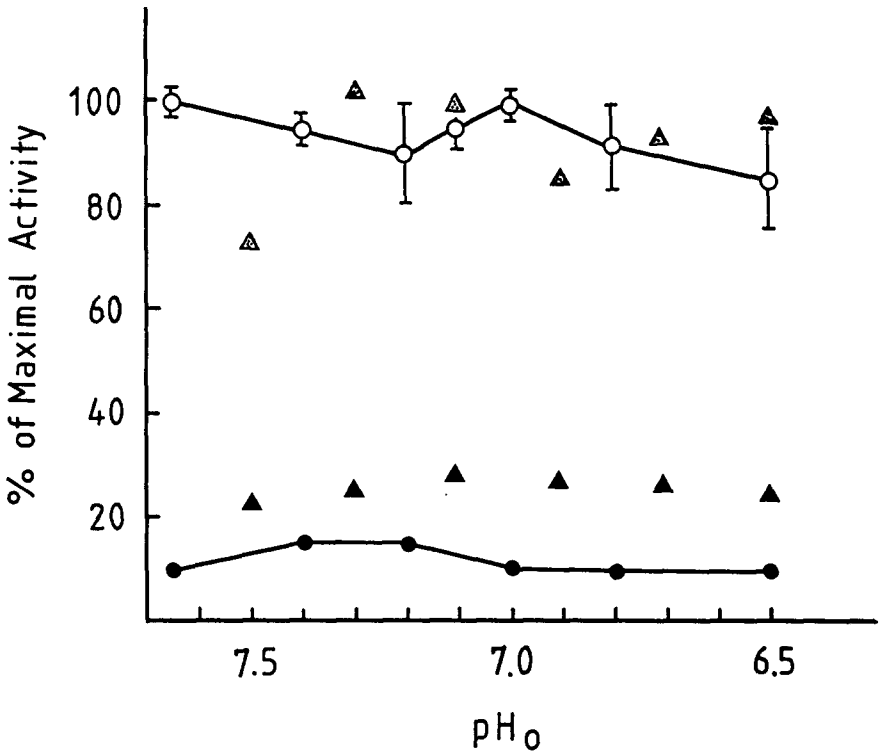

Figure 1. PH_o dependence of the activation of S6 kinase by EGF ZR-75-1 cells were grown in 100-mm² dishes (5.10^6 cells) in Richter's medium and serum-deprived for 16 h as described previously (Novak-Hofer et al., 1987). Cultures were then incubated for 1 h at 37°C in the absence of CO_2 in bicarbonate-free Richter's medium supplemented with 1 mg/ml of BSA and buffered with 25 mM Pipes, or 3-N-morpholino-ethanesulfonic acid (MOPS) or Hepes to the indicated pH as described (Chambard and Pouyssegur, 1986). EGF (10^{-8} M was added for 15 min after which the pH of the medium was measured and designated as pH_o. Cellular extracts were prepared and S6 kinase activity of extracts from controls (solid circles) and from EGF-treated cells (open circles) was measured as indicated in Material and Methods. Results (means ± SEM of 4 independent experiments) are expressed as percent of maximal S6 kinase activity. 100% correspond to 3.2 pmol of phosphate incorporated into S6/min/mg protein. Triangles represent the effect of pH on S6 kinase activity in vitro in cell-free extracts from controls (*solid triangles*) and from EGF-treated cells (*open triangles*). Results are means of two experiments and 100% activity corresponds to 3.5 pmol of phosphate incorporated into S6 min/mg protein.

