Opiates and cultured neuroblastoma × glioma cells

Effect on cyclic AMP and polyamine levels and on ornithine decarboxylase and protein kinase activities

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(Received 20 August 1984/7 December 1984; accepted 2 January 1985)

In cultured NG 108-15 neuroblastoma × glioma cells, opiates decreased cellular cyclic AMP and polyamine levels. This decrease was related to the inhibition of ornithine decarboxylase and cyclic AMP-dependent protein kinase activities during the acute exposure of the cells to the drugs. Growing the cells in the presence of opiates for several days led to drug addiction. In the tolerant-addicted cells, polyamine and cyclic AMP levels were close to normal values as were the activities of ornithine decarboxylase and cyclic AMP-dependent protein kinase. Removal of the opiate from 'addicted' cells, by either washing or by adding the antagonist naloxone, resulted in an increase in cyclic AMP and polyamine levels and the activities of ornithine decarboxylase and cyclic AMP-dependent protein kinase. The effect of opiates was closely related to their biological activities. Inactive enantiomorphs did not affect cyclic AMP or polyamine levels; neither did they decrease ornithine decarboxylase and cyclic AMP-dependent protein kinase activities.

Most of the studies aimed at the elucidation of the mode of action of narcotics are based on animal experiments or on the use of brain slices. The use of laboratory animals has definite limitations, since side effects due to stress or drug excretion are quite common. Brain slices also do not provide an ideal system, because of the heterogeneity of the cell types and the apparent lability of brain enzymes (Puri *et al.*, 1975; Tell *et al.*, 1975). The finding that neuroblastoma × glioma hybrid cells bind morphine (Klee & Nirenberg, 1974) opened new possibilities in studying the mechanisms of drug addiction and withdrawal. Such a system provides a useful experimental model and facilitates well-controlled studies under defined conditions. Indeed, tissue culture experiments indicated that the binding of morphine and other opiates to narcotic receptors results in an inhibition of adenylate cyclase [ATP pyrophosphate-lyase (cyclizing), EC 4.6.1.1] activity (Sharma *et al.*, 1975a; Blosser *et al.*, 1975). Other studies (Sharma *et al.*, 1975b) demonstrated that further incubation with the drug reveals a secondary regulatory process which involves a compensatory increase in adenylate cyclase activity. Cells are now tolerant to morphine

and are also dependent upon the narcotic, since withdrawal of the drug or the addition of a specific narcotic antagonist will raise cyclic AMP levels to abnormally high values. It is conceivable that opiates, which inhibit adenylate cyclase activity, should therefore inhibit cellular cyclic AMP-dependent processes.

We have previously demonstrated (Bachrach *et al.*, 1979) that morphine inhibits the activation of cyclic AMP-dependent protein kinase, as well as the induction of ODC (L-ornithine carboxy-lyase, EC 4.1.1.17), in neuroblastoma × glioma hybrid cells. ODC catalyses the conversion of ornithine into putrescine. This is the rate-limiting reaction in the biosynthetic pathways of the naturally occurring polyamines spermidine and spermine (Bachrach, 1973). These compounds have been implicated in various processes of growth and differentiation (Caldarera & Moruzzi, 1970; Jänne *et al.*, 1978). They also play an important role in the biosynthesis of cellular macromolecules, such as nucleic acids and proteins (Cohen, 1971).

It is the purpose of the present paper to extend our previous studies which dealt with the acute effect of opiates on the hybrid cells (Bachrach *et al.*, 1979). It deals with the fluctuations of cyclic AMP and polyamine levels upon the chronic treatment of the cultured hybrid cells with opiates. Changes in the activities of ODC and cyclic AMP-

Abbreviations used: ODC ornithine decarboxylase; IBMX, 3-isobutyl-1-methylxanthine; PGE₁, prostaglandin E₁; PBS, phosphate-buffered saline.

dependent protein kinase during the chronic treatment of the cells will also be described. These will be compared with the changes induced by drug withdrawal.

