Proc. Natl. Acad. Sci. USA
Vol. 75, No. 3, pp. 1314–1318, March 1978
Biochemistry

Regulation of acetylcholine release from neuroblastoma × glioma hybrid cells

(synapse formation/dibutyryl cyclic AMP/neuron development)

RICHARD MCGEE[ab], PAUL SIMPSON[ac], CLIFFORD CHRISTIAN[d], MARINA MATA[d], PHILLIP NELSON[d], AND MARSHALL NIRENBERG[a]

[a] Laboratory of Biochemical Genetics, National Heart, Lung, and Blood Institute, and [d]Behavioral Biology Branch, National Institute of Child Health and Human Development, National Institutes of Health, Bethesda, Maryland 20014

Contributed by Marshall Nirenberg, January 10, 1978

ABSTRACT **Neuroblastoma × glioma NG108-15 hybrid cells exposed to $N^6,O^{2'}$-dibutyryladenosine 3′:5′-cyclic monophosphate for several days release [^{3}H]acetylcholine in response to serotonin, prostaglandin $F_{2\alpha}$, KCl, or veratridine. NG108-15 cells grown in the absence of dibutyryl cyclic AMP do not respond to an excitatory stimulus by releasing [^{3}H]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 $N^6,O^{2'}$-dibutyryladenosine 3′:5′-cyclic monophosphate (Bt_2cAMP). Culture of NG108-15 cells with Bt_2cAMP 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).[e]

The cells generate both Na^+ and Ca^{2+} action potentials and have endorphin receptors, excitatory muscarinic acetylcholine (ACh) receptors, α-adrenergic receptors, prostaglandin E_1 (PGE_1) receptors, $PGF_{2\alpha}$ receptors, and adenosine receptors that are coupled to shifts in cyclic AMP and/or cyclic GMP levels of the cells (6–14).[e,f,g] Serotonin (13), ACh (13), $PGF_{2\alpha}$ (13),[g] or dopamine (14) can depolarize the cells and initiate action potentials.

In this report, we describe the properties of stimulus-dependent release of [^{3}H]ACh from NG108-15 cells, a response required for synaptic communication, and show that the ability of the cells to release [^{3}H]ACh in response to stimulation can be regulated by Bt_2cAMP. 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 minimal essential medium (DMEM) (GIBCO H-21)/10% fetal bovine serum/100 μM hypoxanthine/1 μM aminopterine/16 μM thymidine] was supplemented with 1 mM Bt_2cAMP, 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 ($1.5–2.0 \times 10^5$ cells per capillary) and incubated for 3 hr at 37° 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.[h] Each U tube then contained 200–400 μg of cell protein.

Measurement of [^{3}H]Choline Uptake and [^{3}H]ACh Release. Cells in capillaries were washed for 3 min by perfusion (0.4 ml/min) with medium A [DMEM without choline, $NaHCO_3$, 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 [*methyl*-^{3}H]choline (10.1 Ci/mmol, Amersham/Searle) as indicated in figure legends. For cells grown in the presence of Bt_2cAMP, 1 mM Bt_2cAMP also was added to the [^{3}H]choline uptake medium but not to the wash medium. After incubation, most of the [^{3}H]choline in the medium was removed by perfusion with medium B (medium A with 20 μM eserine sulfate). When only intracellular [^{3}H]choline, [^{3}H]ACh, and other ^{3}H-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 ^{3}H-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 ^{3}H-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 ^{3}H-labeled compounds extracted from cells were fractionated by high-voltage paper electrophoresis with 1.5 M acetic

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U. S. C. §1734 solely to indicate this fact.

Abbreviations: Bt_2cAMP, N^6,O_2-dibutyryladenosine 3′:5′-cyclic monophosphate; ACh, acetylcholine; PG, prostaglandin; DMEM, Dulbecco's modification of Eagle's minimum essential medium; 5-HT, 5-hydroxytrypamine (serotonin).

[b] Present address: Department of Pharmacology, Georgetown University, Washington, DC 20007.

[c] Present address: Cardiology 111C, Veterans Administration Hospital, San Francisco, CA 94121.

[e] B. Hamprecht, T. Amano, and M. Nirenberg, unpublished data.

[f] S. Sharma and M. Nirenberg, unpublished data.

[g] H. Matsuzawa and M. Nirenberg, unpublished data.

