Activation of mRNA translation in rat cardiac myocytes by insulin involves multiple rapamycin-sensitive steps

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Insulin activates both overall rates of protein synthesis and the translation of specific mRNAs. Among the latter are the so-called 5'-terminal oligopyrimidine tracts (5'-TOP) mRNAs that encode ribosomal proteins and translation elongation factors and that possess a 5'-terminal tract of pyrimidines (hence “5'-TOP”), which confers on them translational upregulation by stimuli such as serum (30, 49). In serum-starved cells, such mRNAs are poorly translated, and stimulation of the cells causes them to “shift” into polyribosomes, presumably as a consequence of enhanced ribosome binding (i.e., enhanced initiation). The regulation of the translation of such mRNAs is believed to involve the 70-kDa protein kinase termed p70 S6k, which is activated by diverse stimuli, including insulin, and which phosphorylates ribosomal protein S6 (29, 63, 74).

p70 S6k lies on a signaling pathway that includes the protein mammalian target of rapamycin (mTOR), which binds and is inhibited by the immunosuppressant rapamycin when this compound is bound to the 12-kDa FK506 binding protein (FKBP12) (59, 75). Also downstream of mTOR lie the eukaryotic initiation factor (eIF) 4E binding proteins (4E-BPs), which interact with eIF4E, the translation initiation factor that binds the 7-methylguanosine (i.e., enhanced initiation). The phosphorylation of such mRNAs is believed to involve the 70-kDa protein kinase termed p70 S6k, which is activated by diverse stimuli, including insulin, and which phosphorylates ribosomal protein S6 (29, 63, 74).

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inactivating protein synthesis (reviewed in Ref. 64). Re-tein is activated by insulin and many other agents that exchange factor eIF2B, and this heteropentameric pro-GTP-bound form is catalyzed by the guanine nucleotide-ribosome bound to GDP. Recycling back to the active

The kinase that phos-phorylates eEF2 is a Ca2+/calmodulin-dependent ki-nase and is now termed eEF2 kinase, since eEF2 is its only known substrate (54). Earlier studies have shown that, in Chinese hamster ovary cells, insulin brings about the dephosphorylation of eEF2 and accelerated rates of elongation (66). Insulin also brings about the inactivation of eEF2 kinase, and all three effects are blocked by treatment of the cells with rapamycin, indicating that the signaling events responsible involve mTOR (66).

A fourth potential control point in translation initia-tion is the recycling of eIF2 between its inactive GDP-liganded and active GTP-bound states. eEF2 transfers the initiator Met-tRNA to the ribosome, a step that is required for all initiation events. During this process, the GTP is hydrolyzed to GDP and eIF2 leaves the ribosome bound to GDP. Recycling back to the active GTP-bound form is catalyzed by the guanine nucleotide-exchange factor eIF2B, and this heteropentameric protein is activated by insulin and many other agents that stimulate protein synthesis (reviewed in Ref. 64). Re-cent data favor a role for glycogen synthase kinase-3 (GSK-3) in regulating eIF2B. GSK-3 phosphorylates a single site in eIF2B (Ser546 in its ε-subunit), leading to inactivation of eIF2B (82). Insulin causes inactivation of GSK-3 [through a signaling pathway involving phos-phatidylinositol 3-kinase (PI 3-kinase; see Refs. 5 and 12); rapamycin interferes with mTOR signaling, as described above; PD-098059 inhibits activation of the mitogen-activated protein (MAP) kinase [extracellular ligand-regulated kinase (Erk)] pathway (3)]. The concentrations used were chosen because other studies have shown that they completely inhibit the relevant target protein but are not so high as to be likely to interfere with other processes.

After treatment, cells were washed twice with ice-cold PBS and homogenized in ice-cold extraction buffer containing (in mmol/l) 50 Tris (pH 7.5), 1 EDTA, 1 EGTA, 1 Na3VO3, 50 NaF, 5 sodium pyrophosphate, 270 sucrose, and 1 DTT and also 1% (vol/vol) Triton X-100, 1 µmol/l microcystin-LR, 5 µg/ml leupeptin, 5 µg/ml pepstatin, 5 µg/ml antipain, and 200 µmol/l phenylmethylsulfonyl fluoride (added immediately before use). Postmitochondrial/nuclear supernatants were prepared by centrifugation of the total cell homogenates at 14,000 rpm for 10 min at 4°C. Total protein concentrations of the lysates were determined by Bradford protein assay, and samples of lysate containing equal amounts of protein were used for protein kinase assays, Western blots, translation factor analysis, and protein synthesis measurement.

Assays for protein kinases. p70 S6k, protein kinase B (PKB), and GSK-3 were assayed (after immunoprecipitation) using specific peptide substrates, as described previously (52, 79, 84). The phosphorylation state of p70 S6k was also assessed by virtue of its mobility on SDS-PAGE, where the more highly phosphorylated, more active species migrate more slowly (21). The activity of eEF2 kinase was measured in the presence of Ca2+ and calmodulin using eEF2 purified from rabbit reticulocytes as substrate (19). In some assays, where indicated, eEF2 was omitted.

The phosphorylation state of PKB (α- and β-) isoforms was assessed by Western blotting using an antiserum specific for PKB phosphorylated at Ser473 (from New England Biolabs). The total level of PKB in each sample was determined on a parallel Western blot using an antiserum that detects PKB irrespective of its state of phosphorylation (also from New England Biolabs).

