EFFECTS OF POLLUTANTS ON VERTEBRATE CELLS IN VITRO

CALIFORNIA UNIV IRVINE DEPT OF DEVELOPMENTAL AND CELL BIOLOGY

M W BERNs 13 JAN 83 AFOSR-TR-83-0126

UNCLASSIFIED

AFOSR-80-0062
Effects of pollutants on vertebrate cells in vitro

Michael W. Berns

University of California
Department of Developmental & Cell Biology
Irvine, CA 92717

Air Force Office of Scientific Research (NL)
Bolling AFB DC 20332

Approved for public release; distribution unlimited.

This three year study has investigated the effects of hydrazine (H₂) and monomethyl hydrazine (MMH) on several different cellular systems in vitro. The major cellular systems studied were cultured fibroblasts, epithelial cells, and rat myocardial cells.

It was demonstrated that both H₂ and MMH had a toxic effect and inhibited cell growth in the concentration range of 0.1 mM - 10 mM.
20. However, of greater significance was the finding that two major cellular target sites of Hz and MMH are the cell membrane and the mitochondria. These studies resulted in the development of a sophisticated laser based fluorescence stimulation and detection system that could be used as a sensitive assay tool for analyzing the cellular effects of many toxic agents. Using this system it was demonstrated that the fluidity of the cell membrane was affected by Hz and MMH treatment. This correlated well with scanning EM studies that revealed a pronounced dose dependent effect of Hz and MMH on the amount of cell surface microvilli.

In addition to the outer membrane being affected by MMH it was demonstrated that ultrastructural alteration in mitochondria occurred. These structural effects correlated with cell surface electrical changes as evidenced by electrophysiological implantation of microelectrodes. Based upon the electrical and electron microscopic observations an alteration of calcium ion flux was hypothesized.

In summary, this three year study has demonstrated that the toxicity of hydrazines may be based upon cell membrane and metabolic (via mitochondrial effects) alterations.
Effects of pollutants on vertebrate cells in vitro

University of California, Irvine
Irvine, CA 92717

Michael W. Berns
B. Research Objectives.

This is the final report for research conducted under AFOSR Grant #0062, "Effects of Pollutants on Vertebrate cells in Vitro."

The overall objective of these studies was to elucidate the mode of action of hydrazines on vertebrate cells and tissues. Six specific goals were stated for the three years of support:

1. Detailed scanning electron microscope analysis of the cell surface of cells treated with different hydrazines.
2. Electrophysiological analysis of cardiac cells treated with hydrazines.
3. Cell surface fluorescence studies to determine effects on membrane fluidity.
4. Internal fluorescence studies on cell cytoplasm (specifically mitochondria).
5. Mutagenic analysis on cell lines and mice.
6. Tumorigenic inducing capacity of hydrazines.

C. Status of Research.

In the course of our studies to elucidate the cellular sites of hydrazine action, considerable evidence was gathered that implicated the cell surface as an initial and primary target site of action. Based on these and subsequent studies, most of the effort over the three year period was focused on the cell surface and cytoplasm (Objectives 1-4) rather than on the mutagenic and carcinogenic aspects (Objectives 5-6).

At the conclusion of the first year (10/31/80) progress in three major areas was evident. First, a method for sophisticated computer-laser fluorescence analysis of cells was developed. This system was subsequently described (Siemens et al., Proc. Natl. Acad. Sci., U.S.A., 70:466, 1982) and demonstrated that laser light could be used to provide analytical measurements on the cell membrane and internal cell structures. Initial studies with the mitochondrial probe, Rhodamine 6G demonstrated an oscillating effect of this compound on the fluorescence emitted from these organelles. Subsequent studies ultimately revealed that this pattern of fluorescence could be altered by treatment with hydrazines, thus implicating mitochondria as target sites of hydrazines in addition to the cell membrane.

