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Enhanced acetylcholine secretion in neuroblastoma × glioma hybrid NG108–15 cells transfected with rat choline acetyltransferase cDNA

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Neuroblastoma × glioma hybrid NG108–15 cells and mouse neuroblastoma N18TG-2 and N1E-115 cells were transiently transfected with the sense cDNA coding for rat choline acetyltransferase (ChAT). All transfected cell lines showed a high level of ChAT activity. ACh secretion was monitored by recording miniature end-plate potentials (MEPPs) in striated muscle cells that had been co-cultured with transfected cells. The number of muscle cells with synaptic responses and the MEPP frequency were higher in co-culture with transfected NG108–15 cells than with control or mock cells. No synaptic response was detected in muscle cells co-cultured with transfected N18TG-2 or N1E-115 cells. The results show that ACh secretion into the synaptic cleft was enhanced due to ChAT overexpression in NG108–15 hybrid cells but not in neuroblastoma cells.

Acetylcholine synthesis; Overexpression; Synapse formation; End-plate potential; NG108–15 cell

1. INTRODUCTION

Cholinergic neurons play an important role in muscle contraction, in learning and memory. Choline acetyltransferase (ChAT; EC 2.3.1.6) is the enzyme that is responsible for the synthesis of acetylcholine (ACh) and is a specific marker for cholinergic neurons. Full-length complementary DNAs (cDNAs) encoding ChAT from various species have been cloned and sequenced [1–4]. These cDNAs were used to express ChAT in oocytes [2,4], CHO cells [4], and neuroblastoma cells [4]. However, it has never been examined whether or not the ACh synthesized from cDNA-expressed ChAT can be utilized for secretion from transformed cells.

Neuroblastoma cell lines are the well established model for cholinergic neurons. Neuroblastoma × glioma hybrid NG108–15 cells possess an endogenous ChAT activity and can secrete ACh [6,7], while little or no ChAT activity has been reported in neuroblastoma N18TG-2 or N1E-115 cells [8,9]. Thus, they are suitable as a recipient of ChAT cDNA to examine the fate of ACh newly synthesized from exogenous ChAT in transfected cells. For monitoring the ACh output from neuroblastoma or hybrid cells, we cultured them with striated muscle cells, as a sensitive bioassay system [10,11]. We recorded depolarizing synaptic potentials due to stimulation of nicotinic ACh receptors in myotubes in response to released ACh. In this report, we show the net increase in ACh secretion into the synaptic cleft by ChAT cDNA transfection in NG108–15 hybrid cells but not in N18TG-2 and N1E-115 neuroblastoma cells.

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Abbreviations: ChAT, choline acetyltransferase; ACh, acetylcholine; cDNA, complementary DNA; MEPPs, miniature end-plate potentials; DMEM, Dulbecco's modified Eagle's medium.

2. MATERIALS AND METHODS

2.1. *Cell culture*

Mouse neuroblastoma N18TG-2 and N1E-115 cells were maintained in Dulbecco's modified Eagle's medium (DMEM, Gibco, Grand Island, NY) supplemented with 5% fetal calf serum, as described previously [8]. Hybrid NG108–15 cells, derived from mouse neuroblastoma N18TG-2 and rat glioma C6BU-1 cells, were maintained in DMEM supplemented with 5% fetal calf serum, 100 μM hypoxanthine, 0.1 μM aminopterin and 16 μM thymidine, as described previously [12].

2.2. *Transient transfection of rat ChAT cDNA*

The expression plasmid pcDL1-ChAT which contains a 2.1 kb fragment for the entire protein-coding sequence of rat ChAT was described previously by Ishii et al. [4]. 1.5×10^6 Neuroblastoma or hybrid cells were plated in three 60-mm dishes. Twenty-four hours later, the cells were transfected with 10 μg pcDL1-ChAT using 25 μg lipofectin (Bethesda Research Laboratories Life Technologies, Inc, Gaithersburg, MD), according to the manufacturer's protocol (see also Felgner et al. [13]). One day after the transfection, one-third of the cells were harvested and co-cultured with rat myotubes as described below for synapse formation experiments. The rest of the transfected cells were incubated for 2 and 3 days and used for ChAT activity assays.

2.3. *ChAT assay*

ChAT activity was measured by a slight modification [4] of the method described by Fonnum [14]. The following is a brief description. Cultured cells were detached by incubation for 5 min in Dul-

becco's Ca^{2+}, Mg^{2+}-free phosphate-buffered saline and collected by centrifugation for 10 min at 1 000 × g. The cells were suspended in 25 mM potassium phosphate buffer, sonicated on ice, and centrifuged for 10 min at 20 000 × g. The supernatant of the cell homogenate was incubated in the presence of ^{14}C-acetyl Co-A (Amersham, 10 mCi/mmol) for 30 min at 37°C. The incubation was terminated by the addition of acetonitrile containing 5 mg/ml tetraphenyl boron, and then toluene was added. After centrifugation, the organic layer was transferred to a scintillation vial and the amount of [^{14}C]acetylcholine formed was determined by liquid scintillation counting.

