Selective Induction of Tyrosine Hydroxylase by Cell–Cell Contact in Bovine Adrenal Chromaffin Cells Is Mimicked by Plasma Membranes

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Abstract. As a first step towards the identification and purification of the molecule(s) that are involved in cell contact–mediated tyrosine hydroxylase (TH) induction in cultures of bovine adrenal chromaffin cells, we have prepared plasma membranes (PM) from bovine adrenal medulla and tested their ability to mimic cell contact–mediated TH induction in low density chromaffin cultures. PM indeed induced TH in a manner similar to that observed in high density cultures. The maximal TH induction reached by PM corresponded to 69% of that of high density cultures, and half-maximal TH induction was obtained with 12 μg of PM per ml of medium. The induction of TH by PM was blocked by α-amanitin as observed in high density cultures. Since acetylcholinesterase was neither induced in high density nor in PM-treated low density cultures, an induction of TH as a result of a general increase in protein synthesis was excluded. The cell contact molecule(s) appear to be intrinsic membrane proteins. They were not removed by high or low salt extraction, but solubilized by 50 mM octylglucoside. They were resistant to 0.1% trypsin and heat denaturation but inactivated by 0.01% chymotrypsin. PM isolated from the adrenal cortex, kidney, and liver also induced TH in low density chromaffin cell cultures, although to a smaller extent than PM of the adrenal medulla. In contrast, muscle and erythrocyte PM were inactive. This shows that the cell contact molecule(s) are not restricted to the adrenal medulla, but are also present in some other but not all tissues.

Materials and Methods

Plasma Membrane Isolation

PM were isolated from bovine adrenal medulla by a modified procedure of Trifaro and Duerr (38). Adrenal glands from freshly slaughtered bulls...
Figure 1. Time course of TH induction in PM-treated low density cultures. Chromaffin cells were plated at either low (3 x 10^4 cells/cm^2) or high density (3 x 10^5 cells/cm^2) and kept in culture for up to 4 d. ●, high density; ○, low density; △, low density cultures plus 30 μg of adrenal medulla PM per ml of medium. Medium with or without PM was added 1, 2, and 3 d after initial plating. For each time point cells were harvested from duplicate wells, and TH and protein content were determined in duplicate assays. Since it was not possible to determine accurately the amount of protein in PM-treated low density cultures, the protein content of untreated low density cultures was used to calculate the specific activity of TH. The specific activity of AChE was not increased, showing that there is no general increase in protein synthesis. dopa, dihydroxyphenylalanine.

were obtained from the slaughterhouse, and the adrenal medullae were immediately dissected from the cortex. All steps were done at 4°C. The medullae were suspended in 0.3 M sucrose, 10 mM Tris-HCl, pH 7.5 (4 ml/g of tissue), homogenized with an Ultra-Turrax (Jankel and Kunkel, IKA-Werke, Staufen, FRG) for 30 s at medium speed, and finally with six strokes in a glass-Teflon pestle homogenizer at 800 rev/min. Cell debris and nuclei were removed by centrifugation at 800 g for 20 min. The supernatant was centrifuged for 1 h at 100,000 g and the resulting pellet ("crude membranes") was resuspended in 0.3 M sucrose (2 ml/g of tissue). The crude membranes (35 ml) were layered over 0.8 M sucrose (35 ml) and centrifuged in a Beckman Ti 45 rotor for 70 min at 100,000 g. The PM were collected from the 0.3-0.8 M interphase, diluted with 1 vol of phosphate-buffered saline (PBS), and finally pelleted by centrifugation at 100,000 g for 1 h. The pellet was suspended in PBS at a concentration of 10 mg/ml of protein. The enrichment for PM was estimated by determining the specific activity of the plasma membrane marker 5’-nucleotidase (38). As compared with crude membranes the specific activity of 5’-nucleotidase was increased by eightfold in the PM with a recovery of 19%. This is in good agreement with the results of Trifaro and Duerr (38). PM from adrenal cortex, kidney, liver, and skeletal muscle were prepared using the same procedure. The PM remained stable for at least 2 mo at −70°C.

