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Dissociated cell culture of cholinergic neurons from nucleus basalis of Meynert and other basal forebrain nuclei

(diagonal band nuclei/medial septal nucleus/action potentials/substance P/glutamate)

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ABSTRACT **Degeneration of cholinergic neurons from the basal forebrain nuclei is suspected to be the cause of Alzheimer disease. We have developed dissociated cultures of cholinergic neurons from these nuclei (the nucleus basalis of Meynert, the medial septal nucleus, and the diagonal band nuclei). Brain slices of the forebrains were made by a vibratome, and the basal forebrain nuclei were dissected out, dissociated, and cultured. Choline acetyltransferase immunocytochemistry and acetylcholinesterase cytochemistry revealed large cholinergic cells (average diameter, 20–25 μm) in these cultures. About 75% of large neurons (20 μm or larger in diameter) were cholinergic. Electrophysiological experiments were performed on these large neurons. The neurons usually did not show spontaneous firing, but steady depolarizations produced trains of action potentials, which adapted quickly. The neurons responded with depolarization to the application of L-glutamic acid. Substance P produced depolarization (sometimes hyperpolarization), and during the depolarization membrane resistance was increased.**

Until recently, little attention has been paid to the cholinergic system in the vertebrate brain. However, this situation has changed dramatically with the introduction of ChoAcTase (choline acetyltransferase) immunohistochemistry. This method is regarded as the most specific marker for cholinergic neurons (1, 2), and, for the past few years, many studies have been published on the structures and the pathways of cholinergic neurons in the brain (2–4). In the basal forebrain there are conspicuous groups of cholinergic neurons that supply cholinergic innervation to wide areas of neocortex and hippocampus. Interest in these cholinergic neurons has increased following the finding that their degeneration may be the primary cause of Alzheimer disease and senile dementia of the Alzheimer type (5, 6). In spite of the importance of these cholinergic nuclei, nothing is known about their cellular physiological properties.

We have recently succeeded in developing dissociated cell cultures of the cholinergic neurons from the basal forebrain nuclei (the nucleus basalis of Meynert, the medial septal nucleus, and the diagnonal band nuclei). The neurons are functionally alive, and we could obtain data on the basic electrophysiological properties of these cells. The main aim of this paper is to report structural and physiological characteristics of these cultured neurons. A part of the results appeared in an abstract (7).

MATERIALS AND METHODS

Culture. We adopted the same culture method as used for culturing the locus coeruleus neurons (8). Brain slices (300–400 μm thick) were obtained from the forebrains of newborn Wistar rats or Long–Evans rats (1–3 day old) by the use of a vibratome (Lancer, 1000) (In one experiment, a 9-day-old rat was used.) From these brain slices, tissue fragments from the following two regions were excised under a dissecting microscope: (*i*) the nucleus basalis of Meynert and (*ii*) the medial septal and the diagonal band nuclei. The dissected tissue fragments were incubated in 0.25% trypsin in a calcium-free balanced salt solution for 15 min at 37°C and then dissociated by trituration in a modified Eagle's minimum essential culture medium with Earle's salt, L-glutamine (2.92×10^{-4} g/ml), glucose (6×10^{-3} g/ml), $NaHCO_3$ (3.7×10^{-3} g/ml), L-ascorbic acid (1×10^{-5} g/ml), penicillin (50 units/ml), streptomycin (50 μg/ml), fetal bovine serum (10%), and heat-inactivated horse serum (10%). In some cultures heat-inactivated rat serum (10%) (9) was used instead of bovine and horse serum.

The culture chamber was a small well (about 1.2 cm in diameter) made at the center of each Petri dish (10). The bottom of the well, which was made of either carbon-coated cover glass or of ACLAR fluorohalocarbon film [5 mil in thickness (1 mil = 25.4 μm), Allied Fibers and Plastics, Morristown, New Jersey], was coated with rat tail collagen and then plated with a feeder layer consisting mainly of astroglial cells (11). Dissociated neurons were plated on the feeder layer at a density of 2×10^4 to 3×10^5 cells per cm^2. The cultures were kept at 37°C in an atmosphere of 10% CO_2 in air for 2–4 wk.

