ADENOSINE 3',5'-MONOPHOSPHATE IN HIGHLY PURIFIED AND MIXED PRIMARY CULTURES OF NEURONS AND NON-NEURONAL CELLS FROM EMBRYONIC CHICK SYMPATHETIC GANGLIA

by

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ABSTRACT

Primary cultures containing $\geq$ 99% neurons, $\geq$ 99% non-neuronal cells (glia), or both cell types were prepared from the sympathetic ganglia of 12-day chick embryos. Levels of cyclic AMP in the non-neuronal cells ($\sim$ 14 pmoles per mg protein) were approximately three-fold higher than levels in the neurons ($\sim$ 4 pmoles per mg protein). Mixed cultures had concentrations of cyclic AMP which fell between the values measured for pure neuronal and pure non-neuronal cultures. The measured cyclic AMP values of mixed cultures were indistinguishable from values predicted by summing the expected contributions of the neurons and non-neuronal cells. Thus, contact between the neurons and non-neuronal cells in these mixed cultures did not appear to alter the level of cyclic AMP in either cell type. Neuronal-glial interactions, such as the specific neuronal stimulation of non-neuronal cell proliferation, occurred independently of any changes in the level of cyclic AMP in the mixed cultures. Cell density was varied in both pure and mixed cultures, and both cyclic AMP concentrations and amounts of ^{3}H-thymidine incorporation into DNA were measured. The cyclic AMP

content of the non-neuronal cells varied inversely with cell density. ^{3}H-Thymidine incorporation was independent of cell density in both neuronal and non-neuronal cultures. Parallel density-dependent decreases in cyclic AMP concentration and ^{3}H-thymidine incorporation were observed in mixed cultures as cell density was increased. The data suggest that there is no relationship between changes in rate of non-neuronal cell proliferation and cyclic AMP levels in these cultures.

The effects of some putative neurotransmitters, analogs of transmitters, and adenosine on levels of cyclic AMP were determined in primary neuronal, non-neuronal, and mixed cultures of embryonic chick sympathetic ganglion cells. Adenosine increased the level of cyclic AMP in the non-neuronal cells but had no effect on levels in either neuronal or mixed cultures. Norepinephrine increased the level of cyclic AMP in neurons, had no effect on the non-neuronal levels, and decreased the cyclic nucleotide content of mixed cultures. Dopamine, isoproterenol, and pilocarpine all caused a reduction in the cyclic AMP content of mixed cultures but had no effect on levels of either neurons or non-neuronal cells. The measured cyclic AMP levels of norepinephrine- and isoproterenol-treated mixed cultures were significantly lower than the values predicted by summing the expected individual contributions of the neurons and the non-neuronal cells. Thus, cells in mixed cultures sometimes responded differently from cells in pure cultures.

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INTRODUCTION

Neurons and glia are intimately associated in the nervous system and are thought to interact with one another. Since cyclic AMP has been implicated as a "second messenger" in numerous aspects of cell-cell communication, it seems possible that this cyclic nucleotide might mediate some of the reported interactions between neurons and glia (BURNHAM et al., 1972; FRIEDE, 1963; LUDUENA, 1973; McCARTHY & PARTLOW, 1976b; NEWBURGH & ROSENBERG, 1972, 1973; PATTERSON & CHUN, 1974; SCHWYN, 1967; VARON & RAIBORN, 1972; VARON et al., 1974). Support for this hypothesis comes from observations indicating that a catecholamine-induced increase in the specific activity of lactate dehydrogenase in at least one kind of glial cell is mediated by an increase in the intracellular concentration of cyclic AMP (DeVELLIS & BROOKER, 1972, 1973). Thus a substance which is normally stored and released by neurons can alter the biochemistry of glial cells by elevation of glial cyclic AMP.

One advantageous system for the study of neuronal-glial interactions utilizes essentially pure ($\geq$ 99%) primary cultures of separated

neurons and non-neuronal cells derived from embryonic chick sympathetic ganglia (McCARTHY & PARTLOW, 1976a). The non-neuronal cells in these cultures appear to be either satellite cells or Schwann cells, both of which are glial elements, while the neurons are thought to be post-ganglionic sympathetic neurons. Using such cultures it is possible to directly compare the biochemistry of each isolated cell type with that of combined cultures formed by mixing pure neurons and pure glia. For example, McCarthy and Partlow (1976b) have recently demonstrated a specific interaction between sympathetic neurons and homologous non-neuronal cells in which the neurons markedly increase the rate of non-neuronal cell proliferation. In addition, non-neuronal cells were shown to promote fiber outgrowth from sympathetic neurons and to increase the specific activity of the neuronal enzyme acetylcholinesterase (McCARTHY & PARTLOW, 1976a).

The studies reported in this paper have utilized the culture system described in the preceding paragraph to obtain information about levels of cyclic AMP in sympathetic neurons and non-neuronal cells. Basal levels of cyclic AMP were quantified in highly purified cultures of sympathetic neurons and non-neuronal cells grown under a variety of conditions in order to determine whether neurons and glia differ in their content of this cyclic nucleotide. In addition, cyclic AMP levels in combined cultures were measured and compared with values obtained in pure cultures. This was done in order to determine whether cyclic

AMP mediates any of the neuronal-glial interactions known to occur in such combined cultures. The neuronal-glial interaction involving neuronal stimulation of non-neuronal cell proliferation was verified by comparing amounts of ^{3}H-thymidine incorporated into DNA by combined cultures with those incorporated by pure neuronal and non-neuronal cultures. These observations also demonstrated varying degrees of contact inhibition in cultures maintained at high cell densities. Thus, it was possible to determine if contact inhibition is related to changes in cellular cyclic AMP in this system.

Data presented in this paper and an earlier abstract (WALLACE et al., 1977) demonstrate that (1) sympathetic glia contain three-fold higher levels of cyclic AMP than sympathetic neurons, (2) neither culture substratum nor time in vitro significantly alters basal levels of the cyclic nucleotide, (3) cyclic AMP does not mediate the neuronal-glial interactions which occur in the combined cultures described in this study, and (4) cyclic AMP does not appear to mediate contact inhibition in this system.

MATERIALS AND METHODS

Culture media and salt solutions. L-15 (Leibovitz) medium (North American Biologicals, Manhattan Beach, CA) was supplemented so that the final preparation contained 10% fetal calf serum (Irvine Scientific, Fountain Valley, CA), 5 mg/ml glucose (analytical reagent, Mallinckrodt, St. Louis, MO), 100 I.U./ml penicillin, and 100 µg/ml streptomycin (Grand Island Biological Co., Grand Island, NY). The fetal calf serum was equilibrated with air before addition to the culture medium by stirring it for two hours at room temperature in a large sterile vessel. The osmolarity of the medium was adjusted to 300 mOsM, and the pH of the supplemented medium was adjusted to 7.15 at 37° C. Medium used in cultures containing neurons was supplemented with 15 ng/ml nerve growth factor (Burroughs Wellcome Co., Research Triangle Park, NC). Puck's saline "G" without phenol red (Irvine Scientific) was supplemented with 5 mg/ml glucose and used as a balanced salt solution. Saline "G" lacking Ca^{++} and Mg^{++} was used during cell separation, while that containing divalent cations was used for washing cells prior to harvest.

Culture surfaces. Polystyrene dishes, either 35 mm Falcon

plastic dishes (Oxnard, CA) or Lux plastic dishes (Newbury Park, CA), were used in all experiments.

In some studies, the surface was coated with rat-tail collagen, which was prepared by the method of Bornstein (1950). The final protein concentration in the collagen solution was 1.5 to 2.0 mg/ml as determined by the biuret method (RUTTER, 1967). Collagen was precipitated by exposure to ultraviolet light in the presence of the catalyst riboflavin monophosphate (Sigma Chemical Co., St. Louis, MO) (MASUROVSKY & PETERSON, 1973). Fifty µl of the collagen-riboflavin mixture were spread evenly over the surface of a 35 mm dish. After a 20 min exposure to ultraviolet light, the dishes were rinsed three times with a balanced salt solution to remove the riboflavin. Dishes were used within 24 hrs of the time they were collagen-coated.

