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Taurine promotes the differentiation of a vertebrate retinal cell type in vitro

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SUMMARY

The retina offers a model system for investigating the mechanisms that control cell type determination and differentiation in the vertebrate central nervous system. Previously, rod photoreceptor development in vitro was found to require a diffusible activity released by retinal cells (D. Altshuler and C. Cepko, *Development* 114, 947-957, 1992). In this report, we show that retinal-cell-conditioned medium and extracts contain two separable activities that influence rod development: a >10 kDa inhibitory activity, and a stimulatory activity that is <1 kDa and heat stable. Taurine was found to be a component of the <1 kDa fraction and to stimulate rod development when added to retinal cultures. Taurine was not the only rod-promoting factor in these retinal preparations, however, as conditioned medium and extracts stimulated a higher level of rod development than did taurine alone. Taurine uptake into cells could be blocked without inhibiting taurine's ability to stimulate rod development, arguing against an osmoregulatory or nutritive mechanism of action. Finally, a competitive antagonist of taurine's bioactivity was identified and shown partially to inhibit rod development in retinal explants, suggesting that taurine may normally act to stimulate rod development in the retina. These results provide evidence for three activities, one of which is taurine, that are candidate regulators of rod photoreceptor development in vivo.

Key words: differentiation, photoreceptor, rod, retina, taurine

INTRODUCTION

The vertebrate central nervous system (CNS) contains a tremendous diversity of cell types. For this complexity to arise from the relatively homogenous neural tube, developing cells must proliferate, choose cell fates, migrate to appropriate positions, enact programs of differentiation and morphogenesis, and connect with synaptic partners. Since the retina is less complicated, better characterized and more experimentally accessible than many other parts of the CNS, it offers a powerful model system for studying the mechanisms that regulate these developmental processes.

The mature retina contains a diverse but limited repertoire of cell types, including sensory neurons (rod and cone photoreceptors), interneurons (horizontal, bipolar and amacrine cells), glia (Müller cells) and projection neurons (ganglion cells) (reviewed in Dowling, 1987). Classical birthdating studies have demonstrated that the seven retinal cell types are generated in an evolutionarily conserved order during development, although multiple cell types are simultaneously produced at any given developmental stage (reviewed in Altshuler et al., 1991) . In addition, lineage mapping studies of single retinal progenitor cells have shown that many, if not all, retinal cells arise from multipotent progenitors (Turner and Cepko, 1987; Holt et al., 1988; Wetts and Fraser, 1988; Turner et al., 1990). Together, these experiments suggest that an individual retinal progenitor cell can give rise to many different cell types, and that the specific cell types produced change throughout retinal development.

Given the multipotency of retinal progenitor cells, it was reasonable to hypothesize that signaling amongst developing cells might contribute to the determination of retinal cell phenotype. To test this hypothesis, we and others have attempted to identify molecules that can regulate the development of progenitor cells into rod photoreceptors in vitro. The goal of these experiments is to characterize interactions and molecules that have the ability to influence rod differentiation. Having used cell culture to identify these candidate signaling molecules, it will ultimately be necessary to determine the relative contribution of each to the regulation of rod differentiation in vivo.

A number of reports have independently identified activities that can influence rod development in vitro. Cell mixing experiments have suggested that a diffusible activity promotes rod differentiation in the rat (Watanabe and Raff, 1990, 1992). Based upon comparisons of photoreceptor development in monolayer and aggregate cultures, a requirement for cell-contact-mediated interactions has been proposed (Harris and Messersmith, 1992; Reh, 1992). The molecules responsible for these in vitro activities have not yet been identified. Two previously identified molecules, bFGF (Hicks and Courtois, 1992) and S-laminin (Hunter et

al., 1992), have been shown to influence rod development in monolayer cultures. However, not all experiments suggest that cell-cell interactions are required for photoreceptor development: cone photoreceptor development appears to occur in the chick as a default pathway of development when retinal cells are grown in sparse monolayer cultures (Adler and Hatlee, 1989).

Previously, we observed that rod differentiation occurred at apparently normal levels when neonatal retinal cells were placed at high cell density in serum-free, collagen gel cultures. Rod development required influences from other retinal cells, however, as a four-fold reduction in cell density completely abolished the appearance of newly differentiated opsin$^+$ cells. In lower density gels, rod development was restored by co-culture with high-density gels, providing evidence for a diffusible, rod-promoting activity. The ability of cells from different developmental stages to stimulate rod development in this assay varied in a manner that was temporally correlated with rod development in vivo (Altshuler and Cepko, 1992).

To identify the molecular components of this rod-promoting activity, we have characterized retinal-cell-conditioned medium (CM) and extracts as sources of factors that influence rod development in the gel culture system. First, two opposing activities were observed in these retinal preparations: a >10 kDa inhibitory fraction, and a <1 kDa, heat-stable fraction that promoted rod development. Second, the low molecular weight rod-promoting activity was found to be partially attributable to taurine, which is present at high levels in the retina as well as other parts of the CNS, and thought to be important for CNS development (reviewed in Sturman, 1988) and photoreceptor function (Hayes et al., 1975) in vivo. Third, the <10 kDa fraction appeared to contain rod-promoting activity in excess of that attributable to taurine. Fourth, pharmacological experiments dissociated taurine uptake from the stimulation of rod development, arguing against an intracellular site of action. Finally, rod development in retinal explants was competitively inhibited by treatment with a novel taurine antagonist. These results identify taurine as one of several factors that might normally play a role in regulating rod photoreceptor development in vivo.

MATERIAL AND METHODS

Gel cultures

Collagen gel cultures were made as previously described (Altshuler and Cepko, 1992), with slight modification. Retinas were dissected from neonatal (<24 hours postpartum) C/D rat pups in Hanks balanced salt solution plus Ca^{2+}/Mg^{2+} (HBSS+) and dissociated (0.1% trypsin(Worthington)/HBSS without Ca^{2+}, Mg^{2+}/1 mM EDTA) for 10 minutes at 20°C. Soybean trypsin inhibitor (STI, from Sigma) was added from a 10× stock to 0.1%, the cells were pelleted (1000 revs/minute in a clinical centrifuge, 5 minutes), and then resuspended in HBSS+/DNAse I (50 U/ml, Sigma). Cells were recentrifuged (as above) and resuspended in culture media (a 1:1 mixture of DME/F-12 (JRH), 1× modified Sato's serum-free culture supplement (Lillien et al., 1988), penicillin/streptomycin (100 U/ml), insulin (5 μg/ml), and Hepes pH=7.3 (5 mM)). Cell concentration was determined with a hemocytometer and adjusted using culture medium as a diluent to give 1.6× the final cell density desired in gels. Hepes (1 M, pH 7.3) and Bovine type 1 collagen (3.9 mg/ml, from Collaborative Research) were added sequentially to the cell suspension to give a final concentration of 1× the desired cell density, 100 mM Hepes, and 1.2 mg/ml collagen. Low-cell-density cultures ranged from 3-5×10^5 cells/100 μl gel, while high-density gels ranged from 1.5-2.5×10^6 cells/100 μl gel. This gel mixture was left at room temperature for 10 minutes, and then 100 μl was pipetted as a ring around the edge of the wells of a 24-well dish (Costar). Only the central 8 wells of each plate were used, as it was previously observed that cultures on the edge of plates were occasionally compromised for rod development. The remaining 16 wells were filled with 1 ml of sterile tissue culture water (JRH). Aliquots of the gel solution were counted in a hemocytometer to measure the actual cell density in each gel mixture. Gels were placed in a 37°C, 5% CO_2 incubator for 2-3 hours to allow the gel to set. Finally, 400 μl of culture medium was gently added to each well. 10% (50 μl) of each well's total volume (gel+medium) was removed daily and replaced with fresh medium.