Analysis of translation factor phosphorylation, association, or activity. The phosphorylation state of 4E-BP1 was assessed using the standard procedure, i.e., by virtue of its mobility on SDS-PAGE, as described earlier (18, 77). To assess the interaction of eEF4 with other proteins such as 4E-BP1 or eIF4G, cell extracts were subjected to affinity chromatogra-phy on 7-methyl-GTP Sepharose, and the bound material was analyzed by SDS-PAGE/Western blotting using appropriate antisera (21, 77). The state of phosphorylation of eEF2 was assessed by Western blotting using an antiserum specific for the form of eEF2 phosphorylated at Ser56 (45). Levels of total eEF2 were ascertained using an antiserum that detects eEF2 irrespective of its state of phosphorylation (65).

Measurement of protein synthesis. ARVC that had been cultured overnight were perfused in fresh medium 199 with or without signaling inhibitors. Cells were then stimulated with insulin (100 nmol/l) for 30 or 60 min before the addition of [35S]methionine (5 µCi/ml) for a further 30 min. Cells were washed three times with cold PBS and then lysed with extraction buffer. Protein was then collected by filtration on 3µm paper filters (Whatman) before precipitation with 5% (wt/vol) TCA and measurement of incorporated radiolabel by scintillation counting.
Alternatively, cells were stimulated with insulin for 30 min in the presence or absence of rapamycin before the addition of \[^3H\]phenylalanine (5 \muCi/ml) for 30 min. Cells were washed three times with ice-cold PBS, and 10% (wt/vol) ice-cold TCA was added at 4°C for 60 min to precipitate the proteins. The precipitates were washed two times with ethanol, and were dissolved in 0.5 mol/l NaOH and 0.1% (wt/vol) SDS. Radioactivity was measured by liquid scintillation counting.

RESULTS

Response of isolated adult rat cardiomyocytes to insulin. In our initial experiments, we examined whether insulin and other agents could activate two major signaling pathways (MAP kinase and p70 S6k) in freshly isolated ventricular myocytes. ARVC were treated with insulin, ANG II, endothelin-1 (ET-1), or phenylephrine (PE), all of which have been reported to activate protein synthesis in cardiomyocytes. In the freshly isolated cells, little or no activation of either pathway was observed (data not shown). To allow the cells to recover from the process of isolation, and any proteolytic or mechanical damage that they may have suffered during this, cells were cultured overnight on laminin-coated dishes in medium devoid of serum. After overnight culture under these conditions, 80–90% of the cells survived, and cells retained their rod-shaped morphology although some rounding of the ends of the cells was noted (data not shown). Cells cultured overnight were treated with the same stimuli, and the activation of MAP kinase and p70 S6k was studied again (Fig. 1, A and B). In this case, substantial activation of MAP kinase and p70 S6k was now observed (the extent depending upon the stimulus used). For example, ANG II, ET-1, insulin, and PE all activated p70 S6k, as indicated by the characteristic reduction in mobility on SDS-PAGE that accompanies the phosphorylation and activation of this enzyme (Fig. 1A and, for insulin, data shown below). In the case of MAP kinase, Fig. 1B shows that ANG II caused a pronounced increase in the phosphorylation of both Erk1 and Erk2 (the two isoforms of MAP kinase), whereas the phorbol ester phorbol myristate acetate (which activates protein kinase C) elicited a smaller effect. Insulin had an even smaller effect on the phosphorylation of the Erks.

Thus, in ARVC that had been cultured overnight, a range of stimuli activate signaling pathways that are implicated in the activation of mRNA translation. We elected to focus on a detailed study of the effects of insulin on signaling pathways and translation factors, since considerable attention has been devoted to elucidating regulatory mechanisms by which it may activate translation, although very little work has been carried out on its actions on these targets in primary cells, as opposed to immortalized cells grown in tissue culture.

Insulin activates p70 S6k in adult rat cardiomyocytes. As mentioned above, insulin treatment of cultured ARVC led to the activation of p70 S6k. Maximal activation required ~30 min and followed an initial “lag” phase during which little or no activation was seen (Fig. 2A). Maximal activation was about fivefold (n = 6). The activation of the enzyme correlated well with the characteristic decrease in the mobility of the protein on SDS-PAGE alluded to in response of isolated adult rat cardiomyocytes to insulin (Fig. 2B).

We tested the effects of insulin on the activation of p70 S6k by a number of signaling inhibitors; these were the immunosuppressant agent rapamycin, LY-294002, which inhibits PI 3-kinase, and wortmannin, a structurally unrelated inhibitor of PI 3-kinase. In the presence of any of these three compounds, insulin was unable to significantly activate p70 S6k in ARVC (Fig. 2, C and D, P > 0.5 vs. activity with inhibitor alone). Because the inhibitory effect of wortmannin was less complete than that of LY-294002, the latter was used in preference to wortmannin in subsequent studies. The inhibitor of the Erk pathway, PD-098059, had no effect on the ability of insulin to activate p70 S6k (Fig. 2C, P > 0.5 for insulin plus PD vs. insulin alone). None of the inhibitors tested had a significant effect on the basal activity of p70 S6k (Fig. 2C, P > 0.2 vs. control).

Insulin activates PKB in cardiomyocytes. Some reports have suggested that PKB lies upstream of p70 S6k and may therefore provide a link between PI 3-kinase and p70 S6k (since PKB is activated by insulin and other agents in a PI 3-kinase-dependent fashion; see Refs. 10 and 34). We therefore examined the ability of insulin to activate PKB. Mammalian PKB exists in three distinct isoforms (α, β, and γ), and we made use of recently developed isoform-specific antisera to examine the regulation of each form in response to insulin (79).

