Proc. Natl. Acad. Sci. USA
Vol. 89, pp. 6443–6446, July 1992
Biochemistry

Platelet activation by simultaneous actions of diacylglycerol and unsaturated fatty acids

(protein kinase C/dioctanoylglycerol/phospholipase A_2)

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Contributed by Yasutomi Nishizuka, April 7, 1992

ABSTRACT **Several cis-unsaturated fatty acids such as oleic, linoleic, linolenic, eicosapentaenoic, and docosahexaenoic acids added directly to intact human platelets greatly enhance protein kinase C activation as judged by the phosphorylation of its specific endogeneous substrate, a 47-kDa protein. This enhancement absolutely requires the presence of a membrane-permeant diacylglycerol, 1,2-dioctanoylglycerol, or a tumor-promoting phorbol ester, phorbol 12-myristate 13-acetate. In the presence of ionomycin and either 1,2-dioctanoylglycerol or phorbol 12-myristate 13-acetate, the release of serotonin from the platelets is also remarkably increased by cis-unsaturated fatty acids. The effect of these fatty acids is observed at concentrations <50 μM. Saturated fatty acids and trans-unsaturated fatty acids are inactive. Titration of ionomycin to induce a release reaction and measurement of the intracellular Ca^{2+} level by the fura-2 procedure indicate that cis-unsaturated fatty acids increase an apparent sensitivity of the platelet response to Ca^{2+}. The results suggest that cis-unsaturated fatty acids, which are presumably produced from phosphatidylcholine by signal-dependent activation of phospholipase A_2, may take part directly in cell signaling through the protein kinase C pathway.**

A previous report from this laboratory (1) described that, in cell-free enzymatic systems, several naturally occurring cis-unsaturated fatty acids greatly potentiate the diacylglycerol (DAG)-dependent activation of protein kinase C (PKC) and allowed the enzyme to exhibit almost full activation at nearly basal Ca^{2+} concentrations. Since receptor-mediated activation of phospholipase A_2 has been suggested (2), it is attractive to surmise that phospholipase C and phospholipase A_2 are both involved in signal transduction through the PKC pathway. A potential role of unsaturated fatty acids in stimulating cellular responses has been postulated by Seifert *et al.* (3), who observed that some unsaturated fatty acids such as linoleic acid show synergistic action with DAG to cause platelet activation. Then, one obvious possibility is that, in stimulated cells, PKC, once initially activated by phosphatidylinositol hydrolysis, may sustain its enzymatic activity even after the Ca^{2+} concentration returns to basal levels, when diacylglycerol (DAG) and cis-unsaturated fatty acids are both available. This paper shows the kinetics of fatty acid action on the PKC pathway to activate human platelets.

MATERIALS AND METHODS

Materials. Human platelet-rich plasma and washed platelets were prepared from the venous blood donated by healthy volunteers by the method described earlier (4).

Phorbol 12-myristate 13-acetate (PMA) was a product of LC Services (Woburn, MA). The stock solution of PMA was dissolved in dimethyl sulfoxide and diluted appropriately to give a final concentration of <0.05% dimethyl sulfoxide. Ionomycin (Calbiochem–Behring) was dissolved in ethanol and diluted to give a final concentration of <0.05% ethanol. The acetoxymethyl ester of fura-2 was obtained from Dojindo Laboratories (Kumamoto, Japan). 1,2-Dioctanoylglycerol (1,2-DiC_8), obtained from Nakalai Tesque (Kyoto), was dissolved in chloroform and stored at −20°C. Radioactive 1,2-DiC_8 (racemic 1,2-di[1-^{14}C]octanoylglycerol; 27 mCi/mmol; 1 Ci = 37 GBq) was a product of Amersham. Although 1,2-DiC_8 was gradually isomerized spontaneously to 1,3-DiC_8 in aqueous solution, DL-1,2-DiC_8 (a mixture of 1,2- and 2,3-diacyl-*sn*-glycerol isomers) was practically stable for at least a month when stored in chloroform at −20°C. The radioactive DiC_8 preparation originally contained DL-1,2-DiC_8 (81.7%), 1,3-DiC_8 (6.3%), and unknown impurities (12.0%) as determined by TLC. Before each use, however, the purity was examined. [^{14}C]Serotonin (5-hydroxy[*side-chain*-2-^{14}C]tryptamine; 57.5 mCi/mmol) was obtained from Amersham. [^{32}P]Orthophosphate (carrier-free; 8500–9120 Ci/mmol) was purchased from DuPont. 1,3-DiC_8, octanoic acid, and various fatty acids were the products of Serdary Research Laboratories (London, ON, Canada). A sample of authentic dioctanoylphosphatidic acid was kindly donated by Y. Nakano (Nippon Oil & Fats, Amagasaki, Japan).

