Biochimica et Biophysica Acta, 1110 (1992) 235–238

BBAMEM 70711

Rapid Report

Inhibitors of the plasma membrane redox system of *Zea mays* L. roots. The vitamin K antagonists dicumarol and warfarin

O. Döring, S. Lüthje and M. Böttger

Universität Hamburg, Institut für allgemeine Botanik, Hamburg (Germany)

(Received 15 July 1992)

Key words: Plasmalemma redox system; Dicumarol; Warfarin; Plasma membrane; Electron transport; (*Z. mays* L.)

The action of the 4-hydroxycoumarins dicumarol and warfarin, antagonists of probable vitamin K type components of the plasma membrane electron-transport system, on plasma membrane redox activity of intact maize roots was compared. Both effectors inhibited electron transfer to extracellular hexacyanoferrate III. While the effect of the strongly lipophilic dicumarol on the electron-transport system was irreversible by rinsing, the inhibition caused by the hydrophilic warfarin could be reverted completely by exchange of the incubation medium. We take these results as possible evidence for the integration of dicumarol into the plasma membrane. The action of warfarin may be confined to enzymic sites freely accessible from the aqueous apoplasmic solution.

Plant plasma membranes (PM) contain an oxidoreductase activity able to transfer electrons to extracellular applied electron acceptors. There are still major uncertainties about the physiological role of this system [1,2]. Involvement in growth regulation (elongation growth [3], cell division [4]), apoplastic radical production [5] and membrane energization [6] has been proposed.

After addition of hexacyanoferrate III (HCF III) as an electron acceptor to the incubation medium of plant tissue, reduction to HCF II, concomitant with membrane depolarization and extracellular acidification, has been found. The mechanism of acidification is still a matter of controversy [1]. Reduction and depolarization have been explained by a trans-PM electron transfer [7–10]. Cytoplasmic oxygen [8], semidehydroascorbate [11] and nitrate [12,13] are the main candidates for the electron acceptor under natural conditions.

According to Refs. 14 and 15, dicumarol, a vitamin K antagonist, inhibits the electron flow of the PM redox-system in vivo. The highly hydrophobic molecule was supposed to enter the lipophilic part of the membrane where it interacts with the electron-transport chain, blocking the electron transfer to HCF III.

To elucidate this proposed mode of dicumarol action on electron flow we compared its effect with that of warfarin, a hydrophilic vitamin K antagonist. Because of its hydrophilicity warfarin should not be able to integrate into the lipophilic part of the membrane.

Dicumarol and warfarin (sodium salt) were obtained from Sigma (Deisenhofen, Germany). All other chemicals were purchased from Merck (Darmstadt, Germany). Dicumarol was added to experimental solutions dissolved in DMSO, warfarin and HCF III were dissolved in buffer (see below).

Seedlings of *Zea mays* L. cv. Sil Anjou 18 (Saatenunion, Hannover, Germany) were grown as described elsewhere [10]. 18 2-day-old seedlings were transferred into plastic vessels containing 15 ml incubation medium (3 mM KCl, 0.5 mM $CaCl_2$, 0.125 mM $MgSO_4$, 50 mM Hepes, adjusted to pH 7.0 with HCl and KOH in distilled water). The medium used within the pH-oxidostat (see below) was prepared without Hepes.

Redox activity was determined in a pH-oxidostat as described in Ref. 16 and also using the method described in Ref. 14. An pH-oxidostat holds pH and HCF III concentration constant by titration, while the rates of net proton extrusion and HCF III reduction are recorded by a computer. For the latter setup the

Correspondence to: O. Döring, Universität Hamburg, Institut für allgemeine Botanik, Ohnhorststraße 18, W-2000 Hamburg 52, Germany.