Insulin caused a rapid but transient activation of the α- and β-isoforms of PKB in ARVC (Fig. 3A). Activation
Fig. 2. Insulin activates 70-kDa ribosomal S6 (p70 S6) kinase in adult rat ventricular myocytes. Isolated ARVC were cultured overnight as described in MATERIALS AND METHODS and then were treated with insulin as described below, and extracts were prepared as described in MATERIALS AND METHODS. Activity of p70 S6 kinase was assessed after immunoprecipitation as described in MATERIALS AND METHODS. Activity is expressed relative to that of untreated control cells (100%). In each case, n = 3 and data are means ± SD. A and C: activity of p70 S6 kinase was assessed after immunoprecipitation as described in MATERIALS AND METHODS. Activity is expressed relative to that of untreated control cells (= 100%). In each case, n = 3 and data are means ± SD. B and D: immunoblots developed using an antiserum to p70 S6 kinase. Data are typical of at least 3 independent experiments performed. In A and B, cells were treated with insulin (100 nmol/l) for the times indicated (min). In C and D, cells were preincubated with rapamycin (R), wortmannin (W), LY-294002 (LY, 30 µmol/l), or PD-098059 (PD), as indicated and as described in MATERIALS AND METHODS, before treatment with insulin (100 nmol/l) for 30 min. C and I, control and insulin-treated cells, respectively.

Fig. 3. Insulin activates protein kinase B (PKB) in isolated adult rat ventricular myocytes. Isolated ARVC were cultured overnight and then were treated with insulin as described below, and extracts were prepared as described in MATERIALS AND METHODS. Activity of PKB was assessed after immunoprecipitation, as described in MATERIALS AND METHODS, using antisera specific for PKBα or PKBβ as indicated. Activity is expressed relative to the protein concentration of the extract, with 1 unit of PKB activity being defined as that amount that catalyzes the phosphorylation of 1 nmol of substrate in 1 min. In A, cells were treated with insulin (100 nmol/l) for the times indicated (min). Data are from 3 independent experiments and are means ± SD. In B, cells were treated with insulin (100 nmol/l) for the times indicated (min). Samples were then subjected to SDS-PAGE, and separate blots were developed using either an antibody that detects PKB irrespective of its state of phosphorylation (bottom) or one that detects PKB only when phosphorylated at the COOH-terminal site, Ser473 (top). Data shown are typical of 3 independent experiments. In C, cells were preincubated with rapamycin (R), LY-294002 (LY, 30 µM), or PD-098059 (PD), as indicated, before treatment with insulin (Ins, 100 nmol/l, 5 min), as described in MATERIALS AND METHODS. Samples were then subjected to SDS-PAGE, and separate blots were developed using either an antibody that detects PKB irrespective of its state of phosphorylation (bottom) or one that detects PKB only when phosphorylated at the COOH-terminal site, Ser473 (top). Similar data were obtained in 2 entirely independent experiments.
was maximal within 5 min, and activity then declined almost to basal levels by 30 min (at which P > 0.1 vs. no insulin control). The activity of the α-isofrom was always greater than that of the β-isofrom, suggesting that it is the major form in ARVC, although, because different antisera were used, it is not possible directly to compare the activities of the two isoforms with one another. The activity of the γ-isofrom was always very low, even after insulin treatment, and we have not studied it further. Activation of the α- and β-isofroms of PKB involves their phosphorylation at a conserved threonine residue in the catalytic domain (Thr308 in PKBα) and a COOH-terminal serine residue (Ser473 in PKBα; see Ref. 2). Consistent with this, insulin increased the phosphorylation of Ser473 in parallel with the activation of PKB, as revealed by Western blotting using an antibody specific for PKB that is phosphorylated at that site (Fig. 3B). The total amount of PKB extracted from the cells did not differ under different conditions, as shown by Western blots using a different antibody that recognizes PKB irrespective of its state of phosphorylation (Fig. 3B). With the use of this antibody, two bands for PKB were evident at certain time points. This mobility shift was only seen at those time points where PKB was activated, as judged by activity measurements or Western blotting using the anti-phospho-PKB antiserum. Activated PKB has previously been reported to display a reduced mobility on SDS-PAGE (1, 4). Thus, as assessed by three different criteria, insulin induces a rapid but transient activation of PKB in ARVC.

As judged from Western blots employing the anti-phospho-PKB antibody, activation of PKB by insulin was blocked by LY-294002, an inhibitor of PI 3-kinase, consistent with current understanding of the regulation of PKB (Fig. 3C; see Ref. 2). An inhibitor of the activation of the MAP kinase pathway, PD-098059, had no effect on the activation of PKB by insulin. Rapamycin also failed to block the activation of PKB (Fig. 3C). A loading control was again provided by a blot using the phosphorylation-insensitive anti-PKB antibody, and again the mobility shift was seen under conditions where PKB was activated. Similar conclusions were drawn from direct measurements of PKB activity (data not shown).

Insulin increases the phosphorylation of 4E-BP1, causes its dissociation from eIF4E, and promotes formation of eIF4F complexes. As described above, insulin activated p70 S6k, an enzyme regulated through a pathway linked to mTOR. The translational regulatory proteins known as 4E-BPs are also regulated through an mTOR-dependent pathway (40), and we therefore examined the effects of insulin on this arm of the translational machinery, in which PKB may also be an upstream component (26). ARVC contained detectable levels of 4E-BP1 but not of 4E-BP2 (data not shown). Because we do not have access to antisera to the third member of the 4E-BP family, 4E-BP3 (60), we have not been able to analyze it. Northern blot analysis of whole heart RNA shows that it is expressed in this organ (60).

