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Modification of Cell Response to Insulin by Membrane-Acting Agents in Rat White Adipocytes: Analysis of Structural Features by Computational Simulation

Kazuto Ohkura[a,b,*] and Hitoshi Hori[a]

[a]*Department of Biological Science and Technology, Faculty of Engineering, University of Tokushima, 2-1 Minamijosanjima-cho, Tokushima 770-8506, Japan*

[b]*Bioagricultural Science, Nagoya University, Furo-cho, Chikusa-ku, Aichi 464-8601, Japan*

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Abstract—The effect of membrane-acting agents, biscoclaurine alkaloids (cepharanthine, tetrandrine, isotetrandrine), carbobenzoxy-D-Phe-L-Phe-Gly (z-FFG), and tyrphostin AG17, on the insulin-involved fatty acid synthesis by an β-agonist (e.g., isoproterenol) in adipocytes was examined. The alkaloids dose-dependently enhanced the insulin-involved fatty acid synthesis in rat white adipocytes, stabilized the C_6-NBD-PC (1-acyl-2-[6-[(7-nitro-2,1,3-benzoxadiazol-4-yl)amino]-caproyl]-sn-glycero-3-phosphatidylcholine) model membrane, and suppressed the phospholipase A_2-induced phospholipid degradation. In contrast, z-FFG had no effect on the fatty acid synthesis or the membrane stability. Tyrphostin AG17 suppressed insulin action, but promoted the model membrane stabilization. In the same culture conditions as for the fatty acid synthesis assay, cepharanthine, z-FFG and tyrphostin AG17 had no effect on the transcript levels of glucose transporter isoforms (GLUT 1, 4) and hexokinase isozymes (HK I, II) in rat white adipocytes. Thus, these membrane-acting agents modify the insulin action via a change in the cell membrane condition, and do not directly act on the insulin-involved glucose metabolism. Then we analyzed the structural conformation of these membrane-acting agents by computational simulations. The alkaloids had an elliptic macrocyclic structure, and the order of ellipticity (cepharanthine > tetrandrine > isotetrandrine) agreed with that of the modifying ability for insulin action. The distribution of electrostatic potential fields of these alkaloids was essentially equal by turn in surrounding with the dipole moments. Both in z-FFG and tyrphostin AG17, the distribution pattern of electrostatic potential fields was different from that of the alkaloids. Judging from these results, we concluded that the electrostatic potential field is a good index of the modification of insulin action, and the elliptic structure in these alkaloids is regarded with the modification of insulin action.

Introduction

Non-insulin-dependent diabetes mellitus (NIDDM) is a common metabolic disease. It is generally accepted that insulin resistance plays an important role in the pathogenesis of NIDDM.[1] Insulin resistance is a term used when a given concentration of insulin does not elicit a normal biological response. NIDDM is the disease classically associated with insulin resistance, but insulin resistance also occurs in other common conditions, including obesity, aging, polycystic ovarian syndrome, hypertension, hyperinsulinemia, dyslipidemia and cardiovascular disease.[2–4] Thus insulin resistance is a complex, multifactorial result of a variety of genetic, cellular and environmental causes.[2–4] In general, cellular metabolic changes induced by hormones have been evidenced to accompany with modifications of cell surface membranes.[5–9] In insulin resistance, the alterations in the molecular organization of the cell surface membranes are expected closely related to the metabolic regulation of cells.

Some membrane-acting agents affect membrane stability in vitro as follows. (1) Cepharanthine, a biscoclaurine-type alkaloid from *Stephania cepharantha*,[10] is known as an agent that stabilizes cell membranes by affecting the fluidity of the lipid bilayer and that causes metabolic changes in several types of cells.[11–13] For instance, it exerts a potent inhibitory action on snake venom-induced hemolysis.[14,15] The alkaloid also affects the activity of anthracycline type anti-cancer agents in leukemia and lymphoma cell lines,[16–20] and enhances

*Corresponding author. Tel.: +81-88-656-7525; fax: +81-88-656-7525; e-mail: kohkura@sag.bekkoame.ne.jp

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the proliferation of skin epidermal cells.[21] (2) Carbobenzoxy-D-Phe-L-Phe-Gly (z-FFG) stabilizes lipid bilayers by raising the bilayer- to hexagonal-phase transition temperature of synthetic phosphatidylethanolamines,[22] and inhibits insulin-activated glucose uptake in adipocytes.[23] (3) Tyrphostin AG17 is a powerful uncoupler with mitochondrial oxidative phosphorylation.[24] Its concentration required for uncoupling is as low as 10 nM, and it acts as a protonophor in the mitochondrial inner membrane by the shuttle mechanism.[24] In the present study, we examined the effect of these membrane-acting agents: biscoclaurine alkaloids (cepharanthine, tetrandrine and isotetrandrine: Fig. 1), z-FFG and tyrphostin AG17, on β-agonist (e.g., isoproterenol) induced-fatty acid synthesis, which is suppressed by insulin, in white adipocytes.[25]

Moreover, we examined the effect of biscoclaurine alkaloids, z-FFG and tyrphostin AG17 on membrane stability using 1-acyl-2-[6-[(7-nitro-2,1,3-benzoxadiazol-4-yl)amino]-caproyl]-sn-glycero-3-phosphatidylcholine (C_6-NBD-PC) model membranes, and discussed the relationship between the membrane condition and the cell responses to insulin (e.g., insulin-involved fatty acid synthesis). We also examined the effect of these agents on phospholipase A_2 (PLA_2)-induced C_6-NBD-PC micelle degradation. The amino-terminal hexapeptide (hGH_{8-13}: positioned at the 8th Arg to 13th Ala: H_2N-Arg-Leu-Phe-Asp-Asn-Ala-COOH) of human growth hormone is known as an insulin-potentiating fragment, and increased the binding of insulin to specific receptors and thus modulated the action of glycogen synthase and phosphorylase, producing hypoglycemia as the result of increased glycogen storage in adipose tissue.[26,27] We had reported on the effect of this hexapeptide on the modification of insulin-involved fatty acid synthesis in rat white adipocytes, and analyzed the three-dimensional structure to determine the essential unit.[28] This essential unit had an elliptic shaped macrocyclic structure, whose major and minor axes were almost equal in size to those of the macrocyclic region in biscoclaurine alkaloids (cepharanthine, tetrandrine, isotetrandrine). The peptide of hGH_{9-13}, in which the 8th Arg residue was removed from hGH_{8-13}, had no effect on insulin-involved fatty acid synthesis, and did not form a macrocyclic structure.[28] Then we examined the effect of hGH_{8-13} and hGH_{9-13} on the stability of C_6-NBD-PC micelles.