Abbreviations: PM, plasma membrane; HCF III, hexacyanoferrate III; HCF II, hexacyanoferrate II; dicumarol, 3,3′-methylenebis[4-hydroxy-2H-1-benzopyran-2-one] (3,3′-methylene-bis[4-hydroxycoumarin] (MI 3075)); warfarin, 4-hydroxy-3-(3-oxo-1-phenylbutyl)-2H-1-benzopyran-2-one (3-(α-acetonylbenzyl)-4-hydroxycoumarin (MI 9852)); Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid.

medium was renewed after a preincubation of 12 h. Measurement of redox activity started 1 h after exchange of the medium.

In order to investigate the persistance of the inhibitory effects caused by warfarin or dicumarol, roots and vessels were rinsed with incubation medium (10-fold volume of the vessel). This rinsing procedure took about 60 s, another 15 s were needed for addition of HCF III containing medium and stabilizing the spectrophotometric reading. Therefore, a gap of 75 s in monitoring PM redox activity exists. After this procedure, we were not able to detect any warfarin in the incubation medium measured as absorbance at 310 nm.

Both dicumarol and warfarin inhibited PM redox activity. Maximum inhibition of PM redox activity with dicumarol was reached at 50 to 100 μM. For dicumarol, such an observation has already been reported [14,15], so we did not investigate the effect of various dicumarol concentrations in detail, but confined the experiments to the concentrations shown in Fig. 1. After washing the roots dicumarol induced inhibition was persistent (see Fig. 3, left). DMSO, used to dissolve dicumarol prior to addition to the incubation medium, did not alter PM redox activity in the concentration range used. Net proton secretion measured in a pH-oxidostat in the presence of HCF III was completely inhibited by 50 μM dicumarol (data not shown). Inhibition of proton secretion could not be reverted by exchange of the incubation medium.

As did dicumarol, warfarin inhibited PM redox activity immediately. The effect was concentration dependent. Maximum inhibition was reached at 200 μM (Fig. 2). The effect was completely reversible by washing with incubation medium (Fig. 3, right). Net proton secretion measured in the pH-oxidostat in the presence of HCF III was completely inhibited by warfarin above 100 μM, but could be reverted by exchange of the incubation medium (data not shown).

Neither with warfarin nor with dicumarol in all concentrations used we were able to totally inhibit PM redox activity. The inhibition caused by dicumarol and warfarin took effect immediately after addition, as can be seen in the experiments in the spectrophotometer. With the pH-oxidostat, which has an instrumental time resolution of about 5 min, we found the inhibition of PM redox activity to be constant for at least 3 h at a given concentration of inhibitor. After removal of warfarin, the PM redox activity recovered to the original rate. An attempt to remove dicumarol from a supposed binding site by consecutive rinsing with incubation medium, incubation medium with 500 μM warfarin and again with incubation medium was without success. The inhibition of PM redox activity caused by dicumarol was not changed by this procedure (data not shown).

