Newly Synthesized Catalytic and Regulatory Components of Adenylate Cyclase Are Expressed in Neurites of Cultured Sympathetic Neurons

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Forskolin- and guanine nucleotide-stimulated adenylate cyclase activities were measured in microdissected sections of neurites from small explants and in dispersed cell cultures of sympathetic ganglion neurons to determine whether a competent system for regulated formation of cAMP, consisting of both catalytic units of adenylate cyclase and regulatory GTP binding proteins, is synthesized during neurite outgrowth and where it is distributed in the neuron. An increase in both guanine nucleotide- and forskolin-dependent activity of adenylate cyclase occurred concomitantly with neurite outgrowth and was directly proportional to neurite length. Separate analysis of adenylate cyclase activity in explant cell bodies or neurites showed that the increased activity was localized entirely in the neurites, while activity in the cell bodies remained virtually constant during growth. Concentric sections of neurites of $\sim 500 \mu\text{m}$ width, which contained similar volumes of neurites as determined with the indicator BCECF (Rink et al., 1982), produced similar levels of cAMP, indicating an even distribution of adenylate cyclase in the neurites. Cell bodies, when stimulated by $\text{GTP}\gamma\text{S}$, produced 236 ± 46 attomol cAMP/min (30°C)/cell body and an additional 52.6 ± 20 attomol cAMP/min (30°C)/neuron were produced with each day of neurite growth ($\sim 400 \mu\text{m}$). Assuming a turnover number of 2000 min^{-1} , cell bodies and neurites were calculated to contain similar densities of catalytic unit molecules on their surface ($9\text{--}28$ molecules/ μm^2). An abundant GTP binding protein, detected by ADP-ribosylation with pertussis toxin, was also widely distributed in the neuron. A competent adenylate cyclase system thus appears to be a constitutive component of neurite membranes.

The phosphorylated state of several proteins of diverse function and localization in invertebrate and vertebrate neurons is altered by activation of specific protein kinases (Nairn et al., 1985). It

has therefore been postulated that phosphorylation reactions, exemplified by cAMP-dependent protein phosphorylation of K^+ channels in molluscan neurons (Kandel et al., 1983; Novak Hofer et al., 1985), are important regulatory mechanisms for modifying the properties of neurons. cAMP and calcium-dependent protein phosphorylation may also be involved in regulating neurotransmitter synthesis in cultured sympathetic neurons during development (Walicke and Patterson, 1981). For a critical assessment of any role for cAMP in regulating development and function of mammalian neurons, it must first be demonstrated that (1) developing neurons can synthesize cAMP, e.g., express adenylate cyclase (AC); (2) cAMP levels in the cell are sufficient to activate protein kinases; (3) such activated protein kinase causes protein phosphorylation. This study examines the first of these questions by analyzing at what stage of development of cultured sympathetic neurons the catalytic unit of AC and its associated regulatory GTP binding proteins are synthesized and where these proteins are located in the neuron.

Recently, it has been demonstrated that cAMP and Ca^{2+} -dependent phosphorylation, as well as cAMP binding proteins, occurs in "growth cone particles" isolated from fetal rat brain (Ellis et al., 1985; Katz et al., 1985). From the early and wide distribution of neuronal substrates for cAMP-dependent protein kinase, and the rapid metabolism of cAMP, which would prevent cAMP diffusion over large distances, it might thus be expected that AC is also synthesized and distributed throughout the neuronal plasma membrane early in development if cAMP is involved in regulating developmental patterns of neurons. Little is known, however, about the mechanisms that control the synthesis and distribution of AC in developing or mature neurons, although experiments with synaptosomal preparations suggest that at least nerve terminals contain AC activity (Dunwiddie and Hoffer, 1982; Mulder and Schoffelfmeer, 1985; Schoffelfmeer et al., 1985). Furthermore, it has not been established whether GTP binding proteins, which are necessary in all cells for the modulation of AC activity by receptors for hormones and neurotransmitters, are functional during early development.

For unequivocal localization of functional AC and GTP binding proteins in neurites, both forskolin- and guanine nucleotide-stimulated AC activities were measured in pure neurite populations microdissected out of explant cultures of rat superior cervical ganglion (SCG). Dispersed cell cultures were used to quantitate the density of AC and GTP binding proteins. It is shown that a guanine nucleotide-responsive AC system is expressed throughout the sympathetic neuron from the earliest stages of growth.

