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Effects of Benzodiazepines on the Transport of Sugars and Ions in Rat Skeletal Muscle *in Vitro*

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SUMMARY

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The benzodiazepine drugs, diazepam and chlordiazepoxide, have a dual effect on sugar and ion transport in intact rat hemidiaphragms *in vitro*. At low concentrations they depress 3-methylglucose transport and $^{45}Ca^{++}$ influx and increase Na^+ and K^+ gradients. At higher concentrations all these effects are reversed. It is suggested that the changes in sugar transport are a consequence of alterations in Ca^{++} fluxes and distribution caused directly or indirectly by these drugs.

INTRODUCTION

The anticonvulsant drug phenytoin (5,5-diphenylhydantoin) has been reported to increase transmembrane gradients of Na^+ and K^+ in brain (1, 2) and muscle (3) under some conditions. Its effect on Na^+ and K^+ gradients and sugar transport in rat diaphragm is twofold (3). Cellular Na^+ was decreased at lower and increased at higher concentrations of the drug; sugar transport was affected in parallel to the intracellular Na^+ level and was apparently related to the coupled Na^+-Ca^{++} exchange mechanism at the cell membrane (4). This and other indirect evidence linking changes in Ca^{++} distribution and sugar transport, has led to the proposal (4, 5) that an as yet unidentified cellular pool of Ca^{++} may function as regulator of the glucose carrier in the muscle cell membrane. It is consistent with this hypothesis that "membrane-stabilizing" drugs, known to interfere with Ca^{++} fluxes or its binding to the cell membrane, also influence sugar transport, often in a biphasic manner (6).

To further study the dependence of sugar transport on ion levels in rat skeletal muscle, we have used as pharmacological tools the two benzodiazepines, diazepam and chlordiazepoxide. Although they are chemically unrelated to phenytoin, some benzodiazepines are also effective anticonvulsants [for review see (7)] and diazepam strikingly resembles phenytoin in spatial conformation (8).

METHODS

The procedures for tissue incubation and sample analysis have been described in detail previously (9). "Intact" rat hemidiaphragms (10) were incubated at 37° in modified Krebs-Henseleit bicarbonate solution, containing 143.5 mM Na^+, 5.8 mM K^+, 1.16 mM Mg^{++}, 1.25 mM Ca^{++}, 124.5 mM Cl^-, 1.16 mM $H_2PO_4^-$, 1.16 mM SO_4^{--}, 25 mM HCO_3^-, pH 7.4, gassed with 95% O_2-5% CO_2. The tissue/medium distribution of the ^{14}C-labeled nonmetabolized glucose analogue 3-*O*-methyl-D-glucose and of [^3H]labelled in-

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ulin and the levels of Na^+ and K^+ were determined in the same tissue samples. Ca^{++} influx was estimated from the tissue/medium distribution of $^{45}Ca^{++}$ in similar experiments. Incubation with 3-methylglucose or ^{45}Ca and other additions was for 30 min, preceded by a preincubation period of 20 min in the presence of drugs to be tested. Concentrations of ^{14}C-sugar, ^{45}Ca, Na^+ and K^+ in the intracellular water space were calculated by correction for the inulin space. Results for sugar and ^{45}Ca uptake are given as "penetration," i.e., the concentration in the intracellular water is expressed as percent of the final concentration in the incubation medium. The mean of paired differences in penetration between control and treated hemidiaphragms is taken as a measure of drug effect. This semiquantitative expression of changes in transporting activity was used because determination of unidirectional influx rates is not feasible in solid tissues (9). Diazepam was added as the commercial injectable solution (Roche) and chlordiazepoxide solution was freshly prepared in a propyleneglycol-containing commercial solvent (Roche). Control tissues were incubated with appropriate amounts of the respective solvents. Ca^{++}-free media were prepared by omitting $CaCl_2$ from the standard medium and replacing it isosmotically with NaCl. To maintain stability of the solution, incubations with chlordiazepoxide were done in the dark. Statistical evaluation was by Student's *t*-test applied to paired samples.

