Laminin Increases Both Levels and Activity of Tyrosine Hydroxylase in Calf Adrenal Chromaffin Cells

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Abstract. We have investigated the effects of substrate-bound laminin on levels of enzymes of the catecholamine biosynthetic pathway in primary cultures of calf adrenal chromaffin cells. Laminin increases the levels of the enzymes tyrosine hydroxylase, dopamine-beta-hydroxylase, and phenylethanolamine-*N*-methyltransferase. This effect is selective, in that levels of other enzymes (lactate dehydrogenase, aromatic amino acid decarboxylase, and acetylcholinesterase) are not increased. The effect of laminin can be blocked by antibodies directed against a fragment of the heparin-binding domain of the molecule, whereas antibodies directed against other fragments do not block the increase in tyrosine hydroxylase. Thus the laminin domain involved in enzyme regulation in chromaffin cells is apparently the same as that previously implicated in laminin's interactions with neurons to potentiate survival and stimulate neurite outgrowth (Edgar, D., R. Timpl, and H. Thoenen, 1984, *EMBO (Eur. Mol. Biol. Organ.) J.*, 3:1463–1468). The increase in chromaffin cell tyrosine hydroxylase levels is preceded by an activation of the enzyme in which the V_{max} (but not the K_m) is altered. The effects of laminin appear to be developmentally regulated, since neither activation nor increased levels of tyrosine hydroxylase occur in adult adrenal chromaffin cells exposed to laminin.

RECENT in vitro studies have shown that the effects of soluble neurotrophic molecules, such as nerve growth factor (NGF)[1] and brain-derived neurotrophic factor, on neuronal survival and neurite outgrowth are influenced by the extracellular matrix. A variety of cultured cells produce molecules that, when attached to the culture substrate, promote rapid neurite outgrowth (4, 9, 17, 21, 27). Although these undefined molecules do not directly support neuronal survival, they can potentiate survival in response to NGF (10). Similarly, the basement membrane protein laminin also stimulates neurite outgrowth (6, 26, 38) and potentiates the survival of appropriate target neurons in response to both NGF and brain-derived neurotrophic factor (11, 24). Interactions of laminin with a variety of other cell types have been shown to evoke multiple effects, including changes in morphology due to a cytoskeletal reorganization and promotion of cell growth and differentiation. Additionally, it has been demonstrated that growth of melanoma cells on laminin leads to an increased synthesis of melanin (19).

Laminin is a well-characterized glycoprotein of molecular weight $\sim 1 \times 10^6$, with a cross-shaped structure as revealed by rotary shadowing (13). It has several distinct structural domains that have been analyzed by producing proteolytic fragments of the molecule (for reviews see references 43 and 48). These fragments have then been used in various biological assay systems, as well as for affinity purification of fragment-specific antibodies from laminin antisera (36, 42, 44). It has therefore been possible to correlate some of the structural domains of laminin with its functional properties. For example, a heparin-binding fragment (fragment 3) of the molecule (33) has been implicated in the interaction of laminin with embryonic chick sympathetic neurons to potentiate NGF-mediated survival and neurite outgrowth (11). Fragments corresponding to other domains of laminin are involved in its binding to type IV collagen, tumor cells, and hepatocytes (36, 42, 44).

Like sympathetic neurons, adrenal chromaffin cells are derived from the neural crest (22), synthesize and release catecholamines, and have NGF receptors (32). However, the response of bovine chromaffin cells to NGF depends on developmental stage: At early stages (early fetus), cells respond to NGF with process formation as well as selective increases in levels of the regulatory enzymes in catecholamine biosynthesis, tyrosine hydroxylase (TH), dopamine-beta-hydroxylase (DBH), and phenylethanolamine-*N*-methyltransferase (PNMT). At later developmental stages (late fetus, calf), only increased enzyme levels are seen in response to NGF treatment. In adult bovine chromaffin cells, despite the presence of receptors, no response has, as yet, been found to NGF treatment (32). In contrast to sympathetic neurons, however,

[1] *Abbreviations used in this paper:* AChE, acetylcholinesterase; DBH, dopamine-beta-hydroxylase; DDC, aromatic amino acid decarboxylase; KRH, Krebs-Ringer Hepes buffer; LDH, lactate dehydrogenase; NGF, nerve growth factor; PNMT, phenylethanolamine-N-methyltransferase; TH, tyrosine hydroxylase.

bovine chromaffin cells are not dependent upon NGF for survival at any developmental stage (32). It is therefore of interest to determine whether laminin would potentiate the effect(s) of NGF in bovine adrenal chromaffin cells as in sympathetic neurons. To this end, we chose to work with calf chromaffin cells to address the following questions: (*a*) Could substrate-bound laminin enable cells to respond to NGF as they had at earlier stages in development, i.e., with process formation as well as with increased enzyme levels? (*b*) Would laminin increase the potency and/or efficacy of NGF to increase enzyme levels? (*c*) Which of the functional domains of laminin (neuron vs. non-neuronal cell binding domain) would be effective in interacting with chromaffin cells? The results show that while laminin does not potentiate the NGF-mediated increase in enzyme levels, it has a direct effect on TH activity by itself, bringing about both activation and increased amounts of the enzyme.