Analysis of 1,2-DiC_8 Metabolites. DL-1,2-[^{14}C]DiC_8 in chloroform was dried under a nitrogen stream and dispersed in H_2O by vigorous mixing followed by sonication for 3 min at 0°C. Platelets were suspended at various densities in albumin-free buffer A (5 mM Tris·HCl at pH 7.5 containing 136.8 mM NaCl, 2.68 mM KCl, 11.9 mM $NaHCO_3$, 1 mM $MgCl_2$, 1 mM EGTA, 5.55 mM glucose, apyrase at 0.5 unit/ml, and 20 nM prostaglandin E_1). The radioactive DiC_8 was added to the platelet suspension at a final concentration of 100 μM (2–3 × 10^5 cpm/ml). After incubation, lipids were extracted from the entire incubation mixture by the method of Bligh and Dyer (5), with 1 M HCl as the upper aqueous phase. The chloroform-soluble material was subjected to TLC on a Merck silica gel 60 plate with chloroform/acetone, 96:4 (vol/vol) as the solvent system. Nonradioactive 1,2-DiC_8 and 1,3-DiC_8 were run simultaneously as authentic markers. Each spot was visualized by I_2 vapor, and the radioactivity corresponding to 1,2-DiC_8 and its metabolites was quantitated with a BAS-2000 Bioimage analyzer (Fuji). The detailed procedures were described in an earlier report (6). For analysis of phosphatidic acid, chloroform/methanol/acetic acid, 65:15:5 (vol/vol), was used as the solvent system. Octanoic acid was detected by TLC with hexane/diethylether/acetic acid, 80:30:1 (vol/vol), as the solvent system.

Analysis of Protein Phosphorylation. Platelets (1 × 10^9 cells per ml) were suspended in buffer A containing [^{32}P]ortho-

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Abbreviations: DAG, diacylglycerol; PKC, protein kinase C; PMA, phorbol 12-myristate 13-acetate; DiC_8, dioctanoylglycerol.

phosphate (10 μCi/ml) for 1 hr, under the conditions described earlier (7). The radioactive platelets were washed three times with buffer A and suspended in buffer A at a density of 1×10^9 cells per ml. The platelets were stimulated and incubated at 37°C as indicated in each experiment described below. The platelets were then treated directly with SDS-containing stop solution and subjected to SDS/PAGE with several protein standards as described (7). After the gel was dried on a filter paper, the radioactive intensity of the 47-kDa protein band was quantitated with a BAS-2000 Bioimage analyzer.

Analysis of Serotonin Release. Platelet-rich plasma was incubated with [^{14}C]serotonin (50 nCi/ml of platelet-rich plasma) for 1 hr at 37°C as described by Haslam and Lynham (8). The radioactive platelets were washed with buffer A and finally suspended in buffer A at a density of 1×10^9 cells per ml. At the time indicated in each experiment, the reaction was terminated by ice-cold formaldehyde (at a final concentration of 0.65%). The formaldehyde-fixed platelet suspension was centrifuged, and the radioactivity of the supernatant was quantitated with a liquid scintillation spectrometer.

Measurement of Ca^{2+} Concentration. The cytosolic Ca^{2+} concentration in platelets was measured by the fura-2 procedure as described (9). The platelets were loaded with the acetoxymethyl ester of fura-2 (final concentration of 5 μM) for 30 min at 37°C. After washing, the platelets were suspended in buffer A at a density of 1×10^9 cells per ml. The fluorescence was measured by using a CAF-100 calcium analyzer (Japan Spectroscopic, Tokyo) using a dual excitation source at 350 and 385 nm.

