Proinsulin-like material in mouse foetal brain cell cultures

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Two main forms of immunoreactive insulin have been identified in cultures of foetal mouse brain using HPLC and gel filtration. The major component which resembled proinsulin was converted by trypsin to the minor form which was similar to authentic pancreatic insulin in chromatographic behaviour. Both components showed immunological properties comparable to insulin and proinsulin including sensitivity of the former to reduction and alkylation.

Proinsulin *Insulin* *Cultured brain cell* *HPLC*

1. INTRODUCTION

The presence and significance of immunoreactive insulin in mammalian brain are topics of some controversy. Studies by two main groups have provided evidence for and against the synthesis of insulin in brain [1–4]. We have recently reported the production of insulin-like material by mouse foetal brain cells in culture [5] and we now describe some of its chemical and immunological properties.

2. EXPERIMENTAL

2.1. *Cell culture*

Cells from 12-day-old post-conception mouse embryos were cultured according to [5] modified as in [6]. In brief, brain tissue was dispersed by trituration, grown for 6 days in Dulbecco's minimum Eagle's medium (DMEM) containing 10% foetal calf serum and 10% heat-inactivated horse serum, then for a further 6 days in DMEM containing 20% heat-inactivated horse serum and arabinosylcytosine, an inhibitor of DNA synthesis. Cells were subsequently cultured in DMEM without added serum. Serum-free medium was extracted and concentrated after 16–18 days using C_{18}-Sep-Paks (Waters Associates) as in [6]. Concentrations of insulin-like material were typically 100 pg rat insulin equivalents/dish per 6 ml culture media. Each dish contained cells derived from half a foetal brain.

2.2. *High performance liquid chromatography*

Culture media (120 ml) was concentrated and the C_{18}-Sep-Pak eluate diluted in 0.1% trifluoroacetic acid, loaded onto a Radial-Pak μ Bondapak C_{18} column (Waters Associates) and chromatographed using a 1 h linear gradient from 90% (v/v) solvent A/10% (v/v) solvent B to 30% (v/v) solvent A/70% (v/v) solvent B [solvent A, 0.1% (v/v) trifluoroacetic acid; solvent B, 80% (v/v) acetonitrile/0.1% trifluoroacetic acid]. Fractions (1 ml) were collected and insulin content was determined by radioimmunoassay [6].

2.3. *Reduction and alkylation*

The C_{18} Sep-Pak concentrate of culture media (138 ml) was lyophilized, dissolved in 0.2 M *N*-ethylmorpholine buffer (pH 8.5) and divided into two aliquots; one was reduced for 1 h under nitrogen with 40 mM dithiothreitol. Both aliquots were then mixed with a 2-fold molar excess of iodoacetamide (over dithiothreitol), flushed with nitrogen, left in the dark at room temperature for a further 2.5 h, then lyophilized before radioimmunoassay.

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2.4. *Gel exclusion chromatography*

Samples were chromatographed on a column (0.9 × 73 cm) of Sephadex G-50 (fine) run in 50 mM phosphate buffer (pH 7.5)/0.3% (w/v) bovine serum albumin.

2.5. *Trypsin digestion*

The major peak of insulin immunoreactivity from HPLC was lyophilized and redissolved in 0.1 M NH_4HCO_3. Half of this material was digested with diphenyl carbamyl chloride-treated trypsin (1%, w/w, with respect to total protein) for 2 h at 37°C. Trypsin was then inhibited with a 10-fold molar excess of phenylmethylsulphonyl fluoride. The digest and untreated insulin-like material were subjected to HPLC as described above. Digestion of pancreatic porcine proinsulin under the same conditions resulted in cleavage to insulin as judged by HPLC.