Materials and Methods

Cell Culture

Calf adrenal medullary cells were isolated and cultivated using a previously described method (3), which involves retrograde perfusion of the intact adrenal gland with collagenase, followed by further mechanical and enzymatic dissociation, and finally centrifugation through a bovine serum albumin (BSA) step-gradient to obtain the final purified cell suspension. Medullary cells were cultured in a defined medium (7), and 90% of the cells were identified as chromaffin cells after 5 d in culture using TH immunofluorescence as a marker (see Fig. 1). The plating densities were as follows (unless otherwise stated): For all the studies addressing the issue of the direct effect of laminin on TH activity, plating density was 1×10^4 cells/cm^2, with the exception of the experiments done with fragment-specific antibodies, where the plating density was 3×10^4 cells/cm^2. In studies in which NGF-mediated TH induction was examined, the plating density was 1×10^5 cells/cm^2. Before the addition of test substances, fresh medium was added to the cells. NGF (40) was diluted in culture medium before addition, as were α-amanitin (Serva, Heidelberg, FRG; final concentration 11 μM) and cycloheximide (Sigma Chemie GmbH, Munich, FRG; final concentration 1 μM). When drugs were present for periods of time shorter than the total duration of the experiment, cultures were washed twice with fresh medium before the addition of drug-free medium. Harvesting was done by scraping the cells off the dish with a rubber policeman, centrifuging the resulting suspension of intact cells at 9,000 *g* for 2 min, and completely removing the medium from the cell pellet before freezing at −20C.

Culture Substrates

All tissue culture dishes (Falcon Labware, Becton Dickinson GmbH, Heidelberg, FRG) were coated with poly-DL-ornithine (polyornithine; Sigma type I-B; 0.5 mg/ml in 0.15 M borate buffer, pH 8.3). The dishes were washed twice with water, and then either used for culture (controls) or further coated with laminin. Laminin, purified from the mouse Engelbreth-Holm-Swarm sarcoma (45), was dissolved at a concentration of 10 μg/ml in Krebs-Ringer Hepes buffer (KRH: 125 mM NaCl, 4.8 mM KCl, 1.3 mM $CaCl_2$, 25 mM Hepes, 1.2 mM $MgSO_4$, 1.2 mM KH_2PO_4, and 5.6 mM glucose, pH 7.4). 1 ml of this solution was then used to coat a 35-mm dish. The dishes were incubated overnight at 4°C, and the laminin solution was removed immediately before culture. Antibodies against laminin fragments were purified from anti-laminin antisera raised in rabbits by affinity chromatography over columns containing bound fragments (11, 33, 39, 42). These purified antibodies (at final concentrations of 8.8 μg IgG/ml [anti-fragment 1–4], 9 μg IgG/ml [anti-fragment 3], 8.4 μg IgG/ml [anti-fragment 1], and 14.2 μg IgG/ml [anti-fragment 4]) were then added to the medium in which the cells were suspended immediately before plating.

Harvesting and Replating

Cells were enzymatically harvested and replated according to a slight modification of the previously described method (1). Briefly, Ca^{++}/Mg^{++}-free KRH containing 0.5 mM EDTA and 0.01% trypsin was added to the dishes, and after a 10-min incubation at 37°C, the reaction was stopped by adding an equal volume of complete KRH containing 1% BSA (Sigma Chemie GmbH, fraction V) and 0.01% soybean trypsin inhibitor (Sigma Chemie GmbH). Cells were then gently washed off the dish, collected by a low speed centrifugation step (100 *g*, 20 min), and resuspended in fresh medium. In the experiments described here, cells were initially plated at a density of 2×10^4 cells/cm^2 on 90-mm polyornithine-coated dishes. They were then replated at a density of 1×10^4 cells/cm^2 on polyornithine- or laminin-coated 35- or 58-mm dishes.

Measurement of Enzyme Activity

Cell extracts were obtained by resuspending the thawed cell pellets in 5 mM Tris-HCl, pH 7.4, containing 0.1% Triton X-100, followed by centrifugation at 9.000 *g*. TH activity was then determined in an aliquot of this extract containing ~1 μg soluble protein using the ^3H_2O-release method, as described in detail previously (3). For the determination of the apparent K_m and V_{max} values for TH, the concentration of the synthetic cofactor 6-methyl-5,6,7,8-tetrahydropterin HCl was varied between 0.05 and 4 mM (5–7 concentrations per experiment), while the concentration of tyrosine remained constant at 120 μM. The kinetic constants were calculated using a regression line fitted to the data plotted on a double-reciprocal plot. The activities of acetylcholinesterase (AChE), aromatic amino acid decarboxylase (DDC), DBH, PNMT, and lactate dehydrogenase (LDH) were measured as previously described (3). Soluble protein content of the samples was measured using the method of Peterson (34), using bovine gamma-globulin as a standard. Specific enzymes activities are expressed as: TH, nanomoles ^3H_2O formed per minute per milligram soluble protein; DBH, nanomoles norepinephrine formed per minute per milligram soluble protein; PNMT, picomoles metanephrine-methyl-^{3}H formed per minute per milligram soluble protein; DDC, nanomoles $^{14}CO_2$ formed per minute per milligam soluble protein; AChE, nanomoles ^{14}C-acetate formed per minute per milligram soluble protein; LDH, units per milligram soluble protein, 1 U being defined as the change in optical density at 340 nm/min.

Immunotitration

Immunotitration of TH activity from the cell extracts was carried out according to a modification of the method of Renaud et al. (37), which has been described in detail previously (1). Briefly, cells were homogenized in 5 mM Tris buffer, pH 7.4, containing 0.1% Triton X-100, 150 mM NaCl, and 2.5% BSA, and aliquots of the resulting cell extracts were incubated first with varying amounts of anti-TH antiserum (IgG fraction; 29), and subsequently with *Staphylococcus aureus* Cowan I strain. After centrifugation, TH activity remaining in the supernatant was determined.

