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METABOLIC EFFECTS OF HYPOGLYCEMIC SULFONYLUREAS—V

IN VITRO EFFECTS OF SULFONYLUREAS ON MITOCHONDRIAL ADENOSINE TRIPHOSPHATASE ACTIVITY*

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Abstract—The ATPase (ATP phosphohydrolase, EC 3.6.1.4) stimulating activity of sulfonylureas has been examined. Sulfonylureas stimulate ATPase to variable extents, and this stimulation is oligomycin-sensitive. They are less active than 2,4-dinitrophenol and their relative stimulating activity parallels their uncoupling potency on oxidative phosphorylation. Carbutamide, which in itself does not stimulate ATPase, inhibits ATPase stimulation by other sulfonylureas. Experiments with combinations of chlorpropamide and P594, two active compounds, suggest that P594 penetrates more readily into mitochondria and is a stronger uncoupling agent. All sulfonylureas inhibit ATPase stimulated by optimal concentrations of dinitrophenol. Possible implications of these observations with regard to the metabolic effects of sulfonylureas are discussed.

SULFONYLUREAS have been shown to be uncouplers of oxidative phosphorylation of the dinitrophenol-type.[1] This classical uncoupler is also a potent stimulator of the latent ATPase activity of mitochondria[2–4] and this stimulation is completely abolished by oligomycin.[5] If sulfonylureas do resemble dinitrophenol in their interactions with mitochondrial metabolism, they should also stimulate mitochondrial ATPase. The effects of various sulfonylureas on mitochondrial ATPase are reported here together with their interference with DNP-stimulated and oligomycin-blocked mitochondrial ATPase.

MATERIALS AND METHODS

Preparation of mitochondria

Mitochondria were prepared from livers of overnight-fasted male Wistar rats (150–200 g) as described previously[1] except that the isolation medium contained 0·25 M sucrose, 0·01 M Tris–HCl pH 7·4 and 1 mM EDTA. The final mitochondrial pellet was suspended in 2 ml of 0·25 M sucrose, 0·01 M Tris–HCl pH 7·4/g liver. Mitochondrial protein was determined according to Lowry *et al.*[6] using bovine serum albumin as standard.

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Abbreviation: DNP = 2,4-dinitrophenol.

Determination of ATPase activity

The ATPase activity of the mitochondrial preparation was measured, in the absence of magnesium, essentially according to Myers and Slater.[7] Mitochondrial suspension (0·05 ml) containing 0·4–0·5 mg protein was added to 0·9 ml of incubation medium containing 0·25 M sucrose, 0·01 Tris–HCl pH 7·4 and 5 mM ATP and the volume was made up to 1·0 ml with either distilled water or appropriate additions. The reaction mixture was incubated for 10 min at 30° with occasional shaking. The reaction was terminated by the addition of 1·0 ml of ice-cold 10% TCA (w/v). After standing for 5 min on ice, precipitated protein was removed by centrifugation. Inorganic phosphate was determined on 1·0 ml of the supernatant according to Lowry and Lopez.[8] 'Zero time' and 'no addition' controls were routinely performed. ATPase activity is expressed as μmoles Pi/mg protein/10 min.

Chemicals

Acetohexamide was obtained through the courtesy of E. Lilly & Co., Indianapolis, Ind. U.S.A. Other sulfonylureas and chemicals were obtained from the same sources and prepared in the same way as described in publication IV of this series.[1]

Statistical treatment of data

Results are given as arithmetical means ± standard error of the mean. The significance of differences between means was established by Student's *t*-test.

RESULTS

Effect of sulfonylureas on ATPase

Table 1 shows the effect of increasing concentrations of various sulfonylureas on mitochondrial ATPase. Chlorpropamide, P1458, P594 and acetohexamide stimulate ATPase although to a lesser extent than DNP, whereas carbutamide has no effect. Tolbutamide has the same effect as chlorpropamide (data not shown). Stimulation of ATPase by P1458 and P594 is maximal at 10^{-4} M drug and falls off sharply at higher concentrations.

Preincubation of mitochondria at 0° in the presence of sulfonylureas did not increase their percent stimulatory activity although the stimulatory effect of 10^{-4} M tolbutamide or chlorpropamide was significant, as well as the effect of 5×10^{-3} M carbutamide. An identical significant effect of 10^{-4} M chlorpropamide was found in the control experiments of Fig. 1 (control values: 0·294 ± 0·003; experimental values: 0·341 ± 0·044; $n = 40$; $P < 0{\cdot}001$; 16 per cent stimulation).

