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Liver Cell Potentials: In Vitro Effects of Metabolic Inhibitors, Cardiac Glycosides, and Hormones

Paul M. Beigelman and Lyell J. Thomas, Jr.

University of Southern California School of Medicine,
2025 Zonal Avenue, Los Angeles, Calif. 90033

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Summary. Inhibition of *in vitro* hepatic cell electrical membrane potentials (RP) by metabolic inhibitors constitutes further evidence that this is an electrophysiologically viable preparation. RP was rapidly and reversibly inhibited by 1 mM cyanide (CN) and 1 mM 2,4-dinitrophenol (DNP). A gradual, irreversible decline of RP occurred following addition of 10 mM iodoacetamide (IA). Digitoxin (1 mM), but not ouabain, markedly inhibited hepatic cell RP, with immediate recovery of RP upon removal of this agent. Insulin and L-thyroxine had no effect on hepatic cell RP. The only significant effect of epinephrine was slight depolarization at a concentration of 0.01 mM/liter.

Abundant data have been accumulated indicating that the resting transmembrane potential (RP) of electrically active tissue, such as muscle and nerve, is due mainly to a gradient of potassium ions across the cell membrane [7]. However, hepatic cell RP does not appear to be generated by the same mechanism [3, 4, 19]. These hepatic cell ionic data have been accumulated utilizing in vitro [3], in vivo [19], and perfused organ [4] preparations, each with its difficulties and limitations. In vivo studies are complicated by neural, endocrine, and circulatory factors, perfused organ experiments by vascular changes, and in vitro studies by alterations in tissue viability. Loewenstein and his associates have demonstrated in vitro hepatic cell viability in their studies of cell input resistance and intercellular communication [12, 14].

The present detailed investigations with metabolic inhibitors were performed to further examine electrophysiological viability of the in vitro hepatic cell preparation. Aside from the short communication of Coraboeuf and Claret [5], utilizing an in situ liver preparation, there has been no previous report of such metabolic inhibitor studies. Inasmuch as the in vitro liver preparation responded to inhibitors as would be expected of viable cells, the effects of certain hormones were also investigated.

Materials and Methods

L-epinephrine, digitoxin, ouabain, iodoacetamide, and 2,4-dinitrophenol were purchased from Calbiochem. Sodium cyanide was purchased from J. T. Baker Company. L-thyroxine was obtained from Dr. John Nicoloff, Associate Professor of Medicine, USC School of Medicine. Insulin was donated by Dr. Otto K. Behrens, Eli Lilly and Company.

Segments of liver were excised from male and female rats of the Spraque-Dawley strain, weighing 140 to 200 g, anesthetized with intraperitoneal sodium pentobarbital (6 mg/100 g rat weight). Peripheral segments of liver, measuring approximately $3 \times 4 \times 0.5$ cm were excised so that penetrations could be made through the thin, 0.2 to 0.3 mm intact outer edge. There was some variation in control levels from animal to animal, but the results from any single animal were quite consistent, and the variation between animals was sufficiently small that calculations were made on the basis of absolute values rather than RP change. The segment of liver was maintained at 30 °C by a constant-temperature water bath, and was equilibrated for 8 to 10 min following any change of solution. Most experiments were concluded within 2 hr after the excision of the liver segment from the living animal.

Glass microelectrodes were prepared using an Industrial Scientific Associates horizontal microelectrode puller. Each microelectrode was filled with 3 M KCl by gentle boiling in fresh 3 M KCl for 45 to 60 min. Ag–AgCl electrodes embedded in agar bridges and a Dynograph Directwriter were employed. The electrometer coupler of the Dynograph was modified so that the microelectrode tip resistance could be measured throughout the period of penetration and tip potentials neutralized. Microelectrodes with a resistance of 10 to 20 MΩ and a tip potential of less than 5 mV were used. Penetration was performed automatically using a motor which advanced the microelectrode at a rate of approximately 10 microns per sec. Results were recorded on the basis of a d-c potential configuration being maintained for at least 4 sec. Krebs-Ringer's bicarbonate (KRB) solution was prepared according to the method of Umbreit, Burris and Stauffer [17], with slight modification, and contained K^+ 5, Na^+ 142, Cl^- 128 and Ca^{++} 2.5 mEquiv/liter, and HCO_3^- 25, HPO_4^- 1.2 and SO_2 1.2 mM/liter.

