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EFFECTS OF NEUROPHARMACOLOGICAL AGENTS ON MICROSOMAL MEMBRANE: INFLUENCE ON DEOXYRIBONUCLEIC ACID–MEMBRANE INTERACTIONS *IN VITRO**

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Summary—Over 20 neurotropic drugs were tested for their ability to interact with microsomal membrane and for their influence on membrane-deoxyribonucleic acid (DNA) associations *in vitro*. About 85% of the compounds tested altered the density of the membrane in caesium chloride equilibrium density gradients, the macroscopic appearance of the membrane band, and the extent of DNA binding. In three out of five cases studied in greater detail, the decrease in membrane density was correlated with a reduced protein content of the membrane. Theoretical significance and potential practical applications of these observations are considered.

In our recent report (KUBINSKI, GIBBS and KASPER, 1972b) we have examined the conditions and the specificity of association between cellular membranes and natural and synthetic nucleic acids *in vitro*. We have found that these interactions are highly specific and reproducible under carefully controlled experimental conditions. Such properties make this a suitable model system for studies on membrane–nucleic acid interactions. During our experiments on the effects of chemical carcinogens we have noted (KUBINSKI and KASPER, 1971; KUBINSKI, ANDERSEN and KELLICUTT, 1972a; KUBINSKI, STRANGSTALIEN, BAIRD and BOUTWELL, 1973) that these compounds can drastically affect the amounts of nucleic acids associated with membranes. At the same time the density and the macro- and microscopic appearance of the membrane band may also be altered. All of these changes can occur independently or in combination with each other. Since membranes are involved in a variety of regulatory and synthetic activities and since their ability to interact with nucleic acids may be related to their functions during cell replication and protein synthesis (KUBINSKI *et al.*, 1972b), we felt that a broad survey of the effects of various classes of biologically active molecules on membrane-deoxyribonucleic acid (DNA) interactions was appropriate.

In the present communication we wish to report on the action of neuropharmacological agents on the microsomal membrane and its interaction with DNA. The number of compounds which are able to alter the microsomal membrane–nucleic acid system (as judged by at least one of the criteria mentioned above) approximates 85% of the drugs studied. Since the techniques used are simple and the results reproducible, we believe that future investigations of this kind may aid substantially our understanding of the mechanisms of action of drugs and other biologically active substances.

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MATERIALS AND METHODS

Methods of purification of microsomal membrane and nucleic acids

Rat liver microsomal membrane was prepared as previously described (KASHNIG and KASPER, 1969). Ethylene glycol was added to a final concentration of 10% to avoid damage during freezing and thawing (KASPER and KUBINSKI, 1971). The membrane preparations were stored at −85°C and thawed immediately before use.

Deoxyribonucleic acid used for this work was obtained from bacteria. We have previously observed (KUBINSKI *et al.*, 1972b) that DNA from this source and that from mammalian cells associate in a very similar manner with cellular membranes. Thus, bacterial DNA was used throughout this study.

Escherichia coli Q13 cells were incubated for more than 10 generations in a semi-synthetic medium (GROSSMAN, 1967) supplemented with radioactive phosphate and the cells were collected by centrifugation. Deoxyribonucleic acid was extracted with the modified (KUBINSKI, OPARA-KUBINSKA and SYZBALSKI, 1966) technique of MARMUR (1961), separated from low molecular weight radioactive material by gel filtration on Sephadex G25, and stored frozen.

Sources of drugs

Several research and commercial organizations provided chemicals tested in this study. We obtained orphenadrine citrate from Riker Laboratories; desipramine from Geigy; phenindamine tartrate and levallorphan tartrate from Hoffman-LaRoche; benzotropine mesylate from Merck Institute for Therapeutic Research; artane from Lederle Laboratories; haldol (haloperidol) from McNeil Laboratories, Inc.; dimethisoquin, chlorpromazine and diethazine from Smith, Kline and French Laboratories; holocaine and tetracaine from Sterling–Wintrop Research Institutes. D-Lysergic acid (LSD), mescaline hydrochloride and neostigmine bromide were purchased from Sigma Chemical Company. Morphine sulphate, anectine, novocaine and dopamine were obtained through the courtesy of Dr. S. Kornguth from this University. All other chemicals of the highest available purity were purchased from commercial sources. The drugs were dissolved in water and dimethylsulphoxide. In a separate series of experiments we have determined that the latter compound in concentrations used in this study neither changed the membrane density in caesium chloride nor interfered with binding of DNA.