In other experiments, polylysine-coated dishes were prepared by adding one ml of one mg/ml polylysine (Nutritional Biochemicals Corp., Irvine, CA) in pH 8.4 borate buffer to each dish (LETOURNEAU, 1975). After an overnight incubation, the polylysine solution was removed, and the dishes were rinsed five times with water. Dishes were used within 24 hrs of the time they were polylysine-coated.

Both non-neuronal and mixed cultures grew well on the polystyrene surface of Falcon dishes, the polystyrene surface of Lux dishes, collagen, and polylysine. In contrast, neurons grown on the various substrata showed differences both in the adhesiveness of the cells to each

surface and the quantity of fiber outgrowth. In terms of neuronal adhesiveness, polylysine was the best, polystyrene of Lux dishes and collagen were intermediate, and the polystyrene of Falcon dishes was the poorest surface. With the exception of the experiments shown in Fig. 1, all non-neuronal and mixed cultures were grown on untreated Falcon plastic, and all neuronal cultures were grown on polylysine.

Culture wells. Culture wells with a small surface area were prepared by sealing glass or plastic rings ranging from 5 to 15 mm in diameter to the surface of the culture dish with Dow Corning (Midland, MI) high vacuum grease. Plastic rings were sections cut from either polypropylene tubing or polyethylene stoppers (Caplugs Division Protective Closures, Buffalo, NY). Rings were rinsed extensively in distilled water and sterilized before being used to prepare culture wells.

Cultures. Pure populations of highly purified neurons and non-neuronal cells were prepared as described by McCarthy and Partlow (1976a). The paravertebral ganglia lying in the thoracicolumbar region were excised from 12-day chick embryos and provided the material for all cultures.

In this paper, cell density refers to the number of cells per unit surface area in a culture well. The total number of cells (2×10^5

neurons, 1×10^5 non-neuronal cells, or 2×10^5 mixed cells) and volume of medium (200 µl) were kept constant in each culture well except as noted below, and the density was varied by using culture wells of different surface areas (i.e., wells prepared with rings of varying diameters). Because it was technically difficult to work with rings with diameters of less than 5 mm, the highest density cultures were prepared in a 5 mm well containing 200 µl of a cell suspension twice as concentrated as that used for the remainder of the cultures.

In the experiments in which cell density was varied, non-neuronal and mixed cultures were grown for four days in vitro. At this time the cells in cultures at higher densities completely covered the surface of the culture well, while the cells in cultures at lower densities covered less than half of the surface of the culture well. Unless otherwise specified, neurons were grown for only two days in vitro because of the tendency of these cells to aggregate with time. After more than two days in vitro, all of the cells in an entire culture were found in a few large multicellular aggregates. Thus, there were differences in the amount of cell-cell contact in two-day cultures of varying densities, but such differences virtually disappeared after longer times in vitro.

A set of culture blanks was included in each experiment in order to serve as controls for all of the assays. "Blank" culture wells containing culture medium but lacking cells were kept in the incubator and

harvested along with the cell cultures.

Harvesting procedure. The culture medium was removed, each culture well was rinsed three times with ice-cold balanced salt solution, and 200 µl of cold 5% trichloroacetic acid (TCA) were added to each well. Removal of medium, washing, and addition of TCA took less than 30 seconds. TCA was left in the culture wells for 30 min to allow time for the cyclic AMP to be completely extracted. Both protein and DNA were precipitated on the surface of the culture well. The TCA solution was removed and centrifuged in a plastic microcentrifuge tube (A.H. Thomas, Philadelphia, PA) for 5 min in a Beckman model 152 Microfuge. This was done to precipitate any macromolecules which might have been removed from the culture well along with the acid solution. The supernatant was removed and washed three times by adding 1 ml of ether, vortexing the mixture, and removing the ether. The washed extract was then frozen in a -80° C freezer until the cyclic nucleotide assay could be performed.

100 µl of 0.1 N NaOH were added to each culture well after removal of the TCA solution. The NaOH solution was left for two hours to dissolve the precipitated macromolecules and then added to the microcentrifuge tube containing the TCA pellet (if any) from the same culture so that all TCA-precipitable material would be included in the NaOH extract. This NaOH extract was also stored at -80° C until analysis.

DNA and protein assays. DNA was quantified by the indole

method as described by Hubbard et al. (1970) except all volumes were scaled down so that 40 µl of the NaOH extract could be assayed.

Cultures grown on collagen were analyzed for protein by a bromosulphophthalein dye-binding assay (BONTING & JONES, 1957) because it does not detect collagen (OH et al., 1975). A micromodification of this method was developed which will be described in a subsequent paper.

The Lowry method (LOWRY et al., 1951) was used to quantify protein in cultures grown on either plastic or polylysine.

The culture blanks gave low uniform readings in both protein assays, and these values were subtracted from the sample readings to obtain the protein content of each culture. Lowry protein values for culture blanks on polylysine-coated dishes were identical to those on untreated dishes.

Mean protein-to-DNA ratios for the cultures described in this paper were 35 $\pm$ 2 (33), 50 $\pm$ 2 (24), and 57 $\pm$ 2 (24) mg protein per mg DNA for pure non-neuronal cells, mixed cultures, and pure neurons, respectively.

Cyclic AMP assay. The ether-washed TCA extract was evaporated to dryness under nitrogen in order to concentrate it. The residue was dissolved and acetylated (HARPER & BROOKER, 1975), and the acetylated mixture was evaporated to dryness. This residue was redissolved and assayed for cyclic AMP by the radioimmunoassay procedure of

Steiner et al. (1972). The culture blanks did not give a detectable signal in this assay.

Thymidine incorporation. ^{3}H-Methyl-thymidine was purchased from New England Nuclear (Boston, MA). 0.05 µCi of the labeled compound (specific activity 28 mCi/mg) were added to the cultures 22 hours prior to harvesting. 20 µl aliquots of the NaOH extract were analyzed for labeled macromolecules as described by McCarthy and Partlow (1976a).

Determination of the fraction of neurons and non-neuronal cells in mixed cultures. The amount of neuronal DNA present in mixed cultures was estimated from (a) the ratio of neurons to non-neuronal cells in the initial plating suspension and (b) the total amount of DNA in mixed cultures harvested soon after the cells had attached to the culture surface (~6 hours). The amount of neuronal DNA was assumed to be constant throughout the culture period since the neurons apparently do not proliferate (McCARTHY & PARTLOW, 1976a). Thus, any increase in total DNA in the mixed culture is assumed to result from the proliferation of the non-neuronal cells.

Statistics. All values reported in this paper are means ± S.E.M. Numbers of samples are given in parentheses. Differences between means were analyzed by the Student "t" test and were considered significant when the probability that they were zero was less than 0.01. All lines shown in the figures were determined by linear regres-

sion of the form $y = a + b \ln x$. Significance of the calculated slopes was determined by the Student "t" test. A slope was considered significant if the probability that it was zero was less than 0.01.

RESULTS

Cyclic AMP levels were compared in highly purified low density cultures of sympathetic neurons and non-neuronal cells grown on a collagen substratum for four days *in vitro* (Fig. 1, C and D). The basal level of cyclic AMP in the non-neuronal cells was three-fold higher than that in the neurons.

Cyclic AMP levels also were determined in pure low density cultures of sympathetic neurons and non-neuronal cells grown for either two or four days *in vitro* on other substrata. Cyclic AMP levels ($\sim$ 14 pmole per mg protein) in both two- and four-day non neuronal cultures grown on polystyrene (Fig. 1, A and B) were nearly the same as that in four-day non-neuronal cultures grown on collagen (Fig. 1, C). Furthermore, cyclic AMP levels ($\sim$ 4 p mole per mg protein) in two-day cultures of neurons grown either on polylysine or polystyrene (Fig. 1, E and F) were nearly the same as that in four-day neuronal cultures grown on collagen (Fig. 1, D). Thus, the basal cyclic AMP levels in both neurons and non-neuronal cells were not altered by varying either the substratum on which the cells were grown or the duration of time *in vitro*.