Indirect Immunofluorescence

Indirect immunofluorescence was carried out on cells in culture and frozen sections of calf adrenal medulla. Cells in culture were fixed in phosphate-buffered saline (PBS) containing 4% paraformaldehyde, pH 7.3, for 15 min at room temperature. Fixed cells were then washed twice with KRH, then incubated for 15 min in 150 mM NaCl, 5 mM EDTA, 50 mM Tris-HCl, pH 7.4, 3% BSA, 0.25% gelatin, and 0.1% Triton X-100 (antibody binding solution), followed by incubation with a mouse anti-TH monoclonal antibody (hybridoma supernatant diluted 1:20 in antibody binding solution) for 1 h at room temperature. The monoclonal antibody was obtained from mice immunized with TH purified from rat PC12 cells (a gift from Dr. J. Thibault). It recognizes a single band of the appropriate molecular weight on an immunoblot of total bovine adrenal medullary protein (data not shown). In addition, the monoclonal binds to protein A-Sepharose, and precipitates TH activity from bovine and avian adrenal homogenates (Acheson, A., H. Rohrer, and J. Thibault, unpublished observations). Adrenal medullary tissue was fixed by perfusing the gland with freshly prepared 120 mM phosphate buffer, pH 7.3, containing 4% paraformaldehyde (30–50 ml) within 1 h of obtaining the gland from the slaughterhouse. The medullary tissue was then dissected out in small pieces, and further fixed by immersion in the paraformaldehyde solution overnight at 4°C. The tissue was then extensively rinsed with 25% sucrose, and 10-μm sections were prepared using a cryostat. Frozen sections were then incubated at room temperature for 2 h in PBS containing 10% goat serum and 0.1% Triton X-100 followed by incubation overnight at 4°C with rabbit anti-laminin antiserum (39) diluted 1:500 in PBS containing 10% goat serum and 0.1% Triton X-100. After extensive rinsing with the PBS-goat serum-Triton buffer (sections) or antibody binding solution (cells), the mouse anti-TH antibodies were detected with fluorescein isothiocyanate-labeled goat anti-mouse antiserum (1:100) (Nordic, Tilburg, Netherlands) and rabbit anti-laminin antibodies with goat anti-rabbit antiserum (1:100) (Nordic) labeled with rhodamine. Goat pre-immune serum was used as the control for nonspecific binding with sections, and a mouse anti-NGF monoclonal antibody (20) (hydridoma supernatant diluted 1:20) was used as the control with cells in culture.

Results

Morphological Effects

When chromaffin cells were plated on laminin-coated dishes, there was a tendency towards an increased flattening of the cells, which are normally spherical and thus extremely phase-bright. This tendency was most pronounced shortly after exposing the cells to laminin (1–2 d) (Fig. 1). Little spontaneous process formation was seen, nor was there any obvious difference in the extent of clumping of the cells over time in culture (Fig. 1). The plating efficiency on laminin was not different from that on polyornithine (data not shown; see Fig. 1). When NGF was added to cells plated on laminin, there was no difference in the extent of process formation over a period of 2–3 d as compared with NGF-treated cells plated on polyornithine (data not shown). In both cases, process formation was rare.

Distribution of Laminin Relative to Chromaffin Cells In Vivo

Frozen sections of calf adrenal medulla stained for laminin revealed clusters of chromaffin cells that were bounded by an intensely stained laminin-positive sheath (Fig. 1). Individual cells were not seen to be completely surrounded by laminin, whereas many chromaffin cells appeared to be contacting one another in clusters of varying size (Fig. 1).

Effect of Laminin on TH Activity

Plating chromaffin cells on polyornithine at low density (1×10^4 cells/cm^2) resulted in a decrease in TH specific activity during the first 30 h in culture to a lower level that subsequently remained constant (see Figs. 2 and 3). When the chromaffin cells were plated at this same low density on laminin-coated dishes, the decrease in TH specific activity was much less pronounced (Fig. 2). Under both of these conditions, as in all subsequent experiments, no change was measured in cellular protein. The ability of laminin to maintain TH activity could be blocked by cycloheximide present during the entire 48 h period, but activity could be partially restored if the cycloheximide was removed after the first 24 h (Fig. 2).

To test if laminin could directly increase TH activity, we first plated the cells on polyornithine at low density for 24 h, at which time TH specific activity had reached its new low level. Cells were then harvested and replated at a similar low density on either polyornithine or laminin-coated dishes. TH specific activity was then measured at various times after replating. The initial response to laminin exposure was a rapid fourfold rise in TH activity (maximal 6–8 h post-replate), which had decreased to a lower value (1–2-fold increase) by 24–48 h post-replate (Fig. 3). Assay conditions (1–3 mM 6-methyl-5,6,7,8-tetrahydropterin HCl, 120 μM tyrosine, pH optimum) were such that a change in the K_m of the enzyme for cofactor would not have been likely to have been detected (2, 30), suggesting that only the apparent V_{max} of TH was altered. Immunotitration carried out at 6 and 48 h post-replate revealed that, at the early time, all of the increased activity was due to enzyme activation, i.e., the equivalence points determined from cells on polyornithine (40 ± 2 μg IgG) versus laminin (36 ± 3 μg IgG) (values determined from three separate experiments, one of which is shown in Fig. 4*a*) were the same. At the later time, however, ~70% of the increased activity was due to an increased number of TH molecules (Fig. 4*b*). Equivalence points determined 48 h post-replate were: polyornithine, 41 ± 3 μg IgG; laminin, 64 ± 2 μg IgG, as determined from three separate experiments, one of which is shown in Fig. 4*b*. Direct determination of the apparent K_m and V_{max} values confirmed that at both 6 and 48 h after replating onto laminin the K_m value was unchanged (at 6 h, K_m is 156 μM; at 48 h, K_m is 152 μM; for control, K_m is 147 μM), whereas the V_{max} increased from 5.1 nmol/min per mg (control value) to 20.2 nmol/min per mg (6 h after replating onto laminin), then subsequently decreased to 12.6 nmol/min per mg (48 h post-replate). Cycloheximide prevented both the early (6 h) and late (48 h) effects, whereas α-amanitin, an inhibitor of mRNA synthesis, only blocked the later increase in levels of TH (Table I). Taken together, these data indicate that exposure of chromaffin cells to substrate-bound laminin resulted in a rapid activation of TH, which gradually disappeared, followed by a slower increase in numbers of TH molecules, which became the predominant effect with increased time of laminin exposure. It should be noted that the harvesting and replating procedure was necessary, since laminin presented in solution during culture could not mimic the effects of substrate-bound laminin (data not shown). In addition, laminin (10 μg/ml) added directly to the cell extract also had no effect on TH activity (data not shown). Similarly, coating the dishes with 10 μg/ml BSA or bovine gamma globulin could not mimic the effect of coating the dishes with laminin (data not shown).

