Inhibition of *S*-adenosylmethionine-linked methylation can lead to neurite extension in neuroblastoma cells

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1. INTRODUCTION

Neuroblastoma cells are a suitable model to investigate some aspects of neuronal development because of their ability to extend neurites in response to a variety of stimuli. There are many agents and conditions which stimulate this neurite outgrowth [1,2]. However, the biochemical mechanisms underlying this event are not yet understood. Our observation that mouse neuroblastoma cells extend neurites when grown in a medium supplemented with delipidated fetal calf serum suggests that alterations of membrane lipid composition may influence cell morphology [3]. Since the discovery of 2 different methyltransferases which catalyse the conversion of phosphatidylethanolamine into phosphatidylcholine using *S*-adenosylmethione (AdoMet) as methyl donor, much attention has been focussed on this biochemical event [4]. This stepwise methylation is involved in the control of numerous cellular functions such as changes in membrane fluidity [5], calcium transport [6], Ca^{2+}-ATPase activity [7], IgE-mediated histamine and arachidonic acid release in rat leukemic basophils [8] and lectin-induced lymphocyte mitogenesis [9]. Hence it appears that phospholipid methylation may be an important event which regulates the transduction of receptor-mediated signals through the cell membrane [4]. Not all receptor-mediated events, however, require this biochemical reaction [4,10]. Inhibition of methylation has also been implicated in developmental cellular events [11]. For example, 3-deaza-adenosine (3-deazaAdo), an inhibitor of methylations, increases the frequency of conversion of 3T3-L1 fibroblasts to fat cells. Here, we report that inhibitors of AdoMet-linked methylation reactions efficiently induce neurite extension in parallel with their inhibition of phospholipid methylation in neuroblastoma cells. Furthermore, chemicals which are able to stimulate neurite extension (the calcium ionophore A23187, mepacrine, cycloheximide, dibutyryl-cyclic AMP (dbcAMP) and 3-isobutyl-1-methylxanthine (IBMX)) also inhibit phospholipid methylation in intact neuroblastoma cells. Thus we suggest that inhibition of AdoMet-linked methylation is a biochemical event which can lead to neurite outgrowth.

Abbreviations: AdoHcy, *S*-adenosylhomocysteine; 3-deazaAdo, 3-deaza-adenosine); SIBA, 5′-deoxy-5′-*S*-isobutyl-thioadenosine; dbcAMP, dibutyryl-cyclic AMP; IBMX, 3-isobutyl-1-methylxanthine

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2. MATERIALS AND METHODS

2.1. *Cell culture and bioassay for neurite outgrowth*

Mouse neuroblastoma cells, clone NB_2A, were grown in Dulbecco's modified Eagle's medium (DMEM) which contained 10% fetal calf serum (FCS) at 37°C in a humidified 10% CO_2—90% air atmosphere. The cells were dislodged with EDTA and cells were inoculated at 3.5×10^4/culture dish (35 mm) containing 2 ml DMEM supplemented with 10% FCS and cultures were incubated as above. After 16—18 h, the medium was replaced by 2 ml DMEM supplemented with tricine (3.6×10^{-2} M) and $NaHCO_3$ (1.2×10^{-2} M)

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(TDMEM), containing 0.8% FCS, which had not been heat-inactivated but dialyzed against 0.9% NaCl solution, filter-sterilized and frozen. The bioassay was done 24 h later by changing the medium to 2 ml fresh TDMEM with and without the substance to be tested. The ionophore A23187 was used as an ethanolic solution and so 4 μl ethanol was added also to control dishes. After 4 h incubation the assay was terminated by aspirating away the medium and fixing the cells with 1.5 ml 2.5% glutaraldehyde in phosphate-buffered saline [3]. The extent of neurite outgrowth was determined as in [12].

