Determination of synaptic phenotype: Insulin and cAMP independently initiate development of electrotonic coupling between cultured sympathetic neurons

(Continued from previous page)

JOHN A. KESSLER*, DAVID C. SPRAY†, JUAN C. SAEZ‡, AND MICHAEL V. L. BENNETT†

Departments of Neurology* and Neuroscience†, Rose F. Kennedy Center for Mental Retardation, Albert Einstein College of Medicine, Bronx, NY 10461

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ABSTRACT Electrotonic coupling between pairs of sympathetic neurons dissociated from superior cervical ganglia of neonatal rats is rare when cells are cultured for 2 weeks in a nutrient medium plus serum and is common when cells are cultured for the same period in serum-free defined medium. This defined medium is the same nutrient medium with five added factors (progesterone, transferrin, putrescine, insulin, and selenium). When added singly to serum-containing medium, insulin and, to a lesser extent, selenium promote the development of electrotonic and dye coupling. The insulin effect is obtained with doses as low as 0.01 μg/ml and is maximal after exposures from 3 to 5 days. The incidence of electrotonic coupling is also enhanced by exposure of cells to dibutyryl cAMP. This effect is obtained with doses as low as 0.1 μM, is faster (being maximal at ~12 hr exposure), and is prolonged in the presence of the phosphodiesterase inhibitor caffeine. Butyrate itself promotes coupling to a small extent, but cAMP involvement is confirmed by similar effects of other membrane permeant analogues. Endogenous levels of cAMP are significantly elevated in cultures grown in the defined medium but not in those in serum-containing medium to which insulin or selenium are added. We conclude that the promotion of coupling by cAMP and by insulin or selenium are independent. The development of coupling in the defined medium thus seems to be a consequence of the addition of promoting substances (insulin, selenium) and the removal of an inhibitory effect of serum on cAMP levels.

Neuronal differentiation and specialization are influenced by the environment, so that the phenotype of a neuron may depend on the extracellular milieu (1–4). For example, it is now well established that neurons may alter the transmitters they synthesize and secrete, and thus alter their phenotypes, under appropriate circumstances (5–7). Alteration of neurotransmitter expression in response to experimental alteration in the environment has been particularly well defined in neural crest-derived sympathetic and sensory neurons. For example, regions of the neural crest that normally give rise to cholinergic neurons can be induced to develop noradrenergic progeny by transplanting them into a region of the crest from which noradrenergic ganglia normally arise (8). Conversely, sympathetic neurons that in vivo express predominantly noradrenergic characteristics become cholinergic when cultivated in the presence of certain non-neuronal cells (5).

Phenotypic modifiability is not restricted to choice of transmitter; it also involves other aspects of neuronal differentiation. For example, Higgins and Burton (9) found that embryonic sympathetic cultivated neurons in a chemically defined serum-free medium become electrotonically coupled, a mode of synaptic transmission not normally utilized by sympathetic ganglia. This observation was confirmed by Wolkinsky et al. (10). The cultured neurons continue to express noradrenergic characteristics, including tyrosine hydroxylase and dopamine β-hydroxylase activities, norepinephrine synthesis, and dense-core vesicles (11). They also continue to function as differentiated neurons, being excitable and responsive to exogenous neurotransmitters (9). Consequently, the emergence of electrical coupling represents a highly specific change in the differentiation of neurons that retain most of their other characteristics.

Although electrotonic synapses have not been demonstrated in sympathetic ganglia in vivo (12), electrotonic coupling and/or gap junctions are prevalent in a variety of neural crest derivatives (13–17). Consequently, the potential to develop electrotonic synapses is apparently present in a lineage of the sympathetic ganglia, although it is not normally expressed. The present study follows the observations of Higgins and Burton (9) and explores the factors responsible for development of electrical coupling between sympathetic neurons in culture. In particular, we report that cAMP, selenium, and insulin initiate formation of electrotonic synapses. cAMP apparently acts via a different mechanism than do insulin and selenium.

A preliminary account of this work is in press (18).

