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Extracellular cAMP Accumulation and Degradation in Rat Cerebral Cortex in Dissociated Cell Culture

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Norepinephrine (NE) stimulated the accumulation of cAMP in embryonic rat cerebral cortex in dissociated cell culture. After exposure to NE for 10 min, the intracellular cAMP content of these cultures went from 22 ± 12 to 202 ± 75 pmol/mg protein. Using selective culturing techniques, evidence was obtained supporting the hypothesis that NE-stimulated production of cAMP is a property associated with the glial rather than the neuronal component of these cultures. Beta adrenergic agonist stimulation of cortical cultures also resulted in the efflux of cAMP into the medium. At the peak of extracellular accumulation of cAMP (following a 40-min exposure to isoproterenol), 180 pmol cAMP/mg protein had been transported into the extracellular medium. The fate of extracellular cAMP was investigated using thin-layer chromatography. Extracellular cAMP was degraded to AMP and adenosine; this degradation did not seem to be due to the presence of serum or serum components, suggesting the existence of an extracellular phosphodiesterase. In response to NE stimulation of glia, in particular astrocytes, cAMP or its metabolites may accumulate at high enough concentrations in the extracellular space in cerebral cortex to affect neuronal function, possibly via adenosine receptors.

The cerebral cortex is one of the major projection areas for the noradrenergic axons of the locus coeruleus, the largest noradrenergic nucleus of the mammalian CNS. In order to understand the function of norepinephrine (NE) in cerebral cortex, it is necessary to identify cortical adrenergic target cells. The ability of beta adrenergic agonists to stimulate cAMP accumulation in the CNS has been previously observed (Drummond, 1984). However, because of the cellular heterogeneity of the CNS, in particular the intermixing of neurons and glia, it has not been possible to directly demonstrate the cell types capable of responding to beta adrenergic stimulation by synthesizing cAMP. This is clearly a matter of importance in trying to understand how NE may affect neuronal function on a cellular and molecular level. Two approaches to this problem have been used employing tissue culture to study beta adrenergic targets in cerebral cortex: receptor binding studies and studies of beta adrenergic agonist stimulation of cAMP accumulation. Membrane binding studies (McCarthy and Harden, 1981) as well as radioligand receptor autoradiography (McCarthy, 1983; Burgess and McCarthy, 1985; Burgess et al., 1985) have shown in tissue culture that a subpopulation of cortical astrocytes, but not neurons, possesses beta adrenergic receptors. Studies of beta agonist stimulation of cAMP accumulation using primary cultures of astrocytes (McCarthy and deVellis, 1978; Ebersolt et al., 1981; Hamprecht, 1986), have shown that cortical glia have a beta agonist stimulated adenylate cyclase.

Davoren and Sutherland (1963) discovered that cells that accumulate cAMP in response to beta adrenergic stimulation also secrete cAMP in response to the same stimulus. This phenomenon has been demonstrated in a variety of cell types including cell lines derived from rat and human gliomas (Clark et al., 1974; Penit et al., 1974; Doore et al., 1975; Nickols and Brooker et al., 1978; Barber and Butcher, 1983). Recently the phenomenon of dopamine-stimulated cAMP efflux in rat striatal slice preparation has been reported (Stoof and Kebabian, 1981, 1982; Schoffelmeer et al., 1985; Headley and O'Shaughnessy, 1986; O'Shaughnessy et al., 1987). Cyclic AMP efflux in primary cultures derived from the CNS has not been demonstrated.

We investigated NE stimulation of cAMP accumulation in rat embryonic cortical cultures. Using selective culturing techniques, we found that the NE-stimulated cAMP accumulation that we were able to demonstrate under our conditions of assay seemed to be associated with glia but not with neurons. Since cAMP efflux might have important signaling functions between cells, especially in a tissue in which only a subpopulation of cells possesses beta receptors, we studied whether cAMP efflux occurs in primary cultures of cerebral cortex. We found that cortical cultures released significant quantities of cAMP in response to beta adrenergic stimulation. By investigating the fate of extracellular cAMP, we also obtained evidence of an extracellular phosphodiesterase in cerebral cortex.

Materials and Methods

Mixed neocortical cultures were prepared from embryonic day 16 (E16) CD rats by the method reported by Dichter (1978) and subsequently modified (Snodgrass et al., 1980; Dichter, 1983) to permit culture on poly-L-lysine- or collagen/poly-L-lysine-coated glass coverslips. Since cerebral cortex contains no catecholaminergic or serotonergic neurons, cultures derived from cerebral cortex would be expected to be lacking these cells as well. In fact, glyoxylic acid histochemistry on these cultures revealed no fluorescent cells. Mixed cultures were plated at 450,000 cells per 35-mm dish (4.7×10^4 cells/cm^2). They were mitotically in-

