Transmembrane Potential Changes in Liver Cells Following CCl_4 Intoxication

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IT IS WELL ESTABLISHED that the administration of carbon tetrachloride (CCl_4) to a variety of animal species results in profound structural and functional changes in intracellular membranes,[1,2] but alterations in the plasmalemma are less well studied. Minor changes in the morphology of the plasmalemma have been described[3-5] and changes in the total liver ion content (as a measure of membrane permeability) have been reported.[6-8] These latter studies indicate that CCl_4 poisoning causes an early and reversible accumulation of calcium, not associated with significant alteration in the concentrations of other ions. It has also been shown that there is an early and significant reduction in the total liver adenosine triphosphate (ATP) concentration,[9,10] these changes precede the appearance of dead cells and occur before the appearance of structural changes in the plasmalemma.

The functional integrity of the plasmalemma is necessary for the maintenance of transmembrane potential. Measurement of membrane polarization may be a sensitive indicator of the structural integrity of this cellular membrane. Ion shifts may reflect alterations in the ability of the membrane to regulate specific ion concentrations and maintain this transmembrane potential. This report describes the sequence of changes in hepatic cell transmembrane potentials following CCl_4 intoxication in the rat.

Methods

Preparation

Male Sprague-Dawley rats weighing 200–275 g were used in these experiments. The animals were fasted 16–18 hr prior to the experiments but were permitted tap water *ad libitum*. The CCl_4 (0.25 ml/100 g body weight) dissolved in an equal volume of mineral oil, was administered to the unanesthetized animals by gastric tube. Control animals received an equal volume of mineral oil.

In situ transmembrane potentials were recorded at various times between 0 and

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72 hr. A minimum of 3 animals were examined at each time period. Measurements of transmembrane potentials were carried out with rats anesthetized by an intraperitoneal injection of phenobarbital, 100 mg/kg. The animal was placed on its back on a rat board and each leg tied to a corner. A midventral abdominal incision was made, the skin dissected away from underlying muscle fascia and reflected laterally. A window was cut in the muscle layer and peritoneum over the right lobe of the liver; a smaller window was cut in the left lower quadrant. A small piece of gauze was placed under the right lobe, to help immobilize the liver. An 8-mm circular plexiglass chamber, attached to a brass rod and mounted on a mechanical manipulator, was placed on the liver. This chamber was filled with a physiologic solution having the following composition: Na^+, 140 mM; K^+, 4.0 mM; Cl^-, 127.70 mM; Mg^{++}, 0.60 mM; Ca^{++}, 1.25 mM; $H_2PO_4^-$, 0.60 mM; HCO_3^-, 25.00 mM; and glucose, 5.00 mM, pH 7.4.[11] The abdominal cavity was filled with this solution. An indifferent ground electrode was placed through the window cut in the left-lower quadrant. The microelectrode was advanced through the solution in the chamber.

Microelectrode Recording

Transmembrane potentials were recorded using standard electrophysiologic techniques.[12] Microelectrodes were prepared with a vertical puller from 1-mm (O.D.) pyrex capillary tubing. Electrodes were filled with filtered 3-M KCl by boiling under reduced pressure. They had resistances of 10–30 megohms; tip potentials were less than 5 mv. The microelectrode was mounted on a micromanipulator (Pfeiffer) with a hydraulic advance (Trent-Wells).

The microelectrode holder, made from plexiglass, contained chlorided silver wire immersed in 3 M of KCl solution, that in turn contacted the large end of the microelectrode. The silver wire was connected to the input amplifier. Tip potentials were measured by comparing the potential recorded when an electrode was in place to that recorded when a broken electrode was used. The indifferent ground electrode was made from glass tubing filled with 3 M of KCl in agar. A chlorided silver wire was placed in the glass tubing. This electrode was placed in the peritoneal cavity via a window cut in the lower left quadrant during the in-situ experiments.

The resting transmembrane potentials were recorded with a dynograph (Offner). Transmembrane potentials were measured by the following criteria modified from Woodbury and Woodbury: [12] Any abrupt negative deflection maintained for 5 sec or more was considered a transmembrane resting potential, and the peak deflection was measured; if the initial value was followed by a low increase, the maximum value was used. A slow increase in potential is frequently seen and is believed to be due to an improving seal between electrode and membrane.