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Materials and Methods

Cell preparation and growth media. SCG from newborn rats (1–2 d postnatal) were excised initially into enriched L15 (Gibco) plating medium (prepared as described by Hawrot and Patterson, 1979) without added glucose. Ganglia were desheathed and transferred to final plating medium containing 50 μ l/ml 30% glucose (0.75% final concentration). Ganglia could be stored in this medium at 4°C for up to a week without apparent loss of the potential to regenerate neurites, but ganglia were normally used immediately or within 12 hr after excision. Preparation of explant cultures was similar to the method of Estridge and Bunge (1978). Ganglia were removed one by one onto the inside of a 3 cm plastic petri dish cover and cut into 6–8 pieces of 200–400 μ m diameter using a sterile 23 surgical blade. Segments from 10–20 ganglia were pooled and 5–6 pieces were placed in 3 cm petri dishes precoated with collagen containing 0.5 ml preheated growth medium (see below), leaving ~1 cm between any 2 explants. The small amount of medium ensured that explants remain as placed until attachment, which was complete in 2–3 hr. Growth medium, 1 ml, was added after 12 hr, and medium was changed every 3 d. Growth medium consisted of L15-CO₂ enriched with glucose, glutamine, vitamins, and antibiotics as described by Hawrot and Patterson (1979), 5% rat serum (prepared sterile from adult rats deprived of solid food overnight), 10 μ M cytosine-1- β -D-arabinoide (Sigma Chemical Co.), stored at -20°C as 100-fold concentrate, and 800 ng/ml purified 2.5 S NGF, prepared by the rapid method described by Mobley et al. (1976) and stored as a 133-fold concentrate in 150 μ l lots at -20°C. Single-cell suspensions were prepared by mechanical dissociation (Hawrot and Patterson, 1979). Cells were teased out of 6–10 ganglia into 200 μ l of L15-air plating medium using forceps and titrated 3 times in a pulled glass capillary (internal diameter ~100 μ m) to form a suspension of mostly single cells. Cells that remained in suspension 5 min after final titration were added in 10 μ l drops into a well formed by adhesion of a sterile ring of 1 cm diameter, cut from the wide end of the plastic disposable tip used with a 1 ml micropipette, to a collagen-coated 3 cm petri dish containing 1.5 ml L15-CO₂ growth medium. Yield of viable neurons was quite low (~10%), but very few non-neuronal cells were observed at plating.

Collagen preparation. Collagen preparation was modified from the method of Bornstein (1958). Sterile tendons from 2 rat tails (150–180 g rats) were dissolved in 25 ml sterile 3 M acetic acid at 4°C by stirring for 12 hr. Solution was diluted to 100 ml, centrifuged for 30 min at 25,000 $\times$ g, and the clear supernatant was further diluted to give 0.3 O.D. at 280 nm. Culture dishes (3 cm diameter) were coated with 200 μ l of this collagen solution, opened, and air-dried in a laminar-flow cabinet for 30–45 min. L15-CO₂ growth medium was added immediately, and dishes were placed in an incubator containing 5% CO₂ and 100% humidity until just prior to plating.

Enzyme assays. Cultures were washed 3 times in L15-air leaving ~500 μ l from the third wash to cover the plate. Explant cell bodies were cut out using a 23 surgical blade, attached by suction to the tip of a pulled capillary, and transferred into the bottom of a 2 ml ice-cold plastic test tube (Luckham). Ice-cold assay mix (50–100 μ l) was added immediately, and the tube was left on ice until all samples were collected. Films of neurites or neurite segments attached to the collagen substrate were collected by forming an incision in the collagen around the perimeter or within the neurite field with the scalpel blade and teasing the outer edges of the cut piece gently inwards until a film of neurites was floated off the plate. This film was transferred by suction to a test tube and treated similarly to the cell body region. When the collagen was of the right consistency films formed easily, as illustrated in Figure 1. Drier, thinner collagen (0.2 ml of 0.1 O.D. collagen dried overnight in desiccator) supported more rapid neurite outgrowth but intact neurites could not be detached from the plate.

AC activity was measured by the method of Salomon et al. (1974). The standard assay mix contained 50 mM Tris-HCl, pH 7.4, 11 mM MgCl₂, 2 mM cAMP, and 0.5 mM ATP (Boehringer), 6 mM creatine phosphate, and 5 units/100 μ l assay mix of creatine kinase (Boehringer), 0.06% Lubrol WX (Sigma), and sufficient α -³²P-ATP (40–80 Ci/mmol; Amersham International) to give a specific activity of 30–70 cpm/pmol. Usually, 5 ml of assay mix was prepared at a time, and aliquots were frozen at -20°C in order to obtain a uniform solution for accurate comparison of early and later growth periods. Additional assay components (such as guanine nucleotides and forskolin) were added in 1–2 μ l volumes to tubes still on ice. Tubes were then transferred to 37°C for

Figure 1. Dissection of cultured explants of rat SCG. *Upper*, 7 d culture before dissection. $\times 60$. Scale bar, 400 μ m. *Lower*, dissected culture in which cell bodies have been removed and neurite field has been divided into 8 equal segments. Collagen with attached neurites is partially detached from some of the cut area, indicated by arrows. This and similar cultures were used to measure G_i activity (see section A, Table 1).

