Effect of Formaldehyde and Glutaraldehyde on Electrical Properties of Cardiac Purkinje Fibers

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ABSTRACT The effects of formaldehyde, glutaraldehyde, 1-fluoro-2,4-dinitrobenzene, and 1,5-difluoro-2,4-dinitrobenzene on the electrophysiological properties of cardiac Purkinje fibers were studied. At concentrations of 2.5 mM the aldehydes produced a transient hyperpolarization, lengthening of the plateau of the action potential, and an increase in action potential overshoot and upstroke velocity. If exposure to aldehyde was continued, the fiber failed to repolarize after an action potential and the membrane potential stabilized at about −30 mv. If exposure was terminated before this, recovery was usually complete. At the time the fibers were hyperpolarized the input resistance was increased without much change in length constant, leading to an increase in both calculated membrane resistance and calculated core resistance. Although it was anticipated that an effect of the aldehydes on the membrane was to increase fixed negative charge, it was difficult to explain all the electrophysiological changes on this basis. The major effects of the fluorobenzene compounds were not the same; they produced a shortening of the action potential and a rapid loss of excitability.

INTRODUCTION

Knowledge of the physiological effects of the various fixatives used in the preparation of tissue for electron microscopy is scanty. As we become increasingly aware of the importance of exact morphological information to an understanding of the function of tissues, it becomes essential to learn what physiological alterations are produced by the fixatives. The cardiac Purkinje fiber is commonly used for electrophysiological investigation. We have studied the effects of formaldehyde and glutaraldehyde on the electrical properties of this tissue.

The lower aldehydes are highly reactive substances in aqueous solution (1). One important chemical reaction resulting from exposure of biological tissue to formaldehyde is a covalent combination with positive amino groups on

protein molecules, resulting in a loss of positive charges (1–3). This reaction has been called the Sorensen reaction, when used to titrate amino acids. Sarcolemmal membranes and myoplasm are thought to contain structural proteins, so that an alteration in their isoelectric point in the positive direction would result in an increase in net fixed negative charge at neutral pH (4). Such a change might have important effects on membrane ionic permeabilities and ionic movements, and change the electrical properties of the tissue. Further, an understanding of the action of these agents might offer some insight into the role of membrane and cellular fixed charges in the control of cellular electrical events.

These agents also affect the activity of various cellular enzyme systems, probably by a similar reaction (5). Some of the reactions of aldehydes produce cross-linkages that tend to make cells more rigid (1). Similar reactions with proteins also occur with 1-fluoro-2,4-dinitrobenzene and 1,5-difluoro-2,4-dinitrobenzene. Some studies were made on the electrical properties of the Purkinje fibers using these fluorobenzene agents to see whether they produced effects similar to those produced by the aldehydes.

METHODS

Cardiac Purkinje fibers of young sheep were used in these experiments. The hearts were removed immediately after death at the slaughterhouse and placed in a thermos bottle containing cold (4°C) oxygenated Tyrode solution. Purkinje fibers were dissected in the laboratory within 30 min after the animals were killed. They were placed in a Lucite chamber through which solutions of the desired composition were perfused. The temperature was maintained at 30°C during the experiment. The control measurements were made in Tyrode solution of the following composition: NaCl, 137 mM, MgCl, 1.05 mM, $NaHCO_3$, 13.4 mM, NaH_2PO_4, 2.38 mM, $CaCl_2$, 1.8 mM, 5 mM glucose, and KCl in concentrations of 2.7 or 5.4 mM. The solution was saturated throughout the experiment with a gas mixture containing 95% O_2 and 5% CO_2 and the final pH was 7.2–7.4. Low chloride solutions were prepared by replacing NaCl by sodium acetylglycinate, which was obtained by dissolving acetylglycine in distilled water in the presence of sufficient sodium hydroxide to yield a pH of 7.4.

The chemicals used were formaldehyde, glutaraldehyde, 1-fluoro-2,4-dinitrobenzene, and 1,5-difluoro-2,4-dinitrobenzene. The formaldehyde solutions were prepared by stirring paraformaldehyde overnight in distilled water and adding the appropriate quantity to Tyrode solution.[1] The pH was examined after addition of formaldehyde and the other chemicals and it was not altered. Preliminary experiments showed that high concentrations of formaldehyde killed the tissue too rapidly to permit measurement of many of the membrane properties. A concentration of 2.5 mM formaldehyde was found to produce a slower development of changes, allowing time for measurements to be made, and this concentration was used for the studies to be

[1] The paraformaldehyde was probably not completely dissociated by this technique, so the concentration indicated should not be taken as an exact chemical activity.

reported. The glutaraldehyde[2] was 70% concentrated and stored with an inert gas. For the same reasons mentioned above for formaldehyde, glutaraldehyde was also used in a concentration of 2.5 mM. The fluorobenzene compounds were dissolved in 1 cc of methanol and added to stirred Tyrode solution drop by drop to yield a concentration of 0.5–2.5 mM.