In unstimulated cells, 4E-BP1 was present as the α- (least phosphorylated) and β- (more phosphorylated) forms, with roughly equal amounts of the two species being seen in most cases, although this was a little variable (Fig. 4, A and B). Insulin induced a pronounced retardation in the mobility of 4E-BP1 on SDS-PAGE, indicative of increased phosphorylation of the protein, such that most of the protein now migrated as the most highly phosphorylated γ-form, with a trace of the β-species also being evident (Fig. 4A). As for the activation of p70 S6k, there was a lag period during which no effect of insulin was seen, although this was shorter than for the effects of insulin on p70 S6k, with the full effect of insulin already being apparent by 15 min rather than 30 min (cf. Fig. 2). The effect of insulin was blocked by pretreatment of the cells with rapamycin, with almost all of the protein now appearing as the α-form (Fig. 4, A and B). In the case of the PI 3-kinase inhibitor LY-294002, 30 µM was found to be insufficient to block completely the effect of insulin, whereas 100 µM both blocked the effect of insulin and also caused dephosphorylation of 4E-BP1 in the control cells (Fig. 4B). Phosphorylation of 4E-BP1 prevents it from binding to eIF4E (27, 41, 57, and also see Ref. 40 for a review); consistent with the above data showing insulin-induced phosphorylation of 4E-BP1, the hormone also caused 4E-BP1 to dissociate from eIF4E (Fig. 4C). This effect, like the phosphorylation of 4E-BP1, was blocked by rapamycin (Fig. 4C).

When m7GTP-Sepharose-bound material from control and insulin-treated heart cells was examined by Western blotting for eIF4E bound to the eIF4F isolated in this way, it was clear, in each of four separate experiments performed, that insulin brought about a pronounced enhancement of the association of eIF4E with eIF4G (Fig. 4D), indicative of the increased formation of eIF4F complexes. Such complexes also contain the helicase eIF4A, and Western blots of material recovered in m7GTP-Sepharose pull-downs revealed that insulin also brought about an increase in the amount of eIF4A bound to eIF4E (Fig. 4D). Thus, in ARVC, insulin brings about the increased formation of initiation factor complexes containing eIF4E, eIF4G, and eIF4A.

Insulin induces inactivation of GSK-3. PKB phosphorylates GSK-3 at a regulatory serine near the COOH-terminus of both the α- and β-isofroms, leading to their inactivation (14, 68, 72, 73), and is thought to mediate the inactivation of GSK-3 by insulin in vivo (14, 16, 69). Because insulin activates PKB in ARVC, we examined whether the hormone also affected the activity of GSK-3, which itself is an important potential regulator of translation initiation, through its phosphorylation and regulation of eIF2B (82).

Insulin caused a marked and rapid inactivation of GSK-3 (Fig. 5). GSK-3 activity (of the α- plus β-isofroms) fell to ~50% of the control value by 5 min of insulin treatment and then gradually returned toward control values (Fig. 5). This matches quite closely the regulation of PKB by insulin in these cells, where a maximal effect was seen within 5 min. However, al-
though in the case of GSK-3 this was followed by an increase in activity, unlike the activity of PKB (Fig. 3A), GSK-3 activity did not return to basal levels, even by 20 or 45 min (Fig. 5). In one experiment in which the activities of the α- and β-isoforms of GSK-3 were monitored separately, insulin decreased the activities of these isoforms to 56 and 45% of the control, respectively, indicating that insulin affects both isoforms of this enzyme to similar extents.

Insulin causes inhibition of eEF2 kinase and dephosphorylation of eEF2. Insulin has previously been shown to bring about the dephosphorylation of eEF2 in Chinese hamster ovary cells and more recently in adipocytes (19, 66). Because phosphorylation of eEF2 inhibits its activity (11, 67), this effect of insulin should contribute to the activation of translation and, indeed, has been shown to correlate with accelerated rates of peptide chain elongation (19, 66). We therefore examined the effect of insulin on the phosphorylation state of eEF2 in ARVC. This was assessed by Western blotting using an antibody specific for the phosphorylated form of eEF2 (45), with the level of total eEF2 being assessed in a parallel blot using an eEF2 antibody that is not sensitive to the phosphorylation state (Fig. 6A). After insulin treatment, the level of phosphor-

Fig. 4. Insulin regulates the phosphorylation of eukaryotic initiation factor (eIF) 4E binding protein (4E-BP1), its binding to eIF4E, and the association of eIF4E with eIF4G in adult ventricular myocytes. Isolated ARVC were cultured overnight as described in MATERIALS AND METHODS and then treated with insulin (100 nmol/l) for various times (min, as indicated). Extracts were prepared as described in MATERIALS AND METHODS. The activity of GSK-3 (α- plus β-isoforms together) was assessed after immunoprecipitation, as described in MATERIALS AND METHODS, and is expressed relative to that of untreated control cells (= 100%) ± SD (n = 3). *P < 0.05 and **P < 0.005.

