Regulation of acetylcholine release from neuroblastoma × glioma hybrid cells

(synapse formation/dibutyryl cyclic AMP/neuron development)

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ABSTRACT Neuroblastoma × glioma NG108-15 hybrid cells exposed to N6,O2'-dibutyryladenosine 3':5'-cyclic monophosphate for several days release [3H]acetylcholine in response to serotonin, prostaglandin E2, KCl, or veratridine. NG108-15 cells grown in the absence of dibutyryl cyclic AMP do not respond to an excitatory stimulus by releasing [3H]acetylcholine but can be shifted to a responsive state by treatment with dibutyryl cyclic AMP. Thus, the reactions that are required for acetylcholine release can be regulated in NG108-15 cells, thereby regulating the ability of cells to form synapses and the efficiency of synaptic communication.

Neuroblastoma × glioma NG108-15 hybrid cells form synapses with cultured striated muscle cells (1-4). Synaptogenesis by NG108-15 cells is greatly increased when hybrid cells are treated for a period of days with N6,O2'-dibutyryladenosine 3':5'-cyclic monophosphate (B2cAMP). Culture of NG108-15 cells with B2cAMP also results in increases in cell body diameter, neurite extension, abundance of clear vesicles 600 Å in diameter (5), membrane excitability, and specific activities of choline acetyltransferase (acetyl-CoA:choline O-acetyltransferase, EC 2.3.1.6) and acetylcholinesterase (acetylcholine hydrolase, EC 3.1.1.7). The cells generate both Na+ and Ca2+ action potentials and have endorphin receptors, excitatory muscarinic acetylcholine (ACh) receptors, a-adrenergic receptors, prostaglandin E1 (PGE1) receptors, PGF2α receptors, and adenosine receptors that are coupled to shifts in cyclic AMP and/or cyclic GMP levels of the cells (6-14). Serotonin (13), ACh (13), PGF2α (13), or dopamine (14) can depolarize the cells and initiate action potentials.

In this report, we describe the properties of stimulus-dependent release of [3H]ACh from NG108-15 cells, a response required for synaptic communication, and show that the ability of the cells to release [3H]ACh in response to stimulation can be regulated by B2cAMP. A preliminary report of this work has appeared (15).

MATERIALS AND METHODS

Cell Culture. The culturing of NG108-15 hybrid cells has been described (1). To switch cells to a more differentiated state, growth medium (90% Dulbecco's modified Eagle's minimum essential medium (DMEM) (GIBCO H-21)/10% fetal bovine serum/100 μM hypoxanthine/1 μM aminopterin/16 μM thymidine) was supplemented with 1 mM B2cAMP, purified as described (1), and the fetal bovine serum concentration was decreased from 10% to 5%. Cells to be used for experiments were dissociated and transferred to 200-μl disposable glass capillary pipettes (total volume, 300 μl) bent in the form of a "U" and connected in series (1.5–2.0 × 105 cells per capillary) and incubated for 3 hr at 37°C to promote cell attachment to the glass. The tubes then were perfused with 2 ml of medium per hr for 2 days unless indicated otherwise. Each U tube then contained 200–400 μg of cell protein.

Measurement of [3H]Choline Uptake and [3H]ACh Release. Cells in capillaries were washed for 3 min by perfusion (0.4 ml/min) with medium A (DMEM without choline, NaHCO3, phenol red, and fetal bovine serum but with 15 mM N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid (Hepes) and 125 mM NaCl, adjusted to pH 7.4 and 340 mOsm/liter). Cells then were incubated without perfusion in medium A supplemented with [methyle-3H]choline (10.1 Ci/mmol, Amersham/Searle) as indicated in figure legends. For cells grown in the presence of B2cAMP, 1 mM B2cAMP also was added to the [3H]choline uptake medium but not to the wash medium. After incubation, most of the [3H]choline in the medium was removed by perfusion with medium B (medium A with 20 μM eserine sulfate). When only intracellular [3H]choline, [3H]ACh, and other [3H]-labeled metabolites of choline were to be determined, the tubes were perfused for 1.5 min (0.7 ml/min) with medium A, a small air bubble was introduced into the line, and the capillary was filled with acetone/1 M formic acid, 85:15 (vol/vol), to precipitate protein and extract [3H]-labeled compounds from cells (16). The acetone/formic acid extract was collected and each tube was washed twice with 100 μl of the acetone/formic acid solution, the extracts and washes then were combined. Greater than 98% of the intracellular [3H]-labeled compounds were recovered. Cell protein in each capillary then was dissolved in 0.4 M NaOH and assayed by a modification of the method of Lowry et al. (17) with crystalline bovine serum albumin as the standard.