Effect of sulfonylureas and oligomycin on ATPase

The effect of sulfonylureas on ATPase was examined in the presence of oligomycin which is a powerful inhibitor of latent ATPase and DNP-stimulated ATPase.[5] Inhibition of ATPase by oligomycin and stimulation by sulfonylureas are shown in Table 2. When oligomycin and sulfonylureas are used together, ATPase activity decreases to the level obtained in the presence of oligomycin alone.

Effect of combinations of sulfonylureas on ATPase

Carbutamide having by itself very little effect on ATPase (Table 1) its effect on the

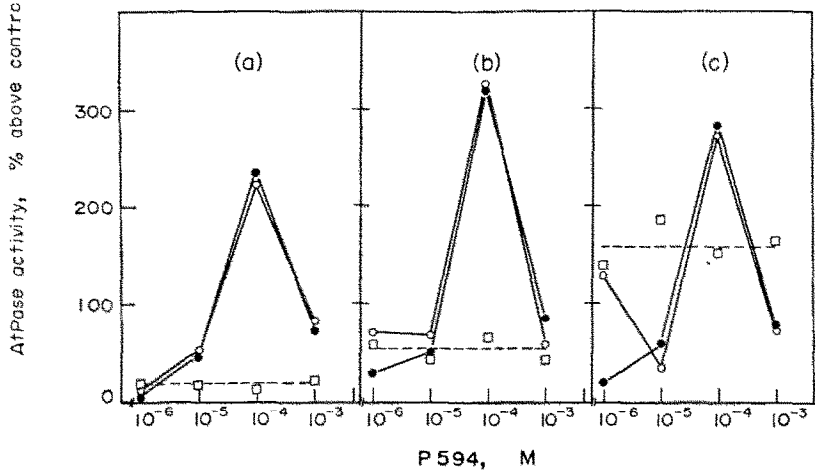

FIG. 1. Mitochondrial ATPase activity in the presence of increasing concentrations of P 594 combined with chlorpropamide 10^{-4} M(a), 10^{-3} M(b) and 5×10^{-3} M(c). Experimental conditions as in Table 1. (□) Chlorpropamide alone (broken line is mean of four values shown). (●) P 594 alone. (○) Chlorpropamide + P 594. Each point represents the mean of ten experiments.

TABLE 1. EFFECT OF SULFONYLUREAS ON ATPASE ACTIVITY OF ISOLATED RAT LIVER MITOCHONDRIA

Sulfonylurea	(M)	Control	Sulfonylurea	Increase above control (%)
		μmoles Pi/mg protein/10 min		
Chlorpropamide	10^{-5} (10)	0·288 ± 0·003	0·299 ± 0·005	4
	10^{-4} (6)	0·285 ± 0·011	0·330 ± 0·018	16
	10^{-3} (6)	0·283 ± 0·011	0·510 ± 0·043*	79
	5×10^{-3} (6)	0·283 ± 0·010	0·738 ± 0·035*	161
Carbutamide	10^{-4} (9)	0·258 ± 0·030	0·314 ± 0·023	22
	10^{-3} (8)	0·258 ± 0·028	0·307 ± 0·026	19
	5×10^{-3} (9)	0·254 ± 0·025	0·299 ± 0·031	18
P 1458	10^{-6} (10)	0·254 ± 0·003	0·376 ± 0·008*	48
	10^{-5} (10)	0·253 ± 0·002	0·629 ± 0·008*	148
	10^{-4} (10)	0·253 ± 0·004	1·194 ± 0·037*	372
	10^{-3} (10)	0·223 ± 0·005	0·331 ± 0·008*	48
P 594	10^{-6} (10)	0·187 ± 0·010	0·239 ± 0·010*	28
	10^{-5} (10)	0·253 ± 0·009	0·429 ± 0·014*	69
	10^{-4} (10)	0·259 ± 0·008	0·908 ± 0·012*	250
	10^{-3} (10)	0·254 ± 0·005	0·391 ± 0·028*	45
Acetohexamide	10^{-6} (10)	0·186 ± 0·003	0·233 ± 0·004*	25
	10^{-5} (10)	0·254 ± 0·007	0·332 ± 0·005*	31
	10^{-4} (10)	0·266 ± 0·002	0·370 ± 0·020*	39
	10^{-3} (10)	0·246 ± 0·010	0·439 ± 0·020*	78
	$2{\cdot}5 \times 10^{-3}$ (10)	0·218 ± 0·011	0·488 ± 0·027*	128
Dinitrophenol	10^{-6} (6)	0·331 ± 0·012	0·495 ± 0·018*	49
	10^{-5} (6)	0·326 ± 0·030	1·050 ± 0·108*	222
	10^{-4} (6)	0·281 ± 0·009	3·105 ± 0·093*	1005
	5×10^{-4} (6)	0·292 ± 0·036	0·425 ± 0·041*	45

