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Regulation of dopamine- and adenosine-dependent adenylate cyclase systems of chicken embryo retina cells in culture

(synaptogenesis/plasticity/dopamine supersensitivity)

M. C. F. DE MELLO*, A. L. M. VENTURA*, R. PAES DE CARVALHO*, W. L. KLEIN†, AND F. G. DE MELLO*‡

*Instituto de Biofisica de Universidade Federal do Rio de Janeiro, Centro de Ciencias da Saude, Cidade Universitária, Ilha do Fundão, Rio de Janeiro, 21941 Brazil; and †Department of Neurobiology and Physiology, Northwestern University, Evanston, Illinois 60201

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ABSTRACT **We have obtained evidence that receptor-stimulated adenylate cyclase activity [ATP pyrophosphate-lyase (cyclizing), EC 4.6.1.1] is regulated physiologically in both embryonic and mature neurons. In a series of experiments using cultured retina cells from chicken embryos, we found that dopamine-sensitive adenylate cyclase activity spontaneously desensitized as cultures differentiated. The cellular response to dopamine reached a maximum after 5 days in culture and then decreased to 40% during the next 5 days. This spontaneous desensitization appeared to be caused by functional dopaminergic transmission because it could be blocked by the dopamine antagonist haloperidol. The ability of added dopamine at 100 μM to cause near-complete desensitization is consistent with this conclusion. Pharmacologically induced desensitization required 31 hr for maximal effect and was half-maximal at 1–10 μM dopamine. Analogous desensitization of the adenosine-dependent adenylate cyclase system also was noted. When dopamine was removed from the medium of chronically treated cultures, cells resensitized to subsequent stimulation at a very slow rate. Resensitization likely depended on replacement of dopamine receptors because chronic dopamine treatment caused the disappearance of binding sites for the ligand [^{3}H]spiroperidol. In a second series of experiments, using hatched animals, we found that similar regulation of dopamine receptor binding sites and activity could be elicited by manipulation of environmental light, a treatment thought to influence dopaminergic transmission. Retinas from animals in constant light had less specific [^{3}H]spiroperidol binding (35 fmol/mg of protein) than did retinas from animals in constant darkness (66 fmol/mg of protein) and made less cAMP in response to added dopamine. Our results indicate that regulation of the dopamine receptor system begins early in development and continues to function in mature synapses.**

Persisting biochemical changes in synapses between nerve cells are of considerable interest with respect to the development and functioning of the nervous system. In developing neurons, lasting molecular changes in a presumptive synaptic region give rise to a differentiated synaptic specialization. In mature neurons, controlled changes in the number and position of key synaptic molecules likely play an important role in modulating communication between cells. An emerging idea is that local control over molecular components is an important regulatory process occurring throughout the life of a synapse.

We have been studying the neurochemical and synaptic differentiation of the retina, a part of the central nervous system that captures light and begins the processing of visual information. Many compounds identified in the brain as putative neurotransmitters also have been found in the retina of different species (1). We recently showed the existence of dopamine-stimulated adenylate cyclase [ATP pyrophosphate-lyase (cyclizing), EC 4.6.1.1] in the embryonic retina of the chicken and showed that the tissue response to dopamine is maximally differentiated early in development (2). However, retinas from animals after hatching are significantly less sensitive to dopamine than are retinas from embryos, indicating a developmental desensitization of the dopamine receptor system. We recently have observed a similar developmental desensitization of the adenosine receptor system (3).

One hypothesis that could account for the observed decrease in receptor-stimulated adenylate cyclase is that endogenous receptor activation, during development and subsequently, negatively regulates the receptor systems. A relationship between activity and receptor levels was discovered with hormone systems (4). Regulation of receptors on nerve cells has been observed for muscarinic acetylcholine receptors and β-adrenergic receptors (5, 6), and pharmacologically induced alterations in receptors now have been observed for a number of transmitter systems (7).

In the current work, we have used embryonic retina cells grown in monolayer cultures and also intact, mature retinas to test for the ability of endogenous activity to regulate dopamine receptors. Chicken embryo retinas can be easily dissociated into single cells, and under appropriate conditions the cells will develop many properties of the intact retina, including the formation of synapses (8–10). Our results with the dissociated embryonic cells indicate that active dopaminergic transmission develops in culture, causing a spontaneous desensitization of the dopamine receptor system in the differentiating cells. Regulation of receptors for dopamine, as well as for adenosine, also could be induced pharmacologically. In addition, long-term exposure of hatched animals to light or dark environments, treatments expected to give different levels of dopaminergic communication (11), gave tissue responses supporting the idea that dopaminergic transmission negatively regulates the receptor system *in vivo*.