After 7 days in vitro, gels were dissociated. Culture medium was removed and replaced with 100 μl of HBSS+/0.1% collagenase (Sigma)/0.1% trypsin (Worthington). After 20 minutes at 37°C, this mixture was removed to 500 μl microfuge tube containing 100 μl of HBSS+/STI (0.1%, Sigma)/DNAse I (50 U/ml, Sigma). The contents were triturated, centrifuged and resuspended in 100-500 μl culture medium (the volume calculated to give constant cell concentration regardless of gel density). 80 μl of this solution was pipetted onto poly-lysine-coated slides (8 wells/slide, Teflon-coated, Cel-line Associates) and incubated in a humidified chamber at 37°C. An aliquot of the cell suspension was counted on a hemocytometer to measure cell yield. After 2 hours at 37°C, slides were fixed in 4% paraformaldehyde (in PBS) for 5 minutes at room temperature, followed by 5 minutes in cold (−20°C) 70% ethanol. Slides were either stained immediately, or frozen at −20°C for later analysis.

Immunohisochemistry

Immunohistochemistry was performed as previously described (Altshuler and Cepko, 1992). Cells prepared as above were rehydrated in PBS and then incubated for 15 minutes in a blocking solution consisting of DME, 10% calf serum, 3% donkey serum (Jackson Immunologicals), 0.4% Triton X-100. RETP1, an anti-rhodopsin monoclonal antibody (courtesy Colin Barnstable), was used at a 1:3000 dilution in blocking solution. After 30 minutes in primary antibody, slides were washed for 2 minutes in PBS and 5 minutes in blocking solution. Secondary antibody (Texas-red-conjugated donkey anti-mouse, Jackson Immunologicals) was used at 1:200 dilution in blocking solution containing the nuclear dye DAPI. Incubation and washes were as above. Slides were mounted in gelvatol and observed using a Zeiss Axiophot. At least 200 DAPI$^+$ cells were counted per culture.

Preparation of conditioned medium and retinal extracts

For conditioned medium, 20 retinas were dissected from P4 rat pups and cultured for 4 days in a 10 cm dish containing 10 ml of medium. At this tissue concentration (2-5×10^7 cells/ml), the medium became acidified (yellow) by the end of the 96 hour culture period. Conditioned medium was centrifuged (3000 revs/minute in a clinical centrifuge, 10 minutes), filtered through a 0.22 μm filter, and frozen at −70°C for future use. Medium was size-fractionated using 10 kDa Centriprep filter concentration units (Amicon), according to the manufacturers instructions (filter units were washed with tissue culture water prior to use). The filtration was continued until approximately 90% of the volume had passed through the filter, generating a 10× concentration of the >10 kDa fraction. Fractionated CM was aliquoted and stored at −70°C. When assayed, CM was diluted to 10× the desired concentration in culture medium and then added to each well with the daily 50 μl medium change.

For extracts, P4 retinas were dissected (as above), and homogenized in PBS (20 retinas/10 ml) with either a Dounce homogenizer or a polytron. Insoluble material was removed by a pre-clear spin (2300 revs/minute in a clinical centrifuge, 10 minutes), followed by centrifugation at 100,000 ***g*** for 1 hour. The supernatant was sterilized by filtration through a 0.22 μm filter. Material was fractionated, stored and assayed as described for CM above.

To characterize further the molecular mass of the <10 kDa activity, material was passed through 1 kDa and 3 kDa microsep filter units (Filtron). No loss of activity was detected in dose-response experiments. To determine heat stability, extract was placed in a 95°C heat block for 30 minutes. No loss of activity was observed in dose-response experiments. Extracts were used for the heat-stability experiment, rather than CM, because it was determined that heat treatment of culture medium (unconditioned or conditioned) created an activity that blocked the stimulatory activity of CM. Since extracts did not contain this inhibitor following heat treatment, it is reasonable to assume that the heat-treatment inhibitor was a component of culture medium rather than of retinal origin.

HPLC analysis

The amino acid content of filtered extracts and tissue culture supernatants was examined with two amino acid analyzers, each using different separation and detection methods. A Beckman 6300 amino acid analyzer was used for routine sample analysis. This system is based on cation exchange HPLC and post-column detection with ninhydrin. With the standard sodium buffers and hydrolyzate gradient recommended by the manufacturer, taurine eluted at the beginning of the chromatogram, near cysteic acid, as expected. The ninhydrin color yield for taurine was about two-thirds that for α-carboxylic amino acids. The system was calibrated with 0.5 nmol amino acid standards supplemented with 0.5 nmol taurine. To measure taurine concentrations accurately, analyzed sample volumes were adjusted to give 0.25-1.0 nmol taurine.

Some samples were also examined with an ABI 420 amino acid analyzer to confirm the assignment of taurine. With this system, samples were derivitized with phenyl isothiocyanate, separated by reverse-phase HPLC and detected by absorbance of ultraviolet light. This system was calibrated with 0.1 nmol standard. The HPLC gradient recommended by the manufacturer was modified to improve the separation of taurine and arginine derivatives.

Screen of candidate compounds

Compounds were purchased from Sigma and from Tocris Neuramin (England). Compounds were suspended at 0.1-1 M in water or culture medium and stored at −20°C. In initial experiments (Fig. 2A), compounds were tested at a range of concentrations (0.5-50 μM) over 7 days in vitro. The data in Table 1 were generated by adding each compound to low-density gel cultures at a final concentration of 50 μM over the last 24 hours in vitro. Compounds were selected for further characterization as agonists if addition of 50 μM stimulated rod development to >50% of the maximal level observed with taurine. To test for antagonism, compounds were added to cultures at 50 μM in the presence of 5 μM taurine. Compounds were selected for further characterization as antagonists if they blocked >50% of the response observed to this dose of taurine. Cell survival in these cultures was monitored as above; compounds that were toxic at 50 μM were retested at 10 μM. Compounds that demonstrated reproducible agonism or antagonism were characterized with full dose-response experiments.

Uptake measurements

Tritiated taurine was purchased from Amersham (28 Ci/mmole). [^{3}H]taurine (1 nM) along with appropriate concentrations of taurine, β-alanine and AMS, were added to each culture during the final 24 hours in vitro, following which the culture medium and cells were separated. The cells were processed for immunostaining as above. The radioactivity present in aliquots of medium and cells was measured by scintillation counting. Uptake in the presence of 5 mM unlabelled taurine was substracted as background, and was <2% (40 cts/minute) of the uptake in the absence of unlabelled taurine. For dose-response experiments, a constant amount of radioactive taurine was added to each well (1 nM) and the specific activity recalculated based upon the ratio of [^{3}H]taurine to total added taurine in each well.

Explant experiments

Retinal tissue was dissected from E20, P0 or P3 eyes. An entire retina was placed in each culture well in 3 ml of culture medium. These explant cultures were cultured at much lower cell density than the explant cultures used to make conditioned medium; no acidification of the medium was observed. When retinas were cultured floating, or on polycarbonate filters, no difference was observed in rod development or response to added compounds. For AMS blocking experiments, the two retinas from each animal were matched; one was placed in medium containing AMS (100 μM), the other being in medium without AMS. Cultures were fed each day (10% medium changed) containing the appropriate compounds. Explants were dissociated and stained as for gel cultures, except collagenase was excluded from the dissociation buffer, and cells were resuspended in 1 ml of culture medium prior to plating on slides.