Treatment of the PM

For all treatments PM were thawed and diluted with PBS to 1 mg/ml. PM were digested for 30 min at 37°C with 0.1% tosyl-L-phenylalanine chloromethyl ketone-treated trypsin (Worthington Biochemical Corp., Freehold, NJ) or 0.01% chymotrypsin (Boehringer Mannheim Biochemicals, Indianapolis, IN). Trypsin was inactivated at the end of the incubation by 0.2% bovine trypsin inhibitor (Sigma Chemical Co., St. Louis, MO), and chymotrypsin by 0.02% trasyol (Sigma Chemical Co.). PM were extracted in 1 M NaCl, 25 and 50 mM octylglucoside (Sigma Chemical Co.) for 60 min at 4°C with 1 min of ultrasonication in Bransonic I2 ultrasonic bath after 30 min. Heat treatment was for 10 min at 90°C in a water bath. After all treatments PM were pelleted except for heat-treated PM by centrifugation for 1 h at 100,000 g. The supernatant solutions were dialyzed against PBS for 1 d and the pellets were resuspended in the same volume as before.

Cell Culture and Assay for the Cell Contact Molecule(s)

Primary cultures of bovine adrenal chromaffin cells were prepared as described previously (2). Chromaffin cells were cultured in 24-well Costar dishes (Costar, Cambridge, MA) coated with poly-D,L-ornithine at either low (6 x 10^4 cells per well or 3 x 10^5 cells/cm^2) or high density (6 x 10^5 cells per well or 3 x 10^6 cells/cm^2). In routine assays the medium was changed after 1 and 2 d of initial plating and fresh medium with or without PM was added to the wells. The cells were harvested 3 d after initial plating with a rubber policeman, pelleted by centrifugation, and kept frozen at −20°C until use.

The original procedure developed in this laboratory for monitoring TH induction in primary cultures of bovine adrenal chromaffin cells (3) involved plating the chromaffin cells first at low density to allow maximal decrease in specific TH activity. After 1 d the cells were harvested from the culture dish with 0.01% trypsin and replated at either low or high density. By this protocol maximal increase in specific TH activity was reached 2 d after replating. Apparently the cell contact molecule(s) were not affected by tryp-
sinization. To reduce the amount of time required to assay routinely for the cell contact molecules in PM fractions the above procedure was simplified by omitting the harvesting and replating step. The cells were plated right after isolation at either low or high density. By this procedure TH levels in low density cultures decreased to a low level and remained constant for 4 d (Fig. 1), similar to the results obtained by the original procedure (3). The time course of TH induction in high density cultures differs from that of the original procedure during the first day where TH levels dropped slightly (Fig. 1). After 2 d TH increased significantly and was maximal after 3 d. It may take freshly prepared chromaffin cells 1 d to recover from the isolation procedure, because the cells are incubated for 100 min in 0.05% crude collagenase, as compared with 0.01% trypsin for 8 min for the harvesting and replating procedure. The disadvantage in using the simplified procedure is the lower TH induction of 3.9 ± 0.9-fold (standard deviation with n = 9) which corresponds to 58% of the original procedure. Whenever the original procedure was used similar results were obtained as described previously (data not shown).

Assays
Protein was determined by the Peterson (1977) procedure (28) with γ-globulin as standard. The enzymic activities of AChE (5), 5′ nucleotidase (12), and TH (2) were assayed as described.

Results
Induction of TH by PM
The ability of adrenal medulla PM to induce TH in low density cultures was investigated by adding PM to the culture medium. In the initial experiments 16 and 32 μg of PM per ml of medium was added. Over the culture period of 3 d the number of cells in the presence of PM (Fig. 2, b and c) was the same as in control cultures (Fig. 2 a). As judged by the chromaffin cells, whereas the flat phase-dark cells are mostly nonchromaffin cells. The PM sedimented after 2 d to the bottom of the dish and were visible as dark spots or clumps. The appearance of PM-treated and untreated cells is very similar, except that fewer flat cells can be observed in PM-treated cultures. Bars, 50 μm.
cell morphology and Trypan blue staining no cell death occurred. If PM were added only once after 24 h and the cells were harvested after 3 d in culture, a 2.5-fold increase in TH activity was observed with 32 μg of PM per ml of medium. No increase was seen with 16 μg/ml. If PM were added after both 1 and 2 d of culture and the cells were harvested after one further day, a 2.5- and 3-fold increase was obtained at 16 and 32 μg/ml, respectively. PM added to tissue culture dishes in the absence of cells and harvested by the same procedure had neither TH activity nor interfered with the TH assay (Table I).