The protein content of the cultures was determined by the method of Bradford [15].

2.4. Synapse assay in co-culture dishes

Hindlimb muscle cells were isolated by trypsinization from newborn rats and cultured in DMEM supplemented with 10% horse serum with or without 10% fetal calf serum until forming fused myotubes for 7 days as described previously [10]. Neuroblastoma or hybrid cells that had been transfected with cDNA were plated at a density of 2×10^4 cells/35-mm dish on the already fused muscle cells. The co-culture was maintained for 1–3 days in a medium composed of 90% DMEM, 10% horse serum (Gibco), 10 μM prostaglandin E_1, 1 mM theophylline, 100 μM hypoxanthine and 16 μM thymidine. Synaptic activity was studied electrophysiologically by recording membrane potential of a myotube as described previously [11]. The presence of MEPPs recorded in a myotube at a frequency of 2 events/min was recognized as a synaptic connection-positive pair, according to the previous definition [6,11].

3. RESULTS

3.1. ACh secretion in transfected NG108–15 cells

Neuroblastoma × glioma hybrid NG108–15 cells were transfected by lipofectin with rat ChAT cDNA which had been inserted into a pcDL1 vector in the sense direction [4]. The transfected cells were harvested after 2 and 3 days of culture. ChAT activity in transfected NG108–15 cells at days 2 and 3 was 401 ± 5 (mean ± S.E.M., $n = 5$) and 445 ± 27 ($n = 3$) pmol/min per mg protein, respectively (Fig. 1). The activity was 2.3- to 2.9-fold higher than the endogenous level of NG108–15 cells (152 ± 13, $n = 45$) ($P < 0.001$), or the level of mock cells transfected with the pcDL1 vector alone (164 ± 6, $n = 5$) ($P < 0.001$). These control values are close to that previously reported [6,7]. The results accord well with those of the previous transfection experiments performed with the same cDNA by $CaPO_4$ in NG108–15 cells [4].

When membrane potentials were measured from rat myotubes which had been co-cultured with control, mock-transfected or ChAT-cDNA-transfected NG108–15 cells for 1–3 days, small depolarizing synaptic potentials were recorded as shown in Fig. 2A and C. Transient depolarizing shifts in the muscle membrane potential were inhibited by 0.1 mM d-tubocurarine, the inhibitor of nicotinic ACh receptors (n=3 for each hybrid cell). This suggests that muscle responses are miniature end-plate potentials (MEPPs) that are nicotinic ACh receptor potentials which are elicited by ACh released from the hybrid cells into the synaptic cleft of the hybrid-myotube pairs. MEPPs recorded from muscle cells co-cultured with both control and transfected hybrid cells were generally small, the majority being < 1 mV.

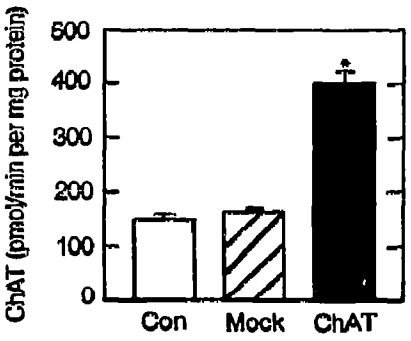

Fig. 1. Expression of ChAT cDNA. ChAT activity was measured in homogenates of non-transfected (con) NG108–15 cells or NG108–15 cells which had been transfected with pcDL1 (mock) or pcDL1-ChAT cDNAs. They were cultured for 78 hrs. Values are the mean ± S.E.M. ($n = 5$). *Significantly different from control and mock at $P < 0.001$ (t-test).

But occasionally large potentials of up to 3–6 mV were concomitantly recorded. Since these potentials showed a smooth rise with a rise time of 2–8 ms and lasted for 5–30 ms as reported previously [6], one muscle depolarizing response may represent one event of vesicular release of ACh from NG108–15 cells.