RESULTS

Retinal-cell-conditioned medium and extracts contain separable activities that either stimulate or inhibit rod development in vitro

Previously, we used cell density and co-culture manipulations to demonstrate that retinal cells produced a diffusible activity that stimulated rod development in vitro. In these experiments, rod development proceeded at in vivo levels in high-density collagen gel cultures, with the proportion of rhodopsin$^+$ cells increasing from 1% to 25% during the first postnatal week. Rod development did not occur in low-density gels, however, as the percentage of opsin$^+$ cells after one week in vitro (≈1%) remained unchanged from that observed in the starting cell population. Co-culture of low- and high-density gels restored rod development to cells grown at low density (22% opsin$^+$), providing evidence for a diffusible, rod-promoting activity (Altshuler and Cepko, 1992).

In order to characterize this activity, culture medium was conditioned for 4 days in vitro by explants of postnatal day 4 (P4) retina, filtered and then added daily (at various dilutions) to low-density gel cultures; rod-promoting activity was defined as causing an increase in the proportion of opsin$^+$ cells in these cultures. As expected, addition of crude CM to low-density gels resulted in a stimulation of rod development. The maximum response to CM, observed with a 1:100 dilution, was an increase from 2% to 8±1% opsin$^+$ cells (Fig. 1A). This stimulation confirmed the presence of a rod-promoting activity in CM, but the magnitude of response was less than that observed when low-density gels were co-cultured with high-density gels (22%, Altshuler and Cepko, 1992). In addition, application of CM at higher concentrations failed to generate a larger response (Fig. 1A). One hypothesis consistent with such a

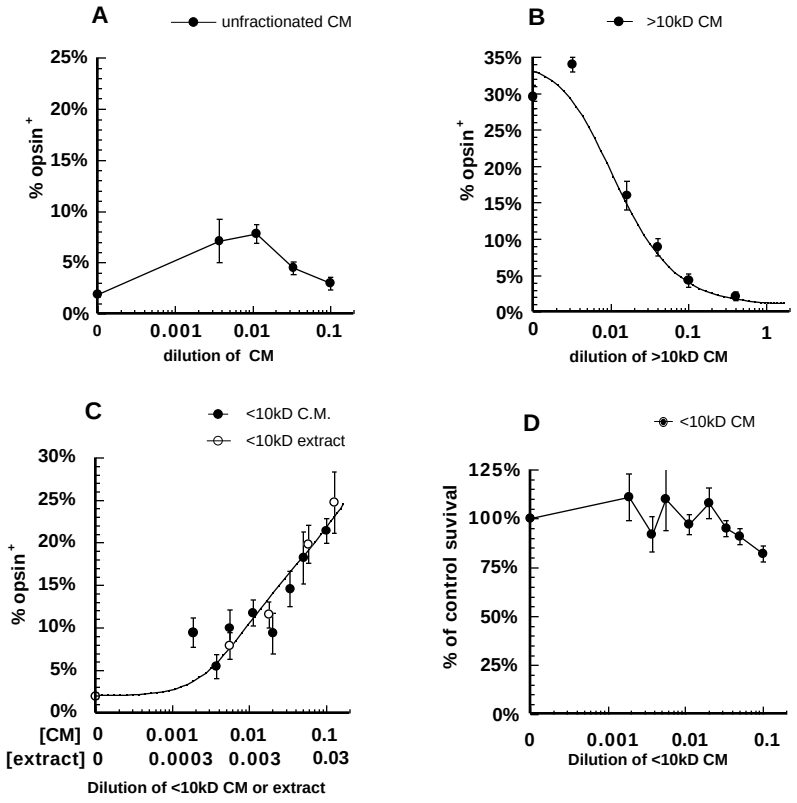

Fig. 1. Rod development is influenced by retinal cell-conditioned medium (CM) and extract. Collagen gel cultures were initiated at P0, and incubated for seven days in vitro. Cells were treated with CM or extract daily at the dilutions indicated. Values in A-C represent the fraction of total cells in the culture that were opsin$^+$. (A) Treatment of low-density gel cultures (4×10^5 cells/100 μl gel) with crude CM. Each datum point represents four cultures from a total of two experiments. (B) Treatment of high-density gel cultures (2×10^6 cells/100 μl gel) with >10 kDa fraction of CM. Each datum point represents four to six cultures from a total of three experiments. (C) Treatment of low-density gel cultures (4×10^5 cells/100 μl gel) with <10 kDa fraction of CM (closed circles) or extract (open circles). The concentration of CM and extract is indicated below the x-axis. Each datum point represents four to fourteen cultures from a total of eight experiments. (D) Cell survival, as a percentage of that observed in control cultures, for gels treated with <10 kDa fraction of CM. Cultures are the same as those in C.

dose-response relationship is that the stimulatory activity in CM might be masked by a component that served to inhibit of rod development.

To separate putative inhibitory and stimulatory activities, filtration was used to fractionate CM into high (>10 kDa) and low (<10 kDa) molecular mass components. Unexpectedly, the >10 kDa fraction displayed potent inhibitory activity. When this fraction was added to high-density cultures, which generated 30±2% opsin$^+$ cells in the absence of CM, a dose-dependent reduction in the development of opsin$^+$ cells was observed (Fig. 1B). This inhibitor was not further characterized in this study, but remains under investigation.

In contrast to the >10 kDa fraction, the <10 kDa CM fraction stimulated development of opsin$^+$ cells when added to low-density gels (closed circles, Fig. 1C). The maximal level of stimulation observed in these experiments was to 21±1% opsin$^+$ cells, a level comparable to that of 22% previously reported for co-culture experiments (Altshuler and Cepko, 1992). Treatment with moderate concentrations of this CM fraction did not cause either an increase or decrease in the overall number of cells present in the cultures, although a trend towards toxicity was observed at the highest concentrations (Fig. 1D). These survival data argue against the hypotheses that the ten-fold increase in opsin$^+$ cells was secondary either to an increase in the viability of rods (which would cause an ≈20% increase in total cell number), or to toxic effects on cells other than rods (which would require an ≈90% decrease in overall cell number).

To determine whether the inhibitory and stimulatory activities were present in vivo, extracts of neonatal retina were separated into high and low molecular weight fractions and assayed as above. As was observed with CM, retinal extracts contained both an inhibitor in the >10 kDa fraction (data not shown) and a stimulator in the <10 kDa fraction (open circles, Fig. 1C). The maximal stimulation observed with the <10 kDa fraction of retinal extract was from 2±1% to 25±4% opsin$^+$ cells (Fig. 1C). Furthermore, the dose-response curves observed for treatment with <10 kDa fractions of CM and extract were parallel (Fig. 1C). When extracts and CM were tested at higher concentrations to see if the dose-response curve continued to rise, cell death was observed (data not shown). Since toxicity may be due to other factors in the CM and extract, the maximal response to the CM/extract rod-promoting activities has not yet been established. Based upon the presence of inhibitory and stimulatory activities in retinal extracts, we conclude that the CM factors (or those with similar characteristics) are normally present in the retina in vivo, and are not produced solely as a response of retinal cells to culture.

In order to characterize further the retinal rod-promoting activity, the <10 kDa fraction of CM was passed through filters with nominal 3 kDa and 1 kDa cut-offs. These filters did not retain the activity to any significant degree (data not shown). In addition, the extract activity was undiminished by heat treatment of 95°C for 30 minutes (data not shown). The activity was not restricted to retina, as conditioned media from cerebellum and olfactory bulb, but not from a 3T3 fibroblast cell line, also contained a <10 kDa stimulatory activity (data not shown).

Taurine is a component of the rod-promoting activity present in retinal conditioned medium and extracts.

