Metabolic Effects of Glibenclamide in Isolated Rat Hepatocytes in the Absence of Extracellular Ca$^{2+}$

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ABSTRACT—We examined the metabolic effects of glibenclamide, a potent second-generation sulfonylurea, in isolated rat hepatocytes incubated in the absence of extracellular Ca$^{2+}$. We first demonstrated in the present study that glibenclamide caused a significant increase in basal glucose release and lactate production without any modification of intracellular Ca$^{2+}$ concentration or cAMP levels in isolated rat hepatocytes. Furthermore, glibenclamide inhibited the noradrenaline-induced increase in cAMP accumulation, while activation of glycogenolysis by noradrenaline was not suppressed by this agent. Our data indicate that glibenclamide exerts its metabolic effects independent of intracellular Ca$^{2+}$ mobilization and cAMP accumulation.

Keywords: Glibenclamide, Cellular cAMP level, Intracellular Ca$^{2+}$ concentration

It is well-established that extrapancreatic actions are implicated in the hypoglycemic actions of sulfonylureas (1). Among them, the effects on hepatic carbohydrate metabolism may be important. Sulfonylureas have been demonstrated to inhibit hepatic glycogenolysis induced by Ca$^{2+}$-dependent hormones (2) and hepatic gluconeogenesis stimulated by glucagon (3) through interference with the effect of mobilized Ca$^{2+}$ and prevention of glucagon-induced cAMP accumulation, respectively. López-Alarcon et al. (4) have reported that glibenclamide actions show a dependency on the presence of Ca$^{2+}$ in the incubation medium. Previously, we have reported that stimulation of $\alpha_1$-adrenoceptors of hepatocytes isolated from fed male rats produces acceleration of glycogenolysis accompanied by both intracellular Ca$^{2+}$ mobilization and cAMP accumulation, irrespective of the presence of extracellular Ca$^{2+}$ (5). The aim of the present study was to clarify whether or not glibenclamide, a potent second-generation sulfonylurea, modifies the $\alpha_1$-adrenoceptor-mediated metabolic actions in rat hepatocytes in the absence of extracellular Ca$^{2+}$.

Male Wistar rats (8–16 weeks of age, 300–500 g; Japan SLC, Inc., Hamamatsu) were used. The rats were allowed free access to water and standard laboratory food. Isolation of hepatocytes, the incubation procedure in the absence of extracellular Ca$^{2+}$, and the determination of metabolites, cAMP and intracellular Ca$^{2+}$ concentration ([Ca$^{2+}$]) have been described previously (5–7). $\alpha_1$-Adrenergic receptors of hepatocytes were stimulated with noradrenaline. In the present study, all incubations contained the $\beta$-adrenergic blocking agent propranolol, phosphodiesterase inhibitor IBMX (3-isobutyl-1-methylxanthine) and Ca$^{2+}$-chelating agent EGTA (ethyleneglycol-bis-(\(\alpha\)-aminoethyl ether)N,N'-tetraacetic acid). In the experiments for the determination of cAMP and metabolites, the effects of agonists were followed for the time indicated elsewhere. When the effects of sulfonylureas on the noradrenaline action were being studied, they were added 1 min prior to noradrenaline addition. When used, lemakalim was added 1 min prior to glibenclamide addition. Incubations for the measurements of [Ca$^{2+}$] were conducted for 345 sec. Agonists were added at 45 sec, and their effects were followed for 5 min. When the effect of glibenclamide on noradrenaline-induced Ca$^{2+}$ mobilization was being studied, it was added at 0 sec. Sulfonylureas and lemakalim were dissolved in dimethyl sulfoxide (DMSO), whose final concentration in the incubation medium was always 1% (v/v). The sources of materials used in this work were as follows: (–)-noradrenaline hydrochloride, tolbutamide, IBMX and EGTA (Sigma, St. Louis, MO, USA); propranolol hydrochloride (Sumitomo Chemical, Osaka); glibenclamide (Yamanouchi Pharmaceutical Co., Tokyo); and lemakalim (SmithKline Beecham, Philadel-
The results are expressed as means±S.E.M. Statistical evaluation of the data was made by means of Student's t-test for paired data.