[h] Details of the capillary culture tube system will appear elsewhere.

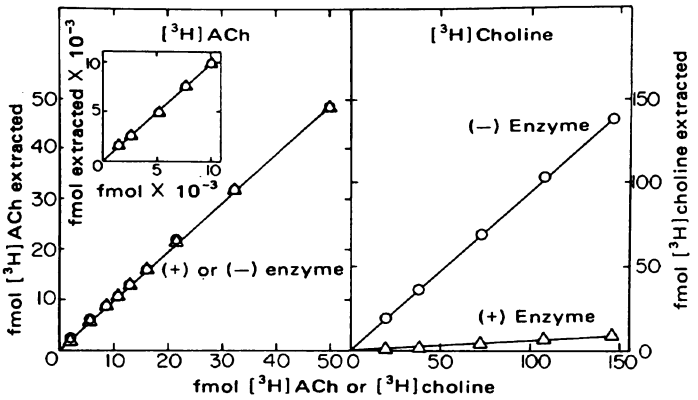

FIG. 1. [3H]ACh was separated from [3H]choline by conversion of [3H]choline to [3H]phosphorylcholine with choline kinase and extraction of [3H]ACh into 3-heptanone/30 mM tetraphenylboron. Indicated amounts of [3H]ACh (*Left*) or [3H]choline (*Right*) (both 10.1 Ci/mmol) were incubated in the absence or presence of 2.5 munits of choline kinase. [3H]ACh or unreacted [3H]choline then were extracted into the organic phase and radioactivity was determined. Each point represents the mean of four values; SEM <4%.

acid/0.75 M formic acid (18). ACh, choline, phospholipids, and a fraction containing phosphorylcholine and CDP-choline were identified by coelectrophoresis with authentic standards. The paper was treated with I_2 vapor to visualize spots, ^{3}H-labeled compounds in each fraction were eluted from the paper with 1 ml of water, the eluate was mixed with 10 ml of Hydromix (Yorktown Research), and radioactivity was determined by using a liquid scintillation spectrometer.

When [3H]ACh release from cells was measured, cells that had been incubated with [3H]choline were washed by perfusion with medium B at 0.4 ml/min for 8–14 min as indicated in the figure legends and fractions then were collected at 2-min intervals unless indicated otherwise. Cells were exposed to different compounds by stopping the peristaltic pump for a few seconds to switch from one perfusion medium to another.

To separate [3H]ACh and [3H]choline in the perfusate, a modification of the method of Goldberg and McCaman (19) was used in which [3H]choline was converted to [3H]phosphorylcholine during incubation with choline kinase, and then [3H]ACh and remaining traces of [3H]choline, but not [3H]-phosphorylcholine, were extracted with 3-heptanone/30 mM tetraphenylboron (Sigma). Choline was converted to phosphorylcholine in a reaction mixture containing, in a final volume of 0.275 ml, the following: 0.25 ml of perfusate, 10 mM NaATP, 10 mM $MgCl_2$, and 2.5 munits of choline kinase (approximately 10 μg of protein, Sigma). Reaction mixtures were incubated for 15 min at 36° and then extracted with 2 ml of 3-heptanone/30 mM tetraphenylboron. The organic phase was removed and part (1.7 ml) was evaporated at 60° under a stream of air, the residue was dissolved in Hydromix, and radioactivity was determined with a liquid scintillation spectrometer. The amount of [3H]ACh or [3H]choline released by cells was calculated with the assumption that the specific radioactivity of [3H]choline was not diluted. Because the [3H]-choline probably is diluted by unlabeled choline of cells, the values reported are lower than true values.

As shown in Fig. 1, >97% of authentic [3H]ACh or [3H]choline is extracted into the organic phase over the wide range of concentrations tested. However, after incubation with choline kinase, 97% of the [3H]ACh, but only 3–5% of the [3H]choline (or other ^{3}H-labeled compounds present), is extracted into the organic phase. Purification of the [*methyl*-3H]choline by paper electrophoresis prior to use decreased the amount of ^{3}H-labeled material extracted after incubation with choline kinase to <1% of the [3H]choline present initially. The amount of ^{3}H-labeled material extracted into the organic phase after incubation of [3H]choline with choline kinase was determined for each experiment and the amount, <25% of the total ^{3}H-labeled compounds extracted was subtracted from the total to calculate the amount of [3H]ACh. The sensitivity of the assay is limited by the specific activity of the [3H]ACh and the quantity of other ^{3}H-labeled compounds which are extracted with [3H]ACh. The maximum sensitivity in our experiments was 2 fmol of [3H]ACh per sample. The assay also is rapid, and as many as 300 samples were analyzed in a single day by one person.