The physical properties of the CM/extract rod-promoting

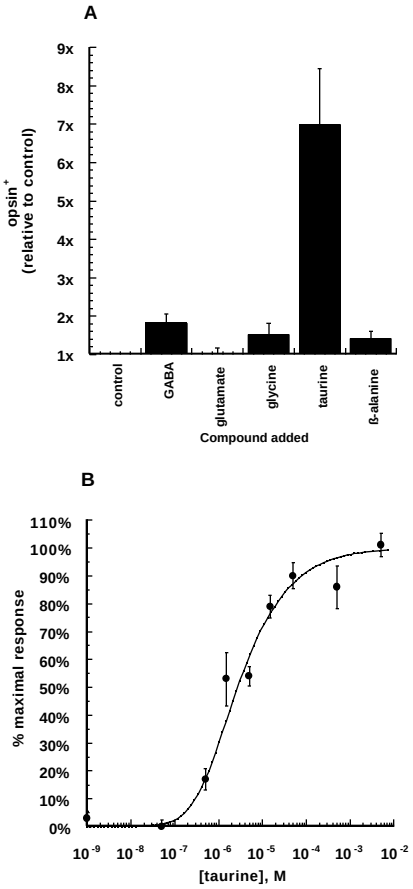

Fig. 2. Rod development is stimulated by taurine. (A) Low-density gel cultures were treated daily with 20-50 μM of the compounds listed. Each value represents the percentage of total cells that were opsin$^+$. Each datum point represents four to ten cultures from a total of six experiments. (B) Low-density cultures were treated with taurine at the range of concentrations indicated. Each value represents the proportion of opsin$^+$ cells, expressed as a percentage of the maximal stimulation by taurine. Each datum point represents eight to eighteen cultures from a total of seven experiments.

Table 1. Summary of pharmacologic screen

Agonism
2-amino ethyl sulphonic acid (taurine)
2-amino ethyl sulphinic acid (hypotaurine)
Piperidine-4-sulphonic acid
Antagonism
amino methyl sulphonic acid (AMS)
Neither
gamma-D-glutamyltaurine
glutamylaminomethyl sulphonic acid
D-homocysteine sulphinic acid
L-homocysteine sulphinic acid
cis-4-amino crotonic acid
2-amino ethyl phosphonic acid
3-amino propane sulphonic acid
L-cysteic acid
L-cysteine sulphinic acid
D-homocysteic acid
L-homocysteic acid
Nipecotic Acid
2-hydroxy saclofen
saclofen
3-amino propyl phosphonic acid
4-amino butyl phophonic acid
3-amino propyl phosphinic acid
Biccuculline methiodide
GABA
β-alanine
glycine
strychnine
glutamate
cysteine
retinoic acid

Compounds listed were assayed on low-density cultures for the ability to stimulate rod development (agonism) and to block the stimulation of rod development by taurine (antagonism). Compounds were added to cultures at a concentration of 50 μM. The antagonism assay was performed in the presence of 5 μM taurine. Compounds were selected for further characterization as agonists if 50 μM stimulated rod development to 50% of the maximal level observed with taurine. Since the half-maximal response to taurine was observed with ≈2 μM, this screen should detect compounds up to 25-fold less potent than taurine. Compounds were selected for further characterization as antagonists if 50 μM blocked >50% of the response to 5 μM taurine. Any compound displaying activity was further characterized in dose-response experiments. Results shown are from a minimum of four cultures from two experiments.

factor suggested classes of molecules that could be responsible for the activity. Amino acids and their derivatives, many of which act as intercellular signals in the mature CNS, are found in vivo prior to synapse formation, where it has been argued they might act to regulate development (for review, Lipton and Kater, 1989). Two approaches were used to identify the rod-promoting component(s) of CM and extract: candidate molecules were tested for rod-promoting activity in the low-density gel culture bioassay, and HPLC was used to detect differences in the amino acid content of unconditioned medium and that conditioned by retinal cells.

Taurine (amino-ethyl-sulphonic acid) was found significantly to stimulate development of opsin$^+$ cells when added to low-density cultures (Fig. 2A). None of the other compounds tested, such as the amino acid neurotransmitters GABA, glutamate and glycine, caused a similar increase in the proportion of opsin$^+$ cells. To date, 30 such compounds have been added to low-density cultures; only taurine and two structurally similar molecules (displaying 10-fold lower potency than taurine) have exhibited rod-promoting activity (Table 1). A dose-response curve for taurine stimulation of rod development (Fig. 2B) showed half-maximal stimulation at 2 μM, a concentration range similar to that at which many amino acid neurotransmitters interact with specific cell-surface receptors. In addition, this concentration is >1000× lower than that at which taurine has been shown to act in most other in vitro assays (Lombardini, 1991). Finally, in a summary of taurine-treated cultures (grown at various cell densities), no statistically significant difference was observed in overall cell number between control and taurine-treated wells, despite an increase in opsin$^+$ cells from 8±1% to 26±3% (Table 2). If taurine treatment acted by increasing the survival of rod photoreceptors, then taurine-treated cultures should contain an increased number of cells equal to the excess rods present in the culture (26%−8%=18%);

Table 2. Summary of cell survival in control and taurine-treated cultures

	Control		Taurine	
	Survival	Opsin$^+$	Survival	Opsin$^+$
Average	36.5%	7.7%	35.3%	26.1%
s.e.m.	1.5%	1.3%	1.8%	2.7%
n=	63	64	46	59

Data were collected from all cultures (grown at various cell densities) in which both cell survival and opsin-immunostaining were quantitated. Data for taurine-treated cultures were taken from wells treated with taurine concentrations producing a maximal response (greater than or equal to 50 μM). Cell survival equals the number of cells harvested from each gel at the end of the week long experiment, expressed as a percentage of the number of cells placed into each gel at the start of the experiment. Opsin-positivity is expressed as the percentage of cells in the culture staining with anti-rhodopsin antibody. No statistically significant difference in cell survival is observed between taurine-treated and control cultures. The hypothesis that taurine-treated cultures contain 18% more cells than do control cultures (the percentage of excess rods) can be rejected at $P<0.01$ using a paired sample *t*-test.

this hypothesis can be rejected for the data in Table 2 at $P<0.01$.

HPLC analysis revealed a small number of peaks that differed between the starting medium and retinal CM (compare middle and bottom panels of Fig. 3). The major peak that consistently was absent in culture medium, but present in conditioned medium, co-elutes with taurine (compare Fig. 3A and C). The identification of this peak as taurine was confirmed using another HPLC based upon a different separation (reverse phase instead of cation exchange, data not shown). As measured with HPLC, the amount of taurine in CM varied between 150 and 350 μM; at this concentration, the taurine in CM would be bioactive at the dilutions used (compare Figs 2B and 1C). Retinal extracts that stimulated rod differentiation were also found to contain appropriate concentrations of taurine. In addition, the rod-promoting activities of taurine and CM were not additive, consistent with a common mechanism of action. In preliminary purification of taurine away from other CM components, rod-promoting activity was observed to co-purify with taurine (D. A., J. L. T. and C. C., unpublished observations). These data are consistent with taurine being a component of the rod-promoting activity present in retinal CM and extracts.

Cultures, CM and extract contain rod-promoting activity in excess of that attributable to taurine

As described above, taurine was found to promote rod development, and to be present at biologically active concentrations in CM and extracts. Three lines of evidence suggest, however, that taurine is not the only stimulatory component in either retinal cell cultures or in CM/extract.

First, if taurine concentration were the only difference between high and low-density cultures, then rod development would be independent of cell density in the presence of high levels of taurine. This behavior was not observed (Fig. 4A). Addition of taurine to low-density cultures failed to restore rod development to the levels observed in untreated high-density cultures. Furthermore, when taurine was added to high-density cultures, a 2-fold increase in the percentage of opsin$^+$ cells was observed, from 32±4% to 61±6% of cells. The response to taurine in high-density gels indicates that taurine levels are not saturating in high-density cultures. More importantly, since rod development remains density-dependent in the presence of saturating taurine, gel cultures must contain activities other than to taurine that are titrated with cell density.