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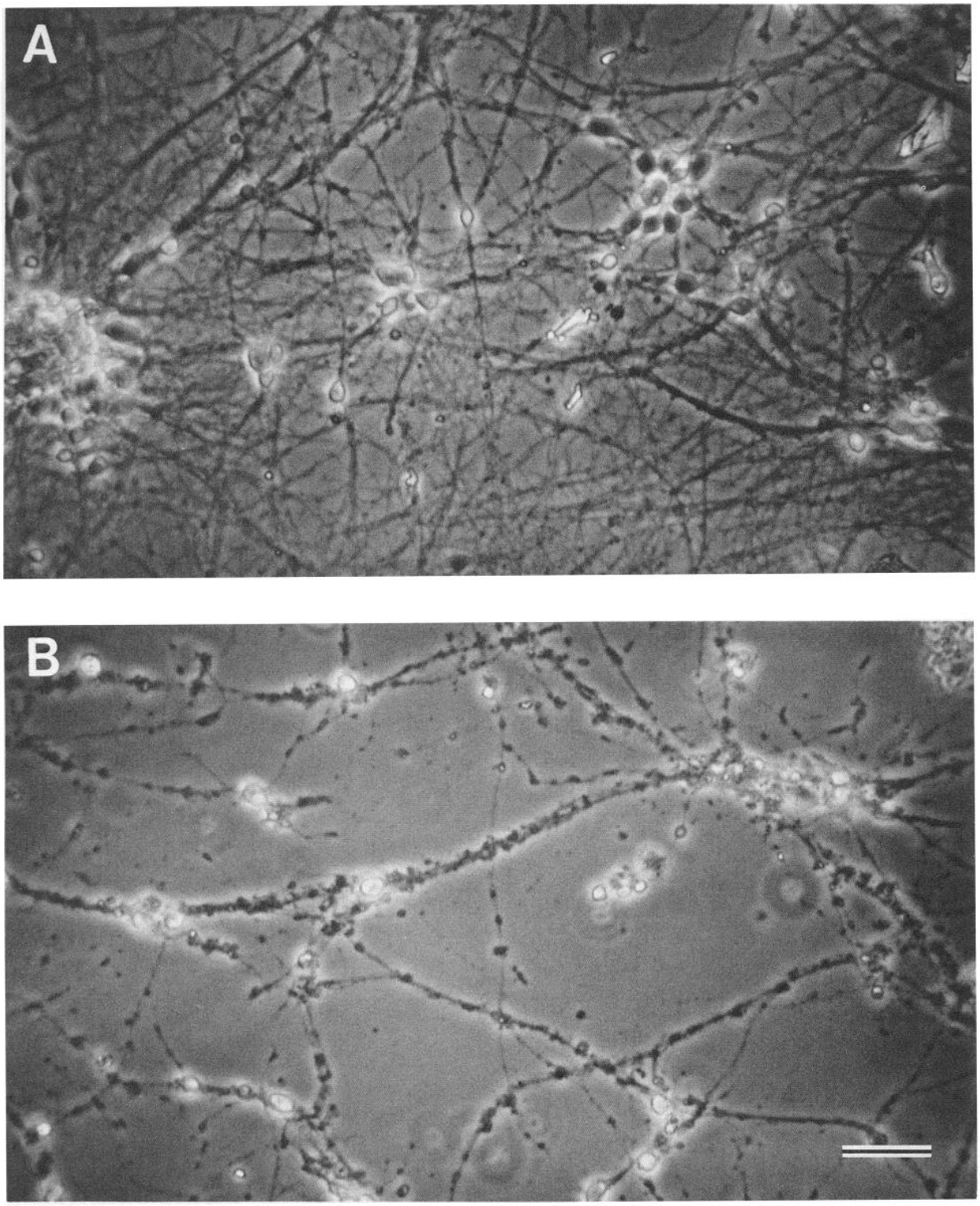

Figure 1. Effect of conditioned media on the survival of neurons in the absence of non-neuronal cells. Rat cerebral cortex was dissociated and plated on five polylysine-coated coverslips per culture dish as described in Materials and Methods. At 4 d in culture, growth medium was changed and cultures were exposed to cytosine arabinoside (5 μM for 2 d) to stop cell proliferation. Two coverslips were then removed and placed in a dish with three coverslip cultures of astrocytes (see Materials and Methods) and fresh growth medium. The three remaining coverslips were placed in a new dish with fresh media. These dishes were placed on wet filter paper in petri dishes to reduce evaporation, and they were not subjected to further media change. These phase contrast photographs were taken one week later. *A,* Neuron-enriched culture growing in the presence of astrocytes. Note the many phase bright neurons and the dense network of processes. *B,* Neuron-enriched cultures growing in the absence of astrocytes. Few neurons survive in the absence of neighboring astrocyte cultures, and the fiber network is sparse and degenerating. A similar effect can be observed by growing neuron-enriched cultures with media conditioned by astrocyte cultures or by mixed cultures (but without astrocyte cultures or mixed cultures actually being present in the dish). Calibration bar, 50 μm.

hibited (5 μM cytosine arabinoside for 2 d) when the glia had proliferated to form a confluent layer across the coverslip, which occurred at 1–2 weeks *in vitro*. Growth medium consisted of MEM supplemented to 11 mM glucose, penicillin (24 U/ml), and streptomycin (24 μg/ml) and contained 5% heat-inactivated (56°C for 30 min) rat serum. Rat serum was obtained from retired breeders. Mixed cultures contained 5–10% neurons based on tetanus/antitetanus immunocytochemistry (Raff et al., 1979). Cultures were grown in 35-mm dishes (Nunc) containing 5 12-mm glass coverslips (Bellco) which were removed from dishes and placed in 24 well plates (Costar) for experiments.

Glial cultures were prepared using cortex derived from P_4 animals that was minced, trypsinized, and plated on collagen/poly-L-lysine-coated glass coverslips (Cummins and Glover, 1976; McCarthy and deVellis, 1980). Oligodendroglia were removed by shaking, and the remaining cells were trypsinized and replated. This procedure yielded cultures that contained no neurons.

Neuron-enriched cultures were prepared from E16 embryos exploiting several observations: (1) Neurons attach well and send out processes on a poly-L-lysine substrate (Bottenstein and Sato, 1980; Yavin and Yavin, 1980); (2) a brief exposure to cytosine arabinoside (5 μM for 48 hr) from 4 to 6 d *in vitro* prevents the proliferation of non-neuronal cells in these cultures; (3) in the absence of abundant glia neurons die, but they may be maintained in culture using either "helper" coverslip cultures of glia placed in the same dish as the neuron-enriched cultures (Banker, 1980), or growth media conditioned by cultures of glia (Banker, 1980), or by mixed cultures of neurons and glia. Figure 1 shows 2 cultures from the same dissection, *A* grown with helper cultures and *B* grown without such helper cultures, shown one week after inhibition. Neuron-enriched cultures were prepared by using poly-L-lysine (Peninsula)-coated glass coverslips (Bellco), inhibiting at 4 d with cytosine arabinoside (5 μM for 2 d), and thereafter changing media 3 times per week using conditioned media (growth medium exposed to mixed cultures for 48 hr). Results similar to those obtained using conditioned medium were achieved using a growth medium based on the serum-free medium developed by Bottenstein and Sato (1979; Bottenstein, 1983) but containing 1% heat-inactivated rat serum (Hoffman et al., 1988). The base for this medium contained F-12 (Gibco #320-1765), MEM (Gibco #320-1090, without glutamine), and DME with glutamine (Gibco #320-1885), 1:5:4. In addition to the "N2" additives suggested by Bottenstein and Sato (1979) were the following: catalase, 1 μg/ml (Walicke et al., 1986); and BSA (Calbiochem #12657), 0.5%. Glucose was supplemented to a final concentration of 11 mM. Penicillin and streptomycin were included. Neuron-enriched cultures were plated at 225,000 cells/35-mm dish. Cultures prepared and maintained in this way routinely survived for 1–2 months *in vitro*. Neuron-enriched cultures at 2 weeks *in vitro* contained 75.2 ± 14.8% cells that were tetanus/antitetanus positive by immunocytochemistry. Some but not all non-neuronal cells were GFAP positive.