Histology

Sections of liver were fixed with neutral buffered formalin and with 2% osmium tetroxide buffer with S-collodine at pH 7.4.[13] Specimens were prepared for both light and electron microscopy. Paraffin embedded tissue was stained with hematoxylin and eosin; thin sections were stained with lead hydroxide and uranyl acetate and examined in a 3G electron microscope (RCA).

Data Analysis

Statistical analysis of the data was done with a LINC computer. The program used calculated means and standard deviations.

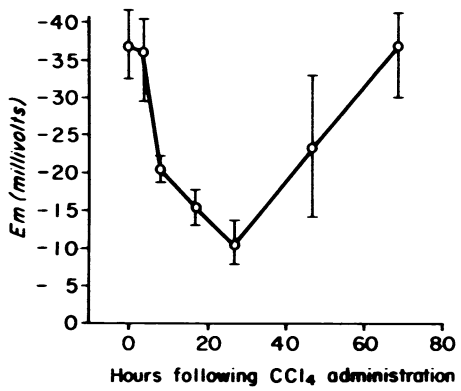
Results

In Situ Resting Potentials

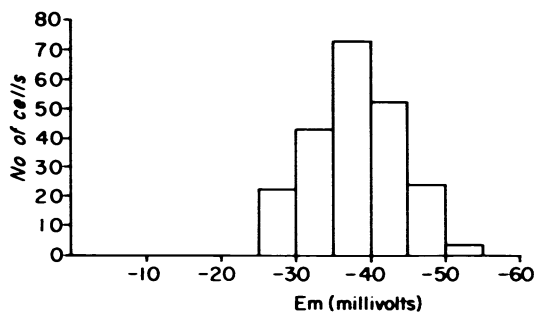
Resting potential measurements were made on 211 liver cells in 5 separate animals. The electrode was advanced into an anterior lobe of the liver and as many as 12 successive layers were sampled. Sharp and usually well maintained negative potentials (−37.0 ± 5.7 mv: mean ± one standard deviation) were recorded as the microelectrode was advanced from the Ringer's solution into the liver. The distribution of resting potentials shown in Text-fig 1 and 2 suggests that there is one population of values.

Effect of CCl_4 on In Situ Resting Potentials of Rat Hepatic Cells

The mean resting potentials of liver cells in CCl_4-poisoned livers did not change significantly from control values during the first 3 hr after administration of the toxin; between 3 and 27 hr there was a progressive depolarization. At 48 hr, a definite repolarization had occurred. Cells surviving at 72 hr had resting potentials not significantly different from control values. These results are summarized in Table 1 and illustrated in Text-fig 1–4.



TEXT-FIG 1. Graphic representation of transmembrane potentials following single intragastric deposition of CCl_4, 0.25 ml/100 g body weight, in Sprague-Dawley rats. Each point represents the mean of 46 or more measurements; vertical bars represent one standard deviation. Values at 3 and 72 hr are not different from controls ($p > 0.5$).



TEXT-FIG 2. Histogram demonstrating range of values of transmembrane potential from control animals.

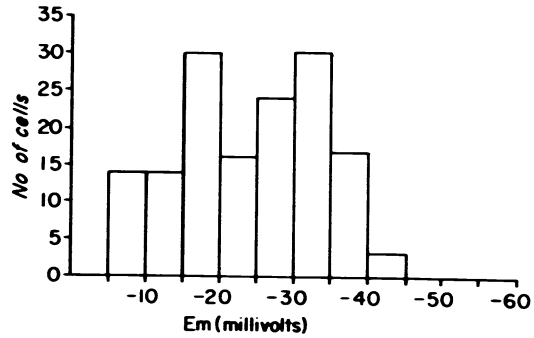
Carbon tetrachloride seems to affect the resting potential of hepatic cells in a uniform manner between 8 and 27 hr after administration since the variation in potential was similar to the variations in control values. Ninety-five percent of the cell potential measurements fell within a 10-mv range. This is contrasted to the results at 48 hr where measured resting potentials had a much larger range (Text-fig 3 is a histogram of the measurements made at 48 hr). There appear to be two cell populations: one (containing the largest number of cells) has potentials mostly in the −15 to −20 range and the other has potentials mostly between −30 and −35 mv, close to control values. Text-fig 4 shows the frequency distribution of potentials at 72 hr. Since these results are not different from control values, the liver has completely recovered as far as transmembrane potential changes are concerned.