Dependence of Laminin Effect on Developmental Stage

Adult chromaffin cells were initially plated at low density on polyornithine, and 24 h later, were harvested and replated at a similar low density on either polyornithine- or laminin-coated dishes. At 6, 24, and 48 h post-replate, specific TH activity was determined. Neither a short-term activation nor long-term increase in TH levels brought about by laminin were detected in adult cells (Table II). This is in contrast to the effect of cell contact, which is present in both calf and adult cells to a similar extent (see below), but is reminiscent of the effect of NGF, which is also absent in adult cells (32).

Influence of Laminin on NGF- and Cell Contact–mediated Effects on TH

We next examined the ability of laminin to alter the dose-response curve for the NGF-mediated increase in TH levels. Cells were cultured on laminin-coated dishes for 24 h before adding varying concentrations of NGF. The specific activity of TH was determined 3 d later. Laminin altered neither the efficacy nor the potency of NGF with regard to increased levels of TH (Fig. 5). Thus, although laminin did not potentiate the long-term effects of NGF in calf chromaffin cells, it has itself a direct effect on TH levels.

As not only NGF but also cell contact regulate TH activity in primary cultures of chromaffin cells (1), we wanted to determine whether there was a relationship between the cell contact–mediated effect and that of laminin. Cells were plated at low, intermediate, or high density, and harvested after 3 d in culture. The increase in TH specific activity due to laminin was pronounced only at low density, was much smaller at

