Structure-Activity Relationship of Reversibly Lipidized Peptides: Studies of Fatty Acid-Desmopressin Conjugates

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Purpose. To synthesize a series of reversible fatty acid-desmopressin (DDAVP) conjugates and to study their structure-activity relationship as anti-diuretic drugs.

Methods. Seven fatty acid conjugates of DDAVP were prepared using various reversible lipidization reagents as described in our previous reports. All products were purified by acid precipitation and/or size-exclusion chromatography. Reversed-phase HPLC was used to evaluate their purity and lipophilicity. The anti-diuretic efficacy of these fatty acid conjugates was assessed in vasopressin-deficient Brattleboro rats. Four selected conjugates, i.e., DPA, DPH, DPD and DPP (acetic, hexanoic, decanoic, and palmitic acid conjugate, respectively), along with DDAVP itself were used in Caco-2 cell uptake studies and their degradation and the regeneration of active DDAVP were investigated using an *in vitro* liver slice metabolic system coupled with a HPLC assay.

Results. All fatty acid-DDAVP conjugates were more lipophilic than DDAVP as examined by HPLC analyses. When cysteine was used as the linker, the capacity index (k′, a measure of lipophilicity) of the conjugates was linearly correlated with the number of carbons in the fatty acid chain. The anti-diuretic activity of the conjugates was correlated with the length of the fatty acid chain, with C10 as the minimal requirement for possessing the enhanced anti-diuretic activity. Among the seven fatty acid conjugates, palmitic acid conjugate was the most potent DDAVP derivative. Removal of carboxyl group from the cysteine linker completely abolished the enhancement of the activity. The extent of cellular uptake also positively correlated with the lipophilicity of the conjugates. The metabolism of DDAVP, DPH, DPD, and DPP by liver slices all followed first order kinetics with half-life of 0.30, 0.01, 0.06 and 3.44 hr, respectively. The degradation rates of DPH and DPD in the liver slice incubation were much faster than that of DDAVP and therefore an accumulation of regenerated DDAVP in the media was observed. In contrast, DPP was metabolized much slower than DDAVP and, consequently, no significant accumulation of regenerated DDAVP could be detected.

Conclusion. Conjugation of DDAVP with fatty acids increased the lipophilicity and the anti-diuretic activity of this peptide drug. The anti-diuretic activity of lipidized DDAVP was dependent on the chain length of the fatty acid, as well as the structure of the linker in the conjugate. The preservation and enhancement of the *in vivo* anti-diuretic activity of the conjugates is most likely due to a combination of an improved pharmacokinetic behavior and a concurrent regeneration of active DDAVP in tissues.

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ABBREVIATIONS: DDAVP, desmopressin; HPLC, high-performance liquid chromatography; AVP, arginine vasopressin; DTT, dithiothreitol.

KEY WORDS: desmopressin; fatty acid conjugates; anti-diuretic activity; SAR.

INTRODUCTION

Desmopressin (DDAVP) is a synthetic therapeutic peptide widely used in the treatment of diabetes insipidus, primary nocturnal enuresis, hemophilia A and Type I von Willebrand's disease (1). It also improves human memory functions (2). DDAVP is a structural analog of the naturally occurring arginine vasopressin (AVP), in which the terminal amino group is removed and Arg^8 is replaced with synthetic *D*-Arg to improve its chemical and metabolic stability (3,4). However, like other protein and peptide drugs, DDAVP is quickly eliminated from the blood circulation ($t_{1/2}$ = 76 min) and multiple daily injections are usually required to obtain optimal effects in patients (1). A derivative with longer duration of action would minimize the number of injections, reduce the medical costs, and significantly improve patient compliance.

We have previously reported a novel method for the reversible lipidization of DDAVP (5). The disulfide bond in DDAVP was reduced by dithiothreitol (DTT) to generate two free thiol groups, which were subsequently treated with a sulfhydryl-reactive lipidizing agent, *N*-palmitoyl cysteinyl 2-pyridyl disulfide (6). The lipidized product, DPP, behaved as a prodrug with DDAVP being regenerated *in vivo* when DPP was administered subcutaneously in mice and rats. Thus, the *in vivo* anti-diuretic activity of DDAVP was fully preserved in DPP and a 250-fold enhancement of the potency was observed (5).

