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Effects of *N*-alkyl-*N,N*-dimethylamine-*N*-oxides on the activity of purified sarcoplasmic reticulum Ca^{2+}-transporting ATPase

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N-alkyl-*N,N*-dimethylamine-*N*-oxides (CnNO, n = 10–20 is the number of alkyl carbon atoms) stimulate the skeletal sarcoplasmic reticulum (SR) Ca^{2+}-transporting ATPase activity at low concentrations and inhibit it at high concentrations. The minimum concentration (c_{min}), at which CnNO inhibits the ATPase, continuously decreases up to n = 16–18 and then increases. The values of c_{min} are smaller than the CnNO critical micelle concentration (cmc) for C10NO-C14NO homologs, but larger than cmc for C18NO-C20NO homologs. The ATPase inhibition is caused by the CnNO-induced lipid bilayer structural perturbation in the ATPase annular region, modulated by the partition equilibria of the CnNO molecules between the bilayer and aqueous phase for short alkyl chain (n = 10–16) CnNO homologs, and between the bilayer, micelles and aqueous phase for long alkyl chain (n = 18–20) CnNO homologs.

1. Introduction

It is well known that the activity of the sarcoplasmic reticulum (SR) Ca^{2+}-transporting ATPase (EC 3.6.1.38) can be modulated by different amphiphilic and hydrophobic drugs (Gutierrez-Merino et al. 1989; Lee et al. 1991; Kutchai et al. 1994; Karon et al. 1999). In particular, we have observed that *N*-alkyl-*N,N*-dimethylamine-*N*-oxides (CnNO, n = number of alkyl carbon atoms) at a constant concentration stimulate the activity in case of short alkyl chain homologs ($n < 12$) but inhibit it in case of long ($12 \leq n \leq 18$) chain homologs (Andriamainty et al. 1997). In the present communication, we extend our previous study to cover a wide range of CnNO concentrations.

2. Investigations, results and discussion

Fig. 1 shows the effect of C12NO on the relative ATPase activity (REA) of purified protein. It is seen that C12NO has practically no effect on the activity up to 20 μmol/l, slightly improves the activity in the region of 30–300 μmol/l, and then inhibits it to zero at higher concentrations. While the increase in activity was observed in each experiment, the value of maximum activity and the C12NO concentration at maximum activity slightly varied between the experiments. Similar dependencies of the activity on the CnNO concentrations were observed with the all CnNO homologs studied. From dependencies similar to that in Fig. 1, the minimum inhibition concentration, c_{min}, was evaluated as the intersection of a linearly approximated decreasing part of the REA dependence on CnNO concentration (full line in Fig. 1) with the horizontal abscissa at REA = 0. The dependence of c_{min} on the CnNO alkyl chain length n displays a typical "cut-off" course – the ability to inhibit the ATPase activity progressively increases with the increase of n up to a maximum potency between n = 16 and 18 and than decreases (Fig. 2). This deviation from the Meyer-Overton rule

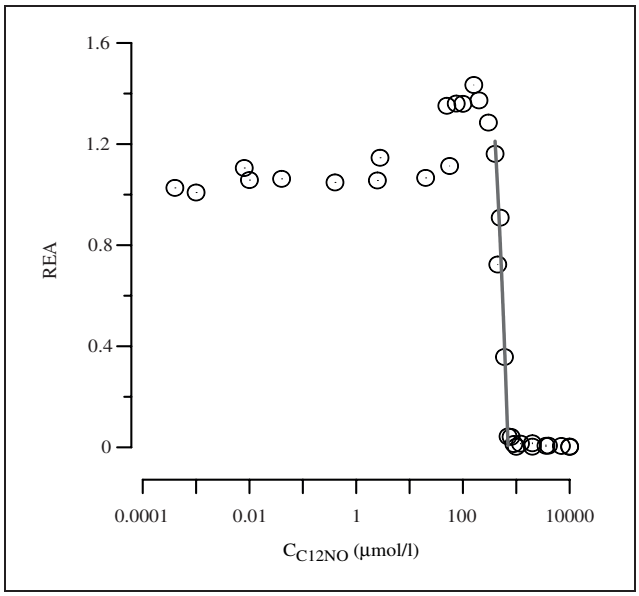

Fig. 1: Dependence of the relative activity (REA) of purified Ca^{2+}-transporting ATPase (3.66 μg/ml) on the C12NO concentration c_{C12NO}. The specific activity at $c_{C12NO} = 0$ was ~16 U/mg