Characterization of Released [3H]ACh. Perfusate fractions from three tubes were pooled and eserine was removed by extraction with equal volumes of $CHCl_3$. The aqueous phase then was warmed to 36° to remove residual $CHCl_3$ and the solution was incubated with choline kinase as described above in the presence or absence of 0.5 unit of acetylcholinesterase (0.5 μg of protein, electric eel, Sigma) in a 0.25-ml reaction mixture. Greater than 95% of authentic [3H]ACh was hydrolyzed under these conditions.

RESULTS

Release of [3H]ACh from Cells Grown with or without Bt_2cAMP. 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 Bt_2cAMP is shown in Fig. 2. NG108-15 cells grown without Bt_2cAMP 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 Bt_2cAMP increased in response to KCl. The basal unstimulated rates of [3H]ACh release from cells treated with Bt_2cAMP were twice those of control cells. Both basal and KCl-stimulated rates of [3H]ACh release increased throughout the 5 days of treatment with Bt_2cAMP. 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 Bt_2cAMP.

KCl also stimulated the release of [3H]choline from both control and Bt_2cAMP-treated cells to approximately the same extent. The rates of basal and KCl-stimulated [3H]choline release decreased between the 1st and 5th days of culture. These results show that Bt_2cAMP has little or no effect on basal or KCl-stimulated release of [3H]choline.

The [3H]ACh released from cells was characterized further by determining the sensitivity of the ^{3}H-labeled material released by hydrolysis catalyzed by acetylcholinesterase (Table 1). After incubation in the presence of acetylcholinesterase, the ^{3}H-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 ^{3}H-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 Bt_2cAMP but had little or no effect on cells grown without Bt_2cAMP (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

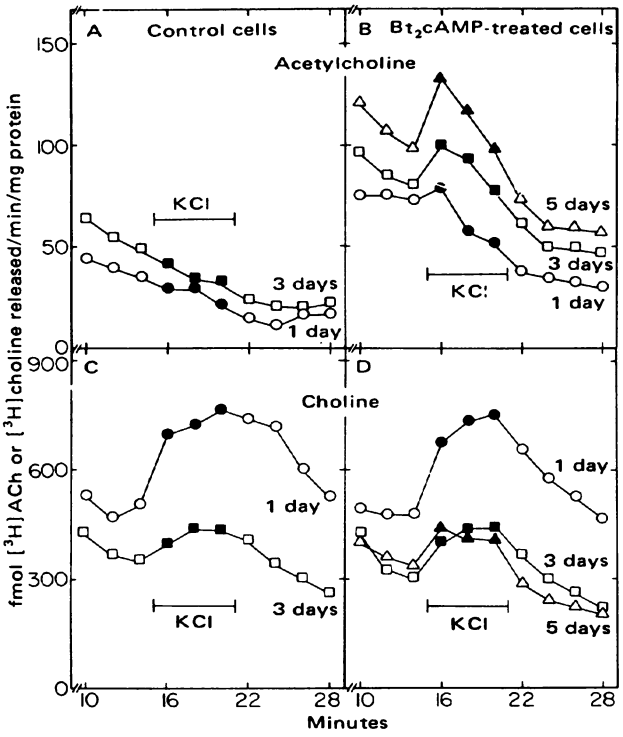

FIG. 2. Effect of culturing NG108-15 cells without or with Bt_2cAMP on KCl-stimulated (solid symbols) release of [^{3}H]ACh (*A* and *B*) and of [^{3}H]choline release (*C* and *D*). A cell suspension (approximately 200 μg of cell protein) was added to each capillary and the tubes then were perfused with DMEM supplemented with 5% fetal bovine serum for 24 hr. Some tubes then were perfused with the above medium supplemented with 1 mM Bt_2cAMP. One (○), 3 (□), or 5 (△) days later, the release of [^{3}H]ACh and [^{3}H]choline from cells was determined. Cells were incubated with 10 μM [^{3}H]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 2-min intervals (0.8 ml per fraction). Stimulation was with 80 mM KCl for 6 min as indicated. Each point represents the mean of four values obtained with separate cultures. Protein ranged from 200 μg per culture on the first day to 550 μg on the fifth day with or without Bt_2cAMP.