In some experiments neuron-enriched cultures were used that were depleted of neurons. As described by Banker (1980), neurons could be eliminated from neuron-enriched cultures maintained using conditioned medium by replacing conditioned medium with fresh medium. Using this procedure, neurons were selectively eliminated from neuron-enriched cultures, leaving only the non-neuronal component of these cultures, which represented approximately 25% of the original culture. In these neuron-depleted cultures there were virtually no morphologically recognizable neurons.

For assay of beta agonist cAMP accumulation, cultures were preincubated for 20 min with a bicarbonate buffered saline without phenol red (NaCl, 120 mM; KCl, 3 mM; $CaCl_2$, 2 mM; $MgCl_2$, 1 mM; $NaHCO_3$, 27.5 mM; glucose, 8 mM; Na_4EDTA, 100 μM). EDTA was included in the saline because it was found that catecholamines were stable for several hours at neutral pH with the addition of EDTA at this concentration, which does not significantly alter [Ca] and [Mg] present at millimolar concentrations (Rosenberg et al., 1985). After preincubation, saline was replaced with one containing 10 μM isoproterenol or norepinephrine. This concentration was chosen to ensure a maximal effect while still within the physiological range. Incubation with agonist was continued for selected times as indicated. At the end of the incubation, the medium was withdrawn and cultures were extracted with ice-cold 0.3 M $HClO_4$. Medium was deproteinized with an equal volume of 0.6 M $HClO_4$. Acid samples were neutralized with 1/10 vol of 3 M $KHCO_3$, incubated for 15 min on ice, and then centrifuged at 2500 × *g*. Cyclic AMP contained in these samples was assayed by the NEN radioimmunoassay kit using ^{125}I-cAMP. A standard curve was constructed for each assay, and sample values were determined by visual interpolation. Standard curves relating cAMP concentration and displacement of radioactivity were sigmoidal. The lowest quantity of cAMP in the series used to construct the standard curve was 0.05 pmol. A zero value (as in Table 2) denotes that no displacement of radioactivity was observed, compared with the blank, with addition of sample. Cyclic AMP detected was found to be linear with added sample volume. Results similar to those obtained with radioimmunoassay were obtained using a binding protein assay (Amersham). The accuracy of this assay was verified using internal standards (Weller et al., 1972).

Extracts were chromatographed by thin-layer chromatography using an ethanol/0.5 M ammonium acetate (5/2) buffer system with PEI-cellulose plates, which provided good separation of adenosine, cAMP, and AMP (Silverman and Epstein, 1975). Extracts were chromatographed together with standards, and the spots were cut out and counted by liquid scintillation. Counting efficiency was 33%.

Proteins were assayed by a solubilizing Lowry technique using a bovine serum albumin standard (Markwell et al., 1978) except as noted. In order to correct for substrate and serum proteins adsorbed to coverslips, proteins were determined on poly-L-lysine- and collagen/poly-L-lysine-treated coverslips that were without cells but were incubated with growth medium and washed in the same way as were coverslips with cultures. These values (1.7 ± 0.9 and 3.5 ± 1.7 μgm, respectively) were subtracted from the values obtained for coverslips with cells. The protein content per coverslip of the 3 types of cultures was: 7.6 μgm/neuron-enriched culture (29 cultures); 66.3 μgm/mixed culture (18 cultures); and 42.8 μgm/glial culture (2 cultures).

Glyoxylic acid histochemistry was performed according to the method of de la Torre (1980) with a 2-min incubation of cultures in the glyoxylic acid solution.

Chemicals were purchased from Sigma Chemical Co. RO 20-1724 was obtained courtesy of Dr. Peter Sorter, Hoffman-LaRoche.

Results

NE-stimulated cAMP accumulation in cerebral cortex in culture

Neuron-enriched cultures were routinely produced by inhibiting at day 4. Because both glial and neuronal precursors proliferate in culture (Kriegstein and Dichter, 1984), it was expected that the timing of inhibition of neuronal cultures might have a significant effect on NE-stimulated cAMP accumulation. Therefore the relationship of day of inhibition to this parameter was characterized.

The results of such an experiment are shown in Figure 2. Cultures were mitotically inhibited at 1, 2, 3, and 4 d *in vitro* by exposure to 5 μM cytosine arabinoside for 2 d. These cultures were then used for experiments comparing responses to NE stimulation at 15 d *in vitro*. NE-stimulated cAMP accumulation increased as mitotic inhibition was delayed over the first 4 d *in vitro*.

The stimulation of cAMP accumulation by NE was studied in neuron-enriched cultures inhibited at 4 d *in vitro* as well as in mixed and in glial cultures derived from rat cerebral cortex (Fig. 3). These data show cAMP content of coverslip cultures following 10-min exposure either to 10 μM NE or to vehicle only. Mixed cultures, glial cultures, as well as neuron-enriched cultures, showed significant ($p < 0.0001$) stimulation of cAMP accumulation by NE. Since the protein content of these 3 types of cultures varied, it was useful to express cAMP content normalized to protein content (Table 1). These results suggest that the enrichment of cultures with non-neuronal elements parallels an increase in NE-stimulated cAMP accumulation.

Further evidence supporting this conclusion came from experiments in which the neurons in neuron-enriched cultures were selectively eliminated by switching to fresh medium 48 hr prior to experiment. In these neuron-depleted cultures, virtually no morphologically identifiable neurons were present. Three

