Effect of sodium butyrate in combination with prostaglandin E_1 and inhibitors of cyclic nucleotide phosphodiesterase on human amelanotic melanoma cells in culture[1]

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Summary. Sodium butyrate and cyclic AMP-stimulating agents (prostaglandin E_1, papaverine, theophylline, and RO20-1724) caused reductions in the cell number (primarily due to reduction in cell division) when added individually to human melanoma cells in culture. However, the combination of sodium butyrate with one of the cyclic AMP-stimulating agents produced a marked reduction in cell number (primarily due to cell death).

An elevation of the intracellular level of adenosine 3':5'-cyclic monophosphate (cyclic AMP) by prostaglandin E_1, inhibitors of cyclic nucleotide phosphodiesterase, or by analogs of cyclic AMP in certain clones of mouse and human neurobalstoma cells increases the expression of several morphological and biochemical differentiated functions which are characteristic of mature neurons[2]. These include formation of long neurites, increase in size of soma and nucleus associated with a rise in total RNA and protein, maintenance of cells in the G1 stage of the cell cycle, increase in activities of specific neural enzymes, increase in stimulation of adenylate cyclase activity by catecholamines. Sodium butyrate causes extensive cell death and produces morphological and biochemical differentiation in human neuroblastoma cells in culture[3]. These studies have led us to suggest that the addition of sodium butyrate and cyclic AMP-stimulating agents to the currently used chemotherapeutic protocols may increase their effectiveness in the treatment of human neuroblastomas. Papaverine, an inhibitor of cyclic nucleotide phosphodiesterase, has been added in the treatment protocol for human neuroblastomas[4]. Sodium butyrate has been used clinically first by Dr Tom Voûte (Spinozastraat 51, Postgiro 2388, Amsterdam) and then by Dr L. Furman Odum (Children's Hospital in Denver, personal communication). Although the clinical value of sodium butyrate cannot be evaluated at this time, high doses (7-10 g per day) of sodium butyrate produce no clinically detectable toxic effect in patients with neuroblastomas.

Cyclic AMP-stimulating agents[5-11] inhibit the growth rate, enhance the melanogenesis, and produce morphological alterations in mouse and human melanoma cells in culture, whereas sodium butyrate reduces the growth rate without causing any change in pigmentation[5]. However, the therapeutic significance of these findings was not apparent, because the cell death was not observed and the growth inhibition was reversible. Because of the potential usefulness of sodium butyrate, we wondered if it, in combination with one of the cyclic AMP-simulating agents, would be effective in producing cell death. We now report that the combination of sodium butyrate with one of the cyclic AMP-stimulating agents causes an extensive cell death in human amelanotic melanoma cells in culture, whereas the individual drugs produced no such effect.

Materials and methods. A human melanoma cell line (amelanotic) was obtained from Dr Giovanella of Houston, Texas. These cells were grown in Falcon flasks or dishes containing MEM with 10% heat-inactivated fetal calf serum, penicillin (100 µg/ml) and streptomycin (100 U/ml) and were maintained at 37 °C in a humidified atmosphere of 5% CO_2. The effect of sodium butyrate and cyclic AMP-stimulating agents on human melanoma cells in culture was studied. Three cyclic nucleotide phosphodiesterase inhibitors, 4-(-3-butoxy-4-methoxybenzyl)-2-imidazolidinone (RO20-1724), papaverine, and theophylline were used. In addition, prostaglandin E_1 (PGE_1), a stimulator of adenylate cyclase, was also used.

To study the effect of drugs on growth, cells (100,000) were plated in Falcon plastic dishes (60 mm) and sodium butyrate (1 mM), RO20-1724 (0.7 mM), theophylline (1 mM), papaverine (25 µg/ml), and PGE_1 (15 µg/ml) were added separately or in combination with sodium butyrate 24 h after plating. 1 set of control cultures received no treatment and another set of control cultures was treated with an equivalent volume of ethyl acohol (final concentration 0.5%) or water. The growth medium and drug were changed every other day. The cell number was counted by a hematocytometer at 6 days after drug treatment. Cells were removed from the dish surface by incubating them in the presence of 1% trypsin solution for 45 min. The relatively long incubation time was needed in order to obtain single cell suspensions for the purpose of counting.

