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DIFFERENTIAL EFFECTS OF DIFLUOROMETHYLORNITHINE ON BASAL AND INDUCED ACTIVITY OF CEREBRAL ORNITHINE DECARBOXYLASE AND mRNA

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Summary—The induction of the activity of cerebral ornithine decarboxylase (EC 4.1.1.17) and mRNA by electrical stimulation exhibits regional differences. The effects of the enzyme inhibitor difluoromethylornithine on these regional variations was examined. Administration of this inhibitor resulted in pronounced depression of both basal and induced activity of ornithine decarboxylase in the hippocampus. Basal activity of the enzyme in the neocortex and the cerebellum appeared to be resistant to difluoromethylornithine but the induced enzyme activity was sensitive to the effects of this inhibitor. Susceptibility to difluoromethylornithine may be directly correlated with a slower turnover rate for ornithine decarboxylase. These results suggest that ornithine decarboxylase in the hippocampus may possess a longer half-life than its counterparts in other regions of the brain. Pretreatment with difluoromethylornithine had no effect on the induced ornithine decarboxylase mRNA in the neocortex. Thus, elevated activity of ornithine decarboxylase enzyme, due to electrical stimulation, appears to not have any effect on either the transcription or the decay rate of the induced ornithine decarboxylase mRNA. These findings support the concept of region-specific regulation of cerebral ornithine decarboxylase.

Key words—mRNA, activity, brain, ornithine decarboxylase, difluoromethylornithine, electroconvulsive shock.

Ornithine decarboxylase is the rate limiting enzyme of the polyamine pathway (Pegg, 1986). Ornithine decarboxylase, a highly inducible enzyme with a very short half-life (<10 min), is present at very small concentrations in the adult brain (Russell and Snyder, 1969). The levels of activity of cerebral ornithine decarboxylase are induced very rapidly in response to a variety of stimuli, such as mechanical injury, intense electrical stimulation or chemical toxicity (Dienel and Cruz, 1984; Pajunen, Hietala, Virransalo and Piha, 1978). The rapid responses of ornithine decarboxylase to any change in the neuronal environment, implies a relationship between these biogenic compounds and the adaptive mechanisms of the central nervous system. The synthesis of the specific irreversible inhibitor for ornithine decarboxylase, difluoromethylornithine (DFMO), has provided a means to ascertain more precisely the biological role of the polyamines (Metcalf, Bey, Danzin, Jung, Casara and Vevert, 1978). The use of this inhibitor has shown that the fluctuations in levels of ornithine decarboxylase and polyamines are not just part of a generalized response to damage but appear to be important for the recovery process itself (Kanje, Fransson, Edstrom and Lowkvist, 1986; Slotkin and Bartolome, 1986; Dornay, Gilad, Shiler and Gilad, 1986).

The cell has evolved a variety of regulatory mechanisms allowing ornithine decarboxylase to alter the levels of its enzymatic activity rapidly. Among the factors responsible for the regulation of ornithine decarboxylase are rates of synthesis of messenger ribonucleic acid (mRNA) and turnover rates of the ornithine decarboxylase protein (McCann, 1980). The extent to which each of these factors are responsible for the fluctuations in the activity of cerebral ornithine decarboxylase is not yet known. Previously, it was found that electroconvulsive shock (ECS) resulted in a region-specific induction of cerebral activity of ornithine decarboxylase (Bondy, Mitchell, Rahmaan and Mason, 1987). Some of the cellular and molecular events that accompany the induction of neocortical ornithine decarboxylase, in response to electroshock-induced seizures, have been described (Zawia and Bondy, 1990). In the neocortex, the induction of enzyme activity was accompanied by a concomitant rise in the ornithine decarboxylase mRNA. The induction of the activity of ornithine decarboxylase was transient, peaking at 5 hr and returning to basal levels by 10 hr. On the other hand, levels of the ornithine decarboxylase mRNA remained elevated for periods longer than 24 hr. This suggested a feedback inhibition of the rate of translation of the ornithine decarboxylase mRNA.

Unpublished observations from this laboratory indicated that the hippocampus exhibited the greatest elevation in enzyme activity, independent of direct changes in the levels of the ornithine decarboxylase mRNA.