Second, CM and extracts were also found to contain activity in excess of that attributable to their taurine content. At low cell densities, the maximal response to <10 kDa fractions of CM or extract was 2- to 5-fold greater than that to saturating concentrations of taurine (Fig. 4A-C). In contrast, at high cell density, the response to CM/extract was equivalent to that observed upon treatment by taurine alone (Fig. 4A). Thus, CM and extract contain an additional activity, response to which is observed following treatment of low- (but not high-) density cultures. This is consistent with an activity that may normally be present in high-density cultures and limiting at low density. A comparison of the dose-response curves to taurine and to CM (on lowest density cells to maximize the difference between CM and taurine) suggested that the additional activity is present at lower titer in CM than is taurine (Fig. 4B). This experiment shows that, at low concentrations, all of the activity of CM can be explained based upon its taurine content, whereas at higher concentrations, CM generates a larger response than does taurine alone (Fig. 4B).

Third, the kinetics of cellular response differed for taurine as compared to CM. With taurine, a maximal response was observed if cells were treated throughout the 7 day culture period or for only the last 24 hours of culture. CM, or extract, on the other hand, required 72-96 hours for maximal response (Fig. 4C). When cells were exposed to CM for only 24 hours, the response was similar in magnitude to that observed with taurine alone (Fig. 4C). This difference in the kinetics of cellular response suggests that the additional CM/extract activity may influence a different developmental process than does taurine. The molecular identity of the additional rod-promoting activity is currently under investigation.

Taurine structural analogs act as agonists/antagonists of taurine's rod-promoting activity

Having identified taurine as an active component of retinal CM and extracts, it is of interest to determine the mechanism through which taurine stimulates rod development. Most important, however, will be to determine whether taurine normally plays a role in regulating photoreceptor development in the retina. Pharmacologic agents that mimic or block cellular responses to taurine are powerful tools to investigate these questions.

Known pharmacologic agents and structural analogs of taurine were tested for rod-promoting activity, and for antagonism of the response to taurine. The specificity of taurine in stimulating rod development is underscored by the rod-stimulation assay, in which only taurine and two structurally related compounds displayed rod-promoting activity (Table 1). Both agonist compounds were approximately ten-fold less potent than taurine in stimulating rod development (data not shown). One compound (amino-methyl-sulphonic

acid, or AMS) was found competitively to antagonize taurine's rod-promoting activity (Fig. 5). AMS is very similar to taurine (amino-ethyl-sulphonic acid) in structure, having one fewer methylene group than does taurine. To our knowledge, this compound has no previously reported pharmacologic activity.

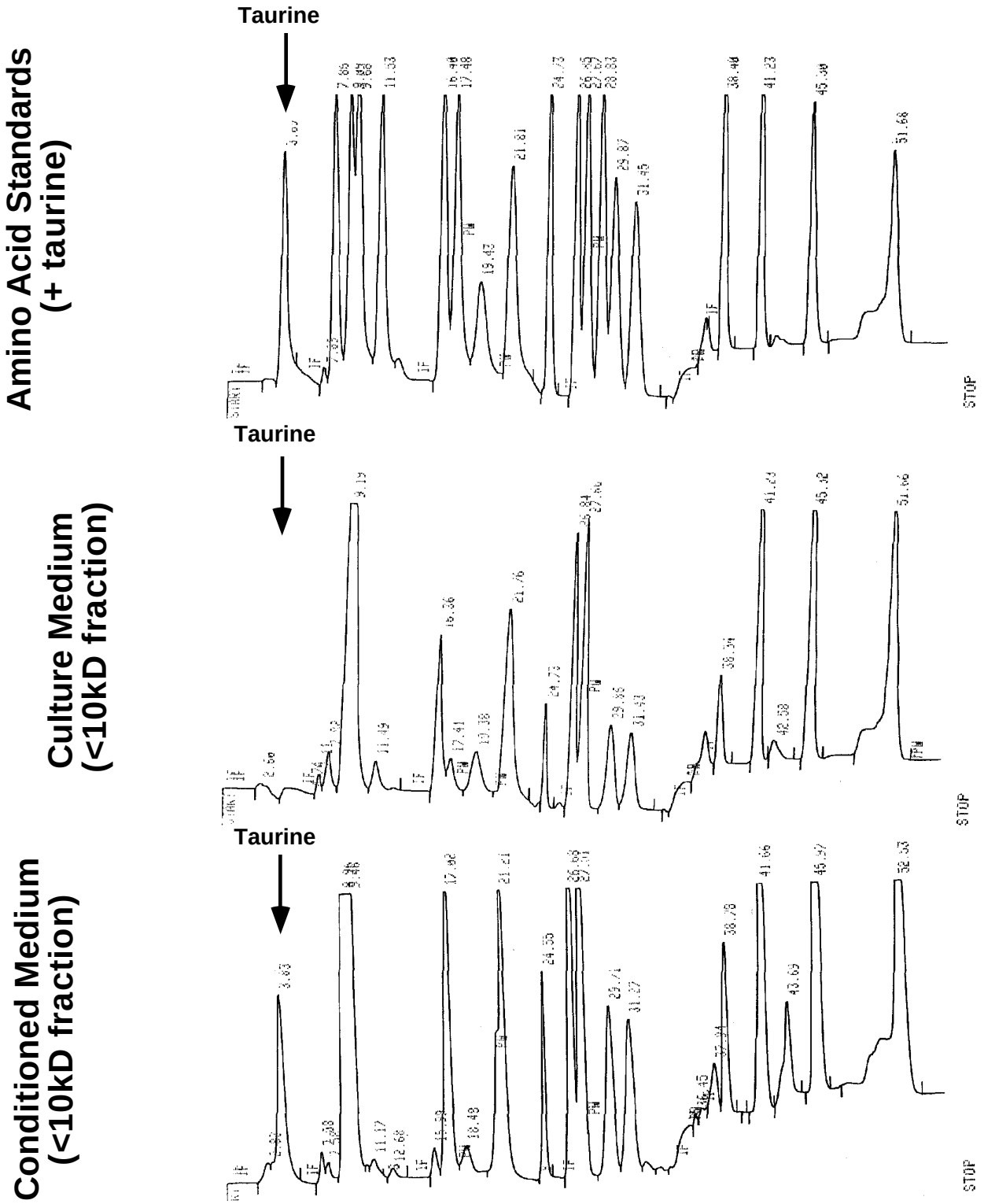

Fig. 3. Conditioned medium contains taurine. Cation exchange HPLC analysis of culture medium and medium conditioned by P4 retinal explants for 4 days in vitro. Peaks are labeled by their elution times, in minutes. (A) HPLC chromatogram of amino acid standards, including taurine. (B) HPLC chromatogram of unconditioned culture medium. (C) HPLC chromatogram of <10 kDa fraction of conditioned medium. The taurine peak is indicated by the arrow.