Dose-Response Curve

A representative dose-response curve of medulla PM added to low density cultures is shown in Fig. 3. The concentration of PM required for half-maximal TH induction was 10 μg/ml in the experiment shown (Fig. 3), and was on the average 12 μg/ml of PM (Table IV). The maximal average induction of TH by PM was 30-fold and corresponded in these experiments to 74% of that observed in high density cultures (Table IV). To examine the time course of TH induction PM (30 μg/ml) were added to low density cultures after day 1, 2, and 3 of culture and cells were harvested every 24 h. The increase in TH of PM-treated low density cultures paralleled that of high density cultures and was maximal after 2 d of culture (Fig. 1).

Selectivity of Enzyme Induction

One of the characteristic differences between the TH induction by cell contact and NGF is that AChE is not induced by cell contact (2, 3). As shown in Table I, PM contained AChE and were therefore inactivated by heat treatment. Although AChE was completely inactivated, the PM still induced TH in low density cultures to the same extent as untreated PM. Heat treatment of the PM reduced the induction of TH on the average by 28% (Table III). As shown in Table II, α-amanitin, a specific inhibitor of RNA polymerase II, inhibited the induction of TH by PM in low density cultures.

Table I. PM Induce TH, But Not AChE in Low Density Cultures

| PM added*       | TH (nmol of dihydroxyphenylalanine/min per mg of protein) | AChE (nmol of choline/min per mg of protein) |
|-----------------|--------------------------------------------------------|---------------------------------------------|
| None*           | 1000                                                   | 1000                                        |
| Untreated       | 360                                                    | 520                                         |
| Heat-treated    | 375                                                    | 98                                          |
| Untreated, no cells | 10                                                      | 350                                         |
| Heat-treated, no cells | 6                                                      | 0                                           |

* Untreated and heat-treated PM were added to low density cultures as described in Materials and Methods. Cells were harvested from duplicate wells and TH and protein content were assayed in duplicate determinations. The curve is derived from one representative experiment and half-maximal TH induction in this experiment was 10 μg of protein per ml of medium. The specific TH activity was 166 nmol of dihydroxyphenylalanine (dopa)/min per mg of protein, as also observed in high density cultures (3). This shows that PM induce selectively TH in chromaffin cultures and that the induction requires de novo transcription.

Characterization of the Cell Contact Molecule(s)

Extraction of PM with 1 M NaCl removed 30% of the protein, but the PM still induced TH (Table III). No effect on TH was observed with the material from the supernatant solution after extraction of the PM with 1 M NaCl. Similarly, extraction without salt in 10 mM Tris-HCl, pH 8, did not remove the cell contact molecules from the PM. In all the following experiments, we used PM that have been extracted with 1 M NaCl. Treatment of the PM with the detergent octylglucoside (50 mM) solubilized the cell contact molecules, because the pelleted material no longer induced TH (Table III). The material solubilized with octylglucoside comprised 80% of the PM protein. In the first attempts the cell contact molecules were only partially recovered after dialysis, most likely because they were precipitated or inactivated after removal of octylglucoside (data not shown). It is interesting to note that octylglucoside at a concentration of 25 mM (35) extracted 34% of the protein, but did not remove the cell contact molecules from the pelleted PM (Table III). The critical micellar concentration of octylglucoside is 25 mM and only if this concentration is exceeded the cell contact molecules are solubilized. These experiments indicate that the cell contact molecule(s) are intrinsic membrane molecules. Trypsin did not inactivate the PM, whereas chymotrypsin inactivated the PM by 73% (Table III). The supernatant solutions after digestion with trypsin or chymotrypsin did not induce TH.

We conclude from these experiments that one or several plasma membrane protein(s) are involved in the formation of cell–cell contacts, because the molecules were only extracted from the PM by a detergent and inactivated by a protease.

TH Induction by PM from Other Tissues

To test whether the cell contact protein(s) are restricted to the adrenal medulla, PM from bovine adrenal cortex, kidney,
Table II. TH Induction by PM Is Inhibited by α-Amanitin

| Cell density* | PM     | α-Amanitin | -Fold increase in specific TH activity |
|---------------|--------|------------|---------------------------------------|
| Low           | −      | −          | 1†                                   |
| Low           | +      | −          | 2.6 ± 0.7†                            |
| Low           | +      | +          | 1.2 ± 0.3†                            |
| High          | −      | −          | 3.8 ± 0.5†                            |

* Cells were plated at low or high density and PM (30 μg/ml) and/or α-amanitin (10 μg/ml) were added as described in Materials and Methods.
† The specific TH activity of untreated low density cultures was set as 1.
§ Average of three experiments with standard deviation.