MATERIALS AND METHODS

Tissue Culture Techniques. Tissue from superior cervical ganglia (SCG) of neonatal Sprague–Dawley rats (CAMM) was dissociated within 12 hr after birth and grown as described on collagen-coated culture dishes (19). Some cultures were placed in a serum-containing medium (SM) consisting of Ham’s nutrient mixture F12/10% fetal calf serum/nerve growth factor (100 ng/ml)/penicillin (50 units/ml)/streptomycin (50 μg/ml). Other tissues were grown in a serum-free defined medium (DM) consisting of F12/transferrin (100 μg/ml)/putrescine (100 μM)/insulin (5 μg/ml)/prostaglandin D2 (20 nM)/selenium (30 nM)/penicillin (50 units/ml)/streptomycin (50 μg/ml)/nerve growth factor (100 ng/ml). Cultures were maintained at 37°C in 95% air/5% CO2 at nearly 100% relative humidity. Ganglion non-neuronal cells were eliminated by treatment with cytosine arabinoside (5 μM) for 24 hr on days 2 and 4 of culture. Cell density ranged from 7000 to 11,000 neurons per 35-mm diameter dish with 1.5 ml of medium.

Cyclic Nucleotide Assay. Cyclic nucleotides were assayed with radioimmunoassay kits (New England Nuclear); the

Abbreviations: SCG, superior cervical ganglia; SM, serum-containing medium; DM, defined medium; Bt2cAMP and Bt2cGMP, dibutyryl cAMP and dibutyryl cGMP. 

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*Present address: Department of Neurology, Albert Einstein College of Medicine, Bronx, NY 10461.
†Present address: Department of Neurology, University of Kentucky, Lexington, KY 40536.
‡Present address: Department of Human and Molecular Genetics, Albert Einstein College of Medicine, Bronx, NY 10461.

6235
acetylated version with a sensitivity of 2.4 fmol was used. To harvest cultures for the assay, the medium was carefully drained, 300 μl of 6% trichloroacetic acid was added, and the cultures were frozen and thawed 5 times to fracture the cells. The trichloroacetic acid was removed and centrifuged for 15 min at 2500 × g, and the supernatants were washed 4 times with 1.5 ml of water-saturated ether. They were incubated briefly in a warm water bath to remove the remainder of the ether and were frozen and lyophilized. The samples were resuspended in assay buffer on the day of the assay. Recoveries of [3H]cAMP added to culture dishes were 80%-85%. Cells were not harvested by scraping or peeling them from the dishes, because even small quantities of the collagen substrate were found to interfere with the radioimmunoassay, spuriously elevating apparent levels of cAMP.

**Electrophysiology.** Pairs of neurons were impaled with electrodes (20–70 MΩ, filled with 3 M KCl) connected to high input impedance electrometers with active bridge circuits (W-P Instruments, New Haven, CT). Current was recorded by a virtual ground current monitor in series with the bath electrode. In some experiments, electrode tips were filled with filtered (0.22 μm) Lucifer yellow CH (2% in 150 mM LiCl) or 6-carboxyfluorescein (2.5% in 1.5 M KCl) and back-filled with 1 M LiCl or 1.5 M KCl for intracellular injection by iontophoresis.

**Assay of Coupling.** Only neurons with contiguous somata (Fig. 1) were impaled; usually there were between 30 and 50 such pairs in the central area of the dish accessible to microelectrodes. Data were only included in the analysis if collected from neurons from which action potentials could be generated by depolarization or in rebound from hyperpolarization. Most measurements of electrotonic coupling were qualitative. Electrotonic coupling was considered present if a response was seen in either cell when current pulses were applied to the other cell sufficient to hyperpolarize it by 100 mV. Coupled cells also showed reciprocal transmission of electrotonic post-synaptic potentials. Typically, neuron input resistances were 10–40 MΩ (in one extensive experiment on cells in defined medium, mean input resistance from 64 cells was 12.71 ± 1.2 MΩ). The resolution of voltage measurements was <1 mV; if the cells were coupled at or near the somata, a coupling conductance of <1 nS would have been detectable. Intracellular recording from soma and major axon with two patch-clamp electrodes connected to voltage-clamp and current-clamp circuits (cf. ref. 20) revealed that the space constant of the major portion of the cell was long compared to its length. Coupling through junctions near the somata was thus unambiguously assessed by application of the Π-T transform to input and transfer voltages obtained in response to currents passed in the two cell bodies (cf. ref. 21).

