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SULPHONYLUREA AGENTS INHIBIT PLATELET AGGREGATION AND $[Ca^{2+}]i$ ELEVATION INDUCED BY ARACHIDONIC ACID

RUOMEI QI, YUKIO OZAKI,* KANEO SATOH, KENJI KUROTA, NAOKI ASAZUMA, YUTAKA YATOMI and SHOJI KUME

Department of Clinical and Laboratory Medicine, Yamanashi Medical University, Yamanashi, Japan

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Abstract—The effects of three hypoglycaemic agents—glimepiride, glibenclamide and gliclazide—were evaluated on platelet aggregation and intracellular Ca^{2+} elevation induced by arachidonic acid. Platelet aggregation was assessed both by the conventional method using changes in light transmission and by a newly-developed procedure using light scattering which allows the detection of small as well as large aggregates. Glimepiride and glibenclamide inhibited the formation of small and large aggregates induced by optimal concentrations of arachidonic acid in a dose-dependent manner. The ID_{50} values for the inhibition of platelet aggregation were approximately one third of those for arachidonic acid metabolism, suggesting that both agents have certain direct inhibitory effects on platelet aggregation unrelated to arachidonic acid metabolism. Gliclazide inhibited the formation of small aggregates induced by low concentrations of arachidonic acid to a limited extent. However, it inhibited the formation of large aggregates but not small aggregates when higher concentrations of arachidonic acid were used. Glimepiride and glibenclamide inhibited $[Ca^{2+}]i$ elevation induced by arachidonic acid in a dose-dependent manner, whereas gliclazide had no inhibitory effect. Taken together, these suggest that gliclazide does not inhibit arachidonic acid metabolism but does have certain direct inhibitory effects on platelet aggregation.

Key words: hypoglycaemic agents; glimepiride; glibenclamide; gliclazide platelet aggregation; intracellular Ca^{2+} elevation; light scattering

Platelets may be hyperactive in diabetes mellitus and contribute to macrovascular complications [1–3]. There have been several reports that the sulphonylurea drug, gliclazide, had an anti-platelet effect. It inhibited platelet adhesion and aggregation induced by ADP and other agonists [4, 5]. When administered to diabetic patients gliclazide suppressed the hyperreactivity of platelets [6, 7]. In previous reports, we showed that two sulphonylurea agents, glimepiride and glibenclamide, had suppressive effects on arachidonic acid metabolism, whereas gliclazide had no effect on the production of arachidonic acid metabolites [8, 9]. Thus, the mechanism by which gliclazide inhibits platelet aggregation remains elusive. A new device employing a particle counting method and light scattering has recently been developed to detect platelet aggregation. It allows quantitative evaluation on the formation of small as well as large aggregates, and thus is able to assess the mechanism of inhibitory effects on platelet aggregation [10]. In this study, we sought to evaluate the effect of three hypoglycaemic agents on platelet aggregation as assessed by this new device along with $[Ca^{2+}]i$† elevation induced by arachidonic acid.

MATERIALS AND METHODS

Agents. The three sulphonylurea agents used in the present study were gliclazide (1-(4-methylbenzenesulphonyl) - (3 - azabicyclo-(3,3,0)octyl)urea), glibenclamide (1-(4-(2-(chloro-2-methoxybenzamide)ethyl - phenyl - sulphonyl) - 3 - cyclohexy)urea), and glimepiride (1-(4-(2-(3-ethyl-4-methyl-2-oxo-3-pyrroline)- carboxamide)- ethylphenylsulphonyl)- 3-(4-methylcyclohexyl))urea. They were dissolved in DMSO at a concentration of 50 mM and stored at −60° until use. Arachidonic acid was obtained from Funakoshi (Tokyo, Japan). Fura-2/AM was purchased from Dojin Laboratories (Kumamoto, Japan). Modified HEPES/Tyrode's buffer containing 129 mM NaCl, 2.8 mM KCl, 0.8 mM KH_2PO_4, 8.9 mM $NaHCO_3$, 0.8 mM $MgCl_2$, 10 mM HEPES pH 7.15, 0.2% BSA and 5.5 mM glucose was passed through 0.45 μm Millipore filters and stored at 4° until use.

