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Partial purification and characterization of a membrane-derived factor regulating neurotransmitter phenotypic expression

(phenotypic plasticity/choline acetyltransferase/cell contact)

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ABSTRACT **Cell membrane contact induces the *de novo* expression of choline *O*-acetyltransferase (CAT; acetyl-CoA: choline *O*-acetyltransferase, EC 2.3.1.6) activity in cultures of virtually pure neonatal rat dissociated sympathetic neurons. To identify molecular mechanisms underlying membrane-associated CAT induction, the responsible membrane component was characterized and partially purified. Substantial CAT-inducing activity was found in membranes from adult rat spinal cord and sensory and sympathetic ganglia. Whole brain membranes demonstrated significantly less activity. CAT induction in sympathetic neurons in response to spinal cord membranes was linear with respect to time, after an initial 6-hr lag. It was also linear with respect to concentrations of spinal cord protein from 2 to 100 μg per ml. CAT-inducing activity was extracted from spinal cord membranes by incubation with 100 mM NaCl and was purified approximately 5000-fold by DEAE ion-exchange and gel filtration chromatography. The active factor appears to be an extrinsic protein with an apparent molecular mass of 27 kDa. It is inactivated by trypsin and chymotrypsin but is moderately thermostable, retaining activity at 60°C but not at 90°C.**

Intercellular interactions are critical determinants of neuronal differentiation during development. In the cascade of cellular processes leading to the stable generation of peripheral ganglia and brain nuclei, migrating neuroblasts coalesce and shortly thereafter express a number of phenotypic characteristics, including neurotransmitter traits. It appears that perikaryal contact during aggregation may provide a stimulus for selective expression of transmitter phenotypes. For example, catecholaminergic traits appear virtually simultaneously with aggregation of neuroblasts to form primitive sympathetic ganglia (1). We have previously reported that cell membrane contact induces the *de novo* expression of choline *O*-acetyltransferase (CAT; acetyl-CoA:choline *O*-acetyltransferase, EC 2.3.1.6) activity in cultures of virtually pure neonatal rat dissociated sympathetic neurons (2, 3). Membrane contact may be provided either by neuronal aggregation in high-density cultures or by addition of crude membranes derived from neonatal sympathetic ganglia. Further, CAT-inducing activity appears to be restricted to membranes derived only from specific neonatal tissues (3).

To identify molecular mechanisms underlying regulation of transmitter expression by neuronal aggregation and membrane contact, we have begun to isolate and characterize a membrane-associated component responsible for stimulation of CAT expression. This factor, which is found in substantial quantities in adult rat spinal cords (4), has been extracted in soluble form by ionic mechanisms and partially purified by ion-exchange and gel filtration chromatography. It appears to be an extrinsic protein with an apparent molecular mass of 27 kDa. It is inactivated by trypsin and chymotrypsin but is only moderately sensitive to heat inactivation, retaining activity at 60°C but not at 90°C. Preliminary reports of these findings have been presented in abstract form (4, 5).

MATERIALS AND METHODS

Experimental Animals and Culture Techniques. Sprague–Dawley rat pups less than 24 hr old were used in all experiments. Superior cervical sympathetic ganglia were dissected under sterile conditions and maintained in saline until dissociation. Methods of dissociation and culture have been described (2, 6) with the following exceptions. Cultures were grown on collagen-coated multiwell culture plates (24 16-mm wells per plate). Dissociated ganglionic single-cell suspensions were seeded at a concentration of approximately 4000 neurons per well in 0.5 ml of medium.

Preparation of Cell Membranes and Extracts. Cell membranes were prepared as described (3) with the following exception. After an initial 500 × *g* spin to remove nuclei and intact cells, the supernatant was recentrifuged at 100,000 × *g* for 1 hr. The membrane pellet resulting from the second spin was suspended in cold (4°C) 20 mM Tris·HCl buffer, pH 7.4/100 mM NaCl at a final protein concentration of 1–2 mg/ml and was mixed gently for 0.5 hr. The suspension was then centrifuged at 100,000 × *g*, and the resulting supernatant was used for purification.

Extraction and Assay Procedures for CAT. CAT was extracted as described (2) and assayed for activity by the method of Fonnum (7); a 1-hr incubation was used to increase sensitivity. The assay proved to be linear to 1.25 hr.

Protein Determination. Protein content of membranes and solubilized extracts was quantified by the method of Bradford (8) with Bio-Rad dye reagent. Membranes were solubilized with 0.1 M NaOH before dye addition.

