Insulin stimulates choline acetyltransferase activity in cultured embryonic chicken retina neurons

(synaptogenesis/neural development/acetylcholine/trophic factors)

JOHN M. KYRIAKIS*, ROBERT E. HAUSMAN, AND SCOTT W. PETERSON†

Biological Sciences Center, Boston University, Boston, MA 02215

Communicated by Herman M. Kalmkar, July 13, 1987

ABSTRACT The effect of insulin on the appearance of the enzyme choline acetyltransferase (ChoAcT; acetyl-CoA:choline O-acetyltransferase, EC 2.3.1.6) in embryonic chicken retina neurons cultured in defined medium was studied. In the presence of a minimal level of insulin (1 ng/ml), ChoAcT activity increased with time in culture. A correspondence between the insulin concentration in the defined medium (1-100 ng/ml) and both the rate of increase and maximum attained level of ChoAcT activity was observed. Maximal ChoAcT activity was 2- to 3-fold greater in cells cultured in the presence of 100 ng of insulin per ml than in cells cultured in the presence of 1 ng of insulin per ml. To elicit maximum ChoAcT activity, insulin at 100 ng/ml was required in the medium for only the first 4 days of the culture period, at which time insulin could be reduced to maintenance levels (10 ng/ml) without affecting ChoAcT activity. Insulin binding assays performed during a 7-day culture period revealed that irrespective of the insulin concentration in the medium during culture, cell-surface insulin receptors decreased by ≈90% between 4 and 7 days in culture. This decrease in insulin binding corresponded to the observed decrease in the sensitivity of ChoAcT activity to insulin. Our findings suggest that insulin plays a role in mediating cholinergic differentiation in the embryonic chicken retina.

Although insulin and insulin receptors are known to be present in the adult and embryonic vertebrate brain (1–4) as well as in cultured vertebrate neurons and glial cells (5–7), the role of insulin in developing neural tissue is not yet known. Aizenman et al. (8) demonstrated that neurons taken from early chicken embryos can survive and extend neurites in culture with only insulin and transferrin as growth factors, while Lindsey and Adler (9) showed that chicken retina neurons require only insulin and linoleic acid for survival in culture. Kessler et al. (10) found that insulin can promote the electrotocoupling of cultured sympathetic neurons, suggesting that insulin may be involved in synaptogenesis.

Although insulin is not required for the appearance of cholinergic markers in developing rat striatal cells (11), insulin may be required for cholinergic synaptogenesis in rat retina cells. Puro and Agardh (12) have shown that insulin can induce the precocious appearance in cultured embryonic rat retina neurons of cholinergic synaptic transmission. Thus, there is some indication that insulin plays a role in the differentiation of retina neurons, including cholinergic neurons.

We have previously demonstrated that there are specific cell-surface insulin receptors on embryonic chicken neural retina cells in vivo and that these receptors decrease in number with embryonic development (13). In this study, we asked whether insulin could effect the appearance in cultured embryonic chicken retina neurons of specific biochemical features of cholinergic differentiation. Using defined medium (14, 15) together with polycornithine-coated culture surfaces (16), we have isolated and cultured neurons from the embryonic chicken retina and studied the effect of insulin on the activity of the acetylcholine synthetic enzyme choline acetyltransferase (ChoAcT; acetyl-CoA:choline O-acetyltransferase, EC 2.3.1.6). ChoAcT was chosen because of the findings of Puro and Agardh (12) concerning the possible role of insulin in rat retina synaptogenesis. It has also been shown that ChoAcT activity in chicken embryo neurons increases with development in vitro and that this increase also occurs in vivo both in neurons cultured in the presence of serum (17, 18) and in neurons cultured in defined medium (15).

In the present study, we also observed that ChoAcT activity increases with time in culture. Furthermore, we observed that the rate of increase as well as the peak ChoAcT activity observed during the 10-day culture period was greater with greater insulin concentrations. To elicit maximum ChoAcT activity, a high concentration of insulin was required in the medium for only the first 4 days of the culture period, at which time insulin could be reduced without affecting ChoAcT activity. Correspondingly, specific cell-surface insulin binding decreased ≈90% starting at day 4 of the culture period. Our data suggest that insulin may be involved in the cholinergic differentiation of retina neurons.