Glass micropipettes were filled with 3 M KCl and selected for resistances of 5–20 MΩ. Membrane potentials were measured as the voltage differences between a pipette within the cell and one adjacent. Each signal was led through a Picometric amplifier[3] for recording. The tracings were displayed on a Tektronix 565 oscilloscope, from which a photographic recording was made. Resting potentials were measured by withdrawal of the electrode from the cell and subsequent reimpalement to confirm the original value. When diastolic depolarization was seen, the maximal diastolic value was used. Cable analysis was performed as described by Weidmann (6) and Fozzard (7). Current was monitored across a 68 K ohms resistor in series with the ground circuit. The dV/dt was obtained electronically with an operational amplifier (Type O, Tektronix). Conduction velocity was measured as the time of conduction between two intracellular electrodes inserted at a distance from each other. Depolarizing current steps were made through an intracellular pipette to measure threshold.

In each case control measurements were made in normal Tyrode solution and at 1 min intervals after the tested chemical was introduced. The fibers were usually exposed to formaldehyde or glutaraldehyde for 10 min. After removal of the drug, the fibers were allowed 30 min or more to recover, until a steady state was reached. For the fluorobenzene compounds the time of exposure was usually shorter.

When experiments were performed in solutions of different ionic composition, control measurements were made first in normal Tyrode solution, then the fiber was maintained in the modified solution for 30 min prior to introduction of the chemical.

RESULTS

The Resting Potential

Upon exposure to 2.5 mM formaldehyde, the transmembrane potential became more negative in every experiment but one, with an average change of 5.6 ± 1.8 mv[4] in 10 fibers (Table I). While the alteration in transmembrane potential began as soon as the formaldehyde reached the tissue, this effect increased for 8-10 min. A typical time course is illustrated in Fig. 1. Recovery occurred after the formaldehyde was excluded from the perfusate, often with a transient depolarization before return to control values after 20–30 min. The effects of glutaraldehyde were similar but smaller, with only a 2.0 ± 0.4 mv hyperpolarization in four fibers. Replacement of chloride by acetylglycinate in six additional experiments did not alter the results.

[2] Ladd Research Industries Company, Burlington, Vt.
[3] Instrumentation Lab, Inc., Watertown, Mass.
[4] Dispersion of experimental results is indicated by ±1 SEM.

The Action Potential

Formaldehyde always caused a striking increase in the duration of the action potential; by 10 min the action potential duration averaged 245 ± 20 % of control duration (Table I). The increase resulted entirely from a change in the slope of the plateau, with the early repolarization phase and the rapid termination of the plateau being affected much less (Fig. 2). A similar effect on the duration of the plateau was seen with glutaraldehyde (Table I). If the exposure to formaldehyde or glutaraldehyde was continued, the fiber eventually failed to repolarize after an action potential, and the transmembrane potential stabilized about −30 mv. Recovery was complete if the exposure to the aldehyde was terminated by 15 min (Figs. 1 and 2). During the early part of the recovery phase, fibers often showed spontaneous activity. Simultaneous with

TABLE I

	Formaldehyde (11 experiments)	Glutaraldehyde (5 experiments)
Action potential duration	245 ± 20%	357 ± 28%
Maximal upstroke velocity	105 ± 7%	No change
Hyperpolarization	5.6 ± 1.8 mv	2 ± 0.4 mv
Increase in overshoot	11.2 ± 2 mv	No change

Values are reported as averages ±1 SEM. The statement "no change" means that any possible changes were smaller than the limits of measurement. The action potential duration was measured as the total time of depolarization beyond resting potential.

the onset of hyperpolarization on exposure to formaldehyde, a rise in the overshoot of the action potential was seen, as in the experiments illustrated in Figs. 1 and 2. This was frequently, but not always, accompanied by an increase in the maximal upstroke velocity. These effects of formaldehyde on the action potential upstroke were not influenced by chloride removal, and they were not seen with glutaraldehyde.

