Wedeloside, a powerful inhibitor and ligand of the mitochondrial ADP/ATP carrier

Martin Klingenberg, Maria Appel and Peter B. Oelrichs+

Institut für Physikalische Biochemie der Universität München, Goethestrasse 33, 8000 München 2, FRG

Received 28 June 1985

The effect of wedeloside, an atractyloside analogue occurring in the Australian weed *Wedelia asperrima*, on the ADP/ATP carrier is investigated on 3 levels; the ADP stimulated respiration, the ADP/ATP exchange, and the binding in competition with atractylate (ATR) and carboxyatractylate (CAT). The inhibition of respiration and of ADP/ATP exchange by wedeloside is nearly uncompetitive with ADP. The competitive binding with [^{3}H]CAT and [^{3}H]ATR reveals a high binding affinity of wedeloside similar to that of CAT. The titration of the ADP/ATP exchange and of the binding with wedeloside gives a titer of 0.35 μmol/g protein for rat liver protein and the titer of the binding gives 2.7 μmol/g protein for beef heart mitochondria. The 1.8-times higher titers than with CAT may indicate that two molecules of wedeloside bind to one ADP/ATP carrier dimer in contrast to the half site reactivity known for the binding of the other ligands.

Wedeloside *Mitochondria* *ADP/ATP carrier* *Inhibition*

1. INTRODUCTION

The ADP translocation system in mitochondria from all sources tested so far is inhibited by certain glucosides containing the diterpenoid type aglycon atractylogenin. The original source for these highly specific and effective inhibitors of the ADP/ATP exchange was the thistle *Atractylis gummifera* from which three closely related glucosides have been isolated and identified (review [1]). It is suggested that CAT is the original product in this plant and that atractyloside and epi-atractyloside are less efficient derivatives obtained by decarboxylation during extraction. Also some other composita are reported to produce CAT [2]. In the *Rubiacea coffea* arabica, atractylogenin glucosides were found in relatively large amounts with a glucuronic instead of glucose moiety [3]. These coffee glucosides are considerably less toxic and act less strongly as inhibitors of the ADP/ATP exchange [4].

In 1980 Oelrichs et al. [5] reported the occurrence in the Australian weed *Wedelia asperrima* of a poisonous glucoside which they called wedeloside (WED). In the chemical structure analysis [5,6] the aglycone was found to be quite similar to atractylogenin, the only difference being an additional hydroxyl group in position 13 of the diterpenoid ring structure. Larger differences were reported for the glucose moiety, consisting in WED of amino glucose with isovaleric acid bound as an amide instead of as an ester. Furthermore, in WED phenylpropionyl acid replaces sulfuric acid in atractyloside and the other sulfuric ester position remains unsubstituted.

Because of the similarity in structure it was suggested quite early that WED might act on mitochondria in a way similar to carboxyatractyloside [8]. ADP-stimulated respiration in liver mitochondria was found to be inhibited by WED but inhibition required about 4-times higher titers than in the case of carboxyatractyloside.

+ Present address: Animal Research Institute, 665 Fairfield Road, Yeerongpilly, Q.4105, Australia

Abbreviations: ATR, atractylate; CAT, carboxyatractylate; WED, wedeloside

Published by Elsevier Science Publishers B.V. (Biomedical Division)
00145793/85/$3.30

The very interesting structural difference of WED compared to ATR and the great value of these compounds in elucidating this important translocation process made it seem worthwhile to investigate more closely the effects of WED on the ADP/ATP exchange system. In all these studies WED type 1 was used.

2. RESULTS

The classical assay for plant glycosides has been the inhibition of ADP-stimulated respiration in rat liver mitochondria. The results from titrating this respiratory activity with increasing amounts of WED are shown in fig.1. To discern a possible competition of WED with ADP, the latter was applied at 3 different concentrations. In exactly parallel experiments the respiration was titrated with CAT. At maximum inhibition the same level (35%) of residual respiratory activity was obtained both with WED and with CAT. A weak competition between ADP and WED is indicated by the increase of WED required for half-maximum inhibition going from 0.3 to 0.38 to 0.46 μmol WED while going from 0.2 to 0.5 to 1 mM ADP. This weak competition by ADP shows that WED binds quite tightly. In comparison, the inhibition of respiration by CAT exhibits an even smaller, barely distinct competition with CAT.