**RESULTS**

**Electrotetonic Coupling of Neonatal Sympathetic Neurons in Culture.** Since electrotonic coupling develops between embryonic SCG neurons cultured in a defined medium (9), we determined whether postnatal neurons couple under similar conditions. SCG neurons were dissociated from ganglia on the day of birth and cultured in SM or DM. After 2 weeks in vitro, pairs of neurons were common in the dishes (Fig. 1), and the presence of electrotonic coupling between contiguous somata was assessed (Table 1). Neurons grown in SM were rarely coupled. In contrast, >28% of neuron pairs cultured in DM were coupled. These studies demonstrate that SCG neurons from neonatal as well as embryonic ganglia rarely become coupled in the serum-containing media but commonly do so in the defined media.

To determine whether neurons cultured in SM retained the capacity to become coupled, SM was removed after 2 weeks and replaced with DM. Two days later the cultures were examined for the presence of coupling. More than 13% of neuron pairs were coupled under these circumstances (12 of 93 pairs; 2 dishes), indicating that exposure to serum does not abolish the capacity of these neurons to become coupled in the appropriate environment.

**Active Ingredients in the Defined Medium.** Two possible hypotheses account for the observation that neurons cultured in DM coupled electrically, whereas neurons grown in SM did not. First, one or more of the ingredients of DM might promote coupling. Second, serum might inhibit coupling. To test the first hypothesis, each of the five primary additives in DM (insulin, progesterone, transferrin, putrescine, and selenium) was added individually to 2-week-old cultures in SM. These cultures were examined after 2–3 days to determine whether coupling was present.

Putrescine (100 μM), transferrin (100 μg/ml), and progesterone (20 nM) did not cause significant coupling, but cou-

![FIG. 1. Exposure of cell pairs to SM with insulin (5 μg/ml) stimulates formation of electrotonic and dye coupling between SCG neurons. (A) Currents (I) injected into cell 1 (first pulse, bottom trace) or cell 2 (second pulse) produced almost equal voltages (V1 and V2) in the two cells, which were excitabile as evidenced by the generation of impulses at the end of the hyperpolarizations. Application of the Π-T transform to these data gave conductances of the nonjunctional membrane of 79 nS and 78 nS and junctional conductance of 330 nS (B and C). In another well coupled cell pair, Lucifer yellow injected into one cell spread to the other cell within a few minutes, as recorded in the fluorescence micrograph in C.](image-url)
Fig. 2. Development of electrical coupling in SM containing insulin. After 2 weeks in SM, insulin (5 μg/ml) was added to the dishes for 48 hr, and coupling was assayed between pairs of contiguous neurons. Points are means of measurements in at least two dishes (actual numbers appear in parentheses); bars represent SEM; 30–50 cell pairs were examined in each dish.

Promotion of Coupling by cAMP. Since cAMP promotes coupling between certain types of cultured non-neuronal cells (22, 23), we assayed its effect on SCG cells. Neurons cultured in SM were treated with the membrane-permeable dibutyryl cAMP (Bt2cAMP). Within 4 hr of treatment, coupling appeared and >30% of neuron pairs were coupled within 12 hr of exposure to 1 mM Bt2cAMP (Fig. 4). The percentage of neurons coupled decreased thereafter, possibly reflecting metabolism of the drug, and only 3% were coupled 48 hr after treatment. Addition of the phosphodiesterase inhibitor caffeine along with Bt2cAMP prolonged the period of coupling, so that >10% of the pairs were coupled 72 hr later (Fig. 4). A similar prolongation of coupling was obtained with isobutylmethylxanthine (not shown). Caffeine (or isobutylmethylxanthine, data not shown) added alone also caused neurons to couple, but to a lesser degree (Fig. 4).