In this report, we describe the synthesis of a series of fatty acid conjugates of DDAVP with different chain lengths and linkers. The *in vivo* anti-diuretic activities were tested in Brattleboro rats, which express a hereditary hypothalamic diabetes insipidus due to the deficiency in vasopressin genes (7). The cellular uptake of selected conjugates was further studied in cultured human enterocyte-like Caco-2 cells and the *in vitro* metabolism and regeneration of parent DDAVP in a liver slice incubation assay was also investigated. Results from these studies will identify the structural requirements and elucidate the structure-activity relationship of these novel fatty acid-DDAVP conjugates.

MATERIALS AND METHODS

Materials

DDAVP was purchased from Penta Biotech (Foster City, California). Cysteine hydrochloric acid, DTT, dithiopyridine, acetic acid, hexanoic acid, decanoic acid, palmitic acid, stearic acid and Sephadex® G 15 gels were obtained from Sigma (St. Louis, Missouri). All chemicals were used without further purification. Brattleboro (*di/di*) rats were purchased from Harlan Sprague-Dawley, Inc (Indianapolis, Indiana). Animal experiments were compliant with the "Principles of Laboratory Animal Care"(NIH Publication #85-23, revised 1985) and approved by the IACUC at USC.

Cell culture medium and fetal bovine serum (FBS) were products of GIBCO-BRL (Grand Island, New York). Caco-2 cells were purchased from American Type Culture Collection (Rockville, Maryland).

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Preparation of Reversible Fatty Acid-DDAVP Conjugates

Structures of all seven lipidized DDAVP are shown in Table I. DPP was synthesized and purified as previously described (5). For the preparation of hexanoic acid (DPH), decanoic acid (DPD), and stearic acid (DPS) conjugates, similar procedures by replacing palmitic acid with other fatty acids were followed. Briefly, DDAVP was dissolved in PBS, pH 7.4 and treated with DTT. The DTT-reduced DDAVP was mixed with respective lipidizing reagent (*N*-hexanoylcysteinyl pyridyl disulfide, Hex-CPD; *N*-decanoylcyteinyl pyridyl disulfide, Dec-CPD; and *N*-stearoylcysteinyl pyridyl disulfide, Ste-CPD) and, subsequently, the reaction mixture was acidified using 1 N HCl. The precipitate formed was isolated by centrifugation and subjected to Sephadex® G 15 chromatography with dimethylforamide (DMF) as the eluent. Fractions containing the product at the void volume of the column were identified by TLC analysis and pooled. Removal of DMF under vacuum afforded DPH, DPD and DPS, respectively. A similar procedure was used to prepare DPA except that Ace-CPD was used as the lipidizing reagent and the HCl precipitation step was omitted since DPA was soluble in water even at acidic conditions. Instead, the reaction mixture was directly processed through a G15 column for purification.

Some of the conjugates were prepared by using linkers, such as cysteamine and methyl ester of cysteine, that were low in hydrophilicity. In those cases, 3-carboxy-4-nitrophenyl disulfide (Ellman's Reagent) was used to prepare the respective water-soluble lipidizing reagents, i.e., 3-carboxy-4-nitrophenyl *N*-palmitoyl 2-aminoethyl disulfide and 3-carboxy-4-nitrophenyl *N*-palmitoyl methyl cysteinyl disulfide for cysteamine and methyl ester of cysteine, respectively. The conjugation reaction and product purification were similar to that of DPP except that HCl was not used because conjugates with cyteamine and cysteine methyl ester, i.e., DPP-DC and DPP-Me, respectively, spontaneously precipitated from the reaction mixture due to their low aqueous solubility.

Reversed-Phase HPLC Analysis

The purity and hydrophobicity of DDAVP and its fatty acid-conjugates were assessed using a reversed-phase High-Performance Liquid Chromatography (HPLC) system consisting of two Ranin Rabbit™ HP pumps, a Ranin pressure module, a Gilson 811B dynamic mixer, an ISCO V^4 UV detector with the wavelength set at 214 nm, a Gilson 506B interface and a Gilson 712 HPLC controller. An ultrasphere protein C4 column (Vydac, California) was employed with a flow rate of 1 ml/min. The mobile phases were solvent A: 0.1% trifluoroacetic acid in distilled water containing 10% acetonitrile; and solvent B: 0.09% trifluoroacetic acid in acetonitrile containing 2% distilled water and 5% tetrahydrofuran. A linear gradient was programmed starting with 10% of solvent B to 90% of solvent B in a period of 25 min and the column was eluted with 90% of solvent B for an additional 5 min.