To study the reversibility of drug effect, fresh growth medium without drug was added at 6 days after treatment, and the culture was observed for 2 weeks. To determine the viability of cells at the time of counting, the attached cells were incubated in the presence of 0.1% trypan blue in saline for 2 min, and the percent of cells without staining was determined.

Results and discussion. The table shows that sodium butyrate by itself reduced the cell number to 28±3% of control. Among phophsodiesterase inhibitors, theophylline and RO20-1724 were equally effective in reducing the cell number; however, papaverine was least effective (53±4% of control). PGE_1 was also relatively less effective (57±4% of control) in reducing the cell number. There was no significant cell death when the culture was treated with the individual agent as apparent by the observation that there were no floater cells in the medium. Therefore, the reduction in cell number in culture treated with the individual drug was primarily due to inhibition of cell division. The combination of sodium butyrate with one of the cyclic

Effect of sodium butyrate in combination with prostaglandin E_1 and inhibitors of cyclic nucleotide phosphodiesterase on the growth of human melanoma cells in culture*

Treatment	% of control cells
Sodium butyrate (1 mM)	28 ±4**
Theophylline (1 mM)	12 ±3
Sodium butyrate + theophylline	1.8±0.3
RO20-1724 (0.7 mM)	15 ±3
Sodium butyrate + RO20-1724	2.9±0.4
Papaverine (25 µg/ml)	53 ±7
Sodium butyrate + papaverine	11 ±1.5
Prostaglandin E_1 (15 µg/ml)	57 ±9
Sodium butyrate + prostaglandin E_1	9.6±1.6

* Cells (100,000) were plated in Falcon plastic dishes (60 mm), and the drug was added 24 h after plating. The drug and medium were changed every other day. The cell number was counted at 6 days after treatment by a hematocytometer. The number of cells in treated culture was expressed as percent of control. The control culture had 7.3×10^5 cells at 6 days after plating. Each value represents an average of 5-6 samples. ** Standard deviation.

AMP-stimulating agents was much more potent in reducing the cell number of melanoma cells in culture than that produced by the individual agent. This was due primarily to an extensive cell death as evidenced by the increased number of floater cells in the medium. The most effective combination was when the cells were treated with either sodium butyrate and RO20-1724 or sodium butyrate and theophylline. On the removal of drug 6 days after treatment the cells renewed cell division and eventually became confluent irrespective of treatment.

The treatment of human amelanotic melanoma cells in culture with sodium butyrate and cyclic AMP-stimulating agents produced varying degrees of morphological alterations. Control cells were large with one or more cytoplasmic processes. Their cellular boundaries were difficult to identify during the growth period and finally became completely fused with each other at confluency. Theophylline and RO20-1724 caused a marked morphological change as evidenced by the increased length of cytoplasmic processes. The entire cell appeared elongated. However, papaverine did not produce such a change; PGE_1 did induce morphological alterations in some cells, but the shape of most cells remains similar to that observed in control culture. Sodium butyrate caused flattening of cells, but the cytoplasmic processes were similar to those seen in control culture.

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2 K.N. Prasad, Biol. Rev. *50*, 129 (1975).
3 K.N. Prasad and P.K. Sinha, In Vitro *12*, 125 (1975).
4 L. Helson, CA *25*, 264 (1975).
5 L. Helson, K. Lai and C.W. Young, Molec. Pharmac. *23*, 2917 (1974).
6 G.S. Johnson and I. Pastan, Nature New Biol. *237*, 267 (1972).
7 J.W. Krieder, M. Rosenthal and N. Lengle, J. natl Cancer Inst. *50*, 555 (1973).
8 J.W. Krieder, D.R. Wade, M. Rosenthal and T. Densley, J. natl Cancer Inst. *54*, 1457 (1975).
9 E. O'Keefe and P. Cautrecasas, Proc. natl Acad. Sci., USA *71*, 2500 (1974).
10 M.L. Steinberg and J.R. Whittaker, J. Cell Physiol. *87*, 265 (1976).
11 G. Wong and J. Pawelek, Nature New Biol. *241*, 218 (1973).