Conduction Velocity, Input Resistance, and Threshold

The effect of formaldehyde on conduction velocity of the action potential was examined in four experiments. It fell slowly reaching an average of 62 ± 11 % of control after 8 min of exposure to formaldehyde. At this time hyperpolarization and increase in overshoot had occurred in each experiment. Input resistance was measured in 12 fibers; this value rose to 151 ± 13 % of control after 8 min. It usually returned to control values 30–40 min after perfusion with formaldehyde was stopped. The current required for stimulation of an action potential was reduced as input resistance increased, but the voltage level for threshold was not changed (Fig. 3). The input resistance was

also found to be increased more than 50 % at the time fibers were depolarized to −20 or −30 mv by prolonged exposure to formaldehyde.

Cable Analysis

In three fibers cable analysis was performed before and during exposure to formaldehyde (Table II). There is a fairly wide scatter in the results, partly because of inherent inaccuracies in the technique (6) and partly because the

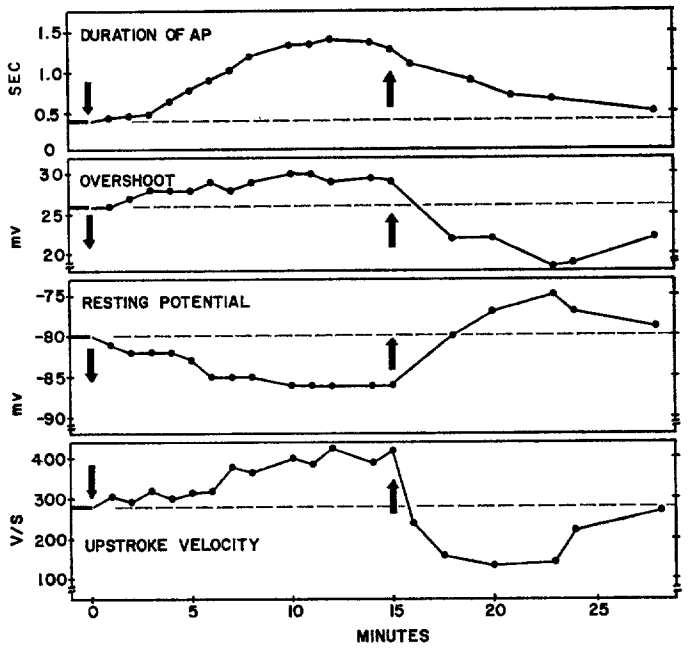

FIGURE 1. Response of a cardiac Purkinje fiber to 2.5 mM formaldehyde. Exposure was begun at the time indicated by the downward pointing arrows and was terminated at the time of the upward pointing arrows. Action potential duration was measured as the total time of depolarization beyond resting potential. Overshoot and resting potential are reported in millivolts. Maximal upstroke velocity of the action potential is reported in volts/second.

measurements in formaldehyde were made while the formaldehyde effect was increasing. Yet there were several consistent findings. The input resistance rose as seen in other experiments, and the membrane time constant tended to increase. There was little change in the length constant. In each experiment calculated specific core resistance rose; the average control value was 202 Ω cm and the average value in formaldehyde was 300 Ω cm. Calculated membrane resistance also rose in each experiment from a control average of 707 Ω cm^2 to 1297 Ω cm^2 after exposure to formaldehyde. Membrane capacitance did not appear to change significantly.

1,5-Difluoro-2,4-Dinitrobenzene

Three fibers were exposed to 0.5–2.5 mM 1,5-difluoro-2,4-dinitrobenzene in Tyrode solution. There was a transient increase in overshoot and action po-

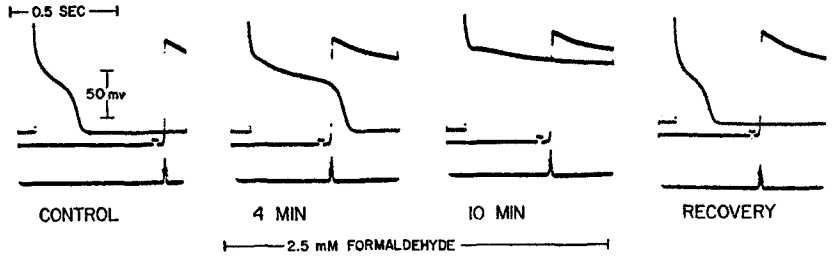

FIGURE 2. Recordings of the action potential and the upstroke velocity are shown before, 4 and 10 min after exposure to 2.5 mM formaldehyde in Tyrode solution, and 20 min after exposure was terminated. At each time the action potential was recorded at a slow sweep speed, 0.5 sec for the period indicated, and at a higher sweep speed, showing only the upstroke phase. For the latter the time period represents 10 msec. The lowest tracing is of the derivative of the action potential.

