Therapy for X-adrenoleukodystrophy: normalization of very long chain fatty acids and inhibition of induction of cytokines by cAMP

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Abstract X-adrenoleukodystrophy (X-ALD) is an inherited fatty acid metabolic disorder with secondary manifestation of neuroinflammatory disease process. We report that compounds (forskolin, 8-bromo cAMP, and rolipram) that increase cAMP and activate protein kinase A (PKA) were found to stimulate the peroxisomal β-oxidation of lignoceric acid ($C_{24:0}$) whereas compounds (H-89 and myristoylated PKI) that decrease cAMP and PKA activity inhibited the peroxisomal β-oxidation of lignoceric acid in cultured skin fibroblasts from X-ALD patients. Consistent with the stimulation of β-oxidation of lignoceric acid, activators of PKA normalized the level of very long chain fatty acids (VLCFA) in X-ALD cultured skin fibroblasts. This normalization of VLCFA in X-ALD cells with forskolin, 8-Br cAMP or with rolipram, an inhibitor of cAMP phosphodiesterase, was realized independent of expression of mRNA or protein of the ALD gene, suggesting that cAMP derivatives can correct the metabolic defect in X-ALD fibroblasts without involving the candidate gene for the disease. Because astrocytes and microglia in demyelinating lesions of X-ALD brain express proinflammatory cytokines such as tumor necrosis factor-α (TNF-α) and interleukin-1β (IL-1β), we examined the effect of cAMP derivatives or rolipram on lipopolysaccharide-stimulated rat primary astrocytes and microglia and found that cAMP derivatives and rolipram inhibited the induction of TNF-α and IL-1β in both astrocytes and microglia. The ability of cAMP derivatives and rolipram to block the induction of TNF-α and IL-1β in astrocytes and microglia and to normalize the fatty acid pathogen in skin fibroblasts of x-adrenoleukodystrophy (X-ALD) clearly identify cAMP analogs or rolipram as candidates for potential therapy for X-ALD patients.—Pahan, K., M. Khan, and I. Singh. **Therapy for X-adrenoleukodystrophy: normalization of very long chain fatty acids and inhibition of induction of cytokines by cAMP.** ***J. Lipid Res.*** **1998.** 39: **1091–1100.**

Supplementary key words cAMP • very long chain fatty acids • X-ALD • glial cells • cytokines

X-linked adrenoleukodystrophy (X-ALD), an inherited peroxisomal disorder, is characterized by progressive demyelination and adrenal insufficiency (1, 2). It is the most common peroxisomal disorder affecting between 1/15,000 to 1/20,000 boys and manifests with different degrees of neurological disability. The onset of childhood X-ALD, the major form of X-ALD, is between the ages of 4 to 8 and then death occurs within the next 2 to 3 years. As yet no proven therapy improves or changes the course of the disease process in X-ALD patients. All forms of X-ALD accumulate pathognomonic amounts of saturated very long chain fatty acids (VLCFA). In fact, levels of VLCFA have been used as a tool for both prenatal and post-natal diagnosis (1, 2).

A number of laboratories, including ours, have previously demonstrated that VLCFA are mainly and preferentially β-oxidized in peroxisomes and that VLCFA in X-ALD accumulate because of a defect in their oxidation in peroxisomes (3–5). Studies with total cellular homogenates and subsequent studies with purified subcellular organelles from cultured skin fibroblasts of X-ALD and control directly demonstrated the deficiency in VLC fatty acyl-CoA ligase in peroxisomes (6, 7). While these metabolic studies indicated lignoceroyl-CoA ligase gene as an X-ALD gene, positional cloning studies led to the identification of a gene that codes for a protein (ALDP), an 84 kDa protein that migrates as a 75 kDa protein in SDS-PAGE, with significant homology with the ATP-binding cassette of the super family of transporters (8). Studies from our laboratory (9) and those of others (10) demonstrated that ALDP is a peroxisomal membrane protein component and that the ATP-binding domain of ALDP, approximately 43 kDa, is oriented toward the cytoplasmic surface of the peroxisomal membrane (11). The normalization of fatty acids in X-ALD cells after transfection of the X-ALD gene (12) supports a role for ALDP in fatty acid metabolism, however, the precise function of ALDP

Abbreviations: X-ALD, X-adrenoleukodystrophy; PKA, protein kinase A; VLCFA, very long chain fatty acids; TNF-α, tumor necrosis factor-α; IL-1β, interleukin-1β; LPS, lipopolysaccharide; ALDP, adrenoleukodystrophy protein; HBSS, Hank's buffered salt solution.

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in the metabolism of VLCFA is not known at the present time. Mutations (missense or nonsense) or gene deletions have been detected in 80% of the X-ALD patients and these mutations were distributed over the whole protein-coding region, except exon 10, and nearly each patient has a different mutation (13–15). Moreover, no relationship could be established between the genotype and the severity of the disease as the same mutation is known to give different types of phenotype (1, 15).

Similar to other genetic diseases affecting the central nervous system, the gene therapy in X-ALD does not seem to be a real option in the near future and in the absence of such a treatment a number of therapeutic applications have been investigated (1, 15). Adrenal insufficiency associated with X-ALD responds readily to steroid replacement therapy, however, there is as yet no proven therapy for neurological disability (15). Two forms of therapies are presently under current investigation. Dietary therapy with "Lorenzo's oil" does normalize the plasma levels of VLCFA, however, it does not seem to improve the clinical status of the X-ALD patients (15–17). These results, in part, may be due to the fact that the fatty acid composition of the brain is not normalized because of a failure of erucic acid to enter the brain in significant quantity (15). Bone marrow therapy also appears to be of limited value because of the complexity of the protocol and of insignificant efficacy in improving the clinical status of the patient (15).

Because X-ALD is a metabolic disorder of VLCFA that eventually leads to an inflammatory bilateral demyelination with marked activation of microglia and astrocytes and accumulation of proinflammatory cytokines (TNF-α and IL-1β) and extracellular matrix proteins (18, 19), we searched for a therapy that would normalize the VLCFA and inhibit the induction of proinflammatory cytokines by astrocytes and microglia. The studies described in this paper demonstrate that the compounds that increase the intracellular levels of cAMP and the activity of protein kinase A (PKA) normalize the levels of VLCFA, possibly by increasing the peroxisomal activity for β-oxidation of VLCFA. Moreover, the same compounds also inhibit the induction of TNF-α and IL-1β in lipopolysaccharide (LPS)-stimulated astrocytes and microglia. These observations demonstrate the therapeutic potential of compounds that increase the activity of PKA in correction of the metabolic defect and inhibition of the neuroinflammatory disease process in X-ALD.

MATERIALS AND METHODS

Reagents

DMEM and bovine calf serum were from GIBCO. Forskolin, 1,9- dideoxyforskolin, 8-Br cAMP, S(p)-cAMP, H-89, rp-cAMP and rolipram were obtained from Biomol, Plymouth Meeting, PA. $C_{18:0}$-CoA, NADPH and N-ethylmaleimide were from Sigma (St. Louis, MO). [2-^{14}C]malonyl-CoA and $K^{14}CN$ (52 mCi/mmol) were purchased from DuPont-New England Nuclear. [1-^{14}C]lignoceric acid was synthesized by treatment of n-tricosanoyl bromide with $K^{14}CN$ as described previously (20).

