24. Pessayre D, Allemand H, Wandscheer JC, Descartoire V, Artigou J-Y and Benhamou J-P, Inhibition, activation, destruction, and induction of drug-metabolizing enzymes by trichloroethylene. *Toxicol Appl Pharmacol* **49**: 355–363, 1979.

25. Yoshida T, Suzuki Y and Hashimoto Y, Sex-related effect of cadmium on hepatic cytochrome P-450 drug-metabolizing enzymes and gamma-aminolevulinic acid synthetase and heme oxygenase in the rat. *Toxicol Lett* **4**: 97–102, 1979.

Biochemical Pharmacology, Vol. 37, No. 19, pp. 3785–3787, 1988.
Printed in Great Britain.

0006-2952/88 $3.00 + 0.00

Phenothiazine-mediated depolarization of the plasma membrane in a renal cell line

(*Received* 11 *January* 1988; *accepted* 12 *April* 1988)

The phenothiazines have been described recently as agents useful for the protection of ischemic tissue [1–4]. Several authors, however, have commented upon the cellular toxicity of phenothiazines, describing increased plasma membrane permeability [2], plasma membrane blebbing [2], mitochondrial abnormalities [2, 5], and decreased cellular proliferation and clonogenicity [6]. Here, we report phenothiazine-induced depolarization of the plasma membrane after exposure to low concentrations of phenothiazines. This depolarization represents an early effect of phenothiazines on the cell membrane.

Materials and methods

Cell culture. LLC-PK1 renal epithelial cells (between passages 209 and 230) were cultured according to methods previously described [2]. The culture medium consisted of Dulbecco's Minimal Essential Medium (DMEM) containing glutamine and 10% fetal bovine serum. Cells were grown at 37° in a humid 5% carbon dioxide atmosphere until utilized in experiments, which were performed at room temperature (20°).

Reagents and incubation techniques. Promethazine, chlorpromazine, trifluoperazine and *N*-(6-aminohexyl)-5-chloro-1-napthalene sulfonamide (W-7) were obtained from Sigma. 1,1',3,3'-Hexamethylindocarbocyanine iodide (DiICl (3)) was obtained from Molecular Probes (Junction City, OR).

For all experiments, approximately 10^6 cells were incubated in 2 ml of Earle's Balanced Salt Solution (EBSS) containing 4 g/L glucose and either 50 μM or 1.8 mM $CaCl_2$ at pH 7.3. Plasma membrane potential was quantitated using the fluorescent probe DiICl(3). This is a cationic fluorescent probe which partitions between the cytoplasm and the extracellular space in accordance with the magnitude of the membrane potential [7, 8]. A stock solution of this dye in dimethyl sulfoxide (2 mM) was stored at 0° and diluted to 1 μM in EBSS prior to use. An equilibrium distribution, as demonstrated by stable cellular fluorescence, was achieved after 15-min incubations of the cells in the dye solution. Phenothiazines were added to the suspension at concentrations varying from 0 to 100 μM, and measurements were made after 30-min exposures of the cells to the phenothiazine.

Cells were harvested using one of two methods in order to produce populations of cells with differing basal membrane potentials: (1) exposure of monolayers to a 0.25% trypsin–EDTA solution at 37° until detachment from substratum, and (2) sequential exposure of monolayers to Ca^{2+}-free EBSS solution at 37° for 30 min followed by exposure to Ca^{2+}-free EBSS solution containing 6 units/ml papain, again at 37° for 5–10 min until detachment.

Flow cytometry. Flow cytometric analysis was performed with an FACS-III fluorescent-activated cell sorter (Becton-Dickenson) using a 488 nm laser operated at 300 mW. Scatter size was set to bracket the LLC-PK1 cell size distribution while excluding both debris and cellular clumps from measurement. A bandpass filter was placed in front of the photomultiplier tube to ensure that only fluorescent light was recorded. Relative membrane potential was measured as described previously [7, 8]. Membrane potential recordings were begun at least 15 min after addition of the fluorescent probe to the cell suspension, at a time when a constant level of dye accumulation had been observed in the cells, indicating equilibrium.

Statistical analysis. All statistical analyses were performed using analysis of variance, followed by the two-tailed *t*-test for unpaired data when the F-test indicated a significant difference between groups. Bonferroni's correction was used, when appropriate, to correct for multiple comparisons.