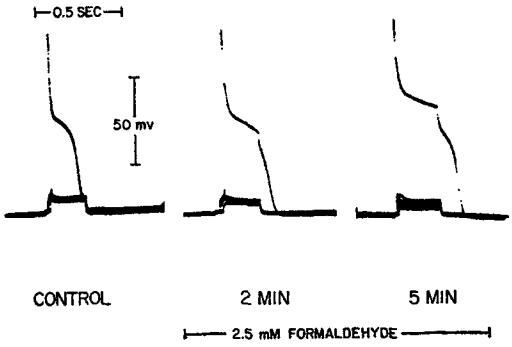

FIGURE 3. Superimposed recordings of 250 msec depolarizing current pulses. As the current was increased a level was obtained that just barely provoked an action potential. No change in the voltage value of threshold was found after exposure to formaldehyde, but the necessary current was reduced because of an increase in the input resistance.

tential duration during the first 2 min of exposure to the drug. With further exposure to the drug the action potential became shorter and the resting potential decreased, resulting in a loss of excitability after 5 min of exposure. The input resistance was increased markedly after 5 min exposure to the drug at the time when the fiber was no longer excitable. These effects were not reversible.

1-Fluoro-2,4-Dinitrobenzene

Four fibers were treated with 0.5–1.25 mM 1-fluoro-2,4-dinitrobenzene in Tyrode solution. The effects were in general similar to those with 1,5-difluoro-2,4-dinitrobenzene with the exceptions that no transient increase in overshoot was observed and that as the fiber became inexcitable it developed a low input resistance. The effects developed more slowly than with 1,5-difluoro-2,4-dinitrobenzene.

TABLE II

Experiment No.	Radius	Time in formaldehyde	V_o/I_o	λ	τ	R_i	R_m
	μ		$k\Omega$	*mm*	*msec*	Ω *cm*	Ω cm^2
1	68	Control	160	1.10	9.5	254	904
		5 min	210	0.92	9.5	480	1206
2	50	Control	106	1.70	5.4	61	699
		12 min	140	1.85	12.0	104	1418
3	55	Control	136	0.70	7.1	290	517
		7 min	140	1.05	9.5	317	1268
		Average values					
		Control	134	1.17	7.3	202	707
		In formaldehyde	163	1.28	10.3	300	1297

The measurement in formaldehyde could not be made at the same time in each fiber because of the complexity of the measurement. V_o/I_o represents the crude input resistance of the fiber. λ is the length constant in millimeters. τ is the time constant in milliseconds; it is equal to R_mC_m and is the time to 84% of the steady voltage at $\chi = 0$. R_i is the specific core resistance and R_m is the resistance times 1 cm^2 of membrane.

DISCUSSION

In concentrations usually employed for tissue fixation, formaldehyde and glutaraldehyde produce membrane depolarization by preventing repolarization after an action potential. Associated with this depolarization is an increase in membrane resistance. These effects develop more slowly at lower concentrations of aldehyde, permitting more detailed observation of the sequence of changes and possibly some insight into the nature of the cell damage.

The initial hyperpolarization of the fibers when exposed to 2.5 mM formaldehyde was small but it was consistent. The measurement is difficult to make, but the problem of instability of the base line was avoided by use of the withdrawal technique. An alteration in the tip or junction potentials only in the myoplasm could have produced such an effect, and this cannot be completely excluded. But other changes in membrane events—the upstroke velocity and

overshoot of the action potential—also occurred and are consistent with a hyperpolarization.

The resting membrane potential is largely dependent on the diffusion potentials of ions in the extracellular and intracellular phases. Normally the diffusion potential in heart muscle is dominated by sodium, potassium, and chloride, and alterations in their relative permeabilities could change the membrane potential. In these experiments chloride ions did not appear to be an important factor, since their replacement by acetylglycinate, a relatively impermeant anion, did not influence the hyperpolarization. A fall in the P_{Na}/P_K ratio would be expected to produce hyperpolarization, although if the ratio is near 0.01, as reported by Page (8) for ventricular muscle, then the effect would not be large. Further, the large increase in membrane resistance seen by cable analysis probably reflects a fall in P_K. With such a fall in P_K, other ions such as Mg^{++} or H^+ become relatively more important, but to produce hyperpolarization their equilibrium potential would need to be more negative than that for potassium. A loss of 20 % of cell water would increase the intracellular concentration of potassium sufficiently to produce hyperpolarization of this magnitude without a change in the P_{Na}/P_K ratio, but there is no evidence that formaldehyde alters cell volume (9, 10).