A major difference between warfarin and dicumarol

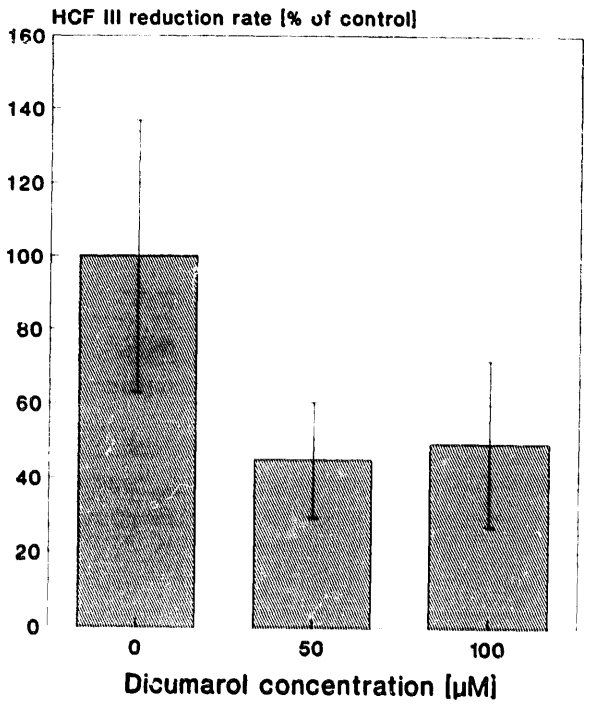

Fig. 1. Concentration-dependence of the inhibition of the PM redox activity by dicumarol in *Zea mays* L. cv. Sil Anjou 18 roots. Because of its poor solubility in aqueous solutions, we did not investigate the effect of dicumarol in concentrations above 100 μM. Each bar represents the mean of at least four experiments. The experimental procedure employed has been described by Döring et al. [14]. Absolute redox activity of the control is 0.546 nmol/(s × gFW).

with respect to our experiments consists in their solubility in water (or in organic solvents, respectively). Since it is not known if warfarin is able to migrate to the site of action of dicumarol, which is supposed to be in lipophilic membrane regions [14,15], it is possible that different parts of the electron-transport chain are affected by these vitamin K antagonists.

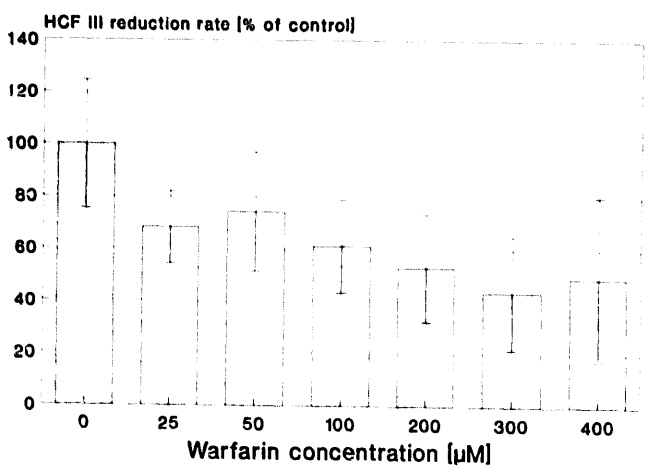

Fig. 2. Concentration-dependence of the inhibition of the PM redox activity by warfarin. Each bar represents the mean of at least four experiments. The experimental conditions are described in the legend to Fig. 1.

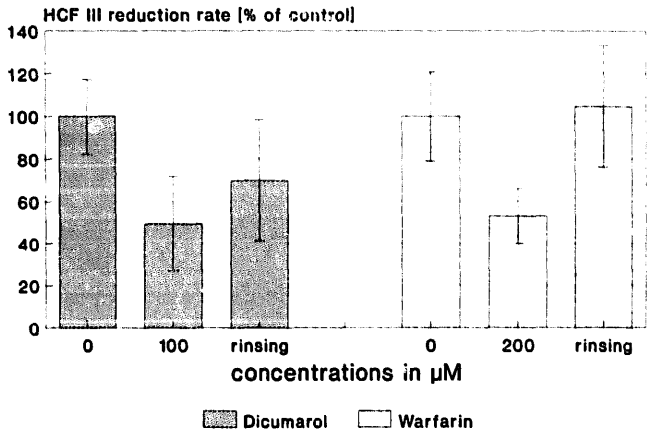

Fig. 3. After rinsing with incubation medium the inhibition of the redox activity of maize roots caused by warfarin (200 μM) disappeared (right part of the figure), while the effect of dicumarol (100 μM) was persistent (left part).

Warfarin and dicumarol may act on the same site within the electron transport chain. Figs. 1 and 2 show that about twice the concentration of warfarin is needed to achieve the same relative inhibition as with dicumarol. One possible explanation would be that this is due to the low lipophilicity of warfarin compared to dicumarol, therefore, a higher concentration is needed to give the same concentration of inhibitor at the site of action, which in this case would be lipophilic. Another explanation of this observation would be the different affinity of warfarin and dicumarol to a component of the electron-transport chain.

