Inhibitors of phospholipase A₂ block the stimulation of protein synthesis by insulin in L6 myoblasts

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Insulin at a concentration close to the physiological range (100 μ -units/ml) stimulated protein synthesis in L6 myoblasts by 17%. Pre-treatment with the phospholipase A₂ inhibitors mepacrine or dexamethasone prevented this stimulation and decreased the release of prostaglandin F_{2 α} , implicating the action of phospholipase A₂ and the subsequent metabolism of arachidonic acid to prostaglandins in the stimulation of protein synthesis by physiological doses of insulin. Higher concentrations of insulin (500–1000 μ -units/ml) stimulated protein synthesis in the presence of mepacrine or dexamethasone, suggesting that an alternative pathway may become important in insulin action when phospholipase A₂ is inhibited.

INTRODUCTION

In isolated skeletal muscle the stimulation of protein synthesis by insulin is positively correlated with prostaglandin F_{2 α} (PGF_{2 α}) release (Reeds & Palmer, 1983), and indomethacin (an inhibitor of cyclo-oxygenase) blocked the stimulation of muscle protein synthesis by insulin both *in vitro* (Reeds & Palmer, 1983) and *in vivo* (Reeds *et al.*, 1985). These observations suggested that the formation of eicosanoids from arachidonic acid was important in the stimulatory action of insulin on muscle protein synthesis. Metabolites of arachidonic acid have also been implicated in the control of protein synthesis in skeletal-muscle myoblasts in culture. Although in one cell line (L8) and in primary chick myoblasts arachidonic acid and PGF_{2 α} failed to affect protein synthesis (McElligott *et al.*, 1988), the stimulation of both protein and RNA synthesis in L6 myoblasts by insulin was completely inhibited by the non-steroidal anti-inflammatory drug indomethacin (Palmer & Bain, 1989). Also, the stimulation by insulin of rRNA accretion and the incorporation of uridine into rRNA were both blocked by indomethacin and ibuprofen (Palmer *et al.*, 1989).

Arachidonic acid for eicosanoid synthesis is released from cell membranes by phospholipases; glucocorticoids are known to inhibit the action of phospholipase A₂ (Hirata *et al.*, 1980) and to induce insulin resistance *in vivo* (Millward *et al.*, 1983). The glucocorticoid hormone dexamethasone also decreased both PGF_{2 α} release and protein synthesis in isolated muscles (Reeds & Palmer, 1984). The object of the present paper was to investigate whether inhibitors of phospholipase A₂ had effects similar to those of cyclo-oxygenase inhibitors on the ability of insulin to stimulate myoblast protein synthesis.

EXPERIMENTAL

L6 myoblasts were obtained from the European Collection of Animal Cell Cultures (PHLS, Porton Down, Salisbury, Wilts., U.K.). All materials for cell culture were obtained from Gibco (Paisley, Scotland, U.K.). PGF_{2 α} radioimmunoassay kits (Dade) were obtained from Steranti Ltd. (St. Albans, Herts., U.K.).

Treatment of the cells and culture conditions have been described previously (Palmer & Bain, 1989). Briefly, on the day before each experiment fresh Dulbecco's modified Eagle's

medium containing 12% (v/v) foetal-calf serum or 0.5 mg of fetuin/ml was added to 50 mm-diam. dishes of sub-confluent cells (2.5 ml/dish). Dexamethasone was added at this time; a final concn. of 100 nM was achieved by addition of 4 μ l of a solution in ethanol. Approx. 18 h later, insulin (Novo; Human Actrapid, 100 units/ml) was added to the medium (final concns. 100–1000 μ -units/ml) for 6 h. Mepacrine was added 30 min before insulin as a solution in ethanol to give concentrations of 12.5 μ M (Expt. 1) or 5 μ M (Expt. 2). Control dishes in all experiments were treated with equivalent amounts of ethanol (4–10 μ l).

Protein synthesis was measured over the final 30 min of incubation by adding L-[2,6-³H]phenylalanine to the medium in aqueous solution to give a concentration of 750 μ M and a specific radioactivity of 2400 d.p.m./nmol. Precisely 30 min later the labelled medium was removed, the cells were rinsed three times with phosphate-buffered saline (Palmer *et al.*, 1989), 3 ml of ice-cold 2% (w/v) HClO₄ was added, and the dishes were stored at 4 °C for 30 min. The HClO₄ was discarded and the cell monolayer was solubilized and transferred into centrifuge tubes with 3 $\times$ 1 ml of 0.3 M-NaOH. After incubation for 30 min at 37 °C, protein was precipitated with 1 ml of 20% HClO₄, centrifuged at 2000 g for 15 min, and re-dissolved in 1 ml of 0.3 M-NaOH. Portions of this solution were used to measure the protein content by a micro-adaptation of the method of Lowry *et al.* (1951) and for scintillation counting (0.5 ml in 10 ml of Optifluor; Packard Instruments, Reading, Berks., U.K.) using the external-standard method of quench correction. Protein synthesis was calculated as d.p.m. of phenylalanine incorporated/30 min per μ g of protein. Significance of differences between treatments was assessed by unpaired *t*-test.