In this study, the *in vivo* effects of administration of difluoromethylornithine on basal and induced activity of ornithine decarboxylase in three regions of the brain were investigated. The levels of the ornithine decarboxylase mRNA in the neocortex have also been followed. The object of these experiments was to determine whether inactivation of ornithine decarboxylase, would alter a subsequent induction of ornithine decarboxylase enzyme activity or levels of the corresponding mRNA.

METHODS

A. Animals and treatment

Male Fischer CD rats, 6–10 week-old were used. The animals were housed at 22°C with free access to food and water, on a 12-hr light–dark cycle. Rats were injected intraperitoneally with either 0.9% saline solution or difluoromethylornithine (500 mg/kg body weight). A single dose of this concentration has been shown to result in optimal inhibition of the activity of ornithine decarboxylase in tissues of the rat, within 4–6 hours (Danzin, Jung, Grove and Bey, 1979). To achieve maximum attenuation of activity of ornithine decarboxylase prior to electroshock, the solutions of difluoromethylornithine were administered in two doses, separated by 5 hours. Electroconvulsive shock was administered through ear electrodes, with saline-moistened contact pads, using a constant-current apparatus. Shock was applied at the following intensities: 85 mA for 1 sec with 1-msec pulses at a frequency of 50 Hz. Control animals were treated similarly, without the application of a current. These intensities of shock have been demonstrated to be without detectable morphological damage to the brain (Dam, 1982). Animals were sacrificed 5 hr after the application of electroshock and the regions of the brain were used for assays of ornithine decarboxylase and analysis of mRNA.

B. Assay of ornithine decarboxylase

The activity of ornithine decarboxylase was determined by measurement of evolved $^{14}\text{CO}_2$ from carboxy- ^{14}C ornithine (55.9 mCi/nmol, New England Nuclear, Boston, Massachusetts). Tissue was homogenized in 19 volumes of 0.04 M Tris–HCl. After centrifugation (26,000 g, 20 min), 0.9 ml of various tissue preparations was added to 50 μl pyridoxal phosphate solution (1 μM) and 50 μl ^{14}C ornithine, in the presence of 0.045 M dithiothreitol. The final concentration of ornithine was 2.5 μM . Incubations were carried out at 37°C for 45 min, in a sealed tube and terminated by injection of 1 ml of 2 M acetic acid into the reaction mixture (Russell and Snyder, 1969). Evolved $^{14}\text{CO}_2$ was trapped on a paper wick, contain-

ing hyamine suspended above the reaction mixture. The decarboxylation process was linear for up to 1.5 hr under these conditions. Decarboxylation, not attributable to ornithine decarboxylase, was determined by running a parallel incubation in the presence of 5 mM difluoromethylornithine, a specific inhibitor of ornithine decarboxylase (Metcalf *et al.*, 1978).

C. Analysis of RNA

Total cellular-RNA was isolated from regions of the brain of control animals and animals treated with difluoromethylornithine and electroshock, using the phenol-sodium dodecylsulphate method (Maniatis, Fritsch and Sambrook, 1982; Soreq, Safran and Eliyahu, 1983). The RNA was fractionated, according to size, in formaldehyde–agarose gels (Fourney, Miyakoshi, Day and Paterson, 1988) and transferred onto nitrocellulose filter paper. The filters were then probed with a ^{32}P -labelled ornithine decarboxylase cDNA plasmid (McConlogue, Gupta, Wu and Coffino, 1984), which had been labelled by nick-translation. Hybridization, between the probe and the RNA on the filter, was allowed to proceed overnight. The filters were then washed 4 times, 5 min each, at room temperature in solution 'a' (a: 2 X sodium citrate sodium chloride + 0.1% SDS + 0.1% pyrophosphate); and twice in solution 'b' for 15 min each at 55°C (b: 0.1 X SSC + 0.1% SDS + 0.1% pyrophosphate). The filters were exposed to X-ray film at -70°C for 5–7 days (Thomas, 1980).

D. Statistics

The results were analyzed using one-way analysis of variance, followed by Fisher's Least Significant Difference Test.

