On a Possible Relationship of Cyclic AMP to the Mechanism of Action of Nerve Growth Factor (36392)

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(Introduced by E. B. Taft)

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Several recent studies have shown that N^6, O^2-dibutyryl-3′,5′-cyclic adenosine monophosphate (dibutyryl cyclic AMP) can transform cells of various tumor lines so that they take on a more normal structural appearance. The initial observations were made by Hsie and Puck (1) using Chinese hamster ovarian tumor cells and by Johnson, Friedman, and Pastan (2) using rat sarcoma and neoplastic mouse fibroblast lines.

Similar observations have been made with tumors of nervous tissue origin. For example, morphologic changes in glioblastoma cells under the influence of dibutyryl cyclic AMP have been reported by Perkins and MacIntyre (3), and neuroblastoma cells have been shown to differentiate, as judged by neurite extension, following exposure to dibutyryl cyclic AMP (4, 5).

An effect of dibutyryl cyclic AMP on the morphology of nonneoplastic mammalian nervous tissue has not yet been reported. The studies presented below reveal that treatment of fetal mammalian sensory ganglia with dibutyryl cyclic AMP produces increased neurite outgrowth and that this outgrowth mimics that observed in cultures exposed to nerve growth factor (NGF). After the work reported here was completed, we learned that Roisen *et al.* (6) had made similar observations with chick embryo ganglia.

Methods. Gasserian ganglia from rat or mouse fetuses, 17–19 days *in utero*, were placed on collagen-coated coverslips in Maximow double coverslip assemblies, as described earlier (7). A drop of F-10 feeding medium (Gibco) containing 25% fetal calf serum and 750 mg of glucose/100 ml was added, and the cultures were incubated at 35° for 24 hr. Dibutyryl cyclic AMP (Sigma) was prepared as a 50 m*M* stock solution in F-10, frozen in aliquots, and thawed and diluted for use as needed. All growth media containing dibutyryl cyclic AMP contained fetal calf serum and glucose at the concentrations given above. Cultures given NGF (3 units/ml, Burroughs Wellcome Co.) were maintained in F-10 medium containing 10% fetal calf serum. Controls for the NGF experiments were fed F-10 containing 10% fetal calf serum. Cultures were studied with the light microscope at various intervals and always at 24 hr, following which they were fixed in 10% formalin and stained by Romanes (8) silver method for axons.

Results. Rat and mouse gasserian ganglia treated with 0.5, 1.0, 5, or 10 m*M* dibutyryl cyclic AMP consistently exhibited longer neurites than did controls (Fig. 1). At 10^{-1} m*M* only occasional cultures showed enhanced neurite extension and at 10^{-2} and 10^{-3} m*M* there was no difference from controls. In the presence of 25 m*M* nucleotide, no cultures showed longer neurite extension than controls.

To facilitate a comparison each of the ganglia treated with 1 m*M* dibutyryl cyclic AMP was randomly matched with a control ganglion. Twenty-seven had longer neurite extensions than did their controls; 4 had questionably longer extensions; and 1 had less.

A semiquantitative measurement of neurite extension was obtained by dividing photo-