Enzyme assay for β-oxidation of lignoceric acid

The enzyme activity of [1-^{14}C]lignoceric acid β-oxidation to acetate was measured in intact cells suspended in Hank's buffered salt solution (HBSS). Briefly, the reaction mixture in 0.25 ml of HBSS contained 50–60 μg of protein and 6 μm [1-^{14}C]lignoceric acid. Fatty acids were solubilized with α-cyclodextrin, and β-oxidation of [1-^{14}C]lignoceric acid was carried out as described previously (3, 6). The reaction was stopped after 1 h with 0.625 ml of 1 m KOH in methanol, and the denatured protein was removed by centrifugation. The supernatant was incubated at 60°C for 1 h, neutralized with 0.125 ml of 6 N HCl, and partitioned with chloroform and methanol. Radioactivity in the upper phase is an index of [1-^{14}C]lignoceric acid oxidized to acetate.

Transport of lignoceric acid into cultured skin fibroblasts

Cultured skin fibroblasts from patients with X-adrenoleukodystrophy were obtained from NIGMS Human Genetic Mutant Cell Repository, USA. These studies were approved by institutional approval (AR# 1128). Cells were incubated for 15 min at 37°C under isotonic conditions in HBSS with [1-^{14}C]lignoceric acid (6 μm) solubilized with α-cyclodextrin as described earlier (3, 5). Then cells were separated from the incubation medium by centrifugation through an organic layer of brominated hydrocarbons (21). This was performed in micro tubes (1.5 ml) containing 50 μl of 0.25 m sucrose in HBSS (as cushion), an organic layer (400 μl) consisting of a mixture of bromododecane and bromodecane (7:4, v/v), and an upper layer (500 μl) of cells in HBSS.

Protein kinase A assay

Cell extracts were assayed for PKA activity as described (22, 23) by measuring the phosphorylation of kemptide (0.17 mm) in the presence or absence of PKI peptide (15 μm). PKA activity was calculated as the amount of kemptide phosphorylated in the absence of PKI peptide minus that phosphorylated in the presence of PKI peptide.

Enzyme assay for fatty acid elongation

The fatty acid elongation activity was assayed by the method of Tsuji et al. (24). Briefly, the assay mixture contained 100 mm potassium phosphate (pH 7.2), 0.5 mm NADPH, 0.05 mm [2-^{14}C]malonyl-CoA, 1 mm N-ethyl maleimide, and 50–60 μg of protein in a total volume of 0.25 ml. The concentration of $C_{18:0}$-CoA was 1 μm. The reaction was started at 37°C by the addition of total homogenate and stopped by the addition of 1.25 ml of 10% (w/v) KOH after 30 min incubation. After saponification at 100°C for 30 min, the solutions were acidified with 1 ml of 4 N HCl and fatty acids were extracted with 2.5 ml of n-pentane three times. The radioactivities incorporated into fatty acids were measured with a liquid scintillation counter.

Measurement of VLCFA in fibroblasts

Fatty acid methyl ester (FAME) was prepared as described previously by Lepage and Roy (25) with modifications. Fibroblast cells, suspended in HBSS, were disrupted by sonication to form a homogeneous solution. An aliquot (200 μl) of this solution was transferred to a glass tube and 5 μg heptacosanoic (27:0) acid was added as internal standard and lipids were extracted by Folch partition. Fatty acids were transesterified with acetyl chloride (200 μl) in the presence of methanol and benzene (4:1) for 2 h at 100°C. The solution was cooled down to room temperature followed by addition of 5 ml 6% potassium carbonate solution at ice-cooled temperature. Isolation and purification of FAME were carried out as detailed by Dacremont, Cocquyt, and Vincent (26). Purified FAME, suspended in chloroform, were analyzed by

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a gas chromatograph GC-15A attached with a Chromatopac C-R3A integrator from Shimadzu Corporation.

Preparation of post-nuclear membranes and Western blot analysis

The membranes were prepared as described previously (11). Briefly, the post-nuclear fraction was diluted with an ice-cold solution of 0.1 m sodium carbonate, 30 mm iodoacetamide, pH 11.5. After 30 min incubation at 4°C, the membranes were sedimented by ultracentrifugation. The sedimented membranes were electrophoresed in 7.5% sodium dodecylsulfate-polyacrylamide gel, transferred to PVDF membranes and immunoblotted with antibodies against ALDP as described (11).

RNA isolation and Northern blot analysis

Cultured skin fibroblasts were taken from culture flasks directly by adding Ultraspec-II RNA reagent (Biotecx Laboratories Inc.) and total RNA was isolated according to the manufacturer's protocol. Twenty micrograms of RNA from each sample was electrophoretically resolved on 1.2% denaturing formaldehyde-agarose gel, transferred to nylon membrane, and cross-linked using UV Stratalinker (Stratagene, San Diego, CA). Full-length ALDP cDNA was kindly provided by Dr. Patrick Aubourg, INSERM, Hospital Saint-Vincent-de-Paul, Paris, France. ^{32}P-labeled cDNA probes were prepared according to the instructions provided with Ready-To-Go DNA labeling kit (Pharmacia Biotech). Northern blot analysis was performed essentially as described for Express Hyb Hybridization solution (Clontech) at 68°C. Actin cDNA probe was used as standard for comparing hybridization signals.

Isolation of rat primary astrocytes and microglia

Astrocytes were prepared from rat cerebral tissue as described earlier (23, 27). Microglial cells were isolated from mixed glial cultures according to the procedure of Giulian and Baker (28). For the induction of cytokine production, cells were stimulated with LPS in serum-free condition.

Determination of TNF-α and IL-1β in culture supernatants

Cells were stimulated with LPS in serum-free media for 24 h in the presence or absence of forskolin or rolipram, and concentrations of TNF-α and IL-1β were measured in culture supernatants by using high-sensitivity enzyme-linked immunosorbent assay (R&D Systems) according to the manufacturer's instructions.