Results

Figure 1 shows the effect of various concentrations of phenothiazines on the plasma membrane potential in LLC-PK1 cells, measured after 30-min exposures to the phenothiazine. The mean concentrations required to depolarize the plasma membrane to 50% of its control value (IC_{50}) were 9 μ3 μM for trifluoperazine, 19 ± 2 μM for chlorpromazine ($P < 0.05$ from trifluoperazine), 39 ± 2 μM

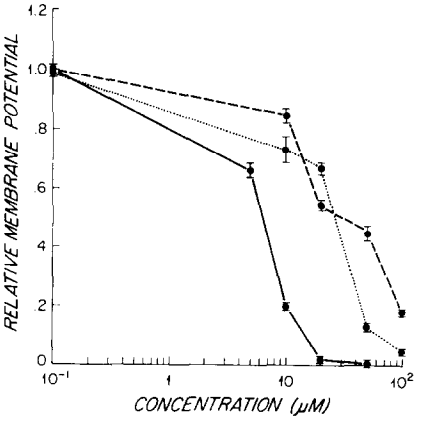

Fig. 1. Effect of various concentrations of phenothiazines upon plasma membrane potential. Relative membrane potential indicates that percent dye content of cells after 30 min of phenothiazine exposure relative to the dye content of control cells at 30 min. Points are means ± SEM, N = 4. Each curve is representative of several experiments. Key: (——) trifluoperazine, (.....) chlorpromazine, and (— —) promethazine.

Table 1. Time course of chlorpromazine-mediated depolarization

Time (min)	% Control potential*
0	100
2	64 ± 5†
14	51 ± 8†
36	51 ± 6†
59	52 ± 3†
95	46 ± 2†‡

The membrane potential of LLC-PK1 cells was monitored as a function of time of exposure to 30 μM chlorpromazine. Values are means ± SEM, N = 3.

* Percent control potential = $\frac{\text{dye content of cells at time t}}{\text{dye content of cells at time 0}} \times 100.$

† $P < 0.01$ from control.

‡ $P < 0.02$ from 2 min.

for promethazine ($P < 0.05$ from chlorpromazine) and 44 ± 10 μM for W-7. A progressive depolarization was observed with time, as indicated in Table 1, although the rate of depolarization was most marked immediately after exposure.

Cells were obtained with differing degrees of membrane injury by harvesting either with trypsin–EDTA or Ca^{2+}-free EBSS-papain solutions. Relative DiICl(3) uptake was determined for each group and the two groups were then exposed to identical graded concentrations of chlorpromazine from 0 to 100 μM (see Fig. 2). Cells harvested using papain–Ca^{2+}-free EBSS demonstrated lower membrane potentials than did those harvested with trypsin–EDTA ($P < 0.01$). Cells with greater degrees of basal membrane injury (as evidenced by a lower membrane potential) demonstrated greater sensitivity to phenothiazine-induced depolarization ($P < 0.001$).

We were unable to determine a consistent influence of extracellular calcium (at concentrations between 50 μM and 9 mM) upon the phenothiazine-induced membrane depolarization. Similar concentrations of chlorpromazine depolarized cells to 50% initial potential in the presence of either 0 (24 ± 2 μM chlorpromazine) or 1.8 (19 ± 2 μM chlorpromazine) mM calcium (not significant).

Trypan blue staining was studied with time of exposure of cells to 0–100 μM promethazine and chlorpromazine. Appreciable staining was observed after a 25-min exposure to 50 and 100 μM concentrations of the phenothiazines, with equivocal staining seen at 20 μM concentrations after a 45-min exposure. In all cases, these changes in permeability to trypan blue were preceded by significant plasma membrane depolarization.

Discussion

The phenothiazines represent a class of calmodulin inhibitors in wide clinical use which have been reported recently to protect ischemic tissue *in vitro* [1–4]. In addition to their calmodulin inhibiting properties, phenothiazines are known to affect membrane permeability by a direct action, likely affecting lateral phase separation [9].

Several toxic effects have been reported in cells exposed to phenothiazines. These include changes in proliferative ability [6], plasma membrane permeability [2], and alterations in mitochondrial membrane potential [5] and ultrastructure [2]. Recent work has suggested that several of these changes constitute earlier markers of cellular injury than others [10–12]. Lemasters *et al.* [11] recently demonstrated that changes in mitochondrial potential precede alterations in membrane permeability. Similarly, we have