Cellular changes noted by light and electron microscopy resemble previously reported results.[2-5] Briefly, by 3 hr the cells in the central zone of the lobules demonstrate a loss of cytoplasmic basophilia, some shrunken nuclei, and an occasional cell with deeply eosinophilic condensed cytoplasm. The midzone shows many markedly dilated swollen cells with fine lacey cytoplasm and in addition, some have pyknotic nuclei. The cells in the portal zones are not altered when assessed by light microscopy. Electron microscopy reveals that the cells in all three zones have a dispersion of ribosomes from the membranes of the endoplasmic reticulum, and the cells in the midzone have a markedly dilated ergastoplasm. Some shortening of the microvilli of the sinusoidal surface is occasionally noted, but distinct evidence of plasmalemmal alteration is not apparent. By 8 hr the cells in the central zone show frequent alteration in the nuclear structure, including clumping and fragmentation, the cytoplasmic changes persisting in all zones. At this time, there are easily recognized alterations in the plasmalemma of cells in this zone with the appearance of frequent plasmalemmal discontinuities.

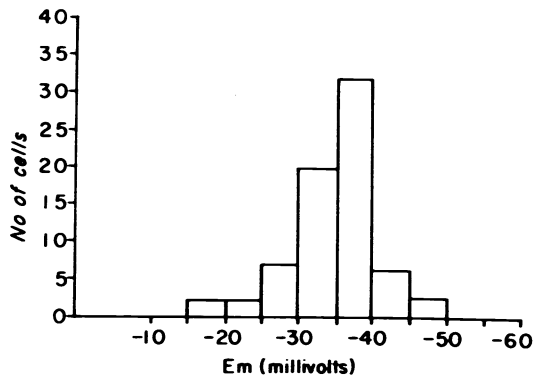
Table 1. Effects of CCl_4 on transmembrane resting potentials of liver cells in situ

No. of animals	No. of cells	Time after CCl_4 admin. (hr)	In situ resting potential (mv ± SD)
5	211	0	−37.0 ± 5.7
4	157	0-3	−34.2 ± 6.2
3	106	7-8	−20.2 ± 2.7
3	46	17-18	−15.7 ± 2.2
3	79	26-27	−11.4 ± 3.4
4	148	47-48	−23.7 ± 9.5
3	74	71-72	−34.2 ± 6.0

The cells in the mid- and portal-zones are not changed since the 3-hr period. In addition, at this time there are a number of inflammatory areas present in the central zone.



TEXT-FIG 3. Histogram demonstrating range of values of transmembrane potential from rats 48 hr after CCl_4 administration. There appear to be two populations of values.



TEXT-FIG 4. Histogram demonstrating range of values of transmembrane potential from rats 72 hr after CCl_4 administration. There appears to be narrowing of the range of values.

By 18 hr the lesion in the central area is well defined, most of the cells within the rim of swollen cells are clearly dead (have condensed nuclei and shrunken cytoplasm), and by electron microscopy show plasmalemmal discontinuities. The cells in the more peripheral areas demonstrate a restoration of ribosome-membrane configuration to some degree, while those on the midzone often show a mixture of markedly distorted intracellular organelles and fat droplets.

By 24 hr, the central zones of dead cells are defined and surrounded by many cells in which there are mitotic figures but are otherwise not remarkable histologically. There is an abundance of fat in many, but by electron microscopy the plasmalemma fail to show distinct alterations. From this time on there is a progressive loss of material from the central zones and a restitution of the normal architecture.