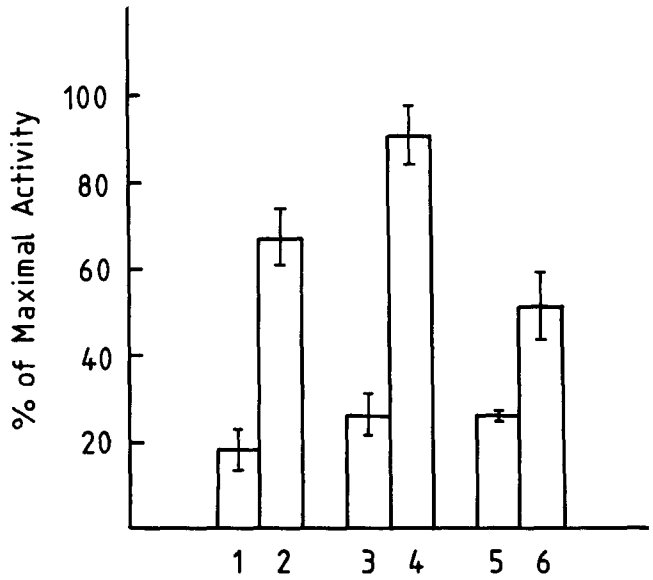

Figure 2. $Ca^{2+}{}_o$ and $Ca^{2+}{}_i$ dependence of the activation of S6 kinase by EGF. Serum-deprived ZR-75-1 cells were incubated for 40 min at 37°C in the absence of CO_2 in a medium consisting of 120 mM NaCl, 5 mM KCl, 1.4 mM $CaCl_2$, and 1.2 mM $MgSO_4$, supplemented with 10 mM Hepes, pH 7.3, and 1 mg/ml BSA (Fig. 2, bars 1 and 2) or in the same medium in which $CaCl_2$ was replaced by either 0.5 mM EGTA (bars 3 and 4) or by 0.5 mM EGTA and 100 μM of quin2/AM (bars 5 and 6). After this preincubation EGF (10^{-8} M) was added and cells were incubated for an additional 15 min. Extracts were prepared and S6 kinase activity was measured in extracts from control cells (bars 1, 3, and 5) and EGF-stimulated cells (bars 2, 4, and 6) as described in Materials and Methods. Results (mean ± SD of four independent experiments) are expressed as a percentage of maximal S6 kinase activity. 100% correspond to 0.93 pmol of phosphate incorporated into S6 min/mg protein.

nase by EGF was consistently enhanced ~25% (Fig. 2, cf. bars 2 and 4), indicating that Ca^{++} influx from extracellular medium is not required for EGF activation of S6 kinase.

When $Ca^{++}{}_i$ was buffered using the permeable Ca^{++} chelator quin2 it was found that after preincubation of cells in the presence of 0.5 mM EGTA and 100 μM quin2 the response of S6 kinase to EGF was inhibited to ~50% (Fig. 2, cf. bars 4 and 6). To investigate whether this inhibition could be due to a general toxic effect of quin2, a wash-out experiment was performed. Quin2-pretreated cells were washed once, reincubated in new buffer for 15 min, and then stimulated with EGF for 15 min. The inhibitory effect of the quin2 pretreatment persisted which argues against a general toxic effect (data not shown). This result indicates that redistribution of $Ca^{++}{}_i$ is involved in the activation of S6 kinase by EGF. When the effect of Ca^{++} in vitro on S6 kinase activity was measured in cell-free extracts from EGF-treated cells, Ca^{++} concentrations (50 μM–5 mM) were found to be inhibitory (data not shown).

Effect of Extracellular Na^+ ($Na^+{}_o$) and Extracellular K^+ ($K^+{}_o$) on the Stimulation of S6 Kinase by EGF

Reinitiation of DNA synthesis by growth factors requires the presence of Na^+ in the medium (Schuldiner and Rozengurt, 1982; Rozengurt, 1986), an increase in $Na^+{}_i$ in re-

