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INHIBITION BY POLYAMINES OF METHYLTHIOPROPYLAMINE-INDUCED ORNITHINE DECARBOXYLASE IN HUMAN LYMPHOID LEUKEMIA MOLT 4B CELLS

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Abstract—Methylthiopropylamine (MTPA), an inhibitor of spermidine synthase, markedly induced ornithine decarboxylase (ODC) activity (about 30-fold of the basal level) in human lymphoid leukemia Molt 4B cells. This induction was blocked by the addition of spermidine, spermine or putrescine simultaneously with MTPA. Inhibition by spermidine or spermine of the MTPA-induced ODC activity was larger than that by putrescine. The increase of ODC activity by MTPA led to the large increase of cellular putrescine content. This increase of putrescine content was abolished drastically by the simultaneous addition of spermidine or spermine. The increase of ODC activity was almost completely blocked by the addition of cycloheximide or actinomycin D. This finding suggested that the increase of ODC activity was not due to activation of ODC preformed in Molt 4B cells. The ODC induction by MTPA was dose-dependently blocked by adding the calcium channel blockers (verapamil and nifedipine) or protein kinase C inhibitors (1-(5-isoquinolinesulfonyl)-2-methylpiperazine and palmitoyl carnithine). These results suggested that calcium and protein kinase C (PKC) were involved in MTPA-associated induction of ODC.

Ornithine decarboxylase (ODC; EC 4.1.1.17) is a rate-limiting enzyme in biosynthesis of polyamines (putrescine, spermidine and spermine). This enzyme is subject to regulation by a wide variety of substances [1–5], and it is clear that the polyamines themselves play an important part in this regulation. It is also well documented that the administration of putrescine, spermidine or spermine, which increases cellular polyamine pools, leads to rapid repression of ODC activity [1–4]. Conversely, the decrease in cellular polyamine contents by the use of inhibitors of polyamine synthesis has been shown to result in the induction of the ODC activity [1, 5, 6]. Although there is general agreement on these findings, it has not been entirely clear which polyamine is most effective in the regulation of ODC activity. On the other hand, several investigators have recently demonstrated the involvement of protein kinase C (PKC) in induction of ODC activity in epithelial cells [7] and other cell types [8, 9].

In the present study, we used an inhibitor of spermidine synthase, methylthiopropylamine (MTPA) [10], to determine whether PKC activity is involved in ODC induction in human lymphoid leukemia Molt 4B cells. It was found that the ODC induction was blocked by the calcium channel blockers and inhibitors of PKC activity.

* Abbreviations used: ODC, ornithine decarboxylase; PKC, protein kinase C; MTPA, methylthiopropylamine; DFMO, α-difluoromethylornithine; NSD 1024, 3-hydroxybenzylamine; AG, aminoguanidine; H-7 1(5-isoquinolinesulfonyl)-2-methylpiperazine.

MATERIALS AND METHODS

Chemicals. MTPA was obtained commercially from Tokyo Chemical Industry Co. Ltd (Tokyo, Japan). 3-Hydroxybenzyloxyamine (NSD 1024) was purchased from Smith & Nephew Research Ltd (Essex, U.K.). DL-[1-^{14}C]ornithine (48.8 mCi/mmol) and DL-α[3,4-^{3}H]difluoromethylornithine (20 Ci/mmol) were obtained from New England Nuclear (Boston, MA). Palmitoyl carnithine was purchased from Sigma Chemicals Co (St Louis, MO). 1(5-Isoquinolinesulfonyl)-2-methylpiperazine (H-7) was purchased from Seikagaku Kogyo Co. Ltd (Tokyo, Japan). All other chemicals were products of Nacalai Tesque (Kyoto, Japan).

Cell culture. Human lymphoid leukemia Molt 4B cells were growth at 37° in RPMI 1640 medium (Gibco, Uxbridge, U.K.), supplemented with 10% fetal calf serum, penicillin (50 I.U./ml) and streptomycin (50 μg/ml) in a 95% air–5% CO_2 humidified incubator. Experiments using polyamines were performed in the presence of 1 mM aminoguanidine (AG) and 0.1 mM NSD 1024 as inhibitors of serum diamine oxidase and polyamine oxidase, respectively.

Preparation of ODC. Molt 4B cells (2×10^5 cells/ml) were seeded in 5-ml culture flasks. The cells were harvested at the indicated times, washed twice in 10 ml phosphate-buffered saline and centrifuged (1000 *g* for 3 min). The cell pellets were suspended in 50 mM Tris–HCl buffer (pH 7.2) containing 10 mM dithiothreitol, 0.1 mM EDTA and 50 μM pyridoxal-5′-phosphate. The cell suspension was frozen and

thawed three times, centrifuged at 12,000 *g* for 5 min and the supernatant was taken for ODC assay.