1–2 hr. The assay was stopped by the addition of 0.5 ml 2% SDS (50°C) and vigorous mixing. Activity was linear with time up to 2 hr (in the presence of nonhydrolyzable GTP analogs) and proportional to the number of cultures added per assay. AC activity in dispersed cell cultures was measured after removal of medium by the addition of 150 μ l assay mix directly into the well formed in the petri dish. The assay was stopped by first collecting the supernatant into tubes containing 100 μ l 2% SDS. Rings were then removed and 2 washes of the plate with 0.5 ml 2% SDS each were added to the tubes before cAMP separation. Blank values were 25 $\pm$ 12 (SD) cpm, as compared to 105–117 cpm for the lowest levels of cAMP generated by the dispersed cell cultures (GDP β S, day

Figure 2. AC activity in explants as a function of age and neurite length. *A*, Explant cultures were removed as indicated and AC activity was measured for 60–100 min at 37°C in the presence of 10 μ M GTP γ S and 20 μ M forskolin (O). Neurite length was measured using a grid subdivided into 200 μ m squares (x). *B*, Cell bodies (Δ) and neurites ($\blacktriangle$) were separated and AC activity was measured in each segment as in *A*. x, neurite length (mm). *C*, Neurites from 8 d explant cultures were divided into 4 areas. The numbers in each segment represent AC activity in pmol cAMP/100 min at 37°C/segment. Magnification: $\times 60$. Scale bar, 400 μ m.

zero; Fig. 3), and were between 100–200 cpm, as compared to 800–1200 cpm for the lowest levels of cAMP-generated by explant segments (GDP β S; Table 1). Blank values were always subtracted before calculation of cAMP recovery from the columns.

Tyrosine hydroxylase (TH) activity was measured essentially by the method of Hendry and Iversen (1971). Cell bodies and neurites were collected as described above and transferred into microfuge test tubes containing 15 μ l of 5 mM Tris/Cl, pH 6.0, and 0.1% Triton X-100 (Sigma). Tubes were frozen and thawed in liquid nitrogen, and cytosol was extracted by spinning tubes for 3 min at 4°C in a microfuge. Ten microliters of cytosol extract were added to 10 μ l ice-cold assay mix containing 0.18 mM K/Na P_i buffer, pH 6, 0.32 M β -mercaptoethanol (BDH biochemicals), 0.98 mM 6,7-dimethyl-5,6,7,8-tetrahydropterine hydrochloride (Aldrich), 2 μ g/assay NSD 1055 (a gift from Dr. Piers C. Emson, MRC Neurochemistry Unit, Cambridge), 80 μ M L-tyrosine (BDH biochemicals) containing L-[2,3-side chain 3 H]tyrosine (1560 cpm/pmol; Amersham International), and 2.5 mM Tris/Cl, pH 6, and 0.05% Triton X-100; tubes were transferred to 37°C for 20 min. Assays were linear

between 10 and 20 min with these small amounts of material. 3 H-tyrosine (100 μ l, 1 mCi/ml) was added to 200 μ l of 0.53 mM Tris buffer, pH 6.8, containing 0.32 mM L-tyrosine and incubated with 2 lots of 1 mg neutral alumina (type WN3, Sigma) on ice before being added to the cocktail. Blank values were between 250 and 350 cpm, as compared to 550–600 cpm (the lowest level of activity generated by noncultured explant pieces) to ~ 7000 cpm (29 d culture; Fig. 4). Blanks were subtracted before calculation of DOPA levels.

ADP-ribosylation and analysis of labeled products. Cell bodies and neurite segments were collected separately or together as described above. ADP-ribosylation labeling mixture contained 1.5 μ g pertussis toxin (Public Health Services Laboratory, Porton Down, Salisbury, U.K.) or 6 μ g cholera toxin (Calbiochem), 8 mM dithiothreitol, 100 mM KP_i buffer pH 7, 0.08% Lubrol WX, 1–2 μ Ci α - 32 P-NAD (0.1–0.2 μ M final concentration), 1 mM ATP, 10 mM thymidine, and 0.2 mM GTP (Boehringer) in a final volume of 61 μ l. Toxins were preactivated for 30 min at 37°C in the same tubes used for the labeling in 10 μ l 50 mM dithiothreitol before addition of the other components. After 30 min at

30°C, 60 μ l solubilizing buffer (as described by Laemmli, 1970) was added and samples were boiled for 3 min. Proteins were separated by SDS-PAGE (Laemmli, 1970) and stained with silver by the method of Wray et al. (1981). Fuji RX X-ray film was exposed to dried gels for 1–7 d at -80°C for autoradiography.

