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INHIBITION OF CYTOCHROME OXIDASE ACTIVITY BY LOCAL ANAESTHETICS

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Abstract—Using a polarographic method, we studied the inhibition of mitochondrial electron transport at the cytochrome *c* oxidase site caused by eight local anaesthetics. The diversity of the types of inhibition observed indicate the importance of electrostatic interactions between the anaesthetic molecules and the membrane protein. A linear relationship was recognized between the anaesthetic activity of infiltration and the affinity for the enzyme. We also observed a significant relationship between this affinity and the octanol–water partition coefficient. This result suggests that lipophilic interactions are involved in cytochrome oxidase–anaesthetic binding. We tried to establish a parallel between this binding and the mechanism of anaesthesia involving the nerve membrane proteins.

Local anaesthetics reversibly block the transmission of nerve impulses by decreasing the sodium conductance through the nerve membrane. A wide range of molecules showing different structures block nerve conduction; the exact molecular mechanism of the action of local anaesthetics has not yet been explained satisfactorily. It has been suggested [1, 2] that anaesthetics act by causing perturbations of the membrane structure. Two hypotheses have been postulated to define a unitary mechanism of anaesthetic action for all local anaesthetics. The membrane perturbations are thought to be located either in the lipid bilayer (leading indirectly to modifications of the target protein structures) or alternatively in the protein structures themselves.

Richards *et al.* [3] showed that anaesthetics do not generally act by perturbing the membrane lipid bilayer and that direct interactions between molecules and membrane proteins are responsible for the local anaesthesia. Bradford and Marinetti [4] proposed that specific lipid–protein and Ca^{2+}–phospholipid interactions play a role in the Na^+ channel function and that local anaesthetics can disrupt these interactions.

The purpose of our research was to obtain information on the mechanism of action of local anaesthetics by establishing a relationship between the structure and the pharmacological activity of drugs with different molecular structures. In a previous study [5] we correlated the physicochemical parameters of twelve local anaesthetics to their activity. A Linear relationship was set up between the anaesthetic activity of infiltration and the octanol–water partition coefficient, also taking into account the ionization state of the molecules. The results obtained show that the same number of molecules are required at the membrane to obtain a given anaesthetic effect for the various drugs. Richards *et al.* [3] proposed that this may also support the hypothesis of a unitary mechanism for the anaesthetic action.

We thought that a comparative study of the anaesthetic interaction with a membrane protein would give a fuller understanding of the mechanisms. Recent reports [6] showed that local anaesthetics inhibit mitochondrial electron transport at several points along the respiratory chain, and that an inhibition effect is located at cytochrome *c* oxidase. Previously Singer [7] studied the interaction of cytochrome oxidase with four local anaesthetics (procaine, tetracaine, dibucaine and benzocaine). He chose this membrane protein, as a good model, for its well-defined biological function [8]: the electron transfer from cytochrome *c* to molecular oxygen. Singer [7] observed that the level of oxidase inhibition varied with the effectiveness of the anaesthetic molecules.

The purpose of this paper is to describe the inhibiting action of eight local anaesthetics on cytochrome *c* respiration and to determine certain parameters leading to a comparative study of these molecules. The drugs investigated were procaine, parethoxycaine, lidocaine, prilocaine, carticaine, bupivacaine, pramocaine and quinisocaine; their molecular structures are given in Fig. 1.

MATERIALS AND METHODS

Cytochrome *c* (type VI), sodium ascorbate and cytochrome oxidase were obtained from the Sigma Chemical Co. (St. Louis, MO). TMPD (*N,N,N',N'*-tetramethyl-*p*-phenylene diamine) was purchased from Merck Laboratories (Darmstadt, F.R.G.)

The polarographic assays of oxidase activity were carried out with an oxygraph Gilson K-IC. The polarographic method developed by Chance [9] and already experimented by Yonetani [10] and Ferguson-Miller *et al.* [11] was adopted for our study.