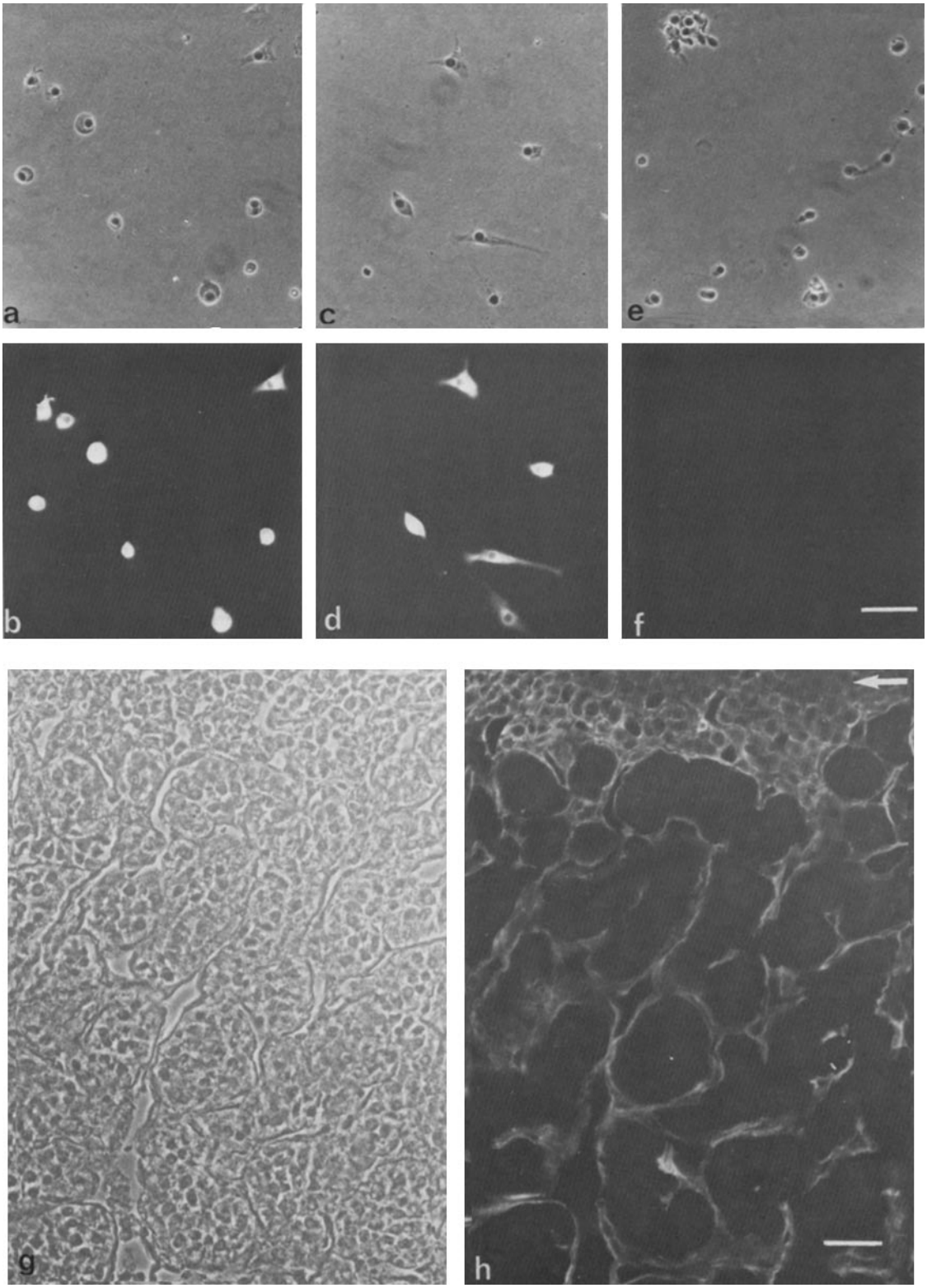

Figure 1. Localization of TH and laminin by indirect immunofluorescence in primary cultures and sections of calf adrenal medullary tissue. (*a–f*) Calf adrenal medullary cells were in culture for 48 h on either polyornithine (*a, b, e,* and *f*) or laminin (*c* and *d*). Chromaffin cells were identified as being TH-positive using indirect immunofluorescence (see Materials and Methods) (*b* and *d*). Nonspecific immunofluorescence is shown in *f*. *a, c,* and *e* show the cells viewed in phase-contrast. Bar, 50 μm. (*g* and *h*) Frozen sections of calf adrenal medulla were stained for laminin (see Materials and Methods). *g* shows a phase-contrast view of the unstained section, in which clusters of chromaffin cells can be seen. *h* shows the pattern of laminin immunofluorescence. Laminin immunoreactivity surrounds the clusters of chromaffin cells. Note that the laminin staining pattern in the adrenal cortex (upper portion of the section, denoted by an arrow in the upper right-hand corner) is completely different from that seen in the medulla, in that individual cells (or smaller clusters of cells) are seen to be surrounded by laminin-positive immunoreactivity. Bar, 100 μm.

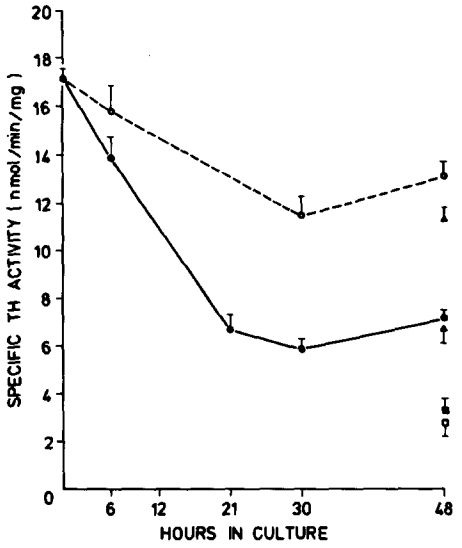

Figure 2. Effect of laminin on the initial decrease in TH specific activity after plating at low density. Chromaffin cells were plated at low density (1×10^4 cells/cm^2) on either polyornithine (●) or laminin (○) coated dishes. The specific activity of TH was determined as a function of time after the initial plating. Cycloheximide (1 μM) was added to some cultures from the beginning of the culture period, and specific TH activity was determined 48 h later (■, polyornithine; □, laminin). In additional cultures, cycloheximide was present only for the initial 24 h, after which time the cultures were washed and incubated drug-free for the remaining 24 h (▲, polyornithine; △, laminin). The values given represent the mean ± SEM of three independent experiments. The value given for zero hours in culture represents in vivo TH activity, as determined in dissociated cells which had not been brought into culture.

intermediate density, and was absent at high density (Fig. 6), showing that the effects of these two stimuli are not additive. Furthermore, increased cell density resulted in selective increases in TH, DBH, and PNMT activities, with no change in DDC, AChE, or LDH specific activities (Fig. 7). Laminin resulted in the same pattern of enzyme induction (Fig. 7). This pattern is in distinct contrast to that of NGF-mediated enzyme induction, where, in addition to increases in TH, DBH, and PNMT, AChE is also induced (3).

Identification of the Laminin Domain Responsible for Its Effects on Chromaffin Cells

To determine which of the domains of the laminin molecule was responsible for the long-term (48 h) increase in TH in calf cells, we added various fragment-specific antibodies to laminin-coated dishes. Without further washing, cells were plated at low density onto the dishes, and TH activity was measured after 2 d. Affinity-purified antibodies directed against the heparin-binding fragment 3, and also against fragments corresponding to whole or part of the previously characterized cell-binding domain, were tested. Only antibodies directed against fragment 3 could fully inhibit the increase in TH activity (Table III). Thus, laminin appears to interact with chromaffin cells via the same structural domain as that previously shown to be responsible for its effects on neurons (11) rather than via the domain corresponding to fragment 1–4 which interacts with a variety of non-neuronal cells (see reference 43 for review).

Discussion

Laminin is known to potentiate the survival and neurite outgrowth of target neurons of both NGF and brain-derived neurotrophic factor at early developmental stages (11, 24). Moreover, it has been demonstrated that a specific domain of the laminin molecule comprising a heparin-binding fragment is associated with its effects on embryonic chick sympathetic neurons (11). In view of laminin's ability to potentiate the effects of NGF without itself promoting survival, it seems that laminin and NGF have distinct but complementary mechanisms of action. It was of interest, therefore, to determine whether laminin could potentiate NGF-mediated enzyme induction in calf adrenal chromaffin cells. These cells provide a suitable system for studying the mechanism of action of NGF, since they possess NGF receptors (32) and respond to NGF treatment with a selective increase in specific enzymes of the catecholamine biosynthetic pathway, TH, DBH, and PNMT, while DDC levels remain unaffected, reflecting the pattern of enzyme induction seen in vivo in the nervous system after NGF treatment (3).