DNA-membrane binding assay

One microgram of labelled DNA was mixed on ice with the amount of the microsomal membrane corresponding to 400 μg of membrane protein. Sodium chloride, magnesium chloride and phosphate buffer at pH 7·2 were added to the final concentration of 0·1, 0·01 and 0·02 M, respectively. Drugs which were to be tested for their effects on the DNA-membrane interaction were added at the same time. The samples were then transferred to a 40°C water bath and incubated with occasional mixing by hand. After 30 min of incubation, the samples were diluted 13-fold with 0·05 M phosphate buffer, pH 7·2, containing 0·1 M NaCl. Saturated solution of caesium chloride (CsCl) was added to adjust the density to 1·18 g/ml (refractive index between 1·351 and 1·353). Centrifugation was performed in a Spinco SW 41 rotor at 28,000 rev/min for 22 hr. Fractions were collected from the bottom of the tubes and refractive indices measured to establish the CsCl concentration along the gradient (ANDERSON and ANDERSON, 1968). Subsequently, aliquots

were pipetted onto planchets or into scintillation vials to determine the distribution of radioactive DNA.

Protein was estimated by the Folin procedure (BAILEY, 1962) and phosphorus by the method of BARTLETT (1959).

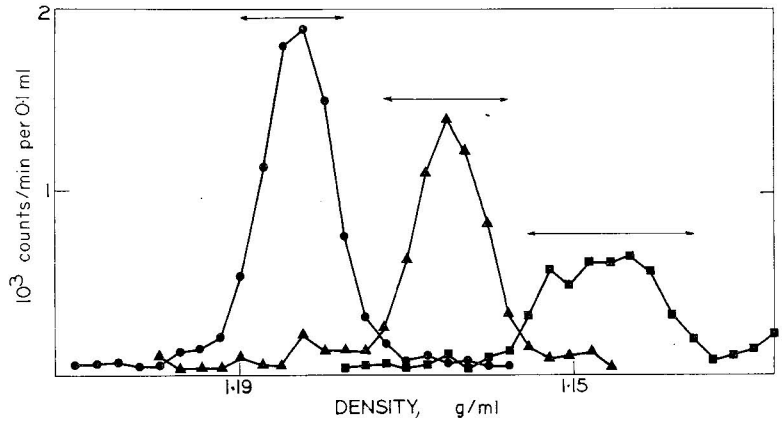

Fig. 1. Effects of chlorpromazine on DNA-microsomal membrane interactions. 1 μg of ^{32}P-labelled DNA was mixed with 400 μg of microsomal membrane (measured as protein). The sample was incubated for 30 min at 40°C, diluted with buffered NaCl and CsCl solutions, centrifuged and the fractions collected and assayed as described in Materials and Methods. In two parallel experiments chlorpromazine was added to the final concentration of 100 and 400 μg/ml and present since the beginning of incubation at 40°C. The double-headed arrows denote the positions of visible membrane material in the gradient. Circles: no chlorpromazine control; triangles: with chlorpromazine, 50 μg/sample; squares 200 μg/sample. Total volume of the sample at the time of incubation at 40°C: 0·5 ml.

RESULTS

Effects of two concentrations of chlorpromazine on the density of the microsomal membrane and its ability to associate with DNA are presented in Figure 1. In control tubes, the membrane associated with the DNA (8–10% of the total DNA present in the sample, see KUBINSKI *et al.*, 1972b) formed a well-defined homogenously dispersed band localized at density 1·180–1·185 g/ml; the unbound nucleic acid was found at the bottom of the gradient. Deoxyribonucleic acid contributed less than 0·1% of the total weight of the complex under our experimental conditions. Thus, no detectable increase in the membrane density due to the binding of the DNA alone (density 1·71 g/ml) was observed.

At the concentration of 100 μg of chlorpromazine per ml, the density of the membrane decreased by more than 15 mg/ml, and the amounts of DNA bound, by almost one-half. The macroscopic appearance of the band also changed: the material appeared aggregated and spread over a somewhat wider range of densities than in the control. Increase in the drug concentration further decreased the density of the membrane and the amounts of DNA associated with it. Macroscopically, all membrane material appeared in large, amorphous clumps, distributed over an even wider range of densities.