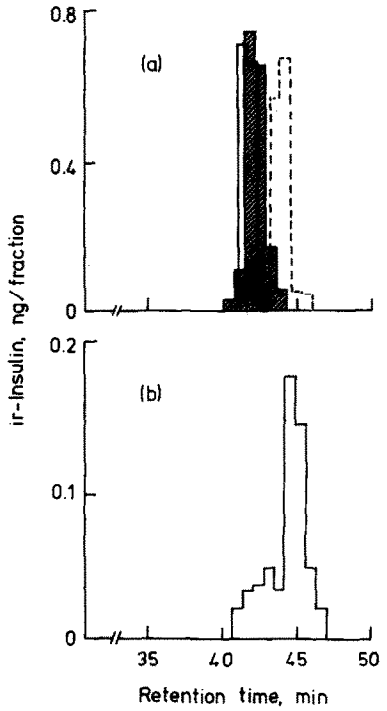

Fig.1. HPLC profile of insulin-like material from cell culture medium. The elution profiles of pancreatic porcine (——) and rat (hatched) insulin and porcine proinsulin (– – –) standards (a) are compared to an extract of culture media chromatographed on the same day (b). The experimental conditions were as described in the text.

3. RESULTS

Analysis by HPLC revealed two peaks of immunoreactivity (fig.1). A minor component had a broad elution profile with a peak retention time slightly greater than those of porcine and rat insulin. The major peak was eluted later, with a retention time similar to that of porcine proinsulin.

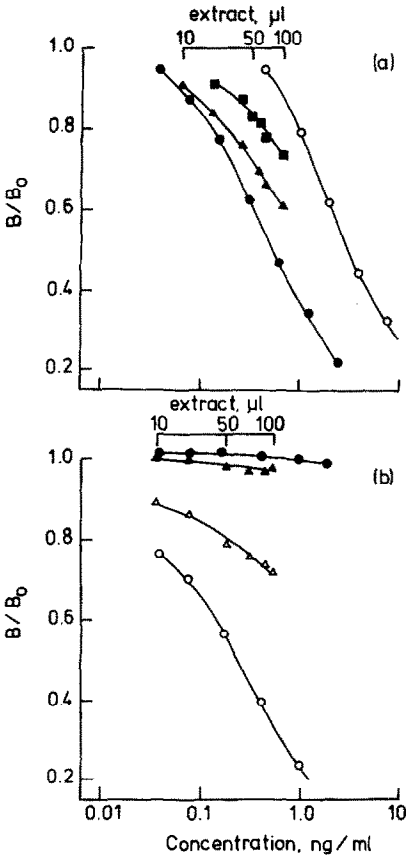

Fig.2. Comparison by radioimmunoassay of insulin-like material with (a) authentic insulin and proinsulin and (b) insulin after reduction and alkylation. Binding of labelled tracer expressed as the B/B_0 ratio is plotted against volume of media extract (top scale) and concentration of standards (bottom scale). The data in (a) are plotted as follows: immunoreactivity of rat insulin (●), porcine proinsulin (○) and early (▲) and late (△) eluting forms of insulin-like material separated by HPLC after concentration of 114 ml culture media using C_{18}-Sep-Paks. (b) Non-reduced insulin (○—○), reduced and alkylated insulin (●—●), non-reduced insulin-like material (△—△) and reduced and alkylated insulin-like material (▲—▲).

Both peaks were compared to rat insulin and porcine proinsulin by radioimmunoassay (fig.2a). Although it was not possible to test immunological activity over the complete concentration range because of logistical difficulties in obtaining sufficient starting material, both forms gave curves similar to those of the standards. The immunological properties of insulin depend upon intact disulphide bonds between the A and B chains; reduction and alkylation of insulin-like material prior to radioimmunoassay resulted in complete loss of immunoreactivity paralleling that of authentic pancreatic insulin (fig.2b).

Gel filtration of concentrates of culture media on Sephadex G-50 (fine) showed major and minor peaks of immunoreactive material similar in M_r to proinsulin and insulin, respectively (fig.3). Gel chromatography of the late-eluting component from HPLC confirmed this to be the high-M_r proinsulin-like form.