The relationship between the number of cells per unit surface area and the cyclic AMP level of sympathetic neurons and non-neuronal cells was examined (Fig. 2). The cyclic AMP concentration of embryonic

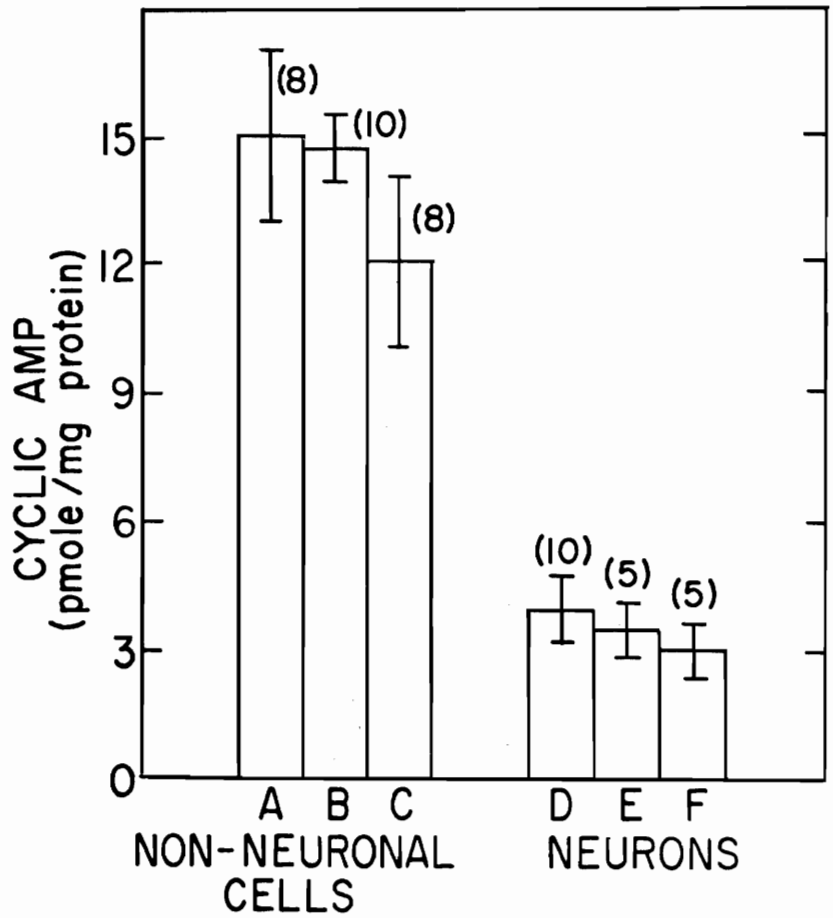

FIG. 1. Cyclic AMP levels in cultures of embryonic chick sympathetic neurons and non-neuronal cells grown on different substrates and for different periods in vitro. Final cell densities were between 1 and 2 ng DNA per mm^2 surface area in all cultures. Surfaces and times in vitro were: A, polystyrene (Falcon), 2 day; B, polystyrene (Falcon), 4 day; C and D, collagen, 4 day; E, polystyrene (Lux), 2 day; F, polylysine, 2 day. Details of experimental procedures are given in Materials and Methods. All values represent means ± S.E.M.; the numbers of samples are shown in parentheses. All non-neuronal levels of cyclic AMP are significantly different from all neuronal levels ($p < 0.01$).

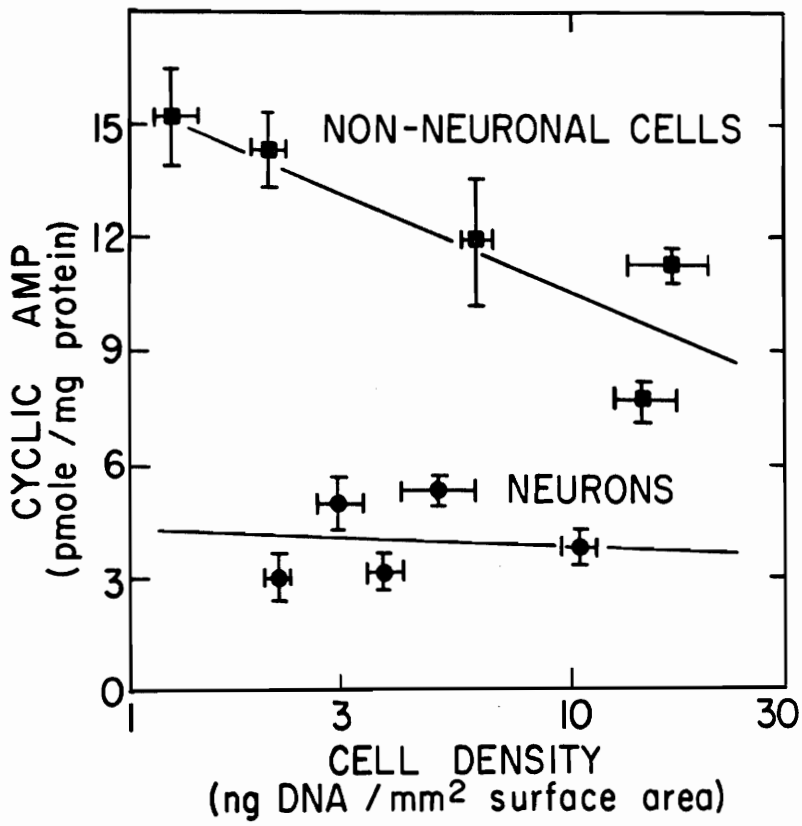

FIG. 2. Cyclic AMP levels of embryonic chick sympathetic neurons and non-neuronal cells grown in vitro at cell different densities. Non-neuronal cells were grown for four days on polystyrene, while neurons were grown for two days on polylysine (see Materials and Methods). All plotted points represent means ± S.E.M. (N = 4 or 5). Linear regression data for non-neuronal cultures are intercept = 15 and slope = -2.1 (significantly different from zero, $p<0.001$). Linear regression data for neuronal cultures are intercept = 4.2 and slope = -0.2 (not different from zero, $p = 0.7$).

sympathetic non-neuronal cells varied inversely with cell density. The cyclic nucleotide levels were 15 ± 1 (5) and 9.7 ± 0.7 (10) pmole per mg protein (490 ± 50 and 330 ± 15 pmole per mg DNA) at cell densities of 1 and 16 ng DNA per mm^2 surface area, respectively. In contrast, the cyclic AMP level of embryonic sympathetic neurons did not change with variations in cell density and was always close to 4.1 ± 0.3 (20) pmole per mg protein (220 ± 20 pmole per mg DNA).

The cyclic AMP levels in mixed cultures containing both sympathetic neurons and homologous non-neuronal cells were determined at several cell densities (Fig. 3). The cyclic AMP concentration of these mixed cultures varied inversely with total cell density. The levels were 9.6 ± 0.4 (5) and 5.6 ± 0.2 (5) pmole per mg protein (450 ± 30 and 250 ± 20 pmole per mg DNA) at cell densities of 2 and 29 ng DNA per mm^2 surface area, respectively. Thus, the cyclic AMP concentration in these mixed cultures always fell between the levels found in pure neuronal and non-neuronal cell cultures. This suggested that contact between neurons and non-neuronal cells might not alter the cyclic AMP concentration of either type of cell. This hypothesis was tested by comparing the cyclic AMP levels of mixed cultures grown at various densities with the values calculated by summing the predicted contributions of the neurons and non-neuronal cells present in the mixed cultures. These calculated values were indistinguishable from the measured values (Fig. 3).