The main difference between the effects of CAT and WED on respiration is the titer, only about 0.15 nmol CAT/mg protein being required, whereas an about 1.8-times higher amount of WED is required for the inhibition of ADP-stimulated respiration.

More directly the effect of WED can be studied on the ADP/ATP exchange between external and internal nucleotides of mitochondria (fig.2A and B). Again 3 different concentrations of ADP were used and the inhibition compared to that of CAT under identical conditions. The percentage of exchange was linearized by using the logarithmic expression, taking into account the first order kinetics of the exchange reaction so that the degree of inhibition can be proportionally evaluated [9,10]. The exchange activity decreases fairly linearly with increasing amounts of WED. The amount required for half inhibition is about 0.22 nmol WED/mg protein and is the same with 50, 150 and 500 μM ADP. The maximum titer extrapolated from the linear range amounts to 0.45–0.4 nmol WED/mg protein, which shows a remarkable independence of the ADP concentration. These values compare with the half maximum titer of CAT at 0.16–0.18 ng/mg protein and the

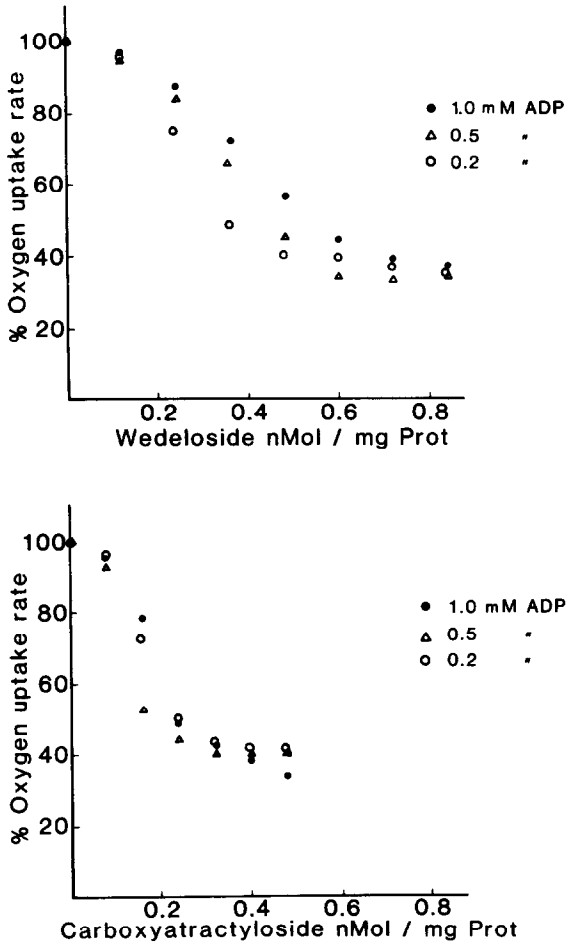

Fig.1. Inhibition of ADP-dependent respiration in rat liver mitochondria by wedeloside. Titration of the oxygen uptake rate measured by oxygen electrode equipped with a differentiator for direct readings of respiration rate. Rat liver mitochondria (0.6 mg protein/0.3 ml medium) incubated in medium consisting of 0.125 mM sucrose, 65 mM KCl, 2 mM P_i, 4 mM MgCl, 10 mM triethanolamine HCl, 10 mM glucose, 4 mM succinate, 4 mM glutamate and 6 μg hexokinase at pH 7.2 and 24°C. First mitochondria are added, then ADP at the concentrations indicated. Wedeloside solution made up in 50% ethanol is injected into the closer vessel at the amounts indicated.