Acetylcholinesterase (AcChoEase) Staining. Cultures were fixed for 15 min by a fixative containing 4% paraformaldehyde, 10 mM $CaCl_2$, and 0.1 M sodium cacodylate buffer (pH 7.3). The cultures were rinsed and then stained for AcChoEase by the method of Karnovsky and Roots (12).

ChoAcTase Localization. Monoclonal antibody to ChoAcTase (IE6), kindly supplied by P. M. Salvaterra and J. E. Vaughn, was used. After cultures were fixed with 4% paraformaldehyde and 0.002% $CaCl_2$ in 0.12 M phosphate buffer (pH 7.2), the material was processed for immunocytochemical reaction to ChoAcTase with the peroxidase-antiperoxidase method (13), following the procedure described by Houser *et al.* (2).

Electrophysiology. Electrophysiological experiments were conducted by a method similar to that described by O'Lague *et al.* (10). During the experiments, cultures were superfused with a Krebs solution (126 mM NaCl/2.5 mM KCl/2.4 mM $CaCl_2$/1.6 mM NaH_2PO_4/1.3 mM $MgCl_2$/25 mM $NaHCO_3$/11 mM glucose) equilibrated with a mixture of 95% O_2/5% CO_2. For the whole-cell patch clamp (14), the patch pipette contained 110 mM (or 95 mM) potassium aspartate, 40 mM NaCl (or KCl), 5 mM (or 10 mM) Hepes/KOH buffer, 5

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Abbreviations: ChoAcTase, choline acetyltransferase; AcChoEase, acetylcholinesterase.

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mM EGTA/KOH, 3 mM $MgCl_2$, and 2 mM ATP (pH 6.7–7.4), and the culture was superfused by an oxygenated Hepes-buffered Krebs solution (148 mM NaCl/2.5 mM KCl/2.4 mM $CaCl_2$/1.3 mM $MgCl_2$/5 mM Hepes buffer/11 mM glucose, pH 7.4). Drugs were applied by pressure ejection from a micropipette (15). Experiments were conducted at 29–35°C (except for a few cells done at 27–28°C).

RESULTS

Cell Culture. The location of magnocellular cholinergic neurons in the basal forebrain of newborn rats was observed in vibratome-sectioned brain slices stained with AcChoEase. Fig. 1*A* is an example of stained brain slices, showing the region of nucleus basalis, which is visible as distinctly dark areas, located in the ventral and medial aspects of globus pallidus (3, 4, 16). After practicing with these stained preparations, we became capable of locating basal forebrain nuclei of unstained slices under the stereomicroscope.

We tested the cholinergic nature of our dissociated cultured neurons by ChoAcTase immunohistochemistry (1, 2). Many neurons revealed positive immunoreactivity (Fig. 1 *C* and *E*). As seen in Fig. 1 *C* and *E*, the ChoAcTase reaction was moderate to strong in perikaryons but was weak in their processes. In Fig. 1*C*, we can see one ChoAcTase-positive neuron and a few cell-like contours. Observations of the same