Effects of this and several other neuropharmacological agents on membrane density and its ability to associate with DNA are listed in Table 1. Concentrations of the compounds shown in Table 1 (the calculated molar concentrations are given in parentheses immediately after the drug's name) were those during the time of incubation at 40°C and before

Table 1. Effects of neuropharmacological agents on the density of rat liver microsomal membrane in CsCl and on the ability of the membrane to interact with DNA. Concentration of the drugs: 400 μg/ml (this concentration in mM, is given in parentheses immediately after the name of the drug). Data average from at least two experiments (INCR = increased; DECR = decreased; NC = no change)

Compound	Main pharmacological activity	Change in membrane density (mg/ml) Main band	Satellite band	Change in binding of labelled *E. coli* DNA (control = 100)
Chlorpromazine (1·26)	Tranquilizer	−42*	No satellite	DECR (41)
Haldol (haloperidol) (1·06)	Tranquilizer	NC	No satellite	INCR (121)
Desipramine (1·52)	Antidepressant	−25	−35 to +51*	DECR (70)
Dopamine (2·61)	Hormone	−20†	−2	INCR (154)
L-DOPA (2·03)	Anti-Parkinson	−25	−25 to +25*	DECR (70)
Diethezine hydrochloride (1·19)	Anti-Parkinson Anticholinergic	−45	−29	DECR (37)
Barbital sodium (1·93)	Sedative	NC	No satellite	NC (109)
Morphine sulphate (10·53)	Analgesic	−10	−10 to −16*	DECR (62)
D-Lysergic acid diethylamide (LSD) (1·24)	Psychomimetic	NC	No satellite	NC (109)
Mescaline (1·87)	Psychomimetic	NC	No satellite	NC (93)
Orphenadrine citrate (0·87)	Anticholinergic	−5	No satellite	NC (108)
Phenindamine tartrate (0·97)	Anticholinergic	−10	No satellite	INCR (132)
Tensilon (Edrophonium) (1·61)	Parasympathomimetic; cholinesterase inhibitor	−5	No satellite	NC (92)
Neostigmine bromide (1·32)	Parasympathomimetic; cholinesterase inhibitor	NC	No satellite	DECR (89)
Decamethonium bromide (0·96)	Neuromuscular blocking	+6	No satellite	NC (102)
(+)-Tubocurarine chloride (0·58)	Neuromuscular blocking agent	−18	No satellite	NC (96)
Anectine chloride (succinylcholine chloride) (0·89)	Neuromuscular blocking agent	NC	No satellite	INCR (117)
Tetracaine hydrochloride (1·33)	Local anaesthetic	−25	No satellite	INCR (110)
Dimethisoquin hydrochloride (1·33)	Local anaesthetic	−14	−14 to −19*	INCR (281)
Novocaine (1·47)	Local anaesthetic	NC	No satellite	INCR (121)
Cyclomethycaine hydrochloride (1·01)	Local anaesthetic	−8	No satellite	INCR (275)
Holocaine hydrochloride (1·13)	Local anaesthetic	−5	No satellite	INCR (115)
Dibucaine hydrochloride (1·05)	Local anaesthetic	−5	No satellite	INCR (316)

* The membrane zone appears aggregated.
† Dark-coloured band.

further dilution and addition of CsCl. We have arbitrarily chosen the concentration of 400 μg/ml, since we had noted that for most chemicals for which systematic studies on the dose–response relationship were carried out, this was the highest concentration beyond which no or very little change in membrane density and binding of DNA was observed.

Changes in membrane density (as compared to the control sample) greater than ±3 mg/ml and changes in nucleic acid binding exceeding ±10% were considered significant. This assessment was based on our observation that for a given membrane preparation, the data

Table 2. Phosphorus/protein ratio in rat liver microsomal membrane treated with five neurotropic drugs; before and after CsCl centrifugation

	Phosphorus/protein (μg/mg)	
	Before CsCl	After CsCl
Control	32 (100)	33 (103)
Desipramine	32 (100)	37 (115)
Diethazine	28 (85)	35 (110)
Dopamine	33 (103)	74 (232)
Tetracaine	42 (132)	46 (144)
Tubocurarine	30 (94)	49 (153)

The concentration of each drug during co-incubation with membrane was 400 μg/ml (see also Table 1). Numbers in parentheses express per cent change compared with the control sample not subjected to CsCl. Data average from two analyses.

were reproducible within these limits (KUBINSKI *et al.*, 1972b). Results obtained with different membrane preparations were qualitatively similar but exhibited slight quantitative fluctuations. For this reason efforts were made to use a single membrane preparation for these comparative studies.

Table 1 includes such chemically and pharmacologically diverse compounds as psychomimetic drugs, local anaesthetics, and neuromuscular blocking agents. Most of them are used in human and veterinary medicine. Out of 23 neuropharmacological agents studied, 15 significantly changed the density of the microsomal membrane and 17 altered the amounts of DNA bound. Only four out of 23 compounds (sodium barbiturate, mescaline, LSD, diphenylpyraline) showed none of the two activities. Several drugs drastically decreased the density of the membrane and the amounts of DNA associated with it. Chlorpromazine, desipramine, morphine and the anti-Parkinson agents (L-DOPA and diethazine) were perhaps the most conspicuous. The two anticholinergic drugs tested, orphenadrine and phenindamine, decreased the density much less drastically and slightly increased the amounts of DNA bound. All six local anaesthetics increased the DNA binding and all but one decreased the density of the membrane. The magnitude of change was different for various drugs: three times as much DNA was bound in the presence of dibucaine, while tetracaine had only a marginal effect. Conversely, the two anaesthetics had similar effects on the density change; however, while tetracaine decreased the density by as much as 25 mg/ml, dibucaine decreased the density by only 5 mg/ml.

