INFLUENCE OF 2-PHENYLETHANOL AND 1,1'-DIMETHYLPHENYLETHANOL ON METABOLIC ACTIVITY AND CELL MEMBRANE FUNCTION IN EHRLICH ASCITES TUMOUR CELLS

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1. Introduction

Since the description of bacteriostatic properties of 2-phenylethanol (PEA) [1] there have been numerous investigations concerning the action of this agent on a variety of biological systems. These include: influence on cell growth in cell cultures [2, 3], isolated cell components [4], bacteria [5–6], fungi [7], and on bacteriophages [8, 9]; effects on the synthesis of macromolecules in bacteria [10, 11], viruses [12], mammalian cells [13], experimental tumour cells [14–17], and *in vitro* systems [18, 19]; action on yeast cells [20, 21]; influence on enzyme activities [22, 23]. Interestingly, it has been found that PEA is produced by the fungus *Candida albicans*, most likely as an autoantibiotic [24].

It is quite reasonable to assume that a substance like PEA may exert a number of different effects on living cells by virtue of its physico–chemical properties. We studied the effect of PEA and its 1,1'-dimethyl derivative (DMPEA) on the metabolic activity of Ehrlich–Lettré ascites tumour (EAT) cells, particularly with respect to possible membrane alterations, since the cell membrane is thought to be a major site of action of PEA (e.g. 6, 7, 25).

2. Materials and methods

For all studies, the glycogen-free strain of the hyperdiploid Ehrlich–Lettré mouse ascites tumour (EAT) was used. The cells were harvested 7 days after transplantation. Since it could be shown that prolonged stirring or shaking results in changes of membrane permeability and eventually in lysis and/or aggregation, all experiments with intact cells were carried out within $1-1\frac{1}{2}$ hr after removal from the animals. Anaerobic glycolysis was measured by 4 independent methods: i) autotitration with 0.005 N NaOH in isotonic NaCl under N_2 at 37°C; ii) enzymatic assay of substrate consumption and lactate production; iii) ion exchange chromatography and liquid scintillation counting with [^{14}C]glucose; iv) Warburg manometry with a CO_2/N_2 gaseous mixture.

Respiration was measured by conventional Warburg manometry.

For the determination of glycolytic rates, the pH-stat method plus enzymatic control measurements proved very convenient, and the results agreed well with those obtained from tracer studies.

Adsorption isotherms were determined with a double-beam recording UV-spectrophotometer. Dye uptake (lissamin green V) by the cells was recorded spectrophotometrically.

Cell-free lysates from EAT cells, prepared by homogenization at 0°C after addition of NAD^+, ATP, and $MgCl_2$, and subsequent centrifugation, were poor candidates fro metabolic studies. With red cell and yeast lysates, however, we were able to obtain unequivocal results [26–28]. Potassium efflux from the cells was measured with an atomic absorption spectrophotometer. Leakage of macromolecules from the cytoplasm was estimated by photometric assay of lactate dehydrogenase (LDH) in the medium; mitochondrial damage was correlated to the efflux of glutamate dehydrogenase (GLDH). Sonication was used in prepar-

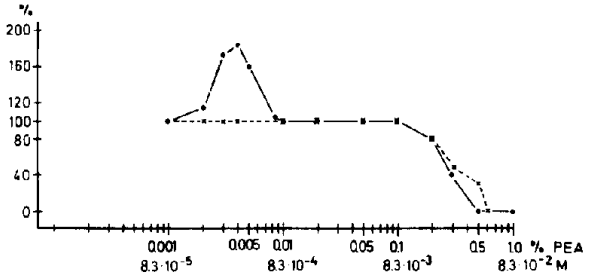

Fig. 1. Anaerobic glycolysis of EAT cells (6.6×10^6 cells/ml) treated with PEA, in % of control. Incubation 15 min at 37°C, pH 7.2. $n = 14$: (•—•—•) Cells washed three times; (X---X---X) unwashed cells.

ing emulsions of PEA and DMPEA in concentrations higher than 10^{-2} M. The octanol/water partition coefficients were determined spectrophotometrically. Possible morphological alterations of the cells were controlled by phase contrast microscopy.

3. Results

3.1. PEA inhibited glycolysis and respiration of EAT cell suspensions significantly when applied in final concentrations of 10^{-2} M and higher (figs. 1 and 2). At a concentration of 4×10^{-2} M (0.48%), metabolic activity was abolished. There was almost full (90%) reversibility of this inhibition up to 2×10^{-2} M, partial reversibility (approx. 30%) at 3.5×10^{-2} M, and sudden irreversibility at higher concentrations. There were,

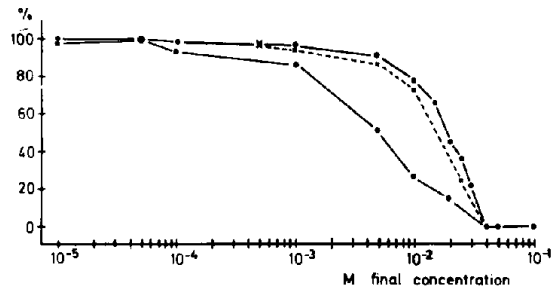

Fig. 2. Respiratory activity of EAT cells (4.5×10^7 cells/ml) treated with 2-PEA and 1,1-dimethyl-PEA, in % of control. Incubation 15 min at 37°C, pH 7.2. $n = 14$: (•—•—•) Unwashed cells, treated with PEA; (X---X---X) cells washed three times, treated with PEA; (o—o—o) unwashed cells treated with 1,1-dimethyl-PEA.