ATPase activity was measured as described under Materials and Methods. The reaction was started by adding the mitochondrial suspension to the incubation medium. Results are presented as means ± S.E.M. of the number of experiments given in parentheses. Significance is indicated as: *$P < 0{\cdot}005$.

TABLE 2. EFFECT OF SULFONYLUREAS AND OLIGOMYCIN ON ATPASE ACTIVITY OF ISOLATED RAT LIVER MITOCHONDRIA

Sulfonylurea	(mM)	μmoles Pi/mg protein/10 min			
		Control	Oligomycin	Sulfonylurea	Oligomycin + Sulfonylurea
Chlorpropamide	5·0	0·257 ± 0·008	0·042 ± 0·009	0·641 ± 0·039	0·042 ± 0·005
Tolbutamide	5·0	0·213 ± 0·021	0·030 ± 0·007	0·605 ± 0·075	0·040 ± 0·010
Acetohexamide	1·0	0·245 ± 0·014	0·055 ± 0·010	0·462 ± 0·023	0·075 ± 0·008
P 594	0·1	0·236 ± 0·011	0·030 ± 0·005	0·670 ± 0·013	0·044 ± 0·001
P 1458	0·1	0·240 ± 0·029	0·038 ± 0·011	0·916 ± 0·098	0·038 ± 0·006

Experimental conditions as in Table 1. Oligomycin was used at the concentration of 2 μg/ml. Results are given as means ± S.E.M. (n = 6).

stimulatory activity of more potent sulfonylureas was examined. The results in Table 3 show that in the presence of 1 mM carbutamide the other sulfonylureas stimulate ATPase to a lesser degree than when used alone. This effect is even more pronounced at 5 mM carbutamide although the residual ATPase activity remains well above control values.

TABLE 3. COMBINED EFFECTS OF CARBUTAMIDE (CB) AND OTHER SULFONYLUREAS ON ATPASE ACTIVITY OF ISOLATED RAT LIVER MITOCHONDRIA

Sulfonylurea	(mM)	μmoles Pi/mg protein/10 min			
		Control	Sulfonylurea	1 mM CB + sulfonylurea	5 mM CB + sulfonylurea
Chlorpropamide	1·0	0·270 ± 0·018	0·397 ± 0·026	0·358 ± 0·027	0·302 ± 0·023*
	5·0	0·242 ± 0·005	0·526 ± 0·005	0·388 ± 0·005†	0·420 ± 0·014†
Tolbutamide	1·0	0·257 ± 0·006	0·425 ± 0·012	0·410 ± 0·014	0·322 ± 0·009†
	5·0	0·183 ± 0·003	0·414 ± 0·004	0·412 ± 0·005	0·309 ± 0·003†
Acetohexamide	1·0	0·196 ± 0·005	0·369 ± 0·006	0·349 ± 0·009	0·302 ± 0·010†
	2·5	0·218 ± 0·011	0·488 ± 0·027	0·414 ± 0·010*	0·338 ± 0·011†
P 594	0·01	0·178 ± 0·004	0·297 ± 0·007	0·277 ± 0·006*	0·218 ± 0·008*
	0·1	0·175 ± 0·006	0·780 ± 0·008	0·693 ± 0·011†	0·538 ± 0·008†
	1·0	0·298 ± 0·067	0·625 ± 0·056	0·609 ± 0·049	0·500 ± 0·033†

Experimental conditions as in Table 1. Results are presented as means ± S.E.M. (n = 10). All values in column 2 are significantly different from their corresponding control values with $P < 0·001$. Data in columns 3 and 4 have been compared with the corresponding value in column 2. Significance is given as: * $P < 0·05$; † $P < 0·001$.

In view of these results the effect of combinations of two more potent ATPase stimulators has been investigated.

