Proc. Natl. Acad. Sci. USA
Vol. 81, pp. 4353–4357, July 1984
Biochemistry

Inhibitors of CDP-choline synthesis, action potential calcium channels, and stimulus-secretion coupling

(phospholipid/methylation/receptors/synapse/neuroblastoma)

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Contributed by Marshall Nirenberg, April 3, 1984

ABSTRACT **The effects of putative transmethylation inhibitors were tested on stimulus-secretion coupling and neurotransmitter secretion at synapses between neuroblastoma × glioma hybrid cells and myotubes. 5′-Deoxy-5′-isobutylthio-3-deazaadenosine or 5′-deoxy-5′-isobutylthioadenosine inhibited CDP-choline synthesis catalyzed by cholinephosphate cytidylyltransferase (CTP:cholinephosphate cytidylyltransferase, EC 2.7.7.15) and thereby decreased the rate of phosphatidylcholine synthesis from CDP-choline, but did not affect the transmethylation pathway for phosphatidylcholine synthesis. These compounds also inhibited $^{45}Ca^{2+}$ uptake by hybrid cells mediated by voltage-sensitive Ca^{2+} channels, acetylcholine secretion at synapses, and signal transduction through cell membranes mediated by myotube nicotinic acetylcholine receptors. In contrast, 3-deazaadenosine or adenosine inhibited the transmethylation pathway for phosphatidylcholine synthesis, but had no effect on Ca^{2+} action potentials, acetylcholine secretion, or signal transduction through cell membranes mediated by nicotinic acetylcholine receptors. These results show that the stimulus-secretion coupling and secretion reactions studied are not dependent on phospholipid methylation and suggest that the activity of action potential Ca^{2+} channels and the rate of neurotransmitter secretion are functionally coupled to the rate of phosphatidylcholine synthesis via the CDP-choline pathway.**

The synthesis of phosphatidylcholine (PtdCho) from phosphatidylethanolamine (PtdEtn) by transmethylation and concomitant translocation of the phospholipid from the inner to the outer leaflet of the plasma membrane has been proposed as a mechanism of signal transduction and secretion across cell membranes (1). Part of the evidence that the transmethylation pathway for PtdCho synthesis plays a role in signal transduction was obtained by the use of adenosine analogs such as 5′-deoxy-5′-isobutylthio-3-deazaadenosine (DZ-SIBA) on the assumption that these compounds are specific inhibitors of transmethylation reactions (2–5). However, with respect to DZ-SIBA the evidence for this assumption is inadequate (6, 7). In addition, questions have been raised about the hypothesized role of phospholipid transmethylation (8, 9).

We have examined the effects of DZ-SIBA and other putative transmethylation inhibitors on secretion of acetylcholine and stimulus-secretion coupling in NG108-15 or NBr-10A neuroblastoma hybrid cells. Cells from both lines synthesize, store, and secrete acetylcholine and form many synapses with cultured striated muscle cells (refs. 10 and 11; H. Higashida and S. Wilson, personal communication; unpublished observations). We wish to report that DZ-SIBA or 5′-deoxy-5′-isobutylthioadenosine (SIBA) inhibits the synthesis of CDP-choline catalyzed by NG108-15 cholinephosphate cytidylyltransferase (CTP:cholinephosphate cytidylyltransferase, EC 2.7.7.15) and thus decreases the synthesis of PtdCho from CDP-choline, but does not inhibit the transmethylation pathway for PtdCho synthesis. DZ-SIBA or SIBA also inhibits action potential Ca^{2+} channels, acetylcholine secretion perceived by myotubes at synapses, and signal transduction mediated by nicotinic acetylcholine receptors.

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METHODS AND MATERIALS

Mouse neuroblastoma × rat glioma NG108-15 hybrid cells were subcultured 13–20 times. Clone NBr10-A cells (J. Minna, personal communication) originated by fusion of mouse neuroblastoma N18TG-2 cells (12) with BRL-30E buffalo rat liver cells (H. Coon, personal communication). Cells were treated with 10 μM prostaglandin E_1 and 1 mM theophylline for 4–7 days to shift the cells to a more differentiated state (unpublished data).

RESULTS

Effects of Adenosine Derivatives on Myotube Responses to Acetylcholine Secreted by NG108-15 Cells at Synapses. *S*-Adenosylhomocysteinase (*S*-adenosyl-L-homocysteine hydrolase, EC 3.3.1.1) catalyzes the hydrolysis or synthesis of *S*-adenosylhomocysteine, a potent inhibitor of transmethylation reactions. Inhibition of *S*-adenosylhomocysteinase by various adenosine derivatives has been shown to increase cellular levels of *S*-adenosylhomocysteine and thereby inhibit transmethylation reactions (13).