RESULTS

Compounds that modulate the intracellular cAMP also modulate the β-oxidation of lignoceric acid and fatty acid chain elongation in X-ALD fibroblasts

First, we studied the effect of cAMP derivatives on lignoceric acid β-oxidation in control human fibroblasts. Cultured skin fibroblasts were treated with different activators and inhibitors of protein kinase A (PKA) and tested for β-oxidation of lignoceric acid. It is apparent from **Table 1** that compounds known to increase cAMP (forskolin and 8-Br-cAMP) stimulated lignoceric acid β-oxidation whereas compounds known to decrease cAMP (H-89 and myristoylated PKI) inhibited lignoceric acid β-oxidation in control skin fibroblasts. The inactive analogue of forskolin, 1,9-dideoxyforskolin, was ineffective in stimulating β-oxidation (Table 1). These results suggest that PKA has a positive modulatory role on lignoceric acid β-oxidation. As the β-oxidation of lignoceric acid is impaired in X-ALD patients, we studied the effect of different activators and inhibitors of PKA on lignoceric acid β-oxidation in cultured skin fibroblasts of X-ALD. **Figure 1** shows that the compounds (forskolin, 8-bromo cAMP and rolipram) known to increase intracellular cAMP stimulated lignoceric acid β-oxidation (Fig. 1A) and activated the PKA activity (Fig. 1C). On the other hand, β-oxidation of lignoceric acid was inhibited by PKA inhibitors (H-89 and myristoylated PKI). A combination of forskolin (activator of PKA) and H-89 or myristoylated PKI (inhibitors of PKA) had relatively little effect on the activation of PKA as well as on the β-oxidation of lignoceric acid. These observations indicate that β-oxidation of lignoceric acid is modulated by cAMP and PKA. However, in contrast to the effects on β-oxidation of lignoceric acid, activators of PKA inhibited the fatty acid chain elongation and inhibitors of PKA stimulated this activity in X-ALD fibroblasts (Fig. 1B). The increase in β-oxidation of lignoceric acid by forskolin (**Fig. 2A**) and its inhibition by H-89 (Fig. 2B) were dose-dependent. To understand the mechanism of cAMP-mediated stimulation of lignoceric acid β-oxidation, fibroblasts of X-ALD were treated with cAMP analogs, and the transport of lignoceric acid into intact cells and β-oxidation of lignoceric acid in cell homogenates of X-ALD were measured. Similar to the modulation of lignoceric acid β-oxidation, activators of PKA also stimulated the transport of lignoceric acid into ALD cells by more than 2-fold whereas inhibitors of PKA inhibited the transport of lignoceric acid by 40–50% (data not shown). Stimulation of lignoceric acid β-oxidation in cell homogenates of ALD fibroblasts as well as in cell suspension (Fig. 1A) suggests that increase in β-oxidation may not be due to an intracellular increase of substrate concentration but possibly to stimulation of the enzyme system for oxidation of lignoceric acid. In the cell, fatty acids are oxidized by mitochondrial and peroxisomal β-oxidation enzyme. Etomoxir, an inhibitor of mitochondrial β-oxidation of fatty acids (29), had no effect on cAMP-mediated stimulation of lignoceric acid β-oxidation (data not shown) suggesting that the observed stimulation

TABLE 1. Effects of different agonists and antagonists of PKA on β-oxidation of lignoceric acid in control human fibroblasts

Treatment	Lignoceric acid β-oxidation
	(pmol/h/mg protein)
Control	565.2 ± 48.3
Forskolin	885.3 ± 62.1
1,9 Dideoxy forskolin	571.4 ± 39.6
8-Br-cAMP	872.0 ± 53.7
H-89	405.6 ± 44.1
Myristoylated PKI	432.3 ± 46.5

Cells were treated for 72 h in serum-containing DMEM with the listed reagents. β-oxidation of lignoceric acid was measured as described in Material and Methods. Media were replaced every 24 h with the addition of fresh reagents. Concentrations of reagents were: forskolin, 4 μm; 1,9 dideoxy forskolin; 4 μm 8-Br-cAMP, 50 μm; H-89, 1 μm; myristoylated PKI, 0.2 μm. Data are means ± SD of three different experiments.

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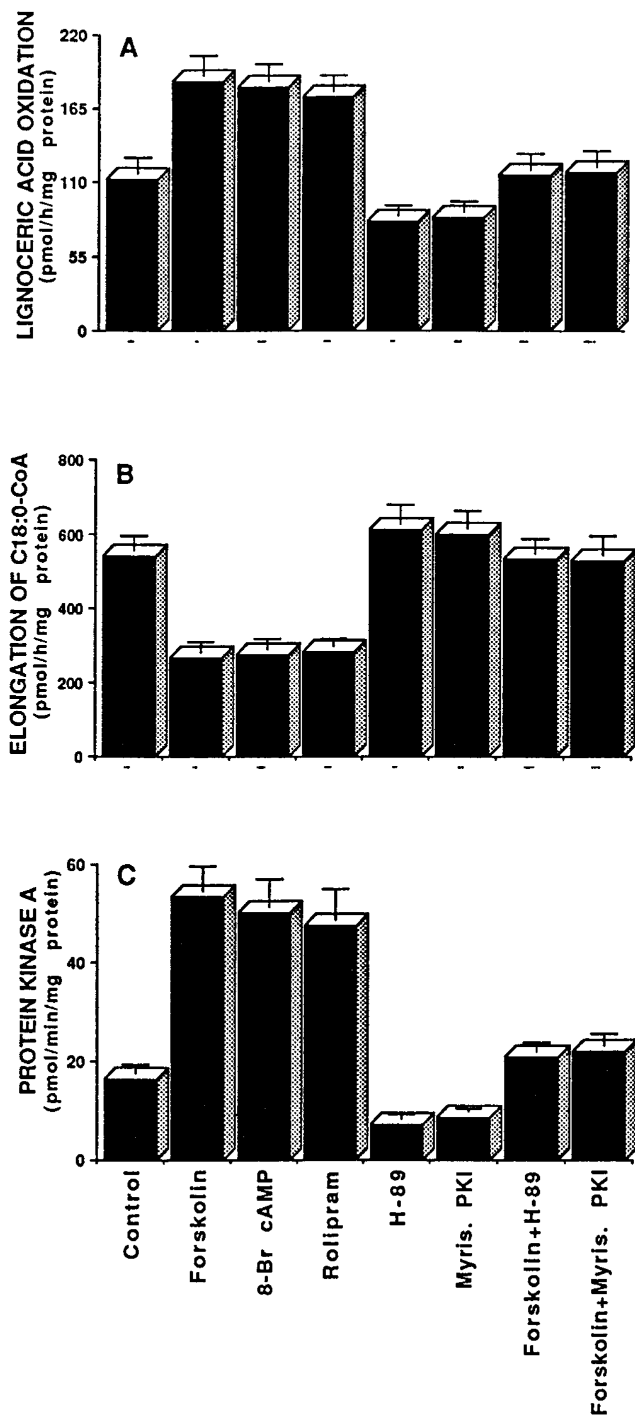

Fig. 1. Activation of PKA correlates with the stimulation of β-oxidation and inhibition of fatty acid chain elongation in cultured skin fibroblasts of X-ALD. Cells were treated for 72 h in serum-containing DMEM with the listed reagents; β-oxidation of lignoceric acid (A), fatty acid chain elongation (B), and PKA (C) activities were measured as described under Materials and Methods. Media were replaced every 24 h with the addition of fresh reagents. Concentrations of reagents were: forskolin, 4 μm; 8-Br-cAMP, 50 μm; rolipram, 10 μm; H-89, 1 μm; myristoylated PKI, 0.2 μm. Data are means ± SD of three different experiments.