MATERIALS AND METHODS

Reagents. cAMP, protein kinase, bovine serum albumin (Sigma); pargyline (Regis Chemical, Chicago, IL); basal medium of Eagle (BME) (GIBCO); 3-isobutyl-1-methylxanthine (Aldrich); dopamine, *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid (Hepes) (Calbiochem); 4-(3-butoxy-4-methoxybenzyl)-2-imidozolidinone (RO 20 1724) (Hoffmann–La Roche); haloperidol (Johnson & Johnson, São Paulo, Brazil); apomorphine (Merck); [^{3}H]cAMP, [^{3}H]spiroperidol (35.9 Ci/mmol; 1 Ci = 3.7×10^{10} becquerels) (New England Nuclear); three times crystallized trypsin (Worthington); and fetal calf serum

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Abbreviation: BME, basal medium of Eagle.

‡To whom reprint requests should be addressed.

(Flow Laboratories, Rockville, MD) were used throughout this study. Fluphenazine hydrochloride was a gift from Squibb (São Paulo, Brazil). All other reagents were of analytical grade.

Biological Materials. Fertilized White Leghorn eggs were obtained from a local hatchery. Retina dissections were performed according to a published procedure (12) and the developmental stages of the embryos were determined according to Hamburger and Hamilton (13).

Incubations and cAMP Assay Procedures. All incubations followed the procedure described before (2). Unless stated otherwise, the incubation time in the presence of each test compound was 10 min. cAMP was extracted with 5% trichloroacetic acid, purified according to Matsuzawa and Nirenberg (14), and assayed by the method of Gilman (15). Protein was determined by the method of Lowry *et al.* (16).

[^{3}H]Spiroperidol Binding Studies. Binding studies were carried out essentially as described by Creese *et al.* (17). The specific [^{3}H]spiroperidol binding was estimated by subtracting the bound spiroperidol in the presence of 0.1 mM dopamine from the binding observed in the absence of dopamine.

Retina Cell Cultures. Primary cultures of retina cells were prepared from retinas obtained from 9-day-old embryos as described (3). Unless stated otherwise all cultures were prepared in 35-mm Falcon plastic Petri dishes.

RESULTS

Retina cells obtained from 9-day-old embryos and maintained in cultures for 6 days accumulated cAMP in response to dopamine in a dose-dependent manner. The dopamine concentration required to promote half-maximal increase in the cAMP level of the cultures was 1 μM, which was the same as the ED_{50} to activate the adenylate cyclase of the intact embryonic tissue (2). Maximal accumulation of cAMP was observed with 10 μM dopamine (Fig. 1). Peak accumulation was reached after 10-min exposure of the cells to 100 μM dopamine (not shown).

The differentiation profile of the dopamine-dependent accumulation of cAMP of retina cells in culture is shown in Fig. 2. Dopamine-elicited accumulation of cAMP was already observed 3 days after retina cells from 9-day-old embryos were plated. A sharp increase in the cellular response to dopamine was observed 5 days after the onset of the cultures, which corresponded to a stage of cell differentiation equivalent to that of retinas from 14-day-old embryos. Cells maintained for 7 and 10 days in culture were less responsive to dopamine, 66% and 40%, respectively, as compared to the maximal response observed on the fifth day. Apomorphine was less effective than dopamine in eliciting cAMP accumulation of cultured cells.

The basal level of the cAMP in cultures incubated in the absence of phosphodiesterase inhibitors (see legend of Fig. 2) also changed as a function of the age of the cultures. The cAMP level of 2-day-old cultures was approximately 9 pmol/mg of protein; the level increased to 24 pmol/mg of protein on day 3 and decreased thereafter (Fig. 2 *Inset*).