Glucose metabolism, the uptake and phosphorylation of glucose, is the major cellular response to insulin. In mammalian cells, four isoforms of glucose transporter (GLUT 1–GLUT 4) are known to facilitate the uptake of glucose through the plasma membranes.[29–33] In

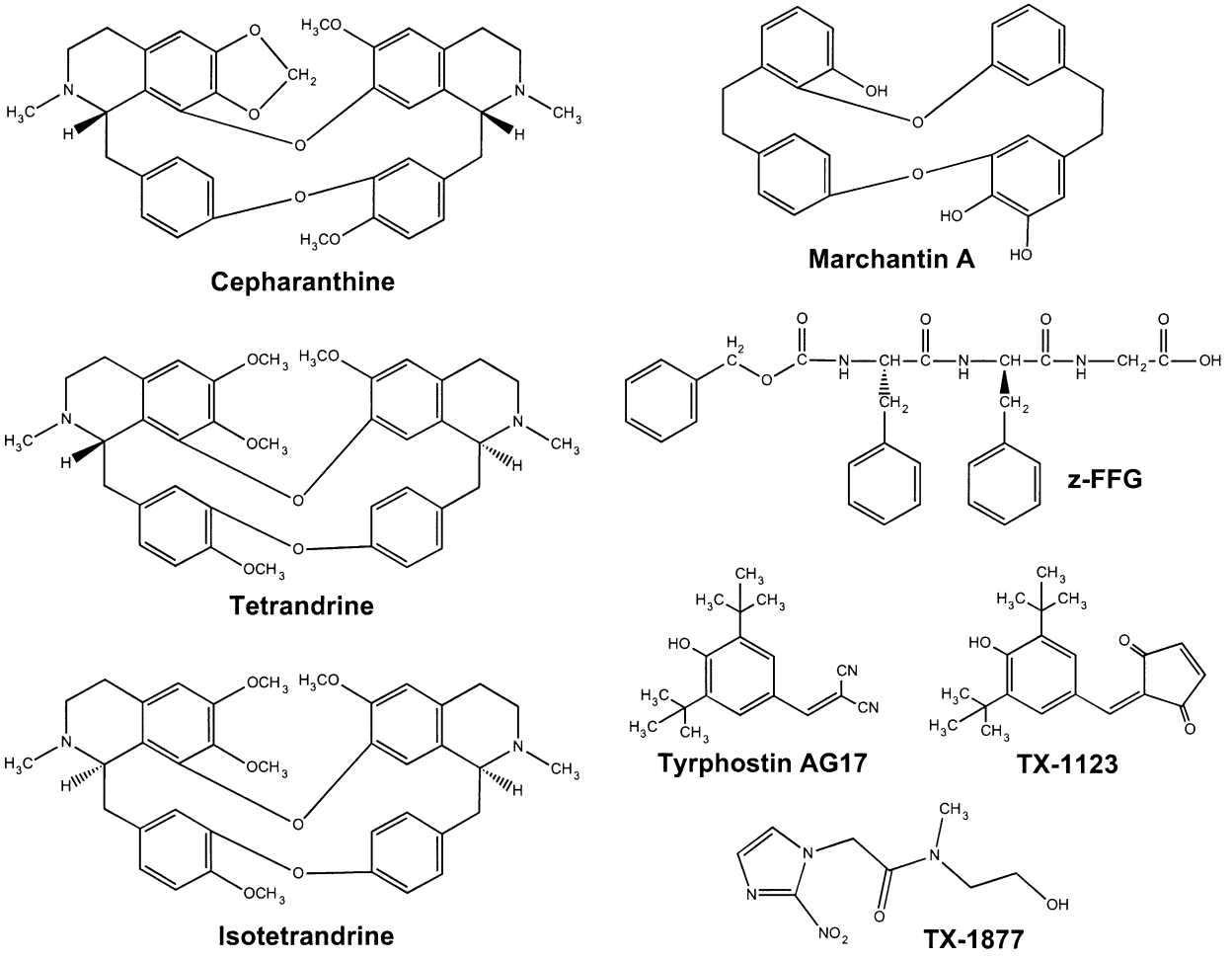

Figure 1. Structure of cepharanthine, tetrandrine, isotetrandrine, marchantin A, z-FFG, tyrphostin AG17, TX-1123 and TX-1877.

addition, the first step of the sequential reactions in glucose metabolism is catalyzed by four isozymes of hexokinase (HK I–HK IV).[34,35] Hence GLUT and HK are important factors in the insulin-involved glucose metabolism. Therefore, we analyzed the effect of cepharanthine, z-FFG and tyrphostin AG17 on the transcript levels of GLUT isoforms and HK isozymes in rat white adipocytes.

Results

Modification of insulin-involved fatty acid synthesis by membrane-acting agents in rat white adipocytes

β-Agonistic drugs (e.g., epinephrine and isoproterenol) stimulate triglyceride lipolysis in white adipose cells, and various β-agonistic drugs were designed and synthesized.[36–41] Insulin effectively counteracts this β-agonist-stimulated activity.[25] In rat white adipocytes, the stimulatory effect of isoproterenol on lipolysis was antagonized by insulin (open circles in Fig. 2A and B). The addition of cepharanthine (1.5 μM, closed circles in Fig. 2A), tetrandrine (1.5 μM, open triangles) or isotetrandrine (1.5 μM, open squares), shifted the insulin–response curve of isoproterenol-induced lipolysis to a lower insulin concentration and enhanced the maximum response elicited by the hormone. When 15 μM of these alkaloids was added, insulin action was markedly enhanced and fatty acid synthesis decreased (Fig. 2B). From the Lineweaver–Burk type plots of Fig. 2 [1/(reduction in synthesized fatty acid) versus 1/(insulin concentration)], we determined the dissociation constant (K_m) of insulin—its receptor complex. Effects of biscoclaurine alkaloids on the K_m value are summarized in Table 1. In the presence of these alkaloids, the affinity of insulin to white adipocytes increased. The order of enhancement was cepharanthine > tetrandrine > isotetrandrine. The affinity with cepharanthine (15 μM) was 23.7-fold greater than the control as well as insulin-potentiating fragment hGH_{8-13} (30 μM).[28] In contrast, z-FFG (1.5 μM, closed triangles in Fig. 2A) suppressed the insulin action, and fatty acid synthesis increased. At 15 μM, z-FFG significantly suppressed insulin action, and the amount of synthesized fatty acid was almost unchanged until 1.97×10^{-10} M of insulin existed (Fig. 2B), and the affinity of insulin to adipose cells decreased 0.2-fold (Table 1).

In the absence of insulin, the alkaloids (cepharanthine, tetrandrine, isotetrandrine) and carbobenzoxypeptide (z-FFG) did not affect β-agonist-induced fatty-acid synthesis in rat white adipocytes (open circles in Fig. 3A–D). When 1.22×10^{-11} M of insulin coexisted, these alkaloids promoted the insulin action and suppressed the fatty acid synthesis dose-dependently (closed circles in Fig. 3A–C). In contrast, z-FFG dose-dependently repressed the insulin effect and increased the fatty acid synthesis (closed circles, Fig. 3D).

As shown in Fig. 4A, tyrphostin AG17 (1.5 μM; open triangles, 30 μM; closed circles) suppressed insulin action and increased fatty acid synthesis similar to z-FFG (closed triangles in Fig. 2). Tyrphostin AG17 did not affect β-agonist-stimulated fatty acid synthesis in adipocytes without insulin (open circles in Fig. 4B). However, in the presence of insulin (1.22×10^{-11} M), tyrphostin AG17 dose-dependently increased fatty acid synthesis (closed circles in Fig. 4B) similar to z-FFG (Fig. 3D). Thus tyrphostin AG17 promoted the insulin action, but did not enhance β-agonist-stimulated fatty acid synthesis per se.

