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INDUCTION OF CALCIUM-ION EFFLUX FROM BRAIN TISSUE BY RADIOFREQUENCY RADIATION: EFFECT OF SAMPLE NUMBER AND MODULATION FREQUENCY ON THE FIELD-STRENGTH WINDOW

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Running title:

ABSTRACT

Changes have been found in calcium-ion binding to brain tissue exposed *in vitro* to a specific field strength (0.83 mW/cm^2) of 147-MHz radiation, amplitude modulated by a 16-Hz sine wave. This report replicates and extends this previous work.

To define more precisely the range of effective field strengths, two different numbers of samples were treated in a Crawford cell with 147-MHz radiation, sinusoidally modulated at 16 Hz. In one series, four brain tissues were exposed at a time; in the other series, four brain tissues plus six dummy loads were exposed together. While the four-sample configuration produced a narrow field-strength window, the ten pseudo-sample configuration resulted in a broader field-strength window. The reason for the sample-number dependence is unresolved, but may be due to interactions between samples and field distortions caused by the close spacing.

The ten pseudo-sample configuration was used to test for the presence and range of a field-strength window at a sinusoidal modulation frequency of 9 Hz. The response curve at 9 Hz was essentially identical to the results for 16-Hz sine-wave modulation.

KEY WORDS

Calcium ions

in vitro

brain tissue

radiofrequency radiation

amplitude modulation

field-strength window

non-ionizing radiation

INTRODUCTION

Bawin et al. (1975) reported that a 147-MHz carrier wave, sinusoidally amplitude modulated between 6 and 20 Hz, can enhance the efflux of calcium ions from brain tissue exposed *in vitro*. We described results (Blackman et al., 1977), consistent with Bawin's report, which revealed an additional and unusual finding: the enhanced efflux of calcium ions occurred only within a narrow range of field-strengths. At the same time, Bawin et al. (1977) reported a similar field-strength window with a 450-MHz carrier wave, amplitude modulated at 16 Hz.

We have conducted further experiments to answer two questions: (1) does the width of the field-strength window depend on the number of brain samples exposed simultaneously in our system? and (2) does the frequency of modulation affect the location or width of this window? These experiments used an improved experimental design; that is, the results of each exposure condition have a companion set of results obtained under identical conditions, except exposure was to zero field-strength radiation.

MATERIALS

Exposure System

Samples were treated in a rectangular transmission line (Crawford cell) exposure system which has been described in detail elsewhere (Weil, 1978; Blackman et al., 1979). The 147-MHz radiofrequency carrier, which was supplied by a crystal controlled signal generator (Wavetek, Model 3000), was amplitude modulated (>95%) with a sine wave at either 16 Hz or 9 Hz from a function generator (Kron-Hite, Model 5200). The modulated signal was then amplified by a 9.5-watt linear-power amplifier (Electronic Navigation Industries, Model 510L). Forward, reflected, and transmitted powers were monitored with two 20 db bidirectional couplers (NARDA Model 3020A) and three identical thermoelectric power meters (Hewlett-Packard, Model 435A) in order to characterize the distribution of energy within the exposure system.

A foamed polystyrene chamber housing the transmission line maintained the air temperature within the chamber at 37 ± 0.2°C by means of a proportional temperature controller (Ali, 1975). Control samples were placed on small shelves within the chamber adjacent to the transmission line so that these were in essentially the same thermal environment as the samples placed within the transmission line.

Biological Samples

The brain tissues were from 1- to 4-day old chicks (*Gallus domesticus*, either Shaver or Young strains) obtained from North Carolina State University.

Media

The medium used in this study was composed of 155 mM NaCl, 5.6 mM KCl, 2.16 mM $CaCl_2$, 2.4 mM $NaHCO_3$, and 11.1 mM glucose. For the radioactive labeling of tissues, this medium was supplemented with calcium-45 at 4.6 μCi/ml (New England Nuclear, 2.1 mCi/mmole). All solutions were prepared on the same day that they were used. The pH of the solutions was 7.6-7.8 and the temperature was 37°C, except for the 20-min water bath treatments at 32° and 41°C.

METHODS AND PROCEDURES

Preparation of Tissues

The chicks were killed by decapitation. The top of the skull and then the forebrain were removed from each chick. Each forebrain was divided at the midline and the two hemispheres then placed into a test tube. The hemispheres of each forebrain were maintained as an exposure-control pair throughout subsequent treatments and analyses.

After four brain-tissue pairs were prepared, one ml of calcium-45 labeled medium was added to each sample tube; all tubes were then placed in a water bath and agitated for 30 min at 37°C. Following the 30-min labeling period, the radioactive solution was aspirated and the tissues rinsed in the following manner. Two ml of unlabeled medium were added to each tube and then poured with the tissues into small plastic sieves held in a rack. The tissues were then rinsed by immersion in two 250-ml volumes of unlabeled medium. The rinsing procedure took approximately 4 min. The tissues, free of any loosely-associated radioactive calcium, were then placed in polystyrene tubes (17 mm x 40 mm) which contained 1 ml of medium.

