Biochimica et Biophysica Acta, 510 (1978) 201—207

BBA Report

BBA 71343

UNCOUPLERS OF OXIDATIVE PHOSPHORYLATION

A STRUCTURE-ACTIVITY STUDY OF THEIR INHIBITORY EFFECT ON PASSIVE CHLORIDE PERMEABILITY

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(Received February 24th, 1978)

Summary

Uncoupling agents inhibit chloride transport in red blood cells, which is a metabolism-independent process. An analysis of the molecular requirements shows that this inhibitory activity is closely correlated with the electronic and the hydrophobic bonding properties of phenols: the more lipophilic and the more electron-attracting the substituent groups are, the greater the activity they confer on the parent molecule. A recent structure-activity study concerning various classes of reversible inhibitors of chloride transport led to the same conclusion (Motais, R. and Cousin, J.L. (1977) in International Conference on Biological Membranes: Drugs, Hormones and Membranes (Bolis, L., Hoffman, J.F. and Straub, R.W., eds.), Raven Press, New York, in the press).

The effects of substituents on the activity of phenols as uncouplers have been recently examined (Stockdale, M. and Selwyn, M.J. (1971) Eur. J. Biochem. 21, 565). The comparison of these results with our data shows that uncoupling depends more on electronic properties of phenols than does chloride inhibition.

When a cellular function is modified by the addition of agents which uncouple oxidative phosphorylation it is generally assumed that this function is metabolism-dependent. However, such a change could result from the fact that uncouplers can affect processes other than phosphorylation in the complex intact cell system. A clear example of such a possibility is given by the experiments of Omachi [1] showing that sulphate permeability is reversibly depressed in human erythrocytes in the presence of 2,4-dinitrophenol or dicoumarol. This effect cannot be due to uncoupling of phosphorylation from respiration since erythrocytes metabolize anaerobically (the mam-

malian red cell does not contain mitochondria) and since in these cells, anions are passively transported [2]. Thus, it appears quite clearly that 2,4-dinitrophenol and dicoumarol act directly at the membrane level to alter permeability. Since this work it has been occasionally reported that some other uncouplers decrease the anion permeability in red blood cells (refs. 3—8 and Cousin, J.L. and Motais, R., in preparation). The powerful capacity of some classical uncouplers to inhibit chloride permeability is illustrated in Table I.

On the other hand, it is well known that the permeability of the red cell membrane to anions can be reversibly and non-competively inhibited by a large number of chemically unrelated compounds [4, 5, 10]. The only common property between these molecules is their amphiphilic structure. The protein in band 3, which is involved in anion transport, is considered to be the site of action [11]. The interaction of such structurally non-specific drugs cannot be directly correlated with chemical structures except to the extent that structure affects physicochemical properties. Recent studies have defined the relationship between physicochemical properties of some of these compounds and their inhibitory properties (refs. 7, 10 and 12 and Cousin and Motais, in preparation).

Hence, the purpose of the present study is two-fold. Firstly it is to determine whether or not the physicochemical characteristics of the agents which are responsible for the uncoupling of oxidative phosphorylation are the same as those required for inhibiting an energy-independent process such as chloride permeability in mammalian erythrocytes. Secondly, in an attempt to gain insight into the molecular nature of the chloride transport system, it is to compare the physicochemical properties involved in the inhibitory action of uncoupling agents and that of the other reversible inhibitors previously studied.

Among uncoupling agents, phenols are particularly suitable for such an investigation since they represent an homologous series of congeners, the physicochemical properties of which can be modified by substituents in the aromatic ring. Therefore, we have tested the ability of a wide variety of phenol derivatives to alter passive chloride permeability in ox red blood cells and have quantitatively correlated their activity with physicochemical properties. Chloride permeability was measured, as described previously [9], by determining the rate of tracer efflux from radioactively-labelled ox red blood cells

TABLE I

THE INHIBITORY EFFECT OF SOME WELL KNOWN UNCOUPLERS OF OXIDATIVE PHOSPHORYLATION ON THE CHLORIDE EFFLUX MEASURED AT DONNAN EQUILIBRIUM

See ref. 9 for technique. The relative activity of these compounds can be expressed as log $(1/I_{50})$, I_{50} being the molar concentration producing 50% inhibition.