MATERIALS AND METHODS

Materials. Fertilized eggs were from Hardy and Son (Essex, MA). [14C]Acetyl coenzyme A (4 mCi/mmol; 1 Ci = 37 GBq), Na125I (100 mCi/mmol), and 125I-labeled insulin (125I-insulin) (100 μCi/μg) were from ICN. Alternatively, 125I-insulin (48 μCi/μg) was prepared from unlabeled porcine insulin and Na125I as described (19). Dulbecco’s modified Eagle’s medium, penicillin/streptomycin solution (5000 units of penicillin per ml, 50 mg of streptomycin per ml), and glutamine solution (200 mM) were from Flow Laboratories. All other chemicals were from Sigma, except where indicated.

Cell Culture. Retinas were removed from Rhode Island Red chicken embryos at day 7 of development. Cells were dissociated with collagenase as described (13). To prepare cultures of retina neurons, cells were suspended in serum-free defined medium, consisting of Dulbecco’s modified Eagle’s medium supplemented with the following components: 25 mM D-glucose, 2.0 mM L-glutamine, 100 units of penicillin per ml, 1.0 mg of streptomycin per ml, 5 μg of conalbumin per ml, 20 nM progesterone, 100 μM putrescine, 1 mM sodium pyruvate, 0.5 mM d-threo-1-(2-naphthyl)-erythritol, 1 mg/ml bovine serum albumin, and 10 mM HEPES. The pH of the medium was adjusted to 7.4, and the medium was supplemented with 10 ng/ml insulin (125I-insulin).

Abbreviations: ChoAcT, choline acetyltransferase; 125I-insulin, 125I-labeled insulin.

*Present address: Howard Hughes Medical Institute/Harvard Medical School, Diabetes Unit, Massachusetts General Hospital, Boston, MA 02114.
†Present address: Arkansas College, Batesville, AR 72501.

The publication costs of this article were defrayed in part by page charge payment. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. §1734 solely to indicate this fact.
and 30 mM Na₂SeO₃ (14, 15). Five insulin concentrations were used (1, 10, 50, 100, or 500 ng/ml). Cells were seeded, at a concentration of 5.0 × 10⁵ cells per cm², onto Falcon 30-mm Petri dishes or onto Falcon 24-well plates. Culture surfaces had been coated previously with 0.1 mg of poly-L-ornithine per ml as described (16). Cultures were maintained in a humidified atmosphere of 5% CO₂/95% air at 37°C. The medium was changed daily. These techniques have been used successfully in our laboratory (15) as well as in other laboratories (14, 16) to isolate neurons in culture. In this study, at all insulin concentrations used, the cell numbers remained constant during the culture period, as did the overall composition of cells (>95% neurons).

**Insulin Binding Assays.** Cells were cultured for 7 days in serum-free defined medium containing 100, 10, or 1 ng of insulin per ml. During the culture period cells were harvested and assayed for insulin binding. Cells were rinsed twice in Tris-buffered saline (25 mM Tris-HCl/25 mM Tris base/100 mM NaCl, pH 8.0) and once in Tris/Hepes buffer (50 mM Tris-HCl/50 mM Hepes/10 mM MgCl₂/2 mM EDTA/10 mM glucose/10 mM CaCl₂/50 mM NaCl/5 mM KCl/0.1% bovine serum albumin, pH 8.0). To dissociate insulin from the medium that was bound to the cells, the cells were washed in Tris/Hepes buffer for 25 min at 22°C prior to the binding assay. This washing step was sufficient to dissociate any unlabeled insulin bound to the cells during the culture period.

Binding assays were modified from Peterson et al. (13). In the single point assays, regardless of the insulin concentration to which the cells were exposed during culture, total insulin binding was determined using a single concentration of [¹²⁵I]insulin (10 ng/ml, 48 μCi/μg). Cells were incubated for 90 min at 15°C with 1 ml of the tracer in Tris/Hepes buffer. For determination of nonspecific binding, cells were incubated with the same quantity of labeled insulin plus an additional 200 μg of unlabeled hormone per ml. Cell culture plates were washed free of unbound hormone by rinsing three times in Tris-buffered saline. The cells were then lifted from the plates by incubating them with 1 ml of 0.25% trypsin (GIBCO) in Tris saline buffer for 15 min at 37°C. One hundred microliters was removed for determination of cell concentration. The remaining cells were then subjected to γ counting. Specific insulin binding was defined as the difference between total and nonspecific binding. Nonspecific binding accounted for an average of 20% of total binding. In competition studies, cells (subjected to the three culture conditions) were incubated as described above with 1.0 ng of [¹²⁵I]insulin per ml (100 μCi/μg) in the presence of various concentrations of unlabeled ligand (0.1-170 nM). Data were analyzed according to the method of Scatchard (20), with the use of a computer-generated linear best-fit model (21).