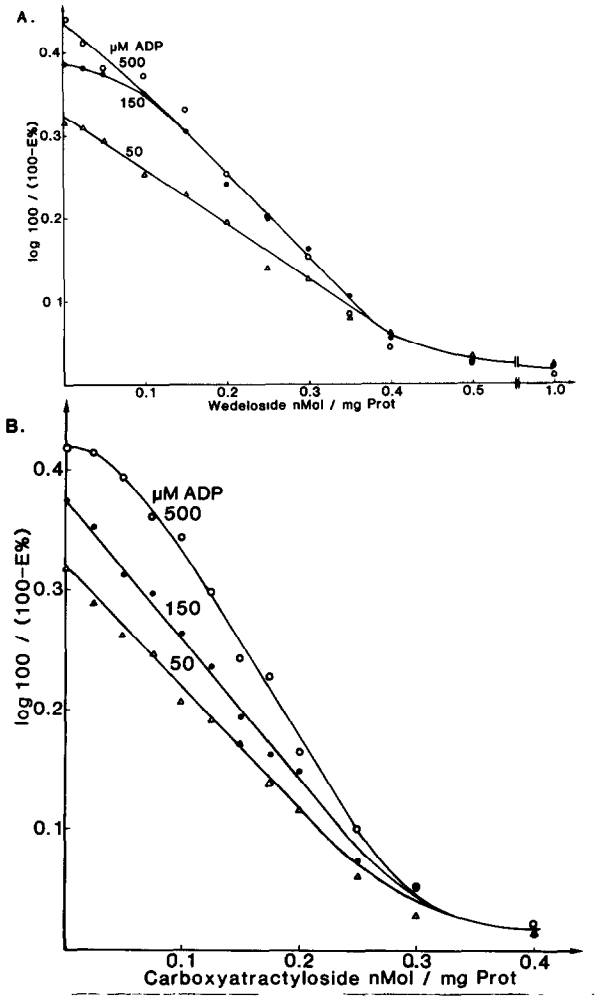

Fig.2. Inhibition of the ADP/ATP exchange rate by wedeloside. (A) Dependence on the concentration and (B) comparison with carboxyatractylate (CAT). Measurements by the back exchange and inhibitor stop method using CAT at stop. Rat liver mitochondria (0.2 mg/0.5 ml medium) incubated in 0.25 M sucrose, 10 mM triethanolamine HCl, 1 mM EDTA, pH 7.2, 10°C. Increasing amounts of CAT or WED were added to the samples prior to the addition of mitochondria. The intramitochondrial adenine nucleotides were prelabeled by [^{14}C]-ADP. 3 min after addition of mitochondria the reaction was started by rapid addition of ADP at the 3 concentrations indicated. After 20 s, 10 mM CAT was added to stop the exchange. The samples were centrifuged and the released [^{14}C]-adenine nucleotides in the supernatant were determined. A blank sample (about 12% of total dpm) was subtracted. E = % exchange as related to total dpm. Log [100/(100 – E)] plotted as a measure for the translocation rate because the exchange follows a pseudo first-order kinetic.

extrapolated maximum titer of about 0.32 nmol CAT/mg protein.

Measuring the effect of an inhibitor on the exchange is not only more direct but also more accurate, if properly performed, than testing its effect on the respiratory activity. This rule is again borne out by the present titrations with WED. The linearity of the effect of different WED concentrations on the exchange rate is in contrast to the nonlinear response to WED of the respiratory rate. Obviously in this case respiration is not limited by the exchange rate. The fact that the exchange inhibition is independent of the ADP concentration, even at 10-fold concentration, shows that there is no competition between ADP and WED similar to that between ADP and CAT. The slight competition of ADP noted when measuring respiration with WED inhibition can be explained by assuming that with high concentrations of ADP the uptake of ADP is slightly less rate-limiting to the respiration than with low concentrations.

To evaluate more directly the affinity of WED to the ADP/ATP carrier sites in comparison to the affinity of CAT and ATR, the interference of WED with the binding of [^{3}H]CAT and [^{3}H]ATR in mitochondrial membranes was studied. For this purpose beef heart mitochondria were used as these have a higher binding capacity and permit more accurate binding determinations [11]. The membranes were incubated with increasing amounts of WED and also with 1.5 nmol of either [^{3}H]CAT or [^{3}H]ATR added before, after or simultaneously with WED. Fig.3 shows the resulting binding, given in percentage of original binding in the absence of WED.