Table I. Structural Information, HPLC Retention Time (t_R) and Capacity Factor (k′) of DDAVP and Its Fatty Acid Conjugates. HPLC Analysis Was Performed at Conditions Describe d in Material and Method Section. The k′ Values were Calculated Using the Formula $k' = (t_R - t_0)/t_0$, Where t_R and t_0 Are the Retention Times of the Test Compound and the Solvent Front, Respectively

	$(R_1\text{-CO-NH-CHR}_2\text{ -CH}_2\text{-S-})_2$-DDAVP			
Compound	Fatty acid (R_1)	Linker (R_2)	t_R (min)	k′
DDAVP	—	—	7.4	1.4
DPA	acetic (CH_3)	cys (COOH)	7.6	1.5
DPH	hexanoic (C_5H_{11})	cys (COOH)	12.7	3.1
DPD	decanoic (C_9H_{19})	cys (COOH)	17.8	4.7
DPP	palmitic ($C_{15}H_{31}$)	cys (COOH)	24.9	7.0
DPS	stearic ($C_{17}H_{35}$)	cys (COOH)	27.2	7.8
DPP-DC	palmitic ($C_{15}H_{31}$)	cysteamine (H)	[a]	[a]
DPP-Me	palmitic ($C_{15}H_{31}$)	methyl cys ($COOCH_3$)	[a]	[a]

[a] The compound was too lipophic to be eluted off the HPLC column.

Anti-Diuretic Activity of DDAVP and Its Fatty Acid Conjugates

Brattleboro rats, which carry the hereditary disease of hypothalamic diabetes insipidus (7), were used to compare the effects of DDAVP and its fatty acid conjugates for alleviating the disease symptoms, i.e., polyuria and polydipsia. A group of three Brattleboro rats were kept separately in three metabolic cages. Their body weight, water intake and urine output were measured daily. DDAVP, DPA, DPH, DPD, DPP and DPP-DC were formulated in 10% Liposyn® II (Abbott, Illinois) and injected subcutaneously into each rat at a dose of 0.5 μg/kg. In a separate experiment, DDAVP, DPP and DPP-Me were tested in a group of four Brattleboro rats at 0.05 μg/kg in 30% Intralipid (Baxter, Illinois) to study the effect of esterification of the carboxylic group of the cysteine linker on the activity of DPP. Statistical analyses were conducted by using student T-test (two-tailed and two-sample equal variance).

Liver Slice Metabolism

A previously described *in vitro* metabolic system was used to investigate the metabolism of the conjugates and regeneration of active DDAVP (8). A fresh liver, taken from a normal Sprague-Dawley female rat, was cut into slices approximately 1 mm in width. DDAVP, DPH, DPD and DPP (0.5 mg/ml) were incubated with the liver slices (1 g of wet tissue/ml of incubation medium) at 37°C in a water bath shaker. The medium consisted of Dulbecco's modified Eagle's medium/F-12 with phosphate buffer (pH 7.4) and 5% FBS. An aliquot of 100 μL was taken from the incubation medium at pre-determined time points and added to 250 μL of methanol. The mixtures were vortexed and supernatant fractions were isolated by centrifugation. One hundred microliters from each of the supernatants was analyzed by HPLC.

Caco-2 Cellular Uptake

Radioiodination of DDAVP and its fatty acid conjugates was carried out using the chloramine-T method (9). Briefly, chloramine-T, 0.4 mg, was added to a solution of 1 mg of DDAVP or its conjugates in 0.5 ml of PBS with 0.5 mCi of $Na^{125}I$. The iodination reaction, presumably on the tyrosine residue in DDAVP, was allowed to proceed for 5 min at room temperature and was terminated with the addition of 0.24 mg of sodium metabisulfite. Iodinated DDAVP or its conjugates

was separated from free iodide and reagents by using a Sephadex G-15 gel filtration column eluted with DMF. The specific radioactivity of the iodinated DDAVP or its conjugates was approximately 1×10^6 cpm/μg.

Confluent, 14-day-old Caco-2 cell monolayers in 6-well cluster plates were washed once and then incubated with serum-free Dulbecco medium at 37°C for 5 min. Subsequently, the incubation medium was replaced with media containing ^{125}I-labeled DDAVP or its fatty acid conjugates (3 μg DDAVP/ml), and the monolayers were incubated for an additional 60 min at 37°C. After incubation, monolayers were washed three times with ice-cold PBS, pH 7.4, and then dissolved in 0.1 N NaOH (1 ml/cell monlayer). The cell extracts were transferred to test tubes (12 × 75 mm) and the radioactivity in each test tube was counted using a gamma counter.