Effect of the alkaloids, z-FFG and tyrphostin AG17 on the stability of the model membrane

When C_6-NBD-PC (1-acyl-2-[6-[(7-nitro-2,1,3-benzoxadiazol-4-yl)amino]-caproyl]-sn-glycero-3-phosphatidylcholine) micelles were incubated at 37 °C in 0.1 M Tris–HCl buffer (pH 7.4), NBD molecules were spontaneously released by the hydrolysis of phosphatidylcholine and the fluorescent intensity increased at a rate of 0.515 arbitrary units per min [column 1 (control) in Fig. 5]. In the presence of 10 μM cepharanthine, tetrandrine, isotetrandrine, the C_6-NBD-PC model membrane was stabilized and the rate of hydrolysis (fluorescent increase) fell to 0.212, 0.289 and 0.360 arbitrary units per min (columns 2, 3, 4), respectively. Ten micromolar

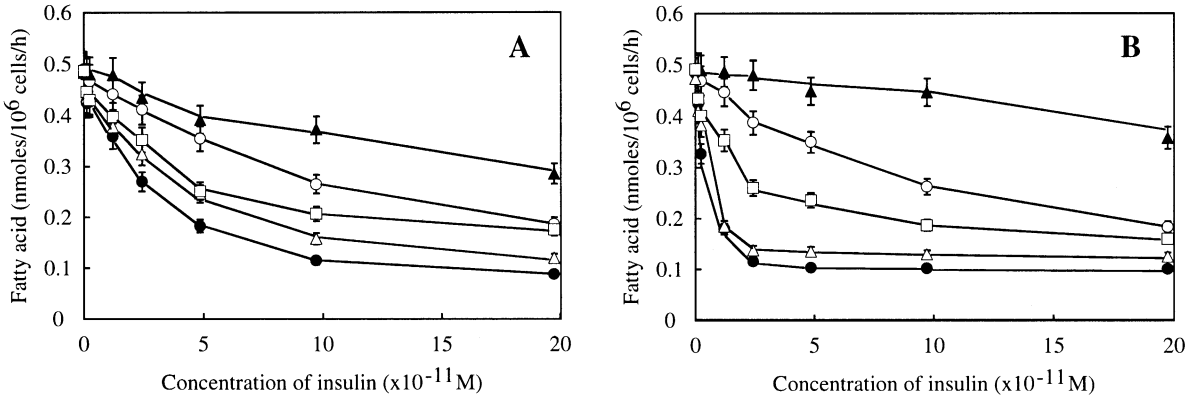

Figure 2. Modification of insulin-involved fatty acid synthesis by biscoclaurine alkaloids and z-FFG in rat white adipocytes. (A) Adipocytes were incubated at 37 °C for 2 h without (open circles) or with 1.5 μM cepharanthine (closed circles), 1.5 μM tetrandrine (open triangles), 1.5 μM isotetrandrine (open squares), or 1.5 μM z-FFG (closed triangles) in the presence of 2 nM isoproterenol and various concentrations of insulin. (B) Adipocytes were incubated at 37 °C for 2 h with 15 μM of each agent as in Figure 2A. The results are the mean (±SE) of three determinations.

z-FFG did not stabilize the model membrane, and the rate of hydrolysis was almost unchanged from the control value (0.525 arbitrary units per min; column 5). Tyrphostin AG17 (10 μM) stabilized the model membrane, and the hydrolysis rate was 0.233 arbitrary units per min (column 6).

In the presence of 10 μM $hGH_{8–13}$, the C_6-NBD-PC model membrane was stabilized and the rate of hydrolysis fell to 0.272 arbitrary units per min (column 7) as with cepharanthine. Ten micromolar $hGH_{9–13}$ did not stabilize the membrane, and the rate of hydrolysis was almost unchanged relative to the control (0.463 arbitrary units per min; column 8).

Table 1. K_m values of rat white adipocytes for insulin

Agent		K_m (M)	Ratio to control
Control		2.684×10^{-11}	1.000
Cepharanthine	(1.5 μM)	4.264×10^{-12}	6.295
	(15 μM)	1.130×10^{-12}	23.752
Teterandrine	(1.5 μM)	4.658×10^{-12}	5.762
	(15 μM)	1.934×10^{-12}	13.878
Isotetrandrine	(1.5 μM)	7.106×10^{-12}	3.777
	(15 μM)	5.152×10^{-12}	5.210
z-FFG	(1.5 μM)	8.454×10^{-11}	0.317
	(15 μM)	1.266×10^{-10}	0.212
Tyrphostin AG17	(1.5 μM)	3.487×10^{-11}	0.770
	(30 μM)	4.436×10^{-11}	0.605
$hGH_{8–13}$[a]	(30 μM)	1.099×10^{-12}	24.422
$hGH_{9–13}$[a]	(30 μM)	2.946×10^{-11}	0.911

In the presence of biscoclaurine alkaloids (cepharanthine, tetrandrine, isotetrandrine), z-FFG, or tyrphostin AG17, the K_m values were determined by Lineweaver–Burk plots from the values of Figure 2.
[a]These values were cited from ref 28.

Effect of membrane-acting agents on the phospholipase A_2-induced phospholipid degradation

Phospholipase A_2 (PLA_2) is the enzyme which degrades phospholipids. NBD-labeled phosphatidylcholine (C_6-NBD-PC) was suspended at 1.29×10^{-5} M in 0.1 M Tris–HCl buffer (pH 7.4) containing 20 mM $CaCl_2$ and formed micelles. PLA_2 degraded the C_6-NBD-PC and NBD molecules released from the micelles, then the fluorescent intensity increased (open circles in Fig. 6A). The alkaloids (10 μM) stabilized the model membrane and suppressed the PLA_2-induced NBD release (Fig. 6A), and the order of suppression was cepharanthine (closed circles) > tetrandrine (open triangles) > isotetrandrine (closed triangles). The PLA_2-induced NBD release was almost unchanged by z-FFG (10 μM) addition (closed squares). Tyrphostin AG17 suppressed the insulin action and increased the β-agonist-induced fatty acid synthesis similar to z-FFG (Fig. 4A), but it stabilized the C_6-NBD-PC model membrane and suppressed the PLA_2-induced phospholipid degradation (open squares in Fig. 6A).

The dose–response curves of these agents for PLA_2 (2.97×10^{-6} M)-induced NBD release are shown in