Bt_2cAMP but tetrodotoxin did not inhibit veratridine-stimulated [^{3}H]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 Bt_2cAMP for 0, 9, or 16 days on [^{3}H]choline metabolism is shown in Table 2. The intracellular concentrations of [^{3}H]ACh and [^{3}H]choline increased and those of phosphorylated ^{3}H-labeled compounds derived from [^{3}H]choline decreased as the time of exposure of cells to Bt_2cAMP was increased. After 16 days of exposure to Bt_2cAMP, NG108-15 cells had 2- and 5-fold higher levels of [^{3}H]ACh and [^{3}H]choline, respectively, than cells not exposed to Bt_2cAMP.

Table 1. Effect of acetylcholinesterase on released [^{3}H]ACh

Stimulation of cells	[^{3}H]ACh released, fmol/mg protein −AChE	+AChE
None (before stimulation)	126	6
80 mM KCl	211	3
None (after stimulation)	136	0

NG108-15 cells grown for 9 days with 1 mM Bt_2cAMP were incubated with 10 μM [^{3}H]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 $CHCl_3$ and the [^{3}H]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.

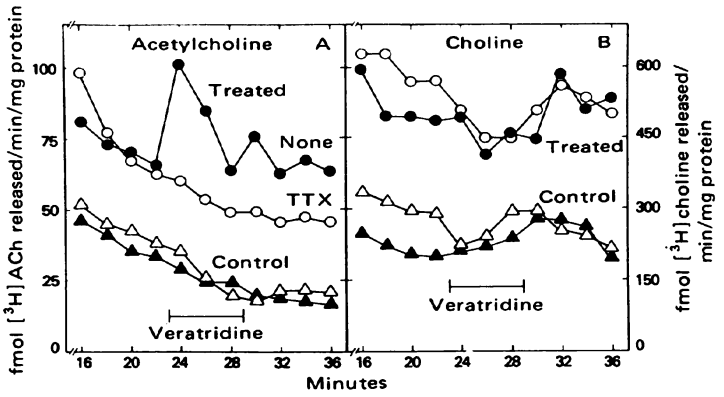

FIG. 3. Effect of culturing NG108-15 cells with or without Bt_2cAMP on veratridine-stimulated release of [^{3}H]ACh (*A*) or [^{3}H]choline (*B*). Cells cultured with or without 1 mM Bt_2cAMP for 7 days were dissociated and transferred to U tubes (about 300 μg of protein per tube) and then perfused with the same growth medium with or without Bt_2cAMP for 2 additional days. Cells were incubated with 10 μM [^{3}H]choline for 45 min and washed for 16 min as described in the legend to Fig. 2. Cells were exposed to 0.2 mM veratridine for 6 min as indicated. ●,○, Cells treated with Bt_2cAMP for 9 days; ▲,△, cells cultured without Bt_2cAMP for 9 days. ○,△, 1 μM tetrodotoxin (TTX) added at the start of the 16-min wash. Each point is the mean of four values obtained with separate cultures.

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

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

Table 2. [^{3}H]Choline metabolism by NG108-15 cells grown with or without Bt_2cAMP

Days with Bt_2cAMP	Intracellular levels, pmol/mg protein [^{3}H]-ACh	[^{3}H]-Choline	Phosphorylated [^{3}H]choline metabolites	Total [^{3}H] compounds
0	19.3	3.3	685	708
9	33.8	11.3	704	750
16	40.0	16.2	519	575

NG108-15 cells grown with Bt_2cAMP for 0, 7, or 14 days were transferred to U tubes and perfused with medium with or without Bt_2cAMP for 2 days. Cells then were incubated with 10 μM [^{3}H]choline for 45 min and washed for 36 min with medium B. ^{3}H-Labeled metabolites in the cells were extracted and subjected to paper electrophoresis. Each value represents the mean of three values obtained with separate cultures.