RESULTS

Rapid Degradation of 1,2-DiC_8. Platelets appeared to metabolize 1,2-DiC_8 very quickly. Analysis of the radioactive products by TLC indicated that a major part of the 1,2-DiC_8 added was hydrolyzed to octanoic acid, and this product was metabolized further (Fig. 1*A*). No appreciable amount of the corresponding phosphatidic acid or 1,3-DiC_8 accumulated. The detailed enzymatic basis of this degradation remains unclear, but it is possible that 1,2-DiC_8 was hydrolyzed by the action of both a nonspecific esterase and DAG lipase, because the radioactive 1,2-DiC_8 employed was a mixture of D and L isomers.

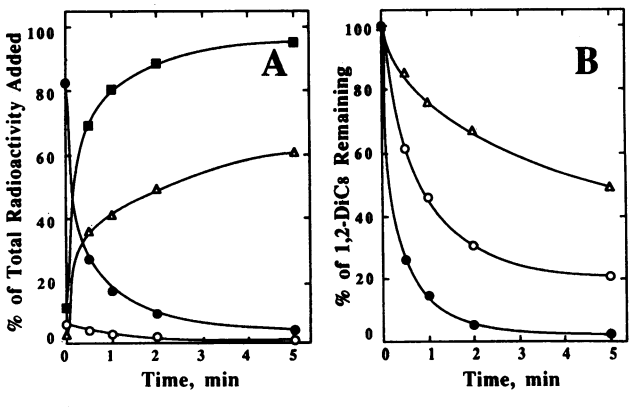

FIG. 1. Metabolism of 1,2-DiC_8 in human platelets. 1,2-[^{14}C]DiC_8 [100 μM (2×10^5 cpm/ml) final concentration] was added to 0.2 ml of a cell suspension (1×10^9 cells in *A* and 1×10^7–10^9 in *B*). At the times indicated, lipids were extracted and separated as described. (*A*) Metabolism of 1,2-DiC_8 and its metabolites. ●, 1,2-DiC_8; ○, 1,3-DiC_8; △, octanoic acid; ■, total metabolites. Results are presented as a percentage of the total input radioactivity. (*B*) Rate of metabolism of 1,2-DiC_8 at a cell density of 1×10^9 (●), 1×10^8 (○), and 1×10^7 (△) cells per ml. Results are presented as a percentage of the 1,2-[^{14}C]DiC_8 originally added.

The rate of disappearance of 1,2-DiC_8 was dependent on the density of platelets in the incubation medium (Fig. 1*B*). At lower platelet densities, 1,2-DiC_8 remained in the incubation medium for a longer period of time and prolonged the activation of PKC.

Phosphorylation of a 47-kDa Protein. When 1,2-DiC_8 was added to platelets, a PKC-specific endogenous substrate, a 47-kDa protein, was rapidly phosphorylated. However, once the phosphate was covalently attached to this protein, it was removed quickly by the action of phosphatases (Fig. 2*A*), presumably because the 1,2-DiC_8 available for maintaining the active PKC disappeared rapidly as described above. Nevertheless, this protein phosphorylation was greatly enhanced by the addition of linoleic acid. The fatty acid alone was completely inactive.

In contrast, when platelets were stimulated by PMA instead of 1,2-DiC_8, a 47-kDa protein was similarly phosphorylated, but the radioactive phosphate remained attached to this protein for a longer period of time (Fig. 2*B*). This is presumably due to the fact that PMA is metabolized slowly and, therefore, prolongs PKC activation. It is worth noting that linoleic acid also significantly enhanced the PMA-induced phosphorylation of the 47-kDa protein.

Release of Serotonin. In the presence of ionomycin plus either 1,2-DiC_8 or PMA, serotonin was released as described (10, 11). The addition of linoleic acid to the platelets further enhanced the release reaction, although linoleic acid alone was inactive unless both 1,2-DiC_8 and ionomycin were present (Fig. 3). When 1,2-DiC_8 was employed, the amount of serotonin released in the medium declined slowly with time (Fig. 3*A*), probably due to reuptake of the serotonin once released, since 1,2-DiC_8 was metabolized rapidly, and thus the intracellular PKC returned to an inactive form.

Fatty Acid Concentration. Unsaturated fatty acids normally show detergent-like properties and damage cell membranes at higher concentrations. Under the present conditions, however, linoleic acid alone was totally inactive, and phosphorylation of the 47-kDa protein and serotonin release described above always required the coexistence of 1,2-DiC_8. Both phosphorylation of the 47-kDa protein (Fig. 4*A*) and serotonin release (Fig. 4*B*) were increased by linoleic acid in a dose-dependent manner up to ≈50 μM. Above 200 μM, the fatty acid alone appeared to damage the membrane to cause the lysis of platelets.