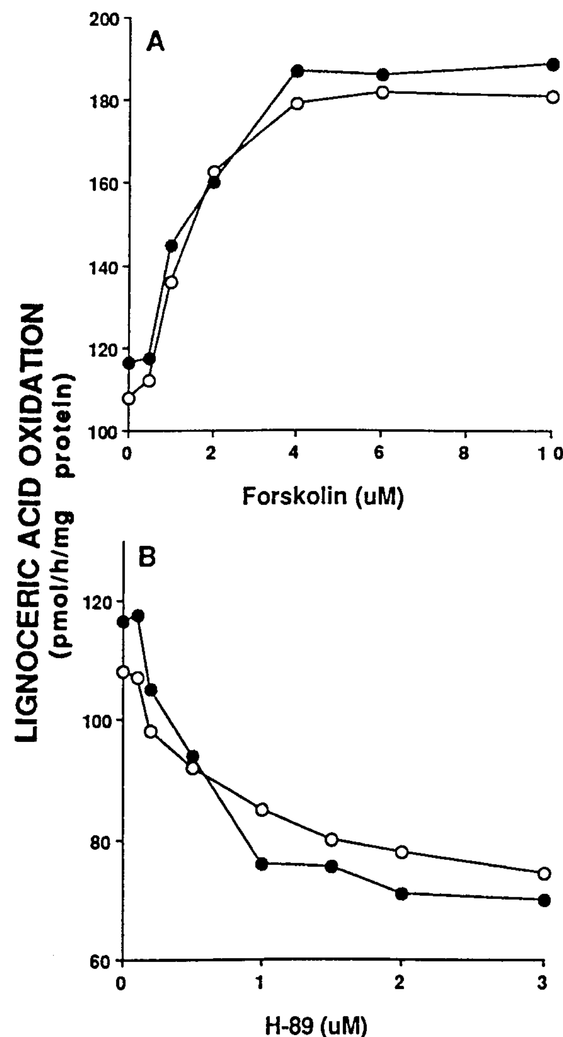

Fig. 2. Forskolin stimulates whereas H-89 inhibits the β-oxidation of lignoceric acid in cultured skin fibroblasts of X-ALD in a dose-dependent manner. Cells were incubated in serum-containing DMEM with different concentrations of forskolin (A) or H-89 (B) for 72 h. Every 24 h, media were replaced with the addition of fresh reagents. β-Oxidation of lignoceric acid was measured in cell suspension as mentioned in Methods (●, experiment 1; ○, experiment 2).

of lignoceric acid β-oxidation was a peroxisomal function. The increase in β-oxidation and transport of lignoceric acid but the decrease in fatty acid chain elongation with the increase in cAMP level and PKA activity and the decrease in β-oxidation and transport of lignoceric acid but the increase in fatty acid chain elongation with the decrease in cAMP level and PKA activity clearly delineate cAMP and cAMP-dependent protein kinase A as important regulators of the metabolism of VLCFA.

Modulation of cellular content of VLCFA in X-ALD and AMN fibroblasts by compounds modulating intracellular levels of cAMP

Because cAMP derivatives increase β-oxidation of lignoceric acid and decrease fatty acid chain elongation, we examined the effect of cAMP derivatives on the level of VLCFA in X-ALD fibroblasts. Treatment of X-ALD fibroblasts with 4 μm forskolin for different time periods (days) resulted in a time-dependent increase in oxidation of lignoceric acid and a time-dependent decrease in the ra-

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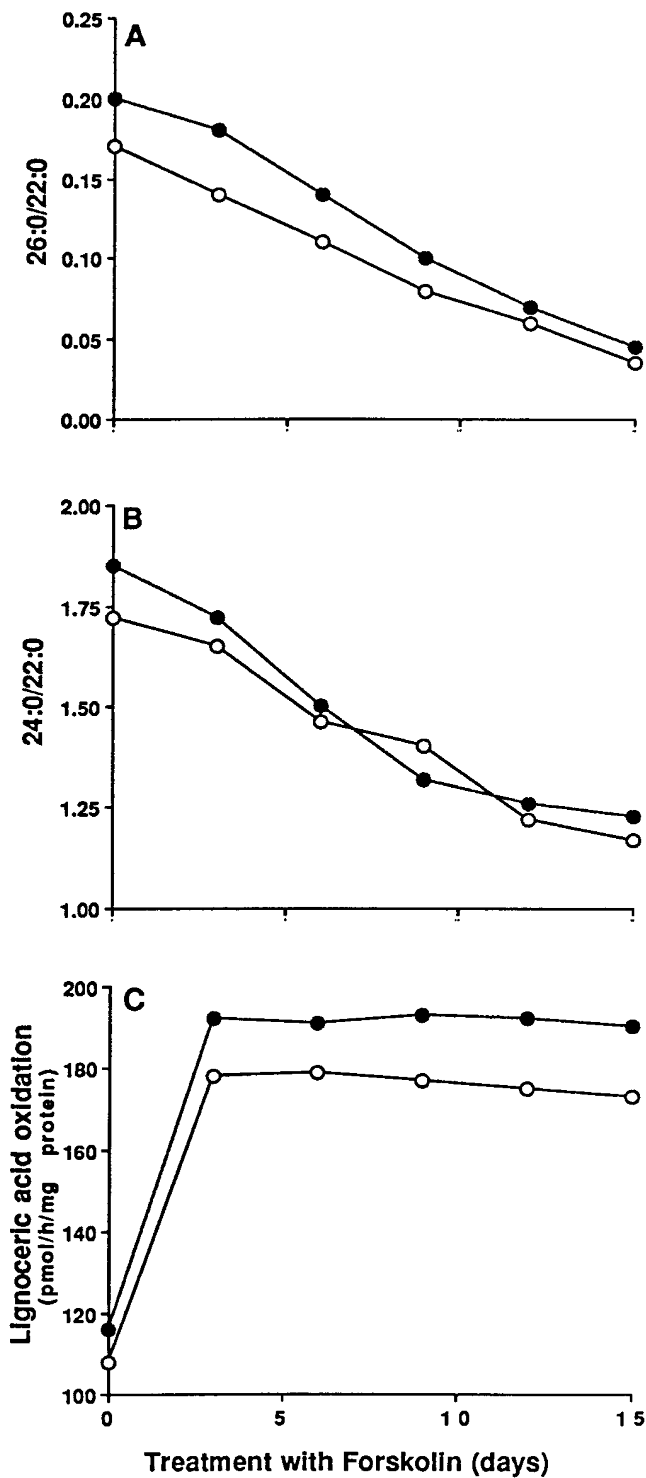

Fig. 3. Time-dependent effect of forskolin on the ratios of VLCFA ($C_{26:0}/C_{22:0}$ and $C_{24:0}/C_{22:0}$) and β-oxidation of lignoceric acid in cultured skin fibroblasts of X-ALD. Cells were incubated in serum-containing DMEM with 4 μm forskolin for different time periods (days), and the ratios of $C_{26:0}/C_{22:0}$ (A) and $C_{24:0}/C_{22:0}$ (B) and β-oxidation of lignoceric acid (C) were measured as described in Methods (○, experiment 1; ●, experiment 2).