To determine if a product-precursor relationship existed between the two forms of insulin-like

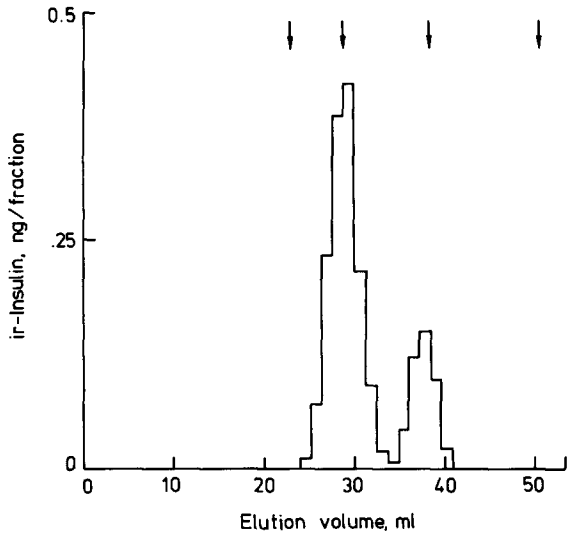

Fig.3. Gel exclusion chromatography of insulin-like material from cell culture media. Lyophilized samples from C_{18}-Sep-Pak concentrates of culture media (240 ml) were redissolved in running buffer and chromatographed as described in section 2. Fractions (1.2 ml) were lyophilized and redissolved in radioimmunoassay buffer (0.5 ml). The arrows (from left to right) indicate the void volume (Blue dextran), porcine proinsulin, ^{125}I-labelled insulin and the salt volume ($Na^{125}I$).

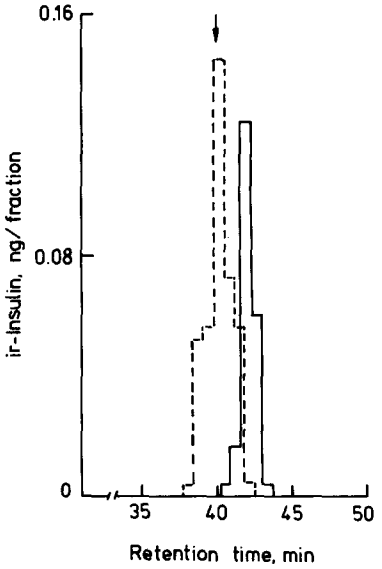

Fig.4. Trypsin digestion of high-M_r insulin-like material. The conditions for enzyme digestion are given in the text. The profiles of insulin immunoreactivity (derived from 180 ml culture media) before (——) and after (– – –) digestion. The arrow indicates the peak elution position of the minor low-M_r form of immunoreactive insulin chromatographed on the same day.

material, the high-M_r species was treated with trypsin and the digest subjected to HPLC (fig.4). This resulted in complete conversion to the lower-M_r form which coincided with an apparent increase in immunoreactivity predicted from the conversion of proinsulin to insulin. From these data it would be expected that the high-M_r form of insulin-like material produced by cultured brain cells contains a peptide sequence (analogous to the C-peptide of insulin) which is bordered by basic amino acids and does not contain any of the essential antigenic determinants.

4. DISCUSSION

Insulin-like immunoreactivity has been described in brain using immunohistochemical techniques [7]. We have recently shown that insulin-like material extracted from whole rat brain behaves as a single component similar to authentic pancreatic insulin by HPLC [6]. However, in cultures of mouse foetal brain cells, the major forms of insulin-like material is immunologically and

structurally related to proinsulin. The proportion of proinsulin-like material was consistently 70–75% of the total insulin immunoreactivity recovered. If this material also has a similar potency in the radioimmunoassay to proinsulin (5–6-fold less potent than rat insulin) then up to 95% of the total immunoreactivity can be accounted for by the high-M_r form. This is in complete contrast to pancreas where proinsulin and partially cleaved intermediate forms represent only up to 5% of the total insulin [8] and may indicate immaturity or late onset of processing enzymes in foetal brain cells.

The presence of proinsulin rather than insulin-like material is a good indication of synthesis by such cells in culture and while one adduces evidence from in vitro experiments with caution, the preponderance of the higher-M_r form suggest a physiological role for the hormone and its precursor in developing mouse brain.

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