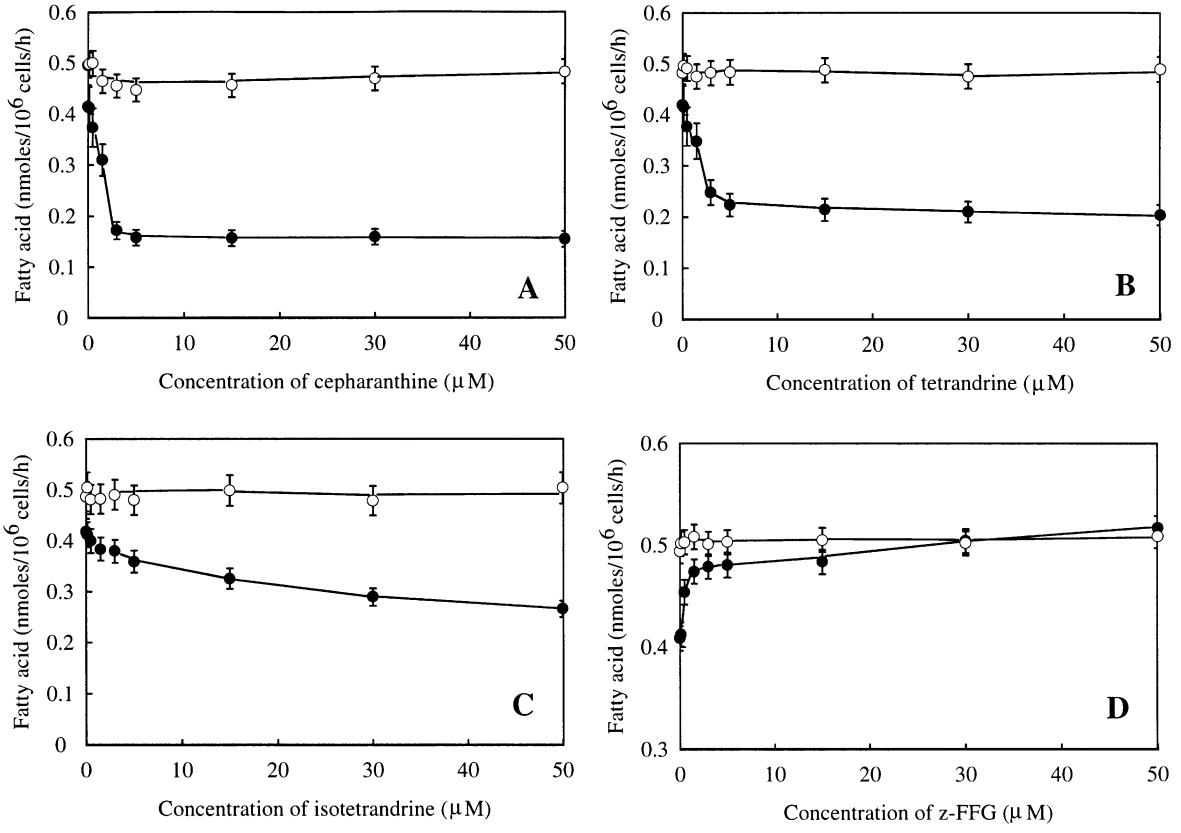

Figure 3. Dose-dependent effect of biscoclaurine alkaloids and z-FFG on insulin-involved fatty acid synthesis in rat white adipocytes. Adipocytes were incubated at 37 °C for 2 h without insulin (open circles) or with 1.22×10^{-11} M insulin (closed circles) in the presence of 2 nM isoproterenol and various amounts of cepharanthine (A), tetrandrine (B), isotetrandrine (C), and z-FFG (D). The results are the mean (±SE) of three determinations.

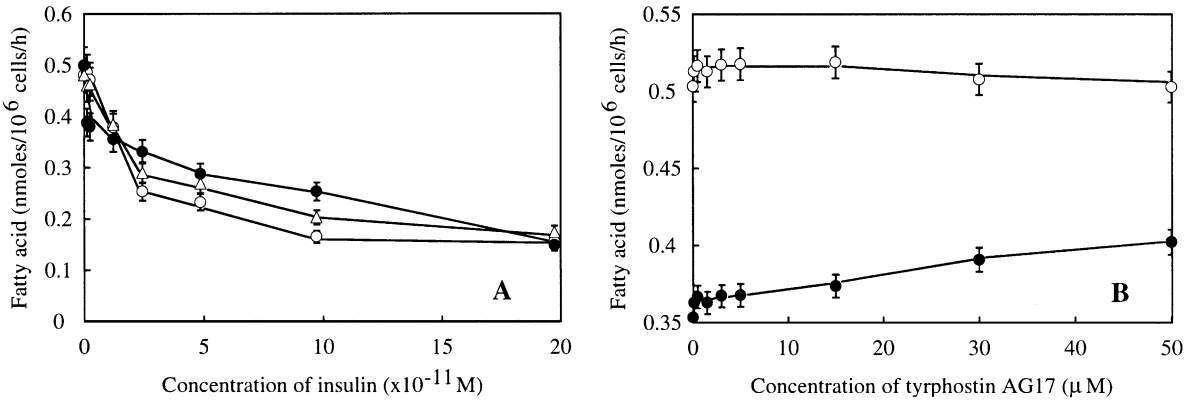

Figure 4. Suppressive effect of tyrphostin AG17 on insulin-involved fatty acid synthesis in rat white adipocytes. (A) Adipocytes were incubated at 37 °C for 2 h without (open circles) or with 1.5 μM (open triangles) and 30 μM (closed circles) tyrphostin AG17 in the presence of 2 nM isoproterenol and various concentrations of insulin. (B) Adipocytes were incubated at 37 °C for 2 h without insulin (open circles) or with 1.22×10^{-11} M insulin (closed circles) in the presence of 2 nM isoproterenol and various amounts of tyrphostin AG17. The results are the mean (±SE) of three determinations.

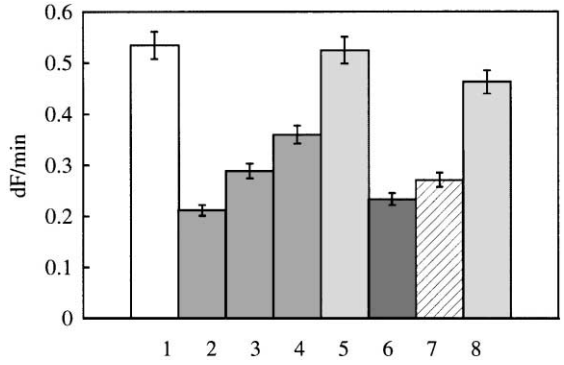

Figure 5. Influence of membrane-acting agents on the stability of the model membrane. The NBD-labeled phosphatidylcholine (C_6-NBD-PC) was suspended at 1.29×10^{-5} M in 0.1 M Tris–HCl buffer (pH 7.4) to form micelles, and then spontaneous NBD release was measured at 37 °C without (lane 1) or with 10 μM of cepharanthine (lane 2), tetrandrine (lane 3), isotetrandrine (lane 4), z-FFG (lane 5), tyrphostin AG17 (lane 6), hGH_{8-13} (lane 7), or hGH_{9-13} (lane 8) in a Hitachi Fluorescent Spectrophotometer Model F-4500 (excitation wavelength, 470 nm; emission wavelength, 540 nm). The results are the mean (±SE) of three determinations.

Fig. 6B. The alkaloids (cepharanthine: closed circles, tetrandrine: open triangles, isotetrandrine: closed triangles) dose-dependently inhibited the PLA_2-induced degradation of phospholipids and the NBD release decreased. The carbobenzoxy peptide z-FFG did not restrain the NBD release (closed squares), and the rate of fluorescence increase was unchanged up to 100 μM of z-FFG. Tyrphostin AG17 suppressed the liberation of NBD to the same degree as isotetrandrine (open squares).

Transcript levels of HK isozymes and GLUT isoforms in rat white adipocytes

Next, we analyzed the transcript levels of four HK isozymes of rat white adipocytes in various culture conditions, which reflect the experimental conditions for fatty acid synthesis. For this, samples of RNA (10 μg) obtained from adipocytes, which were incubated as follows; without (condition 1) or with insulin (1.22×10^{-11} M: condition 2), isoproterenol (2 nM: condition 3), insulin and isoproterenol (condition 4), insulin, isoproterenol and 20 μM agent (cepharanthine, z-FFG or tyrphostin AG17) (condition 5), agent (condition 6), were subjected to electrophoresis and transferred on to nitrocellulose membranes. As shown in Fig. 7A, the transcription of HK I was observed under conditions 1–6, and each intensity was the same. The signal of HK II (B) was detected at the same level in conditions 1–6. Then, these agents (cepharanthine, z-FFG, tyrphostin AG17) did not affect the transcript levels of HK I and HK II. Neither HK III nor HK IV gave a signal in all culture conditions (data not shown).