Fig. 5. Insulin causes the inactivation of glycogen synthase kinase 3 (GSK-3) in isolated ventricular myocytes. Isolated ARVC were cultured overnight as described in MATERIALS AND METHODS and then treated with insulin (100 nmol/l) for various times (min, as indicated). Extracts were prepared as described in MATERIALS AND METHODS. The activity of GSK-3 (α- plus β-isoforms together) was assessed after immunoprecipitation, as described in MATERIALS AND METHODS, and is expressed relative to that of untreated control cells (= 100%) ± SD (n = 3). *P < 0.05 and **P < 0.005.
Ca^{2+}/calmodulin-dependent enzyme whose only known substrate is eEF2 (19, 66). In ARVC, insulin also brought about a marked decrease in the activity of eEF2 kinase (Fig. 6B). Significant inactivation was first observed after 15 min of insulin treatment, and this was maximal at 30 min, although substantial inactivation was still evident after 60 min. This effect was largely blocked by prior treatment of the cells with rapamycin, indicating that, as is the case in the other types of cells where this effect has been studied, the signaling pathway linking the insulin receptor to eEF2 kinase involves mTOR (19, 66). The effect of insulin on eEF2 kinase activity was less marked than its effect on the level of phosphorylation of eEF2, as was also observed in our earlier studies (66). Very similar findings were made in three completely separate experiments. The time courses for the effects of insulin on eEF2 phosphorylation and eEF2 kinase activity were similar but not identical. It is possible that the activity of the phosphatase acting on eEF2 is also regulated, but we have not studied this.

Rapamycin substantially inhibits the activation of total protein synthesis by insulin in ARVC. To study the effect of insulin on overall protein synthesis in ARVC, cells were treated with insulin, and then radiolabeled tracer amino acid ([^{35}S]methionine) was added to monitor the rate of protein synthesis. This was measured as incorporation of labeled amino acid into material which is insoluble in TCA, which is the standard procedure. Insulin treatment of ARVC led to a substantial activation of the rate of protein synthesis, and this was somewhat greater at 60 min (150% above the control rate, Fig. 7) than at 30 min (70% above control, Table 1), which is not unexpected (see DISCUSSION).

![Fig. 6. Insulin decreases the phosphorylation of eukaryotic elongation factor 2 (eEF2) and eEF2 kinase activity in ARVC. Isolated ARVC were cultered overnight as described in MATERIALS AND METHODS and then were treated with insulin (Ins, 100 nmol/l) for various times (min, as indicated). Extracts were prepared as described in MATERIALS AND METHODS. In some cases, cells were pretreated with rapamycin (R) before exposure to insulin for the times indicated. In A, extracts of ARVC were subjected to SDS-PAGE followed by Western blotting. Blot at top was developed using an antiserum specific for the phosphorylated form of eEF2 (45), whereas the blot at bottom shows data for the same samples obtained using an anti-eEF2 antiserum that is insensitive to the phosphorylation state of eEF2 (to allow normalization to the total amount of eEF2 present). Positions of eEF2 are indicated in each case by arrows. Nos. at bottom show the signal due to the phosphorylated eEF2 compared with that for total eEF2, as a ratio (indicative of the relative level of eEF2 phosphorylation). B: data for the activity of eEF2 kinase and the position of eEF2 are again indicated. C+ and C−, lanes in which control extracts were assayed in the presence or absence of added purified eEF2, respectively. In all other assays, eEF2 was present. In B, nos. at bottom show the relative intensity of the phosphorylation of the substrate eEF2 (as % of control) determined by PhosphorImage analysis using a Fuji FLA-2000 Imager, BAS Reader, and Aida software. Similar results for each type of analysis were obtained in 3 independent experiments.

![Fig. 7. Insulin activates protein synthesis in isolated ventricular myocytes. Isolated ARVC were cultered overnight as described in MATERIALS AND METHODS and then were treated with insulin (100 nmol/l). In some cases, where indicated, cells were preincubated with signaling inhibitors (R, rapamycin; LY, 30 µM LY-294002; PD, PD-098059) before treatment with insulin. After 60 min, [^{35}S]methionine was added; 30 min later, cells were extracted, and incorporation of radiolabel in TCA-insoluble material was assessed as described in MATERIALS AND METHODS. Data are shown relative to the untreated control (= 100%). Data are means ± SD, n = 4.](http://ajpheart.physiology.org/)**
To examine the possible roles of individual signaling pathways in the control of protein synthesis in ARVC, cells were pretreated with specific signaling inhibitors before stimulation with insulin. At the 60-min time point, the insulin-activated rate of protein synthesis appeared to be slightly inhibited by pretreatment of the cells with LY-294002 or PD-098059, but this effect was not statistically significant (P > 0.25 vs. insulin alone). Rapamycin had a much larger effect than either of the above compounds, indicating a major role for the mTOR pathway in the regulation of protein synthesis in ARVC (here P < 0.01 vs. insulin alone).

However, to minimize possible effects due to factors other than upregulation of mRNA translation itself (such as the activation of transcription), we elected to focus more closely on the earlier (30 min) time point. Rapamycin had no effect on the basal rate of protein synthesis in control cells (P > 0.5 for rapamycin vs. no addition) but again substantially reduced the activation seen upon insulin stimulation (by ~50%, P < 0.005 for insulin plus rapamycin vs. insulin alone; Table 1). This indicates that events linked to mTOR, such as the activation of p70 S6k, the phosphorylation of 4E-BP1, and the dephosphorylation of eEF2, are likely to play a major role in the activation of protein synthesis by insulin in ARVC.

