Ascorbate suppresses the opiate-induced compensatory increase in cyclic AMP in neuroblastoma × glioma hybrid cells

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In NG108-15 hybrid cells ascorbate suppresses the delayed etorphine-induced compensatory increase in the levels of cyclic AMP. It has, however, no effect on the early response of the cells to etorphine, as manifested in a transient decrease in the levels of cyclic AMP.

In NG108-15 neuroblastoma × glioma hybrid cells opiates regulate the synthesis of cyclic AMP at two different levels (Sharma *et al.*, 1975*a,b*, 1977; Traber *et al.*, 1975). A rapid receptor-mediated inhibition of adenylate cyclase is followed by a delayed and long-lived compensatory increase in its activity. It has been suggested that the latter response may be responsible for narcotic dependence and tolerance (Sharma *et al.*, 1975*b*). It therefore seems reasonable to propose that a selective suppression of the late effect of opiates prevents the development of tolerance. Serum lipids, with linolenic acid as the likely active component, have been implicated in the optimal expression of the compensatory increase in the activity of adenylate cyclase in NG108-15 hybrid cells (Wilkening & Nirenberg, 1980). Reports have also appeared on the stimulation of adenylate cyclase activity in the synaptic membrane preparations from rat brain, accompanied by a parallel increase in the formation of lipid peroxide (Baba *et al.*, 1981).

Results reported in the present paper show that ascorbic acid suppresses the etorphine-induced compensatory increase in the levels of cyclic AMP with little or no effect on the short-term response of NG108-15 hybrid cells to the effector agents. The studies suggest the potential use of ascorbic acid in the prevention of the development of tolerance in therapeutic applications of narcotics as analgesics.

Materials and methods

Growth of cells

NG108-15 neuroblastoma × glioma hybrid cells (obtained from Dr. Marshall Nirenberg, National Institute of Health, Bethesda, MD, U.S.A.) were grown in 60 mm Falcon Petri dishes in a humidified atmosphere of CO₂/air (1:9) at 37°C. The Dulbecco modification of Eagle's minimum essential

medium (Gibco, Grand Island, New York, U.S.A.) supplemented with 0.1 mM-hypoxanthine, 1 μM-aminopterin, 16 μM-thymidine and 10% foetal bovine serum (Gibco) was used as the growth medium (medium A).

Incubation and cyclic AMP assay

NG108-15 neuroblastoma × glioma hybrid cells grown as a confluent sheet covering 70–80% of the surface area were used in the studies. In a set of studies of the effects of ascorbate on the early response to different effector agents, cells were washed three times with 5 ml of medium B {Dulbecco modification of Eagle's minimum essential medium with 25 mM-Hepes [4-(2-hydroxyethyl)-1-piperazine-ethanesulphonic acid; Calbiochem, La Jolla, California, U.S.A.], pH 7.4}. Incubations were carried out in 3 ml of medium B containing 0.5 mM-Ro20-1724 [4-(3-butoxy-4-methoxybenzyl)imidazolidin-2-one; Hoffman-La Roche, Nutley, NJ, U.S.A.] for 15 min at 37°C in the presence of various effector agents as described in the legends to Tables. In another set of experiments cells were grown in medium A containing sodium ascorbate (Sigma Chemical Co., batch nos. 96C-0320 and 89C-0299) at various concentrations, with and without etorphine. The incubation was continued at 37°C in CO₂/air (1:9) for 4 h. The cells were washed with 3 × 5 ml of medium B. Prostaglandin E₁-stimulated increase in the levels of cyclic AMP was determined as described for the determination of basal levels of cyclic AMP involving short-term incubation (15 min). Reactions were terminated by pouring out the medium followed by an addition of 5% trichloroacetic acid at 0–4°C. Cyclic AMP was extracted from the cells by the method of Sharma *et al.* (1975*a*), and determined by the method of Gilman (1970). Protein was determined by the method of Lowry *et al.* (1951) with bovine serum

albumin as standard. Each experiment was performed in triplicate dishes. Values reported correspond to the means of three independent experiments with variations in the range of $\pm 10\%$. Etorphine was a gift from Dr. W. Klee, National Institutes of Health. Naloxone hydrochloride was from Dr. R. Jacobsen (Endo Labs Inc., Garden City, NY, U.S.A.) and prostaglandin E_1 was from Dr. John Pike (UpJohn Co., Kalamazoo, MI, U.S.A.).