The low cellular responsiveness to dopamine observed after the fifth day of culture, as well as the decrease in the basal cAMP level of cultures during the same period of time, might suggest the existence of desensitization mechanisms of the dopamine cyclase system due to the formation of functional dopaminergic synapses between selective populations of cells. We would expect, then, to prevent the occurrence of the observed desensitization by blocking dopamine receptors with specific dopamine antagonists. As predicted, Fig. 3 shows that cells that had been treated for 5 days with 15 μM haloperidol were hyperresponsive to dopamine as compared to nontreated cultures. The cAMP basal level of haloperidol-treated cells was lower than that found in control cultures, consistent with a role for endogenous dopaminergic transmission in influencing cAMP levels. The ratio of the dopamine-stimulated to nonstimulated cAMP levels of haloperidol-treated and nontreated cells is shown in the *Inset* of Fig. 3. Nontreated cultures showed a 5-fold increase in the cAMP level in response to 0.1 mM dopamine 3 min after addition of 0.1 mM dopamine as compared to a 10-

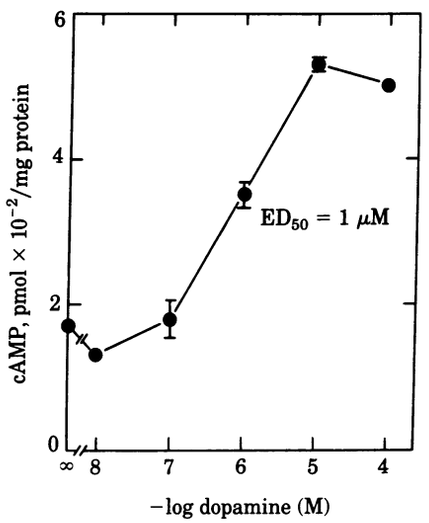

FIG. 1. Dopamine-dependent stimulation of cAMP level of cultured retina cells from chicken embryos. Six days after the cells were plated, the cultures were washed twice with 2 ml of Hepes-buffered BME, pH 7.4 at 37°C. Then 2 ml of BME containing 0.5 mM RO 20 1724, 0.1 mM pargyline, 0.1 mM sodium ascorbate, and 25 mM Hepes (pH 7.4) was added to the dishes, which were preincubated for 10 min at 37°C. The dopamine was added as indicated and the dishes were further incubated for 10 min. The reaction was interrupted by trichloroacetic acid (5% final concentration). Each point represents the mean ± SEM of two cultures. Points without bars indicate that the experimental error bars were within the symbols.

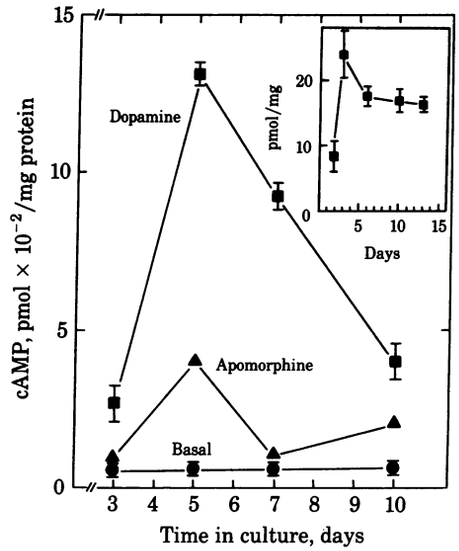

FIG. 2. Developmental profile of dopamine stimulation of cAMP level of cultured retina cells from chicken embryos. The incubation procedure was the same as that described in the legend of Fig. 1. Dopamine and apomorphine were 0.1 mM when present. The results shown in the *Inset* were obtained by washing each 100-mm Falcon plastic Petri dish with 10 ml of BME buffered with 25 mM Hepes, pH 7.4 (37°C). cAMP was extracted from the washed cells with 4 ml of ice-cold 5% trichloroacetic acid. Each point represents the mean ± SEM of two independent cultures; for the apomorphine curve each point represents the result obtained with one culture.