In each experiment a control series of penetrations in KRB were made for approximately 1/2 hr, followed by the addition of the test solution(s) of inhibitor or hormone for approximately 1 to $1\frac{1}{2}$ hr, and terminated with control solution for about 1/2 hr. The inhibitors tested were sodium cyanide (CN), 2,4-dinitrophenol (DNP), iodoacetamide (IA), ouabain, and digitoxin. Insulin, epinephrine, and L-thyroxine were the hormones tested. Inhibitors and hormones, with the exception of digitoxin, were diluted in KRB. The pH was 7.4 (7.3 to 7.5). Only rarely did it require correction. Previous studies have shown that variations in pH from 6.4 to 9.4 had no effect on the *in vitro* rat hepatic RP [2]. The experiments with digitoxin required absolute methanol as the vehicle, with subsequent dilution in KRB. The concentrations of methanol employed were 8% for 1 mM digitoxin, 0.8% for 0.1 mM, and 0.08% for 0.01 mM (v/v). There was a tendency for the digitoxin to precipitate following 30 to 40 min of incubation; thus, fresh digitoxin solutions were substituted every 30 min during an experiment. The control solutions utilized, without digitoxin, were 8%, 0.8% and 0.08% methanol in KRB, as indicated.

Results

As indicated in Table 1, sodium cyanide (CN) in concentrations of 1 to 10 mM profoundly inhibited hepatic RP, decreasing the potential from a mean of 28–29 mV to 19 mV. Subsequent substitution of the control KRB

Table 1. Effect of metabolic inhibitors upon hepatic resting membrane potential (RP)

Inhibitor (mM/liter)		Control preceding		Inhibitor		Control following		P[a]
		No.[b]	Mean ± SEM (mV)	No.	Mean ± SEM (mV)	No.	Mean ± SEM (mV)	
Sodium cyanide								
10	(3)[c]	12	28 ± 2	36	19 ± 1	18	23 ± 1	<0.001
1	(5)	27	29 ± 2	35	19 ± 1	17	29 ± 2	<0.001
0.1	(6)	35	26 ± 1	49	24 ± 1	20	28 ± 1	>0.02, <0.05
0.01	(2)	10	27 ± 2	11	27 ± 2	7	28 ± 1	N.S.[d]
2,4-dinitrophenol								
1	(7)	51	27 ± 1	81	17 ± 0	46	23 ± 1	<0.001
0.1	(7)	50	27 ± 1	60	23 ± 1	34	26 ± 1	<0.001
0.01	(4)	25	30 ± 1	25	29 ± 1	11	27 ± 2	N.S.
Iodoacetamide								
10	(4)	18	26 ± 1	31	20 ± 1	15	13 ± 1	<0.001
1	(4)	18	26 ± 1	39	21 ± 1	19	21 ± 1	<0.001
0.1	(2)	10	25 ± 2	13	24 ± 1	8	25 ± 3	N.S.
Ouabain								
10	(3)	18	25 ± 1	36	24 ± 1	15	23 ± 1	N.S.
1	(6)	34	27 ± 1	44	25 ± 1	18	23 ± 2	N.S.
0.1	(6)	42	26 ± 1	51	23 ± 1	18	24 ± 1	>0.01, <0.02
0.01	(7)	41	27 ± 1	63	25 ± 1	24	24 ± 1	N.S.
Digitoxin								
1	(6)	26	23 ± 1	36	17 ± 1	19	22 ± 1	<0.001
0.1	(4)	19	24 ± 1	26	22 ± 1	15	22 ± 1	N.S.
0.01	(3)	17	22 ± 1	21	24 ± 1	11	21 ± 1	N.S.