In Fig. 1*A* are shown the effects of adenosine derivatives on miniature end-plate potentials (MEPPs) of cultured rat myotubes innervated by NG108-15 cells. Myotube MEPPs were measured with intracellular microelectrodes; presumably, each myotube response is initiated by the secretion of acetylcholine from a single NG108-15 vesicle at a synapse. Mean MEPP frequencies of myotubes were decreased 50% by 60 μM DZ-SIBA or 140 μM SIBA. DZ-SIBA (300 μM) decreased the mean MEPP frequency to 5% of the value found in the absence of the nucleoside, and the inhibition was reversible (not shown). Similarly, 1 mM SIBA or 1 mM 2′,3′-*O*-dinitroadenosine-5′-ethylcarboxamide (744-99) decreased the frequency of myotube responses to 17% of the value found with control cells. In contrast, the mean MEPP frequency increased more than 2-fold in the presence of 1–3 mM adenosine-5′-carboxamide (7199-21), an irreversible inhibitor of *S*-adenosylhomocysteinase (16). However, 3 mM

Abbreviations: DZ-SIBA, 5′-deoxy-5′-isobutylthio-3-deazaadenosine; SIBA, 5′-deoxy-5′-isobutylthioadenosine; 744-99, 2′,3′-*O*-dinitroadenosine-5-ethylcarboxamide; 7199-21, adenosine-5′-carboxamide; DZ-Ado, 3-deazaadenosine; PtdCho, phosphatidylcholine; PtdEtn, phosphatidylethanolamine; MEPPs, miniature end-plate potentials; dV/dt, maximum rate of rise of action potentials.

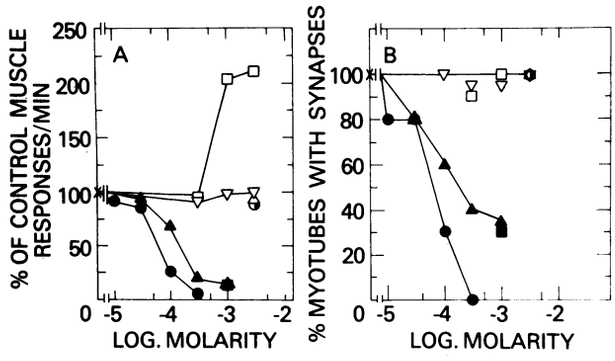

FIG. 1. Effects of adenosine derivatives (*A*) on frequency of rat myotube responses to acetylcholine spontaneously secreted by NG108-15 cells at synapses and (*B*) on percentage of myotubes tested that were innervated. ×, No nucleoside; ●, DZ-SIBA; ▲, SIBA; ■, 744-99; ◑, 2-Cl-3-deazaadenosine; △, adenosine; ▽, DZ-Ado; and □, 7199-21. Each point represents the mean of values obtained from 20 myotubes, except the values for 744-99 (10 myotubes), and for no nucleoside (130 myotubes). One hundred percent on the ordinate corresponds to 25 MEPPs per min per myotube. Myoblasts from hind limbs of newborn Fisher 344 rats were dissociated and cultured for 7–8 days as described (14). Then, 2×10^5 NG108-15 cells were added to myotubes in each 35-mm Petri dish, and cells were cocultured for 3–4 days in 1.5 ml of medium A (90% Dulbecco's modified Eagle's medium/10% horse serum/10 μM PGE_1/1 mM theophylline/0.1 mM hypoxanthine/0.016 mM thymidine). Synapses between NG108-15 and myotubes were detected by intracellular microelectrode recording of myotube MEPPs as described (15). A myotube with 3 or more MEPPs per min was considered innervated. Only recordings from muscle cells with stable resting membrane potentials of −45 to −90 mV were used. Two hours before cells were tested, the medium was replaced by medium B (Dulbecco's modified Eagle's medium without serum adjusted to 3.8 mM $CaCl_2$/129 μM choline chloride/10 μM PGE_1/1 mM theophylline/0.1 mM hypoxanthine/0.016 mM thymidine); nucleosides were added 30 min before cells were tested.

adenosine or 3-deazaadenosine (DZ-Ado), which are converted to the transmethylation inhibitors *S*-adenosylhomocysteine and *S*-3-deazaadenosylhomocysteine, respectively, or 3 mM 2-Cl-3-deazaadenosine had little or no effect on MEPP frequency.

The amplitudes of the depolarizing responses of myotubes to acetylcholine secreted from NG108-15 cells also were decreased in the presence of DZ-SIBA, SIBA, or 744-99 (not shown). DZ-SIBA also decreased the amplitudes of myotube responses to iontophoretically applied acetylcholine in the absence of hybrid cells; however, this effect was not large enough to account for the entire decrease in MEPP frequency of innervated myotubes. The percentage of myotubes tested that were innervated was decreased 50% by 70 μM DZ-SIBA or 200 μM SIBA; whereas little or no effect was detected with 3 mM 7199-21, DZ-Ado, 2-Cl-3-deazaadenosine, or adenosine (Fig. 1*B*).

As shown in Fig. 2*A*, the MEPP frequency decreased rapidly when 300 μM DZ-SIBA was added to the medium (50% decrease in 3.5 min) or when Ca^{2+} ions were omitted from the medium (Fig. 2*A*, *Inset*). Ca^{2+} ions also were required for acetylcholine secretion from NG108-15 cells elicited by electrical stimulation (15) or by depolarization of cells with 80 mM K^+ (S. Wilson and H. Higashida, personal communication; unpublished observations).

