Ischaemia induces changes in the association of the binding protein 4E-BP1 and eukaryotic initiation factor (eIF) 4G to eIF4E in differentiated PC12 cells

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Ischaemia was obtained *in vitro* by subjecting nerve-growth-factor-differentiated PC12 cells to glucose deprivation plus anoxia. During ischaemia the rate of protein synthesis was significantly inhibited, and eIF4E-binding protein (4E-BP1) and eukaryotic initiation factor 4E (eIF4E) were significantly dephosphorylated in parallel. In addition, ischaemia induced an enhancement of the association of 4E-BP1 to eIF4E, which in turn decreased eIF4F formation, whereas no degradation of initiation factor 4G was observed. The treatment of PC12 cells with the specific p38 mitogen-activated protein kinase inhibitor SB203580 induced eIF4E dephosphorylation but did not cause any effect on protein synthesis rate. Rapamycin, the inhibitor of

mammalian target of rapamycin ('mTOR'), but not PD98059, the inhibitor of extracellular signal-regulated protein kinases ('ERK1/2'), induced similar effects on 4E-BP1 phosphorylation to ischaemia; nevertheless, 4E-BP1–eIF4E complex levels were higher in ischaemia than in rapamycin-treated cells. In addition, both protein synthesis rate and eIF4F formation were lower in ischaemic cells than in rapamycin-treated cells.

Key words: initiation factors, mammalian target of rapamycin, mitogen-activated protein kinase, rapamycin, translation initiation.

INTRODUCTION

The phosphorylation and dephosphorylation of translation factors have a key role in protein synthesis regulation that is exerted largely at the level of polypeptide chain initiation. One of the steps in this process, the binding of mRNA to the 40 S ribosomal subunit, is co-ordinated by eukaryotic initiation factor 4F (eIF4F). eIF4F consists of initiation factor 4A (eIF4A, an ATP-dependent RNA helicase), initiation factor 4E (eIF4E, the cap-binding-protein) and initiation factor 4G (eIF4G, which provides docking sites for the aforementioned proteins) [1,2]. eIF4E activity is regulated in two different ways. Direct regulation occurs from eIF4E phosphorylation, which has been correlated with increased translational activity [1,2]. eIF4E phosphorylation *in vivo* is accomplished on activation of the MAPK (mitogen-activated protein kinase) signal-integrating kinase (Mnk1 kinase), a substrate of ERK (signal-regulated kinase) and p38 extracellular MAPKs [3], with eIF4G acting as a docking site for eIF4E phosphorylation [4]. eIF4E levels in cells are limiting and the indirect regulation of eIF4E levels is accomplished by its binding to specific proteins, i.e. 4E-binding proteins (4E-BPs). In the dephosphorylated form, 4E-BPs bind to eIF4E and competitively inhibit its association with eIF4G, thus preventing eIF4F formation and inhibiting the translation of capped mRNA species [5]. A wide variety of mitogens and stress conditions can influence the phosphorylation status of eIF4E-binding protein (4E-BP1; also known as PHAS-I). It has been proposed that stress conditions, such as heat shock, diabetes, amino acid deprivation or hypoxia, result in 4E-BP1 dephosphorylation [6–9]. In contrast, 4E-BP1 phosphorylation

has been correlated with translational stimulation after the treatment of cells with insulin or growth factor [6]. Several kinases have been shown to phosphorylate 4E-BP1, including FKBP12-rapamycin-associated protein/mammalian target of rapamycin (mTOR) and the MAPKs or ERK1/2 [10,11].