Materials and methods

Reagents

DL-[1-¹⁴C]Ornithine (sp. radioactivity 54.4 mCi/mmol), cyclic [G-³H]AMP (sp. radioactivity 42.5 Ci/mmol) and [α -³²P]ATP (sp. radioactivity 2800 Ci/mmol) were from New England Nuclear (Boston, MA, U.S.A.). IBMX was from Sigma Chemical Co. (St. Louis, MO, U.S.A.). Morphine, naloxone, etorphine, levorphanol, levallorphan and dextrorphan were provided by Dr. E. Simon (New York University, New York, NY, U.S.A.). PGE₁ was provided by Dr. John Pike, Upjohn Co. (Kalamazoo, MI, U.S.A.).

Cell line

Neuroblastoma $\times$ glioma hybrid cells NG 108-15 (kindly provided by Dr. M. Nirenberg) were grown in 100 mm plastic dishes in Dulbecco's modified Eagle's minimum medium (GIBCO catalogue no. H-21) supplemented with 10% foetal calf serum, 0.1 mM-hypoxanthine, 1 μ M-aminopterin and 12 μ M-thymidine, in an atmosphere of air/CO₂ (9:1).

Experimental procedures

Confluent NG108-15 hybrid cells were washed twice with prewarmed PBS (0.8% NaCl, 0.02% KCl, 0.115% Na₂HPO₄, 0.02% KH₂PO₄, pH 7.2). Thereafter, a serum-free fresh medium containing the reagents was added to the cells for different incubation times.

Cyclic AMP levels were determined by the method of Gilman (1970) as follows. At various times the medium was removed from the cultures and 3.0 ml of boiling sodium acetate (50 mM, pH 4.0) was added to each plate. Cells were then scraped off the plates and disrupted by freezing and thawing. After centrifugation at 20000g for 10 min, aliquots of the supernatant fluids were assayed for cyclic AMP in duplicate. Bovine adrenal cortex protein kinase served as the binding protein (Brown *et al.*, 1972). The pellets were dissolved in 0.1 M-NaOH and protein was assayed by the method of Lowry *et al.* (1951). Cyclic AMP-dependent protein kinase activity was determined by a modification of the procedure described by Maeno *et al.* (1971) as follows. Cells were washed twice with cold PBS, scraped off the plates and suspended in 1.5 ml quantities of 0.05 M-Tris/HCl buffer, pH 7.5, supplemented with 5 mM-dithiothreitol. After freezing and thawing, cellular debris was removed by centrifugation at 20000g for

10 min. Samples of the supernatant fluids were assayed for protein kinase activity by measuring the transfer of ³²P from [α -³²P]ATP to histone. The activity of cyclic AMP-dependent protein kinase represents the difference between phosphate transfer measured in the presence and absence of cyclic AMP. The activity of ODC was determined as described previously (Bachrach, 1975).

Polyamines were extracted from the cells with 3% HClO₄. Their dansyl derivatives, prepared according to Seiler (1970), were separated and identified by t.l.c. on 300 μ m thick silica gel G plates, using ethyl acetate/cyclohexane (2:3, v/v) as the solvent. Quantification of the various polyamines was done fluorimetrically. Once identified, dansyl-polyamines were scraped off the plates, extracted with 4 ml of ethyl acetate and assayed in a Turner model III Fluorometer (excitation 365 nm, emission 505 nm). The fluorescence corresponding to the scraped spots was compared with that of known standards.

Effects of agonists and antagonists

The effect of the various agents on cyclic AMP and polyamine levels and on cyclic AMP-dependent protein kinase or ODC activities was studied by adding 100 μ l of aqueous solutions (or PGE₁ in ethanol) to confluent cultures in 100 mm dishes containing 10 ml of Dulbecco's modified Eagle's medium. Unless otherwise stated, medium was changed 48 h before the beginning of the experiment. All the cultures were incubated with the phosphodiesterase inhibitor IBMX (0.5 mM). PGE₁, which increases adenylate cyclase activity, was added to some cultures.