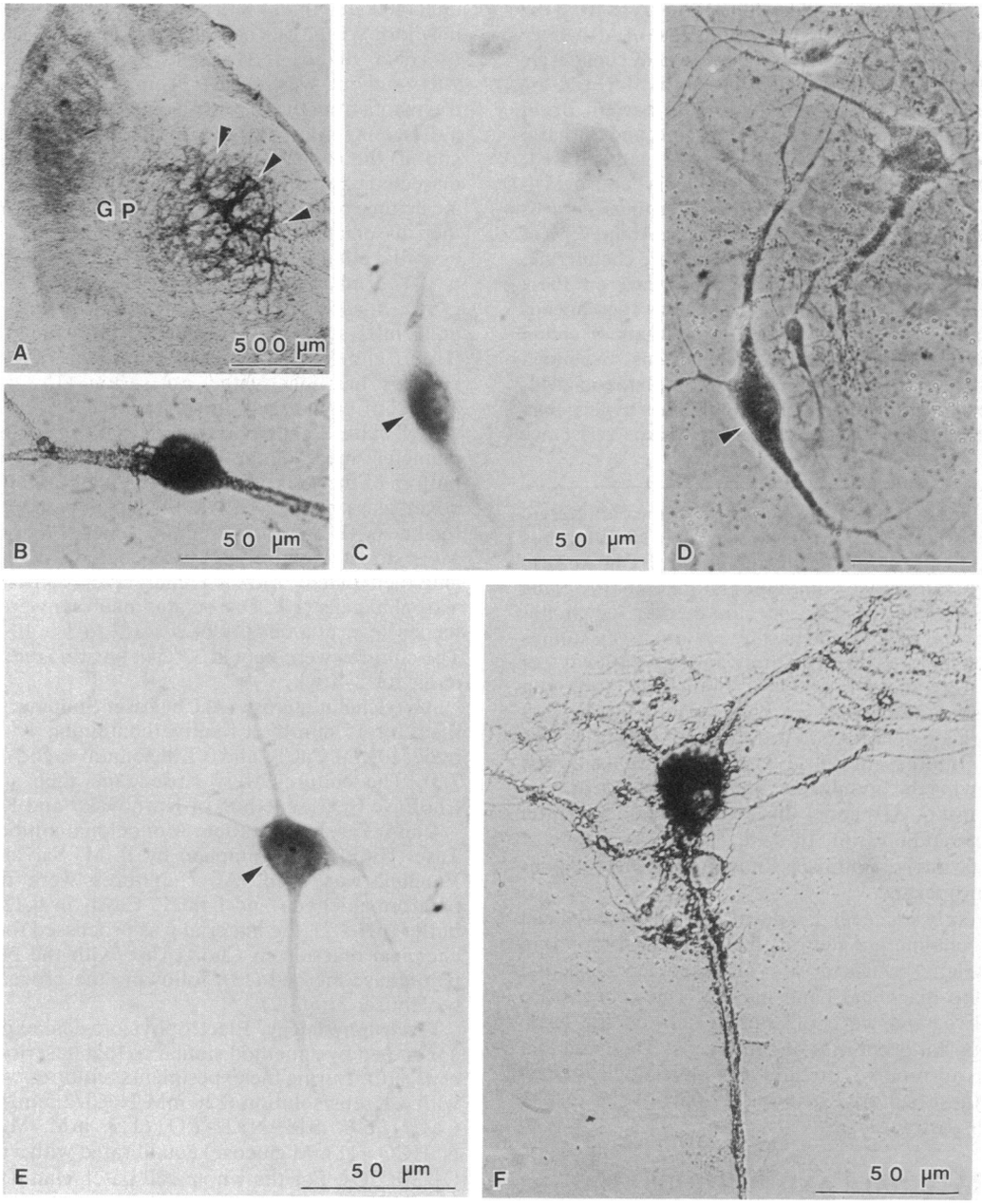

FIG. 1. (*A*) Coronal vibratome slice (150 μm thick) of the forebrain of a 2-day newborn rat. The slice was stained for AcChoEase. The dark areas are positive to AcChoEase stain (arrowheads) and are located at the medial aspect of the globus pallidus (GP). They are neurons homologous with the nucleus basalis of Meynert in humans and primates. (*B* and *F*) Cultured neurons from the nucleus basalis that show intense staining for AcChoEase. (*B*) Cultured for 21 days; taken with conventional microscopy optics. (*F*) Cultured for 27 days; taken with Nomarski optics. (*C–E*) Cultured neurons from the nucleus basalis treated for ChoAcTase immunocytochemistry. Cultured for 24 days; *C* and *E* were taken with conventional microscopy optics and *D* was taken with phase-contrast optics. ChoAcTase positive neurons are indicated by arrowheads.

specimen under the phase-contrast microscope (Fig. 1*D*) clearly revealed all ChoAcTase-negative cells as well as the ChoAcTase-positive cell. In the control, in which the antibody was eliminated or replaced by normal mouse serum, neurons showed slight background staining.

