Ultrasonic production of reversible changes in the electrical parameters of isolated frog skin*

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Isolated frog skin was irradiated with 1-MHz noncavitating focused ultrasound, intensity range 1–100 W/cm^2, in an adaptation of the Üssing chamber providing continuous perfusion of the specimen. The electrical parameters of the skin, viz., the membrane potential (MP) and the short circuit current (SCC), were monitored continuously before, during, and after the acoustic exposure. The magnitudes of the ultrasonically induced changes, in both parameters, were proportional to the acoustic intensity, though the MP decreased while the SCC increased. Unlinking of the two electrical parameters occurs in that their time courses in response to the ultrasound differ, i.e., the SCC increases continuously for exposures of 0.5 sec and longer while the MP reaches its maximum within 0.5 sec and is not altered with increased duration of exposure.

Subject Classification: [43]80.40;[43]35.75.

INTRODUCTION

Recent reports exhibit the continuing interest investigators have in elucidating the physical mechanisms involved in ultrasonically producing reversible and irreversible, structural and functional changes in living systems. Though agreements in dose-response regimes have emerged, with adoption of common techniques and careful approaches,[1] controversy remains with regard to the importance and range of influence of the thermal, mechanical, and cavitation mechanisms.[2] The thermal mechanism has, no doubt, been investigated far more thoroughly than the others.[3–5]

An attending topic of considerable importance deals with the level of structure at which the physical forces act to produce the subsequently observed biological alteration. It has been established that molecular structures in solution can be reversibly and irreversibly affected.[6] However, while large polymers such as nucleic acids appear to have been degraded in the absence of cavitation,[7] this mechanism is a necessary condition for irreversible alteration of structures having molecular weights of the order of those of the globular proteins.[8] Further, it has long been known that tissues can be affected reversibly and irreversibly. An example of the former is the reversible suppression of electrical conduction in mammalian brain without evidence of structural or functional damage.[9] Irreversible tissue changes in response to exposure to intense ultrasound (10^2 to 10^4 W/cm^2, 10 to 10^{-3} sec, respectively) have been studied in great detail for the mammalian central nervous system showing that, in the absence of cavitation, approximately 10 min. elapses before histological evidence of lesion presence is manifest by optical microscopy.[1,10]

The latter finding is a strong suggestion that the initial site of the action of ultrasound occurs on structures of dimensions considerably smaller than the cellular level of structural organization and that appreciable time is required for damage to become manifest at the cellular level. Alternatively, as the environment of biomacromolecules in aqueous (or other) solution is considerably different than that obtaining *in vivo*, there may be very little that can be gleaned from such studies that lend insight to events occurring in tissues exposed to ultrasound. The greatly altered molecular conformation existing during such investigations requires that results be reinterpreted and extrapolated to the tissue situation with extreme caution, if at all. It thus emerges that the level of structure between those of macromolecular and of cellular organization, i.e., the level at which specific macromolecular arrangements occur, may be the interaction site, strongly implicating, thereby, the biological membrane.

The present study was undertaken to obtain quantitative information about short-term reversible changes elicited from a biological specimen exhibiting membranelike properties in response to intense noncavitating ultrasonic exposure. Though a structured multimolecular unit would be the preparation of choice for this study, with the most obvious specimen being the cell membrane or membrane model, their fragility and the mechanical difficulties of handling obviated their use. However, a model system exhibiting some membrane parameters, i.e., membrane potential (MP) and Na-linked short-circuit current (SCC), could be useful. Expedience dictated that the specimen be relatively tough physically, easily prepared for mounting in the sample chamber, and possess a transmembrane potential maintainable well above the background noise and thereby eliminating the need for extensive electrical isolation. As degassing of the bathing solution would be necessary for suppression of cavitation, knowledge of the specimen membrane behavior under nearly anaerobic conditions needed to be well known. Also, the thermal response should be known, or readily determinable, since biological media absorb ultrasound at relatively high rates and thereby provide the opportunity for the temperature of the specimen to increase during irradiation.[11]

The abdominal skin of the frog *Rana pipens*, which has been investigated intensively and employed in many research programs[12] concerned with rapid changes in ion transport, was selected for this study. Its transmembrane potential is determined by the sodium con-