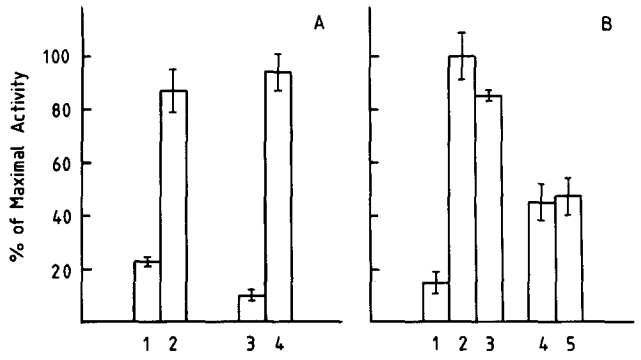

Figure 3. $Na+_o$ and K^+_o dependence of the activation of S6 kinase by EGF. (*A*) Serum-deprived ZR-75-1 cells were incubated for 40 min at 37°C in the absence of CO_2 in a medium consisting of 120 mM NaCl, 5 mM KCl, 1.2 mM $MgSO_4$ supplemented with 10 mM Hepes, pH 7.3, and 1 mg/ml BSA (bars 1 and 2) or in a medium in which NaCl was replaced by 120 mM choline chloride (bars 3 and 4). Cultures were treated with 10^{-8} M EGF for 15 min, extracts were prepared and S6 kinase activity was measured in extracts from control cells (bars 1 and 3) and EGF-stimulated cells (bars 2 and 4). Results (mean ± SD of four independent experiments) are expressed as a percentage of maximal S6 kinase activity. 100% correspond to 1.95 pmol of phosphate incorporated into S6 min/mg protein. (*B*) Before EGF addition, cells were preincubated for 40 min in the absence of CO_2 in a medium consisting of 125 mM choline chloride, 1,2 mM $MgSO_4$ (bars 1 and 2) or 120 mM choline chloride, 5 mM KCl, 1.2 mM $MgSO_4$ (bar 3) or 5 mM choline chloride, 120 mM KCl, 1.2 mM $MgSO_4$ (bar 4) or 120 mM choline chloride, 5 mM KCl, 1.2 mM $MgSO_4$ and 4 μM valinomycin (bar 5). Media were all supplemented with 10 mM Hepes, pH 7.3, and 1 mg/ml BSA. After EGF addition (10^{-8} M) incubation was continued for 15 min. Extracts were prepared from control cells (bar 1) or EGF-stimulated cells (bars 2–4) and S6 kinase activity was measured as described. Results (mean ± SD of three independent experiments) are expressed as a percentage of maximal activity, 100% corresponding to 2.0 pmol of phosphate incorporated into S6 min/mg protein.

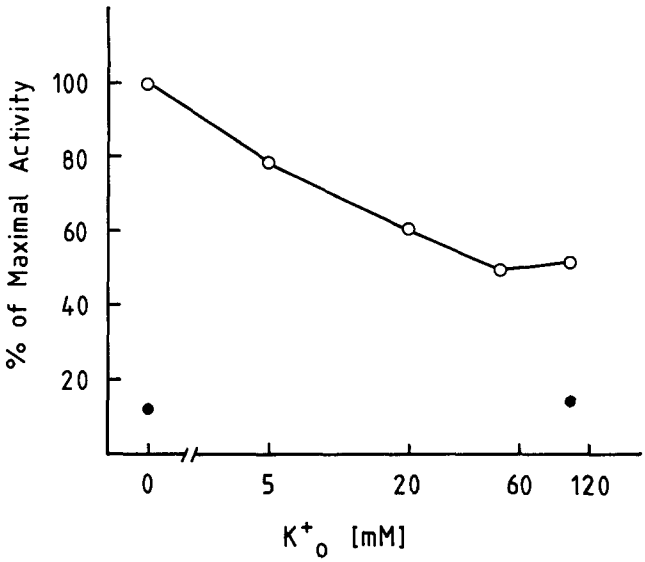

Figure 4. Inhibition of activation of S6 kinase by EGF with increasing K^+_o concentration. Serum-deprived ZR-75-1 cells were incubated for 40 min at 37°C in the absence of CO_2 in media consisting of the indicated KCl concentrations which were compensated with choline chloride to a total concentration of monovalent cations of 125 mM and supplemented with 1.2 mM $MgSO_4$, 10 mM Hepes, pH 7.3, and 1 mg/ml BSA. Cultures were then stimulated for 15 min with 10^{-8} M EGF. Extracts were prepared from either control cells (*solid circles*) or EGF-treated cells (*open circles*) and tested for S6 kinase activity as described. Results are means of two independent experiments and are expressed as a percentage of maximal S6 kinase activity. 100% correspond to 1.02 pmol of phosphate incorporated into S6 min/mg protein.

sponse to growth stimulation having been observed early on (Smith and Rozengurt, 1978). We have investigated whether a requirement for Na^+_o exists for the activation of S6 kinase by EGF. When NaCl was replaced by equimolar amounts of choline chloride, basal S6 kinase activity was somewhat lowered (Fig. 3 *A*, cf. bars 1 and 3), whereas stimulation of S6 kinase by EGF was found to be unimpaired in Na^+-free medium (Fig. 3 *A*, cf. bars 2 and 4).