We also stained our culture for AcChoEase (12). Fig. 1 *B* and *F* show neurons with strong AcChoEase positivity. These cells have perikarya filled with a dark brown reaction product and processes, particularly their outer borders, exhibiting strong positivity. Using the AcChoEase stain, we found that the neuronal processes of most of these AcChoEase-positive cells had many extensive arborizations (Fig. 1*F*). The AcChoEase stain in our culture seems to represent the specific AcChoEase, since using *S*-butyrylthiocholine iodide as the substrate did not produce a positive reaction.

Levey *et al.* (17) investigated the colocalization of AcChoEase and ChoAcTase in the *in situ* preparation and found that, at least in basal forebrain nuclei, all neurons with strong AcChoEase reaction also showed ChoAcTase positivity. Thus, the neurons with strong AcChoEase positivity in our culture, such as those in Fig. 1 *B* and *F*, are most probably cholinergic neurons.

Table 1 summarizes the structural data of our cultured neurons. Cholinergic neurons, as identified with ChoAcTase, accounted for 29% (nucleus basalis) and 19% (medial septal and diagonal band nuclei) of the total neuron-like cells. The size of cholinergic neurons was larger than that of noncholinergic neurons (Table 1). If we select neurons 20 μm or larger in soma diameter, we find that 83% (nucleus basalis) and 71% (medial septal and diagonal band nuclei) of these neurons in our culture were ChoAcTase-positive (not shown in Table 1). Similarly, 73% (nucleus basalis) and 74% (medial septal and diagonal band nuclei) of neurons 20 μm or larger were AcChoEase ++ (not shown in Table 1). The mean size of ChoAcTase-positive neurons from nucleus basalis (23 μm in diameter) is larger than that from the medial septum and the diagonal band (19 μm) (Table 1). These morphometric data of our culture agree, by and large, with those of the basal forebrain ChoAcTase-positive neurons in the *in situ* preparations (2–4).

We can conveniently classify the cultured cholinergic neurons into three types: multipolar, bipolar, and triangular

Table 1. ChoAcTase and AcChoEase stain of neuron-like cells

Stain	Nucleus basalis		Medial septal and diagonal band nuclei	
	Frequency, %	Diameter, μm	Frequency, %	Diameter, μm
ChoAcTase				
+	29	23.0 ± 4.4	19	19.2 ± 3.2
−	71	14.9 ± 3.4	81	13.6 ± 2.9
AcChoEase				
++	23	27.0 ± 4.0	16	20.7 ± 3.8
+	40	17.5 ± 3.2	53	15.8 ± 2.5
−	37	14.9 ± 2.3	31	13.9 ± 2.2

For each of the four groups, 263–310 neuron-like cells from three cultures (14- to 23-day culture) were measured. Diameter was defined as a geometrical mean ($Mm^{1/2}$) of the major (M) and minor (m) soma diameters; values are presented as mean ± SD.

cells. Multipolar neurons had stellate-shaped, ovoid-shaped, or sometimes fusiform somata with more than three processes emanating from the soma (Fig. 1 *D* and *E*). Bipolar neurons had spindle-shaped or spherical perikarya with processes arising from approximately opposite poles of the soma (Fig. 1*B*). Triangular neurons (pyramidal-like) had a triangular somata and an apical dendrite from one apex with a few processes emerging from the base (Fig. 1*F*). On the average, multipolar and triangular cells were larger than bipolar cells (24 μm vs. 20 μm in the nucleus basalis). Among the cholinergic neurons, multipolar neurons predominated (69%) in the nucleus basalis culture, whereas both multipolar and bipolar cells were encountered with roughly the same frequency (41% vs. 48%) in the medial septal and diagonal band cultures. A similar distribution of these different classes of cells has been reported in the *in situ* brain preparation (3).