Synapses at days 1, 2 and 3 were detected in 76, 82 and 75% of muscle cells that had been co-cultured with ChAT-cDNA-transfected NG108–15 cells (Fig. 3A). The percentage of muscle cells with MEPPs co-cultured with non-transfected NG108–15 cells was one-third to

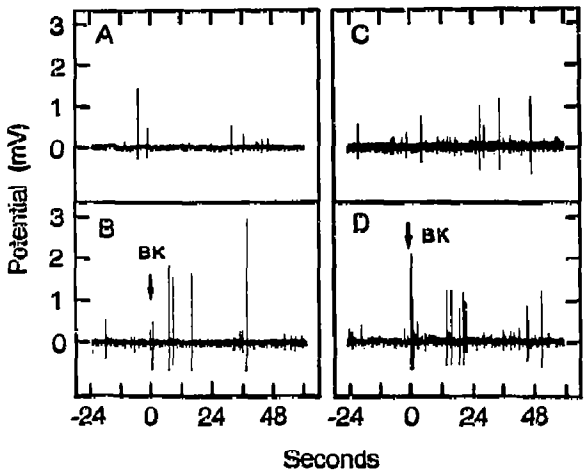

Fig. 2. Examples of MEPPs and bradykinin (BK)-dependent increase in frequency of MEPPs recorded from myotubes co-cultured with control NG108–15 cells (A,B) or ChAT-cDNA transfected NG108–15 cells (C,D) for 2 days. Traces represent high-gain AC-coupled recordings of membrane potentials of the muscle cells. Upward deflections indicate depolarizing synaptic responses due to ACh released from hybrid cells. A and C are typical voltage traces of MEPPs recorded for 84 s from two myotubes co-cultured with control or transfected NG108–15 cells, respectively. MEPP frequency is higher in C than A. Traces B and D show facilitation of ACh secretion induced by the application of BK. BK (3 ml, 10 mM) was applied on the surface of the recording medium at the times indicated by arrows

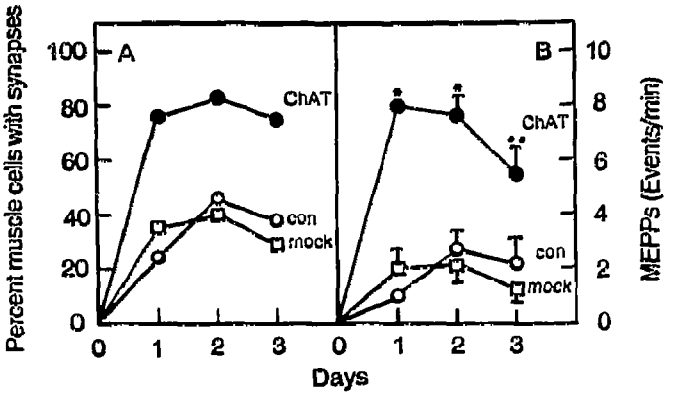

Fig. 3. Rate of synapse formation (A) and mean number of spontaneous synaptic responses in muscle cells (B). Control (○) and mock (□) NG108–15 cells or ChAT-cDNA transfected NG108–15 cells (●) were cultured with myotubes for the indicated time. More than 2 MEPPs/min were accounted for synapse-positive muscle cells. Each point in A was calculated from 12–82 recordings (mean 42) in 34 identical experiments. Each point in B is the mean frequency of MEPPs in all muscle cells. Error bars indicate S.E.M. **Significantly different from both control at $P < 0.001$ (t-test). *Significantly different from mock ($P < 0.05$) but not from a non-transfected control.

one half (24, 46 and 38% at days 1, 2 and 3). The time course of synapse formation by mock NG108–15 cells was similar to that by the control cells.

The mean synaptic event in myotubes co-cultured with control or mock NG108–15 cells at day 1 was 1.0 ± 0.2 ($n = 76$) or 2.0 ± 0.7 ($n = 55$) MEPPs/min, respectively, while that with ChAT-cDNA transfected NG108–15 was 8.0 ± 1.3 ($n = 58$) (Fig. 3B). The increase in MEPP frequency in ChAT-transfected cells at days 1 and 2 is significant ($P < 0.001$), though the difference in MEPP frequency became small at day 3. Therefore, these results show that a much higher amount of ACh was secreted into the synaptic cleft by the ChAT-cDNA-transfected hybrid cells than by the control cells.

To test the possibility that ACh secretion can be evoked after stimulation, we used bradykinin which has been shown to facilitate ACh release, as a result of the activation of the phosphoinositide metabolism in NG108–15 cells [16]. Bradykinin applied to transfected NG108–15 cells produced a characteristic MEPP facilitation, as shown in Fig. 2D. The number of MEPPs evoked by bradykinin was higher in transfected NG108–15 cells than in control cells (Fig. 2B and D; Table I). However, the increase in MEPP frequency after bradykinin application to transfected NG108–15 cells was 1.8-fold, which is lower than in control cells (2.9-fold).