In the course of this study the Krebs–Ringer buffer was finally simplified to a buffer consisting of isotonic choline chloride and 1.2 mM $MgSO_4$ (in contrast to Na^+, Ca^{++}, phosphate, and bicarbonate, $MgSO_4$ was found to be important for stimulation of S6 kinase to occur). In this most "minimal" medium without K^+_o the effect of EGF on S6 kinase was found to be enhanced ~20–30% compared with the same buffer with the normal 5 mM KCl (Fig. 3 *B*, cf. bars *2* and *3*). This observation suggested that because the absence of external K^+ increases the activation, depolarizing conditions (high external K^+) might reduce the response to EGF. Indeed, when cells were stimulated in a buffer containing high K^+ and low Na^+ concentrations, activation of S6 kinase by EGF was reduced ~50% (Fig. 3 *B*, bar 4). A stepwise increase in K^+_o concentrations, while isotonicity was maintained with choline chloride led to a dose-dependent inhibition of the EGF response (Fig. 4). To test the hypothesis that the membrane potential maintained by the K^+ gradient across the membrane regulates the response to EGF, cells were stimulated after preincubation with the K^+ ionophore valinomycin. This treatment destroys the normal K^+ gradient across the membrane and reduced the effect of EGF on S6 kinase to the level observed at the high K^+ concentration (Fig. 3 *B*, bar 5). The nonspecific monovalent cation ionophore Gramicidin S or low doses of digitonin or Triton X-100 showed a similar effect (data not shown). Control experiments on the effect of increasing NaCl and KCl concentrations in vitro on S6 kinase activity from EGF-treated cells showed inhibition of S6 kinase activity starting at 10 mM and full inhibition at 150 mM NaCl or KCl in the assay (data not shown).

Effect of Glucose and Extracellular Bicarbonate on the Stimulation of S6 Kinase by Insulin and IGF1

The experiments on the ionic requirements of the EGF stimulation of S6 kinase were performed in bicarbonate-free buffers, because no effect of bicarbonate (or phosphate) on this response had been observed. However, we noticed that insulin stimulation of S6 kinase was impaired in such buffers. This observation as well as recent reports on the role of extracellular bicarbonate on insulin action in the adipocyte (Shechter and Ran, 1986) led us to investigate the role of bicarbonate in the stimulation of S6 kinase by insulin. ZR-75-1