Platelet separation. Citrate anti-coagulated venous blood was obtained from healthy human donors who had not received any medication for a minimum of 2 weeks preceding the experiment. The blood was centrifuged at 60 *g* for 15 min to obtain platelet-rich plasma. Platelets were washed twice with the HEPES/Tyrode's buffer with 0.2 μM prostaglandin I_2 and resuspended in the HEPES/Tyrode's buffer

* Corresponding author: Yukio Ozaki, M.D., Department of Clinical Laboratory Medicine, Yamanashi Medical University, Shimokato 1110, Tamaho, Nakakoma, Yamanashi 409-38, Japan. Tel. 81-552-71-1111; FAX 81-552-73-6713.

† Abbreviations: $[Ca^{2+}]i$, intracellular Ca^{2+} concentration; fura-2/AM, fura-2 acetoxymethyl ester.

containing 100 μM Ca^{2+} and 0.2% albumin at a concentration of 10^8 cells/mL unless otherwise stated.

Platelet aggregation. We have developed a new procedure which can simultaneously measure platelet aggregation by two different methods [10]. One is the conventional method using changes in light transmission of a platelet suspension. The other is based upon the particle counting technique using light scattering. An optical device designed to focus on a limited area of a platelet suspension measured the intensity of light scattered by particles passing through the area, thereby minimizing multiple light scattering. The intensity of light scattering detected by this new device provides information as to the number and size of aggregates in a suspension. Briefly, 300 μL of a platelet suspension was poured into a cuvette and was incubated for 5 min with various concentrations of the sulphonylurea agents. After incubation, 10–20 μM arachidonic acid was added to the suspension, and platelet aggregation assessed by these two methods was continuously monitored.

$[Ca^{2+}]i$ *measurement.* Fura-2/AM, at a final concentration of 3 μM, was added to platelet-rich plasma and the mixture was incubated for 30 min at 37°. After incubation, platelets were washed twice with the HEPES/Tyrode's buffer and resuspended in the same buffer at a concentration of 10^8 cell/mL. Fura-2 fluorescence was measured with a Hitachi F2000 fluorescence spectrophotometer with the excitation wavelength being changed alternately every 0.5 sec from 340 to 380 nm while the emission wavelength was set at 510 nm. The platelet suspension was kept at 37° by constant stirring throughout measurement. A computer (NEC 9810 EX2; NEC, Tokyo, Japan) was connected up with the spectrophotometer, and fura-2 fluorescence was processed by a computer program developed by Dr H. Koshi (Hitachi Corporation, Tokyo, Japan). Platelet suspensions were incubated with various concentrations of sulphonylurea agents for 5 min, then arachidonic acid was added to activate platelets. $[Ca^{2+}]i$ values were determined from the ratio of fura-2 fluorescence intensities at 340 nm excitation and 380 nm excitation, as described by Grynkiewicz *et al.* [11].

Statistics. Significant differences between the control and the sample data were determined by the Student's *t*-test.

RESULTS

Arachidonic acid induced platelet aggregation at a concentration range between 2 and 40 μM. At low concentrations (2–5 μM), arachidonic acid induced only the first wave of aggregation with the small aggregate formation which was reversible. With higher concentrations of arachidonic acid, the first wave aggregation was followed by the second wave of aggregation with the large aggregate formation (Fig. 1).