After pre-treatment with insulin, mepacrine or dexamethasone as described above, PGF_{2 α} release into the medium was measured over a 5 h period after the addition of fresh medium containing insulin, mepacrine or dexamethasone at the same concentration (100 μ -units/ml, 5 μ M and 100 nM respectively).

RESULTS AND DISCUSSION

Effects of serum and insulin on protein synthesis

Replacement of serum with fetuin (0.5 mg/ml) resulted in a 25% decrease (*P* < 0.001) in the rate of protein synthesis (Table

Abbreviation used: PGF_{2 α} prostaglandin F_{2 α} .

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Table 1. Effect of serum on response of protein synthesis to mepacrine (12.5 μ M)

Protein synthesis was measured 24 h after fresh medium containing either 12% foetal bovine serum or fetuin (0.5 mg/ml) was added to the dishes. Mepacrine was added 18 h later for 6 h; protein synthesis was measured by addition of a flooding dose of [3 H]phenylalanine for the final 30 min of incubation. Values are mean protein specific radioactivities ($\pm$ S.E.M.; $n = 5$): by Student's t test, * $P < 0.01$, ** $P < 0.001$.

	Sp. radioactivity (d.p.m./ μ g)		
	Control	Mepacrine	% change
12% serum	8.4 $\pm$ 0.6	6.9 $\pm$ 0.7	-18*
0% serum + fetuin	6.3 $\pm$ 0.1	5.0 $\pm$ 0.6	-21*
% change	-25**	-27*	

1) in comparison with cells given fresh medium containing 12% serum. These data are consistent with the observations by Ballard (1982) that protein synthesis in L6 cells was half-maximally stimulated by the addition of only 1% serum, and by Gulve & Dice (1989), who showed a significant effect on protein synthesis of 0.1% serum. The foetal bovine serum which was used in the experiments described here contained low levels of insulin and glucocorticoids: when the dilution of the serum in the culture medium was accounted for, the concentrations of these hormones present in the cultures were 1–2 μ -units of insulin/ml and 1–2 ng of corticosterone/ml. Thus the stimulation of protein synthesis by serum is unlikely to be due to insulin, and may result from the action of other factors such as thyroid hormones or insulin-like growth factors.

Protein synthesis was stimulated (+17%) by 100 μ -units of insulin/ml (Table 2). This was not apparently due to an effect of insulin on amino acid transport, since incorporation of phenylalanine into the intracellular pools was very rapid and unaffected by insulin. At 2 min after addition of 750 μ M-phenylalanine to the medium, the intracellular pool contained $14.9(\pm 0.6) \times 10^3$ and $15.4(\pm 1.1) \times 10^3$ d.p.m./dish ($\pm$ S.E.M., $n = 4$) in the absence or presence of insulin respectively. After 30 min incubation with [3 H]phenylalanine, these values were not significantly changed [$15.6(\pm 0.8) \times 10^3$ and $16.7(\pm 1.1) \times 10^3$ respectively]. The possibility that the effect of insulin was due to a change in the labelling of the tRNA pool has been investigated by Airhart *et al.* (1982). In that study the specific radioactivity of the tRNA was shown to be between that of the intracellular and extracellular free amino acid pools, and to be unaffected by the addition of 120 μ -units of insulin/ml, a concentration similar to that used here.

Effects of mepacrine and dexamethasone

Dexamethasone and mepacrine both inhibit the action of phospholipase A_2 , but act by quite different mechanisms. Glucocorticoids are known to exert their effects on cell metabolism by stimulating the synthesis of specific proteins, and it has been suggested that their action on prostaglandin production is by the synthesis of phospholipase A_2 -inhibitory proteins (lipocortins) (Hirata *et al.*, 1980). Mepacrine forms complexes with the phospholipid substrate, protecting it from degradation by phospholipase A_2 (Blackwell & Flower, 1983). Both inhibitors decreased protein synthesis in L6 cells, although there were differences in their actions. Mepacrine decreased protein synthesis in both the presence and the absence of foetal-calf serum (by 18 and 21% respectively; Table 1). Mepacrine also inhibited ($P < 0.001$) the ability of 100 μ -units of insulin/ml to stimulate protein synthesis, both in the absence of serum (Table 2) and when

Table 2. Effect of mepacrine (5 μ M) and dexamethasone (100 nM) on the stimulation of protein synthesis by insulin in L6 cells