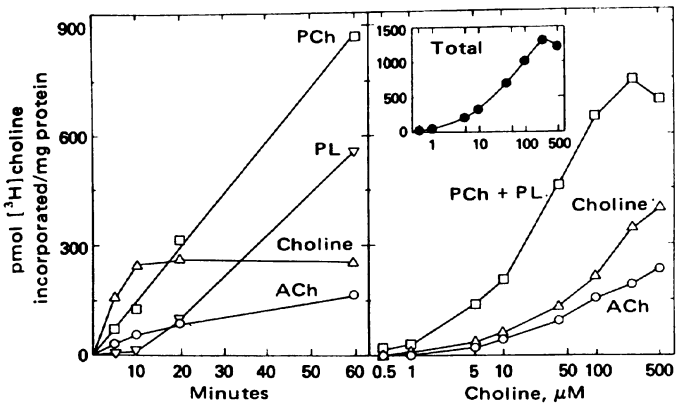

FIG. 4. Choline uptake and metabolism by Bt_2cAMP-treated NG108-15 cells. (*Left*) Cells that had been grown with 1 mM Bt_2cAMP for 16 days were incubated with 10 μM [^{3}H]choline for the times indicated and then washed for 1.5 min at a perfusion rate of 0.7 ml/min. The cells then were extracted with acetone/1 M formic acid, 85:15 (vol/vol), the ^{3}H-labeled compounds extracted were separated by high-voltage paper electrophoresis, and the protein content of each culture was determined. Each point represents the mean of three values from separate cultures. PCh, [^{3}H]phosphorylcholine and [^{3}H]CDP-choline; PL, [^{3}H]phospholipids; choline, [^{3}H]choline; ACh, [^{3}H]ACh. (*Right*) Cells that had been treated with Bt_2cAMP for 11 days were incubated with [^{3}H]choline at the concentrations indicated for 15 min and then washed; then, [^{3}H]choline and metabolites were extracted as for *Left*. PCh + PL, phosphorylcholine, CDP-choline, and phospholipid; choline, [^{3}H]choline; ACh, [^{3}H]ACh. (*Inset*) Total uptake of [^{3}H]choline by cells.

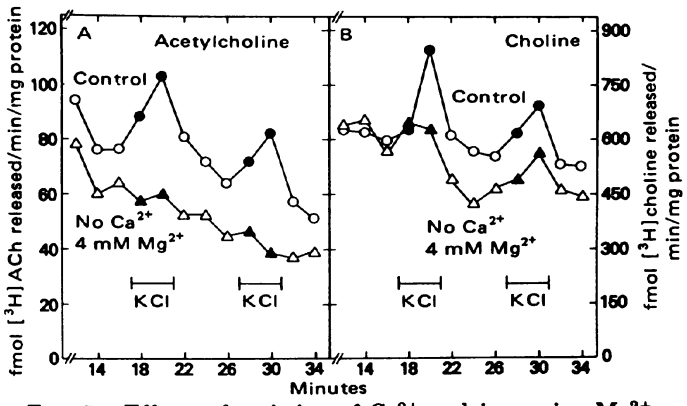

FIG. 5. Effects of omission of Ca^{2+} and increasing Mg^{2+} on KCl-stimulated release of [^{3}H]ACh (*A*) and [^{3}H]choline (*B*) from NG108-15 cells. Cells grown with 1 mM Bt_2cAMP for 35 days were incubated with 13 μM [^{3}H]choline for 45 min and washed, as described in the legend to Fig. 2. Cells then were perfused with control medium (○,●) (1.8 mM Ca^{2+}, 0.8 mM Mg^{2+}) or medium without Ca^{2+} and adjusted to 4 mM $MgCl_2$ (△,▲). Cells were stimulated with 80 mM KCl for 4 min as indicated (solid symbols). Perfusate fractions were assayed for [^{3}H]ACh and [^{3}H]choline. Each point represents the mean of three values from separate cultures.

total ^{3}H-labeled compounds increased as the extracellular choline concentration was increased and plateaued at 250 μM [^{3}H]choline. However, [^{3}H]choline and [^{3}H]ACh levels in cells increased over the entire range of extracellular [^{3}H]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 show that, as the extracellular choline concentration is increased, the levels of intracellular [^{3}H]choline and [^{3}H]ACh increase at approximately the same rate. The distribution of ^{3}H-labeled compounds accumulated by the cell remained constant between 0.5 and 250 μM extracellular [^{3}H]choline. These results suggest that the synthesis of [^{3}H]ACh is not preferentially coupled to the high- or low-affinity choline uptake systems.