We first evaluated the effect of the sulphonylurea agents on the second wave of aggregation induced by 10–20 μM arachidonic acid. Glimepiride effectively inhibited platelet aggregation induced by arachidonic acid. It was equally effective on the formation of small and large aggregates and the changes in light transmission. The formation of large aggregates correlated with the changes in light transmission

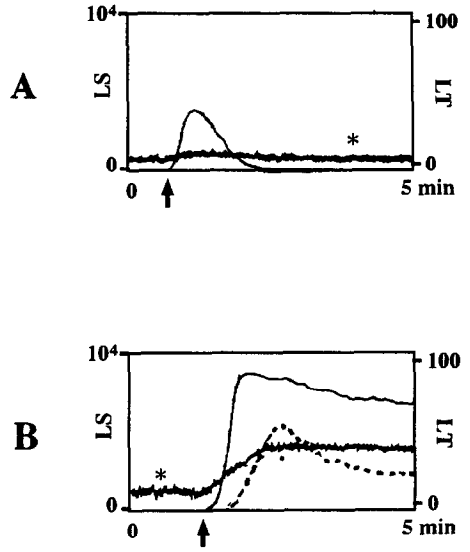

Fig. 1. Changes in light scattering or light transmission induced by low or high concentrations of arachidonic acid. Platelets in platelet-rich plasma were activated by (A) 3 μM or (B) 20 μM arachidonic acid added at the time indicated by arrowheads. Light scattering intensity (LS) and changes in light transmission (LT) were simultaneously monitored for the same sample. Solid and dotted lines represent light scattering produced by small aggregates (< 100 cells) and by large aggregates (> 100 cells), respectively. Fluctuating lines with asterisks represent changes in LT. The ordinates are expressed in arbitrary units. Traces are representative of at least five experiments.

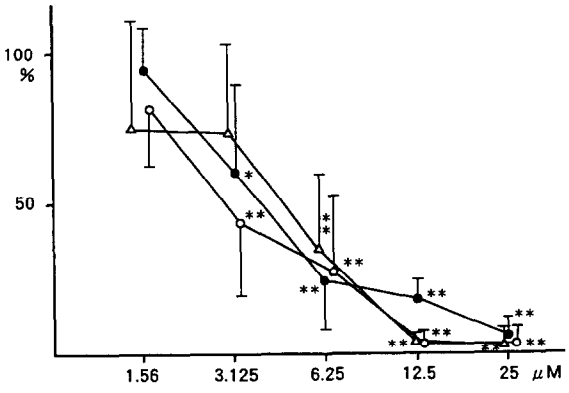

Fig. 2. Effects of glimepiride on arachidonic acid-induced platelet aggregation. Platelets were preincubated with various concentrations of glimepiride for 5 min, then activated by 10–20 μM arachidonic acid. Platelet aggregation was assessed by the formation of small aggregates (△—△), large aggregates (○—○), and changes in light transmission (●—●). The data are presented as the mean ± SD of six experiments (*, P = 0.05; **, P = 0.01).

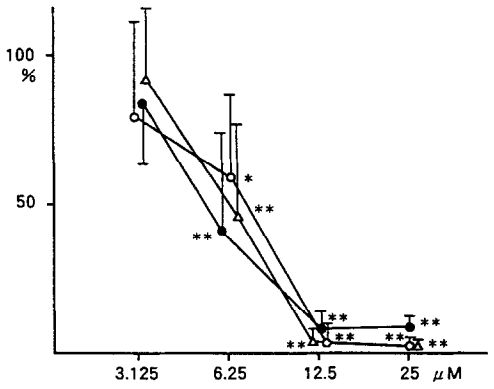

Fig. 3. Effects of glibenclamide on arachidonic acid-induced platelet aggregation. Platelets were preincubated with various concentrations of glibenclamide for 5 min, then activated by 10–20 μM arachidonic acid. Platelet aggregation was assessed by the formation of small aggregates (△—△), large aggregates (○—○), and changes in light transmission (●—●). The data are presented as the mean ± SD of six experiments. (*, P = 0.05; **, P = 0.01).