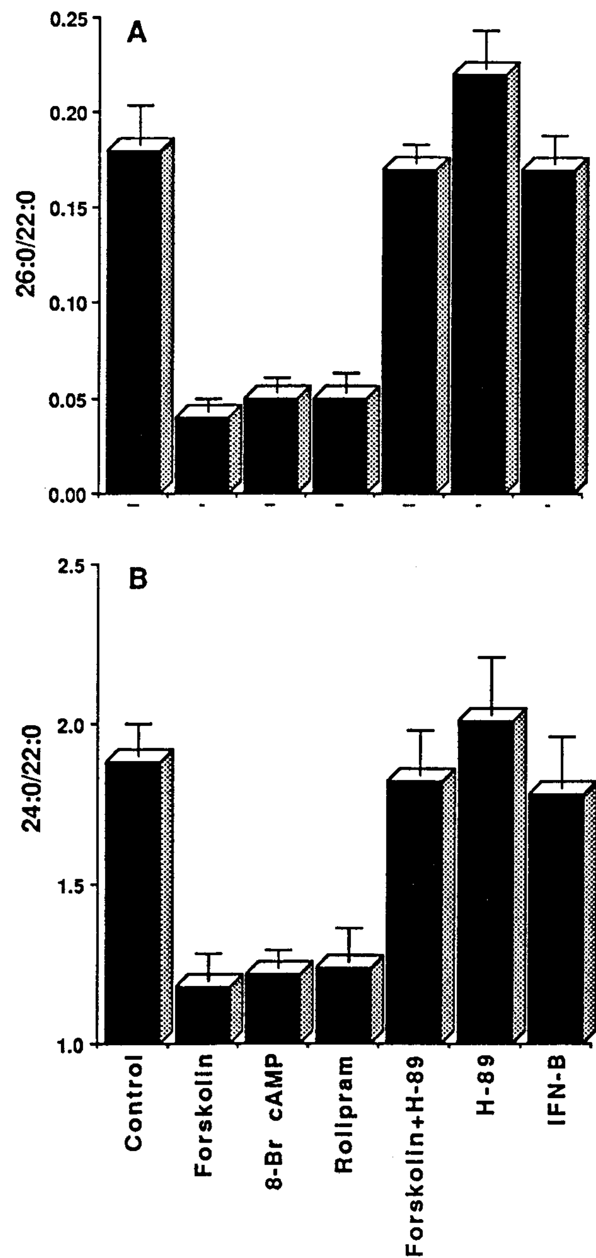

Fig. 4. Effect of cAMP derivatives, rolipram and IFN-β on the ratios of VLCFA ($C_{26:0}/C_{22:0}$ and $C_{24:0}/C_{22:0}$) in cultured skin fibroblasts of X-ALD. Cells were incubated in serum-containing DMEM for 15 days with the listed reagents, and the ratios of $C_{26:0}/C_{22:0}$ (A) and $C_{24:0}/C_{22:0}$ (B) were measured as described in Methods. Concentrations of reagents were: forskolin, 4 μm; 8-Br-cAMP, 50 μm; rolipram, 10 μm; H-89, 1 μm; myristoylated PKI, 0.2 μm; IFN-β, 50 U/ml. Data are means ± SD of three different experiments.

tios of $C_{26:0}/C_{22:0}$ and $C_{24:0}/C_{22:0}$ as shown in **Fig. 3**. Within 12 to 15 days of treatment, the ratios of $C_{26:0}/C_{22:0}$ and $C_{24:0}/C_{22:0}$ in X-ALD fibroblasts decreased to the normal level. The $C_{26:0}/C_{22:0}$ and $C_{24:0}/C_{22:0}$ values in control fibroblasts in these culture conditions were 0.04 ± 0.01 (n=8) and 1.32 ± 0.20 (n=8), respectively. This decrease in the ratios of $C_{26:0}/C_{22:0}$ and $C_{24:0}/C_{22:0}$ was also associated with the decrease in the absolute amounts of $C_{24:0}$ and $C_{26:0}$ whereas no significant change was observed in the levels of $C_{22:0}$ (behenoic acid) (data not shown). To decipher the possible mechanism of this dramatic decrease of VLCFA, we treated X-ALD fibroblasts with different activators of PKA (forskolin, 8-Br-cAMP, and rolipram)

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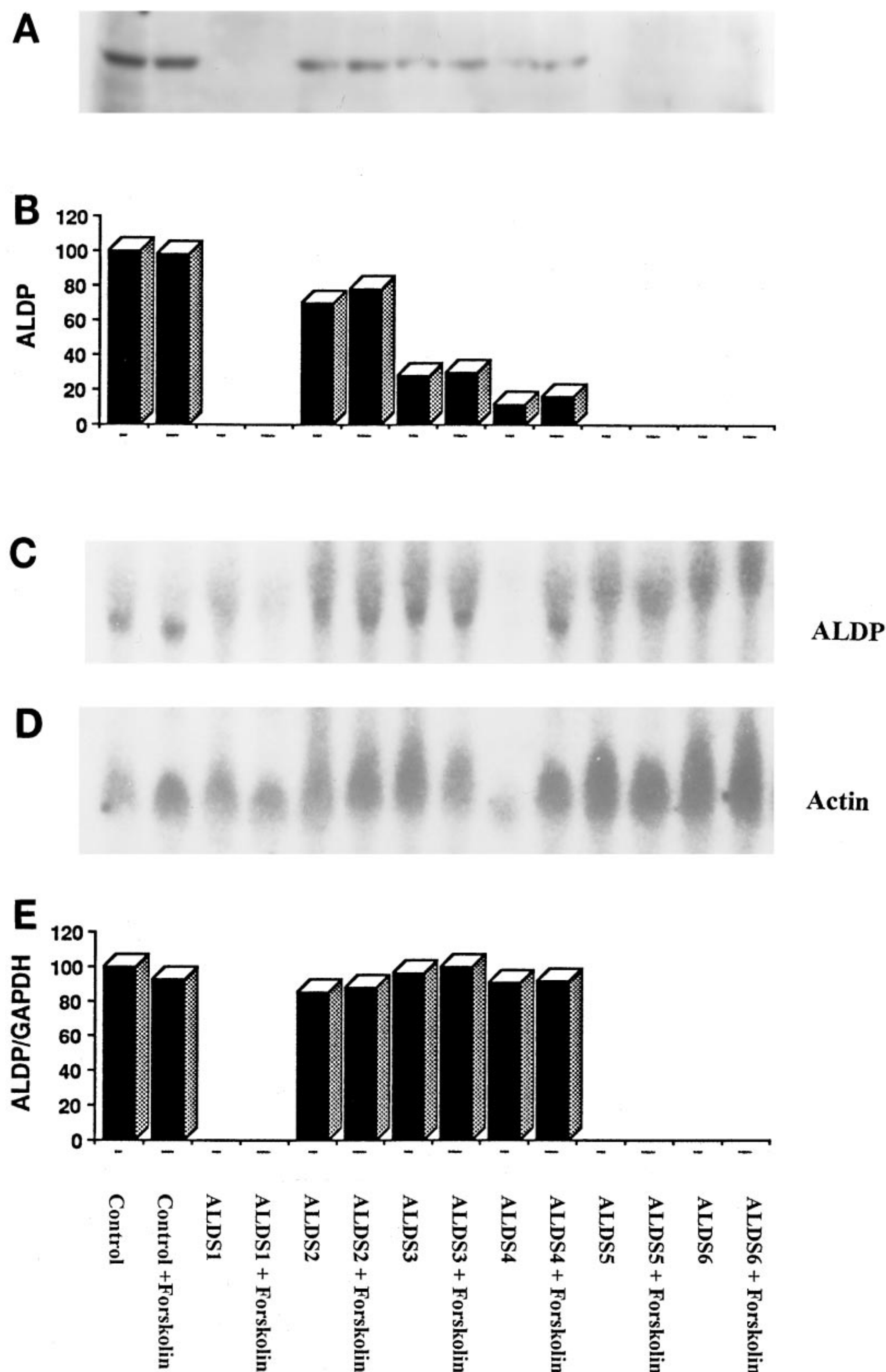