[a] Probability of significance of difference of means of inhibitors compared with control preceding addition of inhibitor.
[b] Number of penetrations.
[c] Number of rats.
[d] Not Significant.

was followed by a partial restoration to the original control level, at 10 mM CN, to 23 mV, and total restoration, following 1 mM CN, to 29 mV. At 1 and 10 mM concentrations, the change was highly significant ($P<0.001$) with a minimal, rather unimpressive, decrease from 26 to 24 at 0.1 mM CN, which was, however, statistically significant ($P>0.02$, <0.05). At the lowest concentration, 0.01 mM CN, there was no effect upon hepatic cell RP. 1 mM 2,4-dinitrophenol (DNP) had a magnitude of effect similar to that of CN, decreasing electrical potential from the control level of 27 mV to 17 mV

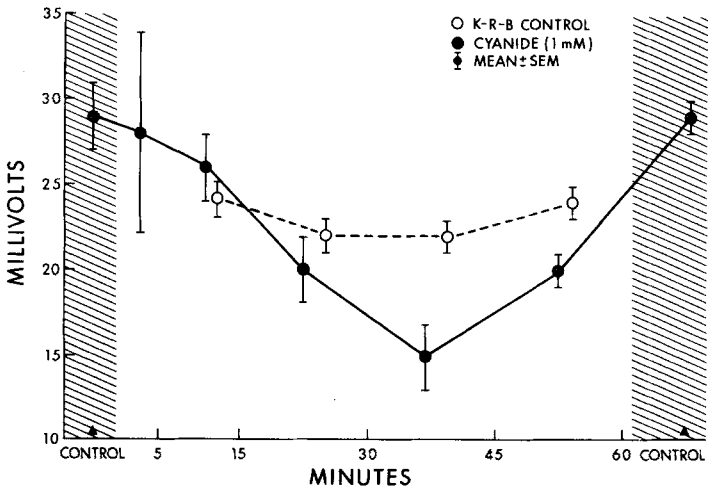

Fig. 1. Effect upon hepatic cell RP (mV) of 1 mM cyanide (CN) as a function of time (min). Control levels before and after addition of CN indicated. Effect of Krebs-Ringer's bicarbonate (KRB) upon hepatic cell RP also indicated as a function of time. Vertical bars indicate SEM

and partial restoration of RP to 23 mV occurring as DNP was replaced by control KRB. 0.1 mM DNP had a smaller, but still highly significant effect ($P<0.001$), decreasing the mean from 27 mV to 23 mV. Nearly complete restoration of the potential to 26 mV occurred after the inhibitor was replaced by KRB control. 0.01 mM DNP had no effect upon hepatic cell RP. Iodoacetamide (IA) at concentrations of 1 to 10 mM significantly ($P<0.001$) decreased hepatic cell RP from control levels of 26 mV to 20 to 21 mV. Replacement of IA by control KRB did not restore RP to its original levels, and the tissue previously treated with 10 mM IA continued its decline of RP to a mean value of 13 mV. Ouabain, at concentrations of 0.01, 1 and 10 mM, had no significant effect on RP. 0.1 mM ouabain had a slight, unimpressive effect, potential decreasing from 24 to 21 mV, which was statistically significant ($P>0.01$, <0.02). However, 1 mM digitoxin caused a marked decrease of hepatic RP from 23 mV to 17 mV, which was statistically significant ($P<0.01$). Lower concentrations, 0.1 and 0.01 mM, of digitoxin had no effect on RP.

Fig. 1 indicates a rapid decline of hepatic cell RP in response to 1 mM CN, the lowest value being 15 mV, with immediate restoration to control levels after removal of CN. In Fig. 2, the decline of hepatic cell RP in response to IA was delayed, and no restoration of RP occurred following removal of IA. Fig. 3 indicates immediate decline of hepatic cell RP in response to both