Mepacrine was added to cells 18 h after the addition of serum-free medium and insulin was added 30 min later. Dexamethasone was added to cells at the same time as fresh medium containing 12% serum; insulin was added 18 h later. In both experiments insulin was present for 6 h and protein synthesis was measured by the addition of a flooding dose of [3 H]phenylalanine for the final 30 min of incubation. Results are expressed as a percentage of the corresponding control group, as means $\pm$ S.E.M. for the numbers of replicates shown in parentheses. By Student's t test: * $P < 0.01$, ** $P < 0.001$, versus control; † $P < 0.01$ versus mepacrine only; ‡‡ $P < 0.001$ versus dexamethasone only.

Insulin (μ -units/ml)	Control	Mepacrine
0	100 $\pm$ 4 (9)	79 $\pm$ 3 (10)**
100	117 $\pm$ 4 (9)*	77 $\pm$ 3 (10)**
500	121 $\pm$ 5 (10)*	90 $\pm$ 4 (10)†

	Control	Dexamethasone
0	100 $\pm$ 2 (10)	88 $\pm$ 4 (10)*
100	117 $\pm$ 3 (10)**	85 $\pm$ 5 (10)*
1000	126 $\pm$ 6 (5)**	113 $\pm$ 4 (5)‡‡

serum was present (results not shown). Similar effects of mepacrine on prolactin-stimulated protein synthesis in mouse and rabbit mammary tissue *in vitro* were reported by Rillema *et al.* (1986).

Dexamethasone also decreased protein synthesis (by 12%) and completely abolished the effects of 100 μ -units of insulin/ml (Table 2). However, these effects were only apparent when serum was present; dexamethasone did not suppress protein synthesis and failed to block the stimulatory effect of insulin in serum-free medium.

Effects on $PGF_{2\alpha}$ release

Both mepacrine and dexamethasone also decreased the release of $PGF_{2\alpha}$. After an 18 h preincubation the dexamethasone-induced decrease in $PGF_{2\alpha}$ release during the subsequent 5 h was 67%, from 5.7 ± 0.8 to 1.9 ± 0.4 ng/dish ($P < 0.01$; $n = 10$, $\pm$ S.E.M.). After a preincubation of only 30 min, mepacrine effected a 37% decrease over the same period, from 4.1 ± 0.3 to 2.6 ± 0.2 ng/dish ($P < 0.001$; $n = 15$). Conversely, over a 5 h period, insulin stimulated the release of $PGF_{2\alpha}$ from 4.2 ± 0.2 to 5.2 ± 0.3 ng/dish in the absence of serum ($P < 0.05$; $n = 10$).

The effects of mepacrine and dexamethasone in inhibiting the ability of insulin to stimulate protein synthesis in L6 cells is similar to that of the cyclo-oxygenase inhibitors, which block insulin stimulation both of protein synthesis and of the synthesis and accretion of RNA in the same cell line (Palmer & Bain, 1989; Palmer *et al.*, 1989). Similar effects on protein synthesis have been observed in isolated rabbit muscle (Reeds & Palmer, 1983), and the suggestion that the stimulation of muscle protein synthesis by insulin is dependent on activation of phospholipase A_2 and subsequent eicosanoid production from arachidonic acid (Reeds & Palmer, 1983) is supported by the present work. The inability of arachidonic acid or $PGF_{2\alpha}$ to influence protein synthesis in L8 cells or primary myoblast cultures (McElligott *et al.*, 1988) may not be incompatible, suggesting that myoblasts have sufficient arachidonic acid in their membranes such that added arachidonic acid has no stimulatory effect on growth, but that the enzymes responsible for the release and metabolism of arachidonic acid may be important in mediating the action of insulin, just as it is in isolated muscles (Reeds & Palmer, 1983).

The inhibition by mepacrine of the effect of insulin was partially overcome at higher insulin concentrations; 500 μ -units of insulin/ml stimulated protein synthesis by 14% ($P < 0.01$ compared with mepacrine alone: Table 2). Similarly the dexamethasone-induced inhibition of protein synthesis was overcome by higher insulin concentrations: no significant effect was observed at 500 μ -units/ml, but at 1000 μ -units/ml insulin induced a 28% increase in protein synthesis ($P < 0.001$; Table 2). The ability of supra-physiological concentrations of insulin to overcome phospholipase A₂ inhibition suggests either that high concentrations of the hormone can act on protein synthesis by a prostaglandin-independent mechanism or that, in the presence of phospholipase A₂ inhibitors, an alternative route for PG release is activated. This may involve the release of diacylglycerol by phospholipase C and the subsequent metabolism of arachidonoyl diacylglycerols to release arachidonic acid.

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