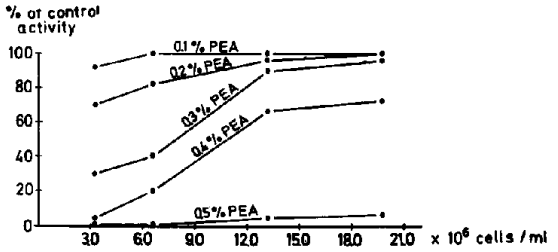

Fig. 3. Dependence of glycolytic activity of EAT cells on the cell concentration at constant PEA concentrations. $n = 14$.

however, no gross morphological alterations of the cells, as judged from phase contrast microscopy.

3.2. DMPEA acted roughly 5–10 times more strongly than PEA, (see fig. 2 for respiration; for glycolysis and potassium efflux similar differences were found).

3.3. The octanol/water partition coefficients were found to be 21.0 for PEA and 60.4 for DMPEA. According to [29] the membrane/buffer partition coefficients were 4.2 and 12.1, respectively.

3.4. Significant leakage of potassium and macromolecules from the cells was brought about only by high concentrations. Concomitantly, dye uptake occurred only at the same high concentrations.

3.5. PEA-induced reduction of glycolysis at lower concentrations was paralleled by a decrease of intracellular [^{14}C]glucose. Thus, metabolic reduction by 40% was accompanied by a 30% decrease of intracellular [^{14}C]glucose, whereas at high PEA concentrations intra- and extracellular levels were equal, irrespective of the external concentrations.

3.6. Untreated cells which were washed 3 times possessed only about 50% of the metabolic activity of unwashed cells. There was marked leakage of macromolecules from these cells, as indicated by the release of large amounts of LDH from the cytoplasm and even of GlDH from mitochondria. The triple washing procedure also depleted the cells by 75% of their total potassium content. Interestingly, LDH efflux could largely be abolished by treating the cells with low doses of PEA (from 2×10^{-4} M–2×10^{-3} M, depending on the cell number). Higher concentrations failed to counteract this leakage.

3.7. The degree of metabolic depression by the agents was not only a function of the concentration of the latter, but also of the cell number (fig. 3).

3.8. When PEA acted upon 6.6×10^6 cells/ml in a final concentration of 8.3×10^{-5} M (0.001%), 90% of the agent was adsorbed by the cells, whereas at 4.15×10^{-4} M (0.005%) 48% of the substance could be recovered from the supernatant. With rising PEA concentrations, additional adsorption decreased. At 4.15×10^{-2} M (0.5%) there was no further adsorption, i.e. saturation occurred. Unwashed cells adsorbed 30% more agent than washed cells.

3.9. Interestingly, cells which were washed 3 times prior to incubation exhibited a marked stimulation of anaerobic glycolysis at very low PEA concentrations, whereas unwashed cells remained completely unaffected. The optimum effect was found at a final concentration of about 3.3×10^{-4} M (0.004%) with 6.6×10^6 cells/ml (fig. 1). No such enhancement could be observed with respiration.

3.10. The dose–response curves for the metabolic alterations produced by the two agents remained essentially unchanged when glucose was replaced by other metabolic substrates, such as fructose, sorbitol, succinate, and pyruvate or when these substrate concentrations were increased up to 30-fold.

4. Discussion

Our findings strongly suggest that the metabolic effects of PEA and its derivative are related to a physico–chemical action on the cell membrane. If penetration into the cell were the decisive event one should expect the effects to be solely dependent upon the concentration of the agent, i.e. the transmembranal concentration gradient. This does not seem to be the case, since all effects studied were a function of the cell concentration as well. Adsorption studies were in good agreement with these findings. The chemical nature of the substances indicates a possible mechanism of action: The hydrophobic moiety of the molecule adsorbs to membrane lipids and/or lipoproteins whereas the hydrophilic group associates with polar residues. Thus, intercalation with membrane constituents may lead to an impairment of membrane function, e.g. glucose uptake, either by simple 'crowding' or by conformational changes of membrane-bound molecules which are engaged in transport and/or regulatory mechanisms. This is in agreement with the fact that lower concentrations which reduce metabolic activity by approx. 40% bring about a concomitant reduction of intracellular glucose by approx. 30%. Higher concentrations would allow for more hydrophobic interactions between agent molecules, eventually leading to micelle formation [30], membrane emulsification and complete membrane barrier breakdown, as indicated by irreversible metabolic inhibition, dye uptake, and free diffusion of electrolytes, substrate molecules, and macromolecules.

The apparently curious finding that very small amounts of PEA increase the glycolytic rate of washed cells – and only of these – may be explained by the surface activity of the agent: As described for local anaesthetics on erythrocyte membranes [30], small doses may produce a 'strengthening' effect by lowering the membranal surface tension. Since washed cells have been damaged mechanically, as indicated by the loss of macromolecules and a reduction of the metabolic rate by 50%, the 'strengthening' effect would lead to a recovery to almost 100% activity, as compared with unwashed cells. The same mechanism may be responsible for the reduction of LDH leakage during washing procedures in the presence of low PEA concentrations. Higher concentrations would fail to counteract metabolic reduction by simultaneously precluding substrate from the cells. Respiration, on the other hand, could not recover because the loss of mitochondrial enzymes caused irreversible damage. Finally, one should expect the 1,1′-dimethyl derivative to act more strongly than the parent compound because of its higher octanol/water partition coefficient. This, in fact, was shown for all parameters.

Investigations on other compounds in this class of substance should be of interest, especially with respect to structure–function relationships.

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