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β_1-Adrenoceptor mRNA Levels Can Be Increased Via β-Adrenoceptor-independent Events

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Abstract

The drug (−)-oxaprotiline has been used as a tool to study the regulation of the β_1-adrenoceptor in rat C_6 glioma cells.

Treatment with (−)-oxaprotiline for 30 min results in an increase in steady-state β_1-adrenoceptor mRNA levels. The effect is β-adrenoceptor-independent, is not additive or synergistic with isoprenaline treatment, and does not involve activation of adenylate cyclase.

The data show that (−)-oxaprotiline can affect β_1-adrenoceptor mRNA levels via a mechanism that bypasses the receptor, perhaps involving direct activation of protein kinase A.

Regulation of the density and function of β-adrenoceptors is under tight control. The β_2-adrenoceptor is down-regulated by an agonist-mediated mechanism that involves protein kinase A, β-adrenoceptor kinase and the protein β-arrestin in a complex interaction (Benovic et al 1988). It is the β_1-adrenoceptor that is predominantly down-regulated by antidepressant drugs (Heal et al 1989; Ordway et al 1991), suggesting a role of the β_1-adrenoceptor system in affective disorders.

Recent evidence suggests that most of the β-adrenoceptors in mammalian brain are on glial cells (Stone & Ariano 1989; Marin et al 1990); and the suggestion has been made that glial cell β-adrenoceptors are the major targets in the central noradrenergic system (Stone & Ariano 1989). Surprisingly, glial β-adrenoceptor density can be affected by treatment with antidepressant drugs (Hertz et al 1981; Fishman & Finberg 1987; Manier et al 1992), suggesting that these drugs can affect β-adrenoceptors via a mechanism yet to be characterized.

We have previously shown in C_6 cells that (−)-oxaprotiline has no effect on β-adrenoceptor density after 30 min of treatment, but that the β_1-adrenoceptor mRNA levels are increased (Manier et al 1992). We now show that this phenomenon is β-adrenoceptor- and cAMP-independent, and may be part of a β-adrenoceptor-mediated signal transduction cascade distal to the receptor.

Materials and Methods

Cell culture conditions and treatments

Rat C_6 glioma cells (CCL 107) were obtained from American Type Culture Collection. The cells were grown in T-75 flasks at 37°C in a humidified chamber containing 5% CO_2. Complete medium (changed three times per week) consisted of Ham's F-10 medium supplemented with 15% (v/v) horse serum (Hyclone Labs, Logan, UT), 2·5% (v/v) foetal calf serum (Hyclone Labs), 2 mM glutamine (Life Technologies, Gaithersberg, MD), 2 mg L^{-1} amphotericin B (Sigma Chemical Co., St Louis, MO), 50 000 units L^{-1} penicillin and 50 mg streptomycin (both antibiotics, Life Technologies). The presence of mycoplasm was minimized by treating the cells with anti-PPLO agent (Life Technologies) before experimental use (1 passage after removal from liquid nitrogen). Only cells between passages 40 and 50 were used. Cell viability was determined by trypan blue exclusion and cell numbers quantified with a haemocytometer.

For subculturing, cells were washed with phosphate-buffered saline (PBS) and trypsinized until they loosened from the flask. The cells were immediately diluted with 50 mL of complete medium and 1 mL was added to 25 mL of fresh medium in each new flask. Drugs were added directly to the complete medium in the flask when the cells had grown to approximately 95% confluency. For harvesting, cells were scraped off the flask surface with a disposable cell scraper.

Isoprenaline (Sigma Chemical Co.), (−)-oxaprotiline (a gift from Ciba-Geigy Ltd, Basel), and (±)-propranolol (Sigma Chemical Co.), were added to complete medium in the flask at the indicated times.

For cAMP determinations, cells were washed twice with PBS, serum-free medium was added, and the cells incubated for 30 min in the presence of the appropriate drug. Incubations were terminated with 0·3 M perchloric acid. Following harvesting, the cells were homogenized and centrifuged at 5000 *g*. cAMP was measured in the supernatant fraction by RIA (Manier et al 1992).

For protein kinase A determinations, cells were washed twice with PBS, dislodged by scraping, and disrupted with a glass/Teflon cell homogenizer. Supernatants were separated from pellets by centrifugation for 10 min at 900 *g*.

Preparation and analysis of RNA

The β_1-adrenoceptor probe was prepared as described previously (Manier et al 1992). Briefly, cDNA was labelled with [^{32}P]deoxycytidine triphosphate using a random hexamer kit (Life Technologies). The resulting labelled cDNA

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was passed over a Sephadex G-50 spin column and used for hybridizations (sp. act. $2-10 \times 10^8$ counts min^{-1} μg^{-1} cDNA). Labelled probe was boiled for 5 min and placed on ice before addition to the hybridization buffer.