Properties of [^{3}H]ACh and [^{3}H]Choline Release. KCl repetitively stimulated [^{3}H]ACh release from NG108-15 cells perfused with control medium containing 1.8 mM Ca^{2+} and 0.8 mM Mg^{2+}; but the second response to KCl was smaller than the first response (Fig. 5). Omission of Ca^{2+} and increasing Mg^{2+} to 4 mM abolished the KCl-dependent [^{3}H]ACh release and also decreased the unstimulated rate of [^{3}H]ACh release. Omission of Ca^{2+} and increasing Mg^{2+} partially decreased the KCl-stimulated release of [^{3}H]choline from cells.

The relationship between KCl concentration and [^{3}H]ACh release from cells is shown in Fig. 6 *left*. KCl at 30 mM stimulated the release of [^{3}H]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 [^{3}H]ACh compared to the first response to KCl at each concentration of KCl tested. These results suggest that the decreased [^{3}H]ACh release evoked by the second application of KCl may not be due to depletion of an intracellular pool of releasable [^{3}H]ACh.

As shown in Fig. 6 *right*, 10 μM 5-hydroxytryptamine (5-HT) or $PGF_{2\alpha}$ stimulated [^{3}H]ACh release from NG108-15 cells; whereas, PGE_1 stimulated [^{3}H]ACh release only slightly. Perfusate fractions were collected at 1-min intervals since cell responses to 5-HT at $PGF_{2\alpha}$ rapidly desensitized. The addition of 5-HT, $PGF_{2\alpha}$, or PGE_1 had little or no effect on the rate of [^{3}H]choline release from cells (not shown).

DISCUSSION

The results show that the ability of NG108-15 cells to respond to excitatory stimuli by releasing ACh into the medium can be

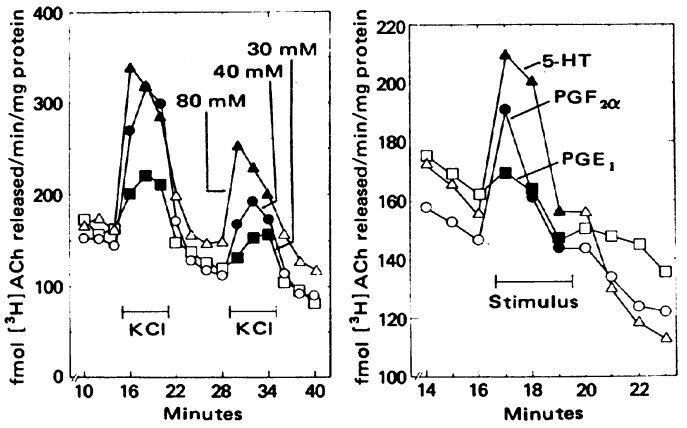

FIG. 6. (*Left*) Effect of KCl concentration on the amount of [^{3}H]ACh released from NG108-15 cells. Cells cultured with Bt_2cAMP for 11 days were incubated with 20 μM [^{3}H]choline for 45 min. The cells then were washed and perfusate fractions were collected and assayed for [^{3}H]ACh as in the legend to Fig. 2. Each point represents the mean of three values from separate cultures. Solid symbols correspond to fractions collected during stimulation with KCl; open symbols correspond to fractions collected from cells before and after the stimulus. △, 80 mM KCl; ○, 40 mM KCl; □, 30 mM KCl. (*Right*) Effects of serotonin (5-HT), $PGF_{2\alpha}$, or PGE_1 on the release of [^{3}H]-ACh from NG108-15 cells. Cells grown in Bt_2cAMP for 9 days were incubated with 35 μM [^{3}H]choline for 45 min and then washed for 14 min as in the legend to Fig. 2; fractions then were collected at 1-min intervals. Cells were stimulated for 3 min (solid symbols) with 10 μM serotonin creatinine sulfate (△), 10 μM $PGF_{2\alpha}$ (●), or 10 μM PGE_1 plus 0.1% ethanol (□). Perfusate fractions were assayed for [^{3}H]ACh. Each point represents the mean of three values from separate cultures.

regulated. Cells grown without Bt_2cAMP do not respond to excitatory stimuli by releasing [^{3}H]ACh but they can be shifted to a responsive state by treatment with Bt_2cAMP. The response to stimulation slowly increases over a period of at least 5 days while cells are cultured with Bt_2cAMP. 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 Bt_2cAMP 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 Bt_2cAMP.