Determination of neurite volume. Single explants were grown on 12 mm glass coverslips precoated with ~ 20 μ l of 0.3 O.D. (280 nm) collagen. A coverslip was placed in a thermostatically controlled (36°C) chamber that was mounted on the stage of a Leitz Diavert fluorescence microscope with Leitz photomultiplier attachment. The explant was incubated with 5 μM of the acetoxymethyl ester of 2',7'-bis(carboxyethyl)-5,6-carboxyfluorescein (BCECF) (Rink et al., 1982) in L15–air medium. After 5 min the solution was replaced with L15–air, which was changed twice in succession. The explant was visualized using a 32 $\times$ objective and the average fluorescence (excitation, 410–425 nm; emission >510 nm) emitted from an area of 150 μm^2 during 30 sec was measured. The microscope stage was used to move the 150 μm^2 window, set by an attachment to the photomultiplier, from the center of the explant to the perimeter in successive steps of 150 μm . This scanning was repeated in several directions. A 0.4% transmission filter was used to prevent bleaching, and frequencies above 0.1 Hz were filtered.

Results

Regional distribution of AC activity

Figure 2 shows an analysis of the distribution of AC activity stimulated by forskolin and GTP γ S in neurites and cell bodies of explant SCG segments as a function of time in culture. Forskolin, which causes direct activation of the catalytic unit of AC (Seamon and Daly, 1981) and GTP γ S, which activates the stimulatory GTP binding protein quasi-irreversibly, were added together to maximize AC activity and to minimize inactivation, which can occur at 37°C over long incubation periods in assay mixed in the absence of guanine nucleotides (Tamir and Tolkovsky, 1985). As shown in Figure 2*A*, total explant AC activity increased by about 7-fold within 17 d in culture. Neurites grew about 6 mm during this time, establishing a constant growth rate of about 400 $\mu\text{m}/\text{d}$ after an initial, slower period of growth during the first 2–3 d. The lag time was similar to that described by Agiro and Johnson (1982) for SCG from older animals, but the rate of neurite extension was usually faster than they described, similar to the rates of neurite extension obtained by Estridge and Bunge (1978). The pattern of growth was highly dependent on the concentration and physical state of the collagen, whose preparation was therefore standardized as described in Materials and Methods. An analysis of activities in separated neurite and cell body regions (Fig. 2*B*) shows that AC activity in the neurites rose at the same rate as AC activity in intact cultures, while levels of activity in the cell bodies remained virtually constant during growth. The level of AC activity expressed in neurites depended directly on neurite length. For example, AC activity in 4-mm-long neurites from a 17 d explant was identical to the activity in neurites obtained from 10 d culture of explants shown in Figure 2, which had also grown by 4 mm.

The linear relationship between average neurite outgrowth and the increase in AC activity suggested that AC activity might be distributed throughout the neurites. It was also possible, however, that AC activity was localized in a discrete area of the neurite, such as the growth cone. To examine the pattern of distribution of AC activity in the neurites, neurites from explant cultures were dissected into 500 ± 134 - μm -wide segments in the form of concentric rings (Fig. 2*C*). AC activity was present in each section, indicating the presence of AC throughout the neurites. The fact that AC activity in each 0.5 mm segment also fell within 1 SD of the average sectional AC activity [1.33 ± 0.34 pmol cAMP/min (37°C)/section in the presence of forskolin

and GTP γ S and Table 1, below] suggested that AC activity may be evenly distributed along the neurites at 0.5 mm resolution. This interpretation assumes an equal volume of neurites in each concentric section. Equal amounts of AC activity per segment would also be obtained, however, if AC activity were kept constant per unit outgrowth despite an increase in branching as neurites extended. (Thus, 0.5 mm of 2 neurite branches preceded by 0.5 mm of an unbranched neurite segment would contain the same amount of total AC activity, but each branch would contain half the density of AC.) To measure the volume of neurites per unit area of outgrowth, neurites were loaded with the fluorescent indicator BCECF (Rink et al., 1982) (Fig. 3*A*). A photomultiplier attached to a fluorescence microscope (Rogers et al., 1983; Richards and Tolkovsky, 1986) measured the fluorescence emitted from a constant area (150 μm^2) of neurites. Sections of neurites were scanned in steps of 150 μm from the center to the perimeter of the explant. If n is the number of steps from the center to the perimeter of the explant, the fluorescence/150 μm^2 in segment n will be smaller by $1/(2n - 1)$ relative to the fluorescence/150 μm^2 in segment $n = 1$ if neurites grow in a radial pattern and the average volume per concentric section is constant. Figure 3*B* shows that the fluorescence/150 μm^2 was a linear function of $2n - 1$ ($r^2 = 0.9135$ for a linear regression), indicating that the volume of neurites per concentric segment does not increase with increasing neurite outgrowth from these explants.