In contrast to the decrease in MEPP frequency found with DZ-SIBA, incubation of cells with 1.5 mM 7199-21 slowly and progressively resulted in a 6.5-fold increase in MEPP frequency (Fig. 2*B*).

Effect of Adenosine Derivatives on $^{45}Ca^{2+}$ Uptake by Hybrid Cells. Since the permeability of the hybrid cells to Ca^{2+} ions regulates the rate of acetylcholine secretion by the cells,

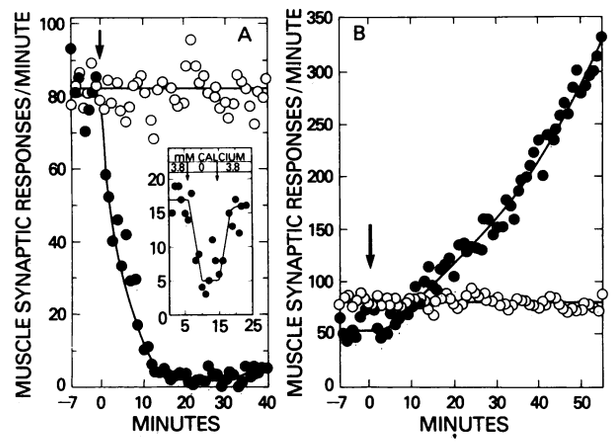

FIG. 2. (*A*) DZ-SIBA dependent inhibition of synaptic responses of a myotube innervated by an NG108-15 cell is shown as a function of time. The arrow at 0 min represents the addition of DZ-SIBA (●) [final concentration in medium B (see Fig. 1), 300 μM], or medium B alone (○). (*Inset*) MEPPs per min (ordinate) of a myotube innervated by NG108-15 in medium B with or without Ca^{2+} ions and without DZ-SIBA; minutes is shown on the abscissa. Between 0 and 6 min, a 35-mm Petri dish with 2 ml of medium B was perfused with medium B (3.8 mM Ca^{2+}) at 2 ml per min. At 6 min (first arrow), the medium was changed to medium B without $CaCl_2$; at 14 min (second arrow), the medium was changed to medium B with 3.8 mM $CaCl_2$. (*B*) 7199-21 (●) (1.5 mM) in medium B or medium B alone (○) were added at zero time (arrow).

the effects of the nucleosides on $^{45}Ca^{2+}$ uptake via voltage-sensitive Ca^{2+} channels of hybrid cells were determined. The cells were incubated with 5 and 80 mM K^+ to determine basal $^{45}Ca^{2+}$ uptake and $^{45}Ca^{2+}$ uptake via voltage-sensitive Ca^{2+} channels, respectively (Fig. 3). 744-99 or DZ-SIBA had little effect on $^{45}Ca^{2+}$ uptake and/or binding to the surface of cells at 5 mM K^+, but $^{45}Ca^{2+}$ uptake due to activation of voltage-sensitive Ca^{2+} channels by 80 mM K^+ ions was inhibited 50% with 65 μM 744-99, 120 μM DZ-SIBA, or 220 μM SIBA. As shown in Fig. 3*C*, 7199-21 increased $^{45}Ca^{2+}$ uptake slightly at 5 mM K^+ and at 80 mM K^+, but had no effect on $^{45}Ca^{2+}$ uptake due to cell depolarization by 80 mM K^+. DZ-Ado (1 mM) or adenosine (1 mM) had little or no effect on $^{45}Ca^{2+}$ uptake at 5 or 80 mM K^+ (not shown).

The effects of adenosine derivatives on NG108-15 Ca^{2+} action potentials elicited by electrical stimulation also were determined by intracellular microelectrode recording (Fig. 4*A*). The mean maximum rates of rise of Ca^{2+} action potentials were decreased 50% by 150, 190, and 780 μM 744-99, DZ-SIBA, or SIBA, respectively, with little effect on voltage-sensitive Na^+ or K^+ channels. DZ-Ado (3 mM) or adenosine (3 mM) had little or no effect on the maximum rate of rise of Ca^{2+} action potentials.

The concentration of nucleoside that resulted in 50% inhibition of $^{45}Ca^{2+}$ uptake due to cell depolarization also inhibited by ≈50% Ca^{2+} action potentials, acetylcholine secretion at synapses (MEPP frequency), and the percentage of myotubes tested that were innervated. Nucleoside potencies as inhibitors, in order of decreasing effectiveness, were as follows: 744-99 > DZ-SIBA > SIBA.