(Meyer 1899a; Meyer 1899b; Overton 1901) is a general phenomenon and it has been observed in various biological and toxic activities in practically every amphiphile homologous series as a function of the length of linear hydrocarbon substituent (Ferguson 1939; Balgavý and Devínsky 1996). In Fig. 2, the c_{min} values are compared with the critical micelle concentration (cmc) of the CnNOs studied. It is seen that between $n = 10-14$, the values of c_{min} and cmc change in parallel (linear correlation $r^2 = 0.974$) and $cmc > c_{min}$, while $c_{min} \geq cmc$ is observed for $n \geq 16$. This means that the C10NO-C14NO homologs interact with the purified Ca^{2+}-ATPase as monomers, while the C16NO-C18NO homologs form micelles at c_{min}. The same substances display the "cut-off" effect in different biological activities. For example, CnNO homologs show similar quasi-parabolic dependence in antimicrobial (Devínsky et al. 1990) and antiphotosynthetic (Šeršeň et al. 1992) activities (Fig. 2), with the decreasing parts of the potency curves for the last two longest homologs at effective concentrations larger than cmc. The simplest explanation of the "cut-off"-type curves in Fig. 2 could therefore be the change of CnNO partition equilibrium of CnNO monomers between the site of action and aqueous solution for short-chain homologs to a more complicated equilibrium of CnNO between the site of action, aqueous solution and micelles for long-chain CnNO homologs.

The purified Ca^{2+}-ATPase prepared by the cholate method is inserted in bilayer fragments containing about 80 phospholipid molecules per protein molecule (Warren et al. 1974a, 1974b; Shivanna and Rowe 1997); 30–32 phospholipid molecules form a ring around the protein (East et al. 1985; Lee 2003). The structure of these 30–32 "annular" phospholipids critically influences the ATPase activity (Lee 1998). The enzyme activity is highest in the fluid dioleoylphosphatidylcholine bilayers, but lower in fluid bilayers from phosphatidylcholines with shorter or longer monounsaturated acyl chains (Lee et al. 1991). The activity is also low in fluid bilayers of dioleoylphosphatidylserine or dioeloylphosphatidic acid (Dalton et al. 1998) or in phosphatidylethanolamines under conditions where these lipids form a nonbilayer phase (Starling et al. 1996).

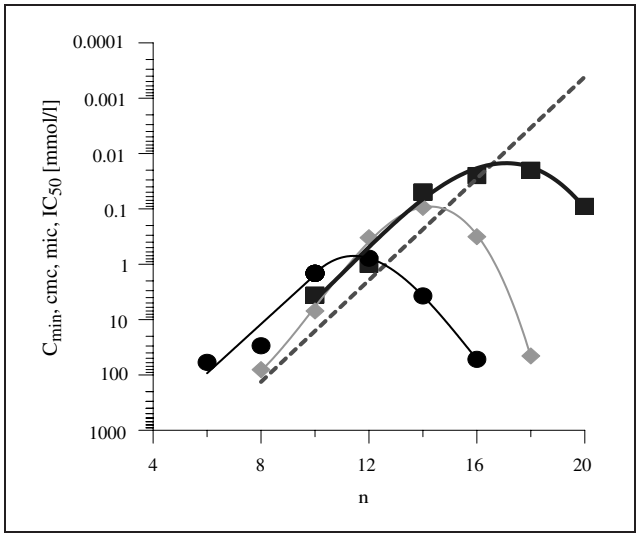

Fig. 2: Dependence of the minimum Ca^{2+}-ATPase inhibition concentration c_{min} (■), of the minimum Escherichia coli inhibition concentration mic (●), of the half-maximum Hill reaction inhibition concentration in spinach chloroplasts IC_{50} (◆) and of the critical micelle concentration cmc (dashed line) on the CnNO alkyl chain length n. The mic and cmc values were taken from (Devínsky et al. 1990) and the IC_{50} values from (Šeršeň et al. 1992)