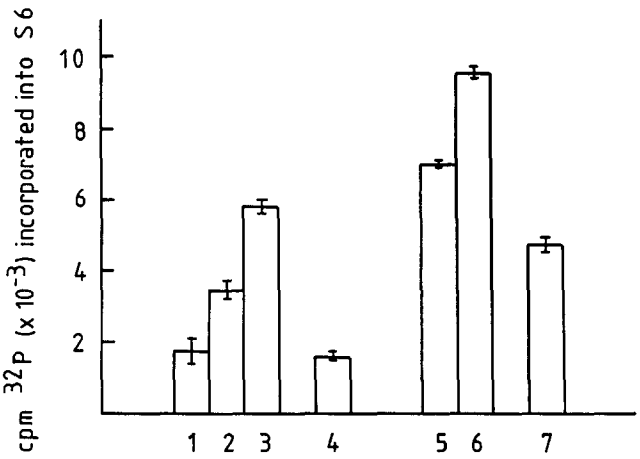

Figure 5. Effect of bicarbonate and glucose on the stimulation of S6 kinase by insulin and IGF1. ZR-75-1 cells (5×10^6 cells) were kept over night in serum-free Richter's medium and then incubated for 40 min at 37°C in a buffer containing 110 mM NaCl, 25 mM $NaHCO_3$, 5 mM KCl, 1.2 mM KH_2PO_4, 1.3 mM $CaCl_2$, 1.3 mM $MgSO_4$, 5 mM glucose and 1 mg/ml BSA; pH was adjusted to 7.3 with HCl (bars 1, 3, and 6). Parallel cultures were incubated in the same buffer without glucose (bars 2 and 5) or in a buffer consisting of 120 mM NaCl, 10 mM Hepes, pH 7.3, 5 mM KCl, 1.3 mM $CaCl_2$, 1.3 mM $MgSO_4$, 5 mM glucose and 1 mg/ml BSA (bars 4 and 7); incubations in this buffer were in the absence of CO_2. After preincubation cells were treated for 30 min with 100 ng/ml insulin (bars 2–4) or 100 ng/ml IGF1 (bars 5–7). Extracts were prepared and S6 kinase activity was measured as described in Material and Methods. Results are means ± SD of two independent experiments, 2000 cpm corresponding to 0.3 pmol phosphate incorporated into S6 min/mg protein.

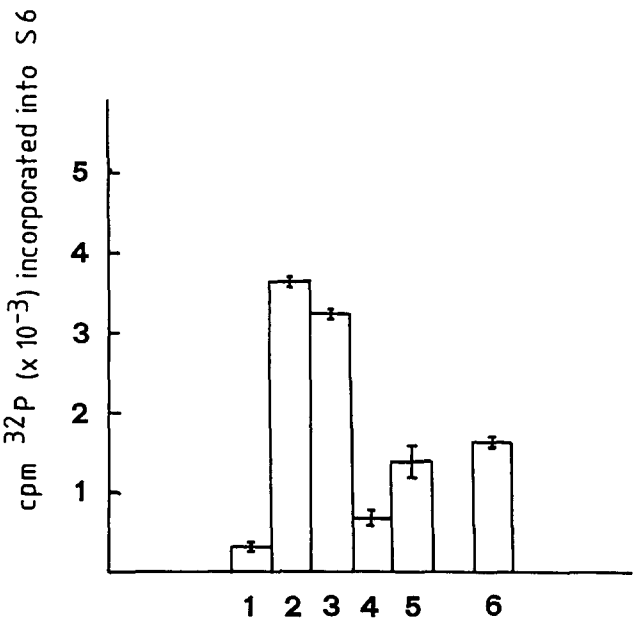

Figure 6. Effect of EGTA, quin2, and high K^+ on the stimulation of S6 kinase by insulin. ZR-75-1 cells were kept overnight in serum-free Richter's medium and then incubated for 40 min at 37°C in a buffer containing 110 mM NaCl, 25 mM $NaHCO_3$, 5 mM KCl, 1.2 mM KH_2PO_4, 1.3 mM $MgSO_4$, 5 mM glucose, and 1 mg/ml BSA; pH was adjusted to 7.3 with HCl (bars 1 and 2) or in the same buffer in which $CaCl_2$ was replaced either by 0.5 mM EGTA (bar 3) or by 0.5 mM EGTA and 100 μM of quin2/AM (bars 4 and 5) or a buffer with 100 mM KCl was used (bar 6). After preincubation cells were left for 30 min longer (controls, bars 1 and 4) or were treated for 30 min with 200 ng/ml insulin (bars 2, 3, 5, and 6). Extracts were prepared and S6 kinase activity was measured as described in Material and Methods. Results are mean ± SD of two independent experiments, 1000 cpm corresponding to 0.1 pmol phosphate incorporated into S6 min/mg protein.

cells were serum-deprived over night and then preincubated for 40 min at 37°C in Krebs–Ringer buffer containing 25 mM $NaHCO_3$ and 1.3 mM KH_2PO_4, or the same buffer without bicarbonate and phosphate and buffered with Hepes to pH 7.3. In some experiments these buffers also included 5 mM glucose. After cells were treated for 30 min with 100 ng/ml insulin and S6 kinase activity was measured it was found that the presence of glucose enhanced insulin stimulation of S6 kinase ~50% (Fig. 5, cf. bars 2 and 3). In the absence of bicarbonate and phosphate insulin had no effect on S6 kinase even in the presence of glucose (Fig. 5, bar 4). This lack of response was due to the absence of bicarbonate and not of phosphate and could be overcome by a 50-fold higher dose of insulin (5 μg/ml) at least partially (data not shown).