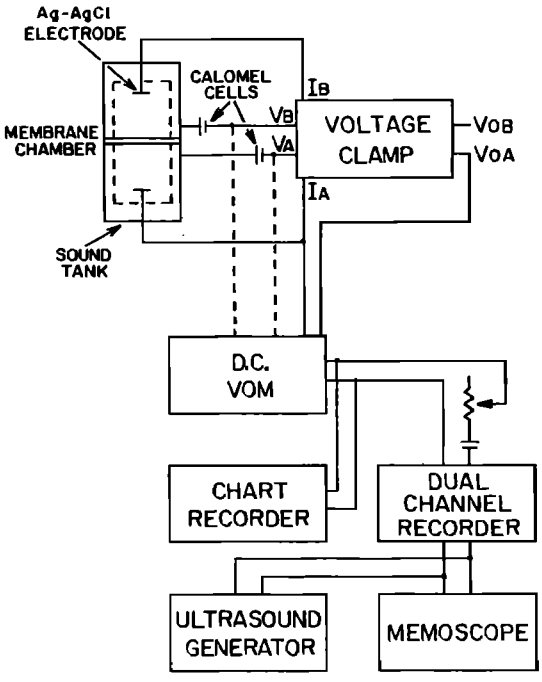

FIG. 1. Block diagram of electrical measuring and recording instrumentation.

centration outside and the potassium concentration inside the skin. With standard frog Ringer's solution bathing both sides of the membrane, the MP is sensitive to solution pH, temperature and membrane metabolic state. The magnitude of the current encouraged to flow when the MP is driven to zero, the SCC, is known, under a variety of conditions to be equal to the sodium flux.[12–15]

I. MATERIALS AND METHODS

Preparation of each specimen consisted of removing the abdominal skin of *Rana pipens* from doubly pithed frogs, washing it in demineralized water and mounting in the membrane chamber, with the serosal surface facing the transducer, except as noted below. The specimen holder is an adaptation of the Üssing chamber fabricated of lucite, but with Saran windows. Thus the chamber provides electrical and fluid isolation while maintaining acoustic continuity between the chamber contents and the source of ultrasound via the sound-tank coupling liquid.[13,16] The skin specimen is clamped with a rubber O ring, insuring that acoustic coupling fluid external to the chamber cannot leak into it, with the frog skin in place. All recording electrodes are placed within the specimen chamber, though not within the acoustic path. Figure 1 is a schematic diagram illustrating the electrical connections between the sample chamber and the monitoring units.

As events were too rapid to carry out normal control of short circuiting of the membrane, the changes were followed with an automatic clamping circuit using FET operational amplifiers. The circuit employed is similar to the one originally used with frog skin by Menninger *et al.*,[17] but with the gain limiting resistors replaced by capacitors.

Phosphate buffering was omitted from the membrane bathing solution in order to avoid the formation of precipitates when degassed, thus inhibiting acoustically induced cavitation. The tris-Ringer's solution of House[18] was used.

The ultrasonic instrumentation has been described in detail elsewhere.[19,20] The transducer is an X cut quartz disk focused with a planoconcave lucite lens. The control units allowed for the production of single acoustic pulses, of rectangular temporal envelopes, of duration from 0.1 to 10 sec, and also trains of pulses with variable pulse duration and variable pulse repetition rate. The focal intensity of the beam was variable from less than 1 W/cm^2 to greater than 10^2 W/cm^2 and was calibrated by the radiation force method described by Fry and Dunn.[20] The values for the acoustic intensity given below were known, thereby, to an absolute accuracy of ±5%. Figure 2 is a block diagram of the instrumentation for the generation and control of the ultrasound. Cooling chambers were clamped to the sides of the sound tank, as needed, to control the temperature and to achieve desired temperatures.

The data obtained with each specimen included the intensity and duration of the acoustic pulses, the MP or SCC and their respective changes, and the tank temperature.

II. RESULTS AND DISCUSSION

The ultrasound was focused, onto the specimen membrane, only for the purpose of developing high intensities. Concurrently, however, a substantial frog skin area was required to provide acceptable magnitudes of the MP and SCC. Further, the electrically active area of the skin could not be established unambiguously, be-