Interpretation of the data obtained with the PI 3-kinase inhibitor LY-294002 and the Erk pathway inhibitor PD-098059 is more complicated. At 30 µM, LY-294002 did not significantly inhibit the basal rate of protein synthesis (P > 0.1 vs. control) but did substantially block its stimulation by insulin (by ~60%, P < 0.005 compared with insulin alone; Table 1). At 100 µM, LY-294002 significantly reduced the basal rate of incorporation of label (P < 0.001) and again also inhibited its activation by insulin. The MAP kinase pathway inhibitor PD-098059 also reduced basal protein synthesis (P < 0.001). When corrected for the fact that it suppressed the rate of protein synthesis in control cells, PD-098059 appears to have little, if any, effect on the ability of insulin to activate protein synthesis in ARVC (P > 0.1; Table 1). The effects of higher concentrations of LY-294002 and of PD-098059 on basal protein synthetic rates may reflect a requirement for a certain level of PI 3-kinase or Erk activity for basal protein synthesis or another effect of these compounds. LY-294002 has been reported to inhibit the protein kinase activity associated with mTOR (9), but because rapamycin, another inhibitor of mTOR, did not affect basal protein synthesis, this potential effect of LY-294002 seems unlikely to be responsible for the reduction in basal protein synthesis rate. PD-098059 has not been reported, as far as we are aware, to interfere with the activity of anything other than mitogen/extracellular signal-regulated kinase 1. Nonetheless, it seems clear that LY-294002 attenuated the effect of insulin, since the stimulation seen in the presence of this compound (relative to the appropriate controls also containing it) was less than one-half that seen in the absence of LY-294002.

To further explore the effects of insulin and inhibitors on amino acid incorporation in ARVC protein, we extended our studies to use a different labeled amino acid precursor, [3H]phenylalanine rather than [35S]methionine. Phenylalanine is transported by system L, which is not subject to acute regulation by insulin or other agents (47), and thus the use of this label should eliminate potential problems arising from possible activation of amino acid transporters such as system A by insulin. Using [3H]phenylalanine as precursor, we obtained data very similar to that obtained with [35S]methionine: insulin activated the incorporation of [3H]phenylalanine by ~50%, and this activation was inhibited by rapamycin (extent of inhibition 40% (P < 0.05 for rapamycin plus insulin vs. insulin alone), taking into account that rapamycin slightly reduced the rate of [3H]phenylalanine incorporation in control cells; Table 2). Thus similar effects were seen using two different labels.

### Table 1. Effects of insulin and signaling inhibitors on protein synthesis in ARVC using [35S]methionine as label

<table>
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<th>Condition</th>
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<th>% of Control</th>
<th>% of Control with inhibitor(s)</th>
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</tr>
<tr>
<td>PD + Ins</td>
<td>3</td>
<td>96.2 ± 5.7</td>
<td>157.7 ± 8.5</td>
</tr>
</tbody>
</table>

Values are means ± SD; n, no. of experiments. Isolated adult rat ventricular cardiomyocytes (ARVC) were cultured overnight as described in MATERIALS AND METHODS and then were treated with insulin (100 nmol/l) for 30 min. In some cases, where indicated, cells were preincubated with rapamycin (Rap), LY-294002 (LY), or PD-098059 (PD) before treatment with insulin. [35S]methionine was added, and, 30 min later, cells were extracted, and incorporation of radiolabel in TCA-insoluble material was assessed as described in MATERIALS AND METHODS.

### Table 2. Effect of insulin and rapamycin on protein synthesis in ARVC using [3H]phenylalanine as label

<table>
<thead>
<tr>
<th>Condition</th>
<th>Rate of [3H]phenylalanine Incorporation</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>100.0</td>
</tr>
<tr>
<td>Insulin</td>
<td>152.2 ± 7.1</td>
</tr>
<tr>
<td>Rap</td>
<td>93.9 ± 1.9</td>
</tr>
<tr>
<td>Rap + Ins</td>
<td>123.9 ± 4.5</td>
</tr>
</tbody>
</table>

Values are means ± SD; n = 4 independent experiments in each case. Isolated ARVC were cultured overnight as described in MATERIALS AND METHODS and then were treated with insulin (100 nmol/l) for 30 min. In some cases, where indicated, cells were preincubated with rapamycin (Rap) before treatment with insulin. [3H]phenylalanine was added, and, 30 min later, cells were extracted, and incorporation of radiolabel in TCA-insoluble material was assessed as described in MATERIALS AND METHODS.
labeled amino acid precursors that are transported on different systems.

**DISCUSSION**

The data reported here represent the first detailed study of the signaling events and the translation factors involved in the activation of protein synthesis by insulin in adult cardiomyocytes, although there have been some earlier reports relating to certain individual translation factors or using neonatal cells (43, 78). Our data demonstrate that insulin activates a number of signaling pathways and steps in mRNA translation. These include several targets linked to mTOR, i.e., p70 S6k, 4E-BP1, and eEF2. This is not only the first study of the regulation of a range of translation factors in adult heart cells but also the first report studying, in a concerted fashion, the regulation of this range of targets of insulin signaling in any cell type.