Exposure of Tissues

Lucite racks holding the tissue samples were arranged symmetrically on either side of the transmission line's center conductor. Similar racks containing the control tissues were placed in the foamed polystyrene chamber on shelves alongside the transmission line at a position where the electric field strength was more than 30 db below the field strength inside the transmission line.

Four chick brains were treated at once, with half of each brain being used as control and the other half as exposed sample. Another

four brains were treated as described above but without microwave radiation. In this set, the samples placed within the transmission line were subjected to a sham exposure. This combined series of eight brain pairs, prepared and treated within one hour, represented one replication of the basic experiment. Several replications were done for each combination of frequency, power density and number of tubes. For some combinations, several replications were done together and then several more were done at a later time; this delay introduced a possible time variable.

The tissues were exposed to 147-MHz radiation for 20 min in a series of experiments at 0-, 9-, or 16-Hz sinusoidal amplitude modulation; power densities of 0.11, 0.55, 0.83, 1.11, 1.38, and 1.66 mW/cm^2 were used. Either four tubes or ten tubes were in the chamber at a time. The four sample tubes were always placed in the same relative locations. For the ten-tube exposures, six additional tubes containing only an equivalent amount of unlabeled medium were also placed in the racks.

Treatment of Tissues at Different Temperatures

To evaluate the influence of temperature on the rate of calcium ion efflux, tissues were prepared as described above. Lucite racks containing four tissue samples were placed in water baths for 20 min at either 32°, 37°, or 41°C; the complementary brain halves were maintained in the 37°C water bath for that time period.

Assay for Calcium Ion Efflux

To estimate the quantity of calcium ions that had been released by each tissue during the treatment period, 0.2 ml aliquots of bathing solution were added to 5 ml of scintillation cocktail (Amersham/Searles, ACS), and counted in a liquid scintillation counter.

Analysis of Data

The relative quantity of calcium ions released by each tissue pair was defined as the ratio of the counts per minute in the treated and control samples (Vt/Vc).

For those combinations of frequency, power density, and number of tubes that had a possible time variable, an analysis of variance for a partially nested design was used to test for time and replication effects. For those combinations that were not done at different times, an analysis of variance for a two-way design was used to test for a replication effect. Since no time and replication effects were found, an analysis of variance for a one-way design was done for each combination. In this analysis, additional data could be included for those combinations that had incomplete replications which could not be easily included in the analyses of variance for the partially nested and two-way designs.

RESULTS

Table 1 gives the sample number (N), mean, and standard error of the mean (S.E.) of Vt/Vc for the sham and exposed groups for each combination of frequency, power density, and number of tubes. The p-value from the one-way analysis of variance is given in the last column of the table. There was a significant ($p < 0.05$) increase in the mean Vt/Vc of the exposed group as compared to the sham group for eight of the combinations of frequency, power density, and number of tubes. These were: 0.83 mW/cm^2 for 16 Hz and four tubes/chamber; 0.55, 0.83, 1.11, and 1.38 mW/cm^2 for 16 Hz and ten tubes/chamber; and 0.55, 0.83, and 1.11 mW/cm^2 for 9 Hz and ten tubes/chamber. For each of the three conditions of frequency or number of tubes, the same power density, 0.83 mW/cm^2, produced the greatest increase in the mean Vt/Vc of the exposed group compared to the sham group ($p < 0.001$).

Table 1 also displays the results of two treatment conditions that were designed to detect other influences on the calcium-ion efflux. The first such treatment condition was to expose samples to unmodulated carrier radiation at the same field-strength that produced the greatest response when modulated at 16 Hz. That exposure, at 0.83 mW/cm^2, showed no difference between the exposed and the sham groups ($p = 0.765$). The second treatment condition was to irradiate the exposed group with zero field-strength, in a manner analogous to the sham group treatment. This procedure tested the effect of the order in which the exposed and sham groups were alternated. Again no difference between groups was found ($p = 0.888$).

Figures 1, 2 and 3 are bar graphs of the same data, showing the mean Vt/Vc for 16 Hz (four tubes) and 16 Hz (ten tubes), and 9 Hz (ten tubes), respectively, as a function of incident power density. The number of samples treated at each condition is shown at the top of the standard error bars.

For those tissues maintained at other temperatures during the calcium-ion-efflux period, the results demonstrated an influence of temperature on the amount of efflux. The efflux for the tissue halves was 9% lower at 32°C and 15% higher at 41°C than the efflux for the corresponding tissue halves at 37°C.

DISCUSSION

The results we presented in an earlier publication (Blackman et al, 1979) described a narrow range of field strengths, at appropriate frequency conditions, in which calcium-ion efflux could be altered. Because the effective field strengths were too low to produce measurable bulk heating (the estimated specific absorption rate was less than 0.075 mW/g), we decided to investigate this effect using an improved experimental design which would provide more effective control over possible artifacts. With this design, data was accumulated for paired samples of exposed and control tissue and for paired samples of sham-exposed and control tissue, in alternate time periods of 30 min each, throughout the course of a day. By comparing the relative efflux values for the exposed and the sham groups obtained in this manner, any changes that occurred during the day or between days would affect both groups and tend to cancel each other.