A second accomplishment of the first year was the demonstration by scanning electron microscopy that both Hs and MMH affect the cell surface by promoting a dose dependent decrease in cell surface microvilli in the ranges of 0.01 - 1.0 mM concentration. However, it did appear that the cell surface microvilli were more sensitive to Hs than MMH.

A third accomplishment of the first year was a demonstration that MMH suppressed cell growth in vitro in a dose dependent fashion (0.1 - 2.0 mM) similar to that previously described for the parental hydrazine compound.
Based upon the results of the first year, the second year of support (ending 10/31/81) focused on elucidation of cell membrane fluidity effects of the hydrazines and cytoplasmic (particularly mitochondrial) effects. In addition the growth studies initiated in the previous year were finished and published (J. Toxicol. and Appl. Pharmacol.55:378, 1980). Of particular interest was the demonstration that the cell surface probe concanavalin A increased in mobility when the cells were treated with hydrazine and monomethyl hydrazine. More increase in receptor mobility was detected with hydrazine than MMH, but both clearly affected the fluidity of the cell membrane.

With respect to internal fluorescent patterns of mitochondria treated with Rhodamine 6G, it was also demonstrated that both hydrazines were able to reduce the amount of oscillating fluorescence in myocardial cells. This study was carried out in over 100 cells, thus assuring statistical significance.

However, in the second year our most significant results were those demonstrating an electrophysiological effect on the cell membrane and an apparent ultrastructural effect on the mitochondria that was correlated with a distinct modification of intracellular calcium. These results were strongest for MMH and less evident for Hz. It was felt that these observations demonstrated a difference between the physiological action of these two compounds.

The third and final year of support on this project was devoted to a more thorough analysis of the electrical, ionic, and ultrastructural effects of monomethyl hydrazine.

The normal, spontaneous electrical activity of contracting neonatal myocardial cells in culture is characterized by a regular pattern of action potential discharge (Fig. 1a). Normal resting potentials average -68 mV (S.E.± 1.10mV;N=800). Rhythmically contracting ventricular cells can be divided into two general categories according to their spontaneous electrical activity. The first category comprises the pacemaker cells, characterized by the presence of a spontaneous diastolic slowly rising depolarization or pacemaker potential preceding the discharge of an action potential. These cells are autoactive. The second category of cells, non-pacemaker cells, are characterized by the absence of pacemaker potentials and show a steady level of membrane potential during diastole. These cells are driven by excitation from other cells.

A sequential pattern of changes (Fig. 1) in the spontaneous electrical activity and contractility of cultured cells during continuous exposure to MMH was regularly observed. During the first 15 to 20 minutes of exposure to MMH (1mM-1.5mM) an increased rate of action potential discharge was consistently evident (Fig. 1b). Although resting membrane potentials usually remained at normal levels, a slight depolarization (5mV) was sometimes observed. These changes in intracellular activity were paralleled visually by an increased rate and strength of mechanical contraction. After this time period, a progressive hyperpolarization ensued, with a concomitant reduction of discharge rate below the control frequency (Fig. 1c,d,e,e'). Continued exposure to MMH frequently stabilized the membrane at a hyperpolarized level (average 12 mV more negative than control) with severe reduction (Fig. 1e) or total block (Fig. 1e') of spontaneous discharges. Action potentials when present were always accompanied visually by strong mechanical contractions. This condition persisted for
up to 25 min at which time the cells underwent a sudden depolarization to approximately -15mV.