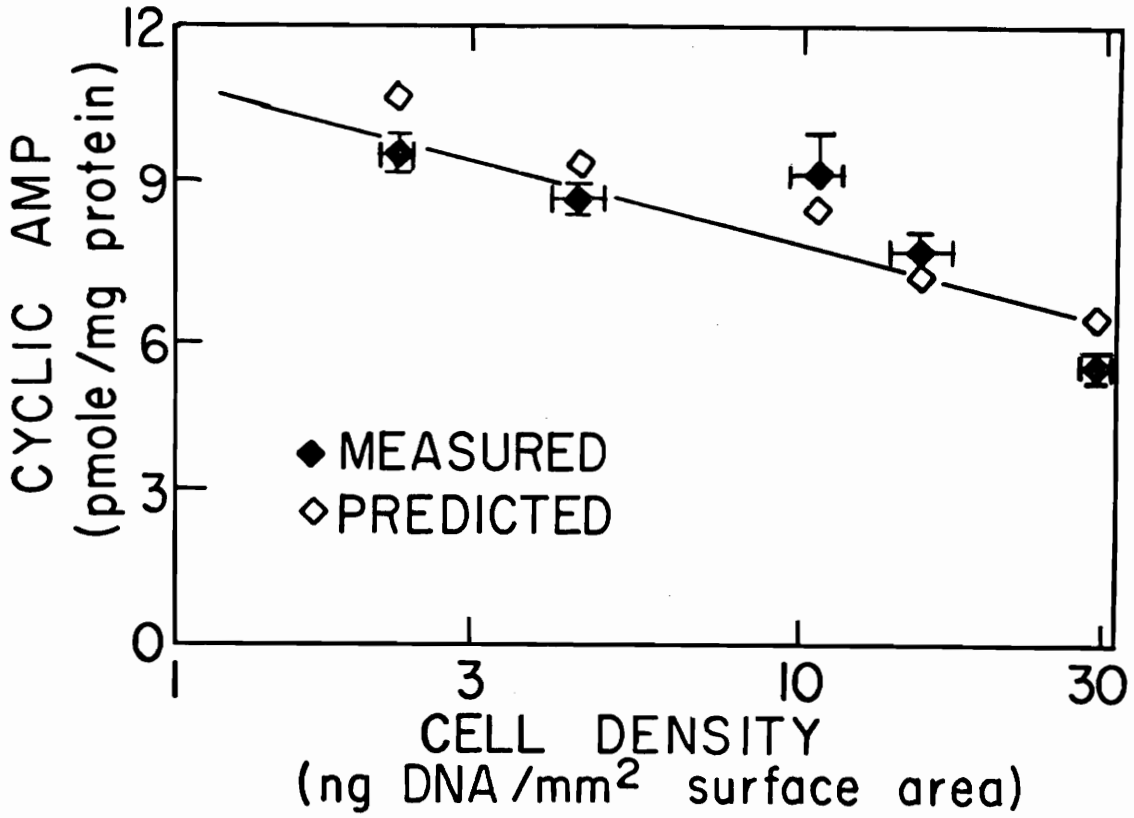

FIG. 3. Cyclic AMP levels of mixed cultures containing both embryonic chick sympathetic neurons and non-neuronal cells grown at different cell densities. Cultures were grown for four days on polystyrene. All plotted points are means ± S.E.M. (N = 4 or 5). Linear regression data are intercept = 11 and slope =-1.3 (significantly different from zero, $p < 0.001$). The predicted values (PV_{cAMP}) were calculated as follows: $PV_{cAMP} = (F_{NN} \times cAMP_{NN} + F_N \times cAMP_N)$, where $cAMP_{NN}$ and $cAMP_N$ are the values of non-neuronal and neuronal cyclic AMP read from Figure 2 at the corresponding cell density and F_{NN} and F_N are the fractions of non-neuronal cells and neurons in the mixed cultures at the time of harvest. Details of experimental procedures are given in Materials and Methods.

Thus, the cyclic AMP content of combined cultures can be predicted when the total cell density and the proportions of neurons and non-neuronal cells are known.

The rate of cell proliferation was estimated by quantifying the amount of ^{3}H-thymidine incorporated into DNA in the same cultures in which the cyclic AMP levels were measured. In both pure neuronal and pure non-neuronal cell cultures, the rate of DNA synthesis was relatively slow and did not change appreciably with variations in cell density (Fig. 4). The ^{3}H-thymidine incorporation into DNA of neuronal cultures is probably due to the exceedingly rapid proliferation of the few sympathetic non-neuronal cells ($\leq$ 1%) which contaminate the neuronal cultures (see Discussion).

Contact between sympathetic neurons and the homologous non-neuronal cells resulted in a marked stimulation of ^{3}H-thymidine incorporation into DNA (Fig. 5). The levels of thymidine incorporation in the mixed cultures were much greater than the values calculated by summing the predicted contributions of the neurons and non-neuronal cells present in the mixed cultures. This difference between the measured and predicted values must be due to a stimulation of non-neuronal cell proliferation as autoradiographic studies on mixed cultures have shown that almost all the non-neuronal cell nuclei are labeled while few if any of the neuronal nuclei are labeled (data not shown).

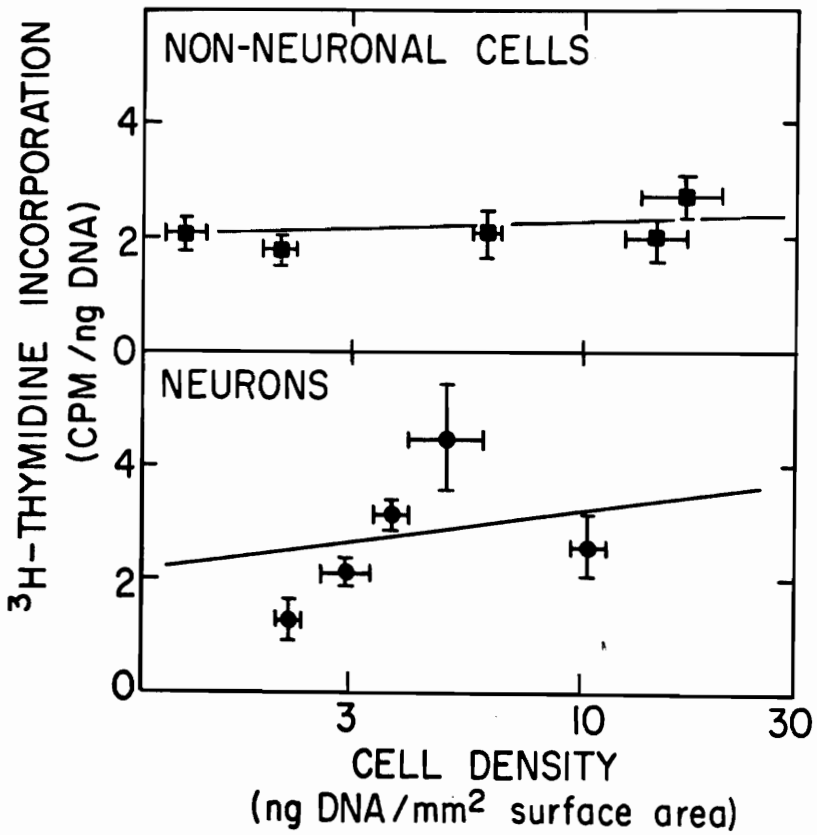

FIG. 4. ^{3}H-Thymidine incorporation into DNA of embryonic chick sympathetic neurons and non-neuronal cells grown in vitro at different cell densities. Non-neuronal cells were grown for four days on polystyrene, while neurons were grown for two days on polylysine (see Materials and Methods). All plotted points are means ± S.E.M. (N = 4 or 5). Linear regression data for non-neuronal cultures are intercept = 1.7 and slope = 0.25 (not different from zero, $p = 0.10$). Linear regression data for neuronal cultures are intercept = 1.7 and slope = 0.43 (not different from zero, $p = 0.13$).