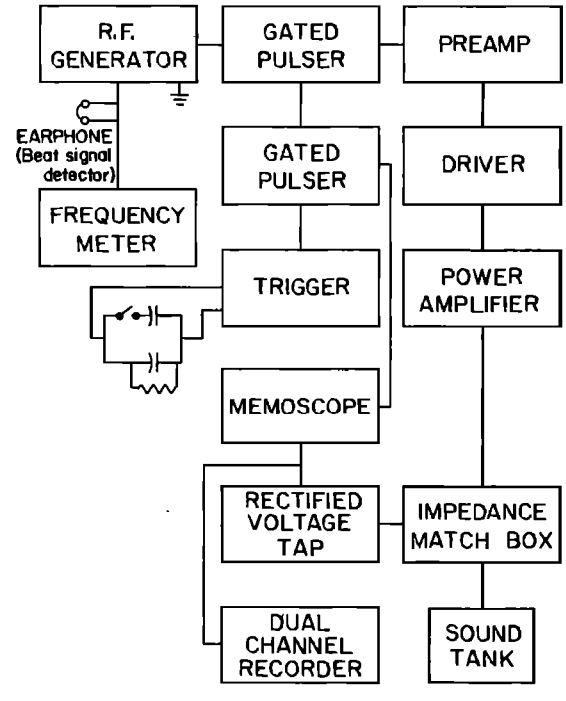

FIG. 2. Block diagram of ultrasonic generation and control instrumentation.

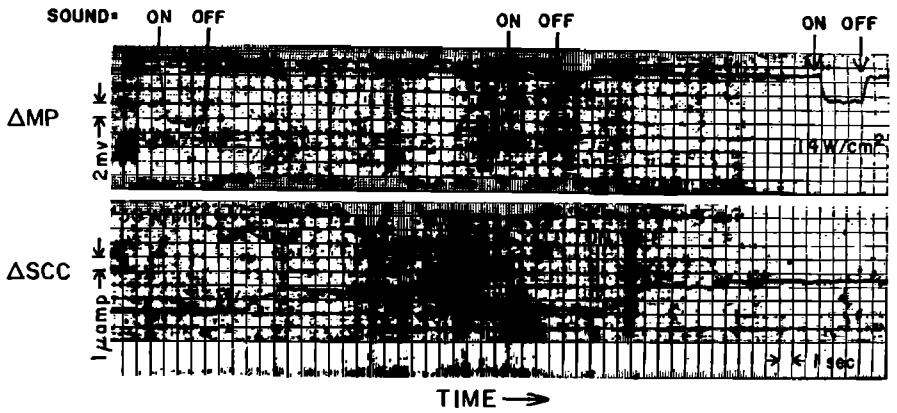

FIG. 3. MP (top, composite of three traces) and SCC (bottom trace) in response to exposure to ultrasound of 1 MHz.

cause of edge damage by the O ring, nor determined for any specimen. Thus, the ratio of the ultrasonically involved area of the specimen to its electrically active area was always unknown, though expected to vary from sample to sample. This situation dictated that each specimen serve as its own experimental control, since reproducibility is easily achievable with a single specimen, but the range of results among all specimens is appreciable.

Figure 3 illustrates a major finding in this study, viz., the opposing behavior, in response to exposure of the frog skin to ultrasound, of changes in MP and SCC. In addition to the MP decrease and SCC increase during each pulse, the time courses of these two parameters are also substantially different in that the former reaches its plateau rapidly and maintains that level, while the latter increases nearly linearly with time, for the duration of the pulse. Reversing the orientation of the skin, such that the external surface faced the transducer, had the effect of reversing the direction of the MP changes, i.e., the MP increased. The magnitude of the change with respect to the exposing acoustic amplitude, however, was preserved. The SCC changes appeared more complex. For example, for a 2-sec exposure, a decrease in SCC occurred for most of this period with an increase occurring near the end of the pulse. These reversing events appear to be consistent with the findings of Nutbourne,[21] who showed that small hydrostatic pressure gradients, of the order of 1–2 mm of water, produced changes in SCC which were dependent upon the magnitude and orientation of the gradient.

Figures 4 and 5 show the absolute and relative changes of both parameters as functions of the acoustic intensity. The linear dependence of the ΔSCC and the ΔSCC/SCC was general while the MP exhibited nonlinear behavior, for the corresponding quantities, which varied among the specimens. A striking example is seen in Fig. 6 where unequal hydrostatic pressures across the two sides of the specimen resulted in different sets of curves for differing pressure gradients. Bulging of the skin also occurred in response to the pressure gradient.