Effect of Adenosine Derivatives on PtdCho Synthesis. In higher organisms, most PtdCho is synthesized by the sequential conversion of choline to phosphorylcholine, CDP-choline, and then to PtdCho. In addition, some PtdEtn is converted to PtdCho by three successive transmethylation reactions. In Fig. 5 *A* and *C* are shown the effects of adenosine derivatives on the incorporation of [1,2-^{14}C]choline into [^{14}C]PtdCho and [^{14}C]lysolecithin, respectively, via the CDP-choline pathway. Fig. 5 *B* and *D* shows the effects of the nucleosides on the transfer of [^{3}H]methyl groups from

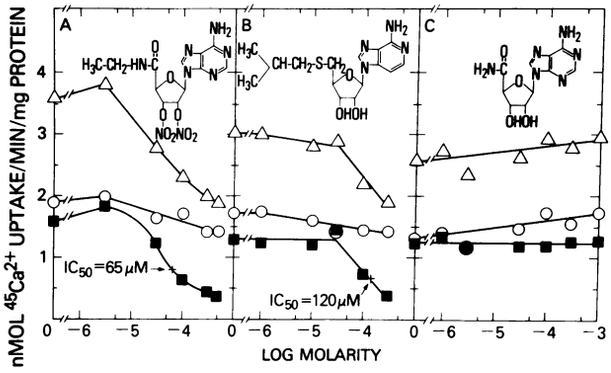

FIG. 3. $^{45}Ca^{2+}$ uptake by NBr10-A cells incubated with 744-99 (*A*), DZ-SIBA (*B*), or 7199-21 (*C*). Symbols represent medium C (described below) with the following: ○, 5 mM KCl and 114 mM NaCl; △, 80 mM KCl and 39 mM NaCl; or ■, $^{45}Ca^{2+}$ uptake dependent on cell depolarization (i.e., $^{45}Ca^{2+}$ uptake at 80 mM K^+ − $^{45}Ca^{2+}$ uptake at 5 mM K^+). The assay will be described elsewhere (A. Rotter and R. Ray, personal communication; unpublished data). Briefly, cells were incubated for 5–7 days in medium with 1 mM dibutyryl cAMP in multiwell dishes (2-cm^2 surface area per well), then for 20 hr in fresh medium with 0.8 mM L-[3,4-^{3}H]valine (250 cpm/nmol), for 2 hr in unlabeled medium with 0.8 mM L-valine, and for 30 min in 0.5 ml of medium C per well (114 mM NaCl/5 mM KCl/50 mM Hepes, sodium salt, pH 7.4/1.8 mM $CaCl_2$/0.8 mM $MgCl_2$/0.9 mM NaH_2PO_4/25 mM glucose/0.1 mM hypoxanthine/0.001 mM aminopterin/0.016 mM thymidine/1 mM dibutyryl cAMP) and an adenosine derivative, where indicated. Then cells were incubated for 60 sec at 37°C in fresh medium C containing $^{45}CaCl_2$ (3500 cpm per nmol), or in medium C adjusted to 80 mM KCl and 39 mM NaCl with $^{45}CaCl_2$. Cells then were washed 4 times with medium without labeled $CaCl_2$ at 3°C. $^{45}Ca^{2+}$ and ^{3}H radioactivities were determined and corrected for spillover. Portions of samples were pooled, assayed for protein (17), and $^{45}Ca^{2+}$ and ^{3}H radioactivities were determined; the cpm/mg of ^{3}H protein values were used to convert cpm of ^{3}H protein to μg of ^{3}H protein. Each $^{45}Ca^{2+}$ uptake value shown is the mean of three to six values obtained with replicate wells. Mouse neuroblastoma × rat liver NBr10-A hybrid cells, which synthesize acetylcholine, generate both Ca^{2+} and Na^+ action potentials when stimulated and form many synapses with myotubes, were used for $^{45}Ca^{2+}$ uptake studies because fewer cells detached from dishes when monolayers were washed compared to NG108-15 cells.

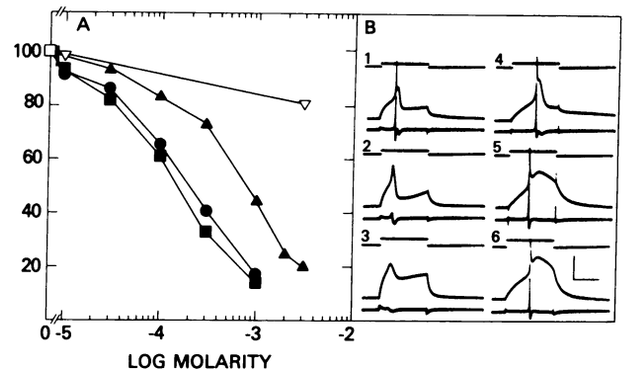

FIG. 4. (*A*) The relationship between nucleoside concentration and mean *dV/dt* of NG108-15 Ca^{2+} action potentials determined with intracellular microelectrodes. The percentage of the control *dV/dt* is shown on the ordinate; 100% (□) represents 4.6 V/sec. Other symbols represent the following: (▽) DZ-Ado, (▲) SIBA, (●) DZ-SIBA, and (■) 744-99. Each symbol represents the mean *dV/dt* obtained from 11–15 cells. Cells were incubated at 37°C in medium D (170 mM Tris·HCl, pH 7.4/1.8 mM $CaCl_2$/0.81 mM $MgCl_2$/5.4 mM KCl and 25 mM glucose). Nucleosides were added 30 min before cells were tested. (*B*) Oscilloscope traces of Ca^{2+} or Na^+ action potentials. The upper, middle, and lower traces correspond to the stimulating current, action potential, and the first derivative of the action potential, respectively; the vertical bar in *B6* represents 5 nA, 40 mV, and 40 V/sec, respectively and the horizontal bar represents 100 msec. Traces in *B1–B3* are from the same cell in medium E (medium D adjusted to 153 mM NaCl/15 mM Tris·HCl, pH 7.4) supplemented as follows: *B1*, no addition; *B2*, 5 μM tetrodotoxin; *B3*, 5 μM tetrodotoxin and 300 μM DZ-SIBA. Traces in *B4–B6* are from another cell; *B4*, medium E; *B5*; medium E without $CaCl_2$ with 0.5 mM EGTA and 2.61 mM $MgCl_2$; *B6* same as *B5* except with 300 μM DZ-SIBA.