RESULTS

Basal ornithine decarboxylase

Assaying for activity of ornithine decarboxylase in different regions of the brain revealed a higher level of activity in the hippocampus and the lowest activity in the cerebellum (Fig. 1). After pretreatment with difluoromethylornithine, the inhibition of activity of the enzyme was only detectable in the hippocampus, which exhibited a 35% reduction in basal activity (Fig. 1). The effect of difluoromethylornithine on basal activity of ornithine decarboxylase in the neocortex or cerebellum was not apparent.

Induced ornithine decarboxylase

In vivo administration of difluoromethylornithine largely inhibited the electroshock-induced rise in activity of ornithine decarboxylase (Fig. 2). The electroshock-induced elevation of ornithine decarboxylase was similarly attenuated by difluoromethylornithine in both the neocortex (45%) and cerebellum (41%). Difluoromethylornithine also

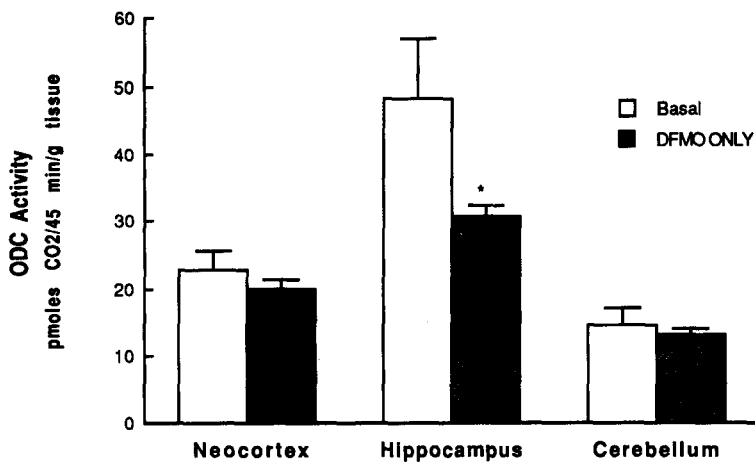


Fig. 1. Basal activity of ornithine decarboxylase (ODC) in various regions of the brain before (open bars) and 5 hr after the administration of difluoromethylornithine (DFMO) (closed bars). Values given are for the mean \pm SE for 9 determinations. *Denotes that values differ significantly from corresponding basal values ($P < 0.001$).

reduced induced activity of ornithine decarboxylase in the hippocampus by about 62%.

The ornithine decarboxylase mRNA

Electroshock-induced seizures in the neocortex resulted in an increase in the ornithine decarboxylase mRNA, which peaked at about 5 hr (Fig. 3). This was consistent with earlier reports (Zawia and Bondy, 1990). *In vivo* administration of difluoromethylornithine, prior to electroshock, had no significant effect on levels of the induced ornithine decarboxylase mRNA (Table 1).

DISCUSSION

These data suggest that the regulation of levels of ornithine decarboxylase in different regions of the brain may be by differing mechanisms. Difluoro-

methylornithine is an enzyme-activated synthetic inhibitor of ornithine decarboxylase, which binds irreversibly to active enzyme molecules (Metcalf *et al.*, 1978). This inhibitor *in vitro* blocks the activity of ornithine decarboxylase very potently and is used as a blank in the assay of ornithine decarboxylase to subtract out any non-specific decarboxylations of ornithine. However, in these *in vivo* experiments, the effect of difluoromethylornithine on the basal activity of ornithine decarboxylase was only detectable in the hippocampus.

There are no published observations that suggest that there are regional differences in the pharmacokinetics of difluoromethylornithine in the brain. However, regional differences in pharmacokinetic parameters may not be responsible for these regional susceptibilities to difluoromethylornithine. This is supported by the fact that, in the same area (e.g.