The effect of opiate agonists such as morphine (10 μ M), levorphanol (10 μ M) and etorphine (1 μ M), or opiate antagonists like naloxone (20 and 50 μ M), levallorphan (20 μ M) or the inactive enantiomorph of levorphanol, dextrorphan (20 μ M) were studied under the above described conditions. We selected the concentrations of the opiates according to published K_d (narcotic-receptor complex dissociation constant) values. Sharma *et al.* (1975a) reported that the K_d values for dextrorphan and morphine were 10000 and 4000, respectively. Therefore we selected concentrations of 20 μ M and 10 μ M for dextrorphan and morphine. Etorphine, which has a higher affinity for the receptors than has morphine, was used by Sharma *et al.* (1977) at a concentration of 1 μ M compared with 10 μ M for morphine. Naloxone was also used in the range 10–20 μ M. The concentrations used were therefore not selected arbitrarily.

To obtain a withdrawal effect, morphine (10 μ M) or etorphine (1 μ M) were added to 2-day-old cultures. After incubation for another 2 days, cells

were washed three times with 5 ml of Dulbecco's medium, and fresh medium (without serum) containing IBMX and/or PGE₁ and/or opiates was added.

Cyclic AMP levels were determined after 10 min incubation. The activities of cyclic AMP-dependent protein kinase and ODC were measured after incubation for 45 min and 6 h respectively. Polyamine levels were measured after different incubation times.

Results

Acute effect of opiates on cyclic AMP levels and on ODC activity

The acute effect of various opiates on cyclic AMP levels and ODC activity was studied. After cells have reached confluency, old medium was removed and fresh medium containing IBMX + PGE₁ or IBMX + PGE₁ + opiates was added to the respective cultures. It may be seen (Table 1) that a short exposure of the cells to 10 μ M-morphine, 10 μ M-levorphanol or 1 μ M-etorphine resulted in a decrease in cyclic AMP levels by 65%, 57% and 65%, respectively. Addition of 20 μ M-naloxone (a morphine antagonist) or 20 μ M-levallorphan (a levorphanol antagonist) hardly affected cellular cyclic AMP levels. Similar results were obtained when the cells were treated with the inactive isomer dextrorphan (20 μ M).

After confirming that opiates had a stereospecific effect on cyclic AMP levels, their effect on ODC activity was next studied. Table 2 shows a parallelism between the effect of opiates on cyclic

AMP levels and ODC activity. Again, the agonists morphine, levorphanol and etorphine reduced ODC, unlike dextrorphan which neither reduced ODC activity nor did it affect cyclic AMP levels. On the other hand, naloxane inhibited ODC activity by approx. 40% (Table 2) while cyclic AMP levels were not affected (Table 1).

In order to study the kinetics of ODC inhibition by opiates, cells were treated with 1 μ M-etorphine. This drug is a potent opiate agonist with high affinity for opiate receptors. It may be seen (Fig. 1) that in the untreated control cultures ODC activity started to increase 3 h after the addition of the

Table 2. *Effects of opiates on ornithine decarboxylase activity*

The experimental conditions were those given in the legend to Table 1, except that ODC activity was determined after incubation for 6 h. Results are mean values of duplicates of four experiments.

Opiates	ODC activity	
	(pmol/h per mg of protein)	(% of control)
Control	700 $\pm$ 80	100
Morphine (10 μ M)	230 $\pm$ 30	33
Naloxone (20 μ M)	420 $\pm$ 40	60
Levorphanol (10 μ M)	230 $\pm$ 27	33
Levallorphan (10 μ M)	560 $\pm$ 68	80
Dextrorphan (20 μ M)	630 $\pm$ 74	90
Etorphine (1 μ M)	252 $\pm$ 35	36

Table 1. *Acute effect of opiates on cyclic AMP levels*

NG 108-15 hybrid cells were grown to confluency in Dulbecco's modified Eagle's medium supplemented with foetal calf serum, hypoxanthine, aminopterin, and thymidine. After 4 days, old medium was removed and serum-free fresh medium containing IBMX + PGE₁ or IBMX + PGE₁ + opiates was added to the respective plates. The cyclic AMP levels were determined after incubation for 10 min. Results are mean values of duplicates of four experiments.