Figure 1 shows that noradrenaline (10 μM) produces a progressive increase in cellular cAMP levels during the 15-min time-course. We found that the addition of glibenclamide (100 μM) significantly inhibited the noradrenaline-induced increase in cAMP accumulation (Fig. 1). In the presence of glibenclamide, the maximum cAMP levels were attained at 2.5 and 5 min after noradrenaline addition, and the cellular cAMP levels were progressively reduced during the following 10 min (Fig. 1). The results indicate that glibenclamide accelerates the disappearance of cellular cAMP once it is formed. Glibenclamide may stimulate cAMP phosphodiesterase (8), although the incubation medium is supplemented with IBMX (100 μM) in the present study. It was observed that the inhibition by glibenclamide of noradrenaline (10 μM)-induced increase in cAMP accumulation was dose-dependent (cAMP levels at 15 min after noradrenaline addition: DMSO + noradrenaline, 1960±257 pmol/g wet weight; glibenclamide (30 μM) + noradrenaline, 1687±253 pmol/g wet weight; glibenclamide (100 μM) + noradrenaline, 1054±166 pmol/g wet weight, n=6). We also found that tolbutamide (1 mM), a first-generation sulfonylurea, caused a significant (P<0.05) but less marked inhibition of noradrenaline-induced stimulation of cAMP accumulation at 15 min after noradrenaline addition (cAMP levels at 15 min after noradrenaline addition: DMSO + noradrenaline, 773±213 pmol/g wet weight; tolbutamide + noradrenaline, 629±167 pmol/g wet weight, n=5). Neither glibenclamide nor tolbutamide affected the basal cellular cAMP levels (data not shown).

Despite the significant inhibition by glibenclamide of noradrenaline-induced stimulation of cAMP accumulation, noradrenaline-induced increase in glucose release was not suppressed by glibenclamide but rather significantly increased (Table 1). Indeed, glibenclamide alone was found to exert a stimulatory effect on basal glucose release and lactate production and effects of glibenclamide on noradrenaline-induced increase in glucose release and decrease in lactate production in isolated rat hepatocytes.

Table 1. Effects of glibenclamide alone on basal glucose release and lactate production and effects of glibenclamide on noradrenaline-induced increase in glucose release and decrease in lactate production in isolated rat hepatocytes

| Addition                        | Glucose release (pmol/15 min/g wet wt.) | Lactate production (pmol/15 min/g wet wt.) |
|---------------------------------|----------------------------------------|-------------------------------------------|
| DMSO (control)                  | 33.8±3.9 (7)                           | 5.6±0.5 (7)                               |
| Glibenclamide (30 μM)           | 37.4±4.0* (7)                          | 9.6±1.6* (7)                              |
| Glibenclamide (100 μM)          | 40.2±4.0* (7)                          | 11.5±0.9* (7)                             |
| DMSO + Saline (control)         | 33.0±5.4 (4)                           | 5.6±1.3 (4)                               |
| DMSO + Noradrenaline (10 μM)    | 48.4±5.7* (4)                          | 1.2±0.7* (4)                              |
| Glibenclamide (100 μM) + Noradrenaline (10 μM) | 53.4±7.2* (4) | 4.6±0.6* (4) |

Hepatocytes were incubated for 15 min in the absence of extracellular Ca²⁺. Glibenclamide or noradrenaline were added at 0 min. When effects of glibenclamide or noradrenaline on metabolic effects were studied, it was added 1 min prior to noradrenaline addition. Results are expressed as means±S.E.M. Values in parentheses are the number of experiments using different hepatocyte preparations. *P<0.05, compared with the corresponding control values. **P<0.05, compared with noradrenaline addition.
established that an increase in \([Ca^{2+}]\), leads to an acceleration of glycogenolysis (9) and glycolysis (6) in hepatocytes. Thus, we determined whether glibenclamide increases \([Ca^{2+}]\) in hepatocytes. Figure 2A shows that glibenclamide fails to change \([Ca^{2+}]\). Furthermore, glibenclamide did not modify noradrenaline-induced \(Ca^{2+}\) mobilization (Fig. 2, B and C).