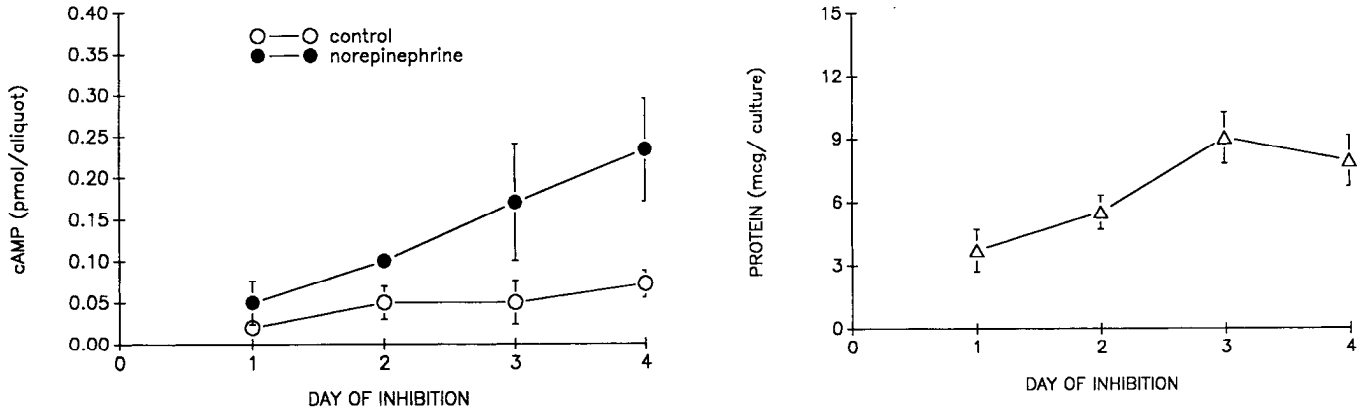

Figure 2. Effect of day of inhibition of cell division with cytosine arabinoside on NE-stimulated cAMP accumulation. At 24 hr, cultures were partially media changed with 1.5 ml growth medium (leaving behind approximately 0.5 ml). At 1, 2, 3, and 4 d, cultures were inhibited with 5 μM cytosine arabinoside added from a stock solution to the cultures. Medium was replaced completely after 48 hr. Cultures were medium changed on a M–W–F schedule using conditioned medium (growth medium exposed to mixed cultures for 48 hr), 1 ml/dish. At 15 d *in vitro,* cultures were used for experiment. Cultures were washed once by being dipped into a beaker containing 20 ml bicarbonate buffered salt solution and placed in wells containing 300 μl medium/well. Cultures were incubated for 20 min at 37°C. Medium was then replaced. NE or vehicle was added to appropriate wells. Final concentration of NE was 10 μM. Incubation was continued for 10 min. Media were aspirated off cultures, and ice-cold 0.3 M perchloric acid was used to extract the cultures. Cyclic AMP was assayed by radioimmunoassay. The mean ± SD for four coverslip cultures is represented by each data point. *A,* Cyclic AMP content of coverslip cultures mitotically inhibited at 1, 2, 3, and 4 d *in vitro. B,* Protein content of cultures mitotically inhibited at 1, 2, 3, and 4 d *in vitro.* Protein was assayed using a dye binding technique (Biorad) with a bovine gamma globulin standard. Cultures were solubilized by sonication using 400 μl 0.2 M NaOH/culture. This experiment was representative of three similar experiments that were performed.

experiments were performed in which responses to NE in these neuron-depleted cultures were compared with responses in neuron-rich sister cultures. In neuron-enriched cultures maintained with conditioned medium, NE stimulated cAMP accumulation (Table 2). Similarly, in neuron-depleted cultures, NE also stimulated cAMP accumulation. These data suggest that in the neuron-enriched cultures the non-neuronal component was responsible for producing the NE-stimulated cAMP accumulation that was observed.

Accumulation and secretion of cAMP in rat cerebral cortex in dissociated cell culture

Figure 4 shows the effect of 10 μM isoproterenol on cAMP accumulation both intracellularly and in the medium. Four-week-old mixed cultures were used. Isoproterenol stimulated the accumulation of intracellular cAMP, as expected of a beta adrenergic agonist (Lefkowitz et al., 1984). The peak occurred at 10 min and then declined over the next 60 min. Isoproterenol also stimulated the efflux of cAMP and subsequent accumulation of this substance in the medium. This was a slower process that peaked between 40–60 min. The quantity of cAMP extractable from cultures at the intracellular maximum was similar to the quantity of cAMP present in the medium at the extracellular maximum. Over the first 40 min of this experiment, these cultures secreted 180 pmol cAMP/mg protein (18.48 pmol/coverslip; protein = 100.2 ± 9.7 μg/coverslip in these experiments). This quantity of extracellular cAMP produced a concentration of cAMP in the medium of 62 nM. Because it does not include secreted cAMP that may have been degraded extracellularly, this figure represents, if anything, an underestimate.

Fate of extracellular cAMP in cerebral cortex

The experiments described above suggested that NE-stimulated adenylate cyclase was predominantly localized in non-neuronal cells in cerebral cortex in culture and that cultures of cerebral cortex were capable of transporting large quantities of cAMP into the extracellular medium. The data shown in Figure 4 also suggested that extracellular cAMP was either taken up or was degraded by cortical cultures. In order to pursue this observation, we studied the fate of extracellular cAMP.

The degradation of extracellular cAMP was directly demonstrated (Fig. 5). Mixed cultures of neurons and glia were incubated with ^{3}H-cAMP at 120 nM. Media were sampled at 0, 5, 10, 20, 30, and 60 min. Cyclic AMP, AMP, and adenosine were separated by thin-layer chromatography. ^{3}H-cAMP decayed to 74% of its original concentration at 30 min, and the decay of ^{3}H-cAMP was accompanied by the appearance of material that co-chromatographed with adenosine and with AMP.

Cyclic AMP secreted by glia might be taken up by neighboring neurons or glia. This possibility was tested directly using neuron-enriched cultures. When neuron-enriched cultures were incubated with [8-^{3}H]-cAMP (383 nM, specific activity 26.1 Ci/mmol) for 30 min, it was found that 0.19% of the total counts were taken up at 25°C. Thin-layer chromatography on the tracer showed that 0.73% of the tracer itself (before access to the cultures) did not migrate as cAMP. The small fraction of total radioactivity taken up by the cultures made likely the possibility that a contaminant of the tracer or a metabolite was the chemical species transported rather than cAMP. Since AMP and adenosine were demonstrated metabolites of cAMP in the tissue culture media (Fig. 5), the uptake of cAMP, AMP, and adenosine was compared. Under comparable conditions, uptake of radioactivity into neuron-enriched cultures incubated for 30 min with ^{3}H-AMP and ^{3}H-adenosine was, respectively, 6.3 and 36.4 times the uptake into cultures incubated with ^{3}H-cAMP.