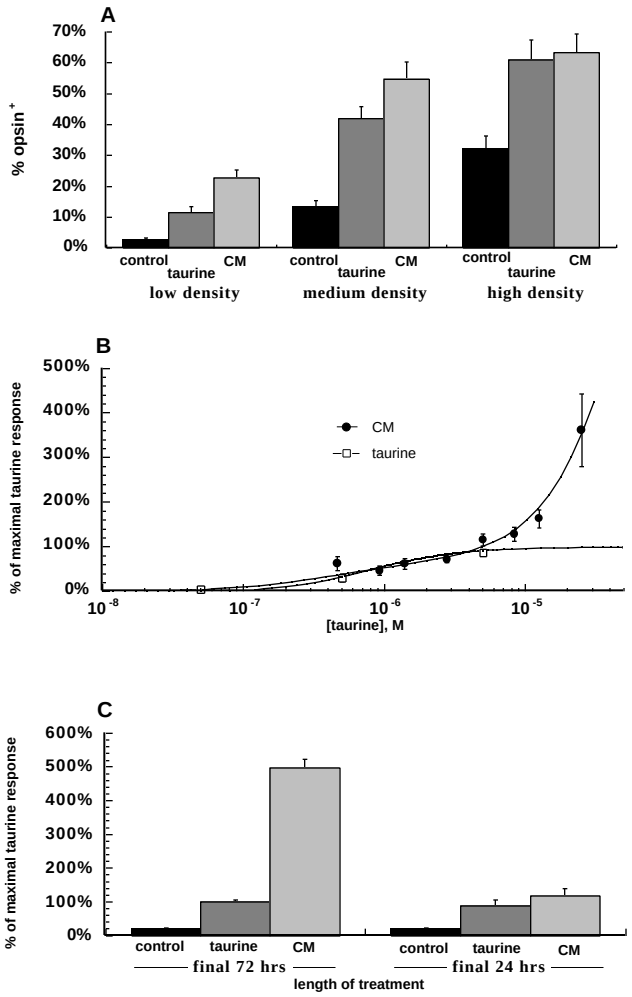

Fig. 4. Cultures and CM/extract contain rod-promoting activity in excess of that attributable to taurine. (A) Response to taurine and CM/extract varies with cell density. Cultures were grown at a range of cell densities: low density (3-5×10^5 cells/100 μl gel), medium density (≈10^6 cells/100 μl gel), and high density (1.5-2.5×10^6 cells/100 μl gel). Taurine data is for treatment with 50 μM taurine, a concentration that generated a maximal stimulation in the experiments shown. CM stimulation represents the maximal concentration used in each experiments (CM dilutions 1:10-1:40). Each value represents the proportion of opsin$^+$ cells in each culture., and represents six to twelve cultures from a total of four experiments. (B) Dose-response to taurine and CM. Low-density cultures were treated with taurine, or with CM. Concentration of CM was normalized to its taurine content, based on HPLC measurement. Values represent the proportion of opsin$^+$ cells, expressed as a percentage of the maximal stimulation observed to taurine alone. Each datum point represents four to ten cultures from a total of five experiments. (C) Kinetics of response to taurine and CM. Low-density cultures were treated with 50 μM taurine or CM (1:10-1:40) for either the last 24 hours of culture, or the last 72-96 hours of culture. Values represent the proportion of opsin$^+$ cells, expressed as a percentage of the maximal stimulation observed to taurine alone (72-96 hours of exposure). Treatment with CM for 72-96 hours generated an equivalent response to treatment for the full seven days of culture (data not shown). Each datum point represents six to eight cultures from a total of three experiments.

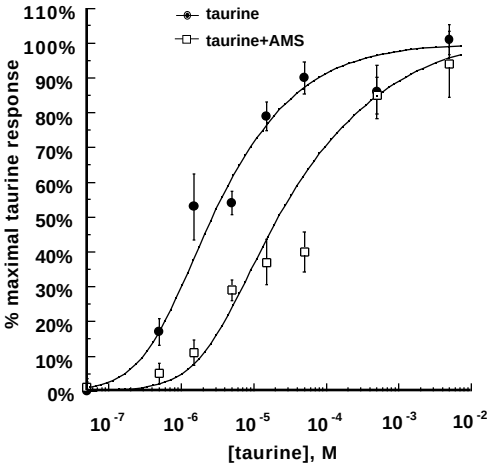

Fig. 5. Aminomethylsulphonic acid (AMS) competitively antagonizes the stimulation of rod development by taurine. Low-density gel cultures were treated with taurine at a range of concentrations, in the presence or absence of 0.5 μM AMS. Each value represents the proportion of opsin$^+$ cells, expressed as a percentage of the maximal stimulation observed to taurine alone. Each datum point represents eight to eighteen cultures from a total of seven experiments.

Taurine does not appear to stimulate rod development through a previously characterized pathway

With pharmacologic agents in hand, it was possible to begin investigating the site of action through which taurine stimulates rod development. No evidence was obtained to support a previously characterized site of taurine action. First, receptors with which taurine is known to interact, such as those for glycine and GABA, do not appear to be involved in the rod-promoting action of taurine. Agonists for those receptors, including GABA and glycine themselves, do not mimic taurine's effect on rod development (Fig. 2A; Table 1). Similarly, antagonists to these receptors do not block taurine bioactivity (Table 1). These results do not support a role for these receptors in the stimulation of rod development by taurine.

Second, studies of taurine uptake do not support a role for this process in the promotion of rod differentiation. One possibility is that taurine's site of action is inside the cell. In this case, the amount of intracellular taurine would determine the magnitude of cellular response and blocking uptake would therefore diminish taurine's ability to stimulate rod differentiation. β-alanine, a compound similar to taurine in structure (amino-ethyl-carboxylic acid), has been reported to block the uptake of taurine into cells (reviewed in Lombardini, 1991). Retinal gel cultures grown in the presence of 500 μM β-alanine displayed a >90% block in taurine uptake (Fig. 6A); the stimulation of rod differentiation by taurine was apparently unchanged in the face of this uptake block (Fig. 6B). In addition, AMS (the competitive antagonist described above) shifted the dose-response curve for rod stimulation without blocking taurine uptake (compare Figs 6A and 5). Thus, taurine uptake can be diminished without blocking its ability to stimulate rod differen-

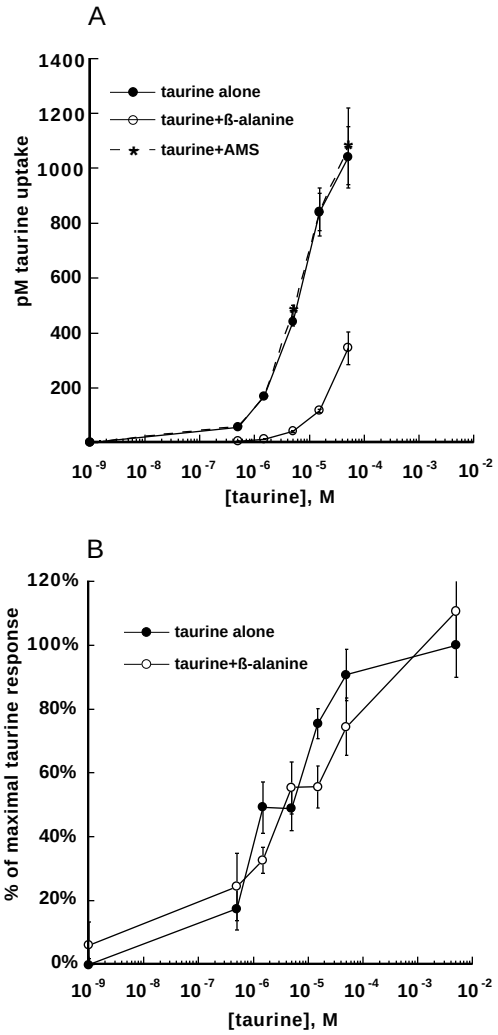

Fig. 6. Taurine uptake is separable from stimulation of rod development. Retinal cells were grown (6×10^6 cells/100 μl gel) in the presence of 1 nM [^{3}H]taurine for 24 hours. Unlabeled taurine, β-alanine (500 μM) and AMS (0.5 μM) were added as indicated. (A) Uptake of taurine is blocked by 500 μM β-alanine, but not by 0.5 μM AMS. Each value represents the amount of taurine (pmoles) taken up per well of cultured retinal cells. Reduction of taurine uptake by β-alanine was >90% for concentrations below 15 μM taurine. Each value represents eight cultures from a total of three experiments. The cells used to measure uptake in the presence of AMS were assayed for rod development and included in Fig. 5. (B) Stimulation of rod development is not blocked by 500 μM β-alanine. The same cells used in A were assayed for stimulation of rod development. Each value represents the proportion of opsin$^+$ cells, expressed as a percentage of the maximal response to taurine. Each value represents eight cultures from a total of three experiments.

tiation, and bioactivity can be reduced without affecting uptake. These experiments indicate that taurine uptake may not be directly required for the stimulation of rod development, arguing against mechanisms that are nutritive or osmoregulatory in nature.