Apart from phosphorylation, translation initiation factor activity can be modulated by other reversible or irreversible modifications of initiation factors. An important example that occurs as a result of infection with certain picornaviruses is eIF4G cleavage, which is responsible, at least in part, for the shutting down of host protein synthesis after viral infection [12]. It is generally agreed that the severe decrease in protein synthesis observed during early recovery after global ischaemia occurs because of an impairment of the initiation step [13]. Results from our laboratory show eIF4E dephosphorylation during global ischaemia [14]; progressive eIF4E degradation during decapitation ischaemia has also been reported [15]. Moreover, evidence of a calpain-mediated eIF4G proteolysis after an ischaemia insult has been provided [16]. All three mechanisms might influence the translation of mRNA species during reperfusion.

The rat pheochromocytoma cell line PC12 displays phenotype traits associated with both adrenal chromaffin cells and sympathetic neurons. On treatment with nerve growth factor (NGF), PC12 cells develop features of sympathetic neurons, i.e. they arrest cellular division and develop neuritic processes as well as electrical excitability and increased choline acetyltransferase levels [17,18]. Different models of combined oxygen and glucose deprivation followed by reoxygenation with PC12 cells have been used as an experimental device for ischaemia studies [19–21]. With a similar model, we have studied the role of 4E-BP1

Abbreviations used: eIF4E, eukaryotic initiation factor 4E; eIF4EP, phosphorylated eIF4E; 4E-BP1, eIF4E-binding protein (also known as PHAS-I); ERK, extracellular signal-regulated kinase; I, ischaemia; IEF, isoelectric focusing; MAPK, mitogen-activated protein kinase; m⁷GTP, 7-methylguanosine triphosphate; mTOR, mammalian target of rapamycin; R30, recovery for 30 min; R2h, recovery for 2 h; LSMD, low-serum medium; NGF, nerve growth factor.

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phosphorylation status as well as its ability to regulate eIF4E availability in ischaemia-induced translational inhibition. In addition, by using different inhibitors, the participation of the different transduction pathways possibly involved in eIF4E and 4E-BP1 phosphorylation has also been investigated.

MATERIALS AND METHODS

Cell culture

PC12 cells were maintained as monolayer cultures in 75 cm² tissue-culture flasks in complete medium [DMEM (Dulbecco's modified Eagle's medium) supplemented with 10% (v/v) horse serum and 5% (v/v) foetal bovine serum, 100 units/ml penicillin, 100 µg/ml streptomycin and 25 µg/ml amphotericin], in a humidified air/CO₂ (19:1) incubator at 37 °C.

Cells were subcultured either in collagen-coated 24-well plates at a density of 5×10^4 cells per well for viability assays and protein synthesis rate or in six-well plates (2.5×10^5 cells per well) for the other experiments. Differentiation of cells was performed in low-serum medium [LSMD, consisting of DMEM supplemented with 1% (v/v) horse serum, 100 units/ml penicillin, 100 µg/ml streptomycin and 25 µg/ml amphotericin], in the presence of 100 ng/ml NGF-7S (Alomone Labs, Jerusalem, Israel) as described elsewhere [22]. The medium was changed twice (on days 2 and 4) during the experiment (5-day period).

Ischaemia model *in vitro*

To provide a glucose-free environment, differentiated cells were washed with glucose-free DMEM medium (DMEM₀). To provide an additional anoxic environment, the cells in DMEM₀ were then placed in oxygen-free medium [DMEM₀ bubbled with N₂/CO₂ (19:1) for 30 min]. The anoxic environment was maintained in a closed chamber without oxygen and gassed with N₂/CO₂ (19:1) for 4 h. At the end of the exposure period, cells were processed (ischaemia, no recovery) or placed in LSMD in an air/CO₂ (19:1) incubator for the different recovery periods: i.e. 30 min (I/R30) and 2 h (I/R2h). Inhibitors (rapamycin, SB203580 and PD98059 from Alexis Biochemicals, San Diego, CA, U.S.A.) were added at the times and concentrations indicated in the Figure legends; 0.1% (v/v) DMSO, used as vehicle, was added to control cells.