L-[*methyl*-^{3}H]methionine to PtdEtn ultimately forming [^{3}H]PtdCho via the transmethylation pathway, and the formation of [^{3}H]lysolecithin, respectively. Both [^{14}C]PtdCho and [^{3}H]PtdCho were synthesized by NG108-15 cells; however, 99.6% of the radioactive PtdCho detected was synthesized by incorporation of [1,2-^{14}C]choline into [^{14}C]PtdCho. The incorporation of [1,2-^{14}C]choline into [^{14}C]PtdCho was inhibited 64% by 300 μM DZ-SIBA and 35% by 300 μM SIBA; whereas 1 mM DZ-Ado or 7199-21 had little or no effect on [^{14}C]choline incorporation. Ninety percent of the ^{14}C products recovered after thin-layer chromatography exhibited the chromatographic mobility of authentic PtdCho. The synthesis of [^{14}C]lysolecithin, presumably by catabolism of [^{14}C]PtdCho, and the synthesis of [^{14}C]PtdCho were inhibited by DZ-SIBA or SIBA to approximately the same extent, which suggests that DZ-SIBA or SIBA inhibit [^{14}C]PtdCho synthesis rather than stimulate the catabolism of [^{14}C]PtdCho.

The conversion of PtdEtn and L-[*methyl*-^{3}H]methionine to [^{3}H]PtdCho by the transmethylation pathway was completely inhibited by 1 mM DZ-Ado or 7199-21; whereas DZ-SIBA or SIBA had little or no effect on [^{3}H]PtdCho synthesis. The results with SIBA confirm the report by Schanche *et al.* (19) that SIBA does not inhibit phospholipid methylation. Only 30% of the ^{3}H compounds recovered after thin-layer chromatography were identified as phospholipids; [^{3}H]PtdCho comprised 17%, and [^{3}H]lysolecithin, [^{3}H]phosphatidyl-*N*-monomethylethanolamine, and [^{3}H]phosphatidyl-*N*-*N*-dimethylethanolamine together comprised 13% of the ^{3}H compounds recovered. The inhibition of [^{3}H]PtdCho synthesis did not result in an increase in the accumulation of the [^{3}H]monomethyl- or [^{3}H]dimethyl-PtdEtn intermediates (not shown). Similar results were obtained with rats treated with DZ-Ado (20). A relatively high proportion of [^{3}H]PtdCho was converted to [^{3}H]lysolecithin compared to the conversion of [^{14}C]PtdCho to [^{14}C]lysolecithin, which suggests that the pool of [^{3}H]PtdCho synthesized via the transmethylation pathway is separate from and turns over more rapidly than the pool of [^{14}C]PtdCho synthesized via the CDP-choline pathway. These results show (*i*) that >99% of the PtdCho synthesized by NG108-15 cells is synthesized by the incorporation of choline into PtdCho, presumably via the CDP-choline pathway; (*ii*) that DZ-SIBA or SIBA inhibits the incorporation of choline into PtdCho but not the transmethylation pathway for PtdCho synthesis; and (*iii*) that DZ-Ado or 7199-21 inhibits the transmethylation pathway for PtdCho synthesis.

Effect of Adenosine Derivatives on ^{35}*S*-Adenosylhomocysteine Accumulation in NG108-15 Cells. As shown in Table 1, intracellular ^{35}*S*-adenosylhomocysteine accumulation in NG108-15 cells incubated with [^{35}S]methionine increased markedly in the presence of 1 mM 7199-21, DZ-Ado, or adenosine, but not in the presence of 0.33 mM DZ-SIBA or SIBA. Intracellular ^{35}*S*-adenosylmethionine increased slightly, if at all, in the presence of DZ-SIBA, SIBA, 7199-21, DZ-Ado, or adenosine. 7199-21 is a potent irreversible inhibitor of *S*-adenosylhomocysteinase; whereas, DZ-Ado is a weak irreversible inhibitor of the enzyme and also a substrate that is converted to *S*-3-deazaadenosylhomocysteine. Both *S*-3-deazaadenosylhomocysteine and *S*-adenosylhomo-