RESULTS

Fig. 1 shows that sugar transport was significantly depressed by low concentrations of diazepam and stimulated by higher ones. This biphasic effect was much more pronounced in the presence of insulin, as was earlier also found for phenytoin (3). The effective concentrations of diazepam were fairly high, around 0.3 and 1.0 mM for

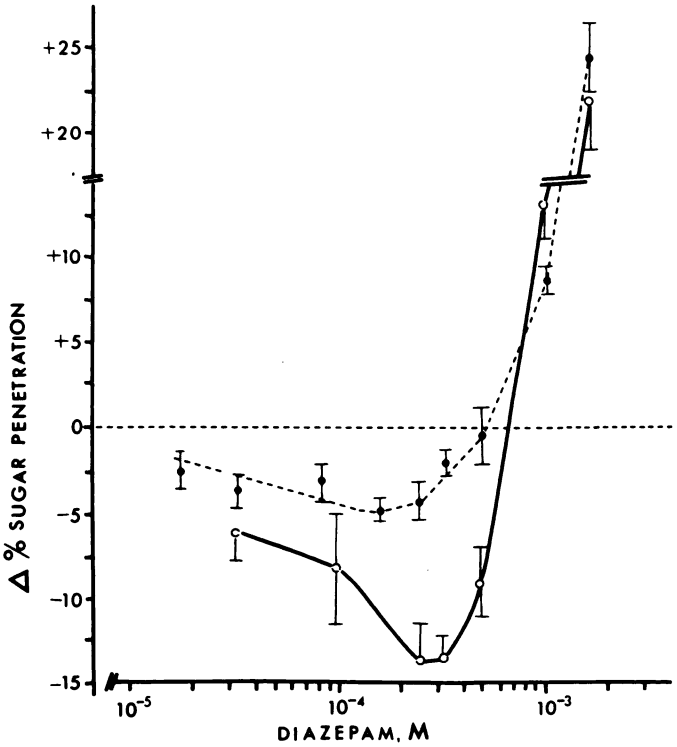

FIG. 1. *Effect of diazepam on the intracellular penetration of ^{14}C-3-methylglucose in rat hemidiaphragms* The data are means of paired differences (± standard error). N = 6 to 12 pairs. 3-methylglucose concentration, 5.0 mM. ●——● basal uptake, ○——○ uptake in the presence of insulin, 0.25 m-unit/ml. All differences from paired control hemidiaphragms, except those at 1.7×10^{-5} M and 3.3×10^{-5} M, are highly significant, $p < 0.01$.

the inhibitory and the stimulatory effects, respectively. The effects of two representative concentrations on sugar and ion transport are summarized in Table 1. With 0.35 mM diazepam there was a small but significant increase in Na^+ and K^+ gradients; in contrast, with 1.05 mM the ion gradients were greatly decreased. The changes in ion content were unaffected by insulin, and the data shown are pooled from experiments with and without the hormone. The increase in Na^+ and K^+ gradients was less marked at lower drug concentrations but was significant down to 3.3×10^{-5} M diazepam (not shown), in parallel to the depression of sugar transport shown in Fig. 1.

^{45}Ca influx was significantly depressed at the lower concentration of diazepam and increased at the higher concentration of the drug, suggesting a positive correlation between activity of the sugar transport system and Ca^{++} influx. However, sugar transport in Ca^{++}-free medium was affected in the same manner as in standard medium, indicating that the alterations in sugar transport are not dependent exclusively on the influx of Ca^{++} into the muscle cells.

Comparison of control values indicates that higher concentrations of the commercial solvent for diazepam (which was added to all control flasks in appropriate concentrations) decreased the stimulation of sugar transport by insulin. This decrease could be overcome by raising the insulin concentration. As this effect did not prevent the rise in sugar transport elicited by high levels of diazepam, it was not investigated further.

As shown in Table 2, another benzodiazepine drug, chlordiazepoxide, had essentially the same biphasic effects on basal sugar transport, intracellular levels of Na^+ and K^+ and influx of ^{45}Ca. However, both the low and the high concentration of the drug inhibited insulin-stimulated sugar transport although, like diazepam, the high concentration depressed Na^+ and K^+ gradients and increased ^{45}Ca influx.

DISCUSSION

The working hypothesis mentioned above (4, 5) links sugar transport regulation in muscle to the binding of Ca^{++} to specific regulatory sites and predicts that any increase in cytoplasmic Ca^{++} available for binding should activate sugar transport. The two aspects of the dual effect of benzodiazepines on sugar transport will be considered in relation to this concept.