If cAMP were taken up by neuron-enriched cultures, then one would expect that it would be possible to demonstrate intracellular ^{3}H-cAMP accumulation into cultures incubated with ^{3}H-cAMP in the medium. Neuron-enriched cultures were in-

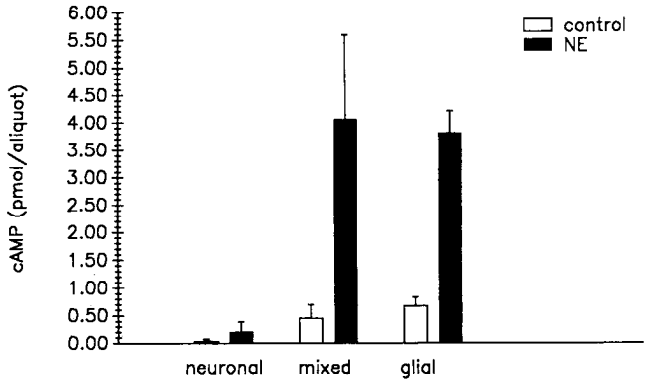

Figure 3. NE stimulation of cAMP accumulation in neuron-enriched, mixed, and glial cultures. Neuron-enriched cultures were all mitotically inhibited on the fourth day *in vitro,* using 5 μM cytosine arabinoside for 48 hr. Mixed cultures were inhibited at confluence (10–14 d *in vitro*). Neuron-enriched and mixed cultures were used at 13 d *in vitro.* Astrocytes were 6 weeks *in vitro.* Cultures were preincubated for 20 min in bicarbonate buffered saline. Medium was replaced and NE or vehicle was added. Final NE concentration was 10 μM. Cultures were returned to incubator for 10 min at 37°C. They were then briefly agitated, and medium was removed and replaced with 0.3 ml ice-cold 0.3 M $HClO_4$ for 1 hr, on ice, with agitation. 250 μl was removed and 25 μl 3 M $KHCO_3$ was added to neutralize and precipitate perchlorate. After 15 min on ice, samples were spun at 2500 × *g* for 15 min, and supernatant was decanted. Cyclic AMP (NEN) assays were performed on 100 μl aliquots of this supernatant. Values are mean ± SD.

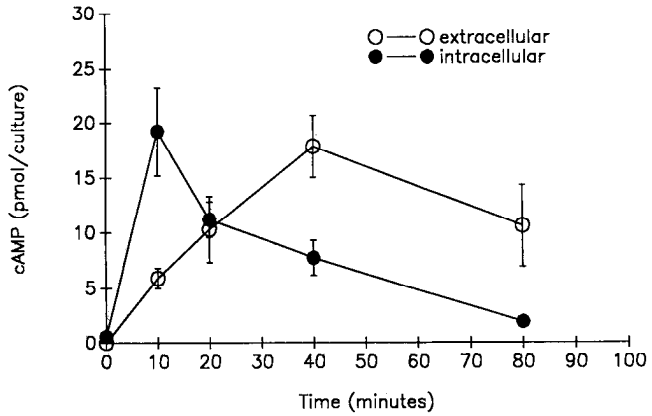

Figure 4. Intracellular and extracellular cAMP accumulation in isoproterenol-stimulated mixed cortical cultures. Mixed cultures were used at 34 d *in vitro.* Experiment was set up in duplicate. Coverslip cultures were preincubated in a balanced salt solution for 20 min. Medium was replaced with fresh medium containing 10 μM isoproterenol. Incubations were continued for 0 (no isoproterenol), 10, 20, 40, and 80 min. Incubation periods were terminated by replacing media with 300 μl ice-cold 0.3 M perchloric acid. Media samples were added to equal volumes of ice-cold 0.6 M perchloric acid. Media samples and tissue extracts were neutralized and assayed for cAMP. Protein assay was performed on extracted culture using a solubilizing Lowry procedure with a BSA standard. Average protein was 100.2 ± 9.7 μg/coverslip. Error bars indicate standard deviation. This experiment was representative of two similar experiments that were performed.

cubated with ^{3}H-cAMP for 5, 10, 15, and 30 min; the cultures were washed and extracted using perchlorate, and the extracts were then chromatographed. Figure 6 shows the results of such an experiment. There was in fact no evidence for the accumulation of ^{3}H-cAMP intracellularly. However, material that co-chromatographed with AMP (i.e., that remained at the origin in this system) showed progressive accumulation during the time course of this experiment. The fact that this accumulation of radioactivity showed no evidence of saturating during the experiment argues for this accumulation representing intracellular accumulation rather than surface binding.

IBMX and RO 20-1724 (Kinnier and Wilson, 1977), a methylxanthine and an imidazolidinone phosphodiesterase inhibitor, respectively, were compared for their effect on the uptake into neuron-enriched cultures of radioactivity derived from ^{3}H-cAMP. At 300 μM, these inhibitors blocked phosphodiesterase, as demonstrated by the reduction in the formation of AMP to 16% and 38% of control, respectively. However, neither inhibitor increased the amount of intracellular radioactivity migrating as cAMP.

Table 1. Cyclic AMP content of neuron-enriched, mixed, and glial cultures

	cAMP content (pmol/mg protein)	
Culture	Basal	Stimulated
Neuron-enriched	15 ± 18 (n = 37)	89 ± 78 (n = 39)
Mixed	22 ± 12 (n = 16)	202 ± 75 (n = 19)
Glial	52 ± 12 (n = 3)	294 ± 31 (n = 4)

Data from Figure 2 have been normalized with respect to protein content of the respective culture types. Nine experiments were performed using neuron-enriched coverslip cultures, 7 using mixed cultures, and 2 using glial cultures. Pooled data from these experiments are expressed here as means ± SD. n, number of coverslip cultures assayed for cAMP content in a given category.

Discussion

NE-stimulated cAMP accumulation has never been directly demonstrated in neurons of the CNS. Beta adrenergic stimulation in primary cultures of astrocytes, however, has been shown

Table 2. Effect of selective elimination of neurons from neuron-enriched cultures on NE-stimulated intracellular cAMP accumulation

Experiment no.	cAMP content (pmol/100 μl) Basal	Stimulated	Protein (μg)	p
Conditioned medium				
1	0.02 ± 0.02	0.17 ± 0.19	8.3 ± 1.4	N.S.
2	0.03 ± 0.01	0.19 ± 0.09	13.5 ± 3.6	<0.005
3	0	0.36 ± 0.25		<0.01
Fresh medium				
1	0	0.45 ± 0.02	4.0 ± 2.1	<0.005
2	0.02 ± 0.01	0.10 ± 0.08	<1	<0.05
3	0.01 ± 0.02	0.07 ± 0.03		<0.0005

To prepare cultures depleted of neurons, 11-d *in vitro* cultures were used. Half the coverslips were placed in wells containing 0.5 ml conditioned medium/well supplemented to 8 mM glucose, and the other half were washed in their dishes twice with 2 ml MEM and then placed into fresh growth medium, 0.5 ml/well, and returned to the incubator. Twenty-four hours later neurons were phase dark, many were floating, and processes had broken up and had lost their smooth appearance. These cultures were used for experiments at 48 hr. Three experiments were performed. Experiments were conducted as described. Data given are cAMP (pmol)/100 μl aliquot. The cAMP content of 4 coverslip cultures in each category was assayed. Values are means ± SD. The data from each experiment are displayed. N.S., not significant.