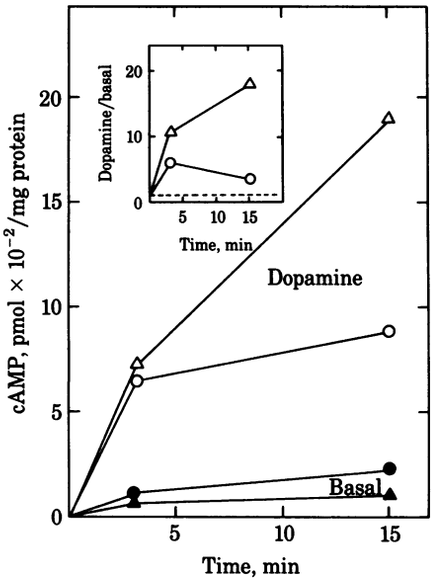

FIG. 3. Haloperidol-induced dopaminergic supersensitivity of cultured retina cells from chicken embryo. Two days after the cells were plated, the cells in one group of dishes were fed with medium (BME/5% fetal calf serum) containing 15 μM haloperidol (△, ▲). The cells in the other dishes were fed with normal medium (○, ●). The medium was changed every other day in both experimental groups. Five days after the onset of haloperidol treatment the cells were washed three times with 2 ml of BME at 37°C, without serum, buffered with 25 mM Hepes, pH 7.4. Then each dish was incubated with 2 ml of BME containing 0.5 mM isobutylmethylxanthine, 0.1 mM pargyline, 0.1 mM ascorbate, and 0 or 0.1 mM dopamine. The reaction was interrupted at the time intervals indicated, with the addition of trichloroacetic acid (5% final concentration). Each point is the average result from two independent cultures, in which individual determinations deviated less than 13% of the values shown. (*Inset*) Data plotted as ratio of cAMP levels in dopamine-stimulated cells to levels in nonstimulated cells.

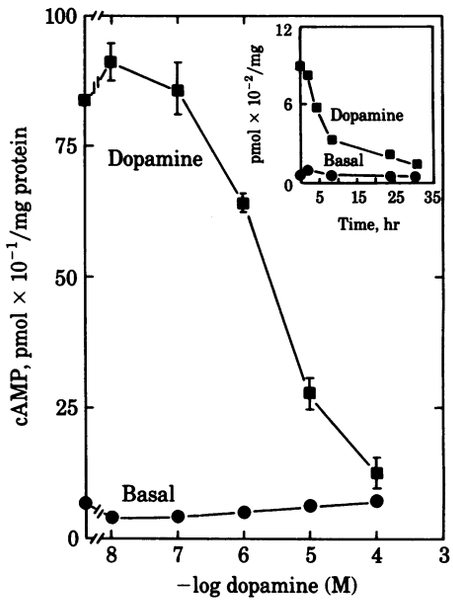

FIG. 4. Dopamine-induced dopaminergic desensitization of cultured retina cells from chicken embryos. Five days after plating the cells in one group of dishes were fed with BME/5% fetal calf serum containing 0.1 mM pargyline, 0.1 mM sodium ascorbate, and 0.1 mM dopamine. The control group was fed with the same medium without dopamine. Control and treated cultures were fed 24 hr later with freshly prepared medium, without or with dopamine (0.1 mM) as above; 48 hr after the onset of dopamine treatment the cells were washed three times with 2 ml of BME buffered with 25 mM Hepes, pH 7.4, at 37°C. Then 2 ml of BME containing 0.1 mM pargyline, 0.1 mM sodium ascorbate, 25 mM Hepes (pH 7.4), and 0.5 mM RO 20 1724 at 37°C was added. The cultures were incubated for 10 min at 37°C, then 20 μl of 10 mM dopamine (freshly prepared in BME) in 20 μl of medium (basal) was added and cultures were further incubated for 10 min. The reaction was stopped as described in the legend of Fig. 3. Each point is the mean ± SEM from two independent cultures. (*Inset*) Time course of dopamine-induced dopaminergic desensitization of chicken embryo retina cells on culture day 6. Dopamine (0.1 mM) was added in one group of dishes at time zero and pulse stimulation of washed cells with dopamine was performed as above at the times indicated. Each point represents the mean ± SEM for two cultures. Points without bars indicate that the experimental error fell within the symbols.

fold increase in haloperidol-treated cells. The cAMP concentration increased progressively thereafter, reaching 18-fold the basal level at 15 min of incubation with dopamine. These results show that long-term inactivation of dopamine receptors blocks the dopaminergic desensitization observed in the cells as a function of culture age (Fig. 2).