RESULTS

Characterization of Fatty Acid-DDAVP Conjugates

All of the conjugates were prepared with a purity >90% as demonstrated by TLC (5) and HPLC analysis. Under the conditions of HPLC as described in the Materials and Methods Section, DDAVP and its fatty acid conjugates with a cysteine linker were eluted from the C-4 column with retention times ranging from 7.4 min to 27.2 min while DPP-DC and DPP-Me were too lipophilic to be eluted off the C4 column (Table I). The HPLC capacity factor (k′) is often used to assess the relative lipophilicity of a series of compounds (10–12). Thus k′ values were calculated for each compound using the formula $k = (t_R - t_0)/t_0$, where t_R and t_0 are the retention times of the test compound and an unretained peak (solvent front), respectively. As anticipated, higher lipophilicity was obtained when a longer fatty acid was conjugated to DDAVP via a cysteine linker. When k′ values are plotted against the number of carbons (N) in the fatty acid, linear regression analysis reveals a perfect correlation (Fig. 1):

$$k' = 0.72 + 0.39N \quad (r = 1.00)$$

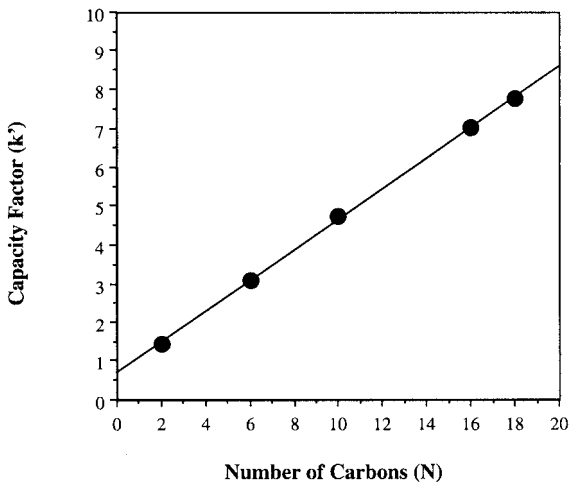

Fig. 1. Relationship between capacity factor (k') of DDAVP conjugates and chain length (number of carbons) of attached fatty acid. Cysteine is the linker in this series.

Anti-Diuretic Activity of Fatty Acid-DDAVP Conjugates

Figure 2 shows a typical response to DDAVP treatment at a dose of 0.5 μg/kg, which would maintain the rats at a symptom-free level for less than 24 h The activities of DPA and DPH did not differ significantly from DDAVP ($p = 0.754$ and 0.377, respectively). On the other hand, enhancement of activity was observed with DPD, where the symptoms were relieved even on the second day. The maximal effect was achieved by DPP. At an identical dose of 0.5 μg/kg, the rats remained symptom-free for more than 3 days. A further increase in the length of the fatty acid chain reduced the enhancement, as DPS is significantly ($p = 0.002$) less potent than DPP on the third day after the administration (Fig. 2).

The importance of the carboxylic group in the linker cysteine is also demonstrated in Figure 2. By deleting the carboxylic group, i.e., replacing cysteine with cysteamine, the conjugate DPP-DC became more lipophilic than DPP as demonstrated by the fact that DPP-DC was not eluted from the HPLC column by the solvent system as described in Materials and Methods. However, the enhanced potency was completely lost in DPP-DC with an activity very similar to that of the unconjugated DDAVP (first day effect, $p = 0.198$).

In a separate experiment, the effect of esterification of the carboxylic group in the cysteine linker of DPP was investigated. Like DPP-DC, the methyl ester derivative of DPP, DPP-Me, was more lipophilic than DPP as demonstrated by the same observation in HPLC retention as DPP-DC. However, the activity of DPP-Me was not as potent as DPP ($p = 0.005$, DPP vs. DPP-Me for the second day effect), even though the anti-diuretic effect of DPP-Me was significantly higher than that of DDAVP ($p = 0.002$, DPP-Me vs. DDAVP for the first and second day effects) (Fig. 3).