The rapid inward current that is associated with the upstroke of the action potential was not reduced by formaldehyde, and it may have been increased, since the overshoot and upstroke velocity were often greater. However, the increase in overshoot and upstroke velocity could have resulted from the hyperpolarization, since inward sodium current during a depolarization is dependent on the preceding membrane voltage (11). Threshold voltage for inward current was not changed. A striking effect of the exposure to aldehyde was the lengthening of the plateau phase of the action potential. Since the ionic mechanism of the cardiac action potential plateau is still a matter of controversy (12–15), it is difficult to conclude how the plateau was altered in these experiments. An important factor in plateau generation is the low P_K during this phase, characteristic of inward rectification of the potassium current, and if such a reduction in P_K was produced by the aldehyde during the plateau, the action potential would be prolonged.

Increase in input resistance was a consistent finding in these experiments. Since this occurred in spite of a hyperpolarization, it was important to understand what cellular changes were responsible for this effect, and cable analysis was performed in three fibers. Little change in the length constant was found, in spite of the increase in input resistance. These measures led to a calculated rise in both core resistance and membrane resistance, with each increasing about 65 %. Accurate measurement of cell surface area is not possible in Purkinje fibers, since membrane folds, clefts, and possible transverse tubules appear to be present. However, the proportional change in the cable analysis

values is independent of the actual surface area and radius, provided they did not change on exposure to formaldehyde. The minimal change in total membrane capacity suggests that there were at least no large changes in surface area as a result of exposure to formaldehyde. The rise in core resistance is able to account for at least part of the fall in conduction velocity. The conductivity of the myoplasm results mostly from its potassium ion content (17), and it is not apparent by what means formaldehyde could alter this property. However, in heart muscle the internal resistance is thought to be largely the result of the transversely oriented membranes of the intercalated disk (16, 17). If the surface membrane and the intercalated disk membrane were affected similarly by the formaldehyde, then an increase in surface membrane and core resistance would be expected.

Fixed charges in the membrane probably influence transmembrane potential and membrane conductances in several ways (18). First, charges lining membrane pores would affect ion movement in the pores. An increase in fixed negative charge in the pores would inhibit anion movement and promote cation movement, an effect that appears to have been shown for red blood cells treated with difluorodinitrobenzene (19). In cardiac tissue chloride conductance is already low (15, 20), so that complete loss of chloride conductance would not be expected to increase membrane resistance more than 20 %. Further, replacement of chloride with an impermeant anion did not block the rise in input resistance upon exposure to formaldehyde or glutaraldehyde. It therefore seems that the aldehyde effect cannot be simply explained by an increase in fixed negative charge in membrane pores. The boundary potentials—Donnan potentials at the interfaces between extracellular fluid, intracellular fluid, and the membrane phase, are usually equal and opposite in sign at the two boundaries, so that they contribute to the membrane potential only indirectly by altering ionic permeabilities. However, an asymmetrical alteration of fixed charge by aldehyde might produce a transient asymmetrical change in these boundary potentials. The reduction in total membrane conductance could have resulted from the ability of formaldehyde to form cross-linkages between proteins in the membrane, altering the pathway or carrier for ion movements. A similar effect of the fluorobenzene compounds has been seen in squid axons, where both active sodium and active potassium currents were blocked (21). Formaldehyde is capable of binding to positive amino groups of proteins not only in the membrane but also in the cytoplasm. Such an effect could result in an increase in the "fixed" anion in the cytoplasm by release of protons from these proteins.

As mentioned above, 1-fluoro-2,4-dinitrobenzene and 1,5-difluoro-2,4 dinitrobenzene react readily with free amino groups, and would be expected to alter membrane fixed charge. Fibers were treated with these compounds to see whether effects could be produced similar to those seen with formaldehyde.

While the action potential plateau did lengthen slightly and briefly, there was no hyperpolarization and the action potential was rapidly abolished. These agents also probably interfere with various enzyme systems in muscle such as creatine phosphokinase (22), and the effects seen in the Purkinje fibers may have been produced by interference with enzymatic activities.

These experiments demonstrate remarkable effects of formaldehyde and glutaraldehyde on the membranes of cardiac Purkinje fibers. It was difficult to explain all the effects by an alteration in fixed membrane charges. It seems likely that, in addition to an increase in fixed negative charge, some chemical combination occurs within the membrane, reducing its permeability to both sodium and potassium. An awareness of the dramatic effects of these agents on the excitable properties of cardiac tissue may assist in the proper interpretation of morphologic studies when these chemicals are used.

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