The [3H]-labeled compounds extracted from cells were fractionated by high-voltage paper electrophoresis with 1.5 M acetic acid.
Compounds in each fraction were eluted from the paper with paper was treated with 12 vapor to visualize spots, 3H-labeled acid/O.75 M formic acid (18). ACh, choline, phospholipids, and identified by coelectrophoresis with authentic standards. The fraction containing phosphorylcholine and CDP-choline were using a liquid scintillation spectrometer.

Indicated amounts of [3H]ACh (Left) or [3H]choline (Right) (both 10.1 Ci/mmol) were incubated in the absence or presence of 2.5 munits of NaATP, 10 mM MgCl2, and 2.5 munits of choline kinase (ap-

The [3H]ACh released from cells was characterized further by determining the sensitivity of the [3H]-labeled material released by hydrolysis catalyzed by acetylcholinesterase (Table 1). After incubation in the presence of acetylcholinesterase, the [3H]-labeled material recovered in the organic phase was <5% of the amount obtained in the absence of acetylcholinesterase. In experiments not shown here, >97% of the [3H]-labeled compounds released from cells exhibited the electrophoretic mobilities of ACh or choline.

Veratridine, which activates action potential Na+ ionophores, also stimulated [3H]ACh release from NG108-15 cells grown in the presence of BtzcAMP but had little or no effect on cells grown without BtzcAMP (Fig. 3A). The response to veratridine decreased with time. Veratridine-stimulated ACh release was abolished in the presence of 1 μM tetrodotoxin, a specific inhibitor of action potential Na+ ionophore activation, but tetrodotoxin had no effect on the basal, unstimulated rate of [3H]ACh release. Veratridine increased the rate of [3H]choline release, after a delay, from cells grown with or without

RESULTS

Release of [3H]ACh from Cells Grown with or without BtzcAMP. NG108-15 cells incubated with [3H]choline release both [3H]ACh and [3H]choline into the medium. The release of [3H]ACh and [3H]choline into the medium from NG108-15 cells grown with or without BtzcAMP is shown in Fig. 2. NG108-15 cells grown without BtzcAMP released [3H]ACh into the medium but 80 mM K+ had little or no effect on the rate of release. In contrast, the rates of [3H]ACh release from cells grown in the presence of 1 mM BtzcAMP increased in response to KCl. The basal unstimulated rates of [3H]ACh release from cells treated with BtzcAMP were twice those of control cells. Both basal and KCl-stimulated rates of [3H]ACh release increased throughout the 5 days of treatment with BtzcAMP. These results show that populations of NG108-15 cells can be shifted from an unresponsive to a responsive state with respect to KCl-dependent ACh release by exposure of cells to BtzcAMP.
and B) and of [3H]choline release (C and D). A cell suspension (approximately 200 µg of cell protein) was added to each capillary and the tubes then were perfused with DMEM supplemented with 5% fetal bovine serum for 24 hr. Some tubes then were perfused with the above medium supplemented with 1 mM BtzcAMP. One (0), 3 (O), or 5 (A) days later, the release of [3H]ACh and [3H]choline from cells was determined. Cells were incubated with 10 nM [3H]choline for 45 min and washed for 10 min with medium without choline but with 20 µM eserine sulfate (wash discarded), and then fractions were collected at 5-min intervals (0.8 ml per fraction). Stimulation was with 80 mM KC1 for 6 min as indicated. Each point represents the mean of four values obtained with separate cultures. Protein ranged from 200 µg to 550 µg on the fifth day with or without BtzcAMP.

BtzcAMP but tetrodotoxin did not inhibit veratridine-stimulated [3H]choline release. This suggests that the increase in choline release due to veratridine is not mediated by the activation of action potential Na+ ionophores.