Cell viability assays

The Trypan Blue exclusion method was used to assess cell viability. In brief, cells (viable plus non-viable) were removed from the dishes in 10 mM sodium phosphate (pH 7.4)/137 mM NaCl/2.7 mM KCl buffer (PBS); 50 µl of cells was then resuspended in 40 µl of PBS and 10 µl of Trypan Blue (0.04%). Live (Trypan-Blue-excluding) cells were counted with a haemocytometer.

Protein synthesis rate measurement

The protein synthesis rate was assayed in multi-well dishes of 24 mm diameter. The growth medium was aspirated and replaced with 0.25 ml of fresh medium containing 0.175 Ci/mmol [³H]methionine (200 µM), then incubations were performed for 30 min as described elsewhere [22]. All results were normalized according to the number of live cells in culture on the day on which the cells were labelled.

Determination of levels and phosphorylation status of initiation factors

Cells cultured on 35 mm multi-well dishes were washed twice with ice-cold buffer A [20 mM Tris/HCl (pH 7.6)/1 mM dithiothreitol/1 mM EDTA/1 mM PMSF/1 mM benzamidine/10 mM sodium molybdate/50 mM NaF/10 mM β-glycerophosphate/120 mM KCl/10 µg/ml leupeptin/10 µg/ml pepstatin A/10 µg/ml antipain] and lysed (50 µl per 10⁶ cells) in the same buffer containing 0.5% (v/v) Nonidet P40 and 0.1% (v/v) Triton X-100. Cell lysate was centrifuged at 12000 g for 10 min and the supernatants were kept at -80 °C until used. Protein determination was performed by the bicinchoninic acid method [23].

To determine the eIF4E phosphorylation level under the different experimental conditions, 30–35 µg of sample was resolved by isoelectric focusing (IEF) slab gels and analysed by protein immunoblotting as described elsewhere [14]. To resolve the three different 4E-BP1 forms, 30 µg of lysates was resolved by SDS/PAGE [15% (w/v) gel] and analysed by protein immunoblotting with a polyclonal anti-(PHAS-I) antibody (diluted 1/1000) obtained from commercial sources (SantaCruz Biotechnology, Santa Cruz, CA, U.S.A.). Blots were developed by using the enhanced chemiluminescence system and Hyperfilm (Amersham, Little Chalfont, Bucks., U.K.). The percentages of the α, β and γ isoforms in relation to total 4E-BP1 were calculated. To determine eIF4G proteolysis, protein lysate (30 µg) was resolved by SDS/PAGE [7.5% (w/v) gel] and analysed by protein immunoblotting with a polyclonal anti-eIF4G antibody (diluted 1/1000) generously provided by Dr S. J. Morley. The whole gels were transferred and blots were developed with alkaline phosphatase reagents. At least duplicate blots were run for each different culture. Stained bands on immunoblots or Hyperfilm were scanned and quantified with an image analyser with the use of the Diversity One software package (PDI, New York, NY, U.S.A.).

4E-BP1–eIF4E and eIF4G–eIF4E complexes

Measurement of the amount of 4E-BP1 and eIF4G recovered when eIF4E was purified with 7-methylguanosine triphosphate (m⁷GTP)-Sephacryl provides a method for assessing the association of 4E-BP1 and eIF4G with eIF4E. Protein lysate (200 µg) was incubated with 30 µl of m⁷GTP-Sepharose for 15 min at 4 °C, then washed with buffer A. Proteins were eluted with SDS sample buffer and then subjected to electrophoresis for eIF4G [7.5% (w/v) polyacrylamide gel] and eIF4E and 4E-BP1 quantification [15% (w/v) polyacrylamide gel]. eIF4G, eIF4E and 4E-BP1 levels in the complex were detected by immunoblot analysis and quantified as described above for the individual factors.