Figure 1a indicates that 10^{-4} M chlorpropamide (broken line) which is slightly but significantly active by itself does not interfere with the effect of increasing concentrations of P594 on ATPase. At higher concentrations (10^{-3} M in Fig. 1b and 5×10^{-3} M in Fig. 1c) chlorpropamide exhibits its own stimulatory activity in the presence of

10^{-6} M P594 but does not influence the effect of P594 on ATPase activity at higher concentrations of P594.

Effect of sulfonylureas on DNP-stimulated ATPase

In order to examine further the mechanism whereby sulfonylureas stimulate ATPase, their effect on DNP-stimulated ATPase was investigated. Figures 2 and 3 (open circles) show that, in the presence of 10^{-4} M DNP, ATPase activity is increased approximately 10-fold compared with basal values of 0·250–0·380 μmoles Pi/mg protein/10 min.

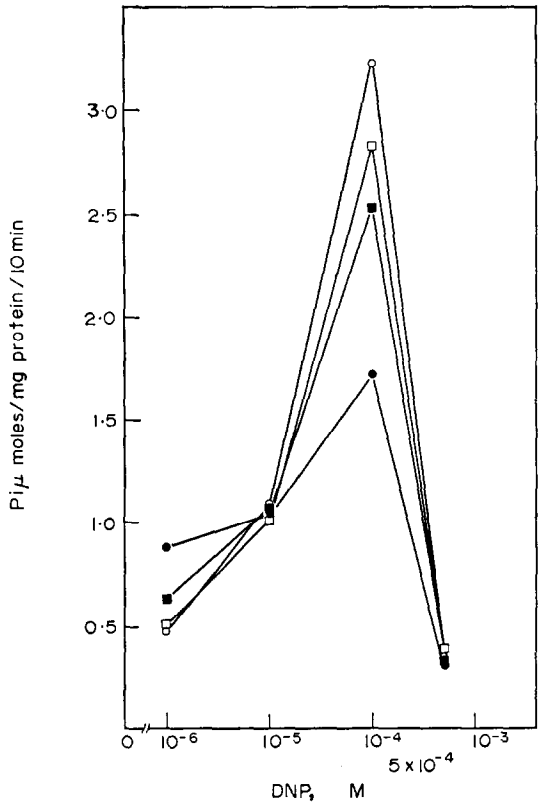

FIG. 2. Effect of combinations of DNP and tolbutamide on ATPase. Experimental conditions as in Table 1. (○) DNP alone. (□) DNP + 10^{-5} M tolbutamide. (■) DNP + 10^{-3} M tolbutamide. (●) DNP + 5×10^{-3} M tolbutamide. Each point represents the mean of ten experiments.

Two patterns of interaction between DNP and sulfonylureas appear on these figures. One is characteristic for the most active stimluators of ATPase and another one for sulfonylureas which are less active or inactive. Tolbutamide (Fig. 2) is a typical example of the first kind of interaction. In the presence of 10^{-6} M DNP, it exerts an additional dose-dependent stimulation of ATPase. At 10^{-5} M DNP no clear effect of tolbutamide can be seen whereas at 10^{-4} DNP the sulfonylurea becomes an inhibitor of DNP-stimulated ATPase. Finally, at 5×10^{-4} M DNP, ATPase activity is no longer stimulated either by DNP alone or in combination with tolbutamide. Chlor-

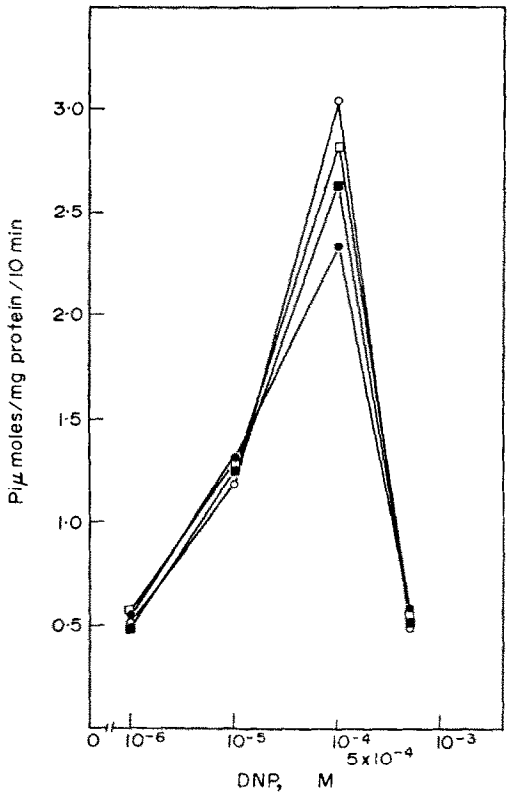

FIG. 3. Effect of combinations of DNP and carbutamide on ATPase. Experimental conditions as in Table 1 (○) DNP alone. (□) DNP + 10^{-5} M carbutamide. (■) DNP + 10^{-3} M carbutamide. (●) DNP + 5×10^{-3} M carbutamide. Each point represents the mean of ten experiments.