The activation of p70 S6k is believed to play a key role in stimulating the translation of the 5'-TOP mRNAs (30, 49) and thus presumably in activating the production of ribosomes and increasing the capacity of the cell for protein synthesis. This is likely to be especially important in the longer-term upregulation of mRNA translation. Insulin also promoted the phosphorylation of 4E-BP1, its dissociation from eIF4E, and, as a consequence of this, the formation of the eIF4E/eIF4G complex, which is considered to be necessary for cap-dependent translation of mRNAs, especially of those that have extensive secondary structure in their 5'-UTRs (reviewed by Refs. 20 and 70). Taken together, these events are likely to be important in activating the translation of specific sets of mRNAs in response to insulin. In contrast, the insulin-induced dephosphorylation (and activation) of eEF2 will contribute to the overall activation of protein synthesis.

A consistent observation in these studies was that the activation of p70 S6k lagged significantly behind the other two mTOR-linked regulatory events studied. Phosphorylation of 4E-BP1, dephosphorylation of eEF2, and inactivation of eEF2 kinase were already maximal or almost maximal after 15 min of insulin treatment, whereas activation of p70 S6k was still very slight at this time and became substantial only after 30 min of insulin treatment. As far as we are aware, this is the first study that has examined, in parallel, the regulation of all three of these targets of mTOR-linked signaling. Because the molecular events underlying the regulation of these different proteins are not yet fully established, it is not clear why activation of p70 S6k should lag behind that of other targets of the mTOR pathway.

As observed in many earlier studies, phosphorylation of 4E-BP1 correlated with its dissociation from eIF4E, and both events were blocked by pretreatment of the cells with rapamycin. Because there are several (at least 5) phosphorylation sites in 4E-BP1, although only three distinct bands are observed on SDS-PAGE, each band must correspond to multiple different phosphorylated forms, and we have not presented a quantification of the proportions in the different forms as this would not provide significant information. The key point is that the dissociation of 4E-BP1 is accompanied by a marked increase in the amount of eIF4G bound to eIF4E (which is consistent with the fact that 4E-BP1 and eIF4G compete for a common binding site in eIF4E (27, 42)). Thus the clear interpretation of the data presented here is that the increase in 4E-BP1 phosphorylation brought about by insulin suffices 1) to bring about the complete release of 4E-BP1 from eIF4E and 2) to permit a marked increase in the binding of eIF4G to eIF4E, which may play an important role in promoting initiation of translation on cap-dependent mRNAs in heart cells. A further indication that insulin promotes formation of eIF4E complexes in ARVC is that it increased the amount of eIF4A that copurifies with eIF4E on m7GTP-Sepharose.

The data for eEF2 show that insulin quite rapidly induced its dephosphorylation and that this was blocked by rapamycin. There have only been two previous studies on the effect of insulin on the phosphorylation of eEF2 in Chinese hamster ovary cells (66) and in 3T3-L1 adipocytes (19). In both cases, and in the present study, the insulin-induced dephosphorylation of eEF2 was accompanied by decreased activity of eEF2 kinase, and, where studied, the effects were blocked by rapamycin. The data presented here demonstrate for the first time in primary cells that eEF2 and eEF2 kinases are targets for mTOR-dependent signaling.

The inactivation of GSK-3 in response to insulin in ARVC is similar to effects observed in several other cell types (8, 14–16, 28, 86). The activity of GSK-3 remained lower than controls up to 20–45 min of insulin treatment, even though the activity of the probably upstream kinase, PKB, had returned to basal levels. This may reflect a slow rate of dephosphorylation of GSK-3. Because GSK-3 can regulate the activity of eIF2B (82), which plays an important role in regulating overall translation initiation (35, 56, 62), it was important to assess whether insulin regulated this regulatory translation factor in heart cells. However, we were unable to assay the activity of eIF2B in extracts of ARVC using our standard procedure, which we have applied to many other cell types (25, 36, 83, 85, 87); no detectable activity was seen in extracts from either control or insulin-treated cells. We attempted to immunoprecipitate the eIF2B from cell extracts before assay using our monoclonal (55) or polyclonal antibodies to concentrate it; we also attempted to remove anything that might interfere with the assay and then measured its activity, but again no activity was detected. It is not clear why assaying eIF2B activity in heart cell extracts presents such difficulties. Given that it is required for translation, heart cells must express eIF2B, and, indeed using immunological approaches (monoclonal antisera), we have detected it in extracts of whole rat hearts (55). However, our earlier study (55) strongly suggested that the level of eIF2B was low in heart relative to the other tissues that we studied, which may offer an explanation of the difficulties experienced in assaying its activity. We have also attempted to use our anti-se-
rum, which is specific for eIF2Bε and which is phosphorlated at the GSK-3 site (82), to examine the phosphorylation state of this protein in heart cell extracts, but again this was unsuccessful.

We tested the effect of rapamycin on the incorporation of labeled amino acid into TCA-precipitable material using two different radiolabeled amino acids that are transported on different systems. The purpose of this part of the study was to assess whether it was likely that the rapamycin-sensitive regulatory events observed here actually contributed to the activation of protein synthesis in ARVC rather than to measure absolute rates of protein synthesis. With the use of either [35S]methionine or [3H]phenylalanine, insulin increased the incorporation of label, and this was partially (40% phenylalanine; 50% methionine) blocked by rapamycin. Because phenylalanine is transported on system L, which is not regulated acutely by insulin, the increased incorporation induced by insulin is presumably not due to increased transport of the label into the cells. With methionine as the labeled precursor, a slightly larger effect of insulin was observed (70% stimulation compared with ca. 50% for phenylalanine). This may reflect the possibility that system A, which is activated by insulin in some cell types, including skeletal muscle, (46, 47), may contribute to methionine uptake. However, because McDowell et al. (46) found that rapamycin did not affect the ability of insulin to activate system A, it is unlikely that the inhibitory effect of rapamycin on methionine incorporation reflects an effect on uptake of this labeled amino acid. It is also possible that rapamycin affects protein turnover in the heart with a consequent effect on pool sizes. We have not examined this aspect of the regulation of protein turnover in ARVC as part of this study, which focused on the regulation of specific translation factor proteins.