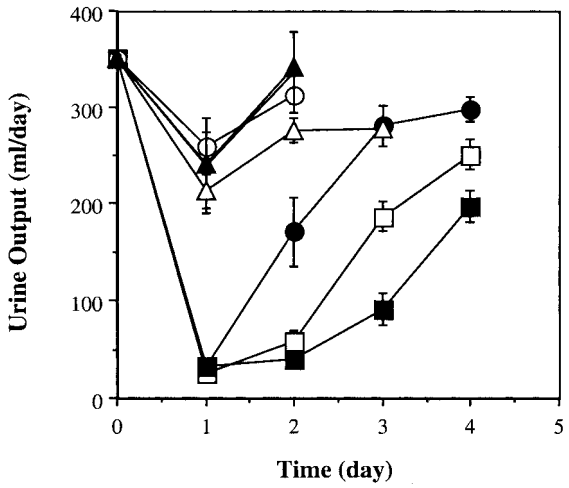

Fig. 2. Effects of DDAVP and its fatty acid conjugates on the urine output of Brattleboro rats. Three Brattleboro rats were subcutaneously injected with 0.5 μg/kg of DDAVP or a conjugate, formulated in 10% Liposyn®. The animals were kept in separate metabolic cages. The daily urine output (DDAVP, ▲; DPA, ✖; DPH, ○; DPD, ●; DPP, ■; DPS, □; DPP-DC, ▲) of each animal was measured and presented as the average ml per day. (Bars indicate standard deviations, n = 3).

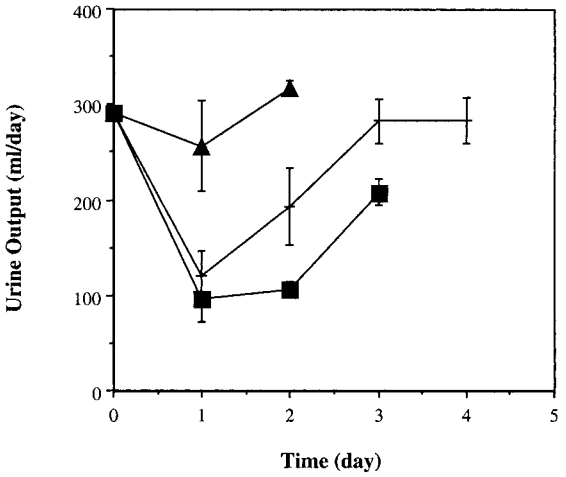

Fig. 3. Effect of esterification of the carboxylic group of the cysteine linker on the anti-diuretic effect of DPP in Brattleboro rats. Four Brattleboro rats were subcutaneously injected with 0.05 μg/kg of DDAVP, DPP or DPP-Me, formulated in 30% Intralipid®. The animals were kept in separate metabolic cages. The daily urine output (DDAVP, ▲; DPP, ■; DPP-Me, +) of each animal was measured and presented as the average ml per day. (Bars indicate standard deviations, n = 4).

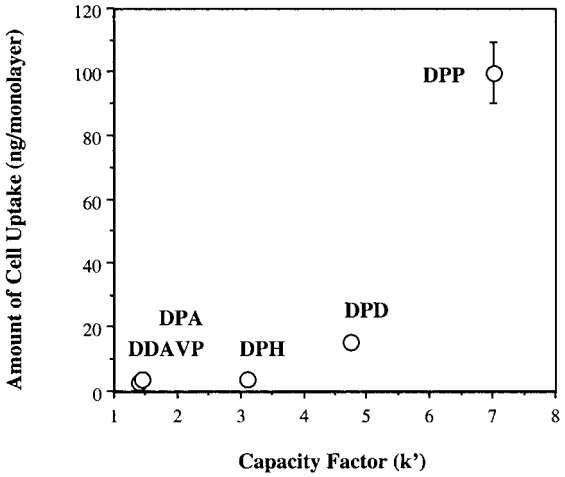

Fig. 4. The relationship between Caco-2 cell uptake of DDAVP, DPA, DPH, DPD and DPP and their lipophilicity. The cell monolayers were incubated with ^{125}I-labed compounds at 3 μg/ml in serum-free Dulbecco medium for 60 min at 37°C. The cell uptake is expressed as the average of three monolayers, with standard deviations indicated by a bar or otherwise smaller than the symbols. The value of capacity factor k' is used as a measure of lipophilicity.