Fatty Acid Specificity. The potentiation of the phosphorylation of the 47-kDa protein by linoleic acid described above

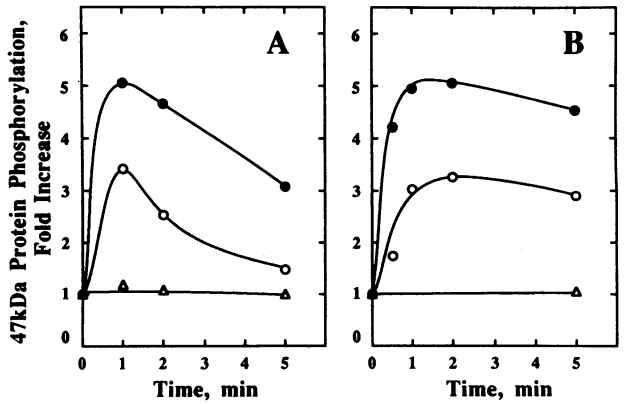

FIG. 2. Effect of linoleic acid on phosphorylation of the 47-kDa protein induced by either 1,2-DiC_8 or PMA. Platelets labeled with [^{32}P]orthophosphate (1×10^9 cells per ml) were stimulated at 37°C for the various times as indicated with 25 μM 1,2-DiC_8 (*A*) or 100 nM PMA (*B*) in the presence (●) or absence (○) of 50 μM linoleic acid. The platelets were also incubated with linoleic acid alone without 1,2-DiC_8 and PMA (△). The phosphorylation of the 47-kDa protein was quantitated as described.

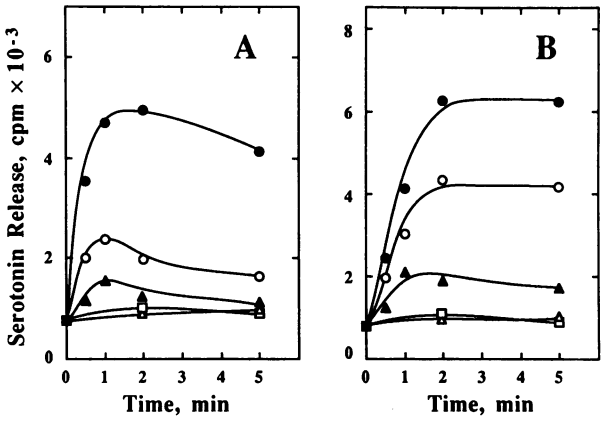

FIG. 3. Effect of linoleic acid on serotonin release. Platelets (1×10^9 cells per ml) labeled with [^{14}C]serotonin were stimulated with 25 μM 1,2-DiC$_8$ (*A*) or 100 nM PMA (*B*), in the presence of 50 μM linoleic acid plus 0.8 μM ionomycin (●), in the presence of ionomycin (○), or in the absence of linoleic acid and ionomycin (▲). The platelets were also incubated with linoleic acid alone (△) or ionomycin alone (□) without 1,2-DiC$_8$ and PMA. The radioactivity released was measured as described.

was also observed for many other naturally occurring cis-unsaturated fatty acids such as oleic, linolenic, eicosapentaenoic, and docosahexaenoic acids. Arachidonic acid was not tested here because the effect of its metabolites such as thromboxane A$_2$ cannot be ruled out. Neither saturated fatty acids such as palmitic and stearic acids nor trans-unsaturated fatty acids such as elaidic and linolelaidic acids were active in enhancing the protein phosphorylation induced by 1,2-DiC$_8$. A similar fatty acid specificity was observed for the enhancement of the release reaction in the presence of both 1,2-DiC$_8$ and ionomycin (Fig. 5).