RNA was prepared by the CsCl/guanidine thiocyanate method (Chirgwin et al 1979), separated on denaturing gels, and transferred to GeneScreen Plus (NEN Research Products, Boston, MA) nylon membranes as described previously (Manier et al 1992). Northern blot analysis was performed using Rapid-Hyb hybridization mix (Amersham Corporation, Arlington Heights, IL). Briefly, blots were washed with diethylpyrocarbonate (DEPC)-treated H_2O, then blotted dry, photographed on Polaroid Type 55 film, and the negatives scanned for ribosomal RNA using a laser densitometer (Duhl et al 1992). The blots were baked for 2 h under vacuum, rehydrated with DEPC-treated H_2O and pre-hybridized in Rapid-Hyb supplemented with 0·1 mg mL^{-1} herring sperm DNA (Promega Corporation, Madison, WI) for 1 h at 67°C in a shaking water-bath. Labelled β_1-adrenoceptor probe (boiled for 5 min) was added (10^6 counts min^{-1} mL^{-1}) and hybridization continued for 2·5 h. After hybridization, the blot was washed twice in 2 × SSC (0·15 M NaCl, 0·015 M sodium citrate)/0·1% SDS (sodium dodecyl sulphate) (w/v) followed by one wash (60°C) in 0·1 × SSC/0·1% (w/v) SDS, then rinsed with H_2O. The moist blot was placed against a dampened paper towel, enclosed in plastic wrap, and placed against Kodak XAR-5 X-ray film at −80°C using intensifying screens.

Protein kinase A assay

The activity of protein kinase A (PKA) was determined by measuring the net incorporation of phosphate from ATP to Kemptide using [^{32}P]ATP as tracer, using the protocols provided by GIBCO/BRL, Gaithersburg, MD. Drug treatments and PKA assays were performed on supernatant fractions. Data from experiments measuring protein kinase A stimulation by (−)-oxaprotiline were analysed using the EZ-FIT program of Perrella (1988) with the EC50 value being determined.

Analytical

Autoradiographs were scanned with an LKB Ultroscan enhanced laser densitometer, and peaks were integrated with supporting software. As internal controls, scans of Polaroid negatives (Type 55 film) of the 28S ribosomal RNA (from the total RNA blotted to the nylon membrane) were integrated and used to calculate the variations in amounts of RNA being analysed (Duhl et al 1992). The ratio of the hybridization probe signal/28S fluorescence was calculated with the control ratio being designated as 100%. Proteins were quantified by the method of Lowry et al (1951).

Statistical analysis was by analysis of variance followed by the unpaired two-tailed Student's *t*-test. Statistical significance is defined as $P \leqslant 0{\cdot}05$.

Results

Time-course of change in steady-state levels of β_1- adrenoceptor mRNA

We have previously shown that (−)-oxaprotiline treatment of C_6 cells results in an increase in the steady-state levels of β_1-adrenoceptor mRNA 30 min after treatment, while the β_1-adrenoceptor number remains unaffected until 48 h after treatment (Manier et al 1992). This observation was expanded to a more complete study of the time-course of response (Table 1).

Table 1. Effect of isoprenaline or (−)-oxaprotiline on steady-state levels of β_1-adrenoceptor mRNA in C_6 cells at various times after treatment.

	Isoprenaline (1 μM)	(−)-Oxaprotiline (50 μM)
5 min	140 ± 11 (5)	131 ± 18 (2)
30 min	193 ± 9* (5)	182 ± 16* (5)
1 h	206 ± 22* (2)	137 ± 4 (3)
3 h	88 ± 7 (5)	105 ± 16 (5)
24 h	124 ± 19 (5)	94 ± 7 (5)
48 h	124 ± 10 (5)	98 ± 9 (5)

Data are expressed as percent of control values ± s.e.m. Numbers in parentheses indicate number of experiments. *$P < 0{\cdot}001$ compared with control values.

Isoprenaline (1 μM) or (−)-oxaprotiline (50 μM) resulted in maximal increases in the steady-state levels for β_1-adrenoceptor mRNA by 30 min. At this combination of dose and time, the cells exhibited no toxic effects of drug as confirmed by trypan blue exclusion. Levels in isoprenaline-treated cells remained increased throughout 1 h of treatment, while the levels in (−)-oxaprotiline-treated cells began to return to control values. Both treatments resulted in a return of the messages to control values by 3 h. At 48 h of treatment, a time when the density of β-adrenoceptors had decreased (Manier et al 1992), no changes in steady-state mRNA levels for β_1-adrenoceptor mRNA were apparent.

(−)-Oxaprotiline- and β-adrenoceptor-mediated signal transduction

The ability of (−)-oxaprotiline to activate the β-adrenoceptor signal transduction cascade was examined. After 30 min of incubation, 1 μM isoprenaline induced a greater than 500-fold increase in levels of cAMP, while (−)-oxaprotiline at 50 μM had no effect (Table 2). Treatment of cells with isoprenaline or (−)-oxaprotiline resulted in an increase in β_1-adrenoceptor mRNA. The increase due to isoprenaline treatment could be prevented by treating the cells with 10 μM propranolol (Table 3). Propranolol treatment, however, had no such effect on (−)-oxaprotiline-induced increases in β_1-adrenoceptor mRNA levels, indicating that the (−)-oxaprotiline-induced changes were not β-adrenoceptor-mediated.