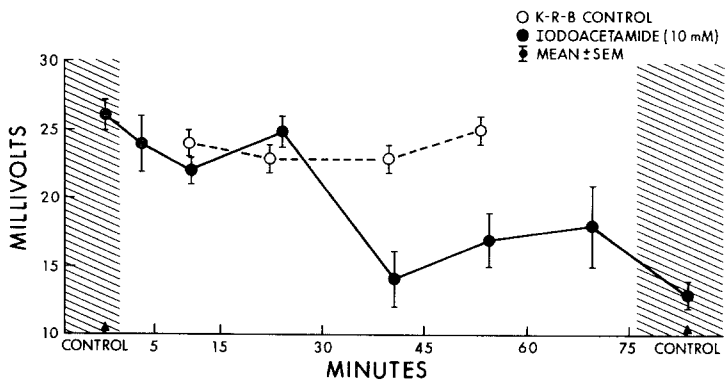

Fig. 2. Effect upon hepatic cell RP (mV) of 10 mM iodoacetamide (IA) as a function of time (min). Control levels before and after addition of IA indicated. Effect of Krebs-Ringer's bicarbonate (KRB) upon hepatic cell RP also indicated as a function of time. Vertical bars indicate SEM

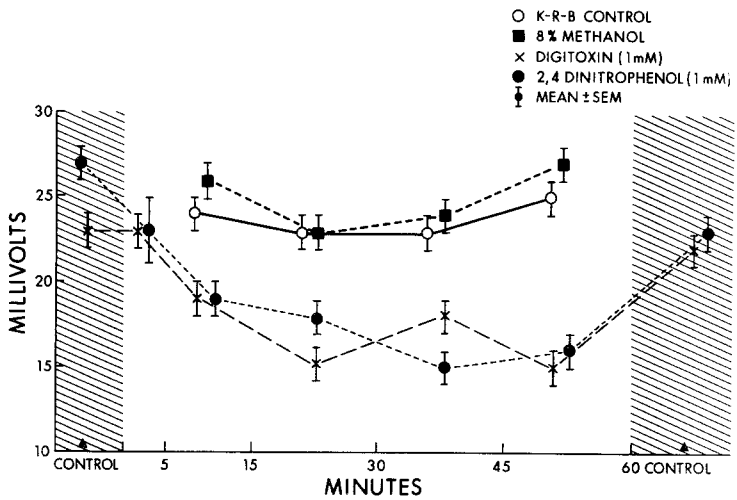

Fig. 3. Effects upon hepatic cell RP of 2,4-dinitrophenol (DNP) and digitoxin as a function of time (min). Control levels before and after addition of DNP and digitoxin indicated. Effects of 8% methanol in Krebs-Ringer's bicarbonate and of KRB also indicated as a function of time

DNP and digitoxin, and a rapid recovery after removal of either inhibitor. Control solutions of KRB and 8% methanol showed little change in the course of these time studies (Figs. 1–3).

Table 2 indicates that insulin, 10 to 1,000 μU/ml, and L-thyroxine, 0.01 to 0.0001 μg/ml, had no effect upon liver RP. Epinephrine, 0.0001 to 1.0 mM, had no effect, with the exception of a small, but statistically significant ($P > 0.02$, < 0.05) effect at a concentration of 0.01 mM.

Table 2. Effect of hormones upon resting membrane potential (RP)

Hormone concentration	RP of control for hormone applied		RP of hormone		RP of control after hormone removed		P
	No.	Mean + SEM	No.	Mean + SEM	No.	Mean ± SEM	
Insulin (μU/ml)							
1,000 (4)	28	23 ± 1	37	25 ± 1	20	27 ± 1	N.S.
500 (5)	28	26 ± 1	37	24 ± 1	25	27 ± 1	N.S.
100 (3)	18	28 ± 1	25	26 ± 1	13	28 ± 1	N.S.
10 (3)	20	27 ± 1	19	27 ± 1	15	27 ± 1	N.S.
Epinephrine (mM)							
1.0 (2)	18	28 ± 1	20	30 ± 1	13	29 ± 1	N.S.
0.10 (6)	33	27 ± 1	41	25 ± 1	34	24 ± 1	N.S.
0.01 (6)	32	26 ± 1	33	23 ± 1	23	24 ± 1	>0.02, <0.05
0.001 (3)	15	25 ± 1	17	26 ± 1	11	28 ± 2	N.S.
0.0001 (4)	20	25 ± 1	35	25 ± 1	17	25 ± 1	N.S.
Thyroxine (μg/ml)							
0.01 (3)	20	24 ± 1	29	25 ± 1	19	24 ± 1	N.S.
0.001 (5)	34	28 ± 0	45	29 ± 3	23	26 ± 1	N.S.
0.0001 (3)	18	25 ± 1	20	27 ± 3	13	25 ± 2	N.S.