When cells were treated with 50 ng/ml IGF1, S6 kinase was activated to a higher extent than with insulin (Fig. 5, bar 5), glucose enhanced IGF1 stimulation somewhat (Fig. 5, bar 6) and ~50% of the IGF1 response were inhibited in bicarbonate-free medium (Fig. 5, bar 7).

Effect of Ca^{++}_o, Ca^{++}_i and K^+_o on the Stimulation of S6 Kinase by Insulin and IGF1

As we had observed that Ca^{++}_i and K^+_o regulate the activation of S6 kinase by EGF (Figs. 2–4), it was investigated if the response to insulin and IGF1 is regulated in a similar manner. Serum-starved ZR-75-1 cells were preincubated in a Krebs–Ringer buffer containing bicarbonate and glucose. When $CaCl_2$ was replaced by 0.5 mM EGTA, insulin stimulation of S6 kinase was not much affected (Fig. 6, bars 2 and 3). In contrast to the response to EGF, insulin stimulation was not enhanced under these conditions. When preincubations were in the presence of EGTA and 100 μM quin2, the effect of insulin on S6 kinase was reduced by more than 50% (Fig. 6, bar 5). Insulin activation in buffers with high KCl and low NaCl concentrations was reduced to a similar extent (Fig. 6, bar 6). The response of S6 kinase to IGF1 was reduced in a similar manner when cells were either incubated in the presence of quin2 or stimulated in high K^+ buffers (data not shown).

Discussion

Binding of EGF to its cell surface receptor generates a number of ionic signals such as increases in pH_i, Na^+_i, and Ca^{++}_i (e.g., Rothenberg et al., 1982; Hesketh et al., 1985; Moolenaar et al., 1986; Pandiella et al., 1987; Hepler et al., 1987) which are believed to participate in transmembrane signaling leading to changes in gene expression and finally increased DNA synthesis. We have investigated the role of such ionic signals in the activation of ribosomal protein S6 kinase by EGF in ZR-75-1 cells. This human mammary tumor cell line possesses a small number of high affinity EGF receptors

(Roos et al., 1986; Fabbro et al., 1986) and responds to EGF with increased DNA synthesis and, within minutes, with an ~10-fold increase in S6 kinase activity (Novak-Hofer et al., 1987). Activation of S6 kinase was studied in simple isotonic buffers where overall activity was about three times lower than in the complex Richter's medium, but where the stimulatory activity by EGF was maintained at the same level. Neither lowering extracellular pH to pH_o 6.5 (Fig. 1) or depleting extracellular Ca^{++} with EGTA (Fig. 2) or replacing extracellular Na^+ with choline (Fig. 3 *A*) inhibited the stimulation of S6 kinase by EGF. Also bicarbonate or phosphate ions were not required for the EGF response (Fig. 1). These results argue against EGF-induced changes in pH_i or Na^+_i or an influx of Ca^{++} from extracellular medium being involved in the activation of S6 kinase. Activation of S6 kinase by EGF was found to be blocked ~50% by buffering intracellular Ca^{++} with quin2 (Fig. 2) and a similar inhibition was observed when in an isotonic buffer consisting of choline chloride and $MgSO_4$ the extracellular K^+ concentration was raised (Fig. 3 *B*, bar 4). On the other hand in a completely K^+-free medium the response to EGF was found to be enhanced (Fig. 3 *B*, bar 2). The observed effects of Na^+_o, K^+_o, or Ca^{++}_i on the EGF stimulation of S6 kinase in intact cells are not likely to be due to an effect of these ions directly on S6 kinase activity. In vitro S6 kinase activity in the assay is inhibited in a similar manner by increasing NaCl or KCl concentrations (100% inhibition at 150 mM), as well as by $CaCl_2$ (inhibition starting at ~50–100 μM). Our results suggest that in the intact cell the membrane potential maintained by the K^+ gradient regulates the response to EGF and this hypothesis is supported by the finding that preincubation with the K^+ ionophore valinomycin led to a similar inhibition of the EGF activation as high K^+ concentrations (Fig. 3 *B*, bar 5). A similar inhibition of the EGF response was observed when cells were permeabilized with low doses of nonionic detergents. None of these treatments however led to a complete inhibition of the activation of S6 kinase by EGF, indicating that part of the activation mechanisms must be regulated in a different, yet unknown manner.