When cells were treated with isoprenaline (1 μM) and (−)-oxaprotiline (50 μM) together, the increase in β_1-adreno-

Table 2. Effect of isoprenaline or (−)-oxaprotiline on cAMP levels in C_6 cells 30 min after treatment.

Control	15·4 ± 1·6 (4)
Isoprenaline (1 μM)	7925 ± 482* (3)
(−)-Oxaprotiline (50 μM)	15·1 ± 1·2 (4)

Data (mean ± s.e.m.) are expressed in pmol cAMP (mg protein)$^{-1}$. Numbers in parentheses indicate number of experiments. *$P < 0{\cdot}0001$ compared with control.

Table 3. Effect of (±)-propranolol on (−)-oxaprotiline- and isoprenaline-induced increases of steady-state levels of β_1-adrenoceptor mRNA in C_6 cells.

Isoprenaline (1 μM)	158 ± 14 (5)
Isoprenaline (1 μM) + (±)-propranolol (10 μM)	105 ± 10* (6)
(−)-Oxaprotiline (50 μM)	143 ± 19 (9)
(−)-Oxaprotiline (50 μM) + (±)-propranolol (10 μM)	132 ± 10[a] (6)
(−)-Oxaprotiline (50 μM) + isoprenaline (1 μM)	143 ± 9[a] (10)
(±)-Propranolol (10 μM)	100 ± 8 (7)

Data are expressed as percent of control values ± s.e.m. Numbers in parentheses indicate number of experiments. *$P < 0.02$ compared with isoprenaline. [a]Nonsignificant compared with (−)-oxaprotiline.

ceptor mRNA levels was not synergistic or additive (Table 3).

Effect of (−)-oxaprotiline on protein kinase A activity

Treatment of the supernatant fractions of broken C_6 cells with 50 μM (−)-oxaprotiline resulted in a 247% increase in endogenous (no added cAMP) protein kinase A activity (Table 4). When the effect of varying the (−)-oxaprotiline concentration was examined, it was determined that the dose of drug required to achieve 50% maximal reponse (EC50 value) was 20 ± 11 nM.

Discussion

Regulation of β-adrenoceptors has been expertly reviewed (Benovic et al 1988; Stadel & Lefkowitz 1991). In rat brain, an increased availability of noradrenaline elicited by chronic treatment with noradrenaline-reuptake blockers, MAO inhibitors and ECT causes a deamplification of the β-adrenoceptor-coupled adenylate cyclase (Vetulani & Sulser 1975) and a down-regulation of β-adrenoceptors (Banerjee et al 1977). This down-regulation of β-adrenoceptors by antidepressants is specific for the β_1-adrenoceptor subtype (Ordway et al 1991). However, the levels of β_1-adrenoceptor mRNA in rat frontal cortex are regulated in a complex manner by these treatments and could not be predicted from the consistent changes in the density of the receptors (Hosoda & Duman 1993). β-Adrenoceptor mRNA levels are also affected by β-adrenoceptor agonists (Collins et al 1989; Hough & Chuang 1990; Port et al 1992).

Our data with (−)-oxaprotiline in C_6 glioma cells demonstrate that steady-state β_1-adrenoceptor mRNA levels can be affected quickly after treatment with drugs that unlike for example desipramine, do not block the uptake of noradrenaline (Mishra et al 1982), do not cause down-regulation of the β_1-adrenoceptor and do not elevate cAMP levels. Treatment of C_6 glioma cells with (−)-oxaprotiline results in a statistically significant increase of steady-state β_1-adrenoceptor mRNA levels by 30 min under conditions in which the drug is not toxic. While this effect seems to mimic the response observed with isoprenaline, the effect is not as prolonged, and the measured mRNA levels return to control values by 1 h after treatment. Unlike isoprenaline, however, (−)-oxaprotiline exerts its action via a mechanism that is receptor-independent. The (−)-oxaprotiline treatment does not result in activation of adenylate cyclase and the effect on mRNA levels cannot be blocked by propranolol. While the mechanism of action of (−)-oxaprotiline is clearly different from that of isoprenaline, the two drugs may act through some common pathway (distal to adenylate cyclase) as is suggested by the lack of additivity or synergy when the two are added together.

The mechanism by which (−)-oxaprotiline exerts its effect on β_1-adrenoceptor mRNA levels is still unknown. It remains to be seen whether the drug increases β_1-adrenoceptor mRNA levels by stimulating transcription, stabilizing the message, or both. The effect is not receptor-mediated and does not involve cAMP, but may exert its action via one of the protein kinases. In fact, the stimulation of protein kinase by (−)-oxaprotiline in broken cells may suggest such a mechanism.

Table 4. Protein kinase A activity in supernatant fraction following (−)-oxaprotiline-treatment (50 μM) of broken C_6 cells.

	+cAMP	−cAMP
Control	2265 ± 116 (17)	688 ± 93 (14)
(−)-Oxaprotiline	1982 ± 211 (13)	1701 ± 376 (10)*

Data are expressed as the means of pmol PO_4 incorporated min^{-1} $(mg\ protein)^{-1}$ ± s.e.m. Numbers in parentheses indicate number of experiments. *$P < 0.02$ compared with control.

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