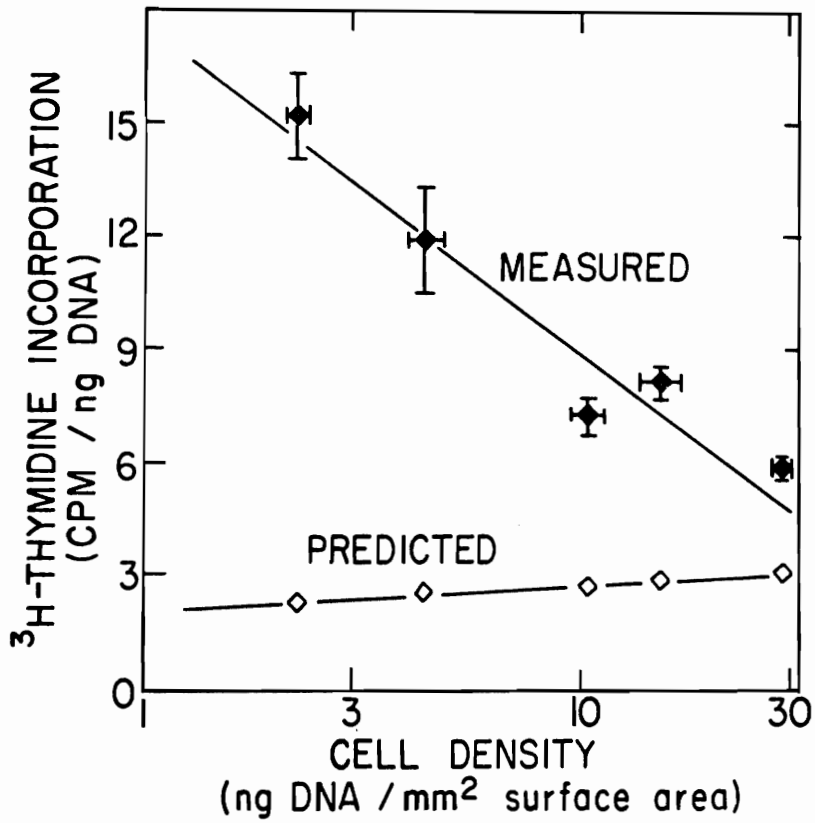

FIG. 5. ^{3}H-Thymidine incorporation into DNA of mixed cultures containing both embryonic chick sympathetic neurons and non-neuronal cells grown at different cell densities. Cultures were grown for four days on polystyrene. All plotted points are means $\pm$ S.E.M. (N = 4 or 5). Linear regression data are intercept = 18 and slope = 3.7 (significantly different from zero, $p < 0.001$). The predicted values (PV_T) were calculated as follows: $PV_T = (F_{NN} \times T_{NN} + F_N \times T_N)$, where T_{NN} and T_N are the values of non-neuronal and neuronal ^{3}H-thymidine incorporation read from Figure 4 at the corresponding cell density and F_{NN} and F_N are the fractions of non-neuronal cells and neurons in the mixed cultures at the time of harvest. Details of experimental procedures are given in Materials and Methods.

^{3}H-Thymidine incorporation by combined cultures of sympathetic neurons and non-neuronal cells decreased with increasing cell density (Fig. 5). A similar decrease was not seen with pure non-neuronal cells even at cell densities that exceeded those of combined cultures showing marked contact inhibition (Figs. 3 and 5). (As a point of reference, confluency was reached at a cell density of approximately 10 ng DNA per mm^{2} surface area.)

Possible involvement of cyclic AMP in the mediation of contact inhibition can be assessed by comparing Figures 3 and 5 for the combined cultures and Figures 2 and 4 for the pure non-neuronal cultures. Thus, cyclic AMP fell by 42% and ^{3}H-thymidine incorporation fell by 60% in going from the lowest to the highest density of mixed cultures. On the other hand, cyclic AMP fell by 35% and ^{3}H-thymidine incorporation was unchanged in going from the lowest to the highest density of pure non-neuronal cultures. Thus, this density-dependent decrease in non-neuronal cyclic AMP occurs without any demonstrable contact inhibition and is sufficient to account for the density-related change in cyclic AMP seen in the combined cultures (Fig. 3).

DISCUSSION

Data are presented in this paper on the levels of cyclic AMP in highly purified ($\geq$ 99%) primary cultures of embryonic chick sympathetic neurons and non-neuronal cells. The basal concentration of cyclic AMP in sympathetic non-neuronal cells was always three-fold higher than that in neurons when expressed per mg protein and two-fold higher when expressed per mg DNA. These basal levels did not vary with either time *in vitro* or culture substratum. Thus, the observed inequality in the concentrations of cyclic AMP in embryonic sympathetic neurons and non-neuronal cells might reflect a basic phenotypic difference between these cell types.

Basal levels of cyclic AMP in neurons and glia have been investigated to a limited extent using other techniques. Neurons appeared to show a weak positive response, while few, if any, satellite cells showed detectable responses when an immunohistochemical technique for cyclic AMP was applied to slices of bovine superior cervical ganglia (KEBABIAN *et al.*, 1975a). Similar immunohistochemical studies on the Purkinje cells in the cerebellum suggest that the basal level of cyclic AMP might be higher in neurons than in glia (BLOOM *et al.*, 1972).

However, studies of this sort are difficult to interpret because of problems in quantification and with selective loss of the cyclic nucleotide from different sites (BLOOM et al., 1972).

Other studies on the relative concentrations of cyclic AMP in neurons and glia have utilized tumor cell lines. The mean cyclic AMP level for five neuronal cell lines was 17 ± 8 pmole per mg protein, while that for eleven glial cell lines was 15 ± 8 pmole per mg protein (SCHUBERT et al., 1976). Thus, neuronal and glial tumor cells do not show the difference in basal levels of cyclic AMP reported in this paper. Since spontaneous genetic changes are known to occur in these cells as they proliferate in vitro (AMANO et al., 1972), cyclic AMP levels of the tumor cells might not represent those of the "parent" neurons and glia from which the cell lines are thought to be derived.

The levels of cyclic AMP reported in this paper for pure and mixed cultures of sympathetic neurons and non-neuronal cells lie between 3 and 15 pmole per mg protein. The range of values in the literature for adult superior cervical ganglia isolated from several species (rat, cat, guinea pig, rabbit, and cow) is 5 to 30 pmole per mg protein (BLACK et al., 1976; KALIX et al., 1974; KALIX & ROCH, 1976; KEBABIAN & GREENGARD, 1971; KEBABIAN et al., 1975b; LINDL & CRAMER, 1974; LINDL et al., 1975; McAFFEE et al., 1971; OTTEN et al., 1974). Thus, the levels of cyclic AMP in our primary cultures of embryonic sympathetic neurons and glia are similar to those found in

intact adult sympathetic ganglia.

Contact between sympathetic neurons and non-neuronal cells does not appear to alter the concentration of cyclic AMP in either cell type. Thus, the level of cyclic AMP in mixed cultures can be predicted by (a) assuming that the constituent neurons and non-neuronal cells each have the same levels of cyclic AMP that they had in pure cultures and (b) summing the weighted contributions from both cell types (Fig. 3). In contrast, contact between sympathetic neurons and non-neuronal cells markedly alters some other biochemical and physiological parameters. For example, neurons greatly stimulate non-neuronal ^{3}H-thymidine incorporation into DNA (McCARTHY & PARTLOW, 1976b; Fig. 5). Also, non-neuronal cells increase the specific activity of the neuronal enzyme acetylcholinesterase (McCARTHY & PARTLOW, 1976a) and promote greater neuronal fiber outgrowth (LUDUENA, 1973; McCARTHY & PARTLOW, 1976a). These neuronal-glial interactions do not appear to be mediated through alterations in the intracellular levels of cyclic AMP since they occur in the absence of detectable changes in the concentration of this cyclic nucleotide.

Sympathetic neurons and non-neuronal cells differ in the way in which their levels of cyclic AMP change with cell density. Cyclic AMP levels of non-neuronal cells in both pure and mixed cultures decreased as cell density was increased. In contrast, the levels of neuronal cyclic AMP in both pure and mixed cultures did not change with cell

density. Thus, a phenotypic difference appears to exist between these cell types in the manner in which cell density affects the regulation of the synthesis and degradation of cyclic AMP.

We are unaware of any other report indicating that glial levels of cyclic AMP vary inversely with cell density. However, a similar inverse relationship has been observed in cultures of 3T3 cells and some of their viral transformed derivatives (BURSTIN et al., 1974; MOENS et al., 1975; OEY et al., 1974).

A comparison of cyclic AMP levels (Fig. 3) and the rate of DNA synthesis (Fig. 5) in mixed cultures demonstrates that cellular cyclic AMP decreased somewhat as non-neuronal cell proliferation was inhibited by high cell density. However, data for pure non-neuronal cultures show that cyclic AMP decreased in these cultures without any inhibition of proliferation (Figs. 2 & 4). Since this density-dependent change in cyclic AMP concentration of non-neuronal cells is sufficient to account for the decrease observed in mixed cultures (Fig. 3), it does not appear that this decrease is associated with contact inhibition. In some studies with other cell types, cyclic AMP levels increased as cell proliferation was inhibited because of high cell density (ANDERSON et al., 1973; HEIDRICK & RYAN, 1971; OTTEN et al., 1971), while in others no change or a slight decrease in cyclic AMP levels was observed when proliferation was inhibited (BURSTIN et al., 1974; MOENS et al., 1975; OEY et al., 1974; SHEPPARD, 1972).