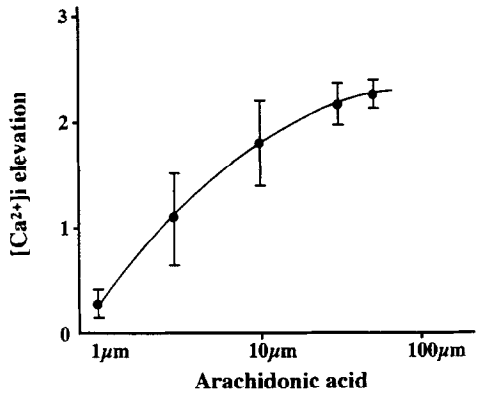

Fig. 5. Dose-dependency of $[Ca^{2+}]i$ elevation on arachidonic acid. Platelets loaded with fura-2 were activated with various concentrations of arachidonic acid, and the fura-2 fluorescence was measured with the excitation wavelength of 340 nm and 380 nm. The ratio of 340 nm fluorescence intensity to that of 380 nm was used to estimate $[Ca^{2+}]i$. $[Ca^{2+}]i$ elevation induced by arachidonic acid was assessed as the maximal increase in $[Ca^{2+}]i$ ratio over the control. The circles and bars represent the means ± SD of three experiments.

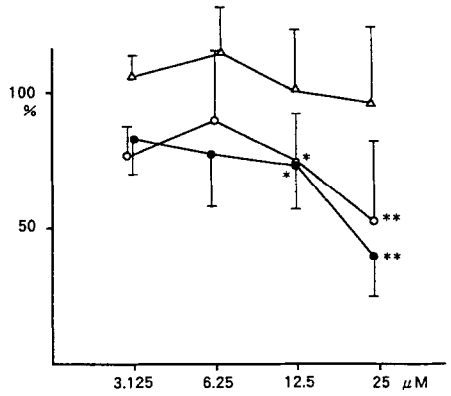

Fig. 4. Effects of gliclazide on arachidonic acid-induced platelet aggregation. Platelets were preincubated with various concentrations of gliclazide for 5 min, then activated by 10–20 μM arachidonic acid. Platelet aggregation was assessed by the formation of small aggregates (△—△), large aggregates (○—○), and changes in light transmission (●—●). The data are presented as the mean ± SD of six experiments (*, = 0.05; **, P = 0.01).

[10]. The ID_{50} value for the inhibition of platelet aggregation was approximately 3 μM (Fig. 2). Glibenclamide also inhibited platelet aggregation in a dose-dependent manner. Its effect was essentially the same for both large and small aggregate formation with an ID_{50} value of approximately 6 μM (Fig. 3). In contrast to the other two agents, the effect of gliclazide was weak. Its inhibitory effect was only evident at a concentration of 25 μM, its maximum inhibitory effect being only 50% of the control. It is of interest that gliclazide inhibited only the formation of large aggregates and corresponding changes in optical density, but not the formation of small aggregates (Fig. 4). We previously provided evidence demonstrating that small aggregates coalesce to form large aggregates [10]. These findings suggest that in platelet activation induced by high concentrations of arachidonic acid the formation of small aggregates is comparatively unaffected by gliclazide and that the formation of large aggregates which results from the fusion of smaller aggregates is hampered by gliclazide.

We then evaluated the effects of the sulphonylurea agents on platelet aggregation induced by low concentrations of arachidonic acid. The modifications in optical changes induced by low concentrations of arachidonic acid were only minimal and were not suitable for quantitative evaluation. The light scattering method, which sensitively detects the formation of small aggregates, did prove useful however. The overall inhibitory profiles of the sulphonylurea agents were similar to those evaluated with higher concentrations of arachidonic acid (data not shown), except for gliclazide which, at a concentration of 25 μM, inhibited the formation of small aggregates by 74 ± 15% (N = 3), while being without effect at lower concentrations. These findings suggest that gliclazide at the concentration of 25 μM exhibits general inhibitory effects on platelet aggregation, regardless of the size of aggregates.