2.2. *Phospholipid methylation*

Neuroblastoma cells were incubated under the same conditions as those of the bioassay for neurite outgrowth with the difference that 10 μCi L-[*methyl*-^{3}H]methionine (91 Ci/mmol, Amersham) dish was added twice, 2 h before and immediately after TDMEM + 0.8% FCS medium had been changed to 2 ml TDMEM with and without the substance to be tested. At each indicated time point the reaction was stopped by removing the medium and adding 1.5 ml 5% trichloroacetic acid. Cells were washed with 1.5 ml same solution and detached from the dishes with a rubber policeman. The dishes were then washed with an additional 1 ml trichloroacetic acid. After addition of 40 μl bovine serum albumin solution (1 mg/ml) and centrifugation at 6000 × *g* for 10 min, the supernatant was discarded and phospholipids were extracted from the pellets with 3 ml chloroform/methanol (2:1, v/v) followed by 2 washes with 2 ml 0.1 M KCl/50% methanol solution as in [13]. The solvent was evaporated with a stream of N_2, the residue was resuspended in scintillation fluid and assayed for radioactivity. In separate experiments phospholipid extracts from several dishes were combined and analyzed by a two-dimensional thin-layer chromatography on silica gel plates as in [14]. The subsequent autoradiography revealed that radioactivity incorporated into the phospholipid fraction was located on 3 spots and that there was no radioactive spot seen at the origin. By co-developing phospholipid extracts with authentic samples the radioactive spots were assigned to phosphatidylcholine, lysophosphatidylcholine and a mixture of phosphatidyl-*N*-methyl- and phosphatidyl-*N*,*N*-dimethylethanolamine.

3. RESULTS

To investigate a possible role of AdoMet-linked methylation in neurite extension by neuroblastoma cells, we first tested the effect of *S*-adenosyl-L-homocysteine (AdoHcy), a specific competitive inhibitor of methylations with AdoMet as methyl donor [15]. To promote neurite extension, 2.4×10^{-4} M AdoHcy was required (table 1). At 2.4×10^{-3} M the effect was greatly enhanced and >80% of cells formed neurites. The possibility that inhibition of methylation could lead to neurite outgrowth was further confirmed by the effect of other methyltransferase inhibitors, 3-deazaAdo [4,11] and 5′-deoxy-5′-*S*-isobutylthioadenosine (SIBA) [16] (table 1). 3-DeazaAdo was the most effective and even at 3.7×10^{-5} M, ~70% of neuroblastoma cells became morphologically differentiated.

To determine whether phospholipid methylation is indeed suppressed by these inhibitors, we in-

Table 1

Effects of different chemicals on morphological differentiation of mouse neuroblastoma cells

	Conc. (M)	Cells with neurites (%)
Control		14.3 ± 5.1
AdoHcy	2.4×10^{-3}	82.5 ± 0.3
	2.4×10^{-4}	44.8 ± 1.4
3-DeazaAdo	3.7×10^{-4}	77.2 ± 1.4
	3.7×10^{-5}	68.1 ± 0.5
SIBA	5.0×10^{-4}	58.0 ± 1.0
	2.0×10^{-5}	40.0 ± 0.5
Ionophore A23187	3.8×10^{-7}	73.5 ± 4.6
	3.8×10^{-8}	69.7 ± 3.3
Mepacrine	2.0×10^{-6}	59.6 ± 7.6
	5.0×10^{-7}	32.4 ± 4.0
Mellitin	3.5×10^{-7}	Toxic
	1.8×10^{-7}	11.1 ± 3.6
	3.5×10^{-8}	20.0 ± 3.5
Cycloheximide	1.2×10^{-6}	70.8 ± 0.3
DbcAMP	1.0×10^{-3}	64.6 ± 1.3
IBMX	0.9×10^{-3}	64.2 ± 3.6
	0.9×10^{-4}	36.2 ± 1.8

With the exception of mellitin, none of the chemicals at the concentrations used here was toxic to the cells. Each value represents the mean ± standard deviation of triplicate samples.