Bt2cAMP is widely used to achieve membrane permeation of cAMP, but it possesses two moieties: cAMP and butyrate. Butyrate is liberated by intracellular hydrolysis (as are protons; cf. ref. 24), and since it has profound effects on some aspects of neuronal metabolism (25–27), we sought to determine whether butyrate was responsible for some or all of the effects of Bt2cAMP. Indeed, treatment with butyrate resulted in an increase in coupling at 12 hr (12% of pairs). Little coupling was observed at 24 hr or thereafter, however (Fig. 4), and we conclude that most of the longer term and more potent effects of Bt2cAMP on establishment of coupling are attributable to the cAMP portion of the molecule. Although treatment with dibutyryl cGMP (Bt2cGMP) resulted in some coupling at 12 or 24 hr (12% and 9%, 3 dishes each, data not shown), the percentage of coupling is consistent with that found for butyrate alone and is therefore attributable to the butyrate moiety.

As a further control against the effects of butyrate addition, cultures were treated with other membrane-permeable cAMP derivatives. Treatment with 1 mM monosuccinyl cAMP or 8-bromo-cAMP also caused coupling (Table 2), supporting the thesis that cAMP itself promotes coupling. Consequently, of the cyclic nucleotides tested it appears that cAMP promotes coupling, whereas cGMP does not.

Table 2. Coupling in 1 mM solutions of cAMP derivatives in SM after 12-hr exposure

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<thead>
<tr>
<th>Cyclic Nucleotide</th>
<th>% Coupled, mean ± SEM</th>
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<tr>
<td>Bt2cAMP</td>
<td>32 ± 2 (n = 15)</td>
</tr>
<tr>
<td>8-Bromo-cAMP</td>
<td>11.8 ± 2.1 (n = 4)</td>
</tr>
<tr>
<td>Monosuccinyl cAMP</td>
<td>22.7 ± 3.1 (n = 2)</td>
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To examine the relationship between the dose of cyclic nucleotide and the development of coupling, neurons grown in SM were exposed to various concentrations of Bt2cAMP for 12 hr. The percentage of coupled neurons was increased even with low doses of Bt2cAMP, and >20% of pairs were coupled at concentrations higher than 1 mM (Fig. 5).

Relationship Between Insulin and cAMP Effects. To determine whether the development of coupling produced by insulin or selenium might be mediated through an increase in cytoplasmic cAMP, levels of endogenous cAMP were measured after treatment with these additives. In addition, cAMP levels were measured in control cultures grown in SM and in cultures grown in defined medium (Fig. 6). Treatment with insulin or selenium in SM did not increase neuronal levels of cAMP above those in SM alone (=0.02 fmol of cAMP per neuron). This result suggests that cAMP does not mediate the effects of insulin or selenium. By contrast, neurons cultured in DM contained significantly more cAMP than those in SM (>0.04 fmol per neuron), suggesting that insulin, selenium, and cAMP are all involved in the development of coupling in DM.

Increased Incidence of Coupling Represents Formation of Electrotonic Synapses. Change in strength of electrotonic coupling can result from altered conductance of either junctional or non-junctional membranes (cf. ref. 27). The development of coupling observed above could have been due either to formation of electrotonic synapses or to a large treatment-related decrease in conductance of nonjunctional membranes (g_j). Although morphological data are lacking, other data indicate that synapses were formed. Transfer of dyes to which non-junctional membranes are impermeable was observed in every pair of electrotonically coupled cells tested (n > 40; cf. Fig. 1 B and C), including cells from DM, insulin, and cAMP cultures. In no case was dye coupling seen where electrotonic coupling was absent (>30 pairs). Furthermore, 72-hr insulin exposure and 12-hr Bt2cAMP treatment did not appreciably alter g_j in coupled cells [16.5 ± 4.5 (n = 10) and 14.7 ± 2.3 MΩ (n = 14) compared to the value of 14 ± 2 MΩ (n = 20) in noncoupled cells in SM].

