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Effect of the Suspected Tumor Promoters Saccharin, Cyclamate, and Phenol on Nerve Growth Factor Binding and Response in Cultured Embryonic Chick Ganglia[1]

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ABSTRACT

The suspected bladder tumor promoters saccharin and cyclamate reversibly inhibited nerve growth factor-induced neurite outgrowth in embryonic chick sensory ganglia, and active concentrations of these artificial sweeteners inhibited binding of ^{125}I-labeled mouse submaxillary gland nerve growth factor as well. The skin tumor promoter phenol also reversibly inhibited neurite outgrowth, while comparable concentrations of the nonpromoting but structurally related compounds benzene and fluorobenzene did not. However, in contrast to findings with saccharin and cyclamate, phenol had little, if any, effect on binding of radioactive nerve growth factor.

INTRODUCTION

Neuronal topography is an important element in nerve cell function, and a better understanding of the processes regulating neurite arborization may contribute to a clearer perception of how the remarkable architecture of higher nervous centers is ordered. An interesting property of NGF[2] is that it can stimulate neurite outgrowth from embryonic vertebrate sensory (9) and sympathetic (33) ganglia. Reviews discussing the chemistry of NGF and its effects on the growth and development of the vertebrate nervous system are available (6, 21, 32, 36, 52). Antagonists could prove useful in studies on the mechanism of NGF action. During the search for antagonists, we found that TPA and other macrocyclic plant diterpene esters that are tumor promoters in the 2-stage mouse skin carcinogenesis system (4, 20, 51) were reversible inhibitors of neurite outgrowth, while nonpromoting structural congeners were not (26, 28).

This study was initiated for 2 reasons. One was to determine whether inhibition of neurite outgrowth is a property common to other non-diterpene ester tumor promoters, because not all suspected promoters are active in the same test system. For example, bile acids are promoters in the colon carcinogenesis system (13, 40) but unexpectedly reduce the incidence of tumors induced by benzo(*a*)pyrene in mouse skin (55). Also, while phorbol ester promoters elevate plasminogen activator activity (56) and decrease activity of large external transformation-sensitive glycoprotein (17) in chick embryo fibroblast cultures, other chemical classes of promoters were found to not always share these effects. The second purpose was to test whether tumor promoters could inhibit morphological differentiation by effects on the receptor system regulating neurite outgrowth.

The results are that the suspected bladder tumor promoters saccharin (1, 3, 8, 12, 16, 18, 23, 24, 38, 41, 47) and cyclamate (24, 29, 38, 46) are reversible inhibitors of neurite outgrowth in cultured DRG and that active concentrations of these artificial sweeteners inhibit binding of ^{125}I-labeled NGF. Comparable concentrations of sucrose and glucose were inactive in both respects. The weak skin tumor promoter phenol (5) was also studied.

MATERIALS AND METHODS

Materials. Sodium saccharin and sodium cyclamate were obtained from Sigma Chemical Co., St. Louis, Mo. Phenol was purchased from J. T. Baker Chemical Co., Phillipsburg, N. J. Other chemicals were of the best commercially available grades.

Ganglia Culture and Bioassay. The procedures for ganglia culture and bioassay have previously been described (26). Briefly, 7- to 10-day-old DRG were explanted onto rat tail collagen-coated plastic wells and incubated in RPMI 1640 containing bovine serum albumin (1 mg/ml) and the β subunit of NGF (2 ng/ml). DRG response was scored on an arbitrary scale of 0 to 5 in which the maximum score was given for a symmetrical halo of nerve fibers when the fiber length exceeded the explant diameter.

Preparation and Iodination of NGF. The β subunit of NGF was prepared from male mouse saliva (10, 53, 54); purity was confirmed by the presence of a single band on isoelectric focusing (pH 3.5 to 10) in 7.5% polyacrylamide gels. Purified β-NGF was iodinated with lactoperoxidase (22).

Binding of ^{125}I-labeled NGF. Assay of binding to intact cells obtained from dissociated DRG closely followed procedures described by others (48), except that cells (2×10^6/ml) were incubated in RPMI 1640 containing bovine serum albumin (5 mg/ml) at 37°. Samples were centrifuged for 30 sec in 400-μl plastic needlenose tubes (Walter Sarstedt, Inc., Princeton, N. J.) in a Beckman Model 152 Microfuge (Beckman-Spinco Instruments, Inc., Palo Alto, Calif.) The microcentrifuge tubes were rapidly frozen in a dry ice-acetone bath, and the tips containing the cell pellet were severed (volume, about 2 μl) and monitored for radioactivity content in a gamma scintillation detector.