Results and discussion

Effects of ascorbate on the early response to etorphine

In these studies we have investigated the effect of ascorbate on the basal levels of intracellular cyclic AMP, and on the stimulatory and inhibitory responses to the effector agents prostaglandin E_1 and etorphine respectively. The data in Table 1 show ascorbate alone led to a 60% increase in the basal levels of cyclic AMP. On the other hand, the increase in the level of cyclic AMP due to prostaglandin E_1 is only marginally affected by ascorbate. Etorphine, a potent opiate agonist, lowered both the basal and prostaglandin E_1 -stimulated cyclic AMP levels. Ascorbate had no effect on the transient inhibitory response of the cells to etorphine manifested as a pronounced decrease in the levels of cyclic AMP.

Naloxone, an opiate antagonist, completely reversed the effect of etorphine, suggesting that the former exerts its influence through an opiate receptor.

Table 1. *The effect of ascorbate on the early receptor-mediated synthesis of cyclic AMP*

NG108-15 hybrid cells (1.2×10^6 ; passage 19) were plated in medium A. When 80–100% confluent, the monolayer cultures were washed with medium B and pre-incubated in 3 ml of medium B with 0.5 mM-Ro20-1724 for 15 min at 37°C; 15 μ l of each effector agent as appropriate was added to the plates, and the incubation was continued for 15 min. Amounts of intracellular cyclic AMP were determined as described in the Materials and methods section. Concentrations of effector agents were: sodium ascorbate, 5 mM; etorphine, 1.6 μ M; naloxone, 1 mM; prostaglandin E_1 , 10 μ M.

Additions during cyclic AMP assay	Cyclic AMP (pmol/mg of protein)	
	Basal	Prostaglandin E_1
—	435 $\pm$ 23	6794 $\pm$ 315
Ascorbate	700 $\pm$ 47	8103 $\pm$ 422
Etorphine	170 $\pm$ 19	2972 $\pm$ 219
Ascorbate + etorphine	138 $\pm$ 11	3725 $\pm$ 397
Naloxone	500 $\pm$ 35	7163 $\pm$ 510
Ascorbate + naloxone	650 $\pm$ 42	6500 $\pm$ 633
Ascorbate + etorphine + naloxone	615 $\pm$ 36	6457 $\pm$ 381

Naloxone, however, had no effect on the observed increase in the levels of cyclic AMP due to ascorbate. The presence of both naloxone and etorphine in amounts adequate for an optimal expression of their mutually antagonistic action again had no effect on the increase in cyclic AMP levels observed with ascorbate alone. Although the mechanism underlying the elevation of cyclic AMP levels owing to ascorbate is not known, the data clearly indicate that this is not mediated through an opiate receptor. Remarkably, this does not seem to have any effect on the observed early response of the cells to etorphine.

Delayed compensatory increase in etorphine-induced cyclic AMP formation in the presence of ascorbate

In these experiments cells were grown in the presence of etorphine at a final concentration of 1.6 μ M with various amounts of ascorbate at 37°C for 4 h. Control cultures maintained under identical conditions permitted an estimation of the compensatory response of the cells to the narcotics. Under our experimental conditions the cells remained adhered to the plates during the incubation period. On the basis of a dye-exclusion test with Trypan Blue, 95% of the cells appeared viable. At the end of the incubation period the cells were washed with medium B. Prostaglandin E_1 -stimulated adenylate cyclase activity was estimated as described above.

In Fig. 1 the compensatory increase in both the basal and prostaglandin E_1 -stimulated formation of cyclic AMP is plotted against ascorbate concentration. In the absence of ascorbate (i.e. at zero concentration) etorphine led to a compensatory increase of 80% in the basal and 65% in the prostaglandin E_1 -stimulated adenylate cyclase activity. A progressive and systematic decrease in the compensatory response is seen as the concentration of ascorbate is raised to 5 mM. Initially, at lower concentrations of ascorbate, the decrease is more pronounced for prostaglandin E_1 -stimulated activity. These observations are in marked contrast with those presented in Table 1, where ascorbate has little or no effect on the early inhibitory response of the cells to etorphine.