Statistics. Data were analyzed with a one-way analysis of variance and the Newman–Keuls test.

RESULTS

CAT Induction by Membranes Derived from Adult Rat Tissue. When neonatal rat organs are used as a source, CAT-inducing activity is observed with only certain tissues (3). Dorsal root sensory ganglia yield membranes with the greatest specific CAT-inducing activity, but their small size makes them unsuitable as a source for purification of a membrane component. To identify a better tissue source for such purification, CAT-inducing activity was determined in membranes derived from adult rats. Membranes from neonatal dorsal root ganglia served as controls (Fig. 1). Membranes from adult rat superior cervical ganglia, dorsal root ganglia, and spinal cord contained substantial CAT-inducing

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Abbreviation: CAT, choline *O*-acetyltransferase.

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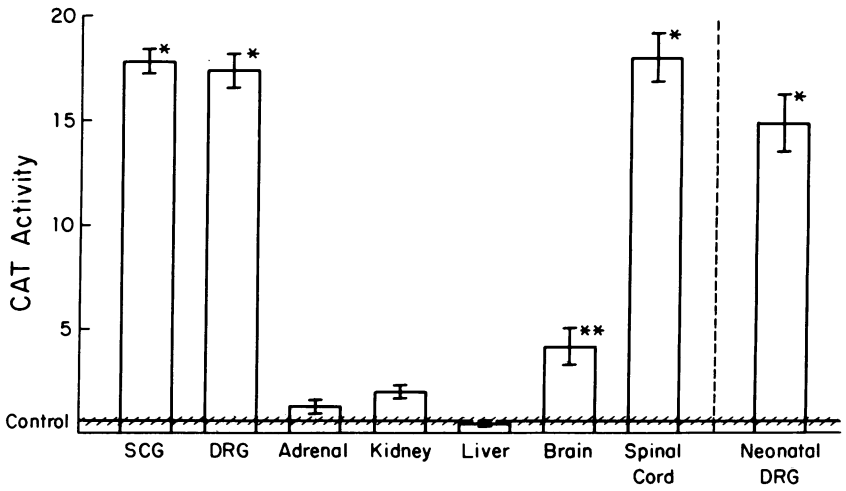

FIG. 1. Membrane-specific induction of CAT activity in sympathetic neurons. Cell suspensions were seeded at approximately 4000 neurons per culture well. Membranes extracted from various adult rat tissues and from neonatal rat dorsal root ganglia were analyzed for protein content and added to groups of six wells (25 μg per well) 8 hr after plating. Three days later, CAT activity was determined. CAT activity is expressed as nmol of product per dish per hr (± SEM). The hatched line represents mean CAT activity from untreated cultures. SCG, superior cervical ganglion; DRG, dorsal root ganglion. *, Differs from all other groups at $P < 0.001$; **, differs from all other groups at $P < 0.025$.

activity per μg of membrane protein added—at least equal to that seen in the neonatal dorsal root ganglion. Whole brain membranes also expressed CAT-inducing activity, although at a considerably lower level than did the other three tissues. Membranes from adrenal medulla, kidney, and liver displayed no activity. Thus, as in the neonate, CAT-inducing activity appears to have a selective tissue distribution. Because of their relatively large size and high specific activity, adult rat spinal cords were selected as the source for purification of the CAT-inducing membrane component.

CAT Induction in Dissociated Sympathetic Neurons: Time Course and Dose–Response. Before beginning purification, it was necessary to define fully the effects of spinal cord membranes on dissociated neonatal sympathetic neurons, the bioassay system used to examine CAT induction. Accordingly, we examined the time course of CAT induction and the relationship of membrane concentration to response. These measurements enabled us to establish optimal assay conditions and permitted comparison with later stages of purification. Cultures of dissociated neurons were treated with 25 μg of membrane protein per well 8 hr after plating. CAT activity was determined at various times thereafter (Fig. 2). After a 6-hr delay, CAT activity rose linearly, increasing more than 5-fold for at least 72 hr, the longest time tested.

To examine the relationship of membrane dose to CAT induction, cultures were exposed to various amounts of membrane protein from 1 to 50 μg per well. CAT activity was measured after 3 days (Fig. 3). CAT-inducing activity was present with membrane protein as low as 1 μg. It then appeared to increase linearly over the entire range tested, indicating that this assay could be used to quantitate putative factor activity during a purification procedure.