RESULTS AND DISCUSSION

Protein synthesis rate and viability

PC12 cells were differentiated into a neuronal phenotype by the addition of 100 ng/ml NGF to the medium for 5 days, this preparation being an excellent tool for studying the regulation of protein synthesis at the level of initiation [22]. To eliminate the possible effect of NGF during ischaemia and recovery [19,20], the experiments were performed with differentiated PC12 cells in the absence of this growth factor. NGF withdrawal has been described to induce dendrite retrieval and apoptosis in differentiated cells, although these effects have been observed only during periods much longer than 6 h [24]. We found that periods

Figure 1 Protein synthesis rate after ischaemia and recovery

Protein synthesis was measured in PC12 cells after ischaemia and recovery as described in the Materials and methods section. Results are expressed as [³H]methionine incorporated (c.p.m. per 10³ cells) and are means $\pm$ S.E.M. for three to six different cultures. Abbreviations: C, control; I, 4 h of ischaemia; I/R30, I/R2h, 4 h of ischaemia followed by 30 min or 2 h of recovery respectively. Statistical significances between control and ischaemia/recovery: * P < 0.05, ** P < 0.01. Numbers in parentheses are percentages of inhibition.

of up to 6 h of incubation in the absence of NGF resulted in an unchanged morphological appearance of differentiated cells and did not change the parameters studied either, suggesting that the results obtained are attributable to ischaemia rather than to NGF withdrawal (results not shown). Cell viability decreased during the ischaemia period, reaching a 59 % of dead cells in cells subjected to ischaemia (I), this percentage being similar to that reported by Boniece and Wagner [19]. No further cell death was observed during the recovery period.

To our knowledge, this is the first time that ischaemia-induced protein synthesis rate inhibition has been reported in cultured cells. Our findings show that after 4 h of ischaemia (glucose deprivation plus anoxia), protein synthesis was significantly inhibited and almost reached control levels after 2 h of recovery (Figure 1). Identical results were obtained when the recovery was done in the presence of NGF (results not shown). Similar translational down-regulation has been reported in hippocampal slices subjected to glucose deprivation plus anoxia [25] and in experimental models of ischaemia *in vivo* [13–15].

Levels and phosphorylation status of eIF4E

As shown in Figure 2(A), phosphorylated eIF4E (eIF4EP) levels in cells subjected to ischaemia were significantly lower than in control cells. No changes in the total levels of eIF4E were observed during ischaemia and recovery. During recovery, eIF4E remained dephosphorylated. This was a striking finding because in the *in vivo* model eIF4E phosphorylation decreases during ischaemia but reaches control values at 30 min of reperfusion [14]. When NGF was added during recovery, eIF4E phosphorylated levels reached control values at I/R30 (results not shown), suggesting that a growth-factor-activated pathway is necessary to induce the factor phosphorylation.

Levels and phosphorylation status of 4E-BP1

Earlier studies have established that phosphorylation of the appropriate sites retards 4E-BP1 electrophoretic mobility [26]. 4E-BP1 from most tissues or cells can be resolved into three species, denoted α , β and γ , by subjecting extracts to one-

Figure 2 Phosphorylation statuses of eIF4E (A) and 4E-BP1 (B) after ischaemia and recovery

(A) Cell lysates (35 μ g) were subjected to IEF electrophoresis; bands corresponding to eIF4E and eIF4EP were analysed by protein immunoblotting as described in the Materials and methods section. Results are expressed as percentages of eIF4EP over total eIF4E. (B) Cell lysates (30 μ g) were subjected to SDS/PAGE; bands corresponding to the three different isoforms of 4E-BP1 were analysed by protein immunoblotting as described in the Materials and methods section. Results are expressed as percentages of each isoform with respect to total 4E-BP1. Values are means $\pm$ S.E.M. for six to ten different cultures. Statistical significances between control and ischaemia/recovery: ** P < 0.01, *** P < 0.001. Abbreviations are as in Figure 1. The insets show representative Western blots. In (B), actin was used as a control for loading homogeneity. The α , β and γ isoforms of 4E-BP1 are indicated.