Opiates	Cyclic AMP	
	(pmol/mg of protein)	(% of control)
Control	3000 $\pm$ 325	100
Morphine (10 μ M)	1050 $\pm$ 195	35
Naloxone (20 μ M)	2700 $\pm$ 310	90
Levorphanol (10 μ M)	1290 $\pm$ 190	43
Levallorphan (10 μ M)	2700 $\pm$ 315	90
Dextrorphan (20 μ M)	2700 $\pm$ 300	90
Etorphine (1 μ M)	1110 $\pm$ 138	37

Fig. 1. *Effect of etorphine on ornithine decarboxylase activity*

NG 108-15 hybrid cells were grown to confluency. After 4 days, the old medium was removed and serum-free fresh medium containing IBMX (0.5 mM) + PGE₁ (10 μ M) was added to the cultures (●). To other cultures fresh medium containing IBMX + PGE₁ + etorphine (1 μ M) was added (△). ODC activity was determined at various times.

serum-free medium containing IBMX + PGE₁ to the cultures. A further increase in activity was observed during the following 3 h. Etorphine caused a definite decrease in ODC activation after stimulating confluent cultures (Fig. 1).

Chronic effect of opiates on cyclic AMP levels and on protein kinase and ODC activities

To study the behaviour of the hybrid cells during narcotic addiction and tolerance, the cultures were treated with the drug for 2 days. Thereafter, the opiate was removed or displaced from the receptors by the antagonist naloxone. The levels of cyclic AMP and the activities of cyclic AMP-dependent protein kinase and ODC were determined during the withdrawal phase. In the control experiments (Tables 3a, 4a and 5a), cells were grown for 4 days without morphine, and then treated with IBMX, PGE₁ and opiates as indicated.

It may be seen (Table 3a) that morphine, after 10 min of incubation, caused a decrease in the cyclic AMP levels. These results resembled those depicted in Table 1. In a parallel experiment, cells were grown in the presence of morphine for 2 days, and then treated as above. Table 3(b) shows that the removal of morphine by washing the cells resulted in a 2-fold increase in cyclic AMP levels. It appears that the opiate was not completely removed from the cells by washing, since naloxone caused an additional increase in the cellular cyclic AMP concentrations (Table 3b).

Under these experimental conditions a typical

Table 3. *Effect of morphine on cyclic AMP levels: withdrawal effect*

(a) The experimental conditions were those given in Table 1, except that some cultures were treated with IBMX (0.5 mM), IBMX + PGE₁ (10 μ M) and IBMX + morphine (10 μ M) or naloxone (20 μ M). (b) Morphine was added to 2-day-old cultures of NG 108-15 hybrid cells. After incubation for another 2 days, cells were treated with serum-free fresh medium containing IBMX, IBMX + PGE₁, or IBMX + morphine or naloxone. The cyclic AMP levels were measured after incubation for 10 min. Results are mean values of duplicates of four experiments.

Addition	Cyclic AMP (pmol/mg of protein)	
	(a) Control	(b) Morphine (2 days)
IBMX	2000 $\pm$ 190	4000 $\pm$ 325
IBMX + PGE ₁	5000 $\pm$ 485	8000 $\pm$ 600
IBMX + morphine	1500 $\pm$ 170	2000 $\pm$ 220
IBMX + PGE ₁ + morphine	3000 $\pm$ 250	5000 $\pm$ 375
IBMX + naloxone	1750 $\pm$ 180	7000 $\pm$ 520
IBMX + PGE ₁ + naloxone	4500 $\pm$ 380	12000 $\pm$ 650

withdrawal effect is obtained. The effect of morphine on the activity of cyclic AMP-dependent protein kinase was also studied. Table 4(a) shows that during an acute effect of the drug the activity of protein kinase was inhibited. When the drug was removed from the addicted cells (either by washing or by the addition of the antagonist naloxone), a 150% increase in protein kinase activity was noticed (Table 4b). Removal of morphine by washing also resulted in a 2-fold increase in the activity of ODC (Table 5). The addition of naloxone caused an additional increase (Table 5). These experiments indicate that the

Table 4. *Effect of morphine on protein kinase activity: withdrawal effect*

The experimental conditions were as given in Table 3 except that the activity of cyclic AMP-dependent protein kinase was determined after 45 min of incubation. Results are mean values of duplicates of four experiments.