We examined the level of the transcripts encoding the four GLUT isoforms in RNA samples obtained under various culture conditions in the same way as for HK isozymes. Northern blot analysis was performed with 10 μg of RNA. The intensity of GLUT 1 isoform bands was the same in culture conditions 1–6 with cepharanthine (Fig. 7C). Either with z-FFG or tyrphostin AG17, transcript level of GLUT 1 isoforms were unchanged by these agents under all culture conditions (Fig. 7C). The GLUT 4 isoform transcript was detected at the same level in all culture conditions (Fig. 7D). No bands of GLUT 2 and GLUT 3 were detected in the culture series, as with HK III and HK IV (data not shown). So cepharanthine, z-FFG, and tyrphostin AG17 had no effect on the transcription of HK I, HK II, GLUT 1, and GLUT4. Figure 7E shows control experiments indicating RNA integrity.

Computational analysis of membrane-acting agents

Figure 8A shows the calculated three-dimensional structures of the membrane-acting agents (a–e) used in this study, as determined by global minimum search using CONFLEX (in vacuum condition, because cell membrane have hydrophobic property). The direction of view was arranged according to the dipole moments (e.g., cepharanthine: 3.678 debye, gray arrow), which were calculated using MOPAC97. Cepharanthine (a) had an elliptic structure (broken arrow), with major and minor axes of 7.816 and 4.175 angstrom, respectively, therefore its ellipticity (major/minor axis) was 1.872. Tetrandrine (b) and isotetrandrine (c) also had an elliptic

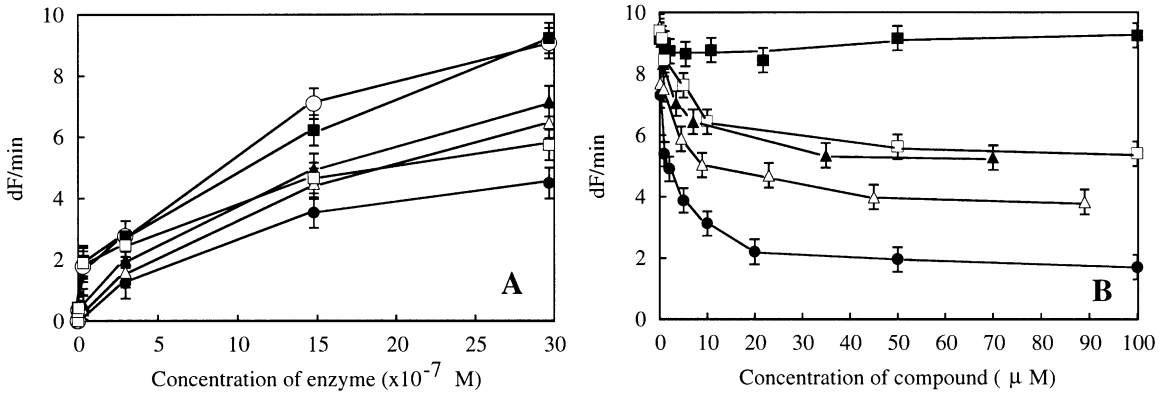

Figure 6. Effect of the alkaloids, z-FFG, and tyrphostin AG17 on phospholipase A_2-induced phospholipid degradation. C_6-NBD-PC was suspended at 1.29×10^{-5} M in 2 mL of 0.1 M Tris–HCl buffer (pH 7.4) containing 20 mM $CaCl_2$ and formed micelles. (A) Indicated amounts of PLA_2 were added to a reaction mixture containing C_6-NBD-PC micelles without (open circles) or with 10 μM of cepharanthine (closed circles), tetrandrine (open triangles), isotetrandrine (closed triangles), z-FFG (closed squares), or tyrphostin AG17 (open squares), and fluorescent intensity was monitored (wavelength of excitation: 470 nm, emission: 540 nm) at 37 °C using a Hitachi Fluorescent Spectrophotometer Model F-4500. (B) In the presence of indicated amounts of membrane-acting agents, 2.97×10^{-6} M of PLA_2 was added to the reaction mixture, and fluorescent intensity was monitored as in Figure 6A. The symbols denote as given in (A). The results are the mean (±SE) of three determinations.

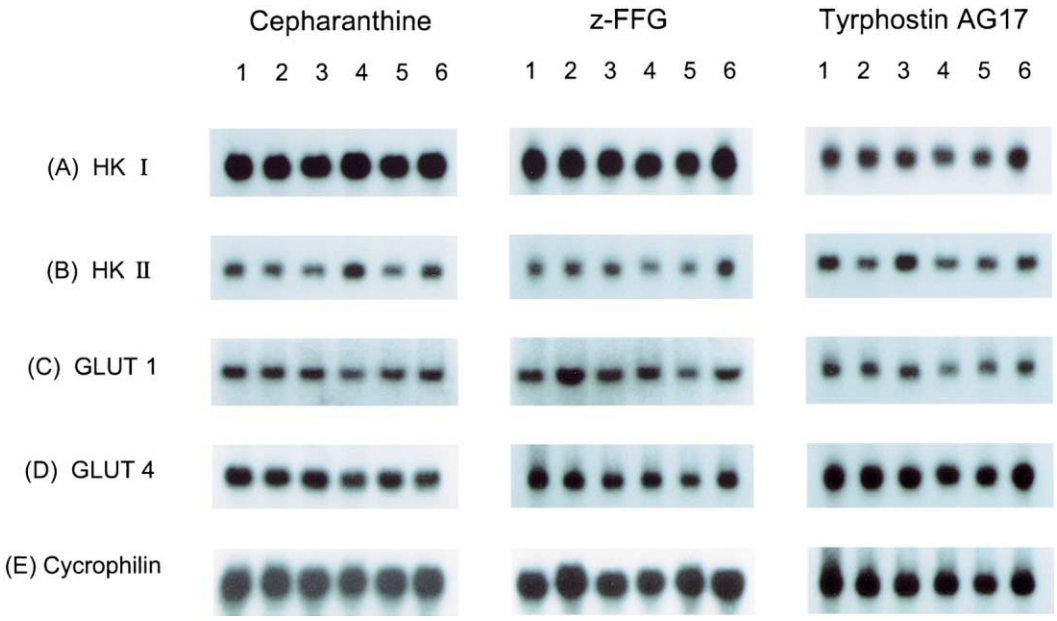

Figure 7. Effect of cepharanthine, z-FFG, and tyrphostin AG17 on transcript levels of HK isozymes and GLUT isoforms in white adipocytes. Samples of 10 μg of RNA obtained from rat white adipocytes, which were incubated as follows; without (condition 1) or with 1.22×10^{-11} M insulin (condition 2), 2 nM isoproterenol (condition 3), 1.22×10^{-11} M insulin and 2 nM isoproterenol (condition 4), 1.22×10^{-11} M insulin and 2 nM isoproterenol, and 20 μM of cepharanthine or z-FFG or tyrphostin AG17 (condition 5), and 20 μM of cepharanthine or z-FFG or tyrphostin AG17 (condition 6) at 37 °C for 2 h, were subjected to agarose gel electrophoresis, transferred on to nitrocellulose membranes and hybridized with the probes for HK I (A), HK II (B), GLUT 1 (C), GLUT 4 (D) and cycrophilin (E).

structure, and an ellipticity of 1.846 and 1.264, respectively. Thus, the order of ellipticity was cepharanthine > tetrandrine > isotetrandrine, and the accelerative efficiency in insulin-involved fatty acid synthesis agreed with this ranking (Fig. 2). The value of the dipole moments of z-FFG (d) and tyrphostin AG17 (e) was almost equal to that of tetrandrine, at 4.675 and 4.637 debye, respectively.