dimensional SDS/PAGE. As shown in Figure 2(B), extracts of control differentiated PC12 cells contained approx. 70 % of the β isoform of 4E-BP1, 13 % of the γ isoform and only 7 % of the unphosphorylated (α) isoform. After 4 h of ischaemia, the most phosphorylated form of 4E-BP1 disappeared and up to 67 % of 4E-BP1 was present in the α form. At I/R30, a rapid increase in the γ isoform, paralleling a decrease in the un-

Table 1 Protein synthesis rate and eIF4EP and 4E-BP1 α levels under ischaemia, anoxia and glucose deprivation

The parameters were measured as described in the Materials and methods section. Results are means $\pm$ S.E.M. for two to five different cultures. Statistical significance of differences between control and experimental conditions: *** $P < 0.001$.

Condition	Protein synthesis rate (% of control)	eIF4EP (% of total eIF4E)	4E-BP1 α (% of total 4E-BP1)
Control	100	60.6 $\pm$ 5.9	6.4 $\pm$ 1.6
Anoxia	79.2 $\pm$ 2.2	55.6 $\pm$ 8.6	14.9 $\pm$ 4.2
Glucose deprivation	88.2 $\pm$ 4.3	50.4 $\pm$ 2.5	34.6 $\pm$ 3.2***
Ischaemia	20.5 $\pm$ 2.2	38.9 $\pm$ 2.7***	67.3 $\pm$ 3.8***

phosphorylated isoform, was observed, whereas the β isoform maintained the same levels as those observed during ischaemia. At I/R2h the three isoform levels almost reached the control values. A non-significant slight decrease in the total levels of 4E-BP1 was observed at I/R30 [19.0 ± 4.8 compared with 34.7 ± 5.7 (means $\pm$ S.E.M.) arbitrary units/mg of protein respectively]. Interestingly, 4E-BP1 phosphorylation during recovery was NGF-independent, suggesting that eIF4E and its binding protein are phosphorylated by different signalling pathways, with both of them being inhibited during ischaemia.

Changes induced by anoxia or glucose deprivation

Differentiated PC12 cells were incubated under anoxic conditions [N_2/CO_2 (19:1)] or in the presence of glucose-free medium in an air incubator, for the same period as the cells that were subjected to ischaemia and recovery. The protein synthesis rate and the eIF4E and 4E-BP1 phosphorylation statuses were determined under both conditions. As shown in Table 1, both glucose deprivation and anoxia induced a slight decrease in protein synthesis rate. eIF4EP levels decreased only 5–10 % under glucose deprivation or anaerobic conditions. A slight decrease in 4E-BP1 phosphorylation was observed under anoxia. 4E-BP1 was significantly dephosphorylated under glucose deprivation, although to a lesser extent than that observed during ischaemia. These findings suggest that ischaemia-induced translation inhibition (anoxia plus glucose deprivation) is not simply induced by hypoglycaemia or anoxia or by the combination of both conditions; they also show that the highest eIF4E and 4E-BP1 dephosphorylation parallels the lowest translational rate.

4E-BP1–eIF4E and eIF4G–eIF4E complexes during ischaemia/recovery

The binding of eIF4E to dephosphorylated 4E-BPs has been shown to prevent eIF4F formation by sequestering eIF4E and to impair cap-dependent but not cap-independent translation [5]. Because ischaemia induces significant dephosphorylation of both eIF4E and 4E-BP1, to establish whether or not eIF4F formation is inhibited during ischaemia, we have determined 4E-BP1 and eIF4G levels that co-purify with eIF4E as a measure of the eIF4F complex. Because eIF4G degradation has been observed in an ischaemia model *in vivo* [16], we first determined total eIF4G levels. The whole gel shown in Figure 3 proves the absence of eIF4G degradation during ischaemia and recovery. Quantification of the unique band detected confirms the lack of changes in eIF4G levels (results not shown).