In another experiment WED and [^{3}H]CAT or [^{3}H]ATR were added simultaneously. There is linear decrease in the binding of [^{3}H]ATR independent of whether WED is added prior or simultaneously. However, in the case of [^{3}H]CAT, WED competes more effectively when added prior to [^{3}H]CAT than when added simultaneously. These differences become still more pronounced when the incubation sequence is reversed. When [^{3}H]CAT or [^{3}H]ATR are added first, excess WED is able to remove only half of [^{3}H]CAT but it can remove 80% of [^{3}H]ATR. This behavior can be well understood when calling to mind that the binding of [^{3}H]CAT is much tighter than that of [^{3}H]ATR. Thus [^{3}H]CAT cannot be removed by

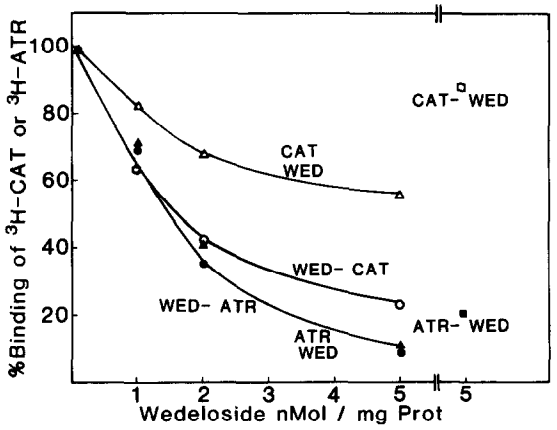

Fig.3. Competition of WED with the binding of CAT and ATR in mitochondria. Beef heart mitochondria (1 mg/ml) incubated in 0.25 M sucrose, 10 mM Pipes, 50 μM ATP and 1 mM $MgCl_2$. Additions, if indicated, are of 1.5 nmol [^{3}H]CAT, 1.5 nmol [^{3}H]ATR and 1–5 nmol WED. As indicated, either [^{3}H]CAT or [^{3}H]ATR were added prior to WED or vice versa. Also [^{3}H]CAT or [^{3}H]ATR was added simultaneously with WED, as indicated. The time differences between additions were: WED, 5 min, [^{3}H]CAT or [^{3}H]ATR, 10 min, followed by centrifugation. [^{3}H]CAT or [^{3}H]ATR and WED simultaneously, for 15 min, then centrifugation. [^{3}H]CAT or [^{3}H]ATR, 10 min, WED, 5 min, followed by centrifugation. The binding of [^{3}H]CAT or [^{3}H]ATR was determined in the sediments after dissolving the pellet of mitochondrial membranes.

excess unlabeled CAT whereas [^{3}H]ATR can largely be competitively displaced by excess ATR or CAT [12]. The results indicate that WED has a binding affinity superior to that of ATR but that it cannot bind quite as tightly as CAT.

The fact that WED can suppress virtually all [^{3}H]ATR binding at only 2- to 3-fold excess argues in favor of a tighter binding of WED. Moreover, the inability of [^{3}H]CAT to displace more than 20% of WED also indicates that the affinity of WED is not much inferior to that of CAT. This is evident also from the competition of WED with [^{3}H]CAT on simultaneous addition.

The extrapolation of the titration with [^{3}H]ATR indicates that WED requires 2-fold excess over [^{3}H]ATR for binding. This may seem to contradict the higher affinity of WED. One explanation could be that the WED preparation used was not uniform and contained only 50–60% of the WED-I compound. This would agree also with the titration by WED of the exchange and respiration, when compared to the titers with CAT.

In view of the tight binding of [^{3}H]CAT and the consequently very slow equilibration with competing ligands, the best approach to ensure a competition equilibrium is the simultaneous addition of WED and [^{3}H]CAT. From this titration curve a ratio of dissociation constants $K_D^{WED}/K_D^{CAT} = 5$ can be evaluated, corresponding to $K_D^{WED} = 5 \times 10^{-8}$ M and $K_D^{CAT} = 10^{-8}$ M. With a smaller content of WED-I the $K_D^{WED} = 3 \times 10^{-8}$ M.

3. CONCLUSION AND DISCUSSION

When comparing the structure of WED with that of CAT, the high affinity of WED to the ADP/ATP translocator seems at first surprising. The sulfate group has so far been believed to be indispensable in high affinity ligands [13] and after comparing other ligands to the ADP/ATP carrier such as bongkrekate, ADP and ATP, it was suggested that at least 3 negative charges are required for effective binding [14]. WED has, however, only two negative charges and none in the glucose moiety. Tighter binding must be strongly supported here by the hydrophobic substitution with phenylpropionic acid. A similar case has been found with a fluorescent AMP derivative. Whereas AMP is non-binding, dimethylamino-naphthoyl-AMP is a surprisingly good ligand with a K_D = 0.5 μM [15,16]. Here the hydrophobic naphthoyl moiety obviously contributes strongly to the binding.