The assay mixture of volume 1.5 ml contained 6 mM sodium ascorbate, 0.6 mM TMPD and 1%

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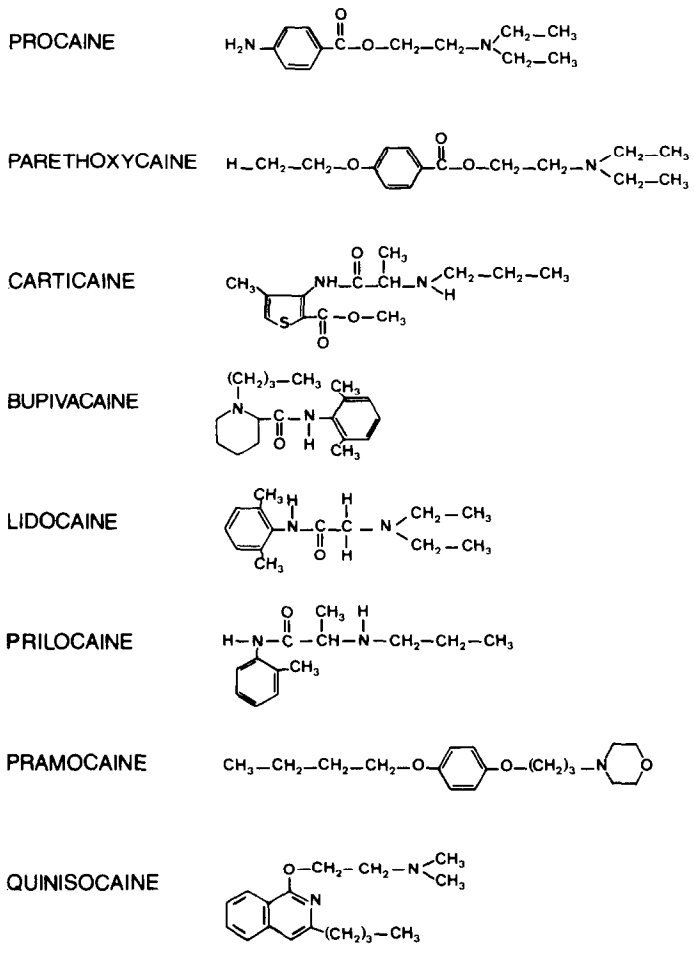

Fig. 1. Molecular structures of local anaesthetics.

Tween 80, all in 67 mM phosphate buffer at pH 7. The temperature was maintained at 37° during experimentation. The pH and temperature chosen were those indicated by Sigma for optimal cytochrome oxidase activity. The solutions were prepared just before use in order to minimize autooxidation (oxygen uptake in the absence of enzyme) of sodium ascorbate and TMPD. The cytochrome oxidase concentration was 0.55 μM. The anaesthetic was added to the assay mixture as soon as the base line was stabilized, and just before the addition of the enzyme.

The rate of oxygen consumption was calculated from the slope of the polarographic trace. Enzymatic activity was expressed as turnover number (TN), which is the number of electron equivalents utilized per sec and per mole of cytochrome oxidase. TN can be calculated from the following equation:

$$TN = \frac{\text{oxygen consumption } (\mu\text{M } O_2/\text{sec}) \times 4 \text{ (electrons*)}}{\text{cytochrome oxidase concentration } (\mu\text{M})}$$

* One mole of consumed oxygen corresponds to four electron equivalents utilized.

TN_{max} values correspond to the values of TN at infinite substrate concentration and are equal to the kinetic parameter V_{max} divided by the enzyme concentration. The oxygen consumption due to cytochrome oxidase activity was obtained by subtracting the oxygen consumption of the ascorbate–TMPD–cytochrome *c* system from that of the complete system including cytochrome oxidase.

RESULTS AND DISCUSSION

Inhibition of cytochrome oxidase activity by the various local anaesthetics investigated is summarized in Tables 1 and 2. The Lineweaver–Burk plot, $1/TN$ vs the reciprocal of the cytochrome *c* concentration, was used to determine parameters K_m and TN_{max}. The control values were obtained in the absence of anaesthetic agents; the kinetic parameters K_m and TN_{max}, measured from control experiments in a 67 mM phosphate buffer, agreed with the low affinity kinetic phase. The TN_{max} values were lower than those published by Singer [7], Yonetani [10] and Ferguson-Miller *et al.* [11]; this may be due to a different method of extraction and purification of the cytochrome oxidase used. Nevertheless, the