Long-term exposure of cells to dopamine led to decreased response of the cells to subsequent pulse stimulation of cultures with saturating concentration of dopamine (0.1 mM). The decay of cell response to dopamine had two components (Fig. 4 *Inset*). The first component had a first-order rate constant of 0.113 hr^{-1} and a $t_{1/2}$ of approximately 6 hr. The second component had a first-order rate constant of 0.032 hr^{-1} and a $t_{1/2}$ of approximately 22 hr. Maximal desensitization was observed 31 hr after the addition of dopamine to the medium. The dopamine-induced dopaminergic desensitization was a function of the concentration of dopamine to which cells were previously exposed for 48 hr. Half-maximal desensitization was observed when the cells were incubated in the presence of micromolar concentrations of dopamine (Fig. 4). Withdrawal of dopamine from the culture media after 48 hr of incubation led to recovery of cell responsiveness to the amine (Fig. 5). However, the recovery time course was longer than that found for the loss of dopamine responsiveness with previous exposure of the cells to dopamine (Fig. 4). The desensitized state observed in cultures exposed for 48 hr to the dopaminergic agonist reflects, at least in part, the loss of specific binding sites for [^{3}H]spiroperidol. While in homogenates of control cultures the level of spiroperidol binding sites was approximately 18.8 ± 0.85 fmol/mg of protein (n = 3), in homogenates of desensitized cultures no specific binding of spiroperidol was detected.

It is known that environmental light increases the turnover rate of dopamine in the rat retina, apparently due to a high rate of dopamine synthesis and release in dopaminergic synaptic contacts of the tissue (11). Fig. 6 shows that the addition of dopamine to intact retinas dissected from light-adapted chickens elicited a 45% increase in the retinal cAMP level above control values. Even without added dopamine, retinas from fluphenazine-treated light-adapted chickens had a 4-fold higher cAMP content. In the latter case, when dopamine was added to the incubation medium, no further increase in the level of cAMP was observed.

Chickens deprived of light for 6 days showed a different pattern of response to dopamine. The basal level of their retinal cAMP was the same as that of animals exposed to light. However, retinas from dark-adapted chickens responded to dopamine by increasing their cAMP content by a factor of 3.8 as compared to 1.45 in light-adapted retinas. The cAMP level of dark-adapted retinas of fluphenazine-treated animals, after incubation in control medium (without added dopamine), did not differ from the basal level of nontreated animals. The addition of 0.1 mM dopamine to these retinas (dark-adapted fluphena-

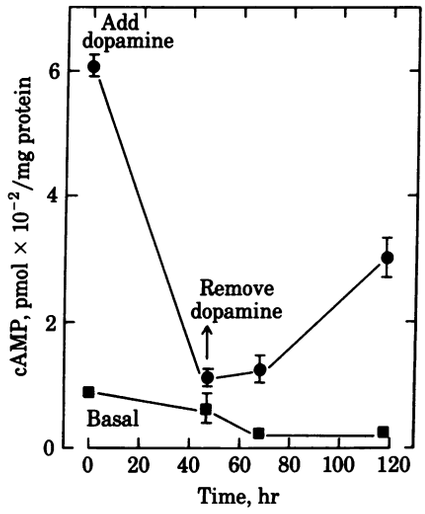

FIG. 5. Recovery time course of dopamine-induced desensitization state of cultured retina cells from chicken embryos. Culture and incubation conditions were as described in the legend of Fig. 4. Dopamine (0.1 mM)-containing medium was added at time zero; 48 hr later one group of dishes was washed twice with 2 ml of dopamine-free BME/5% fetal calf serum and the cultures were allowed to proceed in dopamine-free BME. At the times indicated, previously washed cells were pulse stimulated with 0.1 mM dopamine as described in the legend of Fig. 4. Each point is the mean ± SEM from two independent cultures.