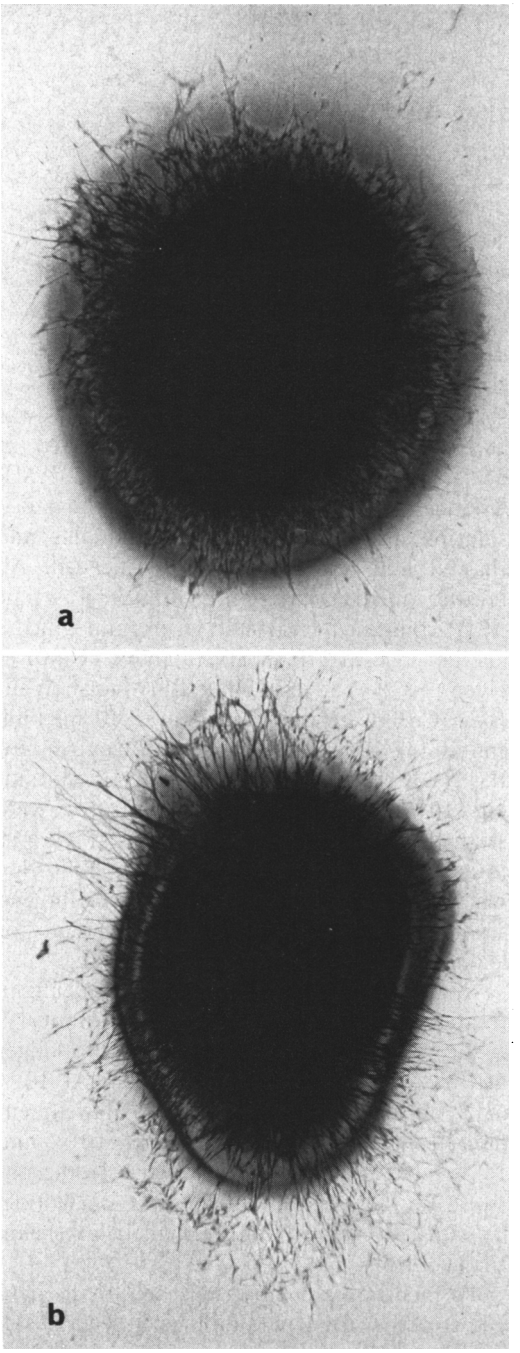

FIG. 1. Photomicrographs of: (a) control fetal rat ganglion, and (b) fetal rat ganglion treated with 1 m*M* dibutyryl cyclic AMP for 24 hr at 35°. The latter exhibits longer neurite extensions, but fewer nonneural cells have migrated out from the explant. The dark ring about each ganglion is an artifact which is probably due to deposition of silver in the collagen matrix. Magnification, 37×; stained with Romanes silver stain.

graphs of individual ganglia into quadrants and selecting the two longest neurites in each quadrant. The average neurite length for four control cultures in a typical experiment was 488 μ while the average for four cultures treated with 1 m*M* dibutyryl cyclic AMP was 674 μ. This represents a 38% increase in neurite extension.

A decrease in migration of nonneural cells from the explants was associated with the enhanced neurite extension. This effect was marked at 1, 5, 10, and 25 m*M* concentrations of the nucleotide, was still present to a lesser degree at 10^{-1} m*M* and disappeared at 10^{-2} m*M*. Outgrowth of all cell types (amebocytes, fibrocytes and spindle-shaped Schwann cells) seemed to be diminished.

Fourteen of 14 NGF-treated cultures showed more extensive neurite outgrowth at 24 hr than did 14 matched controls at 24 hr. The morphologic findings were comparable to those obtained with dibutyryl cyclic AMP.

Discussion. These observations reveal that neurites elongate faster on exposure to an optimal concentration of dibutyryl cyclic AMP than they do otherwise. This acceleration of neurite outgrowth observed *in vitro* raises the question whether cyclic AMP might not play a physiologic role in the maturation of nervous tissue in the intact animal. Also might nerve regeneration *in vivo* be accelerated by cyclic AMP?

Fetal mouse cultures in these experiments have shown a response to NGF directly comparable to that observed with dibutyryl cyclic AMP. In general, studies of NGF action *in vitro* have been performed using less mature sensory ganglia than those studied by us. Indeed, chicken and mouse sensory ganglia taken late in gestation have been reported to be refractory to NGF (9), an observation seemingly in conflict with that reported here. The difference may be more apparent than real. Most prior studies have utilized media containing embryo extract. In our studies, if embryo extract was included in the feeding medium, the NGF effect was obscured. It should be emphasized that fetal mouse cultures, whether treated with NGF or not, (provided they are on collagen-coated coverslips) maintain viability in F-10 medium supplemented with fetal calf serum and are actively engaged in synthesis of microtu-

bular protein after 24 hr *in vitro* (10).

Preliminary studies with cycloheximide suggest that protein synthesis is necessary for neurite extension in our culture system. Addition of 0.2 or 0.07 m*M* cycloheximide to the feeding media markedly decreased neurite and cellular outgrowth in comparison with controls. Dibutyryl cyclic AMP (1 m*M*) failed to reverse the cycloheximide effect. This result parallels that observed with NGF which has been shown to be unable to exert its effect in the presence of cycloheximide (11).

Since NGF is a high molecular weight protein it may act extracellularly upon the cell membrane. It is worth recalling that adenyl cyclase, the membrane-associated enzyme responsible for formation of cyclic AMP from ATP, is activated by macromolecular hormones (e.g., glucagon) when they react with cell surface receptors. It is possible, therefore, that NGF might operate through the intermediary of adenyl cyclase and cyclic AMP.

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