Table I

Bradykinin-induced facilitation of ACh release in control or ChAT-cDNA transfected NG108-15 cells

	Mean MEPP frequency obtained before and after bradykinin stimulation (events/min)			
	Before (a)	After (b)	b/a	(n)
NG108-15	2.3 ± 0.3	6.7 ± 1.8	2.9	(3)
Transfected NG108-15	13 ± 3.6	24 ± 2.6	1.8	(4)

Synapses were recorded 25–30 hrs after starting co-culture with either control or transfected NG108-15 cells. Mean MEPP frequency was calculated from events recorded for 5 min before bradykinin stimulation and for 1 min after bradykinin stimulation. Values are the mean ± S.E.M.

3.2. *ACh release in transfected N18TG-2 and N1E-115 cells*

Using the same protocol for NG108–15 cells, ChAT cDNA was transfected on N18TG-2 and N1E-115 neuroblastoma cells, in order to examine if the cholinergic phenotype including ACh secretion can be acquired by a single transfection of ChAT cDNA. Transfected N18TG-2 and N1E-115 cells possessed ChAT activity at 275 ± 9 ($n = 3$) and 197 ± 19 ($n = 4$) pmol/min per mg protein at day 2, respectively. The activity was 15- to 20-fold higher than that of each control cell ($P < 0.001$).

Although synapses were carefully examined, no synaptic response was recorded at all from 80 myotubes co-cultured with the transfected N18TG-2 and N1E-115 cells. Focal application of a depolarizing agent, KCl (3 ml, 150 mM), on the surface of the recording medium above the cell did not produce any MEPPs in myotubes co-cultured with them (data not shown). Also no response in muscle cells was evoked upon similar applications of bradykinin (3 ml, 10 mM) on transfected N1E-115 cells that had been reported to possess a bradykinin response [17]. The results suggest that transfected N18TG-2 and N1E-115 cells do not discretely secrete ACh into the synaptic cleft, as a electrophysiologically detectable fashion.

ChAT activity, ACh secretion and synapse formation of neuroblastoma cells are intracellular cyclic AMP-dependent processes [6] and also are affected by other factors [18,19], which develop slowly during differentiation. To verify the point at whicht N18TG-2 and N1E-115 cells were not sufficiently differentiated after such a short exposure to the differentiation agent, synapse formation was examined with pre-treated neuroblastoma cells. However, no synaptic activity was detected from myotubes co-cultured for 2 days even with ChAT cDNA-transfected N18TG-2 and N1E-115 cells that had been treated prior to the co-culture with 10 mM prostaglandin E_1 and 1 mM theophylline or 0.25 mM dibutyryl cyclic AMP for 3 days.

4. DISCUSSION

This study shows that ACh synthesized from exogenous ChAT can be used for secretion in NG108–15

hybrid cells but not in two neuroblastoma cells. While ChAT activity and ACh secretion have been reported in control NG108–15 hybrid cells, ACh secretion has never been tested previously in ChAT-defective neuroblastoma N18TG-2 and N1E-115 cells [6,8,9]. Since the ChAT level was significantly higher in all three cell lines transfected with ChAT cDNA, we assume that more ACh may be synthesized by exogenous ChAT within these cells. However, enhanced secretion of ACh is observed only in NG108–15 cells that have been equipped with the secretory process. Although electrophysiological detection of MEPPs is a highly sensitive method for ACh secreted into the synaptic cleft in a discrete fashion (vesicular release). it can not monitor all types of release. Therefore we cannot neglect the possibilities that transfecting N18TG-2 or N1E-115 cells results in the leakage of ACh into the medium or the release of ACh from other areas than the synaptic cleft.

We initially investigated whether ChAT-cDNA transfection can reconstitute ACh secretion in ChAT-defective cell lines by using their intrinsic secretory processes, if they possess. The absense of ACh release in transfected N18TG-2 and N1E-115 cells revealed that they may lack the appropriate synaptic secretion processes. The two neuroblastoma cells could then be newly included in the entity of a group of neuroblastoma or hybrid cells which are mutant cell lines that have defects in synapse formation [6].

Although we have not measured the ACh content in NG108–15 cells, judging from the net increase in the synapse formation ratio and synaptic response that are due to transfection of ChAT cDNA, the amount of releasable ACh seems to be increased. Bradykinin-induced facilitation was similarly observed in transfected hybrid cells as control cells. This may suggest that the increased ACh content in the transfected NG108–15 cells seems to facilitate ACh secretion by increasing the chance of release rather than by changing the (quantal) content of ACh in each event. However, further validation of this point is necessary.

The simplest and most probable explanation for the mechanism underlying the increased number of muscle cells with synaptic responses may reside in the increased number of secretion-ready vesicles which are filled up by uptake of ACh in transfected NG108–15 cells, because of the increased ACh concentration, which subsequently causes the detection of abundant synaptic potentials in many muscle cells.

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