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OPEN CHAIN CROWN-TYPE POLYETHERS AND PYRIDINOPHANE CRYPTANDS ACT AS IONOPHORES UPON FROG MOTOR NERVE AND ISOLATED RAT HEART CELLS

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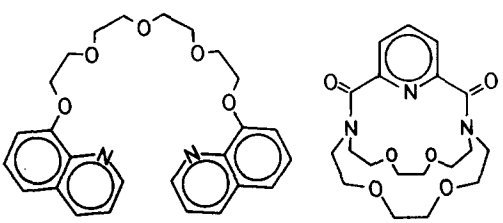
Summary

Frog motor nerves and isolated heart cells from neonatal rats were incubated with solutions of open chain crown-type polyether or pyridinophane cryptand. The following alterations in membrane excitability and energy consumption were found:

1. The non-cyclic ligand stabilizes the resting potential of the frog nerve and reduces the pulsation rate of heart muscle cells. It is reversibly bound at the cell surface and does not affect the energy metabolism of the heart cells.



(I)

(II)

$[2.2.1_{Py}]$-diamide

2. The cryptand 1,12-dioxo-2,11-diaza-5,8,21,24-tetraoxa[12-8^{2,11}](2,6)-pyridinophane) ($[2.2.1_{Py}]$-diamide) is irreversibly bound by the tissues. It facilitates the depolarization of the nerve and shows a positively chronotropic effect upon the heart muscle cells. Single treatment of the cell cultures with 10 μg $[2.2.1_{Py}]$-diamide per ml medium increased the activities of lactate dehydrogenase and of creatine kinase. When the cell cultures were treated three times at 24 h intervals with 10 μg complexone/ml, the creatine kinase activity

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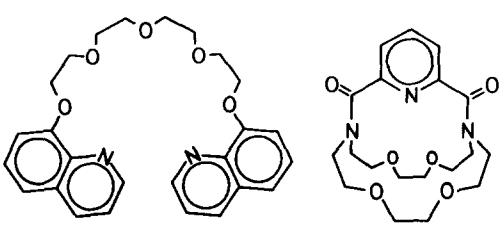
of the heart muscle cells decreased by about 40%.

The physiological properties of the ligands are correlated with the stability of their alkali metal ion complexes and with the rate constants of complex formation. It is concluded that [2.2.1$_{Py}$]-diamide can act as a passive carrier for Na^+ and K^+.

Introduction

Ionophores such as the cyclic depsipeptides are useful models for membrane transport systems, because they form lipid-soluble complexes with polar cations [1—3]. Studies on the molecular basis of the ionophore action suggest two different mechanisms for the permeation of cations across membranes: the transport of the metal ion by complexation with a mobile carrier molecule or through a pore formed by a channel-forming substance [2—4]. Valinomycin [5] and Gramicidin A [6] isolated from microorganisms are the best known examples for the two different mechanisms. "Crown ethers" [7,8] and "cryptates" [9,10] represent another class of model compounds which are able to form stable complexes with alkali and alkaline earth metal ions and to transport cations through apolar media.

We now report physicochemical and physiological properties of a pyridinophane cryptand [11] and of an open chain crown ether [12—15]. In order to be active as carrier molecules these compounds have to form stable complexes with the metal ions in question, but the rate of release of the metal ion must be comparable to the highest rate of ion transport observed in motor nerve axons [16]. These criteria may be established by determinations of the stability constants of the complexes and by measurements of the rate constants of the uptake and release of the metal ion, respectively [3,17—19]. It must also be shown that the compound interferes with biological translocation processes, either by demonstrating that the ionophore induces ion transport across artificial or biological membranes or by proving that the coupling of metabolism and transport is affected by the complexone [1—3]. The non-cyclic ligand (I) and the pyridinophane cryptand (II) shown in Fig. 1 fulfil



(I) (II)

[2.2.1$_{Py}$]-diamide

Fig. 1. Structural formula of compounds (I) and (II). (IUPAC names: ligand (I), 1,11-bis(8-quinolinyloxy)-3,6,9-trioxaundecane; ligand (II), [2.2.1$_{Py}$]-diamide = 1,12-dioxo-2,11-diaza-5,8,21,24-tetraoxa[12.8^{2,11}]-(2,6)pyridinophane).

the physicochemical requirements for a carrier molecule rather well [19] and we, therefore, investigated their action on excitable tissues. Since ionophores such as X537 A or monensin are pharmacologically active on mammalian cardiovascular systems [20—22], we used cultures of isolated heart cells from neonatal rats for the physiological studies. We examined the influence of the two compounds on beating frequency and energy metabolism. In order to check the significance of the results observed for heart cells, the action of the complexones on the excitability of frog motor nerve was investigated qualitatively.