Along the dipole moment (broken arrow), negative (blue mesh) and positive (red mesh) electrostatic potential fields were reciprocally distributed in the cepharanthine molecule, with negative fields located at both ends (Fig. 8B). In tetrandrine and isotetrandrine, negative (blue mesh) and positive (red mesh) electrostatic potential fields reciprocally distributed along by the dipole moments, and both ends of these molecules were negative as in cepharanthine. Thus these alkaloids, which enhanced the insulin effect on β-agonist-induced fatty acid synthesis, had a similar electrostatic potential environment in the axis of the dipole moment. The distribution of the potential field in z-FFG was markedly different from that in cepharanthine, and a negative (blue mesh) or a positive (red mesh) field was located at each end of the axis of dipole moment (broken arrow). In tyrphostin AG17, a positive potential field (red mesh)

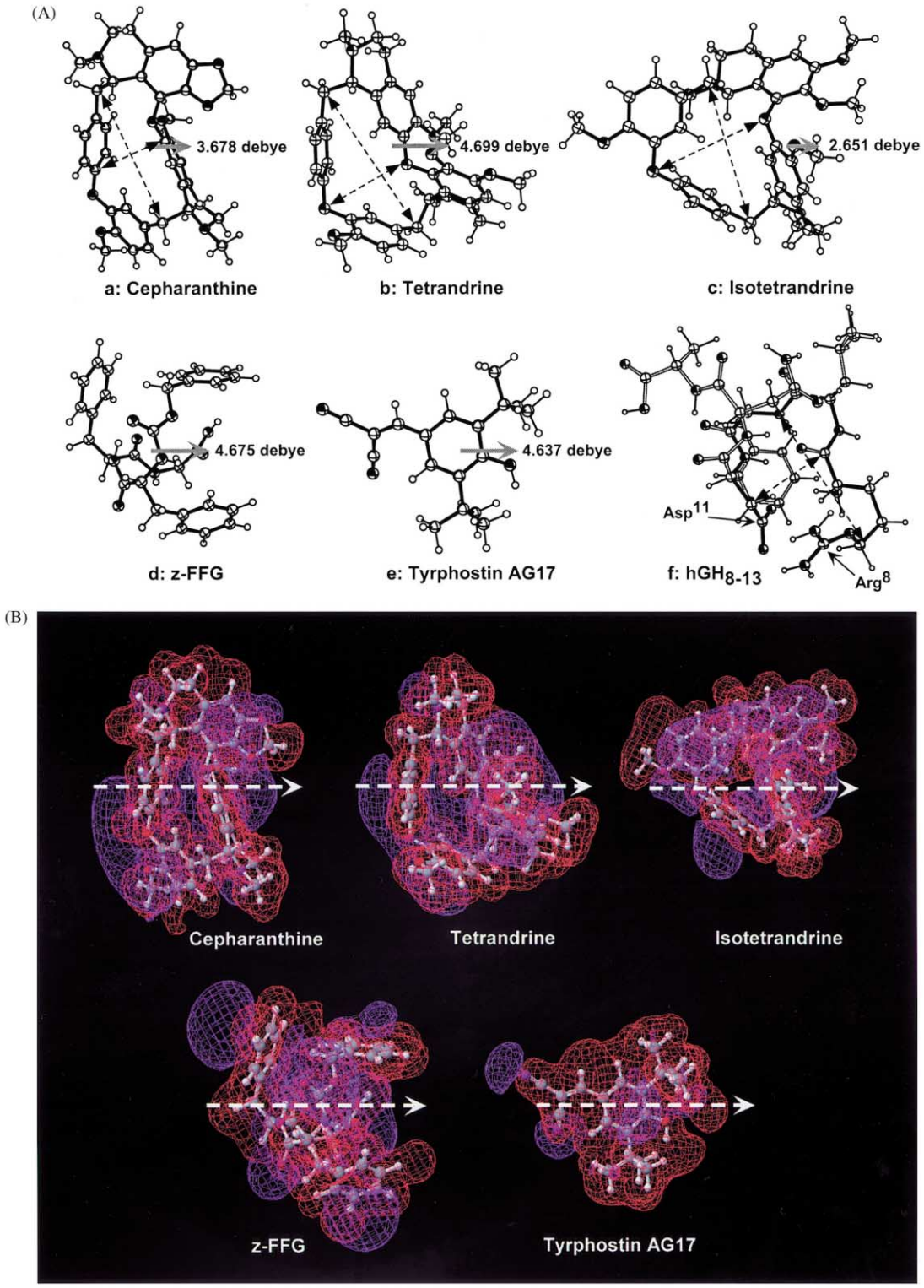

Figure 8. Structural features of membrane-acting agents. (A) Optimized 3-D-projected views of (a) cepharanthine, (b) tetrandrine, (c) isotetrandrine, (d) z-FFG, (e) tyrphostin AG17 and (f) hGH_{8-13},[28] which were calculated using CAChe CONFLEX. The values and direction (gray arrow) of dipole moments indicated were calculated using MOPAC97. The ellipticity (major/minor axis, broken arrow) of cepharanthine, tetrandrine, isotetrandrine and hGH_{8-13} were determined as 1.872, 1.846, 1.264 and 1.941, respectively. (B) The electrostatic isopotential was calculated by MM geometry using the PM3 parameter with the CAChe system. An electrostatic potential isosurface that showed both the polarity of the potential and where in space the potential equals ± 0.030 a.u. (± 18 kcal/mol, ± 75 kJ/mol) was generated. Electrostatic potential is represented by red and blue mesh to indicate positive and negative potential fields, respectively. Broken arrows indicate the axes of dipole moments.

occupied most of the molecule, with negative fields (blue mesh) therein.

Discussion

The human growth hormone (hGH) N-terminal peptide fragments which enhanced the insulin effect, formed a macrocyclic structure in molecule was detected using the MM and MD simulations, and the minimum essential unit for the insulin-involved biological response was identified as hGH_{8-13} (Fig. 8A).[28] The peptide of hGH_{8-13} stabilized the model membrane, and we concluded that membrane conditions affect the sensitivity of target cells to the insulin molecule.[28]

Biscoclaurine-type alkaloids (cepharanthine, tetrandrine, and isotetrandrine) have a macrocyclic structure, and affect the membrane fluidity.[11–13] Cepharanthine had an elliptic ring and was almost equal in size to hGH_{8-13} (ellipticity was 1.941, major and minor-axis were 7.918 and 4.079 angstrom, respectively) (Fig. 8A). In the present study, cepharanthine promoted the insulin-involved fatty acid synthesis in rat white adipocytes as did hGH_{8-13} (Fig. 2). This promotion did not occur without insulin (Fig. 3A). Then, it should be possible to boost the sensitivity to insulin of a target cell by using cepharanthine, and enough of an effect should be obtained at lower concentrations of insulin. Tetrandrine and isotetrandrine did not show insulin-like action per se, but they promoted the insulin effect dose-dependently (Fig. 3B and C). These three alkaloids seem to be enhancers of insulin action.