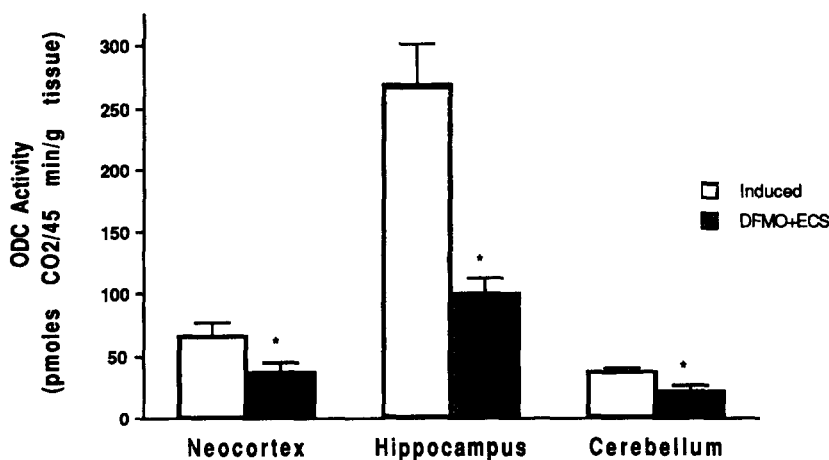


Fig. 2. Inhibition by difluoromethylornithine (DFMO) of electroshock (ECS) induced activity of ornithine decarboxylase (ODC) in different regions of the brain. Animals were pretreated with difluoromethylornithine 10 and 5 hr prior to application of electroshock and then sacrificed 5 hr after administration of electroshock. Values given are for the mean \pm SE of the mean for 9 determinations. *Denotes that values for difluoromethylornithine + electroshock differed significantly from electroshock-induced values ($P < 0.01$).

Table 1. The effect of difluoromethylornithine on basal and electroshock-induced ornithine decarboxylase and actin mRNA levels, 5 hr following treatment with electroshock

mRNA type	DFMO only (% of baseline)	ECS only (% of baseline)	ECS + DFMO (% of baseline)
ODC	102 ± 10	182 ± 15*	192 ± 14*
Actin	100 ± 15	101 ± 9	98 ± 12

Neocortical RNA was isolated and studied by Northern blot analysis, as described in the Methods section. The resulting autoradiograms, such as that shown in Fig. 3, were scanned with a laser densitometer. Levels of mRNA were derived by integrating the area under each peak. The values given are for the mean ± SE of the mean of the resulting areas as a percentage of the basal untreated levels of mRNA. *Values differ significantly from baseline ($P < 0.05$) ($n = 5$).

neocortex), only the induced and not the basal activity of ornithine decarboxylase was detectably affected.

Mammalian ornithine decarboxylase is not known to have different subtypes (Pegg, 1986). Therefore, these different susceptibilities to difluoromethylornithine are not a reflection of different types of interactions between difluoromethylornithine and the ornithine decarboxylase molecule, but rather may be a product of the turnover rate of the ornithine decarboxylase protein.

Ornithine decarboxylase has a very short half-life of only a few minutes. Thus, ornithine decarboxylase molecules inactivated by difluoromethylornithine are rapidly degraded and replaced by fresh enzyme molecules. The different rates of turnover of ornithine decarboxylase are a major determinant of the clinical effects of difluoromethylornithine. The fast turnover rate of mammalian ornithine decarboxylase has undermined the use of difluoromethylornithine as an anticancer agent (Janne, Holttä, Kallio and Kapyaho, 1983). On the other hand, difluoromethylornithine can cure sleeping sickness, because trypanosomal ornithine decarboxylase has a longer half-life (Phillips, Coffino and Wang, 1987).

Thus, susceptibility of ornithine decarboxylase to difluoromethylornithine *in vivo* has been correlated with an increased half-life of the ornithine decarboxylase protein (Phillips *et al.*, 1987). Both basal and induced hippocampal activity were especially subject to inhibition by difluoromethylornithine. Therefore, the half-life of the ornithine decarboxylase protein may be relatively long in this region. Similarly, the ornithine decarboxylase protein would be expected to have a comparatively shorter half-life in the neocortex and cerebellum.

While the basal activity in the neocortex and cerebellum appeared to be resistant to blockade by difluoromethylornithine, there was a clear effect of difluoromethylornithine on the induced activity in these regions. It was found previously that electroshock resulted in the induction of the ornithine decarboxylase mRNA in the neocortex (Zawia and Bondy, 1990). It may be that after electroshock, a build up in newly synthesized ornithine decarboxylase molecules in the neocortex overwhelms the

degradative processes, resulting in increased stability of ornithine decarboxylase. Consequently, inhibition of ornithine decarboxylase by difluoromethylornithine was not obscured by an equally stimulated rapid rate of decay of ornithine decarboxylase. The effect of electroshock on ornithine decarboxylase gene expression in the cerebellum is currently under investigation.