It was not able to substitute dicumarol at a hypothetical binding site with warfarin by rinsing. If dicumarol and warfarin act on the same site in the electron-transport chain, the affinity of dicumarol to this site, therefore, has to be much higher than the affinity of warfarin.

Both, the inhibition of PM redox activity by dicumarol and by warfarin, may either be explained by a HCF III reduction-site which binds these vitamin K_3 inhibitors, dicumarol with high affinity and warfarin with comparatively low affinity. Different sites of action of both inhibitors can also be taken into account. Dicumarol, as well as warfarin, may act on some part of the electron transporting system energetically upstream from the HCF III reduction site, blocking electron flow within the transport chain.

There may be different modes of action for both, dicumarol and warfarin: (1) An effect of warfarin on cytoplasmic processes fueling the PM redox system with NAD(P)H [17] would explain the effects of warfarin, but not of dicumarol, as discussed elsewhere [15]. (2) The existence of a warfarin-, but not dicumarol-metabolizing activity, such as 7-hydroxylases [18,19], may also be an explanation of the reversibility of the warfarin action. However, to our knowledge, there have not been any publications reporting 7-hydroxylase activities in plants.

The complete inhibition of proton extrusion, but not of HCF III reduction indicates that the effects of both inhibitors are not restricted to the electron-transport system. The net PM proton pumping activity is also affected by an unknown mode of action. The inhibitors may act on the H^+-ATPase itself, via its ATP supply or via a change of membrane properties.

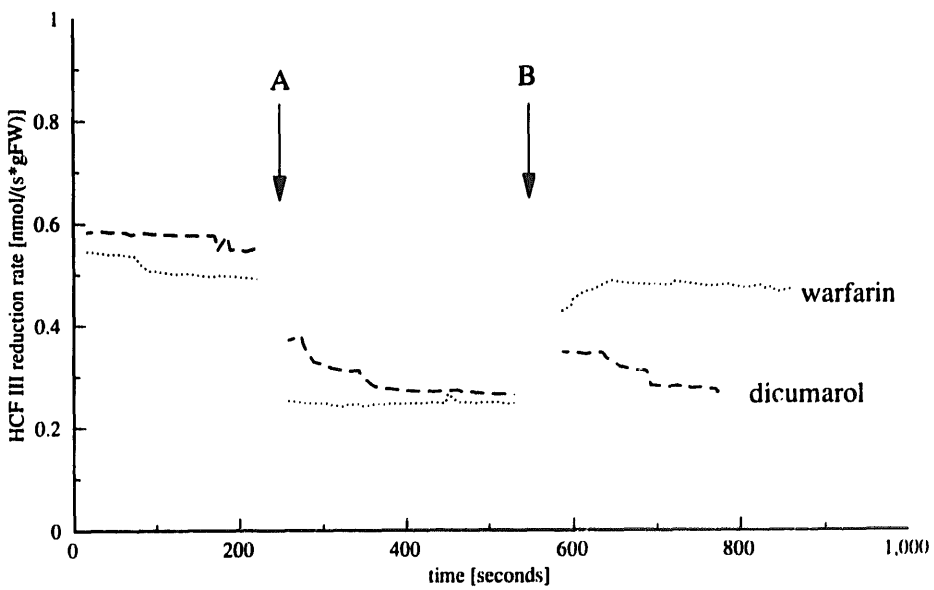

Fig. 4. Inhibition of HCF III reduction rate calculated from typical experiments. The dotted trace represents the inhibition caused by 200 μM warfarin (added at arrow A) and the recovery of HCF III reduction after rinsing (arrow B). The dashed line demonstrates the inhibition caused by 100 μM dicumarol. Dicumarol was added at arrow A, rinsing took place at arrow B. For details of the experimental procedure, see text.