Figure 3 eIF4G levels after ischaemia and recovery

Cell lysates (30 μ g) were subjected to SDS/PAGE; bands corresponding to eIF4G were analysed by protein immunoblotting as described in the Materials and methods section. The molecular masses of markers and eIF4G position are indicated in kDa. The blot is representative of four different cultures. Abbreviations are as in Figure 1.

As expected, the association of 4E-BP1 with eIF4E increased significantly in ischaemia, and returned to control levels in I/R30 (Figure 4A). Because 4E-BP1 competes with eIF4G to bind eIF4E, this increased binding of 4E-BP1 to eIF4E can induce decreased eIF4F complex formation. In fact, as shown in Figure 4(B), a significant decrease in the association of eIF4G with eIF4E was observed in ischaemia. In contrast, complex formation reached levels even higher than those in controls at I/R30. To our knowledge, this is the first study to demonstrate that ischaemia stimulates the association of 4E-BP1 to eIF4E through 4E-BP1 dephosphorylation. As demonstrated by the decreased amount of eIF4G bound to eIF4E, we can hypothesize that ischaemic cells present an impaired eIF4F function. All these findings suggest that during ischaemia, dephosphorylated 4E-BP1 sequesters eIF4E in an inactive complex that prevents the association of eIF4G to eIF4E, thus inhibiting eIF4E recruitment to the ribosome. In addition, our findings suggest that eIF4F complex formation is independent of eIF4E phosphorylation status, because eIF4E remains dephosphorylated at I/R2h, whereas the 4E-BP1–eIF4E and eIF4G–eIF4E complexes reach control values at I/R30.

Effect of rapamycin, PD98059 and SB203580 on protein synthesis rate and 4E-BP1 and eIF4E phosphorylation statuses

To delineate the potential pathway(s) implicated in 4E-BP1 and eIF4E phosphorylation, we investigated the effect of rapamycin, PD98059 and SB203580, specific inhibitors of mTOR, MAPK kinase ('MEK') and p38 MAPK respectively, on protein synthesis rate and 4E-BP1 and eIF4E phosphorylation in both control and ischaemic PC12 cells.

It has been established that eIF4E is phosphorylated after the activation of Mnk1 kinase, a substrate of ERK and p38 MAPKs (reviewed in [3,27]). Nevertheless, our findings showed that whereas eIF4E phosphorylation remained unchanged in PC12 cells treated with PD98059 (Figure 5) and rapamycin (results not

Figure 4 Association of 4E-BP1 (A) and eIF4G (B) to eIF4E after ischaemia and recovery

Cell lysates (200 μ g) were subjected to m^7 GTP-Sepharose chromatography after immunoblotting with antibodies against eIF4E, 4E-BP1 (A) and eIF4G (B). (A) 4E-BP1–eIF4E complexes were measured as the ratio between 4E-BP1 and eIF4E; results are means $\pm$ S.E.M. for five to eight different cultures. (B) Results are expressed in arbitrary units and are means $\pm$ S.E.M. for five different cultures. The insets show representative Western blots. Abbreviations and statistical significances are as in Figures 1 and 2.

shown), SB203580 decreased eIF4E phosphorylation under either control or ischaemic conditions (Figure 5). These results suggest that the p38 MAPK pathway is involved in eIF4E phosphorylation in differentiated PC12 cells. The fact that SB203580 caused a similar eIF4E dephosphorylation in controls to that in ischaemic cells might indicate that ischaemia has no effect on the p38 MAPK pathway. Treatment with SB203580 induced a slight decrease in both 4E-BP1 phosphorylation and eIF4G–eIF4E complex formation only in control cells (results not shown). As already suggested, taken together our findings suggest that eIF4F complex formation is independent of the eIF4E phosphorylation status.