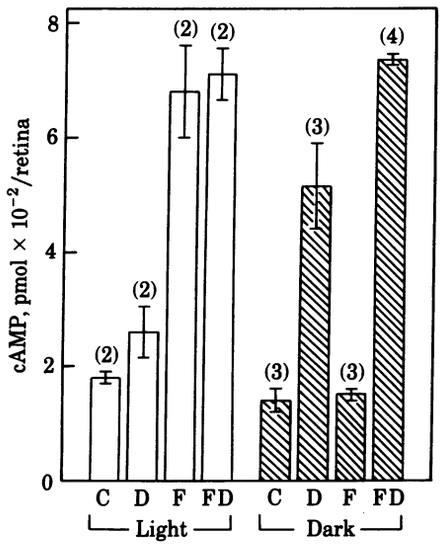

FIG. 6. Light and dark effects upon dopamine-elicited cAMP accumulation of retinas from chickens after hatching. Two-day-old chickens were injected daily with either saline or fluphenazine (180 μg per animal) for 6 days. One group of animals was kept under constant illumination (2,000 lux) during the treatment and the other was kept in the dark. The retinas were dissected out, washed in calcium- and magnesium-free Hanks' solution, and incubated for 10 min in BME containing 0.5 mM isobutylmethylxanthine, 0.1 mM pargyline, 0.1 mM sodium ascorbate, and 25 mM Hepes, pH 7.4. Then dopamine to 0.1 mM final concentration was added and the retinas were further incubated for 5 min. Nonstimulated retinas received only saline solution. Both dissection and incubation procedures were carried out under normal fluorescent illumination. The reaction was stopped by trichloroacetic acid (5% final concentration). The number in parentheses indicates the number of chickens analyzed in each experiment. C, control nonstimulated retina; D, dopamine-stimulated retina; F, nonstimulated retina from fluphenazine-treated animals; FD, dopamine-stimulated retina from fluphenazine-treated animals.

Table 1. Specific [^{3}H]spiroperidol-binding sites in retina homogenates from light- and dark-adapted chickens

Environmental condition	Specific [^{3}H]spiroperidol-binding sites, fmol/mg protein
Light	34.90 ± 6.8 (5)
Dark	66.03 ± 3.0 (5)

Four-day-old chickens were kept either under constant illumination (tungsten bulb, 2,000 lux) or in a dark environment for 5 days, after which time the animals were killed by decapitation and the eyes were removed under normal fluorescent light. The retinas were dissected out in calcium- and magnesium-free Hanks' solution and homogenized for [^{3}H]spiroperidol binding assay. Results are mean ± SEM and the number in parentheses indicates the number of experimental animals analyzed in each case. $P < 0.005$ that the difference between means is due to chance.

zine-treated) promoted a large accumulation of cAMP. Table 1 shows that the number of [^{3}H]spiropheridol-specific binding sites in retina homogenates of the dark-adapted animals, without fluphenazine treatment, was 90% higher than in retinas from light-adapted chicken.

An adenosine-elicited accumulation of cAMP previously has been found in the intact chicken retina, as well as in cultured cells of the embryonic tissue (3). As with the dopamine cyclase system, the adenosine-dependent accumulation of cAMP of cultured retina cells desensitized when adenosine receptors were continuously exposed to specific agonists. Fig. 7 shows the effect of long-term adenosine treatment of cultures on retina cell responsiveness to subsequent pulse stimulation with adenosine (0.1 mM). Adenosine-elicited accumulation of cAMP decreased significantly when the cells were exposed for 72 hr to increasing concentrations of the agonist, with 50% loss of adenosine re-

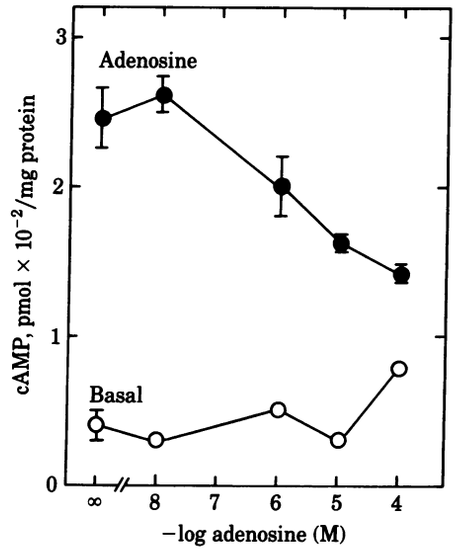

FIG. 7. Adenosine-induced decrease in cultured retina cell responsiveness to pulse stimulation of cAMP accumulation with adenosine. Cultures were prepared with retinas obtained from 9-day-old embryos. For the next 2 days of incubation the cells in one group of dishes were fed every 24 hr with medium containing adenosine in the concentrations indicated. Control cultures were fed with the same medium without adenosine. Three days after the onset of adenosine treatment the cells were washed with BME buffered with 25 mM Hepes (pH 7.4) and incubated for 10 min at 37°C with 2 ml of BME containing 0.5 mM RO 20 1724. Then 20 μl of 10 mM adenosine dissolved in BME was added. Identical volumes of BME were added to control cultures. The cultures were then further incubated for 10 min, and the reaction was stopped by the addition of trichloroacetic acid (5% final). Each point is the mean ± SEM of duplicate cultures.