Neuronal cultures incorporated considerably more ^{3}H-thymidine into DNA than had been expected (McCARTHY & PARTLOW, 1976a,b). The present experiments differ from earlier studies in that the cell density was increased nearly five-fold in order to provide sufficient tissue for assay of cyclic AMP ($\sim$ 0.6 vs. $\sim$ 3.0 ng DNA per mm^2 surface area). This increase in cell density greatly enhances the degree of stimulation of non-neuronal cell proliferation, probably because of increased contact between neurons and non-neuronal cells. Under these conditions, the contaminating non-neuronal cells ($\leq$ 1%) can incorporate up to one hundred times as much ^{3}H-thymidine as a corresponding number of cells in highly purified non-neuronal cultures (HANSON & PARTLOW, in preparation). Furthermore, in autoradiographic studies on pure neuronal cultures, we have not observed label over neuronal nuclei (data not shown). Thus, most, if not all, of the ^{3}H-thymidine incorporated by neuronal cultures is probably due to very rapid proliferation of a few contaminating non-neuronal cells.

In summary, several conclusions can be drawn from the present study. First, basal cyclic AMP levels of cultures of sympathetic non-neuronal cells are approximately three-fold greater than those of sympathetic neurons. This difference in cyclic AMP levels appears to be a basic property of sympathetic ganglion cells <u>in vitro</u> since it was observed when the cells were grown for different lengths of time

on several different substrata. Second, there is an inverse relationship between the cyclic AMP content of non-neuronal cells and the cell density. Third, contact between sympathetic neurons and the homologous non-neuronal cells does not alter the cyclic AMP level of either cell type. Thus, the cyclic nucleotide does not mediate the neuronal stimulation of non-neuronal cell proliferation or several other neuronal-glial interactions known to occur in these cultures. Fourth, contact inhibition in mixed sympathetic ganglion cell cultures does not appear to be mediated by cyclic AMP.

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EFFECTS OF PUTATIVE NEUROTRANSMITTERS ON LEVELS OF ADENOSINE 3',5'-MONOPHOSPHATE IN HIGHLY PURIFIED AND MIXED PRIMARY CULTURES OF NEURONS AND NON-NEURONAL CELLS FROM EMBRYONIC CHICK SYMPATHETIC GANGLIA

INTRODUCTION

The cyclic AMP content of many neuronal preparations is altered by exposure to putative neurotransmitters (for reviews see BLOOM, 1975; CHLAPOWSKI, 1975; DALY, 1975). The identification of the responding cells is difficult because of the heterogeneity of the tissues employed in these studies (e.g., brain slices, intact ganglia, etc.). This difficulty can be overcome by using preparations containing only one type of cell, such as the established tumor cell lines derived from nervous tissues. Recently, primary cultures of pure neurons and pure non-neuronal cells have been prepared from sympathetic ganglia (MAINS & PATTERSON, 1973; McCARTHY & PARTLOW, 1976a). The non-neuronal cells are thought to be either satellite cells or Schwann cells (McCARTHY & PARTLOW, 1976a), while the neurons are postganglionic sympathetic neurons. The present investigation describes the effects of some putative neurotransmitters, analogs of neurotransmitters, and adenosine on the cyclic AMP content of cultures of sympathetic neurons, non-neuronal cells, and mixtures of these two cell types.

MATERIALS AND METHODS

Culture media and salt solutions. Culture media and salt solutions were prepared as described in the preceding paper except that the β-nerve growth factor was a generous gift from Dr. Eric Shooter, Depts. of Genetics and Biochemistry, Stanford University.

Preparation of cell cultures. Preparation of cell cultures was carried out as described in the preceding paper. Cultures were prepared from the paravertebral sympathetic ganglia lying within the thoracico-lumbar region of 12-day chick embryos. Neurons and non-neuronal cells were separated by the method of McCarthy and Partlow (1976a). Highly purified cultures of sympathetic neurons were grown in 12 mm wells on 35 mm tissue culture dishes manufactured by Lux (Newbury Park, CA), while both non-neuronal and mixed cultures were grown in the same size wells on 35 mm tissue culture dishes manufactured by Falcon Plastics (Oxnard, CA). These different polystyrene surfaces were used for reasons that are discussed in the preceding paper. Mixed cultures were prepared with approximately equal numbers of neurons and non-neuronal cells. All cultures were grown for 48 hours prior to exposure to drugs. Culture densities at harvesting were

41 ± 2 (N = 26), 100 ± 3 (N = 29), and 23 ± 2 (N = 25) ng protein per mm^2 surface area for pure non-neuronal, mixed, and pure neuronal cultures, respectively.

Drug treatments. All agents were added to cultures at a final concentration of 0.1 mM because earlier studies have demonstrated that this drug concentration causes a nearly maximal change in the cyclic AMP concentration of neuronal and glial cell lines (CLARK & PERKINS, 1971; GILMAN & NIRENBERG, 1971). Cells were incubated for 15 min at 37° C and harvested by addition of 5% trichloroacetic acid as described in the preceding paper. This time period was chosen as previous investigations have shown that cyclic AMP levels rise rapidly during the first five minutes of exposure to these agents and remain elevated for at least 30 min (BROWNING et al., 1974; CLARK & PERKINS, 1971; GILMAN & NIRENBERG, 1971). Each compound was dissolved in culture medium at 37° C at a concentration of 0.3 mM, and 0.2 ml of the drug-containing medium were added to the culture medium bathing the cells (0.4 ml). An identical volume of drug-free medium was added to control cultures.

The drugs used in this study were adenosine (Boehringer Mannheim, Indianapolis, IN), dopamine hydrochloride (Sigma Chemical Co., St. Louis, MO), isoproterenol hydrochloride (Regis Biochemicals, Morton Grove, IL), noradrenaline hydrochloride (Regis), and pilocarpine hydrochloride (Merck Chemical Co., Rahway, NJ).

Statistics. All values reported in this paper are means ± S.E.M. of groups containing four or five samples. For data sets with calculated values, a pooled S.E.M. was determined by analysis of variance of the data from the neuronal, non-neuronal, and mixed cultures treated with the same drug. The Student "t" test was used to determine statistical significance. The level of significance was set at $p < 0.025$.

RESULTS

Adenosine, dopamine, isoproterenol, norepinephrine, and pilocarpine were tested for effects on the cyclic AMP levels of embryonic chick sympathetic non-neuronal cells (Fig. 6). Adenosine caused a 68% increase in non-neuronal cell cyclic AMP, while none of the other compounds had a significant effect.

The effects of the same agents on the cyclic AMP levels of embryonic chick sympathetic neurons were examined (Fig. 7). Norepinephrine caused a 69% increase in neuronal cyclic AMP, while none of the other compounds had a significant effect.

When these same compounds were added to mixed cultures, adenosine had no effect, while dopamine decreased cyclic AMP levels by 51%, isoproterenol by 55%, norepinephrine by 53%, and pilocarpine by 35% (Fig. 8).

Since some agents caused an increase in cyclic AMP in highly purified cultures and a decrease in mixed cultures, the possibility was examined that neuronal-glial interactions might affect the cyclic AMP levels in mixed cultures treated with these agents. A predicted value for the cyclic AMP content of mixed cultures was calculated by (a)