We then evaluated the effects of these agents on $[Ca^{2+}]i$ elevation induced by arachidonic acid. Arachidonic acid elevated $[Ca^{2+}]i$ in a dose-dependent manner (Fig. 5). Since concentrations of arachidonic acid higher than 30 μM often appeared to lyse platelets, a concentration of 10 μM was used to induce $[Ca^{2+}]i$ elevation. Glimepiride and glibenclamide inhibited $[Ca^{2+}]i$ elevation induced by arachidonic acid in a dose-dependent manner with

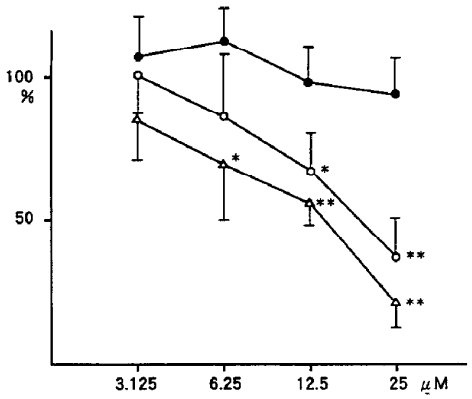

Fig. 6. Effects of three sulphonylurea agents on $[Ca^{2+}]i$ elevation induced by arachidonic acid. Fura-2-loaded platelets were preincubated with various concentrations of sulphonylurea agent for 5 min, then activated by 10 μM arachidonic acid. $[Ca^{2+}]i$ elevation was assessed by the ratio of fura-2 fluorescence at the 340 nm excitation wavelength to that at 380 nm. Glimepiride (△—△), glibenclamide (○—○), and gliclazide (●—●). The data are presented as the mean ± SD of six experiments (*, P = 0.05; **, P = 0.01).

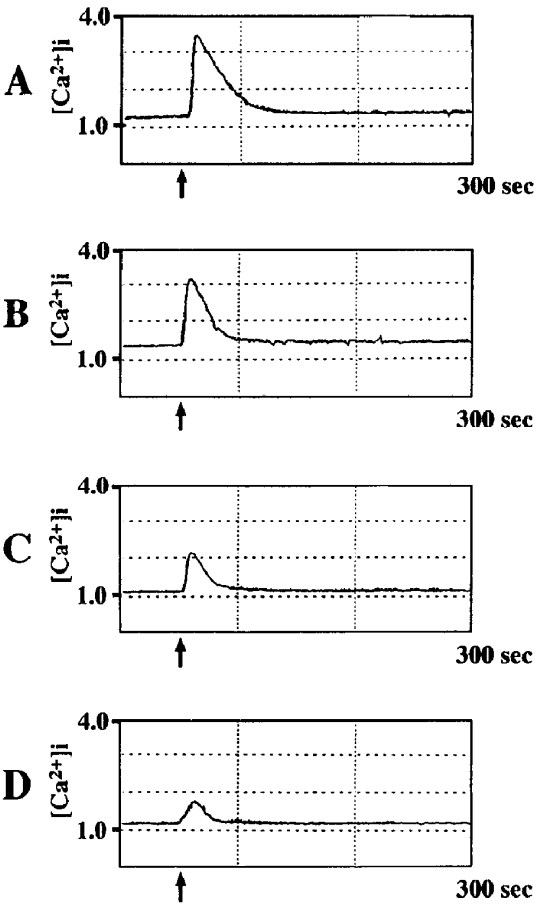

Fig. 7. Time course of $[Ca^{2+}]i$ elevation induced by arachidonic acid and the effects of sulphonylureas. Platelets were incubated with a sulphonylurea agent (25 μM) or with an equivalent volume of dimethylsulphoxide as the control for 5 min. Platelets were then activated with 10 μM arachidonic acid and the changes in $[Ca^{2+}]i$ were monitored for 5 min. A, control; B, 25 μM gliclazide; C, 25 μM glibenclamide; D, 25 μM glimepiride.

similar potency. The ID_{50} value for glimepiride was approximately 15 μM, and that for glibenclamide was 20 μM. It is to be noted that these values are considerably higher than those required for the inhibition of platelet aggregation. Gliclazide had virtually no effect on $[Ca^{2+}]i$ elevation within the same concentration range (Fig. 6). Figure 7 illustrates the trace of $[Ca^{2+}]i$ changes induced by arachidonic acid and the effects of these sulphonylureas. The inhibitory profiles of glibenclamide and glimepiride were almost identical.