sponse occurring when cultures were previously treated with 1 μM adenosine. Maximal desensitization, corresponding to a reduction to 50% in the cell responsiveness to the nucleoside was observed at 0.1 mM adenosine. Complete desensitization was not observed under this condition. However, when cultures were exposed for 48 hr to 0.1 mM adenosine N^1-oxide, an adenosine analog resistant to adenosine deaminase, total loss of cell sensitivity to pulse stimulation with adenosine was obtained (data not shown).

DISCUSSION

Our results show the existence of control mechanisms that regulate dopamine- and adenosine-stimulated adenylate cyclase activities in embryonic and developed avian retina cells. Of particular interest to us is the finding that dopamine receptor desensitization, dependent on spontaneously active dopaminergic transmission, is a natural occurrence in differentiating monolayer cultures. All the data presented are consistent with the idea that decreased cellular sensitivity to neurotransmitters accompanies the onset of neurotransmission during central nervous system synaptogenesis and that control of sensitivity by transmission continues as a natural occurrence in mature neurons as well.

In cultured cells, maximal responsiveness to dopamine occurred 5 days after initial plating. A spontaneous decrease in sensitivity occurred during the next 5 days, even though no changes in protein content per culture were observed during this period (not shown). Most significantly, this spontaneous decrease was blocked by haloperidol, an inhibitor of dopamine receptors (18). The data strongly suggest that the partial loss of dopamine sensitivity that accompanied the maturation of the cultures was due to endogenous dopaminergic activity. Reinforcing this idea is the observation that exogenous dopamine, chronically added, caused a further reduction in dopamine sensitivity, giving pharmacological support for the presence of regulatory mechanisms in the embryonic cells. The ED_{50} for the dopamine-induced loss (1 μM) was the same as observed for the dopamine stimulation of the cyclase systems of the cultured cells (Fig. 1) and of intact embryonic retina (2). Recovery of cell responsiveness to dopamine after withdrawal shows that the loss of the dopaminergic response was not due to cell death. Because the lack of cell responsiveness to dopamine was accompanied by the disappearance of specific [^{3}H]spiroperidol-binding sites, it is likely that the main component of the dopaminergic system affected during regulation was the dopamine receptor. Pharmacologically induced changes in dopamine receptor binding sites have been noted previously in mature animals (17, 19). The long time required for recovery of dopamine sensitivity after dopamine withdrawal may indicate a requirement for *de novo* synthesis of receptor molecules. The rate at which the response decayed in the presence of dopamine was faster than the rate of recovery, and we noted the presence of two kinetic components. Whether two mechanisms exist or desensitization is occurring on different cell types remains to be determined.

The fact that similar changes could be induced for the adenosine-sensitive adenylate cyclase system indicates that regulation may be a general property of developing cells. Further work is needed to characterize the adenosine regulatory response and compare its features with the dopamine system.

Our results with the hatched animals give strong support to the idea that dopaminergic transmission also controls receptor sensitivity after synapses have formed. As an alternative to pharmacological manipulations of dopaminergic activity, we subjected two groups of animals to different environmental lighting. Light stimulation has been found to increase the rate of turnover and release of dopamine in retinas of other species (11). In our experiment, to maximize effects, one group was in constant darkness and the other group was in constant light. We found that light-adapted retinas were less sensitive to dopamine than were retinas of embryonic tissue (2). Light deprivation significantly reduced this desensitization. At least part of the effect of constant light can be accounted for by a decreased number of dopamine receptors. All the data indicate that the dopaminergic system of the retina can be modulated by environmental light through the activity of dopaminergic synapses.

Many behavioral disorders have been ascribed to changes in the efficiency of communication in dopaminergic synapses (20, 21). Further understanding of synaptic control mechanisms for dopamine, and other systems such as adenosine, thus are likely to shed new light on abnormal as well as natural physiological processes. The occurrence of dopaminergic plasticity in embryos indicates also that factors capable of influencing the mechanisms of receptor regulation could be potential hazards to the normal ontogeny of chemical synapses in the central nervous system.

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