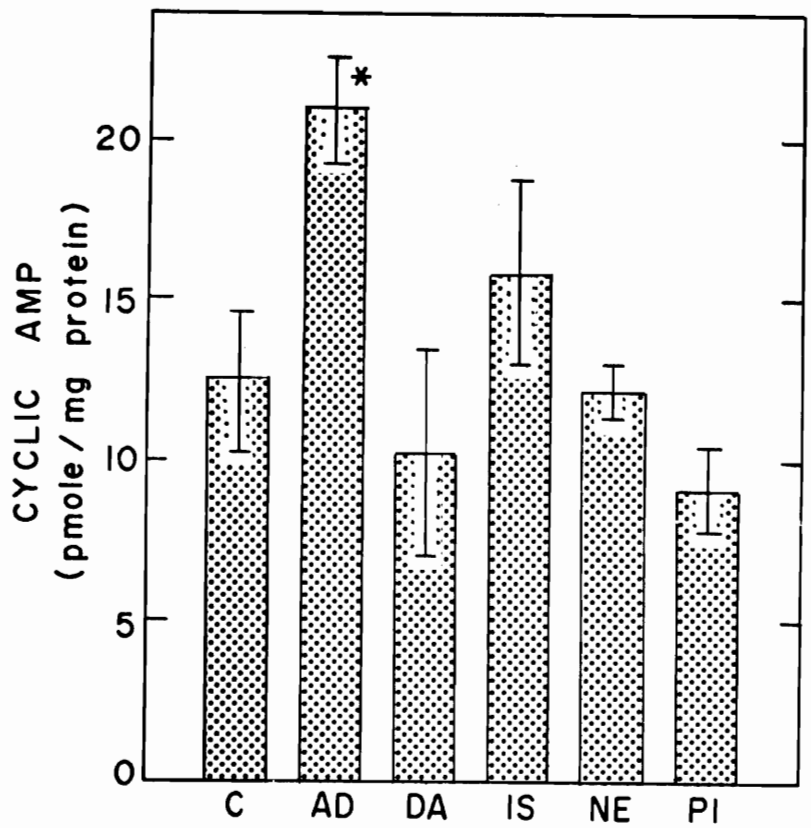

FIG. 6. Effect of putative neurotransmitters, analogs of neurotransmitters, and adenosine on the cyclic AMP content of embryonic chick sympathetic non-neuronal cells. Drugs (0.1 mM) were added 15 min before the cultures were harvested. All values represent means $\pm$ S.E.M. (N = 4 or 5). An asterisk indicates a value that is significantly different from control ($p < 0.025$). Abreviations: C, control; AD, adenosine; DA, dopamine; IS, isoproterenol; NE, norepinephrine; PI, pilocarpine. Experimental details are given in Materials and Methods.

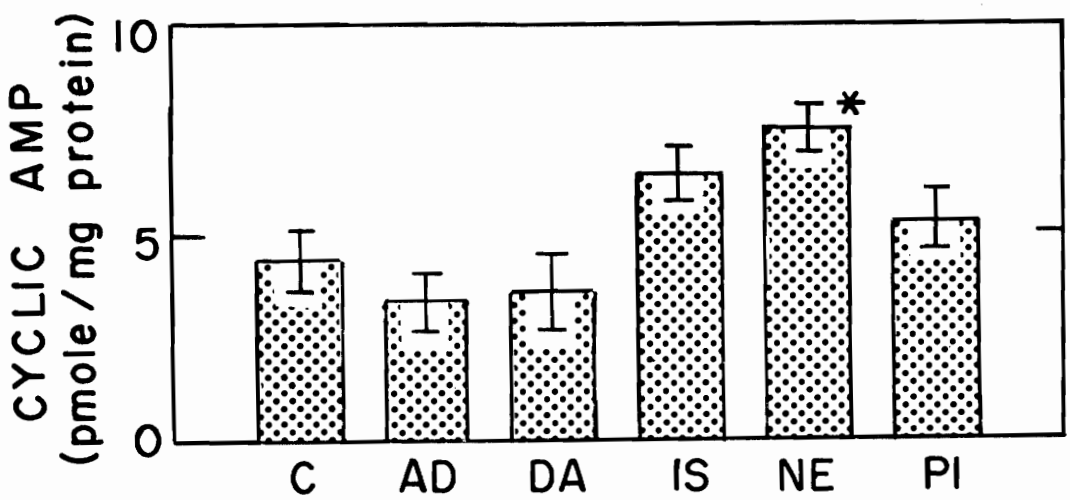

FIG. 7. Effect of putative neurotransmitters, analogs of neurotransmitters, and adenosine on the cyclic AMP content of embryonic chick sympathetic neurons. Drugs (0.1 mM) were added 15 min before the cultures were harvested. All values represent means $\pm$ S.E.M. (N = 4 or 5). An asterisk indicates a value that is significantly different from control ($p < 0.02$). Abreviations: C, control: AD, adenosine; DA, dopamine: IS; isoproterenol; NE, norepinephrine; PI, pilocarpine. Experimental details are given in Materials and Methods.

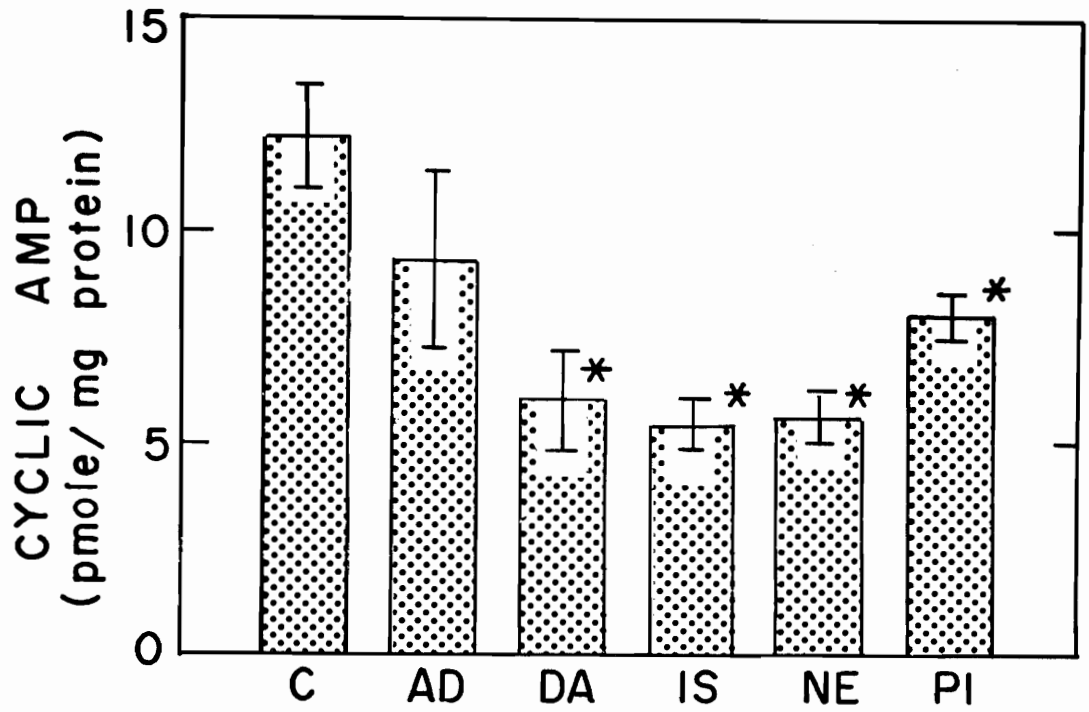

FIG. 8. Effect of putative neurotransmitters, analogs of neurotransmitters, and adenosine on the cyclic AMP content of mixed cultures containing both embryonic chick sympathetic neurons and non-neuronal cells. Drugs (0.1 mM) were added 15 min before the cultures were harvested. All values represent means $\pm$ S.E.M. (N = 4 or 5). An asterisk indicates a value that is significantly different from control ($p < 0.01$). Abreviations: C, control; AD, adenosine; DA, dopamine; IS, isoproterenol; NE, norepinephrine; PI, pilocarpine. Experimental details are given in Materials and Methods.

assuming that the constituent neurons and non-neuronal cells each have the same level of cyclic AMP that they had in pure cultures and (b) summing the weighted contributions from both cell types. Since the mixed cultures contained approximately equal numbers of neurons and non-neuronal cells, the predicted values were simply the mean of the values for pure neuronal and non-neuronal cultures. The measured values were not different from the calculated values for the control or for the cultures treated with adenosine, dopamine, or pilocarpine (Fig. 9). In contrast, the measured cyclic AMP levels were significantly lower than the calculated values in the cultures treated with norepinephrine or isoproterenol (Fig. 9).