Membrane Properties. We performed electrophysiological recordings on the large neurons (diameter range, 22–38 μm). Since the morphological data (Table 1) indicate that about 75% of large neuron-like cells (≥20 μm) are ChoAcTase-positive, we can presume that our physiological data were obtained, with a high probability, from cholinergic neurons. In 13 cells, we processed for AcChoEase staining after physiological data were obtained. Twelve of the 13 cells

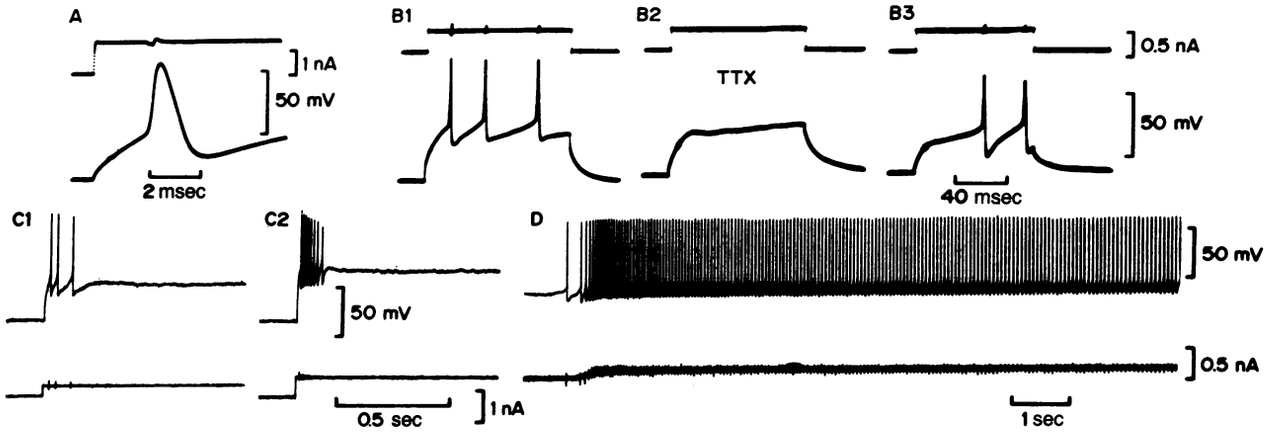

FIG. 2. (*A* and *B*) Action potentials recorded from cultured neurons of the nucleus basalis (*A*) and of the medial septal and diagonal band nuclei (*B*). Resting potential in *A* was −83 mV. *B*1 shows control spikes. In *B*2, the action potentials were abolished with tetrodotoxin (TTX, 3 μM). This effect was reversible, as seen in *B*3. Resting potential was −89 mV. Upper beams are the currents, and lower beams are the voltages (oscilloscope recordings). The overall frequency response is about 5 kHz (3 decibels down). (*C*) Trains of spikes (upper traces) elicited by stepwise constant currents (lower traces) in cultured neurons from medial septal and diagonal band nuclei. The spike trains adapted quickly at all current intensities. Resting potential was −89 mV. (*D*) Train of spikes (upper traces) elicited by depolarizing current (lower trace) in a cultured neuron from the locus coeruleus. Resting potential was −59 mV. In *C* and *D*, the spikes were recorded on an FM tape recorder and played back at slower speed into a pen recorder. The overall frequency response was about 1.3 kHz. In all of the records (*A–D*), the cells were under the current clamp mode by using the whole-cell patch clamp technique.

Table 2. Membrane properties of cultured neurons from basal forebrain nuclei

Technique	*n*	R_{in}, MΩ	V_r, mV	Ap-Amp, mV	Diameter, μm
Microelectrode					
N. basalis	15	80 ± 6	−78 ± 3	61 ± 2	29
Spt-Db	9	179 ± 48	−71 ± 3	57 ± 2	27
Whole-cell patch,					
N. basalis	7	159 ± 19	−88 ± 6	109 ± 7	27

Values are presented as mean ± SEM. In the case of microelectrode results, only the data for the cells with action potential amplitude ≥50 mV and the magnitude of threshold depolarization ≤38 mV were included. N. basalis, nucleus basalis of Meynert; Spt-Db, medial septal and diagonal band nuclei; R_{in}, input resistance; V_r, resting potential, maximal magnitude for each cell was averaged; Ap-Amp, amplitude of action potential. Patch pipette solution (for whole-cell patch clamp data) is given in text, and the values of resting potential were corrected for a liquid junction potential between the pipette solution and the Krebs solution (9 mV; see ref. 18).

produced AcChoEase positivity, a result that corroborates the above presumption.