Colocalization of AC and GTP binding proteins

Regulation of AC activity by receptors for neurotransmitters and hormones is dependent on the function of 2 classes of GTP binding proteins (G proteins) known as G_s and G_i , which promote stimulation and inhibition, respectively, of AC when they bind GTP or nonhydrolyzable analogs of GTP such as GTP γ S or p(NH)ppG. The function of G proteins is inhibited by the GDP analog, GDP β S. The presence of stimulatory and inhibitory G proteins in neurites was therefore examined to determine to what extent neurite AC activity can be modulated by guanine nucleotides early in development. Table 1 summarizes the effects of the nonhydrolyzable GTP and GDP analogs on AC activity in neurite segments after radial (section A) or concentric microdissection (sections B and C; also illustrated in Fig. 2*C*). Activity measured in the presence of GDP β S indicated that AC in all neuron segments has intrinsic (or basal) activity in the absence of stimulatory guanine nucleotides. GTP γ S or p(NH)ppG enhanced basal activity between 3- and 10-fold regardless of whether the neurite segment was derived from a radial or a concentric section. Forskolin enhanced activity between 5- and 15-fold. The activation obtained by the addition of both forskolin and GTP γ S was similar to the sum of the 2 activities measured separately (shown in brackets in Table 1), consistent with the simultaneous expression of active G_s and catalytic subunit of AC throughout the neurites.

Independent structural evidence for the presence of GTP binding proteins was sought by the use of pertussis and cholera toxins. Pertussis toxin catalyzes the ADP-ribosylation of G_i and of another G protein found in brain, known as G_o (Neer et al., 1984; Sternweis and Robishaw, 1984; Wong et al., 1985). Cholera toxin catalyzes the ADP-ribosylation of G_s and some other G proteins specific to brain membranes (Berthillier et al., 1982; Wong et al., 1985). Figure 4*A* shows a gel on which proteins from cell bodies or neurites of 8 d cultures of SCG explants were separated by SDS-PAGE after incubation with either per-

tussis or cholera toxin, GTP, and ^{32}P -NAD. The stained proteins derive from serum and from collagen, as well as from the cells. Neuronal actin was abundant and served as an internal molecular-weight standard for comparison with an actin marker added externally. The corresponding autoradiogram shows heavy labeling of a protein slightly smaller than 40 kDa M_r after incubation of either cell bodies or neurites with pertussis toxin. Figure 4B shows that the same protein was labeled by pertussis toxin in neurites that were divided into 2 unequal sections comprising the anterior portion of the neurites ($\sim 85\%$) and the posterior portion ($\sim 15\%$). There was very slight labeling in the presence of cholera toxin, possibly because the affinity of cholera toxin for NAD and for the 52 kDa α -subunit of G_s is very low (Berthillier et al., 1982; Wong et al., 1985). Cholera toxin did promote, however, some labeling of a protein the same size as the pertussis toxin substrate. Pertussis toxin also promoted intense labeling of a ~ 40 kDa protein when dispersed cultures of dorsal root ganglia neurons were ADP-ribosylated *in situ* using the same labeling cocktail (this experiment was performed in collaboration with Dr. Ann Mudge, University College, London), indicating that both sympathetic and sensory neurons contain large amounts of pertussis toxin substrate during development. The high density of the pertussis toxin substrate, and the fact that it was consistently smaller than creatine kinase, suggests that this protein is primarily G_o (Neer et al., 1984; Sternweis and Robishaw, 1984; Wong et al., 1985), although the presence of G_i cannot be excluded.

Density of catalytic units of AC

A quantitative assessment of the number of active AC and G protein molecules synthesized per cell could not be calculated in experiments with explant cultures since the number of cells that sprouted neurites was not known. AC activity was therefore measured in dispersed cultures of SCG neurons in which the number of neurons growing neurites was counted. Figure 5A shows a typical pattern of development of cultures grown for 29 d. Figure 5B shows that basal, GTP γ S-stimulated, and GTP γ S plus forskolin-stimulated activities of AC in dispersed cells increased as a function of time in culture. The amounts of cAMP produced under these 3 experimental conditions by 9 d cultures of 1800 ± 300 cells were similar to the amounts of cAMP produced by 150 ng of adult rat brain membranes assayed under identical conditions, indicating that AC and G proteins are assembled in a mature state in the growing neurite. The number of molecules of AC in neural cell bodies, and the number of active AC molecules synthesized by a growing neuron per day

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Figure 3. Fluorescence of BCECF from neurite sections as a function of distance from explant center. *A*, Cells loaded with BCECF: *top*, cell bodies; *middle*, edge of small explant with neurites; *bottom*, growth cones (Kodak Tri-X film). $\times 625$. *B*, Six-d-old explants were loaded with BCECF as described in Materials and Methods. The fractional fluorescence (fluorescence (n)/fluorescence ($n = 1$)) is plotted as a function of $2n - 1$ ($\bullet$), where n is the number of a $150 \mu\text{m}^2$ segment in a linear sequence from $n = 1$ in the neurite section most proximal to the cell bodies to $n = 12$ for the most distal segment of neurites. The open circle is the fractional fluorescence in $n = 1$ measured at the end of the experiment (~ 30 min). The average background fluorescence at $\times 300$ magnification used was 15 (in arbitrary units) as compared to 31 for $n = 1$ at the beginning of the experiment. Background was subtracted before relative fluorescence was calculated. The mean $\pm$ SD is shown for 5 determinations. Solid line is derived from a linear regression, $r^2 = 0.9135$; dotted line, theoretical curve.