Materials and Methods

I. Chemicals

The complexones were a gift from Prof. F. Vögtle, Institut für Organische Chemie, Bonn, G.F.R. Their purity was checked by thin layer chromatography. The chemicals for the enzymatic tests were obtained from Boehringer, Mannheim, G.F.R. The trypsin (type 1/250), Eagle's Minimum Essential Medium and newborn-calf serum were from Laborservice, Munich, G.F.R. The minimum essential medium was supplemented with vitamins and amino acids, purchased from Merck AG, Darmstadt, G.F.R., to give medium SM 20-I [23]. Bovine serum albumin (Serva, Heidelberg, G.F.R.) and highly purified DNA from calf thymus (Worthington Biochemical Corp., Freehold, N.J., U.S.A.) were used as protein and nucleic acid standard. All salts "pro analysi" grade were from Merck AG, Darmstadt, G.F.R.

II. Experiments with frog nerves

Frogs were purchased from Koch, Holzminden, G.F.R. The two N. ischiadici A and B of a frog (*Rana temporaria* or *Rana esculenta*) were incubated for 5 min at 25°C in a Ringer solution containing 1 μg complexone/ml. Before and immediately after treatment, and at time intervals of 5 and 40 min later, the excitability of the nerve was checked measuring the absolutely refractory period and the strength duration curve [24,25].

III. Experiments with isolated heart cells

1. Preparation of cell cultures

Cell cultures were prepared as described by Harary and Farley [26] with slight modifications (see below). Ventricles from 1- to 2-day old Wistar rats were dissociated into single cells by repeated mechanical homogenization and trypsination at 37°C (0.125% trypsin in a Ca^{2+} and Mg^{2+}-free phosphate-buffered saline solution [27]). After removal of the trypsin the isolated heart cells were resuspended in synthetic nutrient medium SM 20-I [23] supplemented with 10% calf serum. 4 ml cell suspension ($2 \cdot 10^6$ cells/ml determined with a Coulter Counter) were used for culture in a 15 × 60 mm Falcon-Petri Dish. The cultures were incubated at 37°C in a CO_2 atmosphere saturated with water vapour, and the nutrient medium was replaced ever 24 h.

2. Influence of the complexones on the pulsation rate of the heart cell cultures

On the fourth day the medium SM 20-I was substituted by 2 ml of a phosphate-buffered saline solution containing 0.2—10 μg/ml of the complexones (I) or (II). The solution contained no antibiotics or protein, in order to prevent the adsorption of complexones to components of the medium. The action of the complexones on the cells was studied in one of two ways: either the concentration of ligand was kept constant and the beating frequency of heart cells was observed microscopically over a time interval up to 18 h, or the concentration of the complexone was increased stepwise at time intervals of 6—10 min, and the resulting changes of the pulsation rate were recorded.

3. Influence of the compounds (I) and (II) on the energy metabolism of heart cells

Incubation schemes. (a) Multiple treatment. When the medium was changed after 24, 48 and 72 h, the cells were incubated for 10 or 20 min in 2 ml of antibiotic-free phosphate-buffered saline solution containing 10 μg complexone (I) or (II) per ml.

(b) Single treatment. At the fourth day the cells were incubated for 10, 20, 40, or 80 min with 2 ml of the phosphate-buffered saline solution of 10 μg/ml ligand (I) or (II). After incubation, the cultures were immediately processed as described below.

Analyses. After about 100 h the medium was removed by suction. The cell monolayer was washed twice with ice-cold phosphate-buffered saline solution and was then detached from the Petri dish, resuspended in 1.6 ml ice-cold saline solution, and homogenized. The DNA and protein content in the homogenate was determined by the methods of Burton [28] and of Lowry [29] as modified by Oyama and Eagle [30]. The activities of lactate dehydrogenase (EC 1.1.1.27) and creatine kinase (EC 2.7.3.2) were measured by conventional enzymatic tests [31,32].

Results

The electrophysiological experiments show that the complexones alter the excitability of the motor neuron. Both ligands induce a prolongation of the absolutely refractory period. The non-cyclic ligand (I) makes the depolarization of the nerve more difficult, although 8-hydroxyquinoline, as a constituent moiety of ligand (I), facilitates it. The cryptand $[2.2.1_{Py}]$-diamide increases the excitability. This ligand is irreversibly bound by the tissue.

Addition of 1 μg/ml ligand (I) to the heart cell culture decreases the rate of synchronous pulsations, until at a final concentration between 5 and 10 μg per ml a cessation of cytodynamic activity is observed. The effect is completely reversed by removal of the complexone. $[2.2.1_{Py}]$-Diamide shows a positively chronotropic effect on the pulsation rate of heart muscle cells which even continues after the medium is changed. At a concentration of 10 μg ligand/ml the pulsation rate is increased by a factor of 1.5—2.0 as compared to controls. This effect was clearly established about 30 min after the change of medium and was preserved during the whole observation time of 18 h.