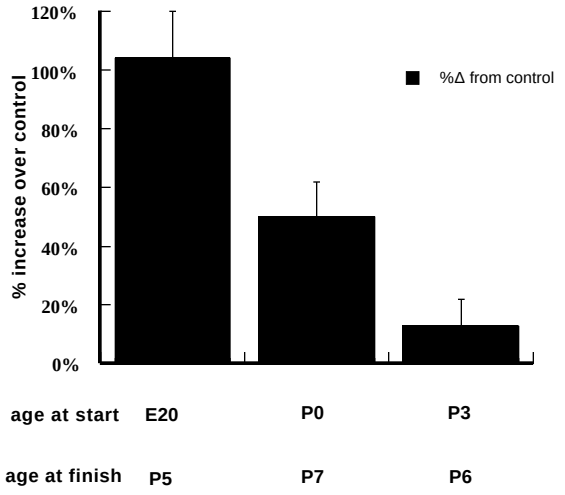

Fig. 7. Response of explant cultures from various ages to taurine. Retinal explants were cultured over the indicated period (E20+7days, P0+7days, P3+3days) with or without the addition of 100 μM taurine. P0 data includes additional cultures not presented in Table 3. Values represent the proportion of opsin$^+$ cells in taurine-treated explants, expressed as a percentage of opsin$^+$ cells in control explants (E20+7=19±1%; P0+7=22±2%; P3+3=19±3%). Each value represents seven to sixteen cultures from a total of two to four experiments.

Rod development in retinal explants is stimulated by taurine, and competitively inhibited by AMS

The most important question raised by the experiments in this report is whether taurine normally plays a role in rod development. As a first step towards answering this question, we took advantage of the characterized ability of retinal tissue to develop organotypically when cultured as explants. Explants utilize intact retinal tissue, and therefore avoid artifacts secondary to dissociation. Moreover, the development of opsin$^+$ cells in explants occurs at levels that are essentially indistinguishable from those observed in vivo (Table 3, P0+7 days ≈25%), and cell survival in these explants is quantitatively similar to that observed in other culture systems (for above compare control values in Table 3 to in vivo and in vitro data in Watanabe and Raff, 1990; Altshuler and Cepko, 1992).

Explants were cultured in the presence of control medium, taurine or AMS (the competitive taurine antagonist), or both. Importantly, addition of taurine stimulated rod differentiation, ruling out the possibility that response to taurine was an artifact specific to dissociated cells, or collagen gel culture (Fig. 7; Table 3). Interestingly, the response of different aged retinas to added taurine varied as a function of age (Fig. 7). When embryonic day 20 explants were treated with taurine for 7 days in vitro, an increase of 104±16% above control was observed. Similar treatment of P0 explants with taurine led to a 50±12% increase over control. Finally, when cultures were initiated on P3 and held for 3 days in vitro, little or no response to taurine treatment was observed (13±9%).

To investigate whether the taurine pathway was stimulating rod development in the absence of exogenous taurine, the

Table 3. AMS causes a competitive inhibition of rod development in explant cultures

		−Taurine				+Taurine		
	Animal	−AMS	+AMS	Effect	Animal	−AMS	+AMS	Effect
Experiment 1	a	21.5	13.0	−40%		n/d	n/d	
	b	21.0	14.5	−31%		n/d	n/d	
	c	22.5	12.5	−44%		n/d	n/d	
Experiment 2	a	30.0	19.9	−34%	g	38.5	32.5	−16%
	b	25.0	21.3	−15%	h	42.0	48.0	14%
	c	25.5	18.0	−29%	i	31.5	31.0	−2%
	d	30.5	22.0	−28%	j	34.5	34.0	−1%
	e	25.5	23.0	−10%	k	39.5	39.0	−1%
	f	27.0	17.5	−35%	l	39.5	40.5	3%
Experiment 3	a	17.2	12.4	−28%	d	24.0	22.5	−6%
	b	20.0	15.0	−25%	e	22.9	27.5	20%
	c	30.0	19.0	−37%	f	33.0	29.5	−11%
Mean opsin$^+$ (%)		24.6	17.3	−30%		33.9	33.8	0%
Standard error		±1.2	±1.1	±3%		±2.3	±2.6	±4%
Number of retinas		n=12	n=12			n=9	n=9	
Mean cell no (10^6)		4.1	4.0	−3%		4.1	3.9	−6%
Standard error		±0.3	±0.3	±7%		±0.3	±0.2	±6%
Number of retinas		m=9	m=9			n=9	n=9	

Whole retinas from P0 rat pups were cultured for seven days in serum-free medium. AMS (100 μM) and/or taurine (10 mM) were included in the culture medium as indicated. The high concentration of taurine (10 mM) was required completely to overcome the inhibition by 100 μM AMS. Retinas were grouped into pairs, such that from each animal one eye was grown without AMS, and the other eye with AMS. The percentage of opsin$^+$ cells in each retina is indicated. Data are shown for individual retinas. Paired sample *t*-test analysis of these data show a statistically significant difference between explants grown in control medium and those grown in AMS (P<0.001); this analysis also indicated that AMS-treated explants contained at least 20% fewer rods (P<0.01). As a control for mitotic or cytotoxic effects of AMS and taurine, the average number of cells found in explants grown in each condition is indicated.

taurine antagonist (AMS) was added to P0 explant cultures. P0 explants cultured in control medium for 1 week contained 24±1% opsin$^+$ cells. When grown in the presence of AMS, a 30±3% reduction in the number of opsin$^+$ cells was observed (17±1%). This difference is significant at P<0.001 (paired sample *t*-test). To test for the specificity of the AMS inhibition, taurine was added and shown to overcome the effect of AMS completely (Table 3). Since high levels of taurine overcome the inhibition of rod development by AMS, AMS must act through the same pathway as does taurine. If AMS blocked rod development through an unrelated pathway, then stimulation with taurine would not be expected to overcome the inhibition. In addition, no change was observed in the number of cells present in each explant (Table 3), indicating that neither taurine nor AMS acts by influencing the survival of cells in retinal explants. The ability of AMS to inhibit rod development competitively in an explant culture is consistent with a role for endogenous taurine in the normal development of rod photoreceptors.

DISCUSSION

Retinal cultures were used to identify molecules normally present in the retina that can influence the development of rod photoreceptors. Study of retinal conditioned medium and retinal extracts provided evidence for at least three distinguishable activities, one inhibitory and two stimulatory. The stimulatory activity is attributable, in part, to the presence of taurine, an amino acid present in high quantities throughout the CNS. Taurine uptake does not appear to be required for this stimulatory action, arguing against an intracellular site of action. Retinal explants cultured in the presence of a taurine antagonist contain fewer differentiated rods, supporting a role for taurine in the development of rods in the intact retina.

These results suggest two main conclusions. First, some endogenous factors that can influence rod differentiation have the properties of small molecules. Second, multiple endogenous activities may both positively and negatively modulate aspects of photoreceptor development. Our results identify taurine as a small molecule that might act together with other cell-cell signals to regulate cellular differentiation in the vertebrate retina.