TABLE 1

Effects of diazepam

Rat hemidiaphragms were incubated with and without diazepam as described in the text. Results are means ± standard error, and the number of paired experiments is indicated in parentheses. Data for Na^+ and K^+ are pooled from experiments with and without insulin (0.25 m-unit/ml); those for ^{45}Ca and ^{14}C-sugar penetration are only with insulin. All experiments except those in the last column were done in standard Krebs-Henseleit medium (see METHODS).

	Intracellular ion level		^{45}Ca penetration	^{14}C-sugar penetration	
	Na^+	K^+		In standard medium	In Ca^{++}-free medium
	mM		%	%	
Control	24.8 ± 1.6	132.6 ± 4.7	65.4 ± 3.0	42.0 ± 1.6	45.9 ± 3.3
Diazepam, 0.35 mM	16.9 ± 0.9	139.4 ± 4.8	50.2 ± 3.0	31.3 ± 3.2	35.0 ± 3.0
Paired difference	−7.9 ± 1.0(35)	+6.8 ± 1.0(35)	−15.2 ± 2.2(16)	−10.7 ± 1.3(23)	−10.9 ± 2.2(8)
Control	21.3 ± 1.6	133.6 ± 5.2	70.1 ± 4.1	32.9 ± 1.7	32.5 ± 3.4
Diazepam, 1.05 mM	71.1 ± 8.4	86.0 ± 8.6	137.0 ± 13.2	44.2 ± 23	44.0 ± 3.1
Paired difference	+49.7 ± 7.1(21)	−47.6 ± 4.5(21)	+67.0 ± 10.0(10)	+13.3 ± 1.7(9)	+11.5 ± 1.4(5)*

* $p < 0.005$, all others $p < 0.001$.

TABLE 2

Effects of chlordiazepoxide

Rat hemidiaphragms were incubated with and without chlordiazepoxide as described in the text. Results are mean paired differences from controls (± standard error). The number of paired experiments is indicated in parentheses.

Chlordiazepoxide	Insulin 0.25 m-unit/ml	Δ Sugar penetration	Δ ^{45}Ca penetration	Δ ion level	
				Na^+	K^+
mM		%	%	*mM*	
Standard medium					
0.3	−	−12.4 ± 2.8(5)[a]	−11.7 ± 3.6(5)[a]	−9.9 ± 2.0(5)[b]	+8.9 ± 2.4(5)[a]
	+	−14.6 ± 1.4(14)	−14.8 ± 3.8(10)[b]	−6.8 ± 1.7(32)	+11.6 ± 1.4(28)
0.9	−	−6.3 ± 1.0(18)		−6.6 ± 1.1(17)	+6.2 ± 1.5(8)[b]
	+	−19.2 ± 1.2(26)	−12.8 ± 2.9(12)[b]	−7.2 ± 1.0(16)	+7.4 ± 2.2(16)[b]
3.0	−	+10.8 ± 1.2(15)		+60.7 ± 2.8(9)	−58.3 ± 2.4(9)
	+	−25.2 ± 1.6(28)	+76.4 ± 7.4(16)	+40.6 ± 2.5(25)	−39.6 ± 2.3(20)
Ca^{++}-free medium					
0.3	+	−14.8 ± 2.3(6)[b]		−8.1 ± 2.0(4)	+11.2 ± 3.4(5)[a]
0.9	+	−21.9 ± 2.6(6)		−6.0 ± 1.3(5)	+11.4 ± 3.9(4)[a]

[a] $p < 0.05$, [b] $p < 0.01$, all others $p < 0.001$.

Sugar transport was decreased by low concentrations of the drugs which decreased internal Na^+ and, consequently, depressed Na_i^+-Ca_o^{++} exchange (11, 12). Conversely, when internal Na^+ levels and Na_i^+–Ca_o^{++} exchange were increased by high levels of benzodiazepines, sugar transport was also enhanced. The observed changes in ^{45}Ca influx are consistent with such a mechanism. Similar effects were also seen with phenytoin (3) or ouabain (4) and with other interventions inhibiting the Na^+ pump. Thus, high concentrations of membrane stabilizers appear to have a "destabilizing effect"; this is expressed in increased permeability to sugars and ions (5, 6), induction of hemolysis in erythrocytes (13), inhibition of Na^+, K^+-ATPase and a decrease in Na^+ and K^+ gradients (2, 11, 12), etc.