Addition	Cyclic AMP-dependent protein kinase (pmol/10 min per mg of protein)	
	(a) Control	(b) Morphine (2 days)
IBMX	80 $\pm$ 10	125 $\pm$ 15
IBMX + PGE ₁	200 $\pm$ 25	300 $\pm$ 30
IBMX + morphine	50 $\pm$ 7	75 $\pm$ 8
IBMX + PGE ₁ + morphine	75 $\pm$ 9	175 $\pm$ 19
IBMX + naloxone	60 $\pm$ 8	190 $\pm$ 21
IBMX + PGE ₁ + naloxone	175 $\pm$ 20	400 $\pm$ 38

Table 5. *Effect of morphine on ornithine decarboxylase activity: withdrawal effect*

(a) The experimental conditions were those given in the legend to Table 2, except that some cultures were treated with IBMX (0.5 mM), IBMX + PGE₁ (10 μ M) or IBMX + morphine (10 μ M) or naloxone (20 μ M). (b) The experimental conditions were those given in Table 3(b), except that ODC activity was determined after incubation for 6 h. Values are the means of duplicates of four experiments.

Addition	ODC (pmol/h per mg of protein)	
	(a) Control	(b) Morphine (2 days)
IBMX	350 $\pm$ 40	400 $\pm$ 38
IBMX + PGE ₁	600 $\pm$ 65	550 $\pm$ 60
IBMX + morphine	200 $\pm$ 18	200 $\pm$ 17
IBMX + PGE ₁ + morphine	400 $\pm$ 35	250 $\pm$ 30
IBMX + naloxone	300 $\pm$ 30	450 $\pm$ 42
IBMX + PGE ₁ + naloxone	450 $\pm$ 50	1200 $\pm$ 98

fluctuations in protein kinase and ODC activities resemble changes in cyclic AMP levels as described (Sharma *et al.*, 1975b).

The kinetics of changes in ODC activity during drug withdrawal were also studied as follows. Cells were grown in the presence or absence of etorphine for 4 days and then fed with fresh medium (with serum), with or without etorphine. After 9 h of incubation, naloxone was added to some cultures. At that time the activity of ODC started to decline (Fig. 2). Naloxone had practically no effect on the activity of ODC in untreated cells (Fig. 2a), whereas a significant increase in ODC activity was observed when etorphine-treated cells are treated with naloxone (Fig. 2b).

Effect of opiates on polyamine levels

After showing the effects of opiates on ODC, we determined cellular polyamine levels in hybrid cells after acute or chronic treatment with opiates. Polyamines were also assayed in the cells after drug withdrawal. Table 6 shows that in control cells the polyamine levels increased after incubation for 4 h with IBMX and that this effect continued until 6 h. After 9 h polyamine levels decreased again. When morphine was present (Table 6) the polyamine levels were below those in control cells. Table 7 shows the effect of opiates on polyamines in cells 'addicted' to the drug. After treating the cells with morphine for 6 h, a significant reduction in polyamine levels was observed (Table 7a). When the cells were treated with morphine for 2 days, the 'addicted-tolerant' cells had polyamines at levels resembling those found in the untreated controls (Tables 7a and 7b). The 'addicted' cells became tolerant to morphine, and a subsequent addition of

Fig. 2. Effect of etorphine on ornithine decarboxylase activity: kinetics of withdrawal effect (a) NG 108-15 hybrid cells were grown for 4 days until confluency. At various times the activity of ODC was determined in normal cells (●) and in cells treated with naloxone (50 μM) (○), starting 9 h after feeding (time 0) with complete medium. (b) Etorphine (1 μM) was added to 2-day-old cultures. After incubation for another 2 days, fresh medium (with serum) containing etorphine (●) was added. To some cultures, naloxone (50 μM) + etorphine (1 μM) (○) were added. At 9 h after feeding (time 0) aliquots were taken at different times for the determination of ODC activity.

Table 6. Acute effect of morphine on polyamine levels

Cells were grown until confluency. Old medium was removed and serum-free medium containing 0.5 mM-IBMX or 0.5 mM-IBMX + 10 μM-morphine was added. At different time intervals the medium was removed, the cells were washed twice with PBS and kept at -20°C for polyamine determination. Results are mean values of duplicates of two experiments (the s.d. is less than 10%) and are expressed as nmol/mg of protein.