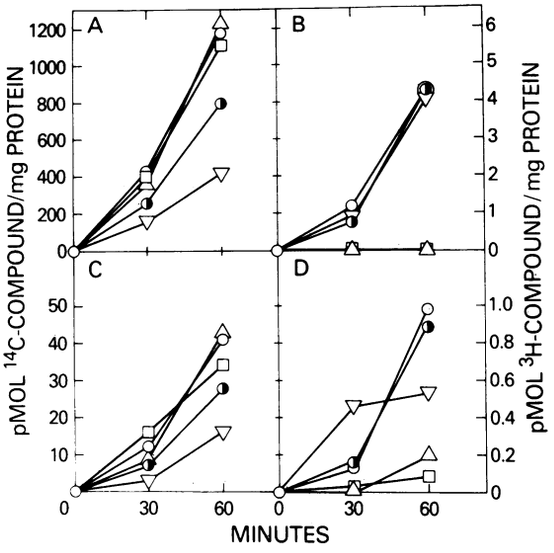

FIG. 5. NG108-15 cells were incubated in medium containing both [1,2-^{14}C]choline and L-[*methyl*-^{3}H]methionine, with or without an adenosine derivative, to determine the effects of the nucleosides on PtdCho synthesis from [1,2-^{14}C]choline via the CDP-choline pathway (*A*), or from [*methyl*-^{3}H]methionine and PtdEtn via the transmethylation pathway (*B*). [^{14}C]- and [^{3}H]lysolecithin are shown in *C* and *D*, respectively. Symbols represent the following: ○, no nucleoside; △, 1 mM 3-deazaadenosine; □, 1 mM 7199-21; ◑, 0.33 mM SIBA; and ▽, 0.33 mM DZ-SIBA. NG108-15 cells were incubated in 1 ml of medium B (see Fig. 1) containing 2.5 mM [1,2-^{14}C]-choline chloride (8.0 cpm/pmol) and 0.2 mM L-[*methyl*-^{3}H]methionine (520 cpm/pmol) per 35-mm Petri dish for 30 or 60 min; then, each monolayer was washed with 1 ml of medium B and 0.5 ml of a solution containing 10% trichloroacetic acid and 10 mM L-methionine at 4°C was added. Precipitates were recovered by centrifugation, and phospholipids were extracted with 3 ml of a solution containing 67% chloroform and 33% methanol as described by Hirata *et al.* (18) and separated by thin-layer chromatography using silica gel G plates and a solvent of chloroform/propionic acid/*n*-propanol/water (2:2:3:1, vol/vol) with authentic phospholipids as standards. Plates were exposed to iodine vapor to visualize the phospholipids; each lane was divided in 1 × 2.7 cm portions and ^{3}H and ^{14}C radioactivities were determined and were corrected for spillover.

cysteine are potent inhibitors of transmethylation reactions.

Effect of Adenosine Derivatives on Cholinephosphate Cytidylyltransferase Activity. A rate-limiting step in the CDP-choline pathway for PtdCho synthesis is the conversion of phosphorylcholine and CTP to CDP-choline and pyrophosphate catalyzed by cholinephosphate cytidylyltransferase (22–24). As shown in Table 2, 1 mM DZ-SIBA or SIBA inhibited cholinephosphate cytidylyltransferase 59% and 18%, respectively; whereas no inhibition was detected with 3 mM DZ-Ado.

DISCUSSION

The results show that DZ-SIBA or SIBA inhibit CDP-choline synthesis, action potential Ca^{2+} channels, acetylcholine secretion from NG108-15 cells perceived by myotubes at synapses, and signal transduction through myotube plasma membranes mediated by nicotinic acetylcholine receptors. DZ-SIBA or SIBA decreased the rate of synthesis of PtdCho via the CDP-choline pathway by inhibiting CDP-choline synthesis; however, DZ-SIBA or SIBA did not inhibit the transmethylation pathway for PtdCho synthesis or affect intracellular levels of the transmethylation inhibitor, ^{35}*S*-adenosylhomocysteine. In contrast, exposure of cells to DZ-Ado resulted in the synthesis of ^{35}*S*-3-deazaadenosylhomocysteine, increased cellular ^{35}*S*-adenosylhomocysteine levels, and profoundly inhibited the transmethylation pathway for PtdCho synthesis; but had little or no effect on $^{45}Ca^{2+}$ uptake via voltage-sensitive Ca^{2+} channels, Ca^{2+} action potentials, or secretion of acetylcholine at synapses. Exposure of cells to adenosine-5′-carboxamide also completely inhibited the transmethylation pathway for PtdCho synthesis; however, $^{45}Ca^{2+}$ uptake by cells increased gradually by a process that was not affected by cell depolarization, and concomitantly, an increase was observed in acetylcholine secretion

Table 1. Effects of adenosine derivatives on ^{35}*S*-adenosylhomocysteine and ^{35}*S*-adenosylmethionine accumulation in NG108-15 cells

Nucleoside added	^{35}*S*-Adenosylhomocysteine, cpm per flask	^{35}*S*-Adenosylmethionine, cpm per flask
None	0	8,468
0.33 mM DZ-SIBA	0	9,578
0.33 mM SIBA	0	10,926
1 mM 7199-21	2586	10,623
1 mM DZ-Ado	2058*	9,200
1 mM adenosine	2318	9,200

NG108-15 cells were incubated in flasks (25 cm^2 each) in medium B containing 0.2 mM L-[^{35}S]methionine (25 mCi/mmol; 1 Ci = 37 GBq) for 2 hr at 37°C. Then, the medium was replaced with medium containing 0.2 mM L-[^{35}S]methionine and the adenosine derivative indicated and cells were incubated for 1 hr. Cells were harvested, pelleted, lysed with 2 ml of a solution containing 1 ml of medium B and 1 ml of 197 mM sulfosalicilic acid, and stored at −20°C. Extracts were thawed, centrifuged at 20,000 × *g* for 10 min, and ^{35}S nucleosides in the supernatant portions were fractionated by high-performance liquid chromatography (21).