Fig. 2 *A–C* show examples of action potentials elicited by depolarizing current pulses, and Table 2 summarizes the data of active and passive membrane properties. The principal ionic currents for the action potential seem to be carried by sodium, since the action potential was abolished reversibly by tetrodotoxin (3 μM) (Fig. 2, *B*1–*B*3). The neurons usually did not produce spontaneous firing of action potentials. When the cell was depolarized by steady current steps of various magnitudes, repetitive spikes were initially elicited, but they adapted quickly and the neuron subsequently ceased to fire within 500 msec (Fig. 2*C*). This fast spike adaptation of cholinergic neurons in culture is in sharp contrast with the property of noradrenergic neurons from locus coeruleus in dissociated culture (8). In the case of the noradrenergic neurons, spike trains (either spontaneously occurring or induced by steady depolarizations) lasted indefinitely without marked adaptation (Fig. 2*D*).

Rarely, we encountered cholinergic neurons (5 of 61 cells) that were producing spontaneous spike activities. Perhaps, these activities did not originate from these neurons but were driven by presynaptic elements, since these rare neurons usually (4 of 5) appeared to be contacted by other neurons, and in 3 of 5 cells spontaneous small electrical activities, resembling post-synaptic potentials, were recorded.

L-Glutamic Acid and Substance P. Drug effects were tested on cultured neurons by using the intracellular microelectrode. L-Glutamic acid (50 μM) always (6 cells) produced fairly quick depolarization (Fig. 3), in agreement with the case of many other excitable tissues (19, 20). Effects of substance P (3 μM) were tested in 20 cells. Of these, 6 cells

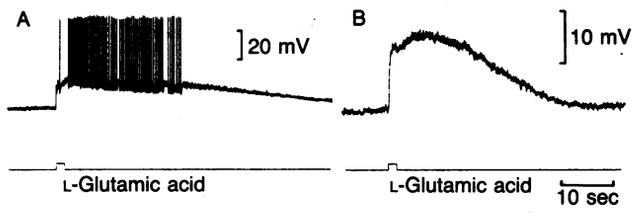

FIG. 3. Effect of L-glutamic acid on a cultured neuron from the nucleus basalis. Upper traces are membrane potential changes recorded with an intracellular microelectrode. Lower traces indicate the opening of the drug-ejection valve: one puff consisted of an ejection of L-glutamic acid (50 μM). Resting potential was −80 mV in *A* and −69 mV in *B*. The overall frequency response was 320 Hz in *A* and 200 Hz in *B*.

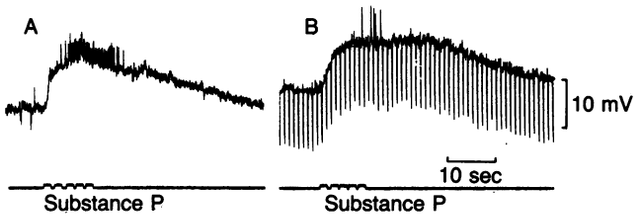

FIG. 4. Effect of substance P on a cultured cell from the medial septal and diagonal band nuclei. Intracellular microelectrode recordings. Substance P (3 μM) was applied by ejection. In *B* membrane resistance changes were intermittently measured by sending hyperpolarizing square-wave current pulses of 0.04 nA. Resting potential was −78 mV. The frequency response of the record was 200 Hz.

responded with depolarization (Fig. 4), 7 cells responded predominantly with hyperpolarization, and 7 cells did not produce noticeable effects. The membrane resistance slightly increased during the substance P depolarization (Fig. 4*B*).

One peculiar phenomenon that we noticed was that sometimes drug-induced depolarizations produced long-lasting repetitive spikes (Fig. 3*A* is an example), despite the fact that these neurons did not produce long-lasting trains of spikes when depolarized by steady electrical currents (described above). Perhaps the adaptation behavior of these neurons is changed by these drugs, as in the case of the acetylcholine effect on hippocampus neurons (21).

In addition, we have tested effects of the following peptides: [D-Ala^2,D-Leu^5]enkephalin, β-endorphin, somatostatin, dynorphin, cholecystokinin, vasopressin, and thyrotropin-releasing hormone. These agents either produced no effects or some capricious effects. At any rate, the number of data is still too small to warrant a discussion at this stage.