Different stresses are potent activators of p38 MAPKs; however, whereas arsenite or anisomycin increase eIF4E phosphorylation, others such as H_2O_2 or heat shock do not

Figure 5 Effect of SB203580 and PD98059 on the phosphorylation status of eIF4E

Cells were preincubated for 30 min in LSMD and maintained for 4 h under control (LSMD) or ischaemic (glucose deprivation plus anoxia) conditions in either the absence (DMSO) or the presence of inhibitors [SB203580 (20 μ M) or PD98059 (30 μ M)]. Cell lysates (35 μ g) were analysed by IEF electrophoresis as described in the legend to Figure 2(A). Results are expressed as percentages of eIF4EP over total eIF4E and are means $\pm$ S.E.M. for two to four different cultures. Statistical significances between addition and no addition of inhibitors: * P < 0.05, ** P < 0.01.

increase eIF4EP levels. It has been hypothesized that these latter stresses could decrease 4E-BP1 phosphorylation, thereby decreasing the availability of eIF4E for phosphorylation. This mechanism might operate during ischaemia, in which the increased level of 4E-BP1–eIF4E complex (Figure 4A) caused by 4E-BP1 dephosphorylation (Figure 2B) could impair eIF4E phosphorylation by its corresponding kinases [28].

Several kinases, including ERK1/2 and mTOR, have been shown to phosphorylate 4E-BP1 [10]; furthermore, the MAPK signalling pathway is activated during ischaemia and recovery [29]. As shown in Figure 6(A), a clear increase in ERK1/2 phosphorylation was found in ischaemia; this was completely abolished when ischaemia was induced in the presence of PD98059. Furthermore, the addition of PD98059 did not change 4E-BP1 phosphorylation status in untreated or ischaemic cells (Figure 6B). It seems clear that MAPK is not involved in 4E-BP1 dephosphorylation that is promoted during ischaemia, because under these conditions both the ERK1 and ERK2 isoforms of MAPK are activated. Supporting our findings, recent evidence demonstrating that MAPK can be either activated or inhibited in cells without affecting 4E-BP1 phosphorylation has led to the conclusion that MAPK is neither sufficient nor necessary for 4E-BP1 phosphorylation [30,31].

In contrast, rapamycin caused a significant increase in the level of the unphosphorylated form of 4E-BP1 in control cells; however, no additive effects were observed in rapamycin-treated ischaemic cells (Figure 6B). Our findings implicate the mTOR pathway in 4E-BP1 phosphorylation in PC12 cells. Similarly, ischaemia treatment in the presence of rapamycin induces 4E-BP1 dephosphorylation; because no additive effect was found, our results suggest that ischaemia and rapamycin might act by a common signalling pathway. We also tested the effect of rapamycin on the ability of 4E-BP1 and eIF4G to bind to eIF4E and, as shown in Figure 6(C), rapamycin increased the amount of 4E-BP1 bound to eIF4E to a smaller extent than did ischaemia (2.2-fold compared with 1.4-fold) and neither effect was additive. As expected, eIF4G–eIF4E complex formation significantly

Figure 6 Effect of rapamycin and PD98059 on 4E-BP1 phosphorylation status and on 4E-BP1-eIF4E and eIF4G-eIF4E complexes

Cells were treated as described in the legend to Figure 5 in the absence (DMSO) or presence of inhibitors [rapamycin (50 nM) or PD98056 (30 μ M)]. **(A)** Cell lysates were subjected to SDS/PAGE [12% (w/v) gel]; bands corresponding to ERK1 and ERK2 were analysed sequentially using antibodies against diphosphorylated ERK1/2 and ERK1/2 (Sigma). Blots are representative of three different cultures. **(B)** Cell lysates were analysed as described in the legend to Figure 2(B). A blot representative of two different cultures is shown. Results represent 4E-BP1 α quantification and are expressed as means $\pm$ S.E.M. for at least two different cultures. **(C)** 4E-BP1-eIF4E complex was analysed as described in the legend to Figure 4(A). Results are expressed as the ratio of 4E-BP1 to eIF4E and are means $\pm$ S.E.M. for two or three different cultures. The insets show representative Western blots. The abbreviations for the insets to **(A-C)** are as in Figure 2. **(D)** eIF4G-eIF4E complex was analysed as described in the legend to Figure 4(B). Results are expressed in arbitrary units and are means $\pm$ S.E.M. for two or three different cultures. Statistical significances between addition and no addition of inhibitors: * $P < 0.05$, ** $P < 0.01$.

decreased with rapamycin treatment, although without reaching the level of inhibition caused by ischaemia (Figure 6D).