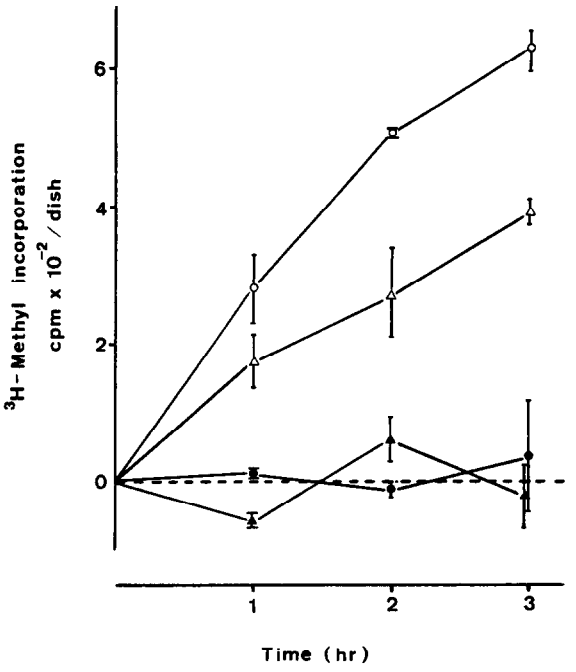

Fig.1. Inhibitory effects of AdoHcy and 3-deazaAdo on the incorporation of [^{3}H]methyl groups into phospholipids. Neuroblastoma cells were incubated with L-[*-methyl-*^{3}H]methionine in the presence of either 2.4 × 10^{-3} M AdoHcy (•) or 3.7 × 10^{-4} M 3-deazaAdo (▲). Controls are given as corresponding open symbols. Results expressed as the difference from the value at time zero represent the mean ± standard deviation of triplicate samples. The [^{3}H]methyl incorporations at time zero were 275 ± 5 cpm/dish in the experiment for AdoHcy and 218 ± 15 cpm/dish in that for 3-deazaAdo.

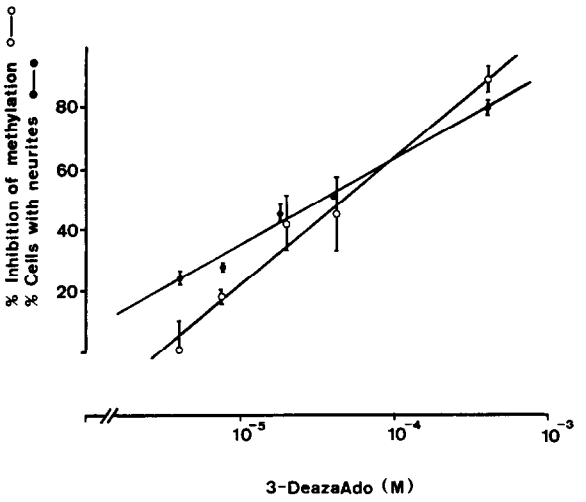

Fig.2. Inhibition of phospholipid methylation (○—○) and neurite outgrowth (•—•) induced by 3-deazaAdo. Mouse neuroblastoma cells were treated with various concentrations of 3-deazaAdo for 2 h. The incorporation of [^{3}H]methyl groups into the phospholipid fraction and the extent of neurite outgrowth were determined as in section 2. In the absence of inhibitor the [^{3}H]methyl incorporation and the percentage of cells with neurites were 420 ± 60 cpm/dish and 22 ± 1%, respectively. Each point is the mean ± standard deviation of triplicate samples.