RESULTS

Effect of Saccharin and Cyclamate on Neurite Outgrowth. DRG cultured in the presence of an optimum concentration of NGF will extend a dense halo of neurites such that the maximum score is attained in about 1 day, whereas in the absence of NGF, neurite outgrowth is undetectable and many neuronal cells do not survive. The presence of the artificial sweetening agents saccharin and cyclamate inhibited neurite outgrowth in

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[2] The abbreviations used are: NGF, mouse submaxillary gland nerve growth factor (the β subunit); TPA, 12-*O*-tetradecanoylphorbol-13-acetate; DRG, embryonic chick dorsal root ganglia; RPMI 1640, Roswell Park Memorial Institute Medium 1640.

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DRG scored at 1 day. However, cultures in comparable concentrations of sucrose and glucose were unaffected (Table 1A). In other experiments, 50 mM NaCl did not inhibit DRG response to NGF, and thus the saccharin and cyclamate effect is not simply an osmotic action. Mice fed high concentrations of sucrose do not produce tumors (42). DRG scores in the continued presence of saccharin and cyclamate were somewhat improved on the second day, suggesting either that inhibition is transient or that neurite outgrowth continues at a reduced rate. However, when the compounds were washed out after 1 day, DRG scores on the second day were not significantly different from those of control cultures (Table 1B), and neurite outgrowth was fully reversible. The relationship between artificial sweetener concentration and inhibition of neurite outgrowth is shown in Chart 1. The onset of inhibition occurred at about 6 to 10 mM; DRG scores were reduced to the half-maximal value at about 33 and 50 mM cyclamate and saccharin, respectively.

To determine whether saccharin and cyclamate could cause established neurites to retract, ganglia were incubated for 1 day with only NGF, and then compounds were added. No detectable decrease in DRG scores was seen on the following day (Table 2). The length of neurites was also measured, and

Table 1

Inhibition of neurite outgrowth by artificial sweetners and recovery after washout in cultured DRG

DRG were cultured in the presence of NGF (2 ng/ml). Cultures in Part A were incubated with the compounds shown, and the extent of neurite outgrowth was scored at Days 1 and 2. Cultures in Part B were incubated with the compounds shown for 1 day and scored. Then, the cultures were washed, and the incubations were continued in the presence of only NGF (2 ng/ml) for another day and scored.

	Compounds	Ganglia response[a] Day 1	Day 2
A.	Control	4.8 ± 0.4[b]	5.0 ± 0.0
	Saccharin (48.8 mM)	0.3 ± 0.5	0.8 ± 0.7
	Cyclamate (55.6 mM)	1.7 ± 0.5	2.8 ± 0.7
	Sucrose (50 mM)	4.5 ± 0.5	4.9 ± 0.3
	Glucose (50 mM)	4.8 ± 0.4	5.0 ± 0.0
B.	Control	4.7 ± 0.2	5.0 ± 0.0
	Saccharin (48.8 mM)	1.0 ± 0.1	4.5 ± 0.3
	Cyclamate (55.6 mM)	2.2 ± 0.3	4.9 ± 0.1

[a] Ganglia were scored on an arbitrary scale ranging from 0 to 5.
[b] Mean ± S.E. (A, $N = 12$; B, $N = 10$).

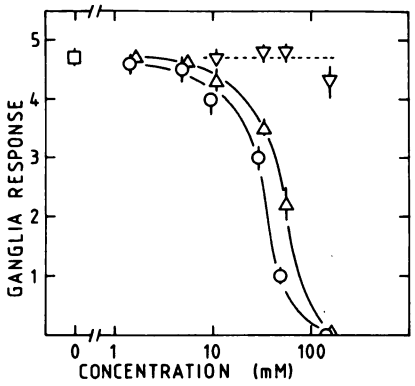

Chart 1. Relationship between sweetener concentration and response. DRG were incubated for 1 day on rat tail collagen-coated wells containing RPMI 1640, bovine serum albumin (1 mg/ml), NGF (2 ng/ml), and the indicated concentrations of sweeteners. The ganglia responses were scored. Values are means ($N = 10$); *bars*, S.E. □, control; ▽, glucose; ○, cyclamate; and △, saccharin.

Table 2

Effect of saccharin and cyclamate on established neurites

Eight-day-old DRG were cultured for 1 day with NGF (2 ng/ml), and ganglia response was scored. Neurite length from the edge of the explant to the growing tip was also measured. The sweetening agents were then added to some cultures, and the incubations were continued for another day and scored for both ganglia response and neurite length.