Choline Metabolism. The effect of growing cells in the presence of BtzcAMP for 0, 9, or 16 days on [3H]choline metabolism is shown in Table 2. The intracellular concentrations of [3H]ACh and [3H]choline increased and those of phospho-

rylated [3H]-labeled compounds derived from [3H]choline decreased as the time of exposure of cells to BtzcAMP was increased. After 16 days of exposure to BtzcAMP, NG108-15 cells had 2- and 5-fold higher levels of [3H]ACh and [3H]choline, respectively, than cells not exposed to BtzcAMP.

The results shown in Table 2 were obtained with NG108-15 cells that had been incubated with [3H]choline and then washed in the absence of extracellular choline for 36 min. The results shown in Fig. 4 were obtained with cells that had been incubated with [3H]choline and washed only 1.5 min prior to the extraction of [3H]-labeled compounds from cells. The intracellular level of [3H]choline increased rapidly during the first 10 min of incubation and then plateaued, whereas the amount of [3H]ACh in cells increased throughout the 60-min incubation period. [3H]Phosphorylcholine accumulated at a rapid linear rate for 60 min. After a short lag, [3H]phospholipids also accumulated rapidly, which suggests a precursor-product relationship between [3H]phosphorylcholine and [3H]phospholipids.

The relationship between extracellular choline concentration and uptake and metabolism of [3H]choline by NG108-15 cells is shown in Fig. 4 right. The accumulation of [3H]-labeled phosphorylated compounds derived from [3H]choline and of

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<th>Table 1. Effect of acetylcholinesterase on released [3H]ACh</th>
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NG108-15 cells grown for 9 days with 1 mM BtzcAMP were incubated with 10 µM [3H]choline for 45 min and washed by perfusion for 10 min. Six-minute fractions were collected before, during, and after stimulation with 80 mM KCl and perfusates from three cultures were then combined. Eserine was then extracted with CHCl3 and the [3H]ACh content of the perfusates was determined after incubation in the presence or absence of acetylcholinesterase (AChE). Each value is the mean of duplicate determinations.
NG108-15 cells. (Left) Cells that had been grown with 1 mM BtscAMP for 16 days were incubated with 10 μM [3H]choline for the times indicated and then washed for 1.5 min at a perfusion rate of 0.7 ml/min. The cells then were extracted with acetone/1 M formic acid, 85:15 (vol/vol), the [3H]-labeled compounds extracted were separated by high-voltage paper electrophoresis, extracted as for Left. PCh + PL, phosphorylcholine, CDP-choline, [3H]ACh, [3H]PH; phospholipids; choline, [3H]choline; ACh, [3H]choline. (Inset) Total uptake of [3H]choline by cells.

The results also showed that, as the extracellular choline concentration is increased, the levels of intracellular [3H]choline and [3H]ACh increase at approximately the same rate. The distribution of [3H]-labeled compounds accumulated by the cell remained constant between 0.5 and 250 μM extracellular [3H]choline concentrations tested (0.5-500 μM). Data analysis by the method of Lineweaver and Burk suggests that NG108-15 cells have both high- and low-affinity choline uptake mechanisms (not shown); however, further work is needed to define more accurately the properties of the choline uptake systems.

The results also showed that, as the extracellular choline concentration is increased, the levels of intracellular [3H]choline and [3H]ACh increase at approximately the same rate. The distribution of [3H]-labeled compounds accumulated by the cell remained constant between 0.5 and 250 μM extracellular [3H]choline. These results suggest that the synthesis of [3H]ACh is not preferentially coupled to the high- or low-affinity choline uptake systems.

Properties of [3H]ACh and [3H]Choline Release. KCl repetitively stimulated [3H]ACh release from NG108-15 cells perfused with control medium containing 1.8 mM Ca2+ and 0.8 mM Mg2+, but the second response to KCl was smaller than the first response (Fig. 5). Omission of Ca2+ and increasing Mg2+ to 4 mM abolished the KCl-dependent [3H]ACh release and also decreased the unstimulated rate of [3H]ACh release. Omission of Ca2+ and increasing Mg2+ partially decreased the KCl-stimulated release of [3H]choline from cells.

The relationship between KCl concentration and [3H]ACh release from cells is shown in Fig. 6 left. KCl at 30 mM stimulated the release of [3H]ACh from cells but not as effectively as 40 or 80 mM KCl. A second addition of KCl resulted in the release of approximately half the amount of [3H]ACh compared to the first response to KCl at each concentration of KCl tested. These results suggest that the decreased [3H]ACh release evoked by the second application of KCl may not be due to depletion of an intracellular pool of releasable [3H]ACh.