Ionomycin Concentration. The release of serotonin required a Ca^{2+} ionophore such as ionomycin, in addition to a membrane-permeant DAG or PMA as described earlier (10, 11). The addition of linoleic acid decreased the concentration of ionomycin that was needed for the release reaction and apparently enhanced the platelet response to 1,2-DiC$_8$ (Fig. 6*A*). Measurement of the actual Ca^{2+} concentration in the platelets with the fura-2 procedure indicated that the fatty

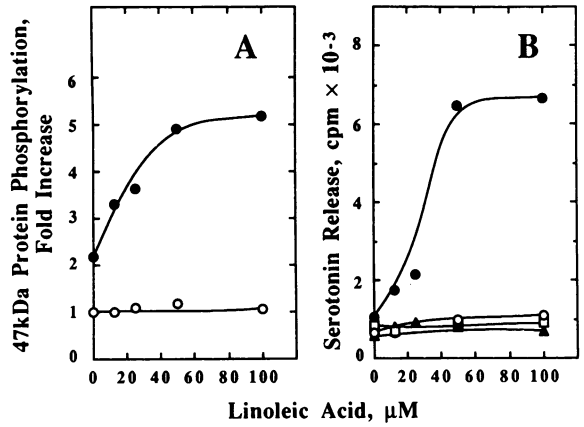

FIG. 4. Effect of the linoleic acid concentration on phosphorylation of the 47-kDa protein and serotonin release. Platelets (1×10^9 cells per ml) labeled with [^{32}P]orthophosphate or [^{14}C]serotonin were stimulated for 1 min at 37°C at various concentrations of linoleic acid. (*A*) Phosphorylation of the 47-kDa protein in the presence (●) or absence (○) of 25 μM 1,2-DiC$_8$. (*B*) Serotonin release in the presence of 25 μM 1,2-DiC$_8$ plus 0.8 μM ionomycin (●), 25 μM 1,2-DiC$_8$ alone (○), 0.8 μM ionomycin alone (□), or in the absence of 1,2-DiC$_8$ and ionomycin (▲).

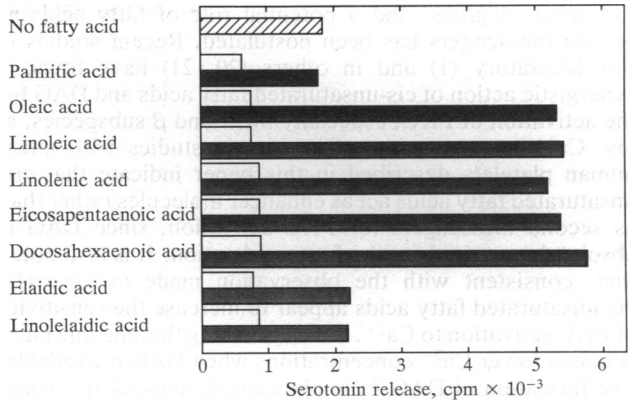

FIG. 5. Effect of various fatty acids on serotonin release. [^{14}C]Serotonin-loaded platelets (1×10^9 cells per ml) were stimulated for 1 min in the presence of 50 μM various fatty acids with (shaded bars) or without (open bars) 25 μM 1,2-DiC$_8$ plus 0.8 μM ionomycin. No fatty acid (hatched bar) represents the serotonin release induced by 25 μM DiC$_8$ plus 0.8 μM ionomycin in the absence of any fatty acid.

acid increased an apparent sensitivity of platelets to Ca^{2+} for the release response (Fig. 6*B*). The fatty acid alone did not increase the intracellular Ca^{2+} concentration.

DISCUSSION

It was shown as early as 1981 by McKean *et al.* (12) that lysophosphatidylcholine is produced in human platelets upon stimulation by thrombin. Burch *et al.* (13) have subsequently presented evidence suggesting that phospholipase A$_2$ and phospholipase C are both activated by a single α_1-adrenergic stimulation through distinct GTP-binding proteins in a cultured thyroid cell line. The physiological role of this phospholipase A$_2$ activation remains largely to be clarified, although these observations have been interpreted as indicating that the enzyme acts to release arachidonic acid for the biosynthesis of various oxygenated products.

Several laboratories (14–19) have shown that cis-unsaturated fatty acids such as arachidonic and oleic acids activate PKC in the absence of phosphatidylserine and DAG