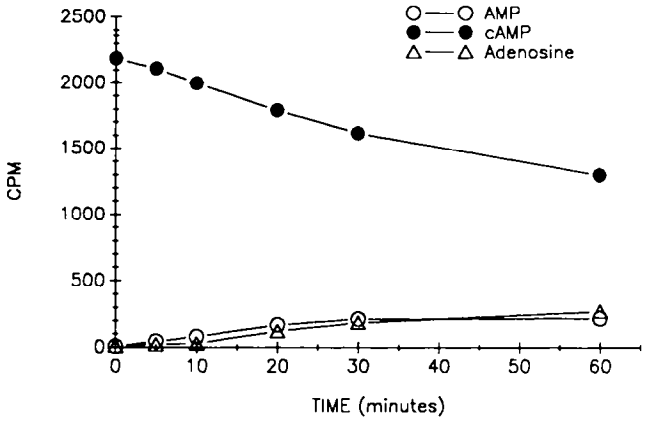

Figure 5. Degradation of ^{3}H-cAMP in culture media in the presence of mixed cultures. Mixed cultures at 55 d *in vitro* were used for these experiments. Cultures were washed once with bicarbonate buffered saline. Coverslip cultures were distributed into wells containing 120 nM [8-^{3}H] cAMP (26.1 Ci/mmol), 250 μl/well. Cultures were incubated for 0 (no tissue), 5, 10, 20, 30, and 60 min. At the end of the incubation period, medium was removed and placed in an Eppendorf tube containing 4 μl 0.5 M EDTA and 10 μl 1 M Hepes, pH 7, and boiled for 3 min. Samples were spun down and stored at 4°C for 48 hr until they were chromatographed using an ethanol/0.5 M ammonium acetate (5/2) system and PEI-cellulose plates with indicator. Five-microliter samples were spotted with cAMP, adenosine and AMP, 5 μl each, 10 mM solutions. Spots were visualized with UV and were cut out, extracted, and counted. There was no significant breakdown of ^{3}H-cAMP in the absence of tissue (zero condition). This experiment was representative of four similar experiments that were performed.

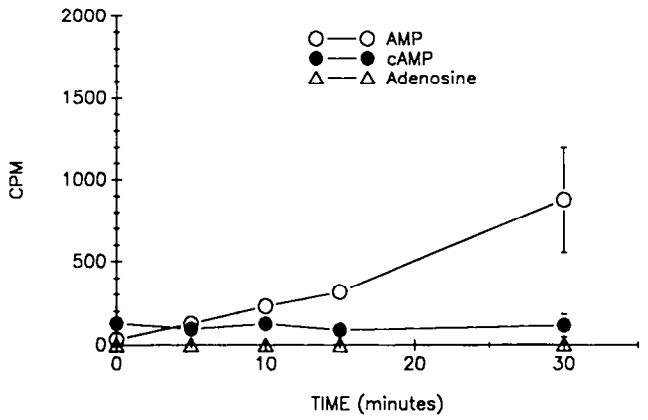

Figure 6. Uptake of radioactivity derived from ^{3}H-cAMP into neuron-enriched cultures. Neuron-enriched cultures at 36 d *in vitro* were used for these experiments. Cultures were inhibited at one day, and medium was replaced 72 hr later. Cultures were gently washed twice using 2 ml bicarbonate buffered saline/wash and were then placed in wells containing medium plus radiolabeled cAMP ([8-^{3}H]adenosine 3',5'-cyclic phosphate, ammonium salt; Amersham TRK.304, 26.1 Ci/mmol); final concentration of cAMP, 383 nM. Cultures were incubated for 0 (no tracer), 5, 10, 15, and 30 min. At end of incubation wells were placed on wet ice, and cultures were washed twice with cold medium, carefully lifting coverslip during each wash. Finally cultures were extracted with ice-cold perchloric acid. To 280 μl extract was added 28 μl 3 M $KHCO_3$. After 30-min incubation on ice, samples were spun and decanted. Ten microliters each of 10 mM adenosine, cAMP, and AMP were added, and samples were lyophilized and then dissolved in 30 μl water. Ten microliters of each sample were chromatographed using an ethanol/0.5 M ammonium acetate (5/2) system and PEI-cellulose plates with indicator. Spots were cut out, extracted in 1 M HCl, and counted. This experiment was representative of two similar experiments that were performed.

in many studies to result in cAMP accumulation (Daly, 1977; Hamprecht, 1986). One function of beta-agonist-stimulated adenylate cyclase in cerebral cortex appears to be the regulation of glycogen metabolism in astrocytes. We found that, similar to the cortical slice preparation (Quach et al., 1978), cortical cultures incubated with ^{3}H-glucose accumulate ^{3}H-glycogen (Rosenberg and Dichter, 1985a) and that this net synthesis of glycogen is inhibited by NE (Rosenberg and Dichter, 1987). The effects of NE on glycogen metabolism in both the cortical slice preparation (Quach et al., 1978; Magistretti et al., 1981) and cortical cultures (Rosenberg and Dichter, 1985b) are blocked by beta adrenergic antagonists. We have also shown cytochemically that glycogen is localized to a subpopulation of astrocytes (Rosenberg and Dichter, 1987). Therefore one of the functions of NE in cerebral cortex appears to be the regulation of cortical glycogen metabolism in a subpopulation of astrocytes.

Biochemical studies using brain tissue to investigate receptors or transmitter-sensitive adenylate cyclase are unable to discriminate neuronal from glial properties. Beta adrenergic properties determined in cortical tissue as well as slice preparations may be properties of astrocytes, since astrocytes possess a beta-agonist-stimulated adenylate cyclase. Therefore a presumed beta adrenergic effect of NE on neurons in cerebral cortex may not be attributable to a direct effect of NE on neurons at all, but rather may be secondary to the interaction of NE with beta adrenergic receptors on astrocytes. In order to understand how NE functions as a neuromodulator in cerebral cortex, it is crucial to determine whether neurons themselves possess adrenergic receptors. In any particular case of an apparent noradrenergic effect on neurons, it is necessary to rigorously exclude the possibility that the effect may be mediated by one or more substances released by noradrenergic stimulation of nearby astrocytes.