cubated neuroblastoma cells with L-[*methyl-*^{3}H]-methionine under the conditions of the neurite outgrowth assay and monitored phospholipid methylation by measuring radioactivity incorporated into the phospholipid fraction. Both AdoHcy (2.4 × 10^{-3} M) and 3-deazaAdo (3.7 × 10^{-4} M) completely prevented incorporation of methyl groups into phospholipids at the concentrations at which they brought an optimal neurite extension (fig.1). The correlation of the inhibition of phospholipid methylation with the induction of neurite outgrowth was more closely examined. Neuroblastoma cells were incubated for 2 h with various concentrations of 3-deazaAdo. The incorporation of [^{3}H]methyl groups into phospholipid and the extent of neurite outgrowth were then determined (fig.2). 3-DeazaAdo affected both events with the same dose–response. The generality of the observed relationship between inhibition of phospholipid methylation and neurite outgrowth was also studied. The short time required to promote neurite extension in our assay condition made it possible to test the effect of substances which could elicit many secondary effects when applied for longer times. We selected various agents known to affect cellular biochemical events in a number of ways and tested their ability to promote both neurite extension and interference with phospholipid methylation in neuroblastoma cells. The calcium ionophore A23187, the antimalarial drug mepacrine (a phospholipase A_2 inhibitor), dbcAMP and IBMX (agents that increase intracellular cyclic AMP content), and cycloheximide (a protein synthesis inhibitor) were chosen for this purpose (table 1 and fig.3). Low concentrations of ionophore A23187 induced ~70% of neuroblastoma cells to extend neurites as in [17]. Phospholipase A_2 activity has been linked to phospholipid methylation [4,8,9]. It is thus interesting that 2.0 × 10^{-6} M mepacrine

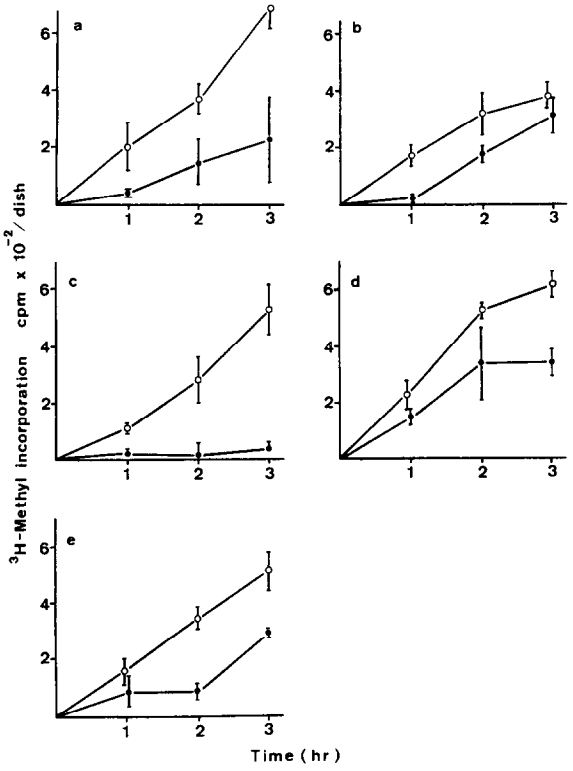

Fig.3. Effects of various chemicals on the incorporation of [^{3}H]methyl groups into phospholipids: (•) experimental; (○) control data. Each point represents the mean ± standard deviation of triplicate samples: (a) 3.8×10^{-8} M ionophore A23187; (b) 2.0×10^{-6} M mepacrine; (c) 1.2×10^{-6} M cycloheximide; (d) 10^{-3} M dbcAMP; (e) 0.9×10^{-3} M IBMX.

was as effective as the transmethylase inhibitors tested in causing neurite outgrowth. By contrast mellitin, a phospholipase A_2 activator, appeared not to have any marked effect on cell morphology (table 1). These results could be the first indication for an involvement of phospholipase A_2 activity in neurite extension. Substances which elevate intracellular cyclic AMP levels promote neurite extension [1,2]. Using our assay, we confirmed that both dbcAMP and IBMX were able to stimulate >60% of cells to form neurites (table 1). An increase in cyclic AMP levels alone, however, does not seem necessary for the initiation of changes in cell morphology. For example, a macromolecular factor released from glial cells promotes neurite outgrowth without significantly affecting intracellular cyclic AMP levels [12].