The peptide z-FFG (10 μM) raised the bilayer- to hexagonal-phase transition temperature in model membranes.[22] In the present study, z-FFG suppressed the insulin action and increased β-agonist-induced fatty acid synthesis (Fig. 2). This suppressive effect of z-FFG was dose-dependently observed with 1.22×10^{-11} M of insulin (closed circles in Fig. 3D). Thus, z-FFG seems to act as an adjustment factor of insulin action. The z-FFG peptide is known to inhibit non-bilayer phase formation as well as vesicle–vesicle and viral fusion. On the other hand, FFG-OBz (D-Phe-L-Phe-Gly-benzyl ester), in which the blocking group was transferred from the amino to the carboxyl end, promoted non-bilayer phase formation.[42] In this way, the FFG region is very useful as a lead compound to design membrane modifying agents. Tyrphostin AG17 suppressed insulin action and increased β-agonist-induced fatty acid synthesis as well as z-FFG (Fig. 4). This effect occurred at 10^{-6}–10^{-5} M of AG17, higher than the concentration range at which AG17 causes uncoupling from the mitochondrial inner membrane (10^{-9} M order).[24] Then we considered that the modifying effects on insulin action both of z-FFG and of tyrphostin AG17 are not mediated by a specific receptor in white adipocytes, and the two agents affected the sensitivity of target cells to insulin via a modification of membrane conditions (e.g., fluidity).

The alkaloids (cepharanthine, tetrandrine, and isotetrandrine) had a stabilizing effect at 10 μM on the model membrane as did hGH_{8-13} (Fig. 5). On the other hand, no such stabilizing effect was observed with z-FFG, consistent with the result that z-FFG had an opposite effect to the alkaloids on insulin-involved fatty acid synthesis (Figs 2 and 3). Tyrphostin AG17 suppressed the insulin action like z-FFG, but it stabilized the C_6-NBD-PC model membrane similar to cepharanthine (Figs 4 and 5).

The three biscoclaurine alkaloids inhibited PLA_2-induced phospholipid degradation dose-dependently (Fig. 6A and B), so they should have stabilizing effect (e.g., radical scavenging effect) on the C_6-NBD-PC model membrane. The insulin-potentiating hexapeptide hGH_{8-13} suppressed the PLA_2-induced C_6-NBD-PC micelle degradation dose-dependently, and NBD release decreased as the level of cepharanthine (data not shown). From these results, we considered that the macrocyclic structure of hGH_{8-13} and cepharanthine, tetrandrine, and isotetrandrine affect the modification of insulin action via stabilization of the cell membrane. Indeed z-FFG did not suppress PLA_2-induced phospholipid degradation, not inconsistent with the fact that z-FFG did not have a stabilizing effect on the C_6-NBD-PC model membrane (Figs 5 and 6). Tyrphostin AG17 (open squares in Fig. 6A and B) suppressed the PLA_2-induced phospholipid degradation to the same degree as isotetrandrine (closed triangles), and consistent with the stabilizing effect on the model membrane (Fig. 5). The uncoupling activity at the mitochondrial inner membrane of tyrphostin AG17 seems to be related to the modification of C_6-NBD-PC micelles. TX-1123 (Fig. 1), a derivative of tyrphostin AG17, suppressed the insulin-involved fatty acid synthesis as tyrphostin AG17 and z-FFG (data not shown). Moreover, TX-1123 did not stabilize the NBD-PC model membrane, and also did not inhibit the PLA_2-induced phospholipid degradation dose-dependently as z-FFG (data not shown). From these facts, we considered that the structure of tyrphostin AG17 may contain the structural factor, which suppressed the insulin action as z-FFG.

The uptake and phosphorylation of glucose are major processes in cellular insulin-involved glucose metabolism. In mammalian cells, four isoforms of the glucose transporter (GLUT 1–GLUT 4) and four isozymes of hexokinase (HK I–HK IV) are important for the sequential reactions in glucose metabolism.[29–35] In previous reports, the mRNA expression of HK isozymes and GLUT isoforms had been examined with 100 nM of insulin, and the enhancement of HK I, II expression and GLUT 1, 4 translation with insulin was reported.[43–48] In the present study, insulin-involved fatty acid synthesis was enhanced by cepharanthine with 10^{-11} M insulin (Fig. 2), but the expression of HK I, II isozymes and GLUT 1, 4 isoforms was not affected by cepharanthine with insulin (1.22×10^{-11} M) in white adipocytes (Fig. 7). Then, we considered that the modification of insulin action by cepharanthine is mediated by neither HK I, II isozymes nor GLUT 1, 4 isoforms. In rat tissues, signals of HK III and GLUT 3 were weakly detected only in brain, and the expression of HK IV and GLUT 2 was observed only in liver.[49] We think that HK III, IV and

GLUT 2, 3 are not involved in the insulin action at 10^{-11} M in rat white adipocytes. Indeed, we did not detect HK III, IV isozymes or GLUT 2, 3 isoforms in rat white adipocytes which were activated by insulin in the presence of cepharanthine. Neither z-FFG nor tyrphostin AG17 affected the mRNA expression of HK I, HK II, GLUT 1 and GLUT 4 similarly to cepharanthine, and we conclude that HK and GLUT did not participate in the modification of insulin (10^{-11} M) action.

The order of the enhancement effect of the alkaloids on insulin-involved fatty acid synthesis was cepharanthine > tetrandrine > isotetrandrine, which agrees with the results of membrane stabilization (e.g., radical scavenging, fluidity modifying),[11–13] so we compared structures using global minimum analysis. The ellipticity (major/minor axis, Fig. 8A) indicated a good correlation with the modifying effect on insulin-involved fatty acid synthesis and the stabilizing effect on the C_6-NBD-PC model membrane, and we considered the elliptic structure to be one of the factors for the modification of insulin action. In our analysis of the electrostatic potential of these alkaloids, negative (blue mesh) and positive (red mesh) electrostatic potential fields were reciprocally distributed along the dipole moment (broken arrow), and negative fields located at both ends of the molecule (Fig. 8B). Thus we are now examining the relationship between the modifying effect on insulin action and the structural conformation (e.g., ellipticity, electrostatic potential) for other macrocyclic compounds. Along the dipole moment (broken arrow), the distribution of electrostatic potential in z-FFG was different from that in cepharanthine, and a negative (blue mesh) and a positive (red mesh) electrostatic potential field, respectively, located at each end of the molecule. In contrast, the electrostatic potential in FFG-OBz, in which the blocking group was transferred from the amino to carboxyl terminal, indicated a cepharanthine-like field pattern (data not shown). Indeed, FFG-OBz had the opposite effect on membrane to z-FFG.[42] Tyrphostin AG17 was almost covered with a positive (red mesh) electrostatic potential field, and the field pattern was significantly different from those of cepharanthine and z-FFG. Tyrphostin AG17 inhibited the insulin action in rat white adipocyte, but it stabilized the C_6-NBD-PC model membrane. From these results, we consider that tyrphostin AG17 has a unique mechanism for membrane stabilization. We are currently analyzing this stabilizing mechanism in detail.