The results of the present study show that exposure to laminin affects neither the efficacy nor potency of NGF with regard to enzyme induction in calf chromaffin cells. Furthermore, the cells do not respond to NGF with process formation when exposed to substrate-bound laminin. However, laminin does alter TH activity in the absence of NGF. The first effect of exposure to laminin is a relatively rapid increase in TH

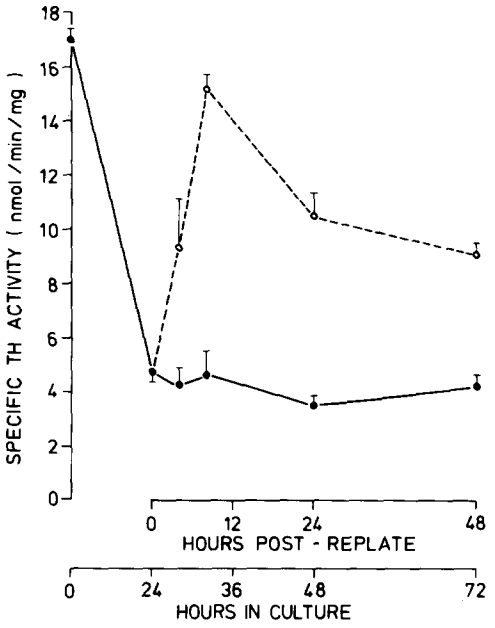

Figure 3. Specific TH activity as a function of time after replating onto laminin-coated dishes. Cells were plated at low density (1×10^4 cells/cm^2) on polyornithine. After 24 h, cells were harvested and replated at a similar low density on either polyornithine (●) or laminin (○) coated dishes. The specific activity of TH was determined as a function of time after replating. Values for the following time ranges were averaged together into single points: 3–5 h, 6–8 h, and 20–24 h. Values represent the means ± SEM obtained from 5–9 independent experiments. The value given for zero hours in culture represents in vivo TH activity (as in Fig. 2), and the value given for zero hours post-replate was determined from cells that were enzymatically harvested but were not brought into culture for a second time.

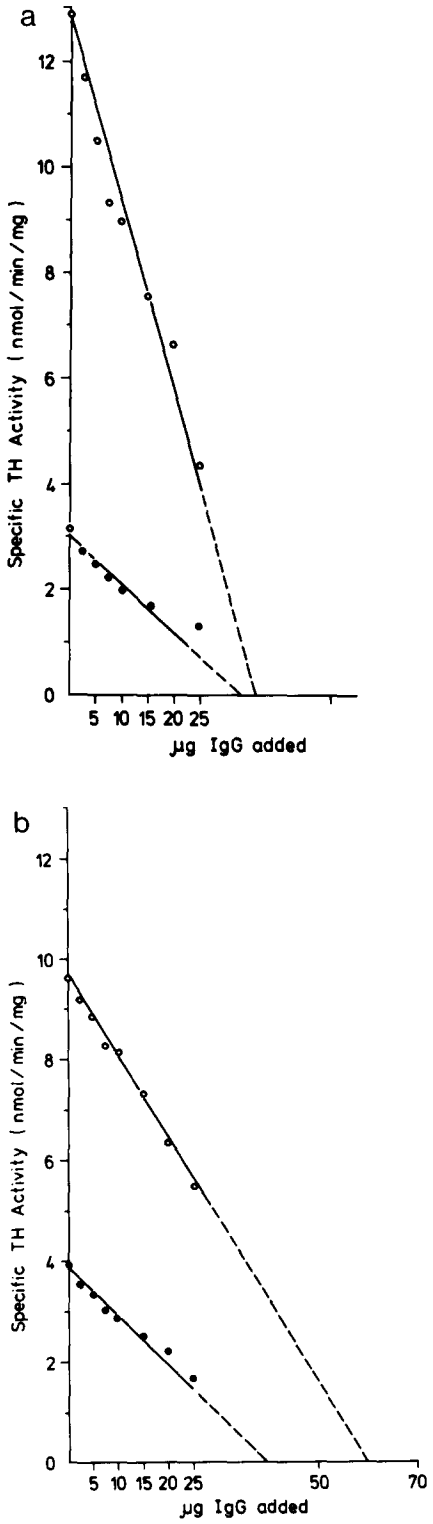

Figure 4. Immunotitration of TH activity from cells harvested and replated onto either polyornithine or laminin. Cells were cultured at low density on polyornithine for 1 d, and were then harvested and replated onto polyornithine (●) or laminin (○) coated dishes as described. Either 6 h (*a*) or 48 h (*b*) post-replate, immunotitration was carried out (see Materials and Methods). Values given are the means of triplicate determinations of TH activity obtained from a single representative experiment for each time point. Regression lines (solid lines) determined from the linear portion of the immunotitration curves were extrapolated (dotted lines) to the abscissa to determine equivalence points.

Table I. Effect of Cycloheximide and α-Amanitin on Laminin-mediated Changes in Tyrosine Hydroxylase Activity

Treatment*	TH specific activity on a substrate of	
	Polyornithine	Laminin
	nmol/min per mg	
6 h post-replate	4.6 ± 0.17	13.2 ± 0.23‡
6 h post-replate + cycloheximide	3.9 ± 0.12	4.3 ± 0.17
48 h post-replate	4.3 ± 0.15	9.2 ± 0.30‡
48 h post-replate + cycloheximide	2.1 ± 0.09	2.7 ± 0.07
48 h post-replate + α-amanitin	2.0 ± 0.07	4.0 ± 0.12‡

* Calf chromaffin cells were cultured at low density for 1 d, and then were harvested and replated onto either polyornithine or laminin as described. Either 6 or 48 h later, specific TH activity was determined. Cycloheximide (1 µM) or α-amanitin (11 µM) were added at the beginning of the replating period, and were continuously present for the time periods indicated. Values represent the mean ± SEM of three experiments.

‡ Significantly different from the corresponding polyornithine value, $P < 0.001$, Student's *t*-test.