Cycloheximide antagonizes the neurite formation induced by dbcAMP [18], whereas cycloheximide alone, when applied in serum-free medium, promotes neurite extension [18]. Under our assay conditions 1.2×10^{-6} M cycloheximide induced 70% of cells to grow neurites. The ways in which phospholipid methylation was affected by these chemicals are summarized in fig.3. At concentrations at which neurite outgrowth was efficiently stimulated, every substance caused a suppression of the incorporation of [^{3}H]methyl groups into phospholipids, even though there were some differences in time course and degree of inhibition. The inhibitory effect of the ionophore A23187 (3.8×10^{-8} M) seems to last ≥ 3 h (fig.3a), whereas the inhibition caused by 2.0×10^{-6} M mepacrine apparently disappears with time (fig.3b). Cycloheximide strikingly interfered with phospholipid methylation (fig.3c). Both dbcAMP and IBMX acted on phospholipid methylation in an inhibitory manner, but their effects were kinetically distinct (fig.3d,e).

4. DISCUSSION

The mechanisms by which neuroblastoma cells are stimulated to form neurites have not been unequivocally identified. Here, we show that inhibitors of AdoMet-linked methylation efficiently induce neurite outgrowth in parallel with their inhibition of phospholipid methylation in neuroblastoma cells. The dose-dependent effect of 3-deazaAdo has not only confirmed a link between both events but also provided indication that neuroblastoma cells might require the inhibition of phospholipid methylation in order to extend neurites. Numerous substances are known to induce neurite outgrowth in neuroblastoma cells. We have selected some of these and found all of them to inhibit phospholipid methylation. This indicates that the correlation between neurite outgrowth and the inhibition of phospholipid methylation is not restricted to typical methylation inhibitors. The inhibition of phospholipid methylation seems rather to be a phenomenon closely linked to the growth of neurites in neuroblastoma cells.

Neurite extension induced by cycloheximide might be attributed to a decrease in activities of crucial methyltransferases as a consequence of the general inhibitory effect on protein synthesis. Our

observations that the ionophore A23187, mepacrine, dbcAMP and IBMX affected phospholipid methylation in neuroblastoma cells are consistent with the reports showing a close link between calcium influx [6,19], phospholipase A_2 activity [4,8,9], cyclic AMP levels [19] and phospholipid methylation in different types of cells. Further studies are necessary to determine whether, in neuroblastoma cells, these biochemical steps act independently or in concert with each other.

In [20], addition of nerve growth factor (NGF) to explants of rat superior cervical ganglia caused a transient increase of the phospholipid methylation localized in the neurites. These findings, when analyzed in detail, do not seem incompatible with our observations. The explants of rat superior cervical ganglia are incubated first for 5 days with NGF. They are then starved of NGF and serum prior to and during the [*methyl*-^{3}H]methionine labeling. The transient increase in phospholipid methylation detected only in the neurites during the 30 s following a new addition of NGF could reflect, for example, a biochemical event related to the uptake of NGF and not to the growth of the neurites. In fact the very short incubation time used in such experiments does not allow phospholipid methylation to be linked to neurite outgrowth. Our experiments monitor the methylation of phospholipids in neuroblastoma cells which have never extended neurites before being triggered. Further, addition of glial conditioned medium known to efficiently induce neurite extension in those cells [12] does not seem to affect phospholipid methylation (not shown). This would indicate that inhibition of phospholipid methylation is not a prerequisite for neurite outgrowth.

We cannot rule out that other methylation reactions (for example, protein methylation) are also affected by the methylases inhibitors or by the substances inducing neurite outgrowth. Nevertheless, our results demonstrate that inhibition of Ado-Met-linked methylation induces neurite outgrowth and many different substances known to trigger this morphological change also inhibit phospholipid methylation in intact cells. Therefore, they suggest that with certain inducers, this biochemical event is closely linked to the membrane changes that must occur prior to or during the growth of neurites.

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