As shown in Fig. 6 right, 10 μM 5-hydroxytryptamine (5-HT)
regulated. Cells grown without Bt2cAMP do not respond to excitatory stimuli by releasing [3H]ACh but they can be shifted to a responsive state by treatment with Bt2cAMP. The response to stimulation slowly increases over a period of at least 5 days while cells are cultured with Bt2cAMP. Because ACh release in response to stimulation is required for synaptic communication, the ability of the cells to form synapses and the efficiency of synaptic communication can be regulated by factors that control stimulus-dependent ACh release. Exposure of NG108-15 cells to Bt2cAMP also results in an increase in cell body diameter, neurite length, number of clear vesicles, membrane excitability, and the specific activities of acetylcholinesterase and choline acetyltransferase (5-14). Thus, many reactions required for synapse formation are regulated, directly or indirectly, by Bt2cAMP.

The properties of evoked ACh release from NG108-15 cells treated with Bt2cAMP are similar to those of neurons. The amount of ACh release due to exposure of cells to KCl is a function of KCl concentration; 40-80 mM KCl evokes maximal ACh release. The response to KCl is abolished by removal of extracellular Ca2+ and elevation of Mg2+ from 0.8 to 4 mM. Veratridine-stimulated ACh release is completely inhibited by 1 μM tetrodotoxin, a specific inhibitor of action potential Na+ ionophore activation. Serotonin or PGF2α-stimulated ACh release ceases rapidly, possibly due to receptor desensitization, whereas veratridine- or KCl-dependent ACh release can be maintained for longer periods (4-8 min). In each case, however, only 1% or less of the intracellular [3H]ACh is released before cell responsiveness to the stimulating agent decreases or disappears.

If [3H]ACh is released into the medium from vesicles that contain approximately 10,000 molecules of ACh per vesicle, then one can estimate that ACh may be released from 10 vesicles per min per cell without stimulation and 15 vesicles per min per cell when stimulated by serotonin. Both basal and serotonin-stimulated rates of ACh release are compatible with muscle responses observed at synapses between NG108-15 cells and striated muscle cells (1-13), but response rates at different synapses vary greatly. Such data suggest that the NG108-15 cell population is heterogeneous with respect to the amount of ACh released and the responsiveness of cells to stimulation. Biochemical measurements of [3H]ACh represent the mean values for the population of cells.

The results suggest that ACh is released from NG108-15 by two mechanisms because Ca2+ is required for KCl-stimulated release of ACh but not for unstimulated release of ACh. KCl stimulates the release of both [3H]ACh and [3H]choline; however, these compounds seem to be released by different mechanisms.

About 15% of the [3H]choline taken up by NG108-15 cells is converted to [3H]ACh at each choline concentration tested between 0.5 and 250 μM. Thus, the synthesis of [3H]ACh is not preferentially coupled to high- or low-affinity choline uptake systems. In contrast, Yavin (20) has observed that an increased percentage of accumulated [3H]choline is converted to [3H]ACh by cultured rat embryo brain cells as the external concentration of [3H]choline is increased. The reasons for the differences between these two systems is unknown at present but both differ substantially from what has been observed with synaptosomes, in which 50-80% of the [3H]choline accumulated is converted to [3H]ACh (21). One major difference between choline metabolism in cultured cells and synaptosomes is that much of the choline probably is taken up by cell bodies rather than nerve terminals and is thus exposed to both acetylation and phosphorylation pathways; little if any phosphorylation of choline is observed with synaptosomes (21).

The results show that stimulus-dependent [3H]ACh release from NG108-15 cells is acquired slowly by cells over a period of at least 5 days while cells are exposed to Bt2cAMP. The slow increase in cell responsiveness to stimuli suggests that one or more components required for ACh release may be formed during this time. Whether regulation of stimulus-dependent ACh release by Bt2cAMP is mediated by cyclic AMP is not known; however, if the process is regulated by cyclic AMP, neurotransmitters, hormones, or other molecules coupled to the activation or inhibition of adenylate cyclase might then regulate both the ability of a neuron to form functional synapses and the efficiency of communication across the synapse.