It is of major significance that the MP responses are not similar in any way to those of the SCC. This finding alone can eliminate thermal processes as the principal mechanism for the action of ultrasound on frog skin since earlier studies showed both parameters to have nearly the same temperature coefficients of +6.9%/°C,

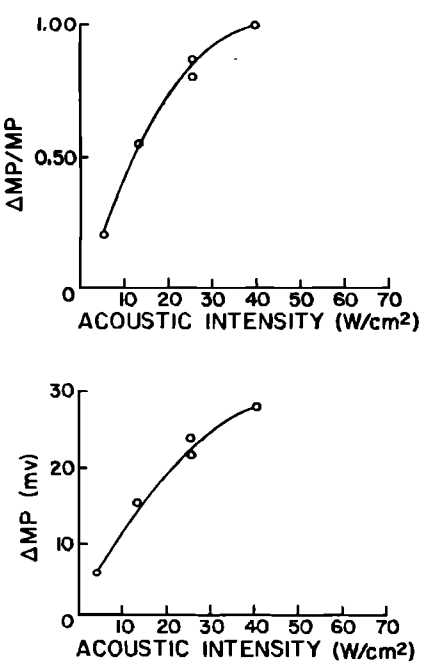

FIG. 4. MP responses to 1-sec pulses of ultrasound vs acoustic intensity.

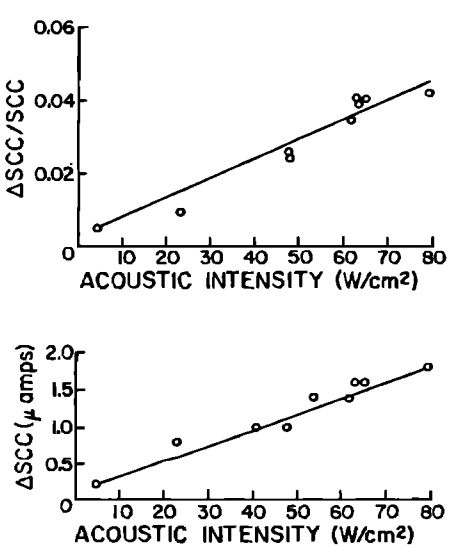

FIG. 5. SCC responses to 1-sec pulses of ultrasound vs acoustic intensity.

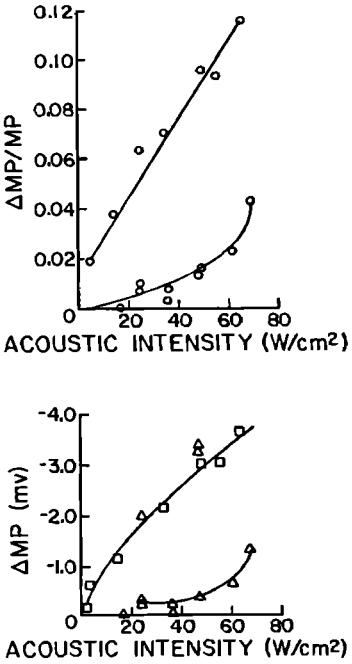

FIG. 6. MP responses vs acoustic intensity (upper traces) for zero pressure difference across membrane and (lower traces) for pressure difference of approximately 5 mm of H_2O.

with the two increasing with increasing temperature to about 27 °C.[14] In an earlier study with frog skin, Lehman and Biegler[22] did produce MP changes with ultrasound that were readily duplicated by heating, using long exposure times and low acoustic intensities.

Dose fractionation was undertaken and produced evidence for significant changes in the response patterns of the MP and SCC. The fractionation considered was the delivery of the same total energy in multipulsed regimes of equally spaced pulses. Except for the situation where the spacing was too short to allow MP or SCC recovery, the magnitudes of their changes was the same as for single pulse exposures of the same delivered energy.

The time courses of the membrane responses suggest that there may be more than one effect of the ultrasound and that the two properties of the skin may not be as closely linked as previously believed. Since earlier investigations reported that temperature, metabolic state, and ion concentration of the bathing solution all produced similar effects on the MP and the SCC, it became assumed that the sources of the two phenomena were the same as, for instance, in elements of an electric circuit.[13] Both MP and SCC were found to be appreciably temperature dependent and to have a similar dependence, viz., decreasing with decreasing temperature. An instance in which the SCC remains unchanged while the MP is altered occurs for the substitution of $SO_4^=$ for Cl^- in the bathing solution.[23] As the membrane is relatively impermeable to $SO_4^=$, the MP is induced to increase without affecting the SCC. The mass transport aspect of the acoustic waves, (microstreaming, radiation force, etc.) may be the agent for altering ion concentration gradients at the two skin surfaces.

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