**ChoAcT Assays.** The procedure used was that of Fonnum (22) with some modifications from Chrisanti-Combes et al. (17). Cells were solubilized in a buffer consisting of 25 mM NaH₂PO₄/25 mM Na₂HPO₄/0.5% Triton X-100/4.5 mM pH 7.4. The protein concentration of the suspension ranged from 500 to 600 μg/ml. The cell suspension was sonicated for 8 sec to ensure maximum release of enzyme. To 8 μl of the cell suspension, 12 μl of incubation buffer was added. The final concentrations in the 20-μl incubation mixture were 40 μM [¹⁴C]acetate Coenzyme A (44 mCi/mmole), 5.0 mM D-glucose, 100 mM NaCl, 0.1 mM eserine salicylate, 4.5 mM pH 7.4, 300 mM NaCl, 25 mM Na₂HPO₄, 25 mM NaH₂PO₄, and 0.5% Triton X-100. The mixture was incubated for 15 min at 37°C and the reaction was stopped with the addition of 100 μl of 3-heptanone containing 10 mg of sodium tetrathylborate per ml. The suspension was mixed with a Vortex mixer; the phases were separated by centrifugation at 1000 × g for 30 sec, and 50 μl of the top organic phase was transferred to scintillation vials containing 3 ml of Aquasol (New England Nuclear). The radioactivity in the vials was counted in a Beckman scintillation counter. Carnitine acetyltransferase activity was corrected for by assaying parallel samples in the presence of 100 μM naphthyl vinyl pyridine, a ChoAcT inhibitor (23). Under these conditions, the radioactivity in the samples was reduced by >85% (data not shown).

**Protein Determinations.** To correct the ChoAcT data for total cell protein, cell homogenates were assayed for protein according to a modification of the method of Lowry et al. (24) described by Kalczer et al. (25). Bovine serum albumin was used as a standard. The difference in the protein content of cells cultured in the presence of 1 ng of insulin per ml vs. that of cells cultured with 500 ng of insulin per ml never exceeded 20% at any time in the culture period.

**Data Analysis.** Where necessary, statistical significance of data was analyzed with a paired Student's t test.

**RESULTS**

**Changes in Insulin Binding with Time in Culture.** Retina neurons from 7-day embryos were cultured for 1–7 days in serum-free defined medium containing 1, 10, or 100 ng of insulin per ml. Single-point insulin binding assays were performed daily. Regardless of the insulin concentration in the culture medium, insulin binding peaked at day 4 of the culture period and decreased thereafter (Fig. 1A). For cells cultured in each of the three insulin concentrations, there was no significant difference in insulin binding at each time point (P > 0.2). Scatchard analysis showed that the changes in binding with time in culture were the result of changes in receptor numbers, not receptor affinities (Fig. 1B; Table 1). For each of the three groups of cells, both the increase in receptors between days 1 and 4 in culture and the decrease in receptors between days 4 and 7 were significant (P < 0.001; Table 1). The single point assays (Fig. 1A) showed an apparent 60% decrease in insulin binding between days 4 and 7 in culture. However, the [¹²⁵I]insulin concentration used in the single-point assays (10 ng/ml) was sufficient to occupy only 5–30% of the receptors (Fig. 1B). The Scatchard data indicated that the decrease in receptor numbers that occurred in this time period was much larger (=90%) (Table 1). We have reported that similar changes in insulin binding occur in neural retina cells taken between days 10 and 18 of embryonic development (13).

**Effect of Insulin on ChoAcT Activity.** To assess the effect of insulin on ChoAcT activity, embryonic chicken retina neurons were cultured in serum-free defined medium containing various insulin concentrations (1–500 ng/ml). A minimum of 1 ng of insulin per ml was required for cell viability. ChoAcT assays, as described in Materials and Methods, were performed daily in parallel with the single-point insulin binding assays described above. At each insulin concentration, ChoAcT activity increased with time in culture (Fig. 2). With increased insulin concentrations in the culture medium, there was both a greater rate of increase in ChoAcT activity with time in culture as well as a greater maximal level of ChoAcT activity (Fig. 2). Cells cultured in the presence of 100 or 500 ng of insulin per ml attained a maximum ChoAcT activity 2- to 3-fold higher than that of cells cultured in the presence of 1 ng of insulin per ml (P < 0.001). During the culture period, the cell concentrations of these cells exposed to different insulin concentrations remained fairly constant, averaging 5.3 ± 0.4 × 10⁵ cells per cm² overall. In all cases, the insulin concentrations in the medium were below those reported to cross-react with the chicken brain insulin-like growth factor type 1 receptor (3).