Compounds	Day 1 Ganglia response[a]	Day 1 Neurite length (mm)	Day 2 Ganglia response	Day 2 Neurite length (mm)
Control	4.8 ± 0.4[b]	0.54 ± 0.07	5.0 ± 0.0	1.98 ± 0.33
Saccharin (48.8 mM)			4.5 ± 0.5	0.63 ± 0.09
Cyclamate (55.6 mM)			5.0 ± 0.0	0.88 ± 0.12

[a] Ganglia were scored on an arbitrary scale ranging from 0 to 5.
[b] Mean ± S.E. ($N = 10$).

the control length at 1 day was close to that expected based on an elongation rate of about 20 μm/hr (45). The nature of the ganglia-scoring system is such that a maximum score is given when the neurite length is equal to or greater than the explant diameter. While the maximum score may be attained in about 1 day, neurite growth continues as seen by the increment in length measured on Day 2. The small improvement in neurite length between Days 1 and 2 in the presence of saccharin and cyclamate suggests that the nature of the inhibition is one in which the rate of fiber outgrowth is greatly reduced but not abolished. It will be shown below that saccharin and cyclamate inhibit binding of NGF. The finding that inhibitors of NGF binding can diminish the rate of neurite outgrowth but do not cause retraction of established neurites suggests that structural elements once formed are relatively persistent. DRG neurites indeed do persist without retraction for 1 or 2 days following washout of NGF (35).

Effect of Saccharin, Cyclamate, and Phenol on Binding of ^{125}I-labeled NGF. The possibility that saccharin and cyclamate inhibit binding of radioactive NGF was tested (Chart 2). The time course of ^{125}I-labeled NGF binding was the same as that reported by Sutter *et al.* (48). Saccharin and cyclamate inhibited the amount of cell-bound radioactivity by about 61 and 64%, respectively. These concentrations of sweeteners caused a substantial decrease in DRG response (Table 1). In other studies (data not shown), sucrose (50 mM) and glucose (50 mM) did not alter binding. NaCl (50 mM) did slightly decrease binding to 3.53 ± 0.09 (S.D.) × 10^3 cpm/10^6 cells as compared to untreated controls (4.10 ± 0.08) but could not explain the substantial inhibition (1.61 ± 0.15) by sodium saccharin (50 mM). Also, all of these findings could be confirmed in RPMI 1640 in which the total Na^+ concentration and the osmolality was kept constant.

TPA inhibits neurite outgrowth but does not inhibit binding of ^{125}I-labeled NGF (26). Therefore, for purposes of further comparison, the effect of phenol, a weak nonphorbol promoter on mouse skin (5), was studied. Phenol (4 mM) was a reversible inhibitor of morphological differentiation, but the same concentration of the nonpromoting congeners benzene and fluorobenzene (5) were inactive (Table 3). However, while saccharin and cyclamate were again found to substantially inhibit ^{125}I-labeled NGF binding, the effect of phenol (3.2 mM), if any, was marginal (Table 4). Another binding experiment with 4 mM phenol, benzene, and fluorobenzene confirmed these results and showed no differences in the amount of radioactive NGF bound relative to control incubations (data not shown). Concentrations

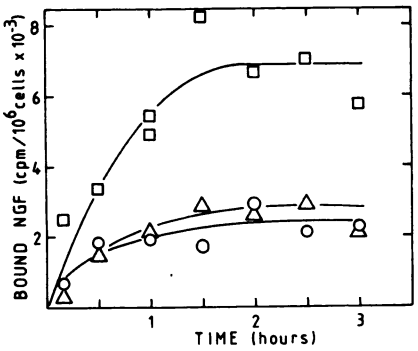

Chart 2. Effect of cyclamate and saccharin on the time course of ^{125}I-labeled NGF binding. A cell suspension obtained from 8-day-old DRG was incubated at 37° in RPMI 1640 containing bovine serum albumin (5 mg/ml), ^{125}I-labeled NGF (specific activity, 58 cpm/pg, 10 ng/ml), and no additions (□) or with cyclamate (55.6 mM) (○) or saccharin (48.8 mM) (△).

Table 3

Effect of phenol, benzene, and fluorobenzene on neurite outgrowth in DRG

DRG were cultured in the presence of NGF (2 ng/ml) and the compounds shown; ganglia responses were scored at Day 1. Cultures were then washed, and the incubations were continued in the presence of only NGF (2 ng/ml) for another day and scored.

Compounds	Ganglia response[a]	
	Day 1	Day 2
Control	4.6 ± 0.5[b] (10)[c]	5.0 ± 0.0 (10)
Phenol (4 mM)	1.9 ± 0.7 (10)	4.8 ± 0.4 (5)[d]
Benzene (4 mM)	4.5 ± 0.7 (10)	5.0 ± 0.0 (10)
Fluorobenzene (4 mM)	4.6 ± 0.7 (10)	5.0 ± 0.0 (10)

[a] Ganglia were scored on an arbitrary scale ranging from 0 to 5.
[b] Mean ± S.E.
[c] Numbers in parentheses, *N*.
[d] Decreased *N* values are due to accidental dislodgement of ganglia during the wash procedure.