Table 1. Distribution of stimulatory G protein (G_s) in cell bodies and neurite segments

Adenylate cyclase activity (pmol cAMP/100 min/segment)						
Sample	GDP β S (100 μ M)	GTP γ S (10 μ M)	for- skolin (20 μ M)	GTP γ S + forskolin		p[NH]ppG (100 μ M)
				10 μ M	20 μ M	
A. Neurites divided into 8 radial sections						
Explant (i)	2.7 $\pm$ 0.3 (3)	34.3 $\pm$ 0.3 (2)	33	77.5	[67.3]	
Explant (ii)	4.1 $\pm$ 0.5 (3)	23.2 $\pm$ 2.2 (2)	54.5	104.4	[77.7]	
Explant (iii)	3.6 $\pm$ 0.4 (3)	9.0 $\pm$ 1.3 (2)	11.1	27.8	[20.1]	
B. Neurites divided into 2 concentric sections						
Cell bodies	10.5	52.6 $\pm$ 5 (2)	151			41.1 $\pm$ 11.1 (2)
Inner n. area	6.5	53.7 $\pm$ 6.1 (2)	141			61.6
Outer n. area	3.5	45.7 $\pm$ 7.6 (2)	117			42.4
C. Neurites divided into 4 concentric sections						
Cell bodies	15 $\pm$ 5 (3)	39 $\pm$ 15 (2)	82	186	[121]	
0–500 μ m	23 $\pm$ 5 (3)	70 $\pm$ 21 (2)	118	14	[188]	
500–1000 μ m	25 $\pm$ 7 (3)	69 $\pm$ 13 (2)	105	174	[174]	
1000–1500 μ m	37 $\pm$ 9 (3)	54 $\pm$ 13 (2)	175	176	[229]	
1500–2000 μ m	38 $\pm$ 3 (3)	96 $\pm$ 3 (2)	136	210	[232]	

A. Neurites of SCG explants (5 d cultures) were divided into 8 radial sections as illustrated in Figure 1*B*. The results in each row are derived from 1 culture and represent AC activity in 1–3 separate $\frac{1}{8}$ segments. *B.* Neurites of SCG explants (6 d cultures) were divided into 2 concentric sections approximately 1 mm wide. AC activity was measured in the cell body region (top row), the inner neurite region (middle row), and the outer neurite region (bottom row). *C.* Neurites of SCG explants (8 d cultures) were divided into 4 concentric sections as illustrated in Figure 2*C*. AC activity was measured in the cell body region (top row) and in 4 neurite sections 500 $\pm$ 134 μ m wide (activity in inner to outer sections in rows 2–5). Enzyme assays were run for 100 min at 37°C. The means and range of values ($n = 2$) or SD ($n = 3$) are followed by the number of replicates (in parentheses); the sum of GTP γ S and forskolin activities is given in brackets.

Figure 4. ADP-ribosylation of explant proteins by pertussis and cholera toxin. *A.* SDS-PAGE of cell body and neurite proteins from 8 d explants. Pertussis toxin (PT) lanes 5, 6, 10, 11; cholera toxin (CT) lanes 2, 3, 8, 9; 4 explant cell bodies (*cb*, lanes 3, 6, 9, 11) or 4 explant neurites (*n*, lanes 2, 5, 8, 10) per lane; lanes 1–7, silver-stained gel (lanes 1, 4, 7: molecular-weight markers; note the actin band in lanes 2, 3, 5, 6, just below the ovalbumin band); lanes 8–11, autoradiography (3 d exposure). The dark, lower region of the autoradiogram was caused by trapping of bulk 32 P compounds at and above the dye front. *B.* Three-day-old explants were divided into 3 segments: cell bodies (*cb*, lanes *a*, *d*); inner neurite area, ~80% of total neurite outgrowth (*ni*, lanes *b*, *e*); and outer neurite area, including growth cones (*no*, lanes *c*, *f*). Two explant segments were used for each lane; autoradiogram was exposed for 24 hr. Molecular-weight markers, lanes *g*, *h*. Note alignment of cellular actin with marker actin (lanes *d*–*f* and *h*).