C12NO predominantly interacts with the lipid component of Ca^{2+}-ATPase membranes (Kragh-Hansen et al. 1998). The partition coefficients between the lipid and aqueous phase increase exponentially in the CnNO homologous series studied (Devínsky et al. 1990; Hrubšová et al. 2003). CnNOs penetrate into phospholipid bilayer and affect its fluidity (Balgavý et al. 1989; Gallová 1993) and thickness (Dubničková et al. 1997; Karlovská et al. 2004); at high concentrations, CnNOs induce formation of nonbilayer phases in the phosphatidylcholine (Uhríková and Stanovská 1990) and solubilize bilayers in unilamellar liposomes via formation of mixed micelles (Uhríková et al. 2001; Hrubšová et al. 2003). At a constant bilayer concentration, the structural bilayer perturbations summarized above are largest for the shortest chain homologs C6NO-C8NO and decrease continuously with the increase of the chain length. One can therefore suggest that the bilayer structural perturbations in the annular region could be the molecular mechanism of the Ca^{2+}-ATPase activity inhibition when the CnNO concentration in the bilayer is modulated by the CnNO partition equilibria between the bilayer and aqueous phase for short chain homologs and between the bilayer, aqueous phase and micelles for long chain homologs. The free-volume theoretical model of the cut-off effect, originally formulated for local anesthetics (Uhríková et al. 1993), has to be extended to include the partition equilibria with micelles.

The cause of the activity increase observed at low CnNO concentrations is less clear. (Kutchai et al. 1994) have observed that hexanol stimulates the Ca^{2+}-ATPase at low concentrations and inhibit at high concentrations. They have concluded from results of the Ca^{2+}-ATPase rotational mobility study that the biphasic effects of hexanol on the activity can be accounted for by biphasic effects of hexanol on the oligomeric state of the Ca^{2+}-ATPase – hexanol promotes the dissociation of larger oligomers into smaller ones al low concentrations, whereas at higher concentrations, hexanol causes larger oligomers to be formed from smaller ones. A similar biphasic effect on the activity has been observed for tertiary amine local anesthetics (Escudero and Gutierrez-Merino 1987; Gutierrez-Merino et al. 1989; Andriamainty et al. 1996) and pentobarbital (Fernandez-Salguero et al. 1990). From these studies, it has been suggested that the activity stimulation is the result of these amphiphilic drugs binding to the protein non-annular binding sites.

3. Experimental

The *N*-alkyl-*N,N*-dimethylamine-*N*-oxides were prepared from corresponding *N,N*-dimethylakylamines by oxidation with hydrogen peroxide and purified as described (Devínsky et al. 1978). L-Histidine was purchased from Calbiochem, dithiothreitol (DTT), pyruvate kinase (from rabbit muscle, EC 2.7.1.40, PK) and Amberlite XAD-2 from Serva, adenosine-5-triphosphate disodium salt (ATP), lactate dehydrogenase (from rabbit muscle, EC 1.1.1.27, LDH), disodium salt of nicotinamide-adenine dinucleotide (reduced, grade I, NADH), phenylmethylsulfonylfluorid (PMSF), and monopotassium salt of phosphoenolpyruvate (PEP) from Boehringer Mannheim, ethylene glycol-bis-(aminoethylether)-*N,N,N′,N′*-tetraacetic acid (EGTA) and *N*-2-hydroxyethylpiperazine-*N′*-2-ethanesulfonic acid (HEPES) from Sigma, anhydrous calcium chloride and the other chemicals were from Lachema. All commercial chemicals and biochemicals were of the highest available purity. The solvents were redistilled before use.