Discussion

We believe that the methods employed in these studies adequately

sample liver cell polarization in all zones of the hepatic lobule. The transmembrane potentials near the hepatic surface were sampled to a depth of 12 cells, estimated from the number of separate negative deflections recorded as the microelectrode was advanced into the liver. Although hepatic lobules are irregular, there are no more than 12 cells in radius [14] in most lobules. It follows that transmembrane potentials have in great likelihood been measured from all three lobular zones. There is a possibility, however, that measurements of another cell type, reticuloendothelial, are included. Parenchymal cells constitute about 90% of liver volume. The contribution of a second population, consisting of no more than 10% of the observed volume, is likely to be small. For these reasons, we believe that these measurements are an accurate estimate of the hepatic parenchymal transmembrane potential. The alterations in zonal structure produced by CCl_4 are relatively uniform throughout the liver, comparing lobule to lobule. Within the lobules, the central zone is more severely damaged than the portal zone. The morphologic similarity of changes in the lobules throughout the liver suggests that our transmembrane potential measurements in superficial lobules are representative, and reflect changes in more deeply situated areas.

Recordings made over the times of 4, 8, 18, 24, 36, 48, and 72 hr following CCl_4 administration revealed a decline and recovery of cell membrane polarization. With the exception of the recording at 48 hr following administration of CCl_4, the variance of the transmembrane potentials that were recorded were relatively small and uniform. The response that we observed was selected from cells that possessed intact cell membranes. Cells with plasmalemmal discontinuities would not have produced a signal when the electrode was advanced into them and hence would not have been recorded. It also appears that the response noted in transmembrane potentials reflects a change that is generalized, and that all cells with intact cell membranes would appear to be affected relatively uniformly. Support for this suggestion comes from analysis of the scatter of values observed in the intervals at 4, 8, 18, 24, and 72 hr. Histograms made at these intervals strongly suggest a single population of values (Text-figs 2 and 4). These cells in all probability represent those with distorted internal structures as well as those that have little alteration in structure, since the changes during this time period are heterogeneous, depending upon the lobular localization of a given cell.

The mechanism by which these changes were brought about is not known. The time sequence of events suggests that significant alteration

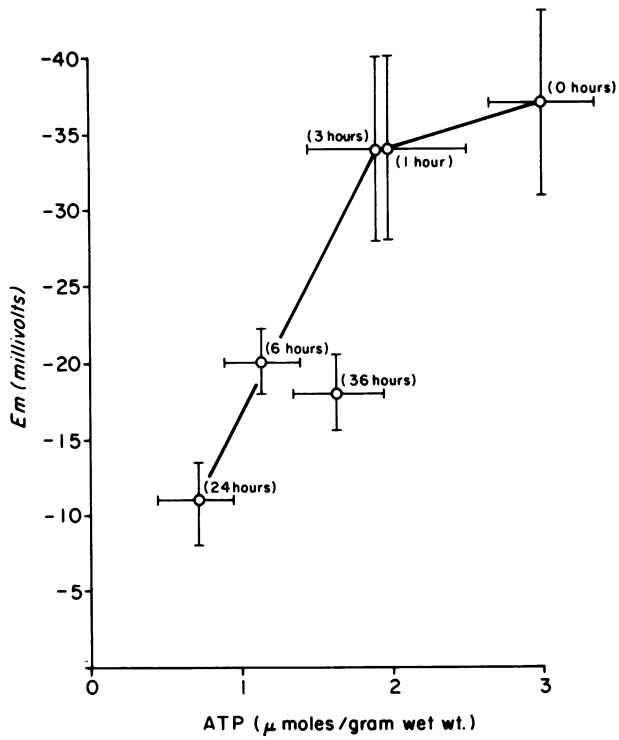
in transmembrane potential occurs considerably later than the histologic and functional changes in intracellular membranes.[1,2,15] It seems unlikely that CCl_4 directly affects the integrity of the plasmalemma; there are no changes in plasmalemma polarization at 4 hr, a time immediately following the highest blood and liver concentrations of CCl_4.[16] Thereafter, the amount of CCl_4 present in the liver and blood decreases to small values by 24 hr, the time of greatest depolarization. The progressive change in polarization makes it doubtful that metabolites of CCl_4 have a direct effect, since further loss of polarization occurs when little CCl_4 is left to be metabolized, but does not exclude the possibility that one or more membranous structures are altered initially and manifest their change later.