Figure 5. AC activity in cultures of isolated SCG neurons. *A*, SCG neurons maintained in culture for 1 (*a*), 3 (*b*), 6 (*c*), and 29 (*d*) d. Magnification: $\times 125$ (*a-c*); $\times 400$ (*d*). Scale bar, 50 μ m. *B*, AC activity measured in the presence of 100 μ M GDP β S ($\circ$), 10 μ M GTP γ S ($\blacktriangle$), or 10 μ M GTP γ S and 10 μ M forskolin ($\bullet$). Each point was obtained from 1800 ± 300 cells, counted from 20 fields at $\times 200$. Zero time points were obtained from the same number of cells counted as viable before seeding. AC activity in a preparation of rat brain synaptosomal membrane protein was measured under identical assay conditions, using the same assay cocktail. Bars represent range of values for 3 replicates. Lines were drawn by eye.

were estimated using a turnover number of 2000/min for GTP γ S-stimulated AC similarly measured at 30°C, based on the assumption that the turnover number is the same for different systems (Pfeuffer et al., 1983); a cell body that produced an average of 236 ± 46 attomol cAMP/min (30°C) would contain approximately $70,000 \pm 10,000$ molecules of AC at the time of excision. The additional 52.6 ± 20 attomol cAMP/min (30°C) produced after 24 hr of growth would be equivalent to the addition of approximately $15,000 \pm 5000$ AC molecules. Furthermore, based on the assumption that AC and G $_i$ are plasma membrane proteins, it was calculated that the surface of a cell body with a ~ 30 μ m diameter (Fig. 5*A*) would contain 22–28 molecules of AC/ μ m 2 and that the surface of a neurite, a cylinder of 0.5 μ m diameter (Fig. 5*A*), would contain 9–15 molecules

AC/ μ m 2 . These results suggest that cell body and neurite membranes contain similar densities of AC coupled to G $_i$.

Reduction of AC levels by tunicamycin

The rapid addition of AC molecules in growing neurites prompted an examination of its rate of turnover. AC is reported to bind wheat germ agglutinin (Pfeuffer et al., 1985), and it was therefore tested whether levels of AC activity would be reduced by tunicamycin, which blocks *N*-asparagine-linked glycosylation. No direct effect of tunicamycin on AC activity was observed, and neurite morphology, as well as the rate of neurite outgrowth, appeared normal after 24 hr in the presence of 2 μ g/ml tunicamycin. Thus, neurite length increased by 20% in both control and tunicamycin-treated cultures but GTP γ S plus forskolin-

Table 2. Effect of tunicamycin on AC activity in explants

Dissected area	Control	+ tunicamycin	% of control
a. AC activity (pmol cAMP/min/section)			
Cell bodies	1.10 ± 0.13	0.61 ± 0.05	55 ± 8 ^a (90–35)
Neurites	3.60 ± 0.61	1.11 ± 0.17	31 ± 7 ^a (67–15)
b. Activity ratios			
Neurites/ cell bodies	3.26 ± 0.68	1.86 ± 0.33	57 ± 33 ^b
c. Elongation (%)			
Neurites	20.8 ± 8	22.2 ± 10	

Explants were cultured for 10 d. A solution of 2 µg/ml tunicamycin was prepared in L15-CO₂ growth medium and was used to replace the normal medium. After 24 hr, cultures were dissected and AC activity measured for 100 min at 37°C in the presence of 10 µM GTPγS and 10 µM forskolin (section a). Ratios of neurite to cell body activities are given in section b. For calculation of the percentage of elongation, the average length of neurites in each culture was measured before and after 24 hr in the presence or absence of tunicamycin (section c). Averages ± SE. Means are given (*n* = 6) with the range of values in parentheses.

^a *p* < 0.01, double tailed Student's *t* test.

^b *p* < 0.05, double tailed Student's *t* test.

stimulated activity was reduced to 55 ± 8% (SEM, *n* = 6) of control (10–65% range of values) in cell bodies and to 31 ± 7% (SEM, *n* = 6) of control (33–90% range of values) in neurites after 24 hr (Table 2). This difference was significant at the 95% level (double-tailed Student's *t* test). After 2–3 d in the presence of tunicamycin neurites disintegrated into a series of beads, similar to the appearance of neurites detached from cell bodies or of cells deprived of NGF. Thus, tunicamycin can reduce the amount of active AC without impairing neurite outgrowth.

Comparison of AC and TH distribution

The distribution of AC and TH activities between cell bodies and neurites and the rate of their appearance were compared for 3 main reasons: (1) TH controls the rate of catecholamine biosynthesis and may be considered to be a specific maturation marker for adrenergic neurons (Black and Patterson, 1980; Wolinsky and Patterson, 1983; but also Iacovitti et al., 1981); (2) the levels of activity and the expression of TH in SCG increases in response to acute and long-term exposure to NGF and other extracellular stimuli (Hefti et al., 1982; Ip et al., 1983), and TH activity is enhanced after phosphorylation by cAMP- and Ca²⁺/calmodulin-dependent protein kinases (Treiman et al., 1983; Niggli et al., 1984); (3) unlike AC, it is a cytosolic rather than an integral membrane protein. Figure 6*A* shows that the rise in TH activity was parallel to the rise in AC activity up to 10 d in culture. After 10 d, TH activity increased more rapidly than the rate of neurite outgrowth, but between 20–29 d the rate of increase in TH activity appeared to decrease; this is similar to the time course of TH induction in cultures of dispersed SCG neurons described by Mains and Patterson (1973). On comparing the distribution of TH and AC activities in cell bodies and neurites during 17 d of growth (Fig. 6*B*), cell bodies were found to retain 85 ± 3% of total cellular TH activity, while the proportion of total cellular AC activity in cell bodies fell rapidly during growth, and only 15 ± 2% was found in cell bodies after 17 d.