**Time Course of Insulin Sensitivity.** Neuron cultures from 7-day chicken embryo retinas were first cultured for 1–5 days
insulin concentration steadied concentration at 10 ng/ml the low ChoAcT activity of um, After in given ng on are incubated with experiments insulin were defined assays, (v) of in embryonic chicken retina Insulin results these embryos and tubulin gene expression in neuroblastoma cultures (28) and promotes the electrotonic coupling of cultured at 4 days or earlier, there was a progressively greater decrease in the maximum observed ChoAcT activity (Fig. 3).

### DISCUSSION

There is increasing evidence that insulin has multiple functions in the central nervous system. Recent studies have indicated that insulin can act as a neuromodulator (26) and a neurotransmitter (27) in the postnatal rat brain. Insulin can accelerate the differentiation in cultured rat retina neurons of cholinergic synapses (12). Insulin potentiates neurite outgrowth and tubulin gene expression in neuroblastoma cultures (28) and promotes the electrotonic coupling of cultured

Table 1. Summary of Scatchard data for insulin binding to embryonic chicken retina neuron cultures as a function of time

<table>
<thead>
<tr>
<th>Time in culture, days</th>
<th>Low affinity ($K_d$) ($10^{10}$ M$^{-1}$)</th>
<th>High affinity ($10^{12}$ M$^{-1}$)</th>
<th>Low affinity</th>
<th>High affinity</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>4.5 ± 0.9</td>
<td>3.2 ± 0.5</td>
<td>26.7 ± 5.5</td>
<td>0.7 ± 0.2</td>
</tr>
<tr>
<td>4</td>
<td>1.0 ± 0.1</td>
<td>2.9 ± 0.4</td>
<td>162.9 ± 18.8</td>
<td>1.2 ± 0.2</td>
</tr>
<tr>
<td>7</td>
<td>7.0 ± 2.1</td>
<td>6.5 ± 2.6</td>
<td>3.9 ± 1.6</td>
<td>0.4 ± 0.1</td>
</tr>
</tbody>
</table>

Cultures of embryonic chicken retina neurons were prepared as described and incubated in serum-free defined medium containing 10 ng of insulin per ml. Cells were harvested after 1, 4, or 7 days in culture and assayed for insulin binding under competitive conditions.

Data were analyzed as described (20). Shown are means ± SEM for three experiments, each with quadruplicate samples. Plots of Scatchard data are shown in Fig. 1B. Parallel experiments performed on cells cultured in the presence of 1 and 100 ng of insulin per ml yielded similar results (data not shown).

in serum-free defined medium containing 100 ng of insulin per ml. After the initial culture period in this high-insulin medium, the culture medium was changed to one containing 10 ng of insulin per ml. Control cells were cultured in either 10 or 100 ng of insulin per ml for the whole 6-day time course. ChoAcT activity was assayed daily; 10 ng/ml was chosen as the low insulin concentration because it was the minimum concentration at which ChoAcT activity was observed to increase steadily for the whole time course (Fig. 2). If the insulin concentration in the medium was reduced from 100 to 10 ng/ml after 4 or more days in culture, there was no significant difference between maximum level of ChoAcT activity of these cells and that of cells cultured only in the presence of 100 ng of insulin per ml ($P > 0.2$; Fig. 3). If the concentration of insulin in the culture medium was reduced

FIG. 1. Insulin binding to cultured embryonic chicken retina neurons as a function of time in culture. Retinas were removed from chicken embryos at 7 days of development and were cultured for 1–7 days in serum-free defined medium containing 100 (●), 10 (○), or 1 ng (■) of insulin per ml as described. (A) Data for single-point insulin binding assays, which were performed daily. Specific insulin binding was defined as the difference between total binding per 10⁶ cells and nonspecific binding per 10⁶ cells. Data are means ± SEM for three experiments with quadruplicate samples. (B) Results of competitive insulin binding studies of cells cultured for 1, 4, or 7 days in serum-free defined medium containing 10 ng of insulin per ml. Cells were incubated with 1.0 ng of [125I]insulin per ml (100 µCi/µg) plus various concentrations of unlabeled insulin (0.10–70 nM). The data were analyzed as described (20). ●, One day in culture; ○, 4 days in culture; ■, 7 days in culture. Parallel experiments performed on cells cultured in defined medium containing 1 and 100 ng of insulin per ml yielded similar results (data not shown). Results for a typical experiment are shown. Scatchard data for the three experiments performed on cells cultured in the presence of 10 ng of insulin per ml are given in Table 1.