Electron microscopy of the cell cultures revealed that mitochondria underwent progressive ultrastructural changes during the period in which electrophysiological changes occurred (Fig. 2). We have found a strict temporal correlation between the progressive alterations in electrical activity (Fig. 1a,b,c,d,e,e') and specific ultrastructural changes occurring in mitochondria (Fig. 2a,b,c,d,e). During the initial 15-20 min of exposure to MMH a statistically significant increase (P < 0.05) in the number of intramitochondrial electron dense granules (Fig. 2b) accompanied the increased frequency of action potential discharge and contractility (Fig. 1b). At this initial stage (15-20 min) intramitochondrial granules were abundant and were distributed both centrally and in the mitochondrial periphery. Concomitant with the progressive cell hyperpolarization that followed this initial stage (Fig. 1c,d,e,e'), mitochondria typically underwent a sequential pattern of ultrastructural changes (Fig. 2c,d,e): 1) a significant decrease (P < 0.05) in intramitochondrial electron dense granules (Fig. 2c), granules only in the mitochondrial periphery, onset of structural disruption of mitochondria; 2) complete loss of intramitochondrial granules (Fig. 2d,e), progressive disruption of closely packed cristae and continued disruption of mitochondrial structure. Progressive cytoplasm degeneration (Fig. 2e) was evident after 35-50 min exposure to MMH. Cells were stabilized at this time at a hyperpolarized level (Fig. 1e, e').

In pacemaker cells hyperpolarized by continuous exposure to MMH, artificial depolarization by current injection (Fig. 3a,b) re-established normal rates of action potential discharge and accompanying mechanical contraction. These cells returned to MMH hyperpolarized levels of resting membrane potential at pulse termination, resulting again in failure of pacemaker potentials to reach action potential triggering threshold (Fig. 3a,b).

Our concurrent electrophysiological and electron microscopic analyses indicate that an induced cytoplasmic [Ca\(^{2+}\)]\(_i\) overload is indeed buffered by mitochondria (presence of dense intramitochondrial Ca\(^{2+}\) deposit granules). A residual, slightly higher than normal [Ca\(^{2+}\)]\(_i\) is still present in the cytoplasm as indicated by the slight depolarization, increased action potential discharge frequency and contractility. This internal free Ca\(^{2+}\) concentration may not be sufficient for Ca\(_{2+}\) activation. Prolonged mitochondrial exposure to increased cytoplasmic [Ca\(^{2+}\)]\(_i\) eventually diminishes their buffering capacity due to intramitochondrial Ca\(^{2+}\) overload and impairment of their normal metabolism. Our findings show that intramitochondrial Ca\(^{2+}\) granules disappear progressively and that mitochondrial structure is progressively altered. Paralleling these ultrastructural changes the intracellular activity of the cells show a progressive resting membrane potential polarization (average 12mV more negative than normal). We propose that the progressive increase in K\(^+\) conductance responsible for cell hyperpolarization is due to a gradual increase to higher than physiological levels in cytoplasmic [Ca\(^{2+}\)] as the mitochondria lose their buffering capacity. As demonstrated, excessive hyperpolarization in pacemaker cells may polarize them beyond action potential threshold. Re-establishment of normal action potential and contraction frequency by depolarizing current injection indicates that the observed changes in electrical activity are due to an active and gradual membrane hyperpolarization induced by the proposed [Ca\(^{2+}\)]\(_i\) elevation.
Our results give further evidence that: 1) mitochondria can act as $\text{Ca}^{2+}$ buffers when the cytoplasmic concentration of $\text{Ca}^{2+}$ increases above normal levels and 2) that the level of free intracellular $\text{Ca}^{2+}$ can affect potassium permeability in cardiac muscle.

We propose that these findings may be generalized to other experimentally induced cardiomyopathies which are attributed to excessive intracellular $\text{Ca}^{2+}$ elevation. Exposure of our cultures to isoproterenol causes similar parallel progressive changes in intracellular electrical activity and ultrastructure as those observed with MMH. These changes are also dependent not only on drug concentration but also on the extent of exposure (unpublished observations). A summary diagram of the concurrent electrophysiological and ultrastructural alterations is presented in Fig. 4.

The noxious and diverse effects of hydrazine and its derivatives on cellular mechanisms are well documented. As such, their use in industry and as a major component of high-energy rocket fuel cells has been criticized as a source of biological hazard. It is possible that the convulsions and seizures often produced by exposure to MMH are mediated by an increase in $[\text{Ca}^{2+}]_i$.