The influence of (I) and (II) on the coupling of energy metabolism and

TABLE I

SINGLE TREATMENT OF HEART CELLS WITH COMPOUNDS (I) AND (II). ENZYME ACTIVITY OF LACTATE DEHYDROGENASE IN DEPENDENCE OF THE TIME OF INCUBATION AFTER THE CHANGE OF MEDIUM

The mean values, which were obtained in each case from three determinations, are expressed in international units of lactate dehydrogenase activity per mg cell protein. The concentration of ligand was 10 μg/ml.

	Time (min)			
	10	20	40	80
Non-cyclic polyether	1.13	0.99	0.83	0.69 *
[2.2.1p_y]-Diamide	1.08	1.00	1.02	0.94
Controls	0.97	0.97	0.93	0.74 *

* Values according to the average enzyme activities found in the absence of mechanical manipulations: 0.77 ± 0.09 I.U. lactate dehydrogenase/mg protein.

transport was studied by determination of the enzyme activities of lactate dehydrogenase and creatine kinase. The activity of lactate dehydrogenase reflects the production of free energy via anaerobic glycolysis of all cell types in heart cell cultures; the activity of creatine kinase, however, indicates the energy consumption of muscle cells only.

The two complexones act very differently on the energy metabolism (Tables I and II). Incubation with ligand (I) does not affect the activity of both

TABLE II

INFLUENCE OF THE OPEN CHAIN CROWN ETHER (I) AND THE CRYPTAND [2.2.1p_y]-DIAMIDE ON THE ENZYME ACTIVITY OF LACTATE DEHYDROGENASE AND CREATINE KINASE OF HEART CELLS

The enzyme activities are expressed in international units per mg protein or DNA. The concentration of ligand was 10 μg/ml. n is the number of samples; values are mean ± S.E.; P is the probability that the differences from the control values occurred by chance; LDH, lactate dehydrogenase; CK, creatine kinase.

Complexone	Time of incubation (min)	n	I.U. LDH / mg DNA	I.U. LDH / mg protein	I.U. CK / mg DNA	$10^2 \times$ I.U. CK / mg protein
Multiple treatment						
Non-cyclic polyether	10	5	26 ± 2	0.88 ± 0.05	0.64 ± 0.09	2.3
Non-cyclic polyether	20	2	27	0.78	0.63	1.8
[2.2.1p_y]-Diamide	10	5	28 ± 3	0.87 ± 0.09	0.81 ± 0.13	2.5 ± 0.6
[2.2.1p_y]-Diamide	20	4	31 ± 5	0.75 ± 0.06	0.46 ± 0.15 ($P < 0.005$)	1.21 ± 0.35 ($P < 0.005$)
Controls	10/20	5	29 ± 2	0.77 ± 0.09	0.75 ± 0.12	2.3 ± 0.5
Single treatment						
Non-cyclic polyether		12		0.91 ± 0.19		2.8 ± 0.8
[2.2.1p_y]-Diamide		12		1.01 ± 0.06 ($P < 0.1$)		3.8 ± 0.4 ($P < 0.005$)
Controls		12		0.93 ± 0.13		2.9 ± 0.3

intracellular enzymes. On the other hand, [2.2.1$_{Py}$]-diamide strongly interferes with the energy metabolism of the cell monolayer:

1. After single treatment of the cells the activity of lactate dehydrogenase remains at the high level of 1 unit/mg protein even 80 min after the change of medium, whereas at the same time the activity of controls has decreased to a value of 0.7 units/mg protein (Table I). The creatine kinase activity with $3.8 \cdot 10^{-2}$ units/mg protein is about 30% higher than the controls.

2. If the cells are incubated three times with cryptand for 20 min after 24, 48, and 72 h, the effect of [2.2.1$_{Py}$]-diamide is reversed when compared with the single treatment. The activity of creatine kinase decreases from 0.75 units/mg DNA for controls to 0.46 units/mg DNA for samples, whereas the activity of lactate dehydrogenase is not influenced (Table II).

Discussion

The non-cyclic ligand (I) and the cryptand (II) differ substantially with respect to their action on biological membranes. Complexone (I) reduces the excitability of nerve and heart cells reversibly. The energy supply from anaerobic glycolysis and creatine phosphate is not affected by the compound. Although the analysis of thermodynamic and kinetic data of the complexation of ligand (I) with alkali metal ions revealed [19] that the complexone meets the physicochemical requirements for a carrier molecule rather well by combining stability with dynamic lability; the complexone is not active as a carrier because it is not taken up by the cells. Instead, the ligand is reversibly bound at the outer surface of the cell membrane stabilizing the resting potential. This may be due to the fact that the mobility of the phospholipids is diminished by electrostatic interactions between the two positive charges of both quinoline moieties and the negatively charged backbones of the phospholipids. In addition, the complexation properties of the ligand may contribute to the reversible alteration of excitability.