FIG. 2. Effect of insulin dosage on the onset of ChoAcT activity in cultured embryonic chicken retina neurons. Retinas were removed from chicken embryos at day 7 of embryonic development. Neuronal cultures in serum-free defined medium containing 1 (●), 10 (○), 50 (■), 100 (▲), and 500 ng (■) of insulin per ml were prepared and assayed for ChoAcT as described. ChoAcT activity for each day in culture was defined as pmol of [14C]acetycholine (ACH) formed per min per mg of total cell protein. Data are means ± SE for four experiments, each with triplicate samples.
which the cells were exposed during culture (Fig. 1B). For example, at day 1 of the culture period, 1 ng of insulin per ml was sufficient to occupy <1% of the available receptors, while 500 ng of insulin per ml was sufficient to occupy >70% of the available receptors (Fig. 1B). Thus, it is possible that differences in insulin receptor occupancy during culture were responsible for the differences in ChoAcT activity shown in Fig. 2. Notably, cells cultured in the presence of 1 ng of insulin per ml showed a 3-day lag in ChoAcT activity in spite of an almost linear increase in the number of available insulin receptors (Fig. 1A).

Interestingly, this correspondence between the insulin concentration in the medium, insulin receptor occupancy, and ChoAcT activity did not extend for the entire time course. When cells incubated in defined medium containing a relatively high insulin concentration (100 ng/ml) were placed after a short culture period (1–4 days) into defined medium with a relatively low insulin concentration (10 ng/ml), the observed increase in ChoAcT activity indeed resembled more that of cells maintained for the entire culture period in low insulin medium. However, this effect became less pronounced the later in the culture period that the cells were switched into low-insulin medium. By day 5 in culture, reducing the insulin concentration in the medium had no effect (Fig. 3). Thus, there was a change in the sensitivity of ChoAcT activity to insulin. Furthermore, there was a correspondence between this change in insulin sensitivity and the onset of decreased insulin binding (Figs. 1A and 3). It is conceivable, then, that there is a causal relationship between these two phenomena, involving a decrease in the sensitivity of ChoAcT activity to insulin as the cells lose their surface insulin binding capacity with time in culture.

It is unlikely that our observations were due to the selective enhancement by insulin of cholinergic cell survival because the cells used in these studies were postmitotic and because there was no observed change in cell concentration with time in culture or with the insulin concentration in the medium. In all cases, daily measurement of cell concentration for the cells exposed to the different levels of insulin revealed an average cell concentration of $5.3 \pm 0.4 \times 10^6$ cells per cm$^2$. The mechanism by which insulin binding ultimately results in stimulation of ChoAcT activity is not known.

We have previously reported that insulin binding to neural retina cells decreases in vivo when measured starting at day 10 of development (13). This result corresponds to findings in the present study that a decrease in insulin binding to retina neurons taken from 7-day embryos begins 4 days into the culture period.

Previous work (15, 29) indicates that embryonic chicken retina neurons can differentiate in culture. Our results on the effect of insulin on the appearance of ChoAcT activity in primary cultures of embryonic chicken retina neurons are consistent with the contention (12) that insulin is a mediator of neuronal cholinergic differentiation. The observed similarities between the changes in ChoAcT activity and insulin binding that occur in cultured embryonic retina neurons and in neural retina cells examined in vivo (13, 17), indicate that primary cultures of retina neurons in defined medium are a valid model system for the study of the role of insulin developing retina and brain. The observation by Large et al. (18) of parallel pre- and postsynaptic development of the cholinergic synapse indicates that it may be worthwhile to investigate the role of insulin in the differentiation of other biochemical features of the cholinergic synapse such as voltage-gated calcium channels and the acetylcholine receptor.

This work was supported in part by National Institutes of Health Grant EYO4461 and National Science Foundation Grant BNS-8511246 to R.E.H.