Fig. 5. Effect of forskolin on protein and mRNA expression of ALDP in skin fibroblasts of X-ALD. Cells were incubated in serum-containing DMEM with 4 μm forskolin for 15 days. Western blot analyses of postnuclear membrane fraction (0.08 mg protein) with antibodies against ALDP were carried out as mentioned earlier (12) (A, Western blot analysis; B, quantitative densitometric data for ALDP protein). Northern blot analyses for ALDP mRNA were carried out as described in Methods (C, Northern blot for ALDP; D, Northern blot for GAPDH; E, quantitative densitometric data for ALDP mRNA). ALDS1, ALDS5, and ALDS6 are X-ALD skin fibroblasts with deletion of the X-ALD gene, whereas ALDS2, ALDS3, and ALDS4 are X-ALD skin fibroblasts with mutation of the X-ALD gene.

for 15 days and analyzed the level of VLCFA. As shown in **Fig. 4**, the treatment of X-ALD fibroblasts with compounds known to increase intracellular cAMP lowered the ratios of $C_{26:0}/C_{22:0}$ and $C_{24:0}/C_{22:0}$ to the normal level. The inactive forskolin analogue, 1,9-dideoxyforskolin, had no effect on the ratios of $C_{26:0}/C_{22:0}$ and $C_{24:0}/C_{22:0}$ (data

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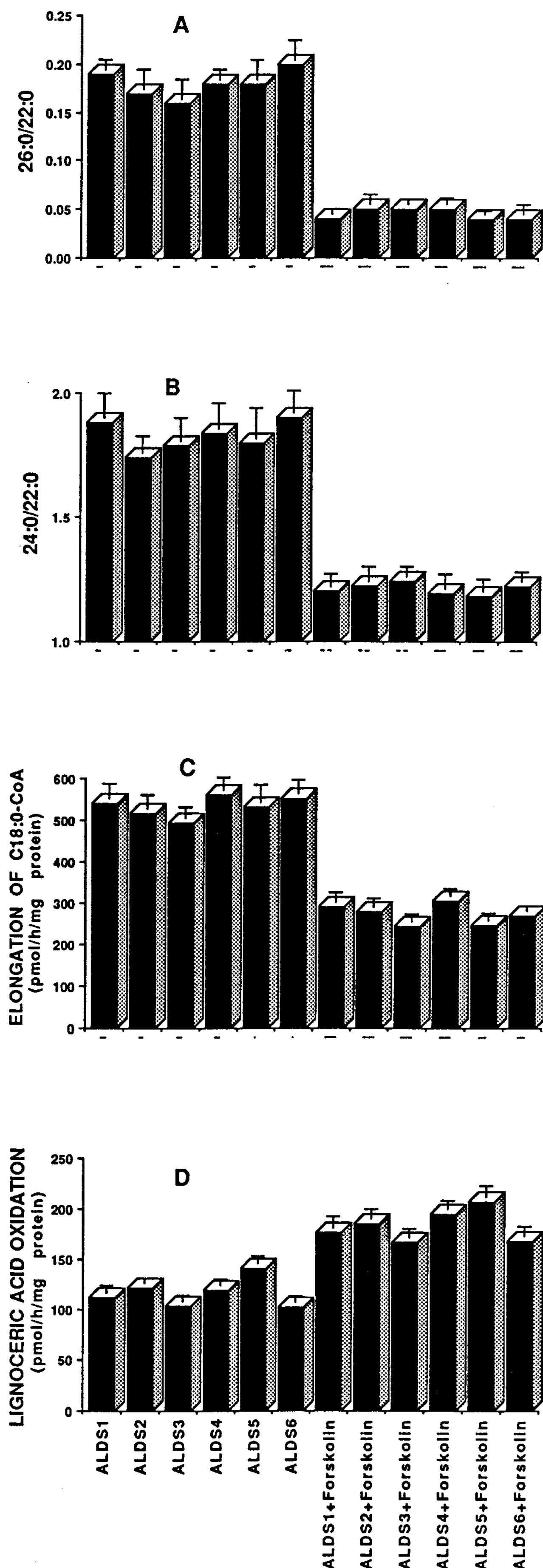

Fig. 6. Effect of forskolin on the ratios of VLCFA ($C_{26:0}/C_{22:0}$ and $C_{24:0}/C_{22:0}$), β-oxidation of lignoceric acid and chain elongation of fatty acids in different skin fibroblasts of X-ALD. Cells were incubated in serum-containing DMEM with 4 μm forskolin for 15 days, and the ratios of $C_{26:0}/C_{22:0}$ (A) and $C_{24:0}/C_{22:0}$ (B), β-oxidation of lignoceric acid (C), and elongation of fatty acids (D) were measured as described in Methods. Results are means ± SD of three different experiments. ALDS1, ALDS5 and ALDS6 are X-ALD skin fibroblasts with deletion of the X-ALD gene, whereas ALDS2, ALDS3, and ALDS4 are X-ALD skin fibroblasts with mutation of the X-ALD gene.

not shown). However, compared to X-ALD fibroblasts, forskolin marginally lowered the ratios of $C_{26:0}/C_{22:0}$ and $C_{24:0}/C_{22:0}$ in control skin fibroblasts. A 2 weeks treatment with forskolin lowered the ratio of $C_{26:0}/C_{22:0}$ from 0.04 to 0.029 and the ratio of $C_{24:0}/C_{22:0}$ from 1.32 to 1.12. Consistent with the effect of H-89 and myristoylated PKI on the β-oxidation of lignoceric acid, these two compounds blocked the observed effect of forskolin on the level of VLCFA when added along with forskolin, suggesting that cAMP analogs lower the level of VLCFA in X-ALD fibroblasts via activation of PKA (Fig. 4). On the other hand, interferon-β, which has been suggested as a possible therapy for X-ALD based on favorable effects found in multiple sclerosis (15), was ineffective in lowering the ratios of $C_{26:0}/C_{22:0}$ and $C_{24:0}/C_{22:0}$ in skin fibroblasts of X-ALD (Fig. 4).