There is evidence for the presence of quinones, such as K-type vitamins in the plasma membrane of plants (see Ref. 20 and Böttger, M., unpublished data). A quinone as component of the PM electron-transport chain, accepting electrons from an unknown hypothetical donor within the chain and transferring them to yet another acceptor (the energetically following link in the electron-transport chain) would compete with quinones (naphthoquinones) which have been integrated into the transport chain. While quinones, such as K-type vitamins are able to accept one electron together with a proton to form a semiquinone radical and a second electron and proton to form the quinol, dicumarol and warfarin may be structurally similar to a natural component of the chain, but are unable to accept electrons and protons and, therefore, would inhibit electron and proton flow. The interference of both warfarin and dicumarol with PM redox activity may, in this sense, be taken as evidence for the involvement of quinolic compounds in PM electron-transport. If this evidence is confirmed by further investigations, then a direct involvement of PM redox activity in H^+-transport must be taken into account, since quinones always binds (release) protons with reduction (oxidation).

We thank F. Hilgendorf and H. Lüthen for stimulating discussions. Supported by Deutsche Forschungsgesellschaft (Bo 537/7-4).

References

1 Crane, F.L., Morré, D.J., Löw, H.E. and Böttger, M. (1991) in Oxidoreduction at the Plasma Membrane: Relation to Growth and Transport, Vol. 2, Plants, pp. 21–33, CRC-Press, Boca Raton.
2 Bienfait, F. and Lüttge, U. (1988) Plant Physiol. Biochem. 26, 665–671.
3 Lüthen, H. (1992) PhD Thesis, Fac. Biol. Univ. Hamburg.
4 Navas, P. (1991) in Oxidoreduction at the plasma membrane: Relation to growth and transport, Vol. 2, pp. 111–120, CRC Press, Boca Raton.
5 Schwacke, R. and Hager, A. (1992) Planta 187, 136–141.
6 Böttger, M., Crane, F.L. and Barr, R. (1991) in Oxidoreduction at the plasma membrane: Relation to growth and transport, Vol. 2, pp. 207–236, CRC Press, Boca Raton.
7 Sijmons, P.C., Lanfermeijer, F.C., De Boer, A.H., Prins, H.B.A. and Bienfait, H.F. (1984) Plant Physiol. 76, 943–946.
8 Böttger, M. and Lüthen, H. (1986) J. Exp. Bot. 37, 666–675.
9 Lass, B., Thiel, G. and Ullrich-Eberius, C.I. (1986) Planta 169, 251–259.
10 Döring, O., Lüthje, S., Hilgendorf, F. and Böttger, M. (1990) J. Exp. Bot. 41, 1055–1061.
11 Morré, D.J., Navas, P., Penel, C. and Castillo, F.J. (1986) Protoplasma 133, 187–193.
12 Ward, M.R., Grimes, H.D. and Huffaker, R.L. (1989) Planta 177, 470–475.
13 Smarelli, J. and Castignelli, D. (1988) Planta 173, 563–566.
14 Döring, O., Lüthje, S. and Böttger, M. (1992) J. Exp. Bot. 43, 175–181.
15 Lüthje, S., Döring, O. and Böttger, M. (1992) J. Exp. Bot. 43, 183–188.
16 Böttger, M. and Hilgendorf, F. (1988) Plant Physiol. 86, 1038–1043.
17 Krüger, S. and Böttger, M. (1988) in Plasma Membrane Oxidoreductases in Control of Animal and Plant Growth, NATO ASI Ser. 157, pp. 105–114, Plenum Press, New York.
18 Rettie, A.E., Korzekwa, K.R., Kunze, K.L., Lawrence, R.F., Eddy, A.C., Aoyama, T., Gelboin, H.V., Gonzalez, F.J. and Trager, W.F. (1992) Chem. Res. Toxicol. 5, 54–59.
19 Honkakoski, P., Arvela, P., Juvonen, R., Lang, M.A., Kiraluona, M. and Pelkonen, O. (1992) Brit. J. Clin. Pharmacol. 33, 313–317.
20 Barr, R., Brightman, A., Morré, D.J. and Crane, F.L. (1990) J. Cell Biol. 111, 389.