Table 1. Effect of local anaesthetics on cytochrome *c* oxidase binding

Anaesthetic	Ionization state* $[I]/[N]$ pH 7	Concentration (mM)	Inhibition type	Regression coefficient b†	Standard error s_b	Inhibition constant K_i (mM)	K_m (μM)	TN_{max} (sec^{-1})
Procaine	36.3	15	Mixed-type	1.47	0.11	12.8	9.54	6.50
		20	Mixed-type	1.86	0.12		10.75	5.78
				0.73‡	0.05‡		7.66‡	10.44‡
Parethoxycaine	12	10	Competitive	1.73	0.10	7.0	21.9	12.66
				0.73‡	0.05‡		9.01‡	12.25‡
		13	Mixed-type	1.56	0.08	11.6	8.55	5.48
				0.73‡	0.05‡		6.80‡	9.26‡
Carticaine	8.1	10	Competitive	1.35	0.17	6.0	8.81	6.52
		14	Competitive	2.37	0.14		18.70	7.87
				0.73‡	0.05‡		5.32‡	7.25‡
Bupivacaine	1.9	5	Competitive	0.74	0.09	5.3	11.23	14.30
		8	Competitive	1.54	0.07		18.68	12.10
				0.65‡	0.06‡		7.82‡	12.10‡
Lidocaine	1.8	15	Non-competitive	0.74	0.07	40.0	4.89	6.60
				0.51‡	0.03‡		4.70‡	9.10‡
		16	Non-competitive	0.84	0.12	30.0	5.60	6.60
				0.65‡	0.06‡		6.60‡	10.20
		18	Mixed-type	1.21	0.11	10.8	7.07	5.85
		20	Mixed-type	1.81	0.27		8.68	4.79
				0.58‡	0.04‡		4.96‡	8.55‡
Prilocaine	2.2	12	Non-competitive	0.73	0.07		8.20	11.20
		14	Non-competitive	0.93	0.10	18.6	8.62	9.23
		16.4	Non-competitive	1.32	0.28		9.00	6.83
				0.51‡	0.03‡		9.10‡	17.70‡
Pramocaine	0.2	1	Competitive	1.48	0.03	1.8	18.50	12.50
				0.73‡	0.05‡		11.90‡	11.74‡
		1.6	Competitive	3.08	0.32	0.6	18.70	6.06
				0.73‡	0.05‡		4.97‡	6.78‡
Quinisocaine	0.2	0.5	Competitive	1.34	0.09	0.62	10.03	7.48
		0.7	Mixed-type	1.46	0.11	0.71	6.97	4.77
				0.73‡	0.05‡		5.58‡	7.60‡

* $[I]/[N]$: Relative amounts of ionized and neutral anaesthetic according to the Henderson–Hasselblach equation $[I]/[N] = 10^{pK-pH}$.

† b is the regression coefficient of the Lineweaver–Burk plot.

‡ Denotes a control value.

measured inhibition constant for procaine was near that measured by Singer [7]: 12.8–12.0 mM. The different types of inhibition induce modifications of the kinetic parameters as follows. In the competitive type of inhibition, the inhibitor acts by increasing the K_m, TN_{max} is unchanged. The inhibitors which have no effect on K_m but act simply by reducing TN_{max} are termed 'non-competitive'. An inhibitor may act on both K_m and TN_{max}, giving a mixture of competitive and non-competitive effects: mixed-type inhibition. The inhibition constant values K_i were determined for purely competitive or non-competitive inhibition by a simple graphical method described by Dixon [12].

For the mixed-type inhibition we used the theoretical model of Friedenwald and Maengwyn-Davies [13], which assumes that the inhibition completely prevents the breakdown of the enzyme–substrate

Table 2. Variation of anaesthetic affinity for enzyme as a function of anaesthetic activity and octanol–water partition coefficient

Anaesthetic	Ionization state $[I]/[N]$ pH 7	Affinity K_i^{-1} (mM^{-1})	Anaesthetic activity of infiltration (AAI)	Log (K_i^{-1})	Octanol–water partition coefficient Log P
Procaine	36.3	0.078	1	−1.11	1.36
Parethoxycaine	12.0	0.086	7.0	−1.06	2.22
Carticaine	8.1	0.167	6.3	−0.78	1.70
Bupivacaine	1.9	0.189	10.1	−0.72	1.60
Lidocaine	1.8	0.093	4.2	−1.03	1.29
Prilocaine	2.2	0.054	4.5	−1.27	1.11
Pramocaine	0.2	0.555	17.7	−0.26	2.09
Quinisocaine	0.2	1.410	55.8	0.15	2.50

complex; in the case of this simple scheme the reciprocal slopes of the $1/TNf(1/s)$ plots vary linearly with the corresponding inhibitor concentration. K_i is deduced from the intersection of this line with the abscissa.