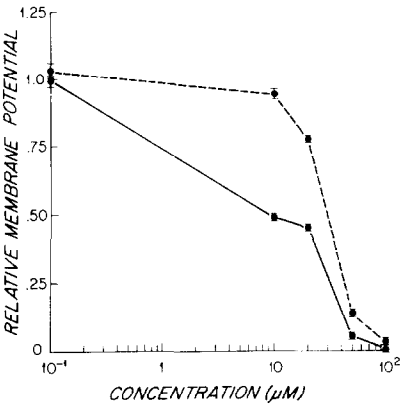

Fig. 2. Effect of cell injury upon membrane potential response to phenothiazine. Relative membrane potential, defined as in Fig. 1, is shown for cells with two different initial membrane potentials exposed to differing concentrations of chlorpromazine. Measurements were made after a 30-min exposure to the phenothiazine. The two cell populations were obtained by harvesting cells with two different enzyme techniques, resulting in different degrees of cellular injury incurred during isolation. Note that the depolarization is more pronounced relative to the initial (unexposed) potential in injured cells (cells with an initially lower dye content). The injured cell group had an initial dye content 31% that of the un-injured group. The dye contents of both groups of cells were normalized (such that the initial potentials, prior to phenothiazine exposure, were equal) to facilitate visual comparison of the two curves. Injured (——) and un-injured (– – –) cells are shown. Points are means ± SEM, N = 3.

shown changes in plasma membrane potential to precede alterations in permeability, using a hydrogen peroxide model of injury [12]. In this paper, we describe alterations in the plasma membrane potential occurring in response to the phenothiazine agents preceding changes in permeability to trypan blue dye.

All three phenothiazines studied significantly reduced the plasma membrane potential at micromolar concentrations. Their effectiveness in producing this depolarization paralleled their potency as calmodulin inhibitors [13], i.e. trifluoperazine ($IC_{50} = 9$ μM) > chlorpromazine ($IC_{50} = 19$ μM) > promethazine ($IC_{50} = 39$ μM). The non-phenothiazine calmodulin inhibitor, W-7, depolarized the cells in a similar concentration range ($IC_{50} = 44$ μM). The depolarization was evident within minutes of exposure (Table 1) and increased slightly with time. Injured cells were more susceptible to depolarization by phenothiazines, as shown by the lower phenothiazine concentration required to depolarize injured cells to 50% of the baseline potential.

Changes in plasma membrane permeability to trypan blue followed depolarization of the plasma membrane after phenothiazine exposure. It is possible that metabolic abnormalities leading to plasma membrane depolarization precede changes in membrane permeability. Also, since the membrane potential is dependent upon the distribution of molecular species (K^+, Na^+) smaller trypan blue, it may be that more subtle changes in plasma membrane integrity produce alterations in the plasma membrane potential. It has been suggested that calmodulin antagonists may act as weak potassium ionophores [5], which might result in membrane depolarization.

We conclude that the phenothiazines altered the plasma membrane potential at micromolar concentrations in LLC-PK1 cells and that this depolarization preceded changes

in plasma membrane permeability to trypan blue. The depolarization was dependent upon both the concentration of, and time of exposure to, phenothiazines, was more pronounced in injured cells, and appeared immediately upon exposure to the drug.

Acknowledgements—Dr. Scott is the recipient of an NIH New Investigator Research Award CA33570. This work was also supported by NIH Ischemia SCOR HL-56212, NIH Grant AM-27401, and by a grant from R. J. R. Nabisco.

* *Department of Radiology and*
‡ *Cardiac Unit*
Massachusetts General Hospital and
Harvard Medical School
Boston, MA 02114, U.S.A.