In general our three year study has indicated that the hydrazines affect the cell membrane and mitochondria. Furthermore we have demonstrated similarities as well as differences between the two hydrazines studied. Finally, we have developed sensitive and sophisticated physiological assay systems that can be applied to the toxicology of other compounds.
FIGURE LEGENDS

Fig. 1. Progressive hyperpolarization and alteration of normal spontaneous electrical activity (a) induced in cultured myocardial cells during continuous exposure to MMH (1mM): 15-20 min (b), 20-30 min (c), 30-40 min (d) and 40-50 min (e,e').

Fig. 2. Progressive changes in the ultrastructure of mitochondria in myocardial cells during continuous exposure to MMH (1mM): (a) control (X 10,000), (b) 15-20 min (X 11,000), (c) 20-30 min (X 11,000), (d) 30-40 min (X 16,000), (e) 40-50 min (X 7600).

Fig. 3. (a) Re-establishment of normal pattern of action potential discharge during injection of depolarizing current. Cell was hyperpolarized by 25 min exposure to 1mM MMH prior to current pulse, (b) Another example of MMH (1.5 mM-20 min exposure) induced hyperpolarization and disruption of spontaneous activity in a pacemaker cell (upper trace) and re-establishment of normal discharge pattern by a 12 mV depolarizing pulse (lower trace).

Fig. 4. Summary diagram of sequential electrophysiological and mitochondrial ultrastructural changes resulting from increased 

$[Ca^{2+}]_{i}$. 
Fig 3
Continuous exposure to agents that mediate 

Increased action potential 

discharge frequency and 

contractility 

Uptake of most of excess 

$\text{Ca}^{2+}$ by mitochondria 

Onset of progressive loss 

of mitochondrial $\text{Ca}^{2+}$ 

buffering capacity 

Functional and structural 

mitochondrial deterioration. 

No $\text{Ca}^{2+}$ buffering capacity 

Eventually deterioration of 

cell function and integrity 

Onset of cell hyperpolarization by $G_{\text{k,ca}}$ 

Cell hyperpolarization beyond action potential threshold 

Sudden cell depolarization
D. Publications.


E. Personnel.

11/01/79 - 10/31/80

M. W. Berns, Professor
A. Siemens, Staff Research Associate
R. Walter, Graduate Student & Research Assistant
J. Barnett, Postgraduate Researcher

11/01/80/ - 10/31/81

M. W. Berns, Professor
M. C. Kitzes, Research Specialist (no charge consultant)
L.-H. Liaw, Staff Research Associate (no charge consultant)
A. Siemens, Staff Research Associate
Man-Hung Chow, Staff Research Associate
R. Walter, Graduate Student & Research Assistant
J. Coulombe, Graduate Student & Research Assistant
11/01/81 - 10/31/82

M. W. Berns, Professor
A. Siemens, Staff Research Associate
Man-Hung Chow, Staff Research Associate
R. J. Walter, Graduate Student and Research Assistant
M. C. Kitzes, Research Specialist (no charge consultant)
L.-H. Liaw, Staff Research Associate (no charge consultant)

F. Interactions.

Hydrazine effects on the cell surface.
AGARD Aerospace Medical Panel on Toxic Hazards in Aviation
(NATO Meeting), Toronto, Canada, September 15-19, 1980.

Ultrastructural and electrophysiological effects of hydrazines
on cells in vitro.
Review of Air Force Sponsored Basic Research in Environmental
Toxicology, Columbus, Ohio, June 2-3, 1981.

Laser-stimulated oscillating fluorescent patterns from single
mitochondria in Rhodamine 6G-treated cultured myocardial cells.
American Society of Cell Biology Meeting, Anaheim, CA, November
9-13, 1981.

G. Inventions.

None.

H. Other.

None.