From the results given in Table 1 the following comments can be made:

(1) The same type of inhibition is encountered for anaesthetics showing a common moiety in their molecular structure, particularly the ester or amide group. The ionization state of the molecules seems to play a role in the type of inhibition observed.

Procaine and parethoxycaine contain an ester function (Fig. 1) and show mixed-type inhibition; parethoxycaine does not inhibit as much as its relatively high anaesthetic activity with respect to procaine would suggest. This may be due to its lower level of ionization. Lidocaine and prilocaine contain amide functions (Fig. 1) and are weakly ionized; they inhibit the oxidase activity by a non-competitive type of inhibition.

Bupivacaine and carticaine contain amide functions (Fig. 1) and are in a higher ionization state than the two former molecules; they act by a competitive type of inhibition. Pramocaine and quinisocaine have molecular structures different from the others (Fig. 1) and present a higher anaesthetic activity of infiltration. They bring about greater inhibition than the six above-mentioned molecules as attested by their low K_i values.

(2) We can also observe that the inhibition type varies with the anaesthetic concentration. At low concentrations the inhibition type is either competitive or non-competitive. For higher concentrations the inhibition is of mixed type; it seems that weakly ionized molecules present a non-competitive type of inhibition at low concentrations; lidocaine and prilocaine do so whereas parethoxycaine does not.

(3) Competitive-type inhibition is encountered for molecules presenting a strong anaesthetic activity and a highly ionized state.

Table 2 and Figs. 2 and 3 report the values of the octanol–water partition coefficient and the anaesthetic activity of infiltration (AAI) reported in a previous paper [5]. They illustrate the comparison of these parameters to oxidase anaesthetic affinity expressed as the reciprocal of K_i. We established a linear relationship between K_i^{-1} and the AAI (Fig. 2):

$$K_i^{-1} = 0.026\,(AAI) - 0.015,\ r = 0.99,\ N = 8,$$

$$t = \frac{r(N-2)^{1/2}}{(1-r^2)^{1/2}} = 17.35,\ t_{0.01[6]} = 3.14$$

The comparison of $\log(K_i^{-1})$ to $\log P$ is shown in Fig. 3 and leads to the following relationship:

$$\log(K_i^{-1}) = 1.03 \log P - 2.42,\ r = 0.99,\ N = 7,$$

$$t = 17.5,\ t_{0.01[5]} = 3.36$$

These two correlations are significant at 1%.

We tested the relationship between $\log(K_i^{-1})$ and $\log P$ for five molecules in a similar ionization state

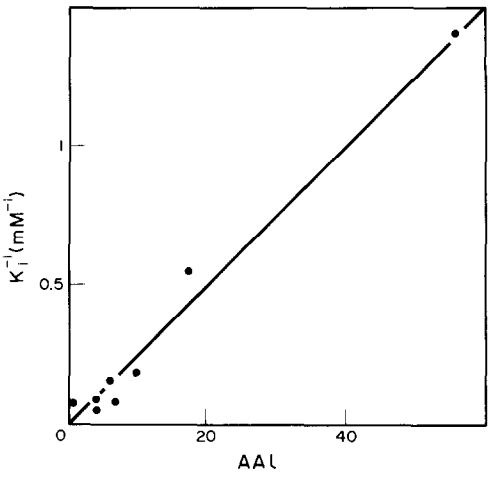

Fig. 2. Relationship between local anaesthetic affinity for enzyme K_i^{-1} and anaesthetic activity of infiltration (AAI).

and obtained the following results:

$$\log(K_i^{-1}) = 1.00 \log P - 2.35,\ r = 0.999,\ N = 5,$$

$$t = 42.6,\ t_{0.0005[3]} = 12.94$$

In this case the correlation is very significant. So a significative linear relationship was shown to exist between oxidase–anaesthetic affinity and anaesthetic activity as well as with the octanol–water partition coefficient. In a previous investigation [5] we observed a significant multilinear correlation between infiltration anaesthetic activity, partition coefficient and dissociation constant for a series of