*In addition, 484 cpm of ^{35}*S*-3-deazaadenosylhomocysteine was found.

Table 2. Inhibition of cholinephosphate cytidylyltransferase activity by DZ-SIBA or SIBA

Addition	[^{14}C]CDP-choline, pmol formed/min per mg of protein	%
Control	117	100
DZ-SIBA		
10 μM	134	114
33 μM	107	91
100 μM	97	83
333 μM	90	77
1000 μM	48	41
SIBA		
10 μM	109	93
33 μM	108	92
100 μM	113	97
333 μM	112	96
1000 μM	96	82
3-Deazaadenosine		
3000 μM	113	97

Each 25-μl reaction mixture contained 100 μg of NG108-15 homogenate protein, 40 mM Tris succinate (pH 7.0), and twice the concentration of nucleoside shown in the table; reaction mixtures were incubated for 15 min at 4°C. Then other components were added so that each final reaction mixture (50 μl) contained 20 mM Tris succinate (pH 7.0), 0.4 mM CTP (disodium salt), 8 mM magnesium acetate, 1 mM [*methyl*-^{14}C]phosphorylcholine (25 Ci/mol), the nucleoside concentration shown in the table, and 100 μg of NG108-15 homogenate protein. Each tube was incubated for 10 min at 37°C, placed in a boiling water bath for 2 min, then 0.87 pmol of unlabeled CDP-choline was added. CDP-choline and phosphorylcholine were separated by thin-layer chromatography (silica gel G) using a solvent of methanol/0.5% NaCl/29.8% ammonium hydroxide (100:100:2, vol/vol) (25). CDP-choline was visualized under UV light, eluted, and the radioactivity was determined. Boiled homogenate was added to some tubes instead of native protein; the mean value was subtracted from each value shown.

at synapses. The adenosine-5'-carboxamide-dependent increases in $^{45}Ca^{2+}$ uptake and acetylcholine secretion probably are not due to inhibition of the transmethylation pathway for PtdCho synthesis, because DZ-Ado also inhibited the transmethylation pathway for PtdCho synthesis but did not affect Ca^{2+} uptake by cells or acetylcholine secretion.

The conversion of CTP and phosphorylcholine to CDP-choline, catalyzed by cholinephosphate cytidylyltransferase, is a rate-limiting step in the CDP-choline pathway for PtdCho synthesis in liver (22), heart (23), and striated muscle cells (24). Preliminary results suggest that DZ-SIBA is a competitive inhibitor of cholinephosphate cytidylyltransferase with approximately the same affinity for the enzyme as CTP. The K_i for DZ-SIBA and the K_m for CTP are approximately 700×10^{-6} M. However, the concentrations of CTP in 35-day-old rat brain (26) and adult rat liver (27) (31 and 83 nmol of CTP per g of tissue, respectively) are lower than the concentration of CTP that was used to determine cholinephosphate cytidylyltransferase activity in NG108-15 homogenates (400 μM CTP). Thus, under the conditions used, lower concentrations of DZ-SIBA or SIBA may be needed for inhibition of cholinephosphate cytidylyltransferase in intact cells than in homogenates.

PtdCho is abundant in NG108-15 cells (99 nmol per mg of protein), turns over slowly (28), and moves rapidly by lateral diffusion in cell membranes above the phase transition temperature (29). If Ca^{2+} uptake by cells via Ca^{2+} action-potential channels were dependent on PtdCho, it seems unlikely that inhibition of PtdCho synthesis by DZ-SIBA or SIBA would rapidly and profoundly inhibit the activity of the channels.

Part of the evidence for the hypothesis that signal transmission through cell membranes is dependent on phospholipid methylation is based on the use of DZ-SIBA as a specific inhibitor of the transmethylation pathway for PtdCho synthesis (1–5). We suggest an alternative hypothesis; namely, that inhibition of cholinephosphate cytidylyltransferase by DZ-SIBA results in a decrease in the level of cellular CDP-choline, and a consequent decrease in the rate of synthesis of PtdCho and perhaps of other compounds derived from CDP-choline. Since the reaction catalyzed by cholinephosphotransferase (CDP-choline:1,2-diglycerol cholinephosphotransferase, EC 2.7.8.2) is freely reversible, a decrease in the intracellular level of CDP-choline would be expected to alter the relative rates of the forward and backward reactions catalyzed by the enzyme and to result, by mass action, in a decreased rate of synthesis of PtdCho and an increased rate of breakdown of PtdCho in cell membranes, thereby increasing the formation of 1,2-diacyl-*sn*-glycerol. Diacylglycerol is known to perturb the structure of cell membranes and might well have pleiotropic effects on membrane functions. The demonstration that DZ-SIBA is a more potent inhibitor than SIBA of cholinephosphate cytidylyltransferase, of PtdCho synthesis from choline, of Ca^{2+} action potential channels, and of acetylcholine secretion at synapses suggests that inhibition of cholinephosphate cytidylyltransferase may be the primary site of action of DZ-SIBA and SIBA in the hybrid cells, and that rates of Ca^{2+} uptake via voltage-sensitive Ca^{2+} channels and neurotransmitter secretion may be functionally coupled to the rate of PtdCho synthesis via the CDP-choline pathway.