A response to monoamines in *Peripatopsis moseleyi* (Onychophora)

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Summary. Preparations of longitudinal muscle respond to catecholamines if the ventral nerve cords are present, and also to 5-hydroxytryptamine. The response to 5HT is complex if the central nervous system is present, but muscle alone is probably inhibited.

The Onychophora are primitive terrestrial arthropods, placed together with myriapods and insects in the phylum Uniramia[3,4]. Physiological knowledge of their nervous system[5,6] and distinctive smooth muscle[7-9] is incomplete. Preparations of longitudinal muscle react to acetylcholine but not to glutamate, gamma-amino butyric acid, picrotoxin or noradrenaline[10,11], suggesting that the motor innervation is cholinergic. In these pharmacological properties Onychophora resemble annelids rather than arthropods. We wished to test further the effects of monoamines.

Materials and methods. Etherized *Peripatopsis moseleyi* were dissected longitudinally. 7 ventral preparations were made of body wall longitudinal muscle including the central nervous system, and 3 with dorsal longitudinal muscle only. A preparation was suspended in 30 ml of Ringer solution[12,13] of pH 7.3, at room temperature (19.5–21 °C), and aerated with 95% O_2, 5% CO_2. Tension was 100–400 mg and contractions were recorded via a Washington T1 isotonic transducer.

The majority (6/7) of ventral preparations showed slow variations in baseline tone with irregular rhythmical contractions (3–8/min) superimposed. The latter are referred to as spontaneous or rhythmical activity. Dorsal preparations without the nerve cords had a stable baseline tone and showed little spontaneous activity.

Test drug solutions were added to the bath by syringe (0.1–0.5 ml). A 15–60 sec contact time was used for acetylcholine responses and 2–5 min for the monoamines. The preparations were washed by overflow and at least 5 min were allowed between doses.

Results. All preparations responded to acetylcholine (ACh: $3\text{–}30\times10^{-6}$ g/ml) with a single smooth contraction. Eserine ($0.8\text{–}1.5\times10^{-6}$ g/ml, 5 applications) evoked similar but longer-lasting contractions. ACh responses were markedly potentiated (mean greater than 200 times) when tested immediately after recovery of previous baseline tone following an eserine response.

Ventral preparations responded to noradrenaline (NA) and dopamine (DA). NA ($5\text{–}80\times10^{-6}$ g/ml, 13 applications in 5 preparations) markedly increased tone, and this continued so long as the drug was in the bath (up to 5 min). DA ($40\text{–}80\times10^{-6}$ g/ml, 13 applications in 6 preparations) raised the tone to a smaller extent but induced repeated or rhythmical contractions. The DA responses tended to reverse within 3–5 min, before the drug had been removed. Dorsal preparations did not respond to NA (up to 16×10^{-5} g/ml, 4 applications) or to DA (up to 24×10^{-5} g/ml, 4 applications).

With ventral preparations 5-hydroxytryptamine (5HT, $10\text{–}100\times10^{-6}$ g/ml) caused a response resembling that to DA in 8 applications, but in 2 applications the existing tone and spontaneous activity were reduced. In 6 other applications both kinds of effect were combined: after an initial increase in tone and spontaneity, both declined. 5HT affected only one of the 3 dorsal preparations and the response consisted of a reduction in tone.

Application of the monoamine oxidase inhibitor iproniazid to 2 ventral preparations ($2\text{–}5\times10^{-4}$ g/ml) evoked an increase in tone and spontaneous activity whilst no re-