Normalization of the levels of VLCFA by forskolin or rolipram in different X- ALD cells with or without deletion of the X-ALD gene

Although the precise function of ALDP, X-ALD gene product, in the metabolism of VLCFA is not known at the present time, accumulation of VLCFA in X-ALD cells with loss or mutations of ALDP and their normalization after transfection of cDNA for ALDP indicate a role for ALDP in the metabolism of VLCFA (12). Therefore, we next attempted to answer the question whether decrease in VLCFA in X-ALD fibroblasts by activators of PKA is mediated through the involvement of the ALD gene. X-ALD cells with mutation or deletion of the ALD gene were treated with forskolin for 2 weeks and tested for the levels of ALDP protein and its mRNA (**Figs. 5A** and **5B**), levels of VLCFA (**Figs. 6A** and **6B**), the rate of chain elongation of fatty acids (Fig. 6C) and the rate of β-oxidation of lignoceric acid (Fig. 6D). It is apparent from Fig. 5 that treatment of X-ALD fibroblasts with forskolin for 2 weeks had no effect on the steady state levels of ALDP and its mRNA in X-ALD cells. However, forskolin normalized the level of VLCFA in X-ALD fibroblasts by decreasing the rate of fatty acid chain elongation and increasing the rate of β-oxidation of lignoceric acid despite the status of mRNA and protein of ALDP. Treatment of X-ALD fibroblasts with rolipram for 2 weeks also increased the oxidation of lignoceric acid between 50–65% and normalized the levels of VLCFA in these cell lines (data not shown) suggesting that rolipram, an inhibitor of cAMP phosphodiesterase, has the same effect on the metabolism of VLCFA in X-ALD cells with nonfunctional ALDP due to a mutation or with absence of ALDP due to a deletion of the X-ALD gene.

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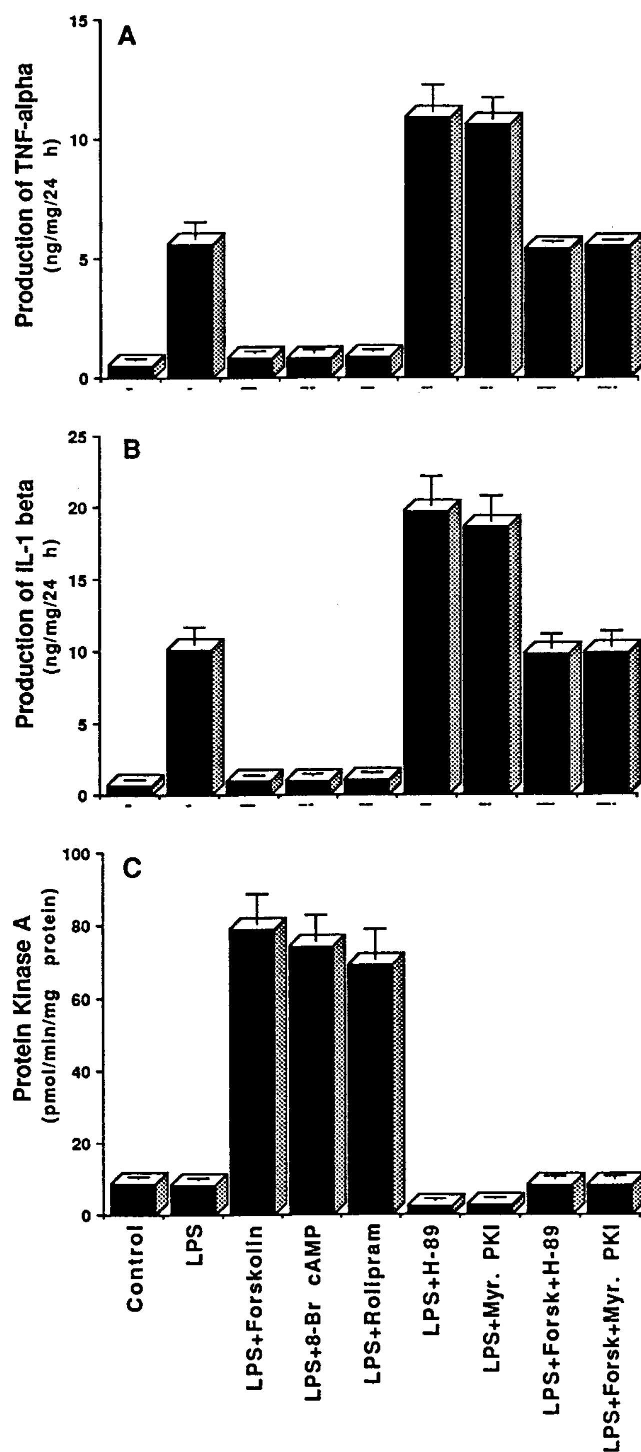

Fig. 7. Modulators of PKA modulate the induction of TNF-α and IL-1β in rat primary astrocytes. Cells preincubated with the listed reagents for 15 min under serum-free conditions were stimulated with 1.0 μg/ml of LPS. After 24 h incubation, concentrations of TNF-α (A) and IL-1β (B) were measured in supernatants as mentioned in Methods. After 1 h incubation, activity of PKA (C) was measured in cell extracts as mentioned in Methods. TNF-α and IL-1β are expressed as ng/24 h per mg protein. Concentrations of reagents were: forskolin, 10 μm; 8-Br-cAMP, 100 μm; rolipram, 20 μm; H-89, 2 μm; myristoylated PKI, 0.4 μm. Data are expressed as means ± SD of three different experiments.

Forskolin and rolipram inhibit the induction of cytokine production in rat primary astrocytes and microglia

As both astrocytes and microglia, reactive glial cells in the demyelinating lesions of X-ALD brain, are reported to express TNF-α and IL-1β (18, 19), we examined the effect of cAMP derivatives on the induction of cytokine production in astrocytes and microglia. Primary astrocytes in serum-free DMEM/F-12 were treated with different activators and inhibitors of PKA for 15 min before the addition of 1 μg/ml of lipopolysaccharide (LPS). **Figure 7** shows that the compounds (forskolin, 8-bromo-cAMP, and rolipram) known to increase intracellular cAMP inhibited the LPS-stimulated production of TNF-α (Fig. 7A) and IL-1β (Fig. 7B), and activated PKA activity (Fig. 7C). On the other hand, LPS-stimulated production of TNF-α and IL-1β were increased by inhibitors of PKA (H-89 and myristoylated PKI). The reciprocal relationship of induction of TNF-α and IL-1β with PKA activity supports the conclusion that PKA may play a pivotal role in the regulation of proinflammatory cytokines in astrocytes. Similar to astrocytes, forskolin or rolipram also inhibited the LPS-induced production of TNF-α and IL-1β, and H-89 stimulated the production of these proinflammatory cytokines in rat primary microglia (**Table 2**).