Gilman and Schrier (1972) demonstrated NE-stimulated adenylate cyclase in primary cultures derived from the CNS. They recognized the potential utility of tissue culture as a means of determining which cells in the brain are targets for NE, but this line of investigation was hindered by the difficulty of maintaining central neurons as the predominant cell type in tissue culture, a problem that can be solved to some extent by using either glia-conditioned medium (Banker, 1980) or a medium based on the serum-free medium devised by Bottenstein and Sato (1979) but containing 1% serum, as described in Materials and Methods. The evidence presented here suggests that in cultures derived from embryonic rat cerebral cortex, NE-stimulated adenylate cyclase is associated with glia but not with neurons. However, the data do not exclude a neuronal contribution, which might have been missed for a number of reasons: a neuronal contribution may be below the level of detection, or the neuronal response may desensitize too rapidly to be detected given the time frame used here. These data, however, are consistent with the data provided by McCarthy and colleagues (cited above) showing that beta adrenergic receptors are located on astrocytes but not on neurons in primary cultures derived from embryonic rat cerebral cortex.

Gonzales et al. (1985) reported the results of investigations designed to determine whether neurons possess alpha adrenergic receptors and found that alpha$_1$-receptor-mediated inositide hydrolysis appeared to be associated with the neuronal component

of the cultures they were using. However, the cultures used by these authors contained catecholaminergic neurons (Sumners et al., 1983). Catecholaminergic neurons in the vertebrate CNS are almost exclusively located in the brain stem. Therefore the cultures these authors used must have contained other brain stem neurons as well. Since $alpha_1$-receptor-mediated responses have been demonstrated on brain stem (McCall and Aghajanian, 1979) and spinal motoneurons (White and Neuman, 1980), it may be that the $alpha_1$-receptor-mediated effect observed by Gonzales et al. was localized to brain stem neurons and not cerebral neurons. In contrast, the cultures used in the present study are derived from cerebral cortex and contain no catecholaminergic cells by glyoxylic acid histochemistry. Whether an alpha adrenergic response associated with neurons can be demonstrated in cultures derived from cerebral cortex and devoid of brain stem neurons is unknown.

The observation first made by Davoren and Sutherland (1963) that cells may secrete cAMP in response to adrenergic stimulation as well as intracellularly accumulate cAMP has previously been made in human (Clark et al., 1974) and rat glioma-derived cell lines (Penit et al., 1974; Doore et al., 1975; Nickols and Brooker, 1978). We have observed this phenomenon in mixed cultures of cerebral cortex. Over the first 40 min of the experiment shown in Figure 4, these cultures secreted 180 pmol cAMP/mg protein (18.48 pmol/coverslip; protein = 100.2 ± 9.7 μg/coverslip). The value reported for C-6 glioma cells at 40 min was approximately 5 nmol/mg protein (Rindler et al., 1978) (taken from Rindler et al., fig. 1).

Recent work suggests that exogenous catecholamines might be toxic to both cortical neurons and glia in dissociated cell culture (Rosenberg, 1988a). It might be argued, therefore, that release of cAMP into the medium was the result of injury to cells exposed to exogenous catecholamines. However, catecholamine toxicity was demonstrated in cortical cells 24 hr after plating but was not demonstrable in mature mixed cultures of the type used for the experiments presented here. Furthermore, in the cultures in which catecholamine toxicity was demonstrated, cell death occurred at concentrations of NE or isoproterenol equal to or greater than 25 μM. In the mature mixed cultures, no toxicity was demonstrable even at concentrations of 250 μM (Rosenberg, 1988b).

Because extracellular cAMP might perform a signaling function, the fate of extracellular cAMP in cerebral cortex is of great interest. There are several mechanisms by which secreted cAMP might modulate neuronal activity: (1) by being taken up by neurons, (2) by interacting with cAMP receptors, or (3) by cAMP or a metabolite interacting with adenosine receptors. We have not been able to obtain evidence for the uptake of extracellular ^{3}H-cAMP (Thomas et al., 1979; Tsukamoto et al., 1980). The data suggest that the uptake of radioactivity that was observed was not associated with cAMP. The data do not rule out the possibility that cAMP was transported but rapidly degraded intracellularly.

Extracellular cAMP might exercise a signaling function through metabolites such as AMP or adenosine. The demonstration that extracellular cAMP is broken down to AMP and adenosine lends credence to this possibility. NE-stimulated extracellular accumulation of cAMP might be a source for extracellular AMP and adenosine that might modulate neuronal activity by way of neuronal adenosine receptors. If one assumes that the specific activity of ^{3}H-adenosine and ^{3}H-AMP was the same as the starting ^{3}H-cAMP in Figure 5, then at 30 min the apparent concentration of adenosine in the medium is calculated to have been 1.9 nM, and that of AMP 2.2 nM. These estimates of adenosine and AMP concentrations represent minimum concentrations, since secretion of unlabeled adenosine and AMP, if it had occurred, would have decreased the specific activity. In tissue culture the extracellular volume is greatly expanded compared to that *in situ*. Because of the much smaller extracellular volume *in situ*, one might expect that higher concentrations of AMP and adenosine could be attained. The smaller extracellular volume *in situ* would lead one also to expect significantly faster kinetics for the appearance of the metabolites of extracellular cAMP.

Previous studies of cAMP efflux using cell lines derived from gliomas have demonstrated that extracellular cAMP was not subject to significant degradation. For example, Penit et al. (1974) incubated their C-6 glioma cultures (7 d *in vitro*) with ^{3}H-cAMP and found that 12.7% hydrolysis occurred starting with 50 nM and 2.8% hydrolysis occurred starting with 1 μM after 120 min. In comparison, using primary mixed cultures (55 d *in vitro*) and starting with 120 nM ^{3}H-cAMP, 41% hydrolysis occurred after 60 min. In addition, the data presented in all the studies using glioma-derived cell lines differ from the data observed using primary cultures in that with the cell lines extracellular cAMP accumulation was shown to continue to increase over a 90-min incubation with isoproterenol (Penit et al., 1974; Doore et al., 1975; Rindler et al., 1978). In contrast, in the primary cultures, extracellular cAMP reached a maximum at 40–60 min, suggesting the presence of mechanisms for clearing extracellular cAMP. An explanation for this difference between the studies with cell lines and those with primary cultures is that the primary cultures contain an extracellular phosphodiesterase.