Compound	I_{50}	Log $(1/I_{50})$
Salicyl anilide	$2.4 \cdot 10^{-5}$	4.60
3,5-Dibromo-3'-trifluoromethyl salicyl anilide	$1.6 \cdot 10^{-6}$	5.80
3,5,4'-Tribromo salicyl anilide	$1.3 \cdot 10^{-6}$	5.87
2,4-Dinitrophenol	$8.2 \cdot 10^{-5}$	4.07
Carbonyl cyanide *m*-chlorophenylhydrazone	$2 \cdot 10^{-6}$	5.71

under steady-state conditions of anion concentration. The inhibitory effect of phenols was studied by suspending erythrocytes in a medium containing the compound to be tested, without any preincubation in presence of this compound. For all the compounds tested, the molar concentration producing a 50% inhibition for chloride transport (I_{50}) was determined. The inhibitory activity of each of them can be expressed as $1/I_{50}$. The alterations of the chemical properties of phenols (liposolubility, ionisation, dipole moment etc.) due to the modification of substituents in the aromatic ring have to be quantitated. Some of these effects on chemical properties can be expressed in terms of substituent constants; two that have proved particularly useful are π, the Hansch's constant* which quantitates the effect of substituents on the hydrophobic bonding properties of the phenol, and σ, the well known Hammet substituent constant** which describes electronic effects within the molecule. The values of the dipole moment μ, of the different phenols was taken from McClellan [13].

Table II shows that all phenols we tested have an inhibitory effect on chloride transport. This inhibition is reversible and instantaneously maximal.

It is well known that lipid solubility has some relation to the uncoupling activity of phenols. As illustrated in Fig. 1A, it appears that also such a relation exists with the inhibitory effect on chloride permeability. In this figure, log $1/I_{50}$ is plotted against π, which quantitates the effect of substituents on liposolubility. It can be seen that the inhibitory activity increases with increasing π over a thousand-fold range. A quantitative description of

TABLE II

PHYSICOCHEMICAL PROPERTIES AND CHLORIDE INHIBITORY ACTIVITIES OF PHENOL DERIVATIVES

σ, Hammett constant; π, Hansch constant (values from ref. 14); μ, dipole moment (values from ref. 13).

Number	Compounds	log $(1/I_{50})$	σ	π	log μ
1	Phenol	3.00	0	0	0.18
2	2-Cl-phenol	3.92	0.23	0.69	0.11
3	3-Cl-phenol	4.28	0.37	1.04	0.33
4	4-Cl-phenol	4.05	0.23	0.93	0.36
5	2,4-Cl-phenol	4.58	0.43	1.60	0.26
6	2,5-Cl-phenol	4.62	0.60	1.74	
7	2,4,6-Cl-phenol	4.88	0.66	2.16	0.18
8	$(Cl)_5$-phenol	5.88	1.07	2.64	0.33
9	2-NO_2-phenol	3.27	0.80	0.33	0.50
10	4-NO_2-phenol	3.50	1.27	0.50	0.70
11	2,4-NO_2-phenol	4.07	2.07	0.04	0.50
12	2,5-NO_2-phenol	3.44	1.50	0.54	
13	2,6-NO_2-phenol	3.40	1.60	0.09	0.54
14	*m*-Cresol	3.22	—0.07	0.50	0.22
15	*p*-Cresol	3.10	—0.17	0.49	0.20
16	2,6-Cl-4-NO_2-phenol	4.85	1.72	1.48	

*π constant can be defined as $\pi = \log P_X - \log P_H$, where P_H is the octanol-water partition coefficient of the phenol, P_X the octanol-water partition of the phenol derivative. Thus, π is the logarithm of the partition coefficient of the substituent x. Octanol-water partition is an index of lipophilicity.

**σ is defined by the equation: $\log k/k_0 = \rho\sigma$, where k is a constant describing a reaction of substituted aromatic compound, k_0 is the constant for the parent compound and ρ is a constant characteristic of the reaction.