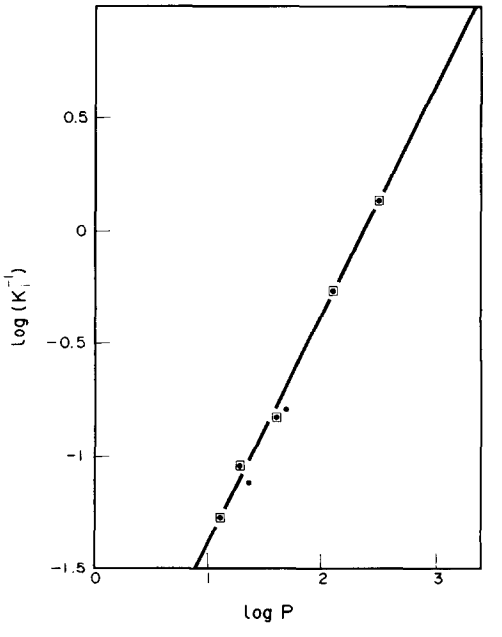

Fig. 3. Relationship between local anaesthetic affinity for cytochrome oxidase and octanol–water partition coefficient. The encircled points correspond to molecules in a similar ionization state.

twelve local anaesthetics. Thus the impact of the ionization state of molecules on their anaesthetic activity was shown; for molecules applied to the outside of the nerve membrane only the neutral drug form seemed to be effective. Hille [14] observed that local anaesthetics presenting a high liposolubility cross the cell membrane more easily. Borchard and Drouin [15] measured carticaine activity at various pH values and obtained a similar result. Otherwise several authors [16–18] have established that the cationic form of molecules affects the membrane permeability to sodium ions only when they are applied to the inside of the nerve membrane.

Lipid solubility of local anaesthetics mainly determines the amount of molecules crossing the membrane, while once inside the cell it is the protonated form that acts in blocking nerve conduction. Borchard and Drouin [15] reported that protein binding seems to reflect partly the interaction with the receptors within the nerve membrane.

These two points have to be correlated to the following comments, which can be made in the light of our studies on local anaesthetic binding to a membrane protein. The extent of the electrostatic interactions involved in the inhibition of oxidase activity by anaesthetic molecules now seems to be clear. Anaesthetics presenting a strong anaesthetic activity and existing in a high ionization state bind to cytochrome oxidase at the cytochrome *c* binding site, or to a site close to it since they act with a competitive-type inhibition; indeed, the binding process of cytochrome *c* oxidase mainly involves electrostatic interactions. We can therefore assume that anaesthetic molecules bind to cytochrome oxidase and inhibit its activity through the same type of interaction.

Singer [7] came to the same conclusion; however, he suggests that the anaesthetic effects cannot be explained by charge–charge interactions alone—hydrophobic interactions may occur between the lipophilic part of the anaesthetics and oxidase-associated phospholipids. Moreover, phospholipids are essential for oxidase effectiveness [19]. As a result of this, we decided to investigate the relationship between the liposolubility of the anaesthetics and their affinity for the enzyme. Our results agree with the assumption of Singer [7]; we observed a linear correlation between the enzyme affinity and the octanol–water partition coefficient of anaesthetic molecules. It is very significant (0.05%) for weakly ionized molecules.

Studies carried out on the interaction of local anaesthetics with proteins led to similar results. So Tanaka and Hidaka [20] investigated the interaction of three local anaesthetics (dibucaine, tetracaine and lidocaine) with calmodulin and showed a significant correlation between affinity for calmodulin and the octanol–water partition coefficient of anaesthetic molecules.

Also Boulanger *et al.* [21] observed the binding of procaine and tetracaine to lipid model membranes; their results suggest that there is at least one binding site for each of the charged and uncharged forms of these molecules. These findings support the proposed mechanisms of anaesthesia; indeed, theories elaborated on the interactions of local anaesthetics with proteins responsible for nerve conduction included structural perturbations of the membrane lipid bilayer.

The results we obtained on the inhibition of cytochrome *c* cytochrome oxidase binding by eight local anaesthetics gave us interesting information about the types of interaction between these species and a mitochondrial membrane protein. The importance of the ionization state and liposolubility of the anaesthetic molecules for cytochrome oxidase anaesthetic binding was particularly stressed.

The relationships between these two parameters and local anaesthetic activity were of great importance in the investigations carried out in order to define a model accounting for the various mechanisms of anaesthetic action. These considerations suggest a similarity between the molecular mechanisms involved in the interactions of anaesthetics with the proteins of the nervous membrane and the molecular mechanisms implied in the inhibition of mitochondrial electron transport at the cytochrome oxidase site by local anaesthetics.

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