DISCUSSION

Features of Our Culture. Recently, there have been reports on organotypic cultures or dissociated cultures of basal forebrain nuclei (22–25). However, these cultures were all from the septal areas, not from the nucleus basalis; furthermore, these cultures have not been used for electrophysiological experiments of the cholinergic neurons. Our cultures are dissociated cell culture from both nucleus basalis and medial septal areas, and we studied them electrophysiologically as well as morphologically. The salient features of our method, originally developed for culturing locus coeruleus neurons by Masuko *et al.* (8), are (*i*) to make vibratome-sectioned brain slices (which give good visibility) and (*ii*) to obtain tissues of any desired regions under the dissecting microscope. This method is particularly useful for making cultures of nucleus basalis of Meynert, which, being situated deep inside the brain, is almost impossible to locate without good visibility. Our culture method gave very good yields of cholinergic neurons [see Hefti *et al.* (22)], probably because the good visibility of thin brain slices minimizes contamination from other regions. The cholinergic neurons of our culture are functionally alive and amenable to physiological experiments. We feel that this dissociated culture will become a useful model system for investigating the cholinergic neurons of the basal forebrain nuclei.

Physiological Properties. Several electrophysiological studies on basal forebrain nuclei have used *in situ* brain preparations (26–28). The main focus of these studies has been to investigate changes of spiking patterns recorded extracellularly in relationship with animal behavior, drug application, etc. Thus, cellular physiological properties of these cholinergic neurons have been unknown.

One important physiological characteristic of our cholinergic neurons is that almost all of them did not generate spontaneous repetitive firing. Steady depolarizing currents produced repetitive firing, but this adapted quickly. On the other hand, noradrenergic neurons from locus coeruleus cultures produced spontaneous trains of spikes indefinitely either with or without depolarizing steady currents. When preparing these two different kinds of culture, we used the same methods and conditions. Thus, this difference of spike adaptation probably reflects innate differences in the character of each class of neuron.

Several investigators have reported that almost all or at least some of the basal forebrain neurons, which project to neocortex or hippocampus, fire spontaneously in *in situ* brain preparations (26–28). In view of the present result that the cholinergic neurons do not produce repetitive firing, it is quite possible that the spontaneous firing of basal forebrain nuclei recorded in the *in situ* preparation is driven from presynaptic neurons rather than originating from the basal forebrain nuclei themselves. In the case of spontaneous firing of the locus coeruleus neurons, it is likely that these noradrenergic neurons themselves act as pacemakers for the spontaneous firing. Since it is always dangerous to reach conclusions regarding the cellular behavior under physiological conditions based solely upon observations of cultured materials, it is necessary to test these ideas by using more physiological preparations.

Drug Application. Our results show that substance P produced either depolarizing or hyperpolarizing responses. The hyperpolarization effect could be an aberrant response caused by unknown conditions. In our more recent series of experiments on these cholinergic neurons, using the patch clamp technique, we have consistently obtained excitatory effects by substance P (29). Whatever the cause of this unexplained discrepancy between the present microelectrode data and the more recent patch clamp data, the depolarizing response is in agreement with the excitatory effects of substance P reported by many investigators on several materials (28, 30–35).

We observed that substance P increased membrane resistance. Furthermore, we found that in the locus coeruleus experiments (using the microelectrode technique; ref. 8), substance P produced a depolarizing effect concomitant with an increase in membrane resistance, and this depolarizing response reversed its sign at a hyperpolarized level (unpublished data). The results of these microelectrode studies do not lead us to any conclusions about the ionic mechanisms of the substance P action. Nevertheless, all of these data are at least consistent with the more recent findings, using the whole-cell patch clamp, that substance P produces excitatory effects on these cholinergic neurons by inhibiting the inward rectification channels (29).

The effect of substance P is of particular interest, since substance P receptors have been found in the basal forebrain nuclei (36). In addition, the substance P content seems to be decreased in the brain of Alzheimer patients (37). It may, therefore, be reasonable to suggest further experiments that may lead to the clinical application of substance P.

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