The Ca^{2+}-ATPase was prepared according to the method outlined by Warren et al. (1974a, 1974b) with slight modifications. A young female rabbit (New Zealand White), weighing about 2.5 kg, was killed and bled. All subsequent operations were carried out at 4 °C. White muscle was dissected from the hindlegs and spinal region, finely chopped on a glass plate and excessive fat and connective tissues removed. A 100 g batch of white muscle was homogenised in a Waring blender for 30 s with 200 ml ice cooled 0.3 mol/l sucrose, 20 mmol/l histidine, 1 mmol/l DTT and 5.1 mol/l PMSF (pH 8.0). The homogenate was centrifuged for 15 min (8000 × g,

4 °C). The undisrupted material was retreated as above. The pooled supernatants were filtered through fine muslin and centrifuged for 90 min (37000 × g, 4 °C). The pellets were resuspended in ice-cooled 0.3 mol/l sucrose, 10 mmol/l histidine, 0.6 mol/l KCl, 1 mmol/l DTT and 5 mmol/l PMSF (pH 8.0, 0.5 ml/g muscle) using a homogeniser tube fitted with a pestle. This homogenate was then centrifuged for 20 min (4000 × g, 4 °C). The supernatant was then centrifuged for 90 min (37000 × g, 4 °C). The pellet was resuspended in 3–5 ml of 0.25 mol/l sucrose, 1 mol/l KCl, 50 mmol/l potassium phosphate (pH 8.0), transferred to dialysis tubing and dialysed overnight against 1 l of the same buffer. To purify (Ca-Mg)ATPase, sarcoplasmic reticulum (SR) was solubilised by adding potassium cholate (100 mg/ml) in the same buffer, to give a final ratio of 0.4 mg cholate/mg protein. This material was loaded onto a discontinuous sucrose gradient. Each gradient tube contained 1 ml of 60% w/v, 7 ml of 30% w/v and 5 ml of 20% w/v sucrose in ice cooled 1 mol/l KCl, 50 mmol/l potassium phosphate (pH 8.0). The solubilised SR was layered onto the gradients to give 40–45 mg of protein/tube. The gradients were then centrifuged for 18 h (95000 × g, 4 °C). The pure enzyme was collected from the 30%–60% interface and was washed by suspending in five volumes of 1 mol/l KCl, 50 mmol/l potassium phosphate (pH 8.0) and centrifuged for 1 h (95000 × g, 4 °C). The pellet was resuspended in 3 ml of the same buffer, transferred to dialysis tubing and dialysed overnight against 1 l of gently stirred 0.25 mol/l sucrose, 1 mol/l KCl, 50 mmol/l potassium phosphate (pH 8.0), containing 10 g of washed Amberlite XAD-2 ion-exchange resin to remove residual cholate. The final preparation was aliquoted and stored at −20 °C. The total (Ca-Mg)ATPase protein concentration was determined by measuring the absorbance at 280 nm according to Hardwicke and Green (1974). The ATPase activity was determined using a slight modification of the coupled assay system outlined by Warren et al. (1974a, 1974b). The ATPase sample was diluted in 1.0 ml of assay buffer containing HEPES (40 mmol/l), $MgSO_4$ (5.1 mmol/l), ATP (2.1 mmol/l), phosphoenolpyruvate (0.53 mmol/l), EGTA (1.02 mmol/l), NADH (0.152 mmol/l), 7.5 IU of PK, and 18 IU of LDH, pH 7.2. The CnNO was contained in the assay buffer. After incubation at 37 °C for 15 min, the reaction was started by the addition of $CaCl_2$ solution in the assay buffer and the decrease of absorbance at 340 nm was measured. Measurements were made at 37 °C using a Hewlett Packard 8452A spectrophotometer and cells of the 10 mm optical path length. ATPase activity (A) in units per mg of ATPase protein (U/mg) was calculated according to the equation $A = A_{340}V_{ass}/6.22m_{prot}$, where A_{340} is the change in absorbance per min, V_{ass} is the final assay volume in ml, and m_{prot} is the amount of ATPase protein in the assay volume in mg. The constant 6.22 takes into account the NADH extinction coefficient. The relative ATPase activity (REA) is defined as $REA = A_{CnNO}/A_0$, where A_{CnNO} is the activity in the presence of CnNO and A_0 in its absence.

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