Table 4

Effect of cyclamate, saccharin, and phenol on binding of ^{125}I-labeled NGF to cells from dissociated DRG

Intact cells from dissociated 9-day-old DRG were incubated at 37° for 1 hr in RPMI 1640 containing bovine serum albumin (5 mg/ml), ^{125}I-labeled NGF (specific activity, 28 cpm/pg, 10 ng/ml) under the conditions indicated, and the amount of radioactivity bound to the cells was assayed. Nonspecific binding was determined in parallel incubations additionally containing excess nonradioactive NGF (10 μg/ml) and subtracted from total binding.

Compounds	Specifically bound ^{125}I-labeled NGF	
	cpm/10^6 cells	%[a]
Control	2005 ± 33[b]	100
Saccharin (48.8 mM)	961 ± 52	48
Cyclamate (55.6 mM)	1120 ± 34	56
Phenol (3.2 mM)	1713 ± 111	85

[a] Percentages were calculated relative to the control value.
[b] Mean ± S.E. for 3 replicate incubations.

of artificial sweeteners that are so high as to completely block binding of NGF might be expected to prevent survival of cultured neurons, but this possibility has not yet been evaluated. With the concentrations used in this study, neurite outgrowth was not completely inhibited, it fully recovered on washout of compounds, and there is no indication that cell survival was affected.

DISCUSSION

While all promoters tested to date are reversible inhibitors of neurite outgrowth, not all promoters are inhibitors of NGF binding. The evidence suggesting that promoters may act by altering the process of cellular differentiation will now be briefly reviewed, and a suggestion will be made as to how promoters might cause such alterations.

Recent studies suggest promoters may act by altering the program of cellular differentiation. Phorbol ester promoters not only inhibit morphological differentiation in DRG (26) and in cultured mouse neuroblastoma cells (28), but also inhibit fusion and myogenesis of cultured chick myoblasts (11), terminal differentiation of Friend erythroleukemia cells (43, 58), and conversion of 3T3 fibroblasts to adipose-like cells (14). These compounds can also induce phenotypic expressions associated with the normal differentiated cell in myeloid leukemia (25, 44) and in melanoma (50) cells. Thus, alteration of cellular differentiation, which has also been reported for mouse skin (39), may be an important property of promoters. The findings of this report further support this interpretation. It is of interest that promoters of widely differing structures and active doses inhibit neurite outgrowth; this assay may then prove useful as a rapid *in vitro* screening procedure for environmental promoters. Further study would be required, however, to determine the frequency of type 1 and type 2 errors, which would limit the utility of any test system.

With respect to active doses, saccharin inhibited neurite outgrowth at between 6 and 100 mM, but a major problem encountered in trying to correlate these findings with *in vivo* studies is that the concentration of promoter at the active site in animal studies is not known. About 5% sweetening agent in the diet is required to demonstrate promotion in a reasonable number of animals (12, 23, 24). Saccharin at about 0.5 mM acts as a promoter in increasing the appearance of type 3 transformed foci in cultured C3H/10T½ cells (37) when tested together with an initiator. However, active doses are higher in *in vitro* systems which do not include initiators. Thus, the threshold dose for increased frequency of sister chromatid exchanges (30, 57) was about 25 mM, and for inhibition of "metabolic cooperation" between cells (49) the dose was about 10 mM saccharin. It can only be said, therefore, that the effects reported here are within the range of doses found active in other studies, and there is presently insufficient knowledge to be able to make an absolute statement about what the proper active concentrations ought to be.

Binding of ^{125}I-labeled NGF to high-affinity sites has been studied (2, 19, 22, 48), but the biological significance of the binding sites is not yet clearly established. The finding that saccharin and cyclamate inhibit both response and binding provides important support for the hypothesis that these sites are indeed the NGF receptors regulating neurite outgrowth. Increasing doses appear to inhibit binding and response in parallel (27). Furthermore, these artificial sweeteners inhibit binding by decreasing the apparent affinity rather than by decreasing the numbers of high-affinity sites. It is hoped that these agents will be useful for other studies on the NGF receptor. As neither phenol nor TPA (26) inhibit binding of ^{125}I-labeled NGF, promoters may antagonize morphological differentiation of neuronal cells by more than one mechanism. Since TPA is reported to inhibit binding of epidermal growth factor and to be mitogenic in other cell systems (7, 15, 31, 34), a common property of promoters may be to alter cellular differentiation by altering the response to cellular effectors or by activating the effector system.

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