Assay of ODC. ODC activity was determined by measuring the release of $^{14}CO_2$ from DL[1-^{14}C]ornithine as described by Seely *et al.* [11]. The assay mixture contained 0.1 mM DL-[1-^{14}C]ornithine (0.2 μCi), 20 μM pyridoxal-5'-phosphate, 5 mM dithiothreitol, 50 mM Tris–HCl, pH 7.2, and enzyme extract in a final vol. of 0.2 ml.

Measurement of the amount of ODC by binding of radiolabelled α-difluoromethylornithine (DFMO) The amount of ODC was determined by measuring the radioactivity of [^{3}H]DFMO bound to ODC as described by Kitani and Fujisawa [12]. The binding of [^{3}H]DFMO to ODC was assayed by nitrocellulose membrane filters. ODC extract was incubated at 37° for 2.5 hr in a total vol. of 10 μl of 10 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid buffer (pH 7.3) containing 1 μM DFMO (0.1 μCi), 0.01% Tween-80, 5 mM dithiothreitol, 0.1 mM pyridoxal-5'-phosphate, and 20% ethyleneglycol. After incubation, 2 ml of 50 mM sodium phosphate buffer (pH 7.0) were added and the mixture was immediately filtered through the nitrocellulose filter (Schleiher & Schuell, Dassel, F.R.G.) had been soaked in 50 mM sodium phosphate buffer (pH 7.0) for more than 3 hr. The filter was washed three times with 5 ml of the same buffer. The radioactivity on the filter was counted in 10 ml of modified Tritosol scintillant [13].

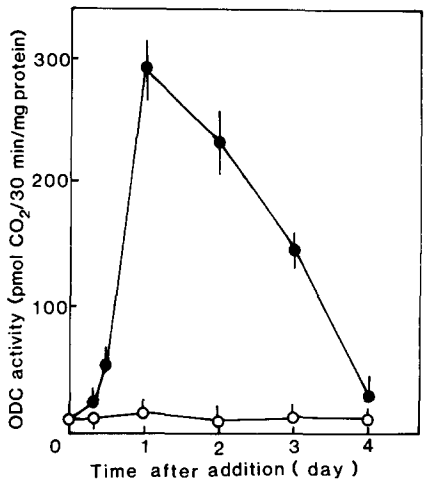

Fig. 1. Time course of induction of ODC activity by MTPA. Molt 4B cells were cultivated in the absence (○) or presence (●) of 2 mM MTPA and at the times shown ODC activity was measured. Each point is the mean of triplicate experiments.

Determination of polyamines. The cells were harvested by low speed centrifugation (1000 *g* for 3 min),

Table 1. Dose-dependent effect of MTPA on ODC activity and the amount of ODC protein measured by [^{3}H]DFMO binding assay and inhibition by polyamines

Addition		ODC actiity (pmol/mg protein/30 min)	[^{3}H]DFMO bound to ODC (pmol/mg protein)
	NSD + AG	12.9 (100)	0.25 (100)
MTPA (1 mM)	+ NSD + AG	164.6 (1276)	3.38 (1352)
MTPA (2 mM)	+ NSD + AG	343.7 (2664)	6.42 (2569)
MTPA (6 mM)	+ NSD + AG	285.7 (2215)	5.22 (2088)
MTPA (12 mM)	+ NSD + AG	138.8 (1075)	2.31 (924)
MTPA (2 mM) + Spd	+ NSD + AG	14.2 (110)	0.27 (108)
MTPA (2 mM) + Spm	+ NSD + AG	20.5 (158)	0.38 (152)
MTPA (2 mM) + Put	+ NSD + AG	216.4 (1677)	4.31 (1724)

Molt 4B cells were exposed to 1–12 mM MTPA, 10 μM spermidine (Spd), 10 μM spermine (Spm), 10 μM putrescine (Put), 0.1 mM NSD 1024 and 1.0 mM AG as shown above and 24 hr later the cells were harvested to determine ODC activity and the amount of [^{3}H]DFMO bound to ODC protein as described in the text. The control activity of ODC and [^{3}H]DFMO bound to ODC in the absence of NSD 1024 and AG were 12.8 pmol/mg protein/30 min and 0.24 pmol/mg protein, respectively. Percent of the control (treatment with NSD 1024 + AG) is shown in parentheses. Each value is the mean of duplicate experiments.