Paradoxical stimulation by sulfonylureas of hepatic glycogenolysis has been reported (4, 10). López-Alarcón et al. (4) demonstrated the dependency of glibenclamide actions on the presence of \(Ca^{2+}\) in the incubation medium. They suggested that an increase in \([Ca^{2+}]\), mediated the effects of sulfonylureas on liver metabolism. However, results of the present study first and clearly indicate that the effects of glibenclamide on hepatic metabolism, i.e., stimulation of glycogenolysis and glycolysis and inhibition of noradrenaline-induced increase in cAMP, are all produced in the absence of extracellular \(Ca^{2+}\) and are not accompanied by intracellular \(Ca^{2+}\) mobilization (Fig. 2). We observed that incubation of hepatocytes with glibenclamide (100 \(\mu M\)) for 15 min produced no alteration in whole-cell ATP content (ATP content in the presence of glibenclamide: at zero time, 1.54±0.10 \(\mu mol/g\) wet weight; at 15 min, 1.58±0.04 \(\mu mol/g\) wet weight, \(n = 3\)). The results indicate that the effects of glibenclamide on hepatic metabolism is not the consequence of a nonspecific toxic effect of this agent. The reason why glibenclamide suppression of the noradrenaline-induced increase in cAMP accumulation did not lead to inhibition of noradrenaline-induced glycogenolysis is not clear. cAMP levels in the compartment where the activation by noradrenaline of glycogen phosphorylase occurs may not be reduced by this agent. Another possibility is that direct activation of the glycolytic process by glibenclamide overcomes the influence of the reduction of cAMP levels. It has been demonstrated that chlorpropamide, a first-generation sulfonylurea, inhibits glucagon-stimulated gluconeogenesis in accordance with the abolition of glucagon-mediated increase in cAMP levels in perfused livers of fasted rats (3).

It is known that sulfonylureas inhibit ATP-sensitive \(K^+\) channels on the pancreatic \(\beta\)-cell membrane, activate voltage-dependent \(Ca^{2+}\) channels and trigger insulin secretion (11). We observed that lemakalim (100 \(\mu M\)), a \(K^+\)-channel opener, did not antagonize the metabolic effects of glibenclamide in isolated rat hepatocytes (data not shown). The existence of \(K^+\) channels in hepatocytes remains unproved (11).

Recently, it has been suggested that sulfonylureas inhibit hepatic gluconeogenesis by raising the intracellular content of fructose 2,6-bisphosphate (F-2,6-P\(_2\)) (12, 13). The increase in hepatic F-2,6-P\(_2\) levels elicited by sul-

**Fig. 2.** Effects of glibenclamide on intracellular \(Ca^{2+}\) concentration (\([Ca^{2+}]_i\)) in isolated rat hepatocytes. Hepatocytes were incubated for 345 sec in the absence of extracellular \(Ca^{2+}\). Panel A: effect of glibenclamide (Glib, 100 \(\mu M\)) alone on \([Ca^{2+}]_i\). Panel B: effect of noradrenaline (Nadr, 10 \(\mu M\)) alone on \([Ca^{2+}]_i\). Panel C: effect of glibenclamide (100 \(\mu M\)) on noradrenaline (10 \(\mu M\))-induced \(Ca^{2+}\) mobilization. Arrows indicate the time when agents are added. Traces shown are representative of five experiments using different hepatocyte preparations.
fonlyureas seems to be related to stimulation of the production of hexose 6-phosphate (10, 13) and inhibition of A-kinase (14, 15). F-2,6-P_2 levels were not determined in the present experiments. However, the significant stimulation of glycogenolysis and glycolysis observed in the present study (Table 1) suggests that this metabolite is also increased by glibenclamide in rat hepatocytes in the absence of extracellular Ca^{2+}.

The molecular mechanism underlying the hepatic actions of sulfonylureas that is independent of intracellular Ca^{2+} mobilization or cAMP accumulation needs to be further investigated.

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