Discussion

The present study shows that SCG neurons synthesize an AC system, consisting of catalytic and regulatory components, con-

Figure 6. TH activity in explants. *A*, TH activity measured for 20 min at 37°C as described in Materials and Methods. *B*, Fraction of total explant TH (○) and AC (●) activities found in cell bodies plotted against time in culture. ×, neurite length (mm).

comitantly with neurite outgrowth and express this system throughout the neuron during development. The rate of increase in AC activity is directly proportional to the rate of neurite outgrowth; it is dependent on neurite length and independent of the number of days in culture. The AC system appears to be widely distributed in the cell, as has been previously shown for Na/K-ATPase in chick dorsal root ganglia neurons (Fambrough et al., 1985) and the voltage-dependent Na channel in various unmyelinated neurons (Rogart, 1983). The presence of a competent AC system in developing neurites suggests that the cAMP-dependent protein kinase found in growth cone particles isolated from the developing fetal brain (Ellis et al., 1985) can be activated by the local production of cAMP.

The decrease in AC activity after exposure to tunicamycin is similar to the decrease of voltage-dependent Na channels observed in cultured muscle cells (Sherman et al., 1985). The somewhat smaller decline in cell body AC after tunicamycin treatment as compared to neurite AC raises the possibility that insertion of newly synthesized AC is more vigorous in the neurite than in the cell body, corresponding to the pattern reported for the insertion of newly synthesized membrane lipids into growing neurites (Pfenninger and Johnson, 1983). Carbonetto and Fambrough (1979), however, found that newly synthesized α-bungarotoxin binding sites on sympathetic neurons were inserted into cell bodies and neurites at an equal rate, despite the fact that the binding sites were more dense on the cell body than

on the neurite. The regulation of the synthesis of the α -bungarotoxin receptor was also different from AC, increasing to a maximum within 3 d but declining to basal levels within 7 d. Thus, mechanisms that are secondary to membrane protein biogenesis may control sites of incorporation of different membrane constituents, similar to the control of H^+ -ATPase and Cl^-/HCO_3^- carrier insertion into apical and basal membranes of kidney tubule cells (Schwartz et al., 1985).

There is only a small amount of data concerning the density of AC in neurons with which to compare the densities of AC calculated here. AC activity has been measured in cell bodies of the defined molluscan neurons D2, E2, and F1 by Deterre et al. (1982). The cell bodies of the molluscan neurons had approximately 10- to 15-fold larger diameters compared to the SCG neurons and contained approximately 20-fold higher AC activity. If the surface area is inferred from the diameter, the calculated density of AC per unit cell surface area is found to be similar for SCG and these neurons. However, it should be pointed out that this comparison is only approximate because the somatic membranes of molluscan neurons are known to be highly convoluted. A density of 9–28 molecules/ μm^2 membrane surface is within the lower range of values given for the density of the voltage-dependent Na channels in various unmyelinated neurons (Rogart, 1983). It is about 100-fold lower than the lowest density given for soybean agglutinin, cholera, or tetanus toxin binding sites on cultured SCG neurons (allowing for 20–60 molecules ligand/gold particle; Schwab and Landis, 1981). This difference may not be surprising, considering the fact that the toxin and the lectin binding sites are gangliosides, not proteins.

The limitation of the technique described for localizing enzymes in neurite fragments at present is the spatial resolution of the microdissection, and methods to obtain higher resolution and a more accurate basis for the measurement of specific activity are now being developed. Having shown that an AC system that can be regulated by guanine nucleotides is maintained in neurites during development, it is now of interest to establish the amount of intracellular cAMP this system generates and to correlate this with endogenous protein kinase activity during development.

Appendix—Abbreviations

The following abbreviations are used: AC, adenylate cyclase; TH or TOH, tyrosine hydroxylase; G protein, GTP binding protein; G_s and G_i , stimulatory and inhibitory GTP binding proteins of the adenylate cyclase systems; G_o , an abundant G protein found in brain membranes; GTP γ S, guanosine 5'-[γ -thio]triphosphate; GDP β S, guanosine 5'-[β -thio]diphosphate; p(NH)ppG, guanosine 5'-[β -imido]triphosphate; CT, cholera toxin; PT, pertussis toxin; BCECF, 2',7'-bis(carboxyethyl) 5-carboxyfluorescein; amol, attomol (10^{-18} mol).

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