The factors responsible for the changes in membrane density are poorly understood. Most of the drugs studied decreased this density and such decrease could be due, at least in part, to a variation in the degree of hydration or to the preferential removal of membrane proteins (average density in CsCl 1·43 g/ml; see HU, BOCK and HALVORSON, 1962). To examine this point, microsomal membrane was exposed to five different neurotropic drugs (Table 2), each of them previously found to decrease membrane density. After incubation with the particular agent, the membranes were diluted in 0·14 M NaCl, isolated by centrifugation and the pellet extensively washed by one additional round of centrifugation in 0·14 M NaCl, since traces of each of the five drugs tested strongly interfered with the protein analysis. One half of each sample was used for protein and phosphorus analyses, while the other half was centrifuged until equilibrium in CsCl buffered with Tris–HCl. The membrane zone was collected, diluted two to three times with distilled water, pelleted and the phosphorus-to-protein ratio determined. Since 97% of the total membrane phos-

phorus is derived from phospholipid (BLACKBURN, 1973) the values give a reliable measure of alterations in relative phospholipid levels.

Table 2 summarizes the results. With the single exception of tetracaine, no increase in the phosphorus/protein ratio was apparent after the treatment of membranes with the individual drugs. However, after centrifugation in CsCl this ratio was drastically increased for samples previously treated with dopamine, tetracaine and tubocurarine. This increase was minimal or absent with membranes exposed to desipramine and diethazine. No increase was detected in control sample.

We conclude, therefore, that while in the case of some neuropharmacological agents (dopamine, tetracaine, tubocurarine) the observed density decrease may be conceivably due to selective removal of membrane proteins or to their labilization and subsequent loss during CsCl centrifugation, with certain other drugs (desipramine and diethazine, both of which drastically decrease the membrane density in CsCl; see Table 1) different mechanisms may be involved.

DISCUSSION

Most of the neuropharmacological agents studied, changed the density of the microsomal membrane and its ability to interact with DNA. We have suggested previously (KUBINSKI *et al.*, 1972b) that the DNA-membrane interaction may play an important regulatory role in cellular metabolism. Effects of various drugs on the cellular membrane observed in this study may reflect their activity *in vivo*; however, the relative significance of this reaction as compared to other pharmacological activities of any particular drug remains to be established.

A cursory examination of Table 1 suggests that agents with limited ability to interact with microsomal membrane and to alter its affinity toward DNA appear to be generally less toxic and have fewer side effects than those which drastically alter membrane properties. For example, chlorpromazine is recognized as a cytostatic agent (VAN WOERT and PALMER, 1969; COHEN, 1973); tetracaine and dibucaine inhibit growth of transformed cells in tissue culture (RIFKIN and REICH, 1971) etc. All of these chemicals either drastically change the membrane's density (tetracaine, chlorpromazine) or its affinity toward DNA (dibucaine, chlorpromazine). If future experimentation confirms this impression, the techniques described in the present report, together with those recently published (ANDERSEN, GIBBS and KUBINSKI, 1974) may become useful as tools for mass screening of new drugs.

Our observations on the effects of neurotropic agents on the density of microsomal membrane suggest that at least one of these chemicals (tetracaine) may bring about the direct release of proteins from membrane. In other cases, the proteins remain bound to the membrane and resist repeated washings; however, upon exposure to CsCl such proteins dissociate from the membrane (labilization). This was most notable in the case of dopamine and tubocurarine.

The specificity of these reactions is unknown at present and future studies are needed to establish whether the protein release is random or whether the patterns differ among various neuropharmacological agents. The apparent lack of correlation between the density change and the amounts of DNA bound by the membrane suggests that these patterns may be different for various chemicals. Our earlier observations established that, in general, basic proteins enhance binding of DNA, while acidic proteins inhibit this reaction (KUBINSKI *et al.*, 1972b). Thus, the release by neurotropic drugs of certain basic proteins may correlate with a decrease in the amount of DNA associated with the membrane while

a similar removal of an acidic protein may conceivably increase the membrane's affinity toward DNA. While the relationship between our *in vitro* observations and the pharmacological activity of these compounds is obscure, the results described in the present report suggests the possibility of selective modification and limited dissection of cellular membranes *in vitro* by neuropharmacological agents.

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