The fact that depolarizing K^+ concentrations inhibit the response suggests that an early consequence of EGF binding may be a shift of membrane potential to a more hyperpolarized state; this could occur through an efflux of K^+ which, in turn, could be linked to the release of Ca^{++} from intracellular compartments. There is no direct evidence linking changes in K^+ fluxes with changes in Ca^{++}_i in this way, but we observed that the inhibition of the EGF effect on S6 kinase by high K^+ and by buffering Ca^{++}_i is not additive (data not shown). Such a mechanism for increasing Ca^{++}_i would not necessarily require the hydrolysis of polyphosphoinositides and formation of inositol 1,4,5-tris phosphate which, in many cell lines, increases in response to growth factors such as platelet-derived growth factor or bombesin (review for example Rozengurt, 1986), but not consistently in response to EGF (Pandiella et al., 1986; Besterman et al., 1986; Magnaldo et al., 1986). In neuroblastoma cells FCSA causes a rapid hyperpolarization, sensitive to K^+_o, followed by a slower depolarization and a prolonged phase of repolarization (Moolenaar et al., 1981). EGF, however, was found to have no effect on membrane potential in human fibroblasts (Moolenaar et al., 1982). Effects of EGF on the electrical properties of ZR-75-1 cells remain yet to be explored.

To find out if the modulation of S6 kinase activation by K^+_o and by Ca^{++}_i was unique for the response to EGF, activation of S6 kinase by insulin and IGF1 was studied. In the course of these studies it was observed that insulin activation and to a much smaller degree also the response to IGF1 was inhibited in bicarbonate-free buffers (Fig. 5). This observation correlates with the finding of Shechter and Ran (1986) that the dose-response curve for insulin actions in the adipocyte is shifted to nearly 100 times higher concentrations in bicarbonate-free media. Because insulin binding is not affected, they suggest that postreceptor effect(s) essential for insulin action must be blocked. Activation of S6 kinase is thus one of these postreceptor responses. Activation of S6 kinase by insulin has been studied (Tabarini et al., 1985; Nemenoff et al., 1986) and it is speculated that phosphorylation by the insulin receptor tyrosine kinase may be involved in the activation mechanism. Possibly the tyrosine kinase activity of the insulin receptor depends on extracellular bicarbonate as it has been found that in bicarbonate-free media insulin did not stimulate tyrosine phosphorylation of a set of cellular proteins in contrast to EGF or α-thrombin (Khono and Pouyssegur, 1986). IGF1 stimulates growth of ZR-75-1 cells (Küng et al., 1986) and activates S6 kinase to a similar extent as EGF (Fig. 5). Although it shows considerable homology to insulin, it exerts its action through a distinct receptor (Massague and Czeck, 1982). In contrast to insulin activation of S6 kinase the response to IGF1 was much less dependent on bicarbonate in the medium (Fig. 5). The fact that high doses of insulin (5 μg/ml instead of 50–100 ng/ml) were able to partially overcome the requirement for extracellular bicarbonate may be due to insulin interacting with the IGF1 receptor. High extracellular K^+ as well as preincubation with quin2 inhibited the activation of S6 kinase by insulin and IGF1 in a similar manner as the response to EGF.

In summary, the pathways by which these different peptides trigger the activation of S6 kinase appear to involve an effect on membrane potential and/or K^+ fluxes as well as on redistribution of intracellular Ca^{++}. Further studies will have to determine how this signal is linked to subsequent steps in the activation mechanism.

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