One possible source for extracellular phosphodiesterase in culture is the serum used to make growth medium (Asano and Hidaka, 1975; Patterson et al., 1975). However, assay for phosphodiesterase activity in rat serum used for tissue culture showed no evidence of cAMP degradation over a 2-hr incubation (data not shown). Presumably, heat inactivation used to prepare the serum destroys any phosphodiesterase activity that may have been present.

Another explanation for these differences between the results using glioma-derived cell lines and those using primary mixed cultures is that the neuronal component of the mixed cultures contains extracellular phosphodiesterase activity. Consistent with this hypothesis are studies that provide evidence of an association of phosphodiesterase activity with cerebral—and, in particular, cortical—neurons. Minneman et al. (1978) showed that kainic acid injections into the neostriatum of rat produced as much as an 84% depletion of phosphodiesterase activity in that structure, suggesting a predominantly neuronal localization. Grab et al. (1981) demonstrated phosphodiesterase activity associated with isolated postsynaptic densities from canine cerebral cortex. Kincaid et al. (1987) immunocytochemically demonstrated that phosphodiesterase was predominantly associated with neurons—in particular, pyramidal cells of hippocampus and neocortex. Ariano and Adinolfi (1977), using ultrastructural cytochemical techniques in the cat caudate and cortex, showed that phosphodiesterase activity was found in the postsynaptic densities of asymmetrical axodendritic synapses. However, their figures show reaction product associated with the presynaptic terminal membrane and the synaptic cleft (see for example their

figs. 5 and 6 for cortex), suggesting that a component of the phosphodiesterase activity at these synapses might be located extracellularly (see also Ariano and Appleman, 1979). These data support the hypothesis that the extracellular phosphodiesterase activity observed in primary mixed cultures is associated with neurons. Recently an extracellular cGMP phosphodiesterase has been described in the interphotoreceptor matrix of bovine and monkey retinas (Barbehenn et al., 1985).

There is no evidence for cell surface cAMP specific receptors in cells of the CNS. However, demonstrating such receptors might be expected to be difficult if cells possessed extracellular phosphodiesterase activity, as was the case for the demonstration of such receptors in slime molds (Devreotes, 1982). There are now several examples of nucleotide modulation of ion channels (Spruce et al., 1987; Nakazawa and Matsuki, 1987; Cook et al., 1987). The locus for these actions of nucleotides is intracellular, but this need not always be the case.

There can be little doubt that NE is a modulator of neuronal activity in hippocampus and neocortex. Many studies have shown that NE modulates both spontaneous and stimulus-evoked activity of cortical neurons recorded *in situ,* in the *in vitro* slice preparation, and in tissue culture (Armstrong-James and Fox, 1983; Rogawski, 1985; Rosenberg et al., 1985). These effects are mediated by both alpha and beta adrenergic receptor coupled mechanisms. Studies in the hippocampal slice preparation using intracellular recording techniques have shown that NE decreases spike accommodation by reducing the calcium-mediated potassium conductance mechanism underlying the slow afterhyperpolarization (Madison and Nicoll, 1982, 1984, 1986a, b; Nicoll, 1988). This effect is mediated by beta adrenergic receptors. A similar phenomenon has been observed in the neocortical slice preparation (Foehring et al., 1986). These studies provide the most direct evidence for a primary neuronal target for NE in the cerebral cortex. One hypothesis concerning the mechanism of action of NE in cerebral cortex that has not been explored postulates a link between the effect of NE on astrocytes and neuronal function. Madison and Nicoll (1986b) carefully developed the evidence that the action of NE on CA1 pyramidal cells is mediated by an increase in intracellular cAMP. They showed that extracellular cAMP or adenosine did not mimic the action of either NE, intracellular cAMP, or the membrane permeable 8-bromo-cAMP. These data make it unlikely that extracellular cAMP plays a role in the action of NE on these cells. However, given that intracellularly injected cAMP may itself be released from cells, and given that access of perfused cAMP to cAMP receptors may be limited by extracellular phosphodiesterase, a mechanism involving the interaction of extracellular cAMP with extracellular cAMP receptors remains conceivable.

If one accepts that the effect of NE on the slow afterhyperpolarization in hippocampal and neocortical pyramidal cells demonstrates that these neurons possess a beta-agonist-stimulated adenylate cyclase, then it needs to be explained why it has not been possible to demonstrate neuronal beta adrenergic receptors or a beta-agonist-stimulated adenylate cyclase in neurons in tissue culture. In addition to possible problems with detection or rapid desensitization already noted, other possibilities include a difference between embryonic and adult tissue, the localization of beta-agonist-stimulated adenylate cyclase to specific populations of neurons which for some reason may not be present under the conditions of culture, or some perturbation (for example, substrate) introduced by the tissue culture paradigm which causes neurons to lose, or fail to develop, the beta-agonist-stimulated adenylate cyclase. In any case, the well-documented fact that astrocytes coexist so intimately with neurons in cerebral cortex (as elsewhere) in itself raises the possibility that some of the actions of NE on neurons actually may be mediated by a primary action of NE on astrocytes and a secondary action on neurons mediated by an extracellular messenger.

Because NE stimulates cAMP efflux, cAMP would be an obvious candidate for an extracellular messenger either itself or via a metabolite. In fact, it has been found that all adenine nucleotides, including cAMP, AMP, ATP, and adenosine, have a depressant effect on both the field EPSP and the population spike response in the hippocampal slice preparation, and that these effects can be blocked by adenosine antagonists (Dunwiddie and Hoffer, 1980; Dunwiddie, 1985). There is abundant evidence that adenosine may be a presynaptic inhibitor of transmitter release (Ribeiro, 1979; Phillis and Wu, 1981; Erulkar, 1983; Proctor and Dunwiddie, 1983; Dolphin and Prestwich, 1985; Dunwiddie, 1985; McAfee and Henon, 1985; Peris and Dunwiddie, 1985–1986; MacDonald et al., 1986; Muller et al., 1987; Silinsky et al., 1987). Extracellular ATP has also been postulated as a possible source of extracellular adenosine (Schubert et al., 1982). Given that beta-agonist-stimulated cAMP efflux occurs in cerebral cortex both *in vitro,* as reported here, and *in situ* (Stone and Egawa, 1988), and given that extracellular cAMP is degraded to AMP and adenosine, NE-stimulated efflux of cAMP needs to be considered as a mechanism mediating some of the actions of NE on cortical neurons. Peptides that stimulate cAMP accumulation in cortical glia may also stimulate efflux of cAMP and may act in part by a paracrine mechanism similar to that being suggested for NE.

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