To assess further the possible implication of the p38 MAPK and mTOR signalling pathways in the effects of ischaemia, we tested the effect of SB203580 and rapamycin on the protein synthesis rate. Supporting the lack of changes in eIF4F formation, SB203580 treatment did not affect the protein synthesis rate in either control or ischaemic cells (Figure 7). In contrast, the observed decrease in eIF4G-eIF4E complex formation in rapamycin-treated control cells was accompanied by a 30% protein synthesis inhibition (Figure 7). Controversial effects of rapamycin on global translational repression have been reported, depending on the cell type studied [9,32-34]. No further protein

synthesis inhibition was observed in ischaemic cells after the addition of the inhibitor (Figure 7).

Translation inhibition during ischaemia is correlated with 4E-BP1-eIF4E complex formation, supporting a role for 4E-BP1 in the regulation of the process. We also demonstrate that the mTOR pathway at least is required for the response to ischaemia. The formation of the 4E-BP1-eIF4E complex obtained in rapamycin-treated cells was lower than that induced under ischaemia, indicating the existence of another mechanism affecting the complex formation in the latter. On the basis of our findings, this unknown mechanism must be independent of either the ERK1/2 and p38 MAPK signalling pathways or changes in eIF4E phosphorylation status. 4E-BP1 is phosphorylated on five

Figure 7 Effect of SB203580 and rapamycin on protein synthesis rate

Cells were treated as described in the legend to Figure 5 in the absence (DMSO) or the presence of inhibitors [SB203580 (20 μ M) or rapamycin (50 nM)] and the protein synthesis rate was measured as described in the legend to Figure 1. The control value corresponds to 65.5 ± 4.8 c.p.m. per 10^3 cells and was taken as 100%. Results are means $\pm$ S.E.M. for two or three different cultures.

serine and threonine residues and mTOR preferentially phosphorylates Thr-36 and Thr-45. The rapamycin-insensitive kinase(s) mediating the phosphorylation of Ser-64, Thr-69 and Ser-82 remain to be determined [35]. In rapamycin-treated cells, 6% of phosphorylated 4E-BP1 was in the γ isoform, whereas undetectable levels of this isoform were found during ischaemia; however, we do not feel that this small difference can explain the difference noted in 4E-BP1-eIF4E complex formation.

We observe a clear parallel between protein synthesis rate and decreased eIF4F formation during ischaemia; however, the impairment of other translation factors should not be excluded. In fact, we have recently found [36] that eIF2(α P) levels are increased in PC12 cells subjected to ischaemia *in vitro*, as has already demonstrated in experimental models of global ischaemia in the rat [14,37,38]. On the basis of our findings, we conclude that ischaemia-induced translation inhibition in differentiated PC12 cells implies the down-regulation of at least two steps, namely complex ternary formation and cap-dependent translation.

We thank Dr S. J. Morley (University of Sussex, Brighton, Sussex, U.K.) for kindly providing the anti-eIF4G antibody, and J. M. Martín Arias for his editorial assistance. The first two authors participated equally in this work, which was supported by grants PM97-0071 from the Ministerio de Educación y Ciencia and 00/0245 from the FIS, Ministerio de Sanidad y Consumo. M.E.M. and F.M.M. are recipients of fellowships from the Comunidad de Madrid.

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Received 5 April 2000/4 July 2000; accepted 3 August 2000