Table II. Effect of Laminin on Tyrosine Hydroxylase Activity in Adult Chromaffin Cells

Time post-replate*	TH specific activity on a substrate of	
	Polyornithine	Laminin
h	*nmol/min per mg*	
0	2.25 ± 0.07	2.00 ± 0.06
6	2.05 ± 0.01	1.83 ± 0.05
24	1.57 ± 0.08	1.22 ± 0.09
48	1.58 ± 0.08	1.78 ± 0.07

* Adult chromaffin cells were cultured at low density (1×10^4 cells/cm^2) for 1 d, and were then harvested and replated at the same low density on either polyornithine or laminin-coated dishes. 0, 6, 24, and 48 h post-replate, cells were harvested and specific TH activity was determined. Values represent the mean ± SEM of five replicate dishes obtained from a single experiment. None of the laminin values are significantly different from the corresponding polyornithine values.

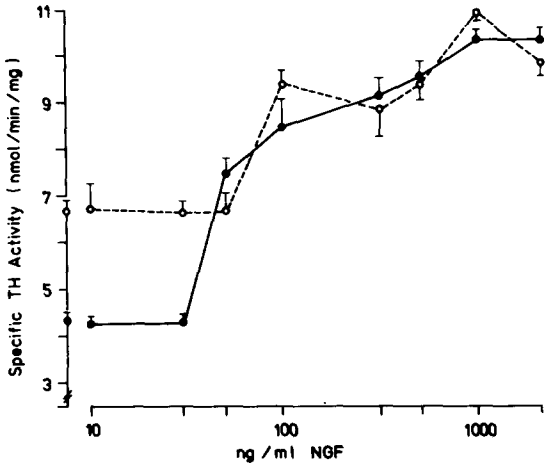

Figure 5. Specific TH activity as a function of NGF concentration in cells plated on either polyornithine or laminin. Cells were plated at a density of 1×10^5 cells/cm^2 on dishes coated with either polyornithine (●) or laminin (○). After 1 d in culture, varying concentrations of NGF (10–2,000 ng/ml) were added to the cells. 3 d later, cells were harvested and specific TH activity was determined. Values represent the mean ± SEM of three experiments. Values on the ordinate represent TH activity determined in the absence of NGF.

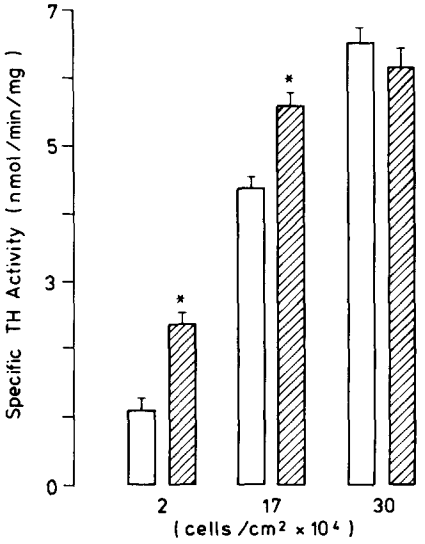

Figure 6. Specific TH activity in cells plated on polyornithine or laminin at varying cell density. Cells were plated at densities of 2, 17, or 30 × 10^4 cells/cm^2 (low, intermediate, or high density) on either polyornithine (□) or laminin (▨) coated dishes. After 3 d in culture, specific TH activity was determined. Values represent the mean ± SEM of values obtained from five dishes in a single experiment. Bars with asterisks are significantly different from the corresponding polyornithine value, $P < 0.001$, Student's *t*-test.

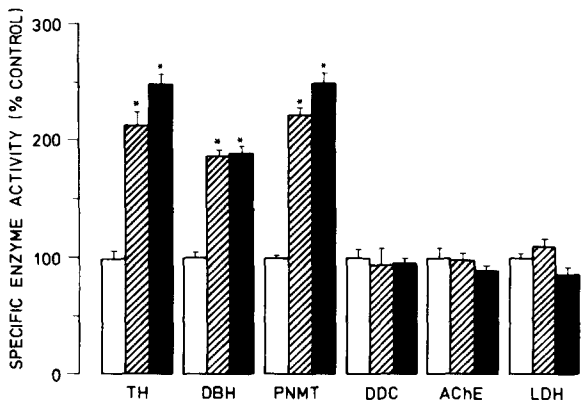

Figure 7. Spectrum of enzymes induced by increased cell-cell contact as compared to laminin. Cells that had been in culture at a density of 2 × 10^4 cells/cm^2 for 24 h were harvested and replated at low density (1 × 10^4 cells/cm^2) (□) or high density (27 × 10^4 cells/cm^2) (■) on polyornithine, or at low density (1 × 10^4 cells/cm^2) (▨) on laminin. 2 d later, cells were harvested and the specific activities of several enzymes were measured. Values are expressed as a percentage of the low density polyornithine value, and represent the mean ± SEM of 5–7 replicates derived from a single experiment (low vs. high density) or from two independent experiments (laminin vs. polyornithine). Values taken as 100% were: TH, 3.44 ± 0.07 nmol/min per mg; DBH, 7.23 ± 0.08 nmol/min per mg; PNMT, 11.96 ± 0.24 pmol/min per mg; DDC, 53 ± 1.2 pmol/min per mg; AChE, 62.25 ± 0.89 nmol/min per mg; LDH, 2.00 ± 0.01 U/mg.

activity, which is maximal after 6–8 h. This is followed by a decrease to a lower level which is reached after about 24 h and is maintained thereafter. The initial effect on TH activity can be accounted for by activation of the enzyme: immunotitration showed that 6 h after laminin exposure, the number of enzyme molecules had not changed as compared with control, despite a fourfold increase in enzymatic activity.