Extraction of CAT-Inducing Activity from Membranes. To proceed with purification of the CAT-inducing membrane component(s), we extracted it from membranes in a soluble form that could be applied to chromatographic columns. When spinal cords were homogenized in low-ionic-strength buffer followed by high-speed centrifugation, CAT-inducing activity was predominantly localized to the membrane pellet. By increasing the ionic strength of the buffer, activity was extracted from this membrane pellet in soluble form (Fig. 4). Incubation of membranes with 100 mM NaCl for 0.5 hr at 4°C resulted in solubilization of more than half of the CAT-inducing activity displayed by the original membrane pellet. Solubilization resulted in a reduction in protein to approxi-

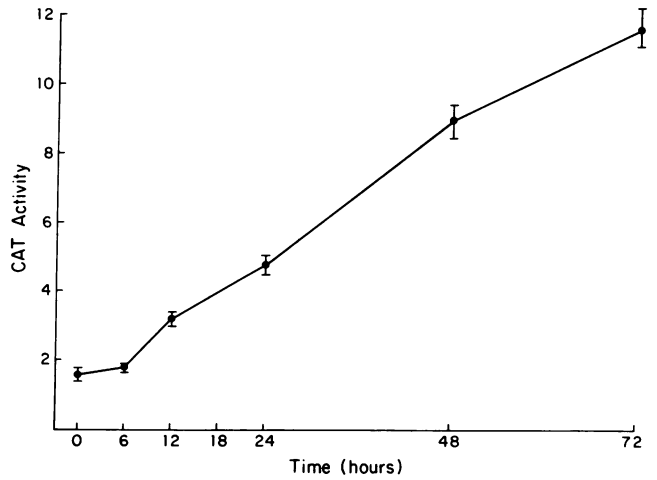

FIG. 2. Time course of CAT induction. Cell suspensions were seeded as in Fig. 1. Eight hours later, 25 μg of membrane protein derived from adult rat spinal cords was added to each well. CAT activity was determined at various times thereafter in groups of six wells. CAT activity is expressed as mean cpm $\times 10^{-3}$ (± SEM).

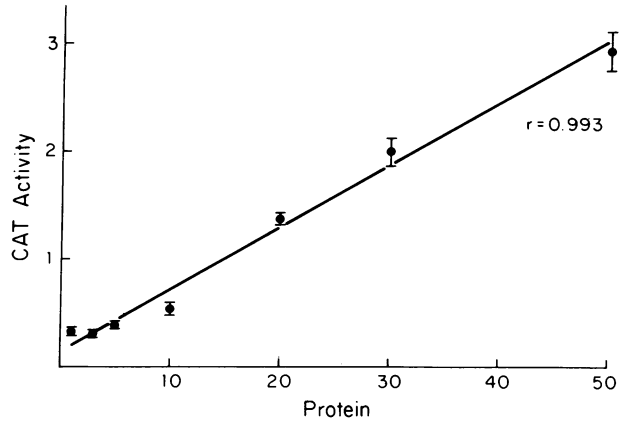

FIG. 3. Relationship of membrane dose to CAT induction. Cell suspensions were seeded as in Fig. 1. Eight hours later, various amounts of adult rat spinal cord membranes were added to groups of six wells. CAT activity was determined after 3 days. Protein is expressed as μg per well. CAT is expressed as in Fig. 2. *r*, Correlation coefficient.

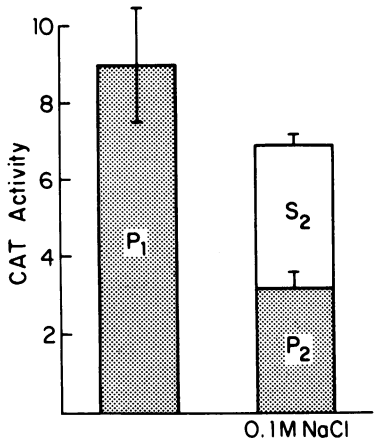

FIG. 4. Salt extraction of CAT-inducing activity. The crude membrane pellet from homogenized adult rat spinal cords (P1) was washed with 100 mM NaCl/20 mM Tris·HCl, pH 7.4, for 30 min. The membrane suspension was then centrifuged at 100,000 × *g* for 1 hr, yielding an extracted supernatant (S2) and residual membranes (P2). CAT-inducing activity was measured in each fraction and is expressed as the total activity (specific activity × total protein) in cpm × 10^{-6} (± SEM). Recovery of CAT-inducing activity from salt extraction was 80%.

mately 1/100th, yielding a 30- to 40-fold purification. Salt concentrations of 900 mM or above in the extraction buffer markedly reduced CAT-inducing activity, suggesting sensitivity to high ionic strength. Further, 5% (wt/vol) octyl glucoside destroyed virtually all activity.