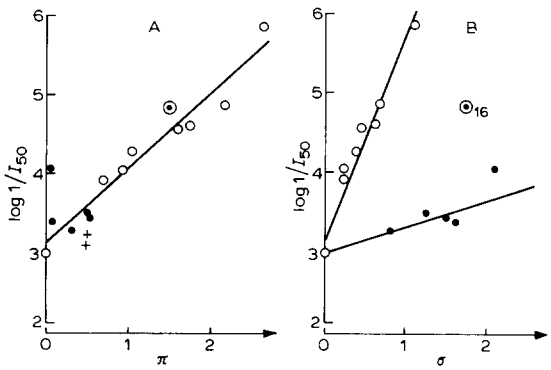

Fig. 1. (A) Relation between inhibition of chloride permeability and the lipophilic character of phenols, quantified by π. Least squares regression line is superimposed on the data. ○, Chlorophenols; ●, nitrophenols; +, cresols. (B) Relation between inhibition of chloride permeability and the Hammett substituent constant, σ for chlorophenols (○) and nitrophenol (●). The number 16 refers to the compounds number in Table II.

this relationship was obtained by least squares analysis of the data given in Table II:

$$\log \frac{1}{I_{50}} = 3.15 + 0.93\pi \quad n=16 \quad r=0.91 \quad s=0.34 \quad F_{1,14} = 67.9 \qquad (1)$$

A value of 0.91 for r, indicates that Eqn. 1 only accounts for 83% ($r^2 = 0.83$) of the variability in the data, suggesting that parameters other than liposolubility of the compounds could be involved in the inhibitory activity. A plot of log $(1/I_{50})$ versus σ for chloro-, and nitrophenols (Fig. 1B) indeed shows that the inhibitory activity also depends on the electronic effect of substituents quantified by σ. The nitrophenols lie on one line, and the chlorophenols on another: for nitrophenols, σ values vary widely but π values are always small (see Table II) in such a way that the slope of the line essentially measures the contribution of σ; on the contrary, for chlorophenols, π and σ closely parallel one another in such a way that the slope of the line measures the mixed effect of π and σ. The intermediate position of 2,6-Cl-4-NO_2-phenol (referred to as number 16 in the figure) points out the dependence of inhibitory activity on both π and σ. Effectively, a least squares analysis in which σ values are also considered greatly improved the correlation between inhibitory activity and physicochemical properties.

$$\log \frac{1}{I_{50}} = 2.89 + 0.94\pi + 0.32\sigma \quad n=16 \quad r=0.95 \quad s=0.27 \quad F_{2,13} = 61.5 \qquad (2)$$

F test indicates that the introduction of σ term in Eqn. 2 is justified at better than 0.99 level of significance when compared with Eqn. 1 ($F_{1,13} = 10.26$, $F_{1,13;0.01} = 9.07$). The fact that σ has a positive coefficient in Eqn. 2 means that groups which attract electrons increase activity.

The capacity of phenol derivatives to inhibit passive chloride permeability could be related not only to their liposolubility and electronic distribution but also possibly to their dipole moment. Indeed, Andersen et al. [8],

showed that nitrophenols and phloretin analogs decrease anion conductances of artificial lipid membranes and suggested that this inhibitory effect could be accounted for by a reduction of the dipole potential of the membrane interior. They assumed that these molecules, which possess a large dipole moment adsorb and orient at the membrane surface to introduce a dipole of opposite polarity to the preexisting one. On the basis of this data, obtained with artificial lipid membranes, they suggested that the inhibitory effect of these compounds on transport in biological membranes results from such an effect on the interfacial dipole fields on the translocators. Concerning phloretin analogs, it has been shown recently [7] that the ability of these compounds to inhibit chloride permeability in erythrocytes depends on the dipole moment and the lipid solubility of the molecules. This result means that their inhibitory action is due, at least in part, to the capacity they have to alter the interfacial dipole potential: the modification in the dipole potential between membrane interior and aqueous phase induced by a compound results both from its dipole moment and its concentration in the membrane, which in turn depends on its lipid solubility.