Table III. The Cell Contact Molecule(s) Are Intrinsic Membrane Proteins

| Treatment of PM* | Specific TH activity (%) of control |
|------------------|------------------------------------|
| None             | 100 ± 21†                          |
| 90°C, 10 min     | 72 ± 27                            |
| 1 M NaCl         | 101 ± 21                           |
| 25 mM Octylglucoside | 121 ± 19                       |
| 50 mM Octylglucoside | 0 ± 20                           |
| 0.1% Trypsin     | 90 ± 9                             |
| 0.01% Chymotrypsin | 27 ± 7                           |

* PM were treated and added to low density cultures of chromaffin cells as described in Materials and Methods. To compare the results from different experiments, the increase in the specific activity of TH obtained by treated PM is expressed as percent of induction obtained with untreated membranes.
† Average of three experiments with standard deviation.

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contribution of membrane lipids in the cell contact–mediated TH induction in chromaffin cells seems to be rather unlikely, since crude lipid extracts from adrenal medullary PM could not induce TH in low density chromaffin cell cultures (unpublished data).

Our experiments have demonstrated that the cell contact protein(s) necessary for the cell contact–mediated TH induction in bovine adrenal chromaffin cells are also present in a variety of other tissues. It remains to be established whether the differences in the maximal induction and in the quantity of protein needed to reach half-maximal TH induction reflect the presence of the same cell contact molecule(s) at a lower concentration or different molecules with similar effects in the PM of adrenal cortex, kidney, and liver. The fact that PM of skeletal muscle and erythrocytes did not induce TH at the PM concentration used demonstrates that the cell contact protein(s) are not ubiquitous membrane protein(s). We have not tested PM from sympathetic neurons because the assay for the cell contact protein(s) requires quantities of PM that are not easily available and certainly not sufficient for the purification of the cell contact proteins by biochemical methods.

A wide tissue distribution was also reported for the cell contact molecules which play a permissive role in the tyrosine aminotransferase induction by dexamethasone in primary cultures of rat hepatocytes. PM prepared from brain, erythrocytes, kidney, and liver could all mimic the effects of high cell density (25, 26). However, the bovine adrenal chromaffin cell contact protein(s) appear to be different from those responsible for the permissive contact effects in hepatocytes. They are not only not present in membranes of erythrocytes, but they are also resistant to trypsin digestion and heat treatment. Both treatments considerably reduced the permissive hepatocyte cell contact effects (25, 26).

With the presently available data it is difficult to evaluate whether the cell contact protein(s) required for cell contact–mediated TH induction are related to the described epithelial and neural cell adhesion molecules (9, 30) except for the neural cell adhesion molecule, N-CAM, and cell adhesion molecule from liver, L-CAM. The possibility that the TH induction by cell contact is mediated by N-CAM is unlikely. Antimouse BSP-2/N-CAM (34) polyclonal antibodies (a kind gift of Dr. C. Goridis) detected small quantities of N-CAM on the surface of bovine chromaffin cells (unpublished results). This observation is in agreement with that of Langley and Aunis (19), who used anti-rat D2/N-CAM (15) antibodies. However, anti-N-CAM antibodies neither induced TH in low density cultures nor inhibited TH induction in high density cultures of chromaffin cells (unpublished results). Moreover, the presence of cell contact molecules in bovine adrenal cortex, kidney, and liver PM makes it unlikely that N-CAM is responsible for the TH induction by cell contact in adrenal medulla. The possible involvement of L-CAM is also unlikely: L-CAM has a different organ distribution (9) and is sensitive to trypsin digestion (11) in contrast to the cell contact protein(s) mediating TH induction in adrenal chromaffin cells. Thus, the cell contact protein(s) involved in TH regulation in adrenal medullary cells are most likely neither related to N- nor L-CAM.

Presently, due to insufficient information, we cannot decide whether the cell contact molecule(s) requires one plasma membrane protein containing both complementary structures that bind to each other (as for instance in the case of N-CAM [9]), or alternatively, two different proteins containing either one of the complementary structures, as seems to be the case for Ng-CAM (9).

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