JAMES A. SCOTT* †
ALAN J. FISCHMAN*
BAN-AN KHAW* ‡
CARLOS A. RABITO*

REFERENCES

1. Schaffer SW, Roy RS and McCord JM, Possible role for calmodulin in calcium paradox-induced heart failure. *Eur Heart J* **4**(H): 81–87, 1983.
2. Scott JA, Khaw BA, Fallon JT, Locke E, Rabito CA, Peto CA and Homcy CJ, The effect of phenothiazines on maintenance of membrane integrity in the cultured myocardial cell. *J Mol Cell Cardiol* **18**: 1242–1254, 1986.
3. Schaffer SW, Burton KP, Jones HP and Oei HH, Phenothiazines protect in calcium overload-induced heart failure: A possible role for calmodulin. *Am J Physiol* **13**: H328–H334, 1984.
4. Barron E, Marshall RJ, Martorana M and Winslow E, Comparative anti-arrythmic and electrophysiologic effects of drugs known to inhibit calmodulin (trifluoperazine, W-7 and beperedil). *Br J Pharmacol* **89**: 603–612, 1986.
5. Snelling R and Nicholls D, The calmodulin antagonist trifluoperazine and R24571 depolarize the mitochondria within guinea pig cerebral cortical synaptosomes. *J Neurochem* **42**: 1552–1558, 1984.
6. Hait WN and Lee GL, Characterization of the cytotoxic effects of the phenothiazine class of calmodulin antagonists. *Biochem Pharmacol* **34**: 3973–3978, 1985.
7. Scott JA, Fischman AJ, Khaw BA and Homcy CJ, Free radical-mediated depolarization in renal and cardiac cells. *Biochim Biophys Acta* **899**: 76–82, 1987.
8. Scott JA, Khaw BA, Homcy CJ and Rabito CA, Oxygen radicals alter the cell membrane potential in a renal cell line (LLC-PK1) with differentiated characteristics of proximal tubular cells. *Biochim Biophys Acta* **897**: 25–32, 1987.
9. Lieber ML, Lange Y, Weinstein RS and Steck TL, Interaction of chlorpromazine with the human erythrocyte membrane. *J Biol Chem* **259**: 9225–9234, 1984.
10. Wolman B, Different modes of cell death and related phenomena: A proposal for meaningful terminology in relation to tests of lethality. *J Theor Biol* **114**: 343–350, 1985.
11. Lemasters JJ, Giuseppi J, Neiminen A-L and Herman B, Blebbing, free calcium and mitochondrial membrane potential preceding cell death in hepatocytes. *Nature* **325**: 78–81, 1986.
12. Scott JA, Fischman AJ, Homcy CJ, Fallon JT, Peto CA, Khaw BA and Rabito CA, Evaluation of plasma membrane injury during oxidant exposure. *Clin Res* **36**: 526A (abstract), 1988.
13. Weiss B, Prozialeck WC and Wallace TL, Interactions of drugs with calmodulin. *Biochem Pharmacol* **31**: 2217–2226, 1982.

† Correspondence: Dr. James A. Scott, Department of Radiology, Massachusetts General Hospital, Boston, MA 02114.

Biochemical Pharmacology, Vol. 37, No. 19, pp. 3787–3790, 1988.
Printed in Great Britain.

0006-2952/88 $3.00 + 0.00

Irreversible inhibition by tyrosine-directed alkylating reagents of muscarinic cholinergic receptors in membranes from rat forebrain and heart

(*Received* 16 *November* 1987; *accepted* 7 *March* 1988)

Muscarinic cholinergic receptors (mAChRs) are composed of several types. The genes of rat cerebral M_1 and cardiac M_2 receptors have been recently cloned and sequenced [1, 2]. Other potential mAChR types have been suggested based on biochemical and functional studies as well as by gene cloning studies [2]. A major characteristic of the mAChR types is their different affinities for selective muscarinic ligands such as pirenzepine (PZ) [3] and AF-DX 116 [4]. Whether or not this difference reflects a variation of the amino acid compositions at the ligand binding site of the different receptor types is a field of active investigation. In the present study, two tyrosine-directed reagents were used to modify the mAChRs from rat forebrain and heart. The results suggest that the existence of a tyrosyl residue is essential for ligand binding to both rat forebrain (mostly M_1) and cardiac (mostly M_2) muscarinic receptors.

Methods and results

Male Sprague–Dawley rats (200–300 g) were killed by decapitation. The forebrains and whole hearts were dissected and homogenized in 20 vol. of ice-cold 50 mM sodium/potassium phosphate buffer (pH 7.4) with a Polytron homogenizer. The homogenates were centrifuged at 1000 *g* for 5 min. The supernatant fractions were recentrifuged at 40,000 *g* for 20 min. The pellets were suspended in the same buffer supplemented with 25 mM $MgCl_2$ and with either 4-fluorosulfonyl-1-hydroxy-2-naphthoic acid (FSNA, Aldrich Chemical Co. Inc.) or *p*-nitrobenzenesulfonyl fluoride (pNBSF, Pierce Chemical Co.) dissolved in absolute alcohol (control samples received an equal amount of alcohol). The final concentration of alcohol in homogenates was 2%. The homogenates were incubated at room temperature for 15 min. In the protection experiments the homogenates were preincubated with muscarinic drugs for 1 hr. The reaction was stopped by immersion of the tubes in an ice-water bath followed by centrifugation. The resuspension–centrifugation process was repeated three times. The final pellets were resuspended in 50 mM sodium/potassium phosphate buffer. An aliquot of the homogenate was incubated with [^{3}H](−)quinuclidinyl benzilate ([^{3}H](−)QNB; 33.2 Ci/mmol, New England Nuclear) at 25° for 2 hr, in the presence or absence of atropine (1 μM) for the determination of specific binding. Bound and free radioligands were separated by rapid filtration through GF/B filters followed by four rinses using 3 ml of ice-cold buffer. The radioactivity