Preliminary Characterization. To determine the most appropriate approach to purification, preliminary characterization studies were performed on the solubilized extract of the spinal cords, designated S2. Further, stability under various conditions was examined to assure proper handling as purification proceeded. CAT-inducing activity was relatively unaffected by repeated freezing or by heating to 37°C or 60°C for 30 min (Table 1). However, heating to 90°C for 5 min destroyed all activity. CAT-inducing activity was destroyed by exposure to trypsin or chymotrypsin, suggesting that protein was a critical component of the active factor(s).

Initial Purification. To purify the solubilized CAT-inducing factor, conventional column chromatography was utilized. On a DEAE-Trisacryl M (IBF) ion-exchange column, the bulk of the total protein in the S2 fraction did not bind to the column (Fig. 5). CAT-inducing activity was eluted at approximately 100 mM on a continuous NaCl gradient from 0 to 300 mM. Less than 10% of the initial protein was eluted in these fractions.

Table 1. Physicochemical characterization

	% activity retained
Thermal stability	
−20°C, 30 min	90
37°C, 30 min	89
60°C, 30 min	72
90°C, 5 min	0
Protease treatment	
Trypsin	0
+ trypsin inhibitor	96
Chymotrypsin	0
+ phenylmethylsulfonyl fluoride	91

Aliquots of S2 (50 μg; the solubilized extract of adult rat spinal cords) in 0.5 ml were incubated under various conditions. For protease treatment, aliquots were incubated at 37°C with either trypsin (1 mg/ml) or chymotrypsin (1 mg/ml). Protease activity was stopped after 30 min by the addition of an equal volume of trypsin inhibitor (3 mg/ml) or phenylmethylsulfonyl fluoride (0.2 mM). For controls, aliquots of S2 were similarly incubated with premixed solutions of the protease + protease inhibitors. CAT-inducing activity was determined by measuring CAT activity in neuronal cultures exposed to either treated or untreated S2. 100% activity represents CAT activity in cultures exposed to untreated S2; 0% activity represents CAT activity in untreated cultures.

Application to an Ultrogel AcA 54 (IBF) gel chromatography column resulted in elution of CAT-inducing activity in a single peak, corresponding to an approximate molecular mass of 27 kDa. The bulk of the applied protein was eluted in the void volume (Fig. 6). Approximately 10% of the total protein was contained in the activity peak.

The overall purification from spinal cord homogenate through gel chromatography was approximately 5000-fold (Table 2). Approximately 11% of the initial activity in the homogenate was recovered. Neither protein nor activity bound to a heparin affinity column (Pharmacia).

DISCUSSION

Our observations have delineated a membrane-associated factor(s) that markedly alters neurotransmitter phenotypic expression *in vitro*. The putative factor(s), which is detectable only in specific neural tissues, may be dissociated from membranes by raising ionic strength, implying that association occurs through electrostatic interactions. However, since the factor does not act by diffusion (2), even though

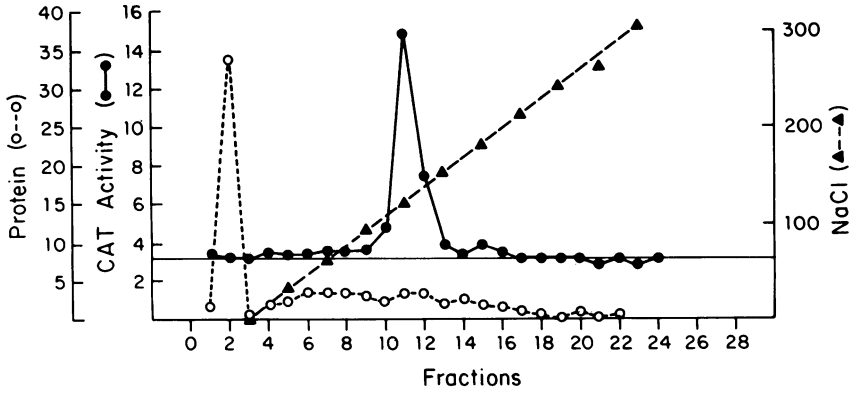

FIG. 5. DEAE ion-exchange chromatography. S2 in 10 mM NaCl was applied to a DEAE-Trisacryl M ion-exchange column. Protein was eluted with an NaCl gradient from 0 to 300 mM. Each 2-ml fraction of the eluate was examined for CAT-inducing activity, expressed as cpm from 0 to 1600 (shown × 10^{-2}), and for protein content, expressed as μg per fraction from 0 to 40. The gradient is expressed as mM NaCl from 0 to 300. The solid horizontal line represents the CAT assay control in cpm.