Benzodiazepines could also influence sugar transport via a more direct effect on Ca^{++} fluxes and distribution. Low concentrations of benzodiazepines may interfere with Ca^{++}-membrane interactions, an effect characteristic of membrane stabilizers (13), and thus influence the availability of Ca^{++} for binding to the regulatory site and also, perhaps, the binding process itself; the latter effect would not cause a change in the influx of ^{45}Ca. Although such a direct effect on Ca^{++} does occur, the present data do not indicate clearly its role in the regulation of sugar transport. It might contribute to the decrease in sugar transport at "stabilizing" (low) drug concentrations but the alterations in sugar transport were also observed in a Ca^{++}-free medium, suggesting that changes in intracellular Ca^{++} distribution not involving influx across the sarcolemma may be involved. However, as incubation of a piece of muscle in Ca^{++}-free medium may not remove all Ca^{++} from the cell surface, the interpretation of these experiments remains ambiguous. It should be noted that inhibition of insulin-stimulated sugar transport by another group of membrane stabilizers, the local anesthetics, is well documented (6).

In the present experiments, an apparent exception to the correlations discussed above is the observation that 3 mM chlordiazepoxide inhibited insulin-stimulated sugar transport although basal sugar transport, ^{45}Ca influx and internal Na^+ level were increased and although the effects of the low drug concentration also paralleled those of diazepam. A possible explanation for this discrepancy could be that the direct effect of the drug on Ca^{++}-membrane interactions may override the indirect effect mediated by the increase in Na^+-Ca^{++} exchange. In other words, the activated state of the sugar carrier may be more sensitive to drug interference at the membrane level than to the rise in cytoplasmic Ca^{++}. A similar difference between effects on basal and on stimulated transport has been ob-

erved with long chain (14) and short chain 9) fatty acids and with the Ca-ionophore, A-23187 (15).

As long as the characteristics of the hypothetical Ca^{++}-binding site and the effective Ca_i^{++} concentrations remain unknown, evidence on the role of Ca^{++} in sugar transport regulation in muscle will of necessity be indirect. All one can do is look for a consistent pattern of changes in sugar transport when Ca^{++} fluxes and distribution are altered by a variety of means. The present data turn out to be largely consistent with the working hypothesis. Although the drug concentrations found effective in these experiments are far higher than those required for the specific central nervous system effects of benzodiazepines, it is of interest that they share with another anticonvulsant, phenytoin, several effects on skeletal muscle, such as the dual effect on Na^+ and K^+ gradients and membrane stabilizing action. The changes in rate of sugar transport appear to follow these more general alterations.

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REFERENCES

1. Woodbury, D. M. (1955) *J. Pharmacol. Exp. Ther.*, **115**, 74–95.
2. Festoff, B. W. & Appel, S. H. (1968) *J. Clin. Invest.* **47**, 2752–2758.
3. Bihler, I. & Sawh, P. C. (1971) *Biochim. Biophys. Acta*, **249**, 240–251.
4. Bihler, I. (1972) in *The Role of Membranes in Metabolic Regulation* (Mehlman, M. A. & Hanson, R. W., eds.), pp. 411–423, Academic Press, New York.
5. Clausen, T. (1975) in *Current Topics in Membranes and Transport* Vol. 6, (Bronner, F. & Kleinzeller, A., eds.) pp. 169–226, Academic Press, New York.
6. Clausen, T., Harving, H. & Dahl-Hansen, A. B. (1973) *Biochim. Biophys. Acta*, **298**, 393–411.
7. Woodbury, D. M. & Fingl, E. (1975) in *The Pharmacological Basis of Therapeutics*, Ed. 5 (Goodman, L. S. & Gilman, A., eds.) pp. 216–218, Macmillan, New York.
8. Camerman, A. & Camerman, N. (1970) *Science*, **168**, 1457–1458.
9. Bihler, I. & Sawh, P. C. (1973) *Can. J. Physiol. Pharmacol.*, **51**, 371–377.
10. Kono, T. & Colowick, S. P. (1961) *Arch. Biochem. Biophys.*, **93**, 514–519.
11. Glitsch, H. G. & Reuter, H. (1970) *J. Physiol.*, **209**, 25–43.
12. Blaustein, M. P. (1977) *Am. J. Physiol.*, **232**, C165–C173.
13. Seeman, P. (1972) *Pharmacol. Rev.*, **24**, 583–655.
14. Bihler, I. & Sawh, P. C. (1972) *Fed. Proc.*, **31**, 287 Abs.
15. Bihler, I. & Sawh, P. C. (1977) *Fed. Proc.*, **36**, 565 Abs.