DISCUSSION

The studies reported in this paper provide evidence that in X-ALD cultured skin fibroblasts, the up-regulation of PKA activity increased the β-oxidation of lignoceric acid, decreased the chain elongation of fatty acids, and lowered cellular content of VLCFA to the normal level, despite the status (mutation or deletion) of the ALD gene. The detailed mechanism leading to the normalization of VLCFA in X-ALD is not known at the present, but is likely to involve cAMP-dependent protein kinase A. Our conclusion is based on the following observations. First, cAMP analogs and rolipram, an inhibitor of cAMP phosphodiesterase, stimulated transport and β-oxidation of lignoceric acid and decreased the chain elongation of fatty acids in X-ALD as well as control skin fibroblasts whereas H-89 and myristoylated PKI, specific inhibitors of PKA, inhibited transport and β-oxidation of lignoceric acid, stimulated chain elongation of fatty acids and blocked the

TABLE 2. Inhibition of lipopolysaccharide-induced production of TNF-α and IL-1β in rat primary microglia by forskolin and rolipram

Production of Cytokines	Treatment		
	LPS Only	LPS + Forskolin	LPS + Rolipram
TNF-α	14.1 ± 2.1	0.9 ± 0.1	1.2 ± 0.09
IL-1β	20.8 ± 2.8	1.9 ± 0.2	2.3 ± 0.3

Cells preincubated with 10 μm forskolin or 20 μm of rolipram for 15 min under serum-free conditions were stimulated with 1.0 μg/ml of LPS. After 24 h incubation, concentrations of TNF-α and IL-1β were measured in supernatants as described in Methods. TNF-α and IL-1ß are expressed as ng/24 h per mg protein. Data are expressed as the means ± SD of three different experiments.

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observed effects in normalization of VLCFA by cAMP analogs. Second, a long-term treatment of fibroblasts of X-ALD with cAMP analogs and rolipram had no effect on protein and mRNA for X-ALD gene but lowered the accumulation of VLCFA to the control level that is also blocked by inhibitors of PKA. These results clearly indicate that increasing cAMP level in fibroblasts of X-ALD normalizes the VLCFA pathogen by a mechanism that is dependent on the activity of PKA but independent of the involvement of the ALD gene product.

Studies from our laboratory (3, 4, 6, 7) and those of others (5) have previously shown that VLCFA (lignoceric and cerotic acids) are preferentially β-oxidized in peroxisomes. The increased transport of lignoceric acid into cAMP-treated cells suggests that the observed increase in β-oxidation of lignoceric acid may be due to higher availability of lignoceric acid in these cells. However, the increase in β-oxidation of lignoceric acid in cell-free extracts or permealized X-ALD cells or cell homogenates demonstrates that normalization of VLCFA is due to increased activity of the fatty acid β-oxidation system. In the cell fatty acids are β-oxidized in mitochondria and peroxisomes (1). The lack of effect of etomoxir, an inhibitor of mitochondrial carnitine palmitoyl transferase-I (29), on the cAMP-stimulated oxidation indicates that the higher lignoceric acid oxidation activity observed in cAMP-stimulated cells was due to an increase in the activity of the peroxisomal β-oxidation system. These observations provide the first evidence that peroxisomal β-oxidation of fatty acids is regulated by intracellular second messenger (cAMP).

The pathogenetic mechanism of X-ALD is poorly understood. The consistent hallmark of X-ALD is an excessive accumulation of VLCFA with subsequent involvement of the central nervous system (CNS) accompanied by induction of proinflammatory cytokines (TNF-α and IL-1β) and extracellular matrix proteins by reactive astrocytes and microglia and demyelination/inflammatory dysmyelination and loss of oligodendrocytes (18, 19, 30). The documentation of immunoreactive TNF-α and IL-1β in astrocytes and microglia of X-ALD brain suggested the involvement of these cytokines in immunopathology of X-ALD and aligned X-ALD with multiple sclerosis (MS), the most common immune-mediated demyelinating disease of the CNS in humans. However, apart from traditionally higher expression of cytokines by microglia than in astrocytes of MS and other neurodegenerative disorders, the expression of TNF-α and IL-1β is more prominent in astrocytes than microglia of X-ALD brain (18). At present it is not known how the inherited metabolic abnormality of accumulation of VLCFA subsequently triggers a neuroinflammatory response in the X-ALD brain. As the metabolic defect appears much prior to the detection of neuroinflammatory disease, the assumption is that these VLCFA by themselves or as a constituent of complex lipids act as a trigger for the inflammatory response that, in turn, becomes the basis for the observed demyelination and loss of oligodendrocytes in X-ALD. Studies reported in this manuscript indicate that cAMP may also inhibit the induction of proinflammatory cytokines in reactive astrocytes and microglia. The treatment of rat brain primary astrocytes or microglia with forskolin or rolipram inhibits the LPS-induced induction of TNF-α and IL-1β. We have also previously shown that cAMP derivatives and rolipram inhibit the cytokine-induced expression of inducible nitric oxide synthase and production of NO in astrocytes (23). Recent studies from our laboratory indicate that proinflammatory cytokines down-regulate the peroxisomal function in the metabolism of VLCFA thereby aggravating the inherited metabolic abnormality by accumulating 4-times higher VLCFA (around the plaque) than in normal looking X-ALD brain and these alterations by proinflammatory cytokines are mediated by NO toxicity (31). The inhibition of induction of cytokines as well as induction of iNOS by compounds that increase the activity of PKA (e.g., cAMP and rolipram) in astrocytes and microglia indicate that these compounds should be beneficial in terms of blocking the induction of proinflammatory cytokines in X-ALD.

Although the gene for X-ALD has been identified, similar to other genetic diseases that affect the central nervous system, the gene therapy in X-ALD patients may not be realistic in the near future. In the absence of such a therapy a number of other therapeutic approaches have been explored (15–17). Two forms of therapy are under current investigation: *1*) "Lorenzo's oil" dietary therapy and *2*) bone marrow transplantation. The dietary therapy to normalize the VLCFA in X-ALD is the use of "Lorenzo's oil", a mixture of trioleate and trieruciate (16, 17). It is based on the observation that unsaturated fatty acids compete with saturated long chain fatty acids ($C_{18:0}$ to $C_{20:0}$) for chain elongation to saturated VLCFA ($>C_{22:0}$), the only known pathogen in X-ALD (16, 17). The dietary therapy with "Lorenzo's oil" does in fact normalize the VLCFA levels in plasma, however, it does not seem to improve the clinical status of X-ALD patients (15). The use of unsaturated fatty acids to block the synthesis of VLCFA was based on the assumption that unsaturated fatty acids are nontoxic. However, a recent study has demonstrated that exogenous unsaturated VLCFA induce the production of superoxide, a highly reactive oxygen radical, by human neutrophils (32). As cerebral demyelination in X-ALD is associated with infiltration of phagocytic cells to the site of the lesion, treatment with unsaturated fatty acids may even be toxic to X-ALD patients. Bone marrow transplantation also seems unrealistic because of complexity of protocol, targeting of nonsecretable proteins/enzymes to the defective cells, and inability to identify presymptomatic children that are likely to develop childhood ALD but not adrenomyeloneuropathy. The cALD patients die in early childhood whereas AMN patients may have a normal life span. We searched for a therapy that would normalize the metabolic abnormality and block the neuroinflammatory process by inhibiting the induction of proinflammatory cytokines. The studies described in this manuscript clearly demonstrate that the compounds (e.g., forskolin, 8-Br-cAMP, rolipram) that increase cAMP and activate PKA meet both of these conditions. Moreover, recent reports (33) showing the prevention of progression of autoim-

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mune encephalomyelitis in mice as well as in marmosets by rolipram indicate that rolipram does cross the blood–brain barrier and inhibits the cytokine-induced neuropathologies in these animal models.

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