The data presented in Figs. 2-5 associate incidence of coupling with drug treatment. In one series of exposures to cAMP and caffeine, we calculated g_j and g_u from input and transfer resistances to determine whether values of g_j followed a time course similar to incidence of coupling. They did not; g_j between coupled cells was maximal at the earliest times measured and remained similar at later times, independent of changes in the incidence of coupling (4-8 hr: 50 ± 3 nS (n = 25); 12-16 hr: 41 ± 5 nS (n = 38); 20-28 hr: 26 ± 3 nS (n = 38)). Moreover, g_u values were similar in the rare cases of coupling that occurred in SM. These data suggest that when cells are stimulated to couple by additives in SM, they rapidly reach a maximal degree of coupling (and that as incidence of coupling decreases, cells either retain their coupling constant or rapidly decrease it below measurable levels).

**DISCUSSION**

Plasticity of neuronal phenotype and formation and remodeling of chemical synapses are well documented. Indeed, transmitter and synaptic modifiability may be a common crucial feature of neurons and neuronal circuits (1-7). The present studies suggest that yet another neuronal capability, the formation of electrotonic synapses, depends on the environment, and that specific chemical signals may govern the extent to which neurons are electrically coupled.

**cAMP and Electrotonic Coupling.** Exposure of sympathetic neurons to any of several membrane-permeable cAMP derivatives resulted in rapid formation of electrotonic synapses. Treatment with phosphodiesterase inhibitors that would increase endogenous cAMP also increased electrotonic coupling. These observations suggest that intracellular cAMP stimulates electrotonic synapse formation by sympathetic neurons. The effect of cAMP on SCG neurons is not confined to the promotion of electrotonic coupling. For example, cAMP increases levels of enzymes required for catecholamine synthesis in these cells (25, 29-31) and tends to suppress cholinergic differentiation. It is not known whether normal environmental stimuli sufficiently increase cytoplasmic cAMP levels to cause development of both noradrenergic traits and electrotonic coupling, but it is intriguing to speculate that the same stimulus might enhance development of both chemical and electrical communication by these neurons.

**Insulin and Electrotonic Coupling.** Insulin, like cAMP, promoted formation of electrotonic synapses in culture, but several lines of evidence suggest that the two factors foster electrical coupling by different mechanisms. Insulin treatment did not increase endogenous levels of cAMP, and the time course of the effects of the two agents differed strikingly; cAMP treatment led to rapid (peak at 12 hr) but short-lived emergence of coupling, whereas insulin-induced coupling developed more slowly (peak at 72 hr) and persisted much longer. Moreover, insulin treatment did not produce the other changes in neuronal function elicited by cAMP, such as increased tyrosine hydroxylase (unpublished observation).
Defined Medium and Electrotonic Coupling. Two of the ingredients of DM, insulin and selenium, promoted electrotonic synapse formation when added to SM. Consequently, these factors are apparently responsible for some of the coupling between neurons cultured in DM. However, cAMP also promoted coupling, and neurons grown in DM contained significantly higher cAMP levels than neurons cultured in the presence of serum. Thus an increase in endogenous cAMP is likely to contribute to development of coupling observed under serum-free conditions. Since insulin and selenium do not increase cAMP when added to SM, some other factor must be responsible for the difference in nucleotide levels in SM and DM cultures. Serum suppresses cAMP levels in cultures of some non-neuronal cells (32–35), and the deletion of serum may be responsible for the increase in cAMP levels. Consequently, electrotonic synapse formation in the DM may result both from the addition of insulin and selenium and from the deletion of serum.

Relevance to Function in Vivo. Electrotonic synapses have not been demonstrated in sympathetic ganglia in vivo (cf. ref. 12), and it is possible that the formation of coupling in vitro represents altered differentiation related to tissue culture conditions. However, even in culture, sympathetic neurons did not form electrotonic synapses except in response to insulin, selenium, or cAMP; consequently, failure to find such coupling in normal intact ganglia is not surprising. Alteration of the neuronal milieu in vitro, however, might lead to the formation of electrotonic synapses and such a phenomenon could play a role in disease processes. Consequently, it will be important to determine whether stimuli that promote electrotonic coupling in vitro also occur and are effective in the intact organism.

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