We thank Dr. Fusao Hirata for discussions on phospholipid methylation and separation methods.

1. Hirata, F. & Axelrod, J. (1980) *Science* **209,** 1082–1090.
2. Hirata, F., Toyoshima, S., Axelrod, J. & Waxdal, M. J. (1980) *Proc. Natl. Acad. Sci. USA* **77,** 862–865.
3. Ishizaka, T., Hirata, F., Ishizaka, K. & Axelrod, J. (1980) *Proc. Natl. Acad. Sci. USA* **77,** 1903–1906.
4. Crews, F. T., Morita, Y., Hirata, F., Axelrod, J. & Siraganian, R. P. (1980) *Biochem. Biophys. Res. Commun.* **93,** 42–49.
5. Hirata, F., Axelrod, J. & Crews, F. T. (1979) *Proc. Natl. Acad. Sci. USA* **76,** 4813–4816.
6. Im, Y. S., Chiang, P. K. & Cantoni, G. L. (1979) *J. Biol. Chem.* **254,** 11047–11050.
7. Robert-Gero, M., Pierre, A., Vedel, M., Enouf, J., Lawrence, F., Raies, A. & Lederer, E. (1980) in *Enzyme Inhibitors*, ed. Brodbeck U. (Chemie, Basel, Switzerland), pp. 61–74.
8. Vance, D. E. & de Kruijff, B. (1980) *Nature (London)* **288,** 277–278.
9. Axelrod, J. & Hirata, F. *Nature (London)* **288,** 278–279.
10. McGee, R., Simpson, P., Christian, C., Mata, M., Nelson, P. & Nirenberg, M. (1978) *Proc. Natl. Acad. Sci. USA* **75,** 1314–1318.
11. Nelson, P., Christian, C. & Nirenberg, M. (1976) *Proc. Natl. Acad. Sci. USA* **73,** 123–127.
12. Minna, J., Glazer, D. & Nirenberg, M. (1972) *Nature (London) New Biol.* **235,** 225–231.
13. Cantoni, G. L. & Chiang, P. K. (1980) in *Natural Sulfur Compounds*, eds. Cavallini, D., Gaull, G. E. & Zappia, V. (Plenum, New York), pp. 67–80.
14. Ruffolo, R. R., Jr., Eisenbarth, G. S., Thompson, J. M. & Nirenberg, M. (1978) *Proc. Natl. Acad. Sci. USA* **75,** 2281–2285.
15. Nelson, P. G., Christian, C. N., Daniels, M. P., Henkart, M., Bullock, P., Mullinax, D. & Nirenberg, M. (1978) *Brain Res.* **147,** 245–259.
16. Chiang, P. K., Guranowski, A. & Segall, J. E. (1981) *Arch. Biochem. Biophys.* **207,** 175–184.
17. Lowry, O. H., Rosebrough, N. J., Farr, A. L. & Randall, R. J. (1951) *J. Biol. Chem.* **193,** 265–275.
18. Hirata, F., Strittmatter, W. J. & Axelrod, J. (1979) *Proc. Natl. Acad. Sci. USA* **76,** 368–372.
19. Schanche, J. S., Schanche, T. & Ueland, P. M. (1981) *Mol. Pharmacol.* **20,** 631–636.
20. Chiang, P. K., Im, Y. S. & Cantoni, G. L. (1980) *Biochem. Biophys. Res. Commun.* **94,** 174–181.
21. Aksamit, R. R., Falk, W. & Cantoni, G. L. (1982) *J. Biol. Chem.* **257,** 621–625.
22. Infante, J. P. (1977) *Biochem. J.* **167,** 847–849.
23. Zelinski, T. A., Savard, J. D., Man, R. Y. K. & Choy, P. C. (1980) *J. Biol. Chem.* **255,** 11423–11428.
24. Sleight, R. & Kent, C. (1980) *J. Biol. Chem.* **255,** 10644–10650.
25. Yavin, E. (1976) *J. Biol. Chem.* **251,** 1392–1397.
26. Mandel, P. & Edel-Harth, S. (1966) *J. Neurochem.* **13,** 591–595.
27. Domschke, W., Keppler, D., Bischoff, E. & Decker, K. (1971) *Hoppe-Seyler's Z. Physiol. Chem.* **352,** 275–279.
28. Yavin, E. & Zutra, A. (1979) *Biochim. Biophys. Acta* **553,** 424–437.
29. Schlessinger, J., Axelrod, D., Koppel, D. E., Webb, W. W. & Elson, E. L. (1977) *Science* **195,** 307–309.