Discussion

Coraboeuf and Claret [5] noted that the maximum intraportal dose, 0.25 mg, of potassium cyanide (KCN), in situ, caused an immediate decrease of liver RP, which returned to baseline within 3 to 6 min. KCN injected into the vena cava produced only slight decrease of liver RP. Injection of DNP, either intraportally or into the inferior vena cava, produced a slower, progressive depolarization. These experiments, being *in vivo*, do not permit an accurate estimate of the KCN or DNP concentration at the hepatic cell surface. Similar results with NaCN and DNP were obtained in this laboratory with the *in vitro* liver preparation. The response to CN *in vitro* indicates this measurement of RP to be dependent, in part, upon oxidative metabolism. Li and McIlwain [11] obtained almost identical results with anoxia produced by substituting N_2 for O_2. Also, Lambotte [10] found that depolarization caused by transient ischemia of the perfused liver, produced by interruption of circulation for 1 hr, was completely reversible. Although the results with CN indicate a key role for oxidative metabolism in the liver RP, the results with DNP are somewhat ambiguous. The rapid depolarization and subsequent recovery with DNP obtained in these studies closely mimic the results

of Abood and Koketsu [1], utilizing skeletal muscle. Koketsu [8] has emphasized that depolarization after addition of DNP, and subsequent repolarization, occur too rapidly to be explained by inhibition of oxidative phosphorylation. He presents the hypothesis that the depolarizing effect of DNP is caused by a dissociation of Ca^{++} bound to membrane lipoproteins. Likewise, the effect of iodoacetamide may not be solely due to inhibition of glycolysis. The delayed onset and the irreversibility of this effect is characteristic of iodoacetamide, which binds irreversibly to SH groups [18].

Effects of cardiac glycosides and epinephrine upon hepatic cell RP have varied according to the liver preparation used. Hyperpolarization of the *in vivo* preparation of Williams, Withrow and Woodbury [19] in response to ouabain contrast markedly with depolarization caused by this agent in Lambotte's [10] perfused liver preparation. Epinephrine depolarized perfused [10] and *in vivo* [16] liver preparations. Orozco and Sabelli [13] noted hepatic cell hyperpolarization, *in vivo*, with small doses of epinephrine, but depolarization with higher doses, which they attribute to ischemia resulting from vasoconstriction effect of epinephrine. Haylett and Jenkinson [6] also reported hepatic cell hyperpolarization, using guinea-pig liver slices, with low concentrations of norepinephrine. In the present study, the only significant effect of epinephrine was slight depolarization at a concentration of 10 μM/liter.

In these studies, ouabain had no effect, but digitoxin decreased hepatic cell RP. This may possibly be explained by the greater lipid solubility of digitoxin which permits it to penetrate into tissues much more readily than ouabain [9]. However, the abrupt reversibility of this digitoxin action following its removal was unexpected since its long duration of action on heart muscle is well known [15]. The high concentration of digitoxin required to obtain an effect in these experiments was not surprising. Rats are known to be quite insensitive to digitalis [15]. Bakkeren and Bonting [2] found that rat liver Na^+,K^+-activated ATPase required as much as 10 mM ouabain for complete inhibition. Because of the rapid reversibility of the digitoxin effect, it is uncertain whether the depression of RP by digitoxin observed in the present studies was due to inhibition of Na^+,K^+-ATPase. Possibly, this particular digitoxin effect was due to a nonspecific action on the cell membrane rather than to a specific inhibition of active transport.

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