Therefore, it is interesting to consider the possibility that the dipole potential is also involved in the inhibiting effect of phenol derivatives. If so, it is expected that when the dipole moment of the compounds, μ, is considered in addition to π and σ, the correlation between inhibitory activity and physicochemical properties will be improved. The values of μ for the sixteen phenols used in Eqn. 2 are not available (see Table II). Therefore, we have derived Eqn. 3, which describes the same correlation as Eqn. 2 for the thirteen compounds for which μ is available.

$$\log\frac{1}{I_{50}} = 2.90 + 0.93\pi + 0.38\sigma \quad n=13 \quad r=0.96 \quad s=0.26 \quad F_{2,10} = 55.0 \qquad (3)$$

Adding $\log \mu$ to Eqn. 3 yields Eqn. 4:

$$\log\frac{1}{I_{50}} = 3.17 + 0.88\pi + 0.57\sigma - 1.04 \log \mu \qquad (4)$$

$$n=13 \quad r=0.97 \quad s=0.24 \quad F_{3,9} = 43.0$$

The F test indicates that addition of $\log \mu$ is not justified ($F_{1,9} = 2.50$, $F_{1,9,0.05} = 5.12$), suggesting that the dipole moment effect of substituent is not significant.

However, we must point out that, for the series of phenols we studied, the variation of dipole moment due to the substituents is relatively small compared to the variations of π and σ (see Table II); thus, an insignificant improvement of the correlation does not necessarily mean that the dipole potential is not involved in the inhibitory process.

The conclusion from this analysis of structure-activity relationship is that inhibitory activity of phenols essentially depends on both hydrophobic bonding properties of compounds and electronic distribution within the molecules (a wider variety of phenols should be used to provide greater variation in dipole moment and allow an answer concerning the role of dipole potential); the more lipophilic and the more electron-attracting the substituent

groups are the greater the activity they confer on the parent molecule. It is interesting to note that a structure-activity study concerning various classes of reversible inhibitors of chloride transport, as different chemically as salicylates, benzoates and ethacrynic acid analogs, comes to the same conclusion [10].

The effects of ring substituents on the effectiveness of phenol derivatives as uncouplers of phosphorylation has also been analysed in terms of the π and σ coefficients substituents. Therefore, it is possible to compare the relative role of electronic and hydrophobic bonding properties of the phenols in their activities either as uncouplers or as inhibitors of chloride permeability. According to Stockdale and Selwyn [14] the uncoupling activity in mitochondria from rat liver is related to π and σ following the equation:

$$\log \frac{1}{U_{50}} = 2.15 + 0.84\pi + 1.13\sigma \quad n=23 \quad r=0.98 \tag{5}$$

In this relation, U_{50} is the molar concentration required to produce 50% inhibition of phosphorylation. It can be seen that the coefficients in this equation are quite similar to those in Eqn. 2, except for σ; in other words, uncoupling depends more on electronic properties of phenols than does chloride inhibition. This is clearly apparent in Fig. 2 in which, for several phenols, log $(1/I_{50})$ is plotted versus log $(1/U_{50})$: the nitrophenols which have high σ values are more efficient as uncouplers than as chloride inhibitors. On the contrary, halophenols, which have relatively small σ values and high π values (ratio approx. 3) have a chloride inhibitory activity which is significantly higher than their uncoupling activity.

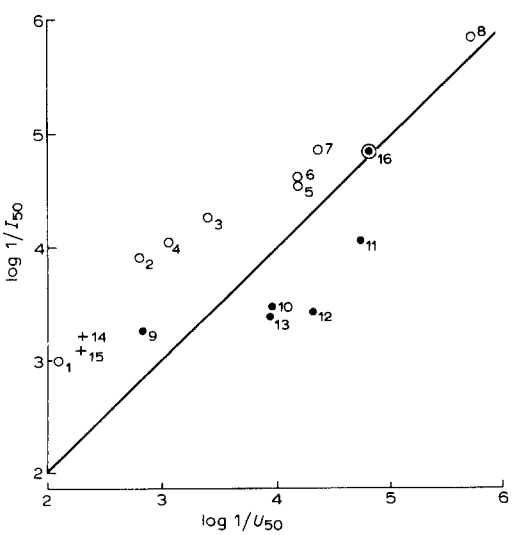

Fig. 2. Comparative activity of several phenols as inhibitors of chloride permeability in red cells (log $(1/I_{50})$) and as uncouplers of oxidative phosphorylation in mitochondria from rat liver (log $(1/U_{50})$). The values of $1/U_{50}$ were obtained from ref. 14. The numbers refer to the compounds in Table II. ○, Chlorophenols; ●, nitrophenols; +, cresols.

This work was supported by Commissariat à l'Energie Atomique (Département de Biologie), C.N.R.S. (E.R.A. No. 495) and D.G.R.S.T.

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