Electroshock in the neocortex leads to a transient elevation in the activity of ornithine decarboxylase, while the corresponding rise in mRNA persists for a very long time (Zawia and Bondy, 1990). Pretreatment with difluoromethylornithine had no significant effect on the level of neocortical electroshock-induced ornithine decarboxylase mRNA (Fig. 3, Table 1). This implies that the stability of the ornithine decarboxylase mRNA was not affected by enhanced activity of ornithine decarboxylase and thus levels of polyamines in the neocortex. Ornithine decarboxylase mRNA in the hippocampus was not used in these studies because it is not induced by electroshock.

It has also been reported that, directly adding large concentrations of putrescine to tissue culture systems, does not affect the levels of the induced ornithine decarboxylase mRNA (Holttä and Pohjanpelto, 1986; Persson, Holm and Heby, 1986). Polyamines may exert a negative feedback inhibition of the rate of translation of the ornithine decarboxylase mRNA. This would account for the rapid decline in levels of ornithine decarboxylase following their elevation after electroshock, despite a sustained high level of the corresponding mRNA (Zawia and Bondy, 1990). Additionally, polyamines can stimulate the degradation of ornithine decarboxylase itself, thereby bringing about a depression of the stimulated activity of ornithine decarboxylase.

These studies of difluoromethylornithine support the concept of regional variations in the regulation of cerebral ornithine decarboxylase. Even though the optimal dose (500 mg/kg) of difluoromethylornithine was used in these studies, it remains to be shown whether the brain responds to difluoromethylornithine in the same dose manner as other organs do. No direct studies were conducted on the actual turnover rate of ornithine decarboxylase in the brain (mainly due to the limitations of doing such studies in neural tissue). However, the present results suggest that turnover rate of ornithine decarboxylase may be one of the factors that contribute to the regional differences in regulation of ornithine decarboxylase in the brain.

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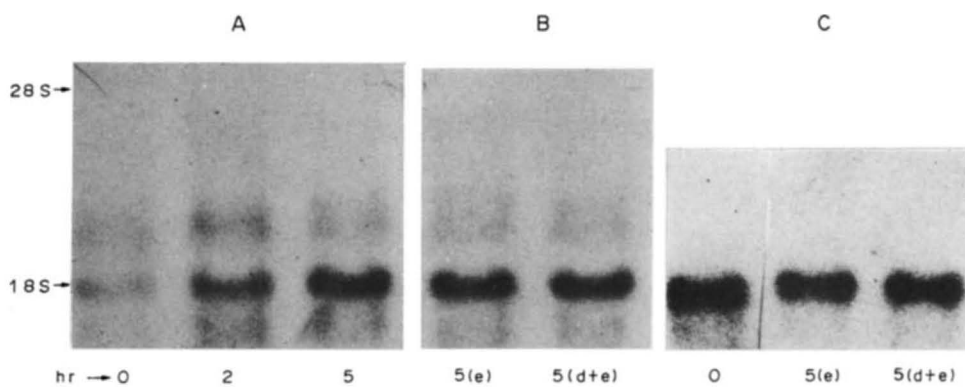


Fig. 3. The effect of electroshock (ECS) and difluoromethylornithine (DFMO) on levels of ornithine decarboxylase mRNA in the neocortex. Total RNA was isolated from animals 5 hr after application of electroshock; with one group pretreated with difluoromethylornithine 10 and 5 prior to the administration of electroshock. The RNA was then probed with the ornithine decarboxylase cDNA plasmid, as described in the Methods section. Representative ornithine decarboxylase mRNA bands are shown after various treatments. (A) The induction of ornithine decarboxylase mRNA at various time points after electroshock. (B) The levels of electroshock-induced ornithine decarboxylase mRNA in animals receiving only electroshock (e), and in animals pretreated with difluoromethylornithine (d + e). (C) The levels of β -actin mRNA basally (0 hr), 5 hr after treatment with electroshock (e) and in animals pretreated with difluoromethylornithine (d + e). These experiments were conducted on 5 different groups of animals. (A), (B) and (C) are from the same group of animals but run on separate gels. The numbers on the left indicate the position of the ribosomal RNA bands. Twenty-five μ g of total RNA was loaded on each lane.

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