When an exogenous peptidal product or a chemical compound is found to influence a biological system, it is logical to ask if the molecule mimics an endogenous substance. The molecule hGH_{8-13} enhanced the insulin action but did not act by itself, thus hGH_{8-13} does not appear to mimic an endogenous substance (e.g., insulin).[22] Biscoclaurine alkaloids (cepharanthine, tetrandrine, and isotetrandrine) also modified the insulin action but they had no insulin-like activity by themselves. From these facts, we concluded that hGH_{8-13} and these alkaloids are good lead compounds to design a new type of anti-diabetes drug. Based on the distribution of the electrostatic potential field, we are designing and synthesizing a steric hindered phenol TX-1123 and 2-nitroimidazole TX-1877 (Fig. 1), and examining the effect of them on the insulin-involved fatty acid synthesis, the membrane condition, and the expression of HK isozymes and GLUT isoforms (submitted for publication). Moreover, to regulate the insulin-involved biological action via a modification of the sensitivity of the receptor protein to insulin, an analysis of the relationship between the condition of the membrane (e.g., fluidity, stability) and the insulin activity is currently underway.

Experimental

Reagents

Reagents used were purchased from the indicated sources: type II collagenase, porcine pancreas phospholipase A_2, Sigma Co., USA; insulin, Novo Nordisk Co., Denmark; 9-anthryldiazomethane (ADAM) and 1-acyl-2-[6-[(7-nitro-2,1,3-benzoxadiazol-4-yl)amino]-caproyl]-sn-glycero-3-phosphatidylcholine (C_6-NBD-PC), Funakoshi Co., Japan; cepharanthine, isotetrandrine, Kaken Syoyaku Co., Japan; tetrandrine, Aldrich Co., USA; Isogen, Nippon Gene Co., Japan; *Taq* DNA polymerase and *Bca*BEST DNA labeling kit, Takara Shuzo Co., Japan; [α-^{32}P]dCTP (specific radioactivity, 111 TBq/mmol), Amersham Ltd., UK. Tyrphostin AG 17 was gifted from Dr. Yasuo Shinohara, University of Tokushima, Japan.[24]

Preparation of adipocytes and lipolysis

White adipose tissue (WAT; epididymal fat tissue) was excised from 8-week-old male Wistar rats, and adipocytes were prepared from the tissue by type II collagenase digestion as previously described.[50] Adipocytes were washed three times with 5 mL of a Krebs–Ringer Hepes buffer (KRH; 12 mM NaCl, 4.7 mM KCl, 2.5 mM $CaCl_2$, 1.2 mM KH_2PO_4, 12 mM $NaHCO_3$ and 30 mM Hepes, pH 7.4). The cells were collected and suspended in KRH containing 10 mM glucose and 0.1% bovine serum albumin (incubation medium) at a density of 1×10^7 cells/mL. Cells (1×10^6 cells) were incubated at 37 °C for 2 h with indicated amounts of the agent and insulin in the presence of 2 nM isoproterenol in 200 μL of incubation medium. Free fatty acids released from the cells were measured according to the method of Dole.[51]

Hydrolysis of NBD-labeled model membrane

NBD-labeled phosphatidylcholine (C_6-NBD-PC) was suspended at 1.29×10^{-5} M in 0.1 M Tris–HCl buffer (pH 7.4) and formed micelles. Fluorescence measurements were performed at 37 °C either with 10 μM of the alkaloids (cepharanthine, tetrandrine, isotetrandrine) or z-FFG or tyrphostin AG17 or hGH derived peptides (hGH_{8-13}, hGH_{9-13}), excitation and emission wavelength at 470 and 540 nm, with a Hitachi Fluorescent Spectrophotometer Model F-4500.

Effect of membrane-acting agents on PLA_2-induced phospholipid degradation

The NBD-labeled phosphatidylcholine (C_6-NBD-PC) was suspended at 1.29×10^{-5} M in 2 mL of 0.1 M Tris–HCl buffer (pH 7.4) containing 20 mM $CaCl_2$ and formed micelles. Indicated amounts of PLA_2 was added to a reaction mixture without or with the alkaloids or z-FFG or tyrphostin AG17, and fluorescent intensity was monitored (wavelength of excitation: 470 nm, emission: 540 nm) at 37 °C using a Hitachi Fluorescent Spectrophotometer Model F-4500.

cDNA probes

All cDNA fragments (HK I–HK IV, GLUT 1–GLUT 4) were gifted from Dr. Yasuo Shinohara, University of Tokushima, Japan.[49]

Preparation of RNA samples from adipocytes

Adipocytes (5.0×10^6 cells) were incubated at 37 °C for 2 h with 20 μM of cepharanthine or z-FFG or tyrphostin AG17 and 1.22×10^{-11} M insulin in the presence of 2 nM isoproterenol in 1.5 mL of incubation medium. After incubation period, total RNA was isolated from adipocytes with Isogen by the method recommended by the supplier, which was essentially based on the report of Chomczynski and Sacchi.[52]

Northern blotting and determination of transcript levels

Northern blotting was carried out essentially as described previously.[49] Briefly, RNA was subjected to electrophoresis in 1.0% agarose containing formaldehyde, transferred to a nitrocellulose membrane, and hybridized by the standard procedure. Samples of 10 μg of total RNA were used for analysis. After hybridization with probes, the membranes were washed three times with 2×saline sodium citrate (SSC) containing 0.1% SDS at room temperature for 10 min and twice with 1×SSC containing 0.1% SDS at 60 °C for 30 min each time and exposed to X-ray film with an intensifying screen at −80 °C.

Computational analysis of membrane-acting agents

The molecular coordinates of biscoclaurine alkaloids (cepharanthine, tetrandrine, and isotetrandrine) were obtained from the X-ray crystallographic structure of tetrandrine and marchantin A (Fig. 1), which has 5-lipoxygenase-inhibitory and calmodulin-inhibitory activity and structural similarity to cepharanthine.[53,54] The initial structure of z-FFG was constructed using CAChe (Fujitsu Co., Japan). Structure of tyrphostin AG17 was constructed by the X-ray crystallographic data.[55] Global minimum conformation search of these agents was using CAChe CONFLEX with MM2 force field (Fujitsu Co., Japan), and molecular orbital calculation were performed using MOPAC97 (Fujitsu Co., Japan). The energy calculations were performed with PM3 Hamiltonian using the MOPAC97,[56] and the stable and transient structures were initially built with general parameters of bond length, bond angle, and dihedral angle, and refined with the eigen-vector following (EF) optimization method. The 3-D-projected view of the optimized structure was drawn by ORTEP-III (Dr. L.J. Farrugia, University of Glasgow, 1996). After energy minimum search with CAChe CONFLEX, the coordinate data of global minimum conformer was extracted, and the electrostatic isopotential was calculated by MM geometry using PM3 parameter with the CAChe system (Fujitsu Co., Japan). An electrostatic potential isosurface that showed both the polarity of the potential and where in space the potential equals ±0.030 a.u. (±18 kcal/mol, ±75 kJ/mol) was generated. Electrostatic potential is represented by red and blue mesh to indicate positive and negative potential fields, respectively.

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