Table III. Effect of Laminin Fragment-specific Antibodies on the Laminin-mediated Increase in Tyrosine Hydroxylase Activity

Treatment*	TH specific activity‡
	nmol/min per mg
Polyornithine control	1.13 ± 0.05
Polyornithine + laminin	2.21 ± 0.085§
+Laminin + anti-fragment 3	1.23 ± 0.07‖
+Laminin + anti-fragment 1–4	2.46 ± 0.07§
+Laminin + anti-fragment 1	1.89 ± 0.05§
+Laminin + anti-fragment 4	2.25 ± 0.075§

* Calf chromaffin cells were plated at a density of 3 × 10^4 cells/cm^2 on either polyornithine or laminin-coated dishes. When antibodies were present, they were added to the dishes simultaneously with the cells.

‡ TH specific activity was determined after 2 d in culture. Values represent the mean ± SEM of three experiments. The presence of antibodies themselves (i.e., in the absence of laminin) did not alter basal TH activity (i.e., that measured in cells plated on polyornithine) (data not shown).

§ Significantly different from control, $P < 0.001$, Student's *t*-test.

‖ Not significantly different from control, $P > 0.99$, Student's *t*-test.

Kinetic analysis of this increase demonstrates that it is due to a change in the apparent V_{max} of TH, with the affinity for its cofactor remaining constant. Such a V_{max} activation of TH is unusual, since most stimuli shown to activate TH in vivo or in vitro alter the apparent K_m of the enzyme for cofactor (see reference 47 for review). Nonetheless, activations due to increased V_{max} have been reported previously in association with depolarization-mediated TH activation in PC12 cells (50), decapitation stress in rabbit portal vein (41), depolarization of striatal slices (12), and activation of TH by short exposure to NGF in PC12 cells (15). It has been assumed that TH activation is brought about by a change in its state of phosphorylation. This is based on the fact that TH acts as a substrate for several different protein kinases in vitro (cAMP-dependent [46], Ca^{++}-calmodulin-dependent [49], and Ca^{++}-phospholipid-dependent [5]), and has been shown to have multiple phosphorylation sites in situ which can be differentially regulated (16). In addition, TH has been shown to exist as a phosphoprotein in vivo (or in situ), the state of phosphorylation of which is altered by several stimuli that also activate it (8, 12, 28, 48). V_{max} activation has been suggested to be due to the action of a Ca^{++}-dependent protein kinase (12, 18). It may therefore be possible to use the laminin-mediated V_{max} activation of TH as a tool to analyze the series of biochemical events that constitute laminin's mechanism of action in these cells, one step of which may involve the activation of a protein kinase. One qualification that must be made to this hypothesis is that cycloheximide was able to block the short-term activation of TH by laminin. This could imply that a TH activator protein is involved, the synthesis of which is stimulated by laminin exposure, rather than that TH is itself directly activated by phosphorylation. The existence of endogenous activator/inhibitor proteins for TH in adrenal medulla has been postulated (31).

The increase in TH enzymatic activity subsequently declines with time of laminin exposure, and is gradually replaced by an increase in the number of enzyme molecules: after 48 h of laminin exposure, immunotitration showed that ~70% of the increased TH activity can be accounted for by an increase in the number of enzyme molecules (i.e., that there had been an approximately onefold increase in the number of TH molecules by this time). This increase can be blocked

by inhibitors of both protein synthesis (cycloheximide) and mRNA synthesis (α-amanitin). Although the laminin-mediated TH increase is not additive with those brought about by either increased cell–cell contact or NGF, nevertheless laminin-mediated and NGF-mediated increases in TH are pharmacologically distinguishable from one another, since the effect of NGF cannot be blocked by α-amanitin (3). Thus, the two stimuli have distinct mechanisms of action, but may nevertheless regulate a common rate-limiting step, i.e., TH synthesis, as has been suggested for NGF and cell contact (3, 32).

The spectrum of other enzymes that are induced by laminin in chromaffin cells is identical to that brought about by cell–cell contact. In addition to TH, DBH and PNMT are induced, whereas DDC, LDH, and AChE remain unchanged. In contrast, the pattern of NGF-mediated enzyme induction is slightly different, in that AChE (in addition to TH, DBH, and PNMT) is also increased (3). Despite the similarity in the pattern of enzymes induced by laminin and cell contact, it is clear that cell contact–mediated enzyme induction is not mediated by laminin. First, laminin was not detectable in cultures of calf chromaffin cells, even at high density, as determined by indirect immunofluorescence (data not shown). Second, the effects of laminin are absent in adult cells, whereas the cell contact effect is qualitatively the same in calf and adult cells (see reference 1). Third, preliminary studies indicate that the molecule mediating the cell contact effect is an intrinsic membrane protein (Saadat, S., personal communication), while laminin is primarily a component of the extracellular matrix (14, 43, 45, 48).

It was previously shown that the heparin-binding domain of the laminin molecule is associated with its interactions with neurons (11). This is of particular interest because a different laminin domain has been shown to be important for laminin's interaction with carcinoma cells and hepatocytes (42, 44), implying that laminin is a bifunctional molecule, able to specifically interact with both neurons and non-neuronal cells. As the increase of TH activity by exposure to laminin is also mediated by a site associated with the heparin-binding domain of the molecule, then chromaffin cells and sympathetic neurons share the property of responding to a distinct domain of the laminin molecule. The simplest explanation for the involvement of different domains of laminin in its interactions with different cell types is that there are different or cell-specific laminin receptors. Laminin binding proteins have already been isolated from tumor cells (25, 35, 42) and from muscle cell membranes (23) and it will be of interest to determine whether the laminin-binding proteins of sympathetic neurons and chromaffin cells differ from those of cells not derived from the neural crest.

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