The extent of activation of amino acid incorporation was greater (150% increase over control) for the later labeling window (60–90 min after insulin) than for the earlier one (70% increase; 30–60 min). This shows that the rate of incorporation is not linear in insulin-stimulated cells, which is to be expected from our data that show 1) insulin does not activate the translational components we have studied immediately but only after a lag period and 2) this lag period differs for different components, with 4E-BP1 and eEF2 being affected fully at 15 min, whereas p70 S6k is not fully activated until 30 min after insulin treatment. Furthermore, the subsequent assembly of initiation complexes, recruitment of mRNAs, formation of polyribosomes, and synthesis of (labeled) polypeptides are all processes that are not instantaneous, contributing to the time required to see increased activation of amino acid incorporation.

The observation that the activation of amino acid incorporation by insulin in ARVC was largely blocked by rapamycin, which inhibits the activation of p70 S6k, the phosphorylation of 4E-BP1, and the dephosphorylation of eEF2, is consistent with the idea that these effects make a substantial contribution to the activation of protein synthesis by insulin in ARVC (Fig. 7 and Tables 1 and 2). In contrast, in many other cell types, rapamycin has only modest effects on the overall rate of protein synthesis (7, 50, 51, 58). For example, in serum-stimulated NIH 3T3 cells, Beretta et al. (7) found that rapamycin inhibited protein synthesis by ~50% over a 24-h period, but the effects at shorter times were much smaller. No data were presented for times as short as those studied here (30–60 min), but it seems likely from their data that the effect would have been very small. The reason for the differences in the degree of inhibition may reflect the fact that almost all of the earlier studies used immortalized cell lines and cells adapted to live in culture, whereas this study involved primary cells. It will be of considerable interest to know whether the stimulation of protein synthesis in other primary cell types also shows marked inhibition by rapamycin. However, it has been reported that rapamycin has little or no effect on the activation of protein synthesis over 4 h in primary T cells (51) but did substantially inhibit protein synthesis in BJAB lymphoma cells (13, 33).

Higher concentrations of LY-294002 reduced the basal level of protein synthesis in ARVC and also partially blocked its activation by insulin. The latter effect may reflect its ability to interfere with the regulation by insulin of the same regulatory events as are affected by rapamycin (as shown here and in other studies, reviewed in Ref. 64). One possibility is that this involves the regulation of eIF2B, which is regulated by a PI 3-kinase–dependent mechanism (87). Unfortunately, as noted above, we have been unable to measure either the activity or the activation of eIF2B in extracts from ARVC, probably due to the low level of eIF2B found in heart (55). Karinch et al. (32) have previously reported that insulin did not affect the activity of eIF2B in whole hearts of diabetic rats.

PD-098059 also inhibited the basal rate of protein synthesis. When this is taken into account, it can be seen (Table 1) that it has no effect on the activation of translation by insulin, implying that the Erk pathway is not involved in this. This is consistent with the observation that insulin has at most a very small effect on the activation state of the Erk pathway in ARVC.

Another recent study (39) suggested that the rate of protein synthesis in cardiomyocytes treated with insulin-like growth factor 1 was inhibited, to differing extents, by PD-098059, wortmannin, or rapamycin, indicating roles for several signaling pathways. The study, however, differed from the present one in several important ways. 1) It employed ARVC from neonatal rather than adult rats. 2) The authors did not examine the effects of the inhibitors on the basal rate of protein synthesis. 3) The effects of the inhibitors on the activation of protein synthesis were studied over an extended period (24 h) rather than acutely, as was the case here. Over such an extended time period, the activation of protein synthesis will almost certainly include contributions due to stimulation of transcription and increased levels of ribosomes (and probably also other translational components).
This study demonstrates that, in ARVC, insulin activates multiple rapamycin-sensitive events involved in the regulation of protein synthesis and that these events appear to play a substantial role in the stimulation of this process by insulin. The present study has also established the methodology for examining the regulation of translation factors and related signaling pathways in ARVC. This will allow us to examine the regulation of these processes in response to other important physiological or pathological conditions, such as treatment with vasoactive agents [which activate protein synthesis in ARVC (22, 23, 48) and appear to be involved in cardiac hypertrophy, which in turn is characterized by enhanced protein synthesis (53)].

We thank Drs. Dario Alessi and Kay Walker (Dundee) for kindly providing the anti-4E-BP antibodies used here for immunoprecipitation, Dr Nick Redpath (Leicester) for purified eEF2, Dr Angus Nairn (Rockefeller University, New York) for the antiserum to the phosphorylated form of eEF2, Dr Simon Morley (Sussex) for anti-eIF4G, and Dr J ackie Vandenheede (Leuven, Belgium) for anti-GSK-3. In some experiments, we used an antibody against 4E-BP1 kindly provided by Dr Adir Thomas (Utrecht, The Netherlands). We are also grateful to Andrew Newman, Sandy Elder, and Carolyn Walker (Dundee) for invaluable help with the isolation of heart cells.

This work was supported by Project Grant 95/112 from the British Heart Foundation.

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Received 27 January 1999; accepted in final form 7 October 1999.

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