Cellular Uptake of DDAVP and Its Fatty Acid Conjugates in Caco-2 Cells

The measurement of cellular uptake in our experiments included both intracellular and membrane-bound DDAVP. Therefore, the cellular uptake represented the total absorption of DDAVP in Caco-2 cells, which was used as an indication of the extent of *in vivo* tissue retention from subcutaneous injection. Results from the studies of Caco-2 cellular uptake of DDAVP and its fatty acid conjugates revealed that both DPA and DPH had a similar affinity to the cells and, consequently, a similar uptake by the cells as DDAVP. However, the increase of lipophilicity in conjugates with a longer fatty acid chain significantly enhanced the cellular uptake. This finding was consistent with our previous report that the increase of cellular uptake of DPP was a result of the binding of the fatty acid moiety of the conjugate to the cell membrane (6). Thus, the uptake of DPD and DPP in cultured Caco-2 cells was 5- and 33-fold higher, respectively, than that of DDAVP (Fig. 4).

Liver Slice Metabolism

To elucidate the difference in enhancing effect on the diuretic activity of fatty acid-DDAVP conjugates, an *in vitro* liver slice metabolic system was utilized to investigate the metabolism of the conjugates, as well as the regeneration of the active parent drug, DDAVP. DPH, which did not possess enhanced anti-diuretic activity, DPD, which had moderate enhancing ability, and DPP, which most effectively enhanced the anti-diuretic activity of DDAVP were included in this study.

As shown in Fig. 5, the metabolism of DDAVP, DPH, DPD, and DPP by liver slices all followed first order kinetics with half-life of 0.30, 0.01, 0.06 and 3.44 h, respectively. The degradation of DPH and DPD in the incubation media was much faster than that of DDAVP, and therefore, the regeneration of DDAVP could be followed by the HPLC assay. The native peptide DDAVP regenerated from DPH peaked at 3 min and degraded similarly to that of the native DDAVP with a terminal half-lives of 0.31 h In the case of DPD, maximal concentration of DDAVP was observed at 15 min and the terminal half-life was 0.37 h (Fig. 6). In contrast, DPP was degraded much slower than DDAVP and, therefore, no significant amount of regenerated DDAVP could be accumulated and detected by the HPLC assay.

DISCUSSION

Lipidization is a simple and effective approach to increase the lipophilicity of peptide drugs with goals of increasing stability against proteases, enhancing transport across bio-

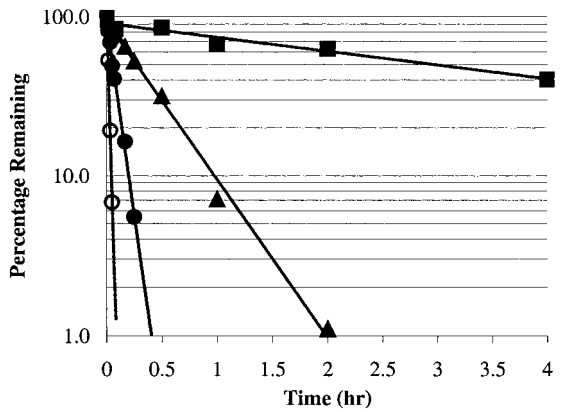

Fig. 5. Liver metabolism of DDAVP and its fatty acid conjugates. DDAVP (▲), DPH (○), DPD (●) and DPP (■) (0.5 mg/ml) were incubated with fresh rat liver slices suspended in Dulbecco's modified Eagle's medium, a phosphate buffer and 5% FBS. Samples at predetermined time points were collected, processed and analyzed by a HPLC assay. Results are presented as the percentage of compound remaining in the incubation mixture vs. time. Curve fitting is accomplished according to first order kinetics.

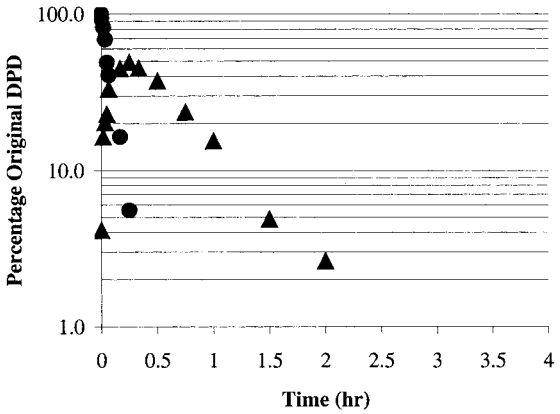

Fig. 6. Disappearance of DPD and concurrent regeneration of DDAVP in liver slice incubation. The disappearance of DPD (●) and the regeneration of DDAVP (▲) in the presence of fresh rat liver slices were measured by a HPLC assay as described in Figure 5.