Table 2. Effect of the addition of spermidine or spermine on the MTPA-induced polyamine contents in Molt 4B cells

Addition		Putrescine	Spermidine	Spermine
		(nmol/mg cellular protein)		
	NSD + AG	0.68 (100)	6.83 (100)	7.80 (100)
MTPA	+ NSD + AG	19.08 (2806)	2.67 (39)	7.62 (98)
MTPA (2 mM) + Spd	+ NSD + AG	<0.05 (<8)	8.13 (119)	7.59 (97)
MTPA (2 mM) + Spm	+ NSD + AG	<0.05 (<8)	6.21 (91)	7.92 (102)

Molt 4B cells were exposed to 2 mM MTPA, 10 μM spermidine (Spd), 10 μM spermine (Spm). 0.1 mM NSD 1024 and 1.0 mM AG as shown above and 24 hr later the cells were harvested to determine polyamine contents. Percent of the control (treatment with NSD 1024 and AG) is shown in parentheses. Each value is the mean of duplicate experiments.

Table 3. Modulation of MTPA-induced ODC activity by the calcium entry antagonists (A), verapamil and nifedipine, and by inhibitors of protein kinase C (B), H-7 and palmitoyl carnithine

Addition	ODC activity (pmol/mg protein/30 min)
(A)	
MTPA	321.5 ± 25.4 (100)
MTPA + verapamil (1 μM)	278.7 ± 20.8 (87)
MTPA + verapamil (10 μM)	215.3 ± 19.5 (67)
MTPA + nifedipine (1 μM)	179.8 ± 15.9 (56)
MTPA + nifedipine (10 μM)	125.4 ± 13.1 (39)
(B)	
MTPA	335.7 ± 31.6 (100)
MTPA + H-7 (1 μM)	281.9 ± 27.5 (84)
MTPA + H-7 (10 μM)	177.9 ± 20.2 (53)
MTPA + palmitoyl carnithine (10 μM)	288.7 ± 27.1 (86)
MTPA + palmitoyl carnithine (50 μM)	174.6 ± 18.3 (52)

Verapamil, nifedipine, H-7 and palmitoyl carnithine were added to the culture medium simultaneously with 2 mM MTPA and 24 hr later the cells were harvested to determine ODC activity. Percent of the control (treatment with MTPA) is shown in parentheses. Values are mean ± SD for three experiments.

washed twice in phosphate-buffered saline, suspended in 0.4 N perchloric acid and disintegrated by freeze-thawing three times. After centrifugation for 30 min at 10,000 *g*, the resulting supernatant fractions were used for polyamine determination by HPLC (Shimazu LC-5A) as described previously [14].

RESULTS AND DISCUSSION

Figure 1 shows the time course of induction of ODC activity by MTPA. The addition of this compound markedly increased ODC activity, reaching a maximum (approximately 30-fold of the basal level) at 24 hr and returned to the initial level by 4 days.

Dose–response effects of MTPA on the induction of ODC activity and ODC protein were examined at 24 hr after the addition of MTPA (Table 1). ODC activity was elevated 13-, 27-, 22- and 11-fold at 24 hr after the addition of 1, 2, 6 and 12 mM MTPA, respectively. The amount of ODC protein measured by [^{3}H]DFMO binding assay was also elevated to about the same levels as the ODC activity. The largest increases of ODC activity and the enzyme protein obtained by 2 mM MTPA were blocked by the simultaneous addition of 10 μM spermidine or spermine. The inhibition by spermidine or spermine of the MTPA-induced ODC activity and protein were larger than that by putrescine. In these experiments NSD 1024 and aminoguanidine (AG), inhibitors of polyamine oxidase and diamine oxidase, respectively, were added to avoid the oxidation of the polyamines. As shown in Table 2, the increase of ODC activity by MTPA led to the large increase of cellular putrescine content. This increase of putrescine content was abolished by the simultaneous addition of spermidine or spermine, whereas the spermidine content was reversed to the control level by this treatment showing the inconversion from spermine to spermidine *in vivo*. The spermine content in the cells was hardly changed by all the treatments.

The MTPA-induced ODC activity was almost completely inhibited by simultaneous addition of 25 μM cycloheximide or 2 μM actinomycin D (data not shown). This finding suggested that the increase of ODC activity by MTPA was not due to activation of ODC preformed in Molt 4B cells.

Mechanism of ODC induction by MTPA was probed by adding the calcium channel blockers such as verapamil and nifedipine and inhibitors of PKC such as H-7 and palmitoyl carnithine simultaneously with MTPA. All these agents blocked the increase of ODC activity by MTPA in a dose-dependent manner (Table 3). These results suggested that calcium and PKC were involved in MTPA-associated induction of ODC.

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