The properties of evoked ACh release from NG108-15 cells treated with Bt_2cAMP 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 Ca^{2+} and elevation of Mg^{2+} 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 $PGF_{2\alpha}$-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 [^{3}H]ACh is released before cell responsiveness to the stimulating agent decreases or disappears.

If [^{3}H]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 (1–4, 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 [^{3}H]ACh represent the mean values for the population of cells.

The results suggest that ACh is released from NG108-15 by two mechanisms because Ca^{2+} is required for KCl-stimulated release of ACh but not for unstimulated release of ACh. KCl stimulates the release of both [^{3}H]ACh and [^{3}H]choline; however, these compounds seem to be released by different mechanisms.

About 15% of the [^{3}H]choline taken up by NG108-15 cells is converted to [^{3}H]ACh at each choline concentration tested between 0.5 and 250 μM. Thus, the synthesis of [^{3}H]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 [^{3}H]choline is converted to [^{3}H]ACh by cultured rat embryo brain cells as the external concentration of [^{3}H]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 [^{3}H]choline accumulated is converted to [^{3}H]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 [^{3}H]ACh release from NG108-15 cells is acquired slowly by cells over a period of at least 5 days while cells are exposed to Bt_2cAMP. 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 Bt_2cAMP 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.

1. Nelson, P., Christian, C. & Nirenberg, M. (1976) *Proc. Natl. Acad. Sci. USA* **73**, 123–127.
2. Puro, D. & Nirenberg, M. (1976) *Proc. Natl. Acad. Sci. USA* **73**, 3544–3548.
3. Christian, C., Nelson, P., Peacock, J. & Nirenberg, M. (1977) *Science* **196**, 995–998.
4. Nelson, P. G., Christian, C. N., Daniels, M. P., Henkart, M., Bullock, P., Mullinax, D. & Nirenberg, M. (1978) *Brain Res.*, in press.
5. Daniels, M. P. & Hamprecht, B. (1974) *J. Cell Biol.* **63**, 691–699.
6. Traber, J., Fischer, K., Latzin, S. & Hamprecht, B. (1975) *Nature* **253**, 120–122.
7. Sharma, S. K., Klee, W. & Nirenberg, M. (1975) *Proc. Natl. Acad. Sci. USA* **73**, 3092–3096.
8. Traber, J., Fischer, K., Buchen, C. & Hamprecht, B. (1975) *Nature* **255**, 558–560.
9. Klein, W. L., Nathanson, N. M. & Nirenberg, M. (1976) *Fed. Proc. Fed. Am. Soc. Exp. Biol.* **35**, 1576 (Abstr.).
10. Traber, J., Reiser, G., Fischer, K. & Hamprecht, B. (1975) *FEBS Lett.* **52**, 327–332.
11. Sabol, S. L. & Nirenberg, M. (1977) *Fed. Proc. Fed. Am. Soc. Exp. Biol.* **36**, 736 (Abstr.).
12. Traber, J., Fischer, K., Latzin, S. & Hamprecht, B. (1974) *FEBS Lett.* **49**, 260–263.
13. Christian, C. N., Nelson, P. G., Bullock, P., Mullinax, D. & Nirenberg, M. (1978) *Brain Res.*, in press.
14. Myers, P. R. & Livengood, D. R. (1975) *Nature* **255**, 235–237.
15. McGee, R., Simpson, P., Christian, C., Mata, M., Nelson, P. & Nirenberg, M. (1977) *Trans. Am. Soc. Neurochem.* **8**, 108.
16. Toru, M. & Aprison, M. H. (1966) *J. Neurochem.* **13**, 1533–1544.
17. Lowry, O. H., Rosebrough, N. J., Farr, A. L. & Randall, R. J. (1951) *J. Biol. Chem.* **193**, 265–275.
18. Potter, L. T. & Murphy, W. (1967) *Biochem. Pharmacol.* **16**, 1386–1388.
19. Goldberg, A. M. & McCaman, R. E. (1973) *J. Neurochem.* **20**, 1–8.
20. Yavin, E. (1976) *J. Biol. Chem.* **251**, 1392–1397.
21. Yamamura, H. I. & Snyder, S. H. (1973) *J. Neurochem.* **21**, 1355–1374.