propamide, which is as potent a stimulator of ATPase as tolbutamide, shows the same pattern of interaction when used in combination with DNP. On the other hand, carbutamide (Fig. 3) which does not stimulate ATPase interferes only with DNP 10^{-4} M and acts as a rather weak inhibitor of DNP-stimulated ATPase. In the case of acetohexamide the stimulatory activity of the drug (Table 1) is no longer seen in the presence of 10^{-6} M DNP whereas acetohexamide appears to be the most potent among sulfonylureas as an inhibitor of DNP-stimulated ATPase (approximately 60 per cent inhibition at $2{\cdot}5 \times 10^{-3}$ M drug and 10^{-4} M DNP).

DISCUSSION

In the preceding publication,[1] sulfonylureas were shown to be dinitrophenol-like uncouplers of oxidative phosphorylation.

In the present study we examined the effect of sulfonylureas on the latent ATPase activity of mitochondria which represents a reversal of oxidative phosphorylation[9] and is strongly stimulated by uncouplers of the dinitrophenol type[2–4,9]

Table 1 shows that sulfonylureas stimulate ATPase to variable extents. They are less active stimulators than 2,4-dinitrophenol and their relative stimulating activity parallels their uncoupling potency.[1] The absence of stimulatory effect by sulfonyl-

ureas in the presence of oligomycin (Table 2) further supports the conclusion that these compounds display a dinitrophenol-like activity.

The dose dependent inhibition by carbutamide of sulfonylurea-stimulated ATPase (Table 3) indicates that this almost inactive compound nevertheless strongly interacts with the processes involved in the stimulatory effect of other sulfonylureas. Another type of interaction between sulfonylureas is apparent from the observation that chlorpropamide, in combination with P594, exerts its dose-dependent stimulation only in the presence of the lowest concentration of P594 while even at high concentrations, chlorpropamide does not interfere with the effect of P594 (Fig. 1). On the other hand, the inactive carbutamide interferes more with the stimulatory effect of P594 than chlorpropamide (Table 3). This points towards complex interactions between sulfonylureas with respect to transport into mitochondria and/or interference with the ATPase complex.

Similar interactions have been observed between sulfonylureas and DNP. In the presence of low concentrations of dinitrophenol, stimulatory sulfonylureas exert their own effect whereas inactive compounds have no effect. All sulfonylureas however inhibit, to variable extents, ATPase stimulated by optimal concentrations of dinitrophenol. This again could be the result of competition for uptake by mitochondria, for interference with ATPase reactions or both. An alternative explanation can be proposed in view of the results obtained at the highest concentrations of P594 (Fig. 1) or dinitrophenol (Figs. 2 and 3). The very limited stimulation of ATPase seen under these conditions can be interpreted as an inhibition of ATP uptake by the high concentrations of uncoupling agent. Indeed, Kraayenhof and Van Dam[10] have shown that stimulation of ATPase activity by increasing concentrations of dinitrophenol increases with the concentration of ATP. The relative inhibition seen at high DNP concentrations is less pronounced at high ATP levels. The authors concluded that DNP inhibits ATP uptake and this might also be the case when sulfonylureas are used in combination with 10^{-4} M dinitrophenol so that the total concentration of uncouplers becomes inhibitory for ATP uptake by mitochondria. The experiments reported here were designed to examine the effect of sulfonylureas on mitochondrial ATPase rather than to unravel the details of the mechanism of their uptake by mitochondria and of their interactions with the membrane-bound multienzymatic systems of oxidative phosphorylation. Detailed kinetic studies would be required in order to establish the mechanisms by which sulfonylureas exert their effects on mitochondria and to determine exactly the kind of inhibition that takes place when they are used in combination with each other or with dinitrophenol.

These results support our earlier conclusions that sulfonylureas, at clinically relevant concentrations, interfere *in vitro* with mitochondrial mechanisms of energy conservation and that this could be important in the understanding of their long-term *in vivo* therapeutic or metabolic effects.

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