Current evidence suggests that transmembrane potential is maintained by virtue of specific ion pumping.[17] Disruption of this polarization may result from increased membrane ion permeability, or inhibition of the Na^+–K^+ pump,* or both. The maintenance of the plasmalemma requires synthetic activity on the part of the cells. In CCl_4 damaged cells, as well as normal cells, the membrane's protein, lipid, and enzymatic constituents are constantly being replaced. The CCl_4-induced decrease in protein synthesis might be a reflection of the cell's inability to maintain and restore these membranes. The early appearance of the polarization changes requires a rapid turnover for these membranes (less than 24 hr) or specific components thereof for inhibition of synthesis to play a significant role in CCl_4-induced injury. Support for the ready restoration of the polarization also suggests a rapid restoration of the essential components for repolarization. The rapid loss and restoration of membrane or membrane component is shorter than estimated half-lives of intracellular membranes.[18] If the plasmalemma has a similar turnover time, the role of altered synthesis seems less likely. The time sequence might also be related to changes in other intracellular regulatory structures. The depolarization may be due to an impairment of the membrane related Na^+-K^+ pump which maintains a high intracellular $[K^+]_i$ and excludes $[Na^+]_i$. It is well established that this pump is energized by ATP.[17] The observed fall in the cellular ATP concentration may be related to decreased pump function, even if the pumping mechanism were intact. Text-fig 5 shows the excellent correlation between the cellular ATP levels and transmem-

* Available evidence indicates that rat liver plasma membranes contain relatively low concentrations of Na^+-K^+ activated ATPase. The significance of the relative amounts of monovalent sensitive and magnesium activated ATPase and the role each plays in transmembrane potential is not known.[19,20]

brane potential; ATP concentration and polarization (Em) are plotted against each other with time as the parameter. The two fall and rise together except for the highest ATP values. The resting potential appears to have leveled off at higher cellular ATP concentrations, suggesting that the pump rate is insensitive to ATP concentration unless it falls below a critical value.

It has been suggested that CCl_4 is cleaved by a free radical mechanism in the liver and that this free radical initiates a secondary process in lipoproteins resulting in the appearance of lipoperoxides.[1,21] Direct assay for the peroxides by measuring their product, malondialdehyde, is unsuccessful because the liver is able to metabolize this material. Direct analysis for diene conjugation, as a reflection of peroxide formation, indicates that there is a progressive appearance of this structure starting 90 min following CCl_4 administration and increasing for the next 24 hr. The time period of this development is coincident with the alteration of the transmembrane potential. If an antioxidant protects



TEXT-FIG 5. Graphic representation of transmembrane potentials and liver ATP levels.[10] Curve was determined by method of least squares. With the exception of control values at 3 μmoles ATP/g liver, a good correlation of transmembrane potential and ATP concentration exists.

against this change, then it is possible to test whether or not the altered membrane potential is related to lipoperoxide production. Further, if this is a generalized phenomenon then the alterations in mitochondrial function and membrane potential might be due to membrane damage. Such damage could increase membrane permeability to Na^+ and other ions and lead to depolarization directly, or indirectly affect the Na^+-K^+ pump by decreased ATP production from altered mitochondria.

The present evidence does not permit a clear selection of the mechanisms of depolarization—that is, altered membrane permeability, or altered Na^+-K^+ pump action. It is probable that measurements of cell membrane resistance and intracellular ion concentration might make a distinction between these possibilities. Regardless of the mechanisms, it seems clear that electrophysiologic techniques are useful in elucidating pathologic changes in cells and these studies indicate that cells can and do survive with a significant alteration in transmembrane potential.

Summary

The administration of carbon tetracholoride results in profound structural and functional changes in intracellular membranes, but structural studies of the plasmalemma have revealed less well defined alterations. The functional integrity of the plasmalemma was measured, in part, by analyzing transmembrane potentials in situ in rat livers following administration of carbon tetrachloride, 0.25 ml/100 g body weight. Control values were -37.0 ± 5.7 mv. Following an initial delay of 4 hr, there was a progressive depolarization of the plasmalemma reaching a minimum at 24 hr (-11.4 ± 3.4 mv). Subsequently there was a recovery by 72 hr to control levels. The technique employed provides an adequate sample of potentials from all lobular zones. The method employed would not record potentials in dead cells. The narrow range of values recorded in control and poisoned livers suggested that a uniform alteration in all cells occurred. A correlation exists between the observed transmembrane potential and total liver adenosine triphosphate values. These studies indicate that carbon tetrachloride produces a generalized change in plasmalemma function, but the mechanism underlying the change is not apparent.

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