The cryptand [2.2.1$_{Py}$]diamide facilitates the depolarization of the nerve and increases the beating frequency of heart cells. Neither effect disappears when the complexone solution is replaced by complexone-free nutrient medium. The compound affects the tissue possibly by irreversibly binding to the individual cells. In addition, a single dose of cryptand induces an increase of the enzyme activities of lactate dehydrogenase and creatine kinase, which corresponds to an enhanced energy requirement of the heart cells. This may be explained by the fact that the [2.2.1$_{Py}$]-diamide is bound to the cell membranes, modifies their structures, and thereby counteracts the energy-dependent ion translocation processes because of its complexation and solubility properties.

[2.2.1$_{Py}$]-Diamide forms 1 : 1 complexes with sodium and potassium ions of considerable high stability. (Stability constants in water, (lg K_{Na^+} = 4.6, lg K_{K^+} = 5.3, lg $K_{Ca^{2+}}$ = 4.8 [19]). The cryptand molecule is soluble in polar as well as in apolar media; therefore it can be deduced that the free ligand and its metal ion complexes can permeate the cell membrane. Since the discrimination factor between sodium and potassium ions, as calculated from the ratio of stability constants, is much lower than the concentration ratio of the two ions

in the intra- and extracellular compartments, mainly sodium ions are complexed and transported into the cell, whereas the reverse is true for the potassium ions. These ion fluxes across the cell membrane are in accord with the increased excitability of nerve and heart muscle cells.

The analysis of kinetic data supports the idea that the complexone $[2.2.1_{Py}]$-diamide represents a useful model for a carrier. As concluded from temperature-jump relaxation experiments [19,33,34] the complexation with Na^+ and K^+ follows a two-step mechanism:

$$M^+ + L \underset{k_{21}}{\overset{k_{12}}{\rightleftharpoons}} [ML^+]' \underset{k_{32}}{\overset{k_{23}}{\rightleftharpoons}} [ML^+]''$$

The first reaction step comprises the encounter of metal ion and ligand as well as the substitution of solvent molecules ($k_{12} = 3 \cdot 10^8\ M^{-1} \cdot s^{-1}$). It is followed by an isomerization process with a frequency of about $10^4\ s^{-1}$. The cryptand fulfils the postulate of Eigen and co-workers [17] that a two-step mechanism is most favourable for carrier-mediated transport. The high rate of the rather unspecific binding and solvent substitution step is combined with the specificity determining consecutive conformational change. The release of the metal ion with k_{off} values of approx. $10^4\ s^{-1}$ accords with the kinetic demands for a carrier molecule. Assuming that the complex moves across the membrane by simple diffusion, the permeation lasts for approx. 10^{-4} s [35]. The lifetime of the complex and the time required for diffusion are of the same order of magnitude.

When the heart cell cultures were treated three times with cryptand solution and were processed 24 h later (Table II), the activity of creatine kinase as a specific indicator of the ATP consumption of the muscle cells decreased by about 40% as compared to controls. On the other hand, $[2.2.1_{Py}]$-diamide increases the frequency of contractions, which consumes most of the cells energy. In our opinion this unexpected result can be explained by an increase of the free intracellular Ca^{2+}-concentration via a Na^+-for-Ca^{2+}-exchange system; the so-called Baker pump [36,37]. $[2.2.1_{Py}]$-Diamide increases the intracellular concentration of sodium ions and overcomes the Na^+-K^+-pump in the plasma membrane. The elevated intracellular concentration of Na^+ induces an increase in intracellular Ca^{2+} via the Baker pump. The rise of the concentration of free calcium improves contractility, e.g. more calcium ions are bound by the complex of the regulatory proteins of the myofibrils. As compared to the Na^+-for-Ca^{2+}-exchange system the direct carrier transport of calcium ions by complexation with $[2.2.1_{Py}]$-diamide contributes only to a small extent to the permation of Ca^{2+} across the cell membrane, because the ligand favours sodium ions by a factor of 18. This figure is calculated from the product of the ratios of stability constants ($K_{Na^+}/K_{Ca^{2+}} = 0.6$) and extracellular concentrations ($c_{Na^+}/c_{Ca^{2+}} = 30$).

Summing up all experimental results which were obtained using different techniques, we conclude that $[2.2.1_{Py}]$-diamide is a passive carrier for Na^+ and K^+. Since this synthetic cryptand might attain pharmacological importance for the treatment of cardiovascular diseases, furter studies of the action of $[2.2.1_{Py}]$-diamide on the cardiovascular system are in preparation.

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