DISCUSSION

In previous reports, we evaluated the effects of the three sulphonylurea agents on arachidonic acid metabolism. Glimepiride was found to have an inhibitory effect on the cyclooxygenase pathway. Glibenclamide appeared to have inhibitory effects on multiple sites of arachidonic acid metabolism including cyclooxygenase [8, 9]. In contrast, gliclazide lacked inhibition on arachidonic acid metabolism. In this study, platelet aggregation was measured by a recently-developed device able to detect platelet aggregation with light scattering. The new device appeared to be particularly sensitive to small aggregate formation, especially those aggregates formed in platelet activation induced by low concentrations of agonists. Furthermore the new method has an advantage over the conventional aggregometry in allowing aggregation size distribution and the extent of aggregation to be estimated. For comparison, we also measured platelet aggregation with the conventional method using changes in light transmission.

All three agents effectively inhibited platelet aggregation induced by arachidonic acid, though to various degrees. Glimepiride was more potent than glibenclamide, and the inhibitory effect of gliclazide appeared to be qualitatively distinct from that of the other two. In our previous study on the effects of these agents on arachidonic acid metabolism, we found that glimepiride was slightly less potent than glibenclamide in inhibiting arachidonic acid metabolism. Therefore, those findings suggest that glimepiride perhaps has an additional inhibitory effect on platelet aggregation unrelated to arachidonic acid metabolism. Furthermore, the ID_{50} values of glibenclamide and glimepiride for the suppression of platelet aggregation are lower than those for arachidonic acid metabolism (glibenclamide, approximately 6 μM for aggregation versus 10–15 μM for arachidonic acid metabolism; glimepiride, 3 μM versus 20–30 μM). These findings imply that in addition to their effects on arachidonic acid metabolism, glimepiride and glibenclamide block platelet aggregation by some mechanism not related

to arachidonic acid metabolism. It is of interest that gliclazide also inhibits platelet aggregation, although to a lesser degree than glimepiride and glibenclamide. More specifically, gliclazide at 25 μM inhibited the formation of small aggregates induced by low concentrations of arachidonic acid, and also suppressed large aggregate formation induced by higher concentrations of arachidonic acid. Our findings are in good accord with previous reports showing that gliclazide inhibits platelet aggregation [4–7, 12]. We have demonstrated that gliclazide has no inhibitory effect on arachidonic acid metabolism [8, 9]. Taken together, these findings suggest that gliclazide has certain direct inhibitory effects on platelet aggregation unrelated to arachidonic acid metabolism.

To further confirm the effects of these agents on platelet activation induced by arachidonic acid, we evaluated the effects on $[Ca^{2+}]i$ elevation induced by arachidonic acid. Glimepiride and glibenclamide are potent inhibitors of $[Ca^{2+}]i$ elevation, whereas gliclazide had no effect on $[Ca^{2+}]i$ elevation. The doses of glibenclamide and glimepiride required for the inhibition of $[Ca^{2+}]i$ elevation are significantly higher than those for platelet aggregation, once again confirming that these two agents have some direct inhibitory effects on aggregation. It has recently been reported that glibenclamide and glimepiride inhibit the action of prostaglandin D_2 and prostaglandin E_2 on rat aorta [13]. However, these two sulphonylurea agents have no inhibitory effects on platelet activation induced by thromboxane A_2 in our system (data not shown), and the mechanism by which these sulphonylurea agents inhibit platelet aggregation remains to be elucidated. The fact that gliclazide did not suppress $[Ca^{2+}]i$ elevation induced by arachidonic acid confirmed that this sulphonylurea does not affect the formation of thromboxane A_2 and further that the intracellular signal transduction as far as $[Ca^{2+}]i$ mobilization is not modified by gliclazide.

In conclusion, we have confirmed that glimepiride and glibenclamide suppress arachidonic acid metabolism in a dose-dependent manner as well as various parameters of platelet activation induced by arachidonic acid. Gliclazide has no effect on platelet activation induced by arachidonic acid metabolism. We have also demonstrated that all these sulphonylurea agents have additional inhibitory effects on platelet aggregation unrelated to arachidonic acid metabolism.

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