A role for taurine in the stimulation of rod development

The central finding of these experiments is that a low molecular weight fraction of retinal CM or extract can stimulate rod differentiation in gel cultures, and that taurine is both a component of CM/extract and can partially mimic its activity. Taurine was also found to stimulate rod differentiation when added to cultures of retinal explants (Fig. 7; Table 3), arguing that responsiveness to taurine is not a result of cell dissociation or gel culture. These results demonstrate that exogenously added taurine can stimulate rod differentiation. In addition, a competitive antagonist of taurine (AMS) was identified; when added to explant cultures AMS caused a significant decrease in the proportion of opsin$^+$ cells (Table 3). This effect was specific, in that addition of taurine completely overcame the inhibition by AMS. The ability of a taurine antagonist to diminish rod development, in the absence of added taurine, indicates that in explant cultures endogenous taurine acts to stimulate rod development.

Given these results, that taurine is present in the retina and

has been shown to be required in vivo for proper CNS development (see below), it is reasonable to hypothesize that taurine normally plays a role in promoting rod differentiation in vivo. Whether taurine might act in a permissive manner, or specifically stimulates rod development, is unclear. In high-density gels treated with taurine, however, >60% of cells become opsin$^+$ (Fig. 4A), more than twice the proportion than would be observed at this stage of development in vivo (≈25%). This apparently supra-normal level of rod differentiation is evidence that taurine is capable of promoting rod development and not solely restoring 'normal' levels of differentiation.

The reduction in rod development observed following AMS treatment was not complete, however (≈30%). AMS might fail to inhibit all rod development because of incomplete taurine blockade by the regimen employed. Alternatively, the 30% reduction could approximate the true phenotype of a complete taurine block. In this case, taurine would not be absolutely required for all rods to develop, and taurine-independent pathways would also act to stimulate rod development. The additional rod-promoting activity present in CM and extract, as well as any other endogenous rod-promoting factors, would be candidates for such taurine-independent activities. The complete lack of rod development observed in low-density cultures (this report, and Altshuler and Cepko, 1992) suggests that some combination of factors is required for rod differentiation to occur.

Taurine function and mechanism of action

Taurine is an intriguing candidate for a developmentally important intercellular signal. Dietary depletion studies have shown that taurine is required in adults for photoreceptor function and survival (reviewed in Lombardini, 1991). A developmental role for taurine has been suggested based upon the offspring of taurine-depleted cats, which display severe brain abnormalities (reviewed in Sturman, 1988). These CNS defects have been described as a failure of differentiation, with persistence into postnatal life of ventricular (progenitor) zones containing mitotic cells in the cerebellum (Sturman et al., 1985) and visual cortex (Palackal et al., 1986). Severely depleted animals displayed extensive CNS defects and fetal inviablility (Sturman et al., 1986).

Despite an extensive literature documenting taurine's role as an essential compound, however, the mechanistic basis of this requirement remains unclear. While taurine is not known to be a precursor of other molecules, it could potentially fulfill a nutritive role. Alternatively, it has been suggested that taurine may act an osmolyte, or as an antioxidant. Finally, it has been hypothesized that taurine may act as an intercellular signal, or to modify signaling by other molecules. In comparing these hypotheses to the data presented in this report, it may be important to consider that most of the bioassays in which taurine has been reported to act require 1-20 mM taurine, concentrations 500-10,000-fold greater than that required for a half-maximal stimulation of rod development in the gel culture system.

If taurine were required for the survival of rods, rather than their differentiation, then an increase in cell number would be expected upon taurine treatment. Alternatively, if taurine increased the percentage of rods by selectively killing cells other than photoreceptors, then a large decrease in overall cell number would be observed. Inconsistent with either of these models are the data in Table 2 that demonstrate unchanged cell number upon taurine treatment. Thus, it is unlikely that taurine acts in this assay through changes in photoreceptor (or non-photoreceptor) viability. The stability of cell number upon taurine treatment also appears inconsistent with the hypothesis that taurine might stimulate rod development by influencing cell proliferation. This hypothesis appears unlikely also because maximal stimulation of rod differentiation was observed with taurine treatment for the last 24 hours of culture, by which time virtually no proliferation was observed in gel cultures (Altshuler and Cepko, 1992). Other intracellular roles for taurine, such as osmoregulation, appear less likely based upon experiments (Fig. 6) that dissociate the uptake of taurine from its ability to stimulate of rod differentiation. Finally, the pharmacology data presented in Table 1 do not support any direct chemical action for taurine, such as antioxidation, since compounds with nearly identical molecular structures failed to mimic taurine's rod stimulatory action.

Our data are consistent with the possibility that taurine acts by mediating intercellular signaling amongst developing retinal cells. In order to confirm this hypothesis, it will be necessary to characterize cell-surface receptors systems through which taurine might act. Attempts to identify such receptors may be facilitated by the pharmacologic agents described in this report.

Multiple factors influence rod development in vitro

Despite the attractiveness of models involving single 'master' regulatory factors, a more complicated picture of rod development may be emerging. In this report, we provide evidence for three distinct endogenous activities that can influence rod development, one inhibitory and two stimulatory. Other investigators have found that bFGF (Hicks and Courtois, 1992), S-laminin (Hunter et al., 1992) and an unknown number of unidentified activities (Adler and Hatlee, 1989; Watanabe and Raff, 1992; Harris and Messersmith, 1992; Reh, 1992) can also regulate this process. Perhaps, as rod development becomes more completely understood, only one or a few of these factors will prove to be critically important regulators. Another possibility, however, is that many signals must act in concert to direct rod development in vivo. It seems reasonable that there might be multiple stages at which rod differentiation can be regulated in a pathway leading from progenitor cell to fully mature rod photoreceptor. Multiple factors might also be required if each regulatory decision required the integration of more than one extracellular input. A single pathway might transduce inductive information, and other factors raise or lower the signaling through this pathway. Alternatively, parallel pathways could independently convey similar information.

With regard to the data in this report, it is not yet clear which specific developmental processes influenced by the three activities found in CM and extract. Some hypothetical interactions between factors can be suggested, however. One possibility is that a density-dependent cell-cell interaction causes cells to become taurine-responsive, since a greater proportion of cells respond to taurine treatment in high- as compared to low-density gels (Fig. 4A). Another hypothe-

sis is that the unknown stimulatory activity in CM/extact influences a different stage of rod development than does taurine, since there were distinct kinetics of response to these two activities (Fig. 4C). The idea that there are multiple steps at which rod development can be regulated has been suggested by studies in *Xenopus* that have provided evidence for at least two distinct stages of photoreceptor differentiation (Harris and Messersmith, 1992).

The idea that complex, intertwining regulatory networks may control vertebrate development has recently gained experimental support from a number of systems. In the case of *Xenopus* mesoderm induction, it has become clear that an unexpectedly large number of extracellular factors (activins, FGFs, WNTs, Noggin) play important roles in patterning mesoderm formation in vivo (reviewed in Moon and Christian, 1992). The study of muscle development, furthermore, has shown that mice lacking either MyoD or Myf-5 develop essentially normal muscle, despite many lines of evidence placing these genes as important regulatory factors in muscle development (Braun et al., 1992; Rudnicki et al., 1992). If networks of overlapping regulatory pathways control many developmental processes, then only by identifying all of the components and simultaneously manipulating multiple pathways will the relative role of each become clear.

Conclusions

We provide evidence that taurine, as well as two additional, unidentified endogenous activities, can influence rod differentiation in vitro, and thus might play a role in regulating rod development in vivo. It will be necessary to continue this work by identifying and characterizing these and other factors that can influence rod development and then to examine critically the role of each in regulating this process in vivo. Furthermore, the cell density assay used to study rod development can be used to investigate at least one other retinal cell type developmental pathway (Chris Austin and C. L. C., unpublished observations). Through the use of in vitro assays to characterize molecularly activities that are capable of influencing retinal development and then by subsequently manipulating each candidate molecule's signaling in the retina in vitro and in vivo, it should be possible to dissect the regulatory pathways that control commitment and differentiation in this simple region of the CNS.

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