Dunlap *et al.* (1979) have shown that, although ascorbate destroyed the stereospecific binding of opiates in brain homogenates, it had no effect on the opiate receptors in intact guinea-pig ileum or in vas-deferens preparations (Leslie *et al.*, 1979). It was of interest to know if the observed effect of ascorbate in preventing the late compensatory increase in the adenylate cyclase activity by etorphine was due to an inactivation of the opiate receptors during prolonged incubation or involved disruption of the functional coupling between the receptor and the enzyme. In one set of experiments the cells were

Fig. 1. The effect of ascorbate on the delayed compensatory increase in etorphine-induced cyclic AMP formation

NG108-15 hybrid cells (1.2×10^6) were plated in medium A. When 60–80% confluent, the culture plates were divided into four groups: (i) control, with no effector agents; (ii) etorphine ($1.6 \mu\text{M}$); (iii) etorphine ($1.6 \mu\text{M}$) and sodium ascorbate (1 mM); (iv) etorphine ($1.6 \mu\text{M}$) and sodium ascorbate (5 mM). The cells were incubated for 4 h at 37°C , washed with medium B and pre-incubated for 15 min in the presence of Ro20-1724. The incubation was continued for another 15 min in the presence of 1 mM -naloxone, with and without prostaglandin E_1 ($10 \mu\text{M}$). Naloxone was added to ensure an optimal expression of adenylate cyclase activity. Amounts of cyclic AMP formed were determined as described in the Materials and methods section. Symbols: ●, basal; ○, prostaglandin E_1 -stimulated.

pre-incubated with ascorbate alone in medium A for 4 h. Table 2 shows that the cells continue to elicit inhibitory and stimulatory responses to etorphine and prostaglandin E_1 respectively. This suggested that the corresponding receptor molecules were not affected by ascorbate.

Susceptibility of ascorbate to oxidation under the assay conditions raises the question as to whether the effects observed here are indeed due to ascorbate or whether they are due to its oxidized products. To answer this question we have examined the stability of ascorbate under our experimental conditions. Ascorbic acid was determined by titration with a redox indicator dye (2,6-dichloroindophenol) under acidic conditions by the method of Henry (1964). At the end of a 4 h incubation period in medium A, 70–75% of added sodium ascorbate could be

Table 2. Functional integrity of opiate receptors after pre-exposure of cells to ascorbate

NG108-15 hybrid cells (1.2×10^6 ; passage 19) were plated in medium A. Cells growing as a monolayer in Petri dishes were overlaid with 3 ml of medium A containing sodium ascorbate at a final concentration of 5 mM . Incubation was carried out at 37°C for 4 h. Cells were washed with medium B. The effects of prostaglandin E_1 and etorphine on the intracellular level of cyclic AMP were determined as in Table 1.

Additions during cyclic AMP assay	Cyclic AMP (pmol/mg of protein per 15 min)	
	Cells pre-incubated without ascorbate	Cells pre-incubated with ascorbate
—	422 ± 21	453 ± 37
Prostaglandin E_1	5524 ± 344	4400 ± 264
Prostaglandin E_1 + etorphine	2000 ± 183	2100 ± 108
Prostaglandin E_1 + naloxone	4500 ± 240	6100 ± 427
Prostaglandin E_1 + etorphine + naloxone	4244 ± 254	5472 ± 328

accounted for. Medium A alone did not show any reduction of the dye, nor did the presence of cells have any effect on the oxidation of ascorbate. Therefore, the effect of ascorbate on the suppression of the etorphine-induced compensatory increase in adenylate cyclase activity is not due to spurious oxidation products of ascorbic acid, or to a destruction of etorphine receptors.

In summary, the results presented suggest that, although ascorbate suppresses the delayed compensatory response to etorphine as manifested in an enhanced synthesis of cyclic AMP in NG108-15 hybrid cells, it has no effect on the short-term inhibitory response of the cells to the drug.

The mechanism by which ascorbate acts is not yet clear. This aspect requires further investigation. We have recently extended these studies to mice. Ascorbate administered along with morphine does seem to prevent the development of tolerance to the drug without significantly affecting its analgesic properties (N. C. Khanna & Shail K. Sharma, unpublished work).

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