Table 2. Purification of the membrane-associated factor

Fraction	Protein, mg	Specific activity, cpm/μg	Total activity, cpm × 10^{-3}	Recovery, %	Fold purification
Homogenate	1092	90	98,280	100	1
P1	330	200	66,000	67	2.2
S2	9.9	3,510	34,749	35	39
DEAE	0.67	16,000	10,720	11	177
AcA 54	0.025	440,000	11,000	11	4889

Adult rat spinal cords (50) were homogenized in 100 ml of 20 mM Tris, pH 7.4. P1 is the membrane pellet before NaCl extraction; S2 is the solubilized extract. Specific activity represents CAT activity induced in neuronal cultures by aliquots of each fraction as determined by radioenzymatic assay (7). All specific activities were determined in cultures of the same neuronal dissociate. Total activity was determined by multiplying specific activity by the total protein in each fraction.

extraction occurs at a lower ionic strength than that of the culture medium, other associative mechanisms may operate in intact cells.

The membrane-associated factor appears to be distinct from other reported cholinergic-inducing agents in terms of source, physicochemical characteristics, and time course of action. Chromatographic characterization suggests that it is acidic, with an approximate molecular mass of 27 kDa. While protein represents a critical constituent, as demonstrated by protease sensitivity, the factor retains activity when exposed to temperatures as high as 60°C, consistent with a relatively stable tertiary structure. The absence of heparin binding suggests that it is not a member of the family of polypeptide growth factors, which include the fibroblast growth factors, that exhibit such affinity (9–13). Finally, induction of cholinergic traits occurs rapidly, within hours of exposure. Three other cholinergic-inducing factors have been reported with distinct molecular characteristics: a basic glycoprotein of approximately 45 kDa molecular mass derived from heart cell-conditioned medium, which has been extensively purified and characterized (14); a 50-kDa heparin-binding protein derived from extracts of rat, bovine, and human brains, which has been partially purified (15); and a 29-kDa glycoprotein extracted from rat spinal cords by high concentrations of salt or detergent, conditions that destroyed CAT-inducing activity in our preparation (16). Cholinergic induction by these agents requires days, in marked contrast to the presently defined membrane-associated factor.

The rapid action of the membrane-associated factor is entirely consistent with the time course of transmitter trait expression subsequent to aggregation and perikaryal contact. *In vivo*, catecholaminergic traits appear shortly after neuroblast aggregation to form primitive sympathetic ganglia (1), while *in vitro*, the time course of substance P and CAT induction is virtually identical to that of perikaryal aggregation observed in time-lapse phase micrographs (2, 3). Indeed, the membrane localization of the factor provides a mechanism whereby cell contact may normally elicit transmitter expression during development. Studies are currently under way to define the specificity of action of the membrane factor.

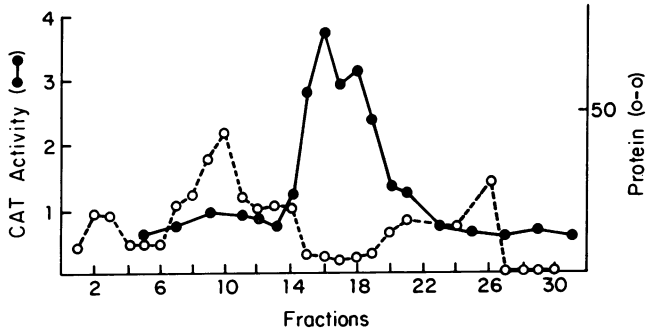

FIG. 6. Gel chromatography. DEAE eluate was applied to an Ultrogel AcA 54 column. Protein was eluted continuously. Each 2-ml fraction of the eluate was examined for CAT-inducing activity, expressed as cpm from 0 to 400 (shown × 10^{-2}), and for protein content, expressed as μg per fraction from 0 to 50.

The CAT-inducing factor appears to be selectively distributed spatially and temporally. Only membranes from neuronal tissue, in the adult, exhibited CAT-inducing activity. Moreover, the differences between neonatal (3) and adult tissues are noteworthy. Membranes from adult spinal cord, sensory, and sympathetic ganglia induced CAT with high and approximately equal specific activity. In contrast, neonatal sensory ganglion membranes also displayed high specific activity, while neonatal sympathetic membranes displayed considerably less, and neonatal spinal cord membranes had no activity at all (3), suggesting that membrane interactions play different roles at various stages of development.

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