The titer obtained for WED seems to indicate that two molecules WED bind for one molecule CAT or ATR. There are some difficulties in excluding impurities or isomers from WED and clear evidence has to await direct measurement of WED binding. It would be most interesting if the 'half site reactivity' found for the highly anionic ligands is absent with a homologous more neutral and hydrophobic ligand.

WED is more easily dissolved in nonaqueous solvents than CAT. The substitution with phenylpropionic acid and the absence of the sulfate group makes this compound more hydrophobic in the glucose moiety. Therefore, it can be expected to be membrane-permeant to a greater extent than CAT, which does not penetrate cell membranes very well. For this reason, and in

view of its high binding affinity, WED could be even more toxic than CAT. Of great importance for toxic action of an atractylogenin derivative is the lack of competition with ATP or ADP. Atractylogenin derivatives with lower affinity, such as those from coffee, could well be poisonous if the high intracellular ADP or ATP concentration did not effectively compete with the binding of these compounds to the ADP/ATP carrier. Even ATR is considerably less poisonous than CAT because of partial competition to its binding by intracellular ADP or ATP.

ACKNOWLEDGEMENT

This work was supported by a grant from the Deutsche Forschungsgemeinschaft (K1 134/23).

REFERENCES

[1] Piozzi, F. (1978) in: Atractyloside: Chemistry, Biochemistry and Toxicology (Santi, R. and Luciani, S. eds) pp.13–32, Piccin Medical Books, Padova, Italy.
[2] Debetto, P. (1978) in: Atractyloside: Chemistry, Biochemistry and Toxicology (Santi, R. and Luciani, S. eds) pp.125–129, Piccin Medical Books, Padova, Italy.
[3] Ludwig, H., Obermann, H. and Spiteller, G. (1974) Chem. Ber. 107, 2409–2411.
[4] Klingenberg, M., unpublished work.
[5] Oelrichs, P.B. and Vallely, P.J. (1980) J. Natural Prod. 43, 414–416.
[6] Eichholzer, J.V., Lewis, I.A.S., MacLeod, J.K. and Oelrichs, P.B. (1981) Tetrahedron, vol.37, 1881–1891.
[7] Lewis, I.A.S., MacLeod, J.K. and Oelrichs, P.B. (1981) Tetrahedron, vol.37, 4305–4311.
[8] Doherty, M.D. and Oelrichs, P.B. (1980) Proc. Aust. Biochem. Soc. 13, 122.
[9] Pfaff, E., Heldt, H.W. and Klingenberg, M. (1969) Eur. J. Biochem. 10, 484–493.
[10] Pfaff, E., Klingenberg, M. and Heldt, H.W. (1965) Biochim. Biophys. Acta 104, 312–315.
[11] Klingenberg, M., Grebe, K. and Scherer, B. (1975) Eur. J. Biochem. 52, 351–363.
[12] Klingenberg, M. (1974) in: Dynamics of Energy-Transducing Membranes (Ernster, L. et al. eds) pp.511–528, Elsevier, Amsterdam.
[13] Vignais, P.V., Vignais, P.M., Lauquin, G. and Morel, F. (1973) Biochimie 55, 763–778.
[14] Klingenberg, M., Buchholz, H., Erdelt, H., Falkner, G., Grebe, K., Kadner, H., Scherer, B., Stengel-Rutkowski, L. and Weidemann, M.J. (1971) in: Biochemistry and Biophysics of Mitochondrial Membranes (Azzone, G.F. et al. eds) pp.465–486, Academic Press, New York.
[15] Schäfer, G. and Onur, G. (1980) FEBS Lett. 109, 197–201.
[16] Klingenberg, M. (1981) in: Structural and Functional Aspects of Enzyme Catalysis (Eggerer, H. and Huber, R. eds) pp.202–212, Springer, Berlin.