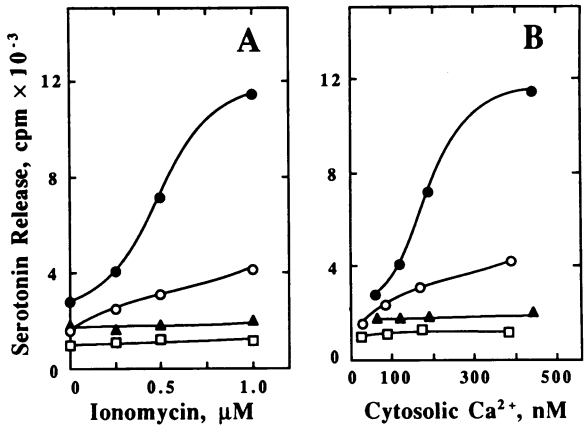

FIG. 6. Effect of ionomycin on serotonin release stimulated by 1,2-DiC$_8$ and linoleic acid. (*A*) [^{14}C]Serotonin-loaded platelets (1×10^9 cells per ml) were stimulated at various concentrations of ionomycin for 1 min with 25 μM 1,2-DiC$_8$ plus 50 μM linoleic acid (●), 25 μM 1,2-DiC$_8$ (○), or 50 μM linoleic acid (▲) or were incubated in the absence of 1,2-DiC$_8$ and linoleic acid (□). Serotonin released was quantitated as described. (*B*) Platelets were stimulated as in *A*. The intracellular Ca^{2+} concentrations after the addition of various concentrations of ionomycin were measured as described. The serotonin released is plotted as a function of the intracellular Ca^{2+} concentration.

to various degrees, and a potential role of fatty acids as second messengers has been postulated. Recent studies in this laboratory (1) and in others (20, 21) have found a synergistic action of cis-unsaturated fatty acids and DAG for the activation of PKC, especially the α and β subspecies, at low Ca^{2+} concentrations. The kinetic studies with intact human platelets described in this paper indicate that cis-unsaturated fatty acids act as enhancer molecules rather than as second messengers for PKC activation, since DAG is absolutely required for the fatty acid action. It is also noted that, consistent with the observation made *in vitro* (1), cis-unsaturated fatty acids appear to increase the sensitivity of PKC activation to Ca^{2+}, thereby making this enzyme more active at lower Ca^{2+} concentrations when DAG is available. The formation of DAG from phosphatidylinositol by signal-dependent phospholipase C activation is normally transient, and it is becoming evident that endogenous DAG nevertheless gradually accumulates, presumably by the action of phospholipase D, especially after stimulation by long-acting signals such as some growth factors (for reviews, see refs. 22 and 23). It is possible, then, that PKC-dependent cellular responses may persist for a prolonged period of time if various phospholipases remain active even when the initial Ca^{2+} signal is transient. Szamel *et al.* (24) have observed that linoleic and arachidonic acids potentiate interleukin 2 synthesis in human lymphocytes, which is induced by the synergistic action of ionomycin and a membrane-permeant DAG, although they interpreted this observation in a different way. Lester *et al.* (25) have described that arachidonic acid and a membrane-permeant DAG synergistically reduced K^+-channel conductance when injected directly into the *Hermissenda* photoreceptor.

It has been reported that fatty acids show some inhibitory action on DAG lipase in cell-free systems (26). The addition of linoleic acid to platelets, in fact, only slightly reduces the rate of disappearance of DAG that is endogenously produced after thrombin stimulation. However, it seems unlikely that the stimulatory effect of fatty acids on platelet activation described above is merely due to the inhibition of endogenous DAG lipase, because the enhancement of platelet activation by fatty acids can also be observed with PMA, which is metabolically stable. Although plausible evidence strongly suggests that cis-unsaturated fatty acids exert their biological action by potentiating the PKC activation directly, other mechanisms of the fatty acid action cannot be ruled out.

The data are taken in part from the dissertation to be submitted by K.Y. to Kobe University School of Medicine in partial fulfillment of the requirement for the degree of Doctor of Medical Sciences. We cordially acknowledge Dr. T. Fujikura (Central Research Laboratories, Yamanouchi Pharmaceutical Company) for his generous support in the chemical synthesis of 1,2-[^{14}C]DiC_8. A generous gift of dioctanoylphosphatidic acid by Dr. Y. Nakano (Nippon Oil & Fats Company, Amagasaki, Japan) is also acknowledged. The skillful secretarial assistance of Mrs. S. Nishiyama, Miss Y. Kimura, and Miss Y. Yamaguchi is cordially acknowledged. This work was supported in part by research grants from the Special Research Fund of the Ministry of Education, Science and Culture, Japan; the Yamanouchi Foundation for Research on Metabolic Disorders; Sankyo Foundation of Life Science; Merck Sharp & Dohme Research Laboratories; Biotechnology Laboratories of Takeda Chemical Industries; and New Lead Research Laboratories of Sankyo Company.

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