logic membranes and prolonging plasma half-life (13–19). In most of the previous reports, the linkage between the peptide and the lipid moiety is a stable amide bond, which limits the *in vivo* regeneration of the parent peptide drug. Therefore, lipidized peptides are generally less potent when compared with the native peptide (19,20), unless the modification is on an amino group that is not essential for the activity (21–24). Recently, we have developed a novel reversible lipidization method for peptide drugs based upon the formation and the *in vivo* reduction of a disulfide linkage (6). By using this method, the anti-diuretic activity of DDAVP was significantly prolonged upon the lipidization with a palmitic acid moiety (5). In this study, we further demonstrate that the enhancement of the anti-diuretic activity is directly correlated to the specific structure of the lipid moiety and the linkage in the conjugates.

One obvious structural variant is the length of the fatty acid chain, which reflects the lipophilicity of the conjugate. In a series of conjugates where cysteine is used as the linker, the enhancing effect begins to appear at C10 (decanoic acid) and peaks at C16 (palmitic acid). Further increasing the carbon number to C18 (stearic acid) fails to increase the potency. In fact, DPS is less effective than DPP. To be biologically active, a lipidized peptide conjugate should be stable at the injection site. Subsequently, the lipidized peptide should be transported into the general circulation, and converted to the active parent peptide in tissues such as the liver (25). Lipophilicity can play important roles in these processes. First, lipophilicity can affect the stability of the conjugates. The increase in stability of a lipidized peptide is most likely due to the increase in binding to serum and/or tissue proteins (22). As shown in Fig. 5, the half-life of DPP in the liver slice incubation was 10-fold longer than that of DDAVP. On the other hand, DPH was less stable than DDAVP, probably due to a combination of the weak binding to serum proteins and the opening of the disulfide ring in the structure. Consequently, no enhancement of the biologic activity was observed in DPH. Secondly, lipophilicity can also affect the release and transport of lipidized DDAVP from the subcutaneous injection site to the blood. Subcutaneously administered macromolecules are effectively transported to the blood via the lymphatic system (26). However, the lymphatic transport system depends not only on the size but also on the lipophilicity of the molecule (27). It is likely that the weak lipophilicity of DPH renders it less accessible for the lymphatic delivery but more accessible for tissue degradation. DPD is also less stable than DDAVP. However, the increased lipophilicity in DPD enhances its binding to local tissues, as indicated by the high uptake by Caco-2 cells, which may sustain the release of DDAVP to the general circulation. The overall effects of lipophilicity on DPD result in a moderated enhancement in the anti-diuretic activity. On the other hand, conjugates with highly lipophilic moieties such as DPS may prolong its tissue retention and/or reduce the regeneration of DDAVP; both cases can lead to a lower potency in the anti-diuretic efficacy.

Another important structural component in the conjugate is the linker connecting the lipid moiety to the peptide drug. Among the three different linkers described in this paper, i.e., cysteine, methyl ester of cysteine, and cysteamine, cysteine is the most effective linker for enhancing the anti-diuretic activity. The superiority of the cysteine linker is possibly due to the reducibility of the disulfide bond, which will determine the rate of *in viv*o DDAVP regeneration. It has been demonstrated that the *in vivo* reducibility of disulfide linkage of immunotoxins in the blood decreases as the hydrophobicity of the linker increases (28). Cys-Cys linkage, a naturally occurring connection in proteins and peptides, is conceivably more reactive towards the enzymatic or non-enzymatic reduction than Cys-Cysteamine or Cys-Cys methyl ester linkage. On the other hand, DPP-Me can be hydrolyzed to DPP by the action of serum esterase(s). The *in vivo* conversion of DPP-Me to DPP may explain the observed enhancing effect of DPP-Me on the anti-diuretic activity.

In conclusion, conjugation of DDAVP with fatty acids increases its lipophilicity and potentiates its anti-diuretic activity. The anti-diuretic activity of lipidized DDAVP was correlated with the chain length of the fatty acid, as well as the structure of the linker. The *in vivo* preservation and enhancement of the anti-diuretic activity in fatty acid-DDAVP conjugates is most likely due to a combination of the pharmacokinetic behavior of the conjugates and the regeneration of DDAVP from the conjugates.

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