Polyamines (nmol/mg of protein)						
Time (h)	(a) Control			(b) Morphine		
	Putrescine	Spermidine	Spermine	Putrescine	Spermidine	Spermine
0	2.3	3.8	4.34	2.3	3.8	4.34
2	2.4	3.7	4.2	1.65	2.6	3.5
4	6.3	10.0	10.8	3.2	6.2	6.5
6	6.5	11.2	12.0	3.5	7.3	7.3
9	2.81	5.6	6.3	3.2	4.3	5.6
16	2.95	4.5	5.2	1.9	4.1	4.5
18	2.77	3.9	4.7	1.85	3.1	3.6
22	2.52	3.6	4.6	1.62	2.8	3.4
24	2.32	3.5	4.2	1.4	2.65	3.7

Table 7. *Chronic effect of morphine on polyamines levels*

(a) The experimental conditions were as given in Table 3 except that naloxone was added to some cultures and polyamine levels were determined after 6h incubation. (b) Morphine was added to 2-day-old cultures. After confluency was reached old medium was replaced by serum-free medium containing the reagents and polyamine levels were determined after incubation for 6h. Results are mean values of duplicates of two experiments (the S.D. is less than 10%) and are expressed as nmol/mg of protein.

Treatment	Polyamines (nmol/mg of protein)					
	(a) Control			(b) Morphine (2 days)		
	Putrescine	Spermidine	Spermine	Putrescine	Spermidine	Spermine
Zero time	2.1*	3.5	4.0	2.4	3.8	5.0
IBMX	7.5	12.3	12.88	8.0	13.0	13.4
IBMX + morphine	4.3	8.2	8.66	6.4	9.6	10.3
IBMX + naloxone	7.2	11.3	11.5	8.3	12.7	13.2

the drug did not lead to a dramatic decrease in polyamine levels (Table 7b).

Discussion

This study shows that opiates regulate cyclic AMP-dependent protein kinase and ornithine decarboxylase activities in a pattern resembling that described for adenylate cyclase (Sharma *et al.*, 1975b). We have previously demonstrated that acute treatment with morphine inhibited the induction of protein kinase and ODC activities in neuroblastoma × glioma hybrid cells (Bachrach *et al.*, 1979). The present study shows that other opiates which affect cellular cyclic AMP levels also inhibited ODC induction (Table 2). Despite the similarity in the effect of the various drugs on cyclic AMP levels and ODC activity one exception was noted: the antagonist naloxone hardly affected cellular cyclic AMP levels (Table 1) but inhibited protein kinase activation (Table 4a) and ODC induction (Tables 2 and 5a). This observation may be explained by the assumption that under certain experimental conditions naloxone behaves like an agonist (Eisenberg, 1980; McMillan *et al.*, 1970).

The effect of opiates on cyclic AMP levels appears to be one of the first steps which occur during drug addiction and withdrawal. Modulations of cyclic AMP-dependent protein kinase activities are apparently the subsequent events that may lead to changes of various cyclic AMP-dependent processes.

Changes in cyclic AMP levels during the acute, tolerant and withdrawal states were followed by similar modulations in the activity of ODC. This finding agrees with published data (Byus & Russell, 1975), suggesting that the induction of ODC is cyclic AMP-mediated. As ODC has an extremely short half-life (10–20 min) it may serve as a good candidate for an opiate-regulated enzyme. Changes in ODC activity during drug

addiction and withdrawal resulted in parallel changes in cellular polyamine levels. Thus, during the acute effect, cellular polyamine levels decrease. Normal values were restored during the tolerant state while a moderate elevation was observed during drug withdrawal (Table 7). Polyamines are known to stabilize nucleic acids and are required for optimal synthesis of nucleic acids and proteins (Cohen, 1971). If opiates indeed affect cellular polyamine levels, a concomitant modulation in macromolecular synthesis could be expected.

Recent studies in our laboratory (D. Benalal, unpublished work) indicate that morphine inhibits the incorporation of [³H]uridine into RNA in hybrid cells. This agrees with the finding that morphine affects the activity of RNA polymerase I (Stokes *et al.*, 1979). Further studies are now required to clarify the effect of opiates on the synthesis of nucleic acids in neuroblastoma × glioma hybrid cells.

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