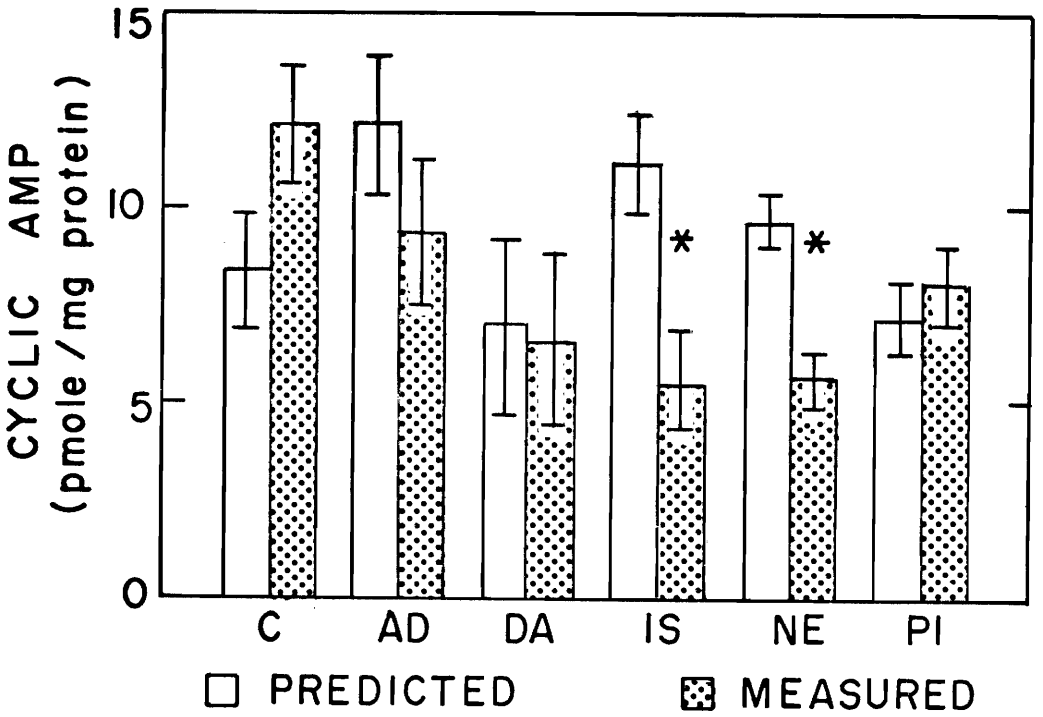

FIG. 9. Comparison of predicted and observed cyclic AMP levels in mixed cultures containing both embryonic chick sympathetic neurons and non-neuronal cells. The predicted values (PV) were calculated as follows: $PV = (F_{NN} \times cAMP_{NN} + F_N \times cAMP_N)$, where $cAMP_{NN}$ and $cAMP_N$ are the cyclic AMP levels in non-neuronal and neuronal cultures treated with the appropriate agent and F_{NN} and F_N are the fractions of non-neuronal and neuronal cells in the culture. An asterisk signifies that the predicted and measured values are significantly different ($p < 0.02$). Abreviations: C, control; AD, adenosine; DA, dopamine; IS, isoproterenol; NE, norepinephrine; PI, pilocarpine. Experimental details are given in Materials and Methods.

DISCUSSION

Treatment with adenosine increased the cyclic AMP content of embryonic chick sympathetic non-neuronal cells but did not affect neuronal cyclic AMP. This effect of adenosine on glial cells might represent a form of neuronal-glial communication since adenosine is released from brain slices following depolarization (SHIMIZU et al., 1970). Adenosine also increases cyclic AMP in some tumor cell lines of glial origin (CLARK et al., 1974), but other glial lines are not responsive to adenosine (GILMAN & NIRENBERG, 1971; SCHULTZ et al., 1972). The lack of an effect of adenosine on neuronal cyclic AMP in the present study is consistent with the results of some studies using neuroblastoma cell lines, which are presumed to be of sympathetic origin (GILMAN & NIRENBERG, 1971). However, other studies on similar neuroblastoma cell lines reported that adenosine increased neuronal cyclic AMP (BLUME et al., 1973).

Treatment with norepinephrine increased the cyclic AMP content of embryonic sympathetic neurons by 69% but did not affect non-neuronal cell cyclic AMP. Since sympathetic neurons normally secrete norepinephrine, this effect on neuronal cyclic AMP might be related to some

kind of a neuronal feedback mechanism. Norepinephrine also promotes an increase in the cyclic AMP content of neuroblastoma cell lines (SAHU & PRASAD, 1975; SCHUBERT et al., 1976). The lack of response of sympathetic glial cells to norepinephrine contrasts with the results of earlier studies on cell lines. Nearly every glial cell line responds to norepinephrine with an increase in cyclic AMP (BROWNING et al., 1974; CLARK et al., 1974; CLARK & PERKINS, 1971; GILMAN & NIRENBERG, 1971; SCHUBERT et al., 1976; SCHULTZ et al., 1972). However, none of these cell lines originated from either sympathetic or embryonic tissue, so their response to norepinephrine might differ from that of glial cells from embryonic sympathetic ganglia.

Isoproterenol did not promote a statistically significant change in the cyclic AMP content of either sympathetic neurons or non-neuronal cells. Studies on neuroblastoma cell lines reveal that the cyclic AMP levels in these cells are unresponsive to isoproterenol (BLUME et al., 1973; GILMAN & NIRENBERG, 1971). In contrast, isoproterenol causes an increase in cyclic AMP levels of glial cell lines (GILMAN & NIRENBERG, 1971; SCHULTZ et al., 1972). However, as mentioned above, glial cell lines might not always be good analogs of sympathetic glia.

Dopamine had no effect on the cyclic AMP levels of either sympathetic neurons or non-neuronal cells in our cultures. Studies examining the effects of dopamine on glial and neuronal cell lines have yielded extremely variable results (GILMAN & NIRENBERG, 1971; SAHU &

PRASAD, 1975; SCHUBERT et al., 1976). A dopamine-induced increase in cyclic AMP in neurons of bovine superior cervical ganglia has been demonstrated using immunocytochemical methods (KEBABIAN et al., 1975). In adult sympathetic ganglia of some species, dopamine released from chromaffin cells is thought to modulate ganglionic neurotransmission (GREENGARD & KEBABIAN, 1974). The lack of a dopamine-mediated increase in cyclic AMP in our neuronal cultures might indicate that dopamine-induced modulation of neurotransmission does not occur in embryonic chick sympathetic neurons. Such an hypothesis is consistent with the observations that in embryonic chick sympathetic ganglia the chromaffin cells comprise only about 5% of the total ganglion cells (JACOBOWITZ & GREENE, 1974) and contain far more norepinephrine than dopamine (GREENE et al., 1976).

Pilocarpine had no effect on the cyclic AMP levels of either sympathetic neurons or non-neuronal cells. This observation agrees with the studies on cell lines as no increase in cyclic AMP has been reported for any glioma or neuroblastoma cell line (GILMAN & NIRENBERG, 1971; SAHU & PRASAD, 1975; SCHUBERT et al., 1976). Furthermore, immunocytochemical methods have been used to demonstrate that acetylcholine does not increase cyclic AMP levels in either neurons or glial cells in superior cervical ganglia (KEBABIAN et al., 1975).

Dopamine, isoproterenol, norepinephrine, and pilocarpine all

caused a decrease in the cyclic AMP content of mixed cultures. In contrast, adult superior cervical ganglia, which are somewhat analogous to our mixed cultures, respond to catecholamines with increased levels of cyclic AMP (CRAMER et al., 1973; GREENGARD & KEBABIAN, 1974; KEBABIAN & GREENGARD, 1971; OTTEN et al., 1974). However, the ability of various brain preparations to increase cyclic AMP levels in response to catecholamines occurs rather late in development (PERKINS & MOORE, 1973; SEEDS & GILMAN, 1971). Thus, the lack of an increase in cyclic AMP in response to catecholamines in our system might be due to the embryonic nature of the sympathetic ganglia used to prepare the cultures.

The responses of mixed cultures to isoproterenol and norepinephrine appear to be affected by neuronal-glial interactions since the measured cyclic AMP values were significantly different from the values predicted by summing the expected contributions of the neurons and non-neuronal cells (Fig. 9). Thus, at least one of the cell types does not respond the same way in mixed cultures as it does in pure cultures. This altered response might be related to one or more of the differences between cells in mixed and pure cultures. For example, nerve fiber production is greatly enhanced in mixed cultures, and glial cell proliferation is markedly stimulated (McCARTHY & PARTLOW, 1976a,b). The identification of the source of the cell-cell interaction which affects the cyclic AMP responsiveness to isoproterenol and norepinephrine

might lead to an increased understanding of the role of cyclic nucleotides in nerve tissue.

In conclusion, the present results demonstrate that putative neurotransmitters, analogs of neurotransmitters, and adenosine can alter cyclic AMP levels in primary cultures of neurons, glia, or combinations of the two cell types. Furthermore, the response of one cell type can be altered by the presence of the other cell type.

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