The results of a field-strength series with a 147-MHz carrier frequency, sinusoidally amplitude modulated at 16 Hz, demonstrated that when four samples were exposed, the intensity of the effective field strength and the limit to the effective intensity range were identical to our initial study. The discrepancy between the 0.75 mW/cm^2 cited in the earlier report and the 0.83 mW/cm^2 cited here is due to a recalibration of the coupling constant of the bi-directional coupler, and to a more precise measurement of the effective cross-sectional area of the transmission line. The earlier value was in error. Thus, the initial finding of a narrow field-strength window was replicated with the improved

experimental design, which resulted in a significant difference between exposed and sham groups at $p < 0.001$, compared to $p < 0.05$ in the earlier study.

Pilot studies indicated that peak and width of the field-strength window would change with the number of samples exposed at one time. To simplify the comparison of efflux values for four tubes in the exposure chamber with a larger number, we elected to use ten tubes in the chamber, with four containing brain tissue as in the four-tube case, and six containing an equivalent amount of medium. In this way the four brain samples would allow us to apply the same statistical test, while the six pseudo-samples would increase the dielectric loading and interaction between samples. The results demonstrated that additional tubes in the exposure apparatus cause a widening of the effective field-strength window to both higher and lower values, without a change in the field strength which produced the maximum difference in efflux. This broadening of the window may be due to interactions between the larger number of tubes in the system which result in field distortions in the vicinity of the brain tissues. This problem is now being investigated.

To determine whether the modulation frequency would affect the magnitude of the efflux or the position of the effective field-strength window, samples were exposed to a field-strength series at 9-Hz modulation. The ten-tube exposure situation, with its broader effective field-strength window, was used to enhance the possibility of detecting a window at 9 Hz. This strategy would have been particularly important if the window had shifted to an appreciably different intensity range. The

results for 9-Hz modulation were essentially the same as the corresponding 16-Hz data. The peak of the field-strength window occurs at the same intensity value, at the same magnitude and level of significance ($p < 0.001$), and the width of the effective field-strengths differs from the 16-Hz data only at 1.38 mW/cm^2, where the group exposed at 9 Hz was not different from the sham group. Thus a comparison of results at 9 and 16 Hz revealed no major differences in the induction of calcium-ion efflux.

Several control experiments were performed to define more carefully the important factors involved in the radiation-enhanced calcium-ion efflux. Exposing samples to unmodulated 147-MHz carrier radiation at the maximally effective field strength produced no change in efflux values. Using zero field-strength radiation to treat both the exposed and the sham groups demonstrated no preferential effect due to the order of treatment. However, the results of these experiments did indicate that conditions were not uniform in the exposure apparatus. The sham-treated brain halves, inside the transmission line, gave higher values for calcium-ion efflux than their corresponding halves in the control positions. The Vt/Vc ratio was approximately 1.06. Although the reason for this result is unresolved, temperature differences between the exposed or sham position and the control location may have affected the amount of calcium ions released into the solution. In testing this thermal hypothesis, experiments using water baths at three different temperatures showed that the efflux was 9% lower at 32°C and 15% higher at 41°C than the efflux at 37°C. Although this result is consistent with the hypothesis, temperature measurements of the medium in tubes

placed in the control and treated positions indicated temperature differences less than 1°C. This small temperature difference alone does not appear to be responsible for the 6% increase in efflux from samples inside the transmission line. Further work is being done to evaluate this result.

In all sixteen exposure conditions conducted with modulated radiation, the efflux ratios, Vt/Vc, were always greater for the exposed samples than for the sham samples. This unusual result may indicate that there is a range of field strengths and modulation frequencies that can stimulate calcium-ion efflux, with particular field strengths within that range being highly effective.

These experiments demonstrate that calcium-ion efflux from brain tissue in vitro can be affected by selected modulation frequencies and field strengths of 147-MHz radiation. Both the mechanism of interaction responsible for this change and the consequences of the biochemical change for the intact animal are yet to be determined.

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Table 1. Mean relative quantity of calcium ions released by brain tissue pairs as a function of exposure to radiofrequency radiation.

LEGENDS

Figure 1 Mean relative quantity of calcium ions released by tissue pairs exposed to 147-MHz radiation, sinusoidally amplitude modulated at 16 Hz, as a function of calculated power density. Four tubes containing brain tissue were exposed at a time. The error bars represent one standard error (SE), the numbers above each bar indicate the number of samples and *** indicates significant differences between the exposed and sham groups at $p < 0.001$.

Figure 2 Mean relative quantity of calcium ions released by tissue pairs exposed to 147-MHz radiation, sinusoidally amplitude modulated at 16 Hz, as a function of calculated power density. Four tubes containing brain tissue and six tubes containing only an equivalent amount of medium were exposed at a time. The error bars represent one standard error (SE), the numbers above each bar indicate the number of samples and * and *** indicate significant differences between the exposed and sham groups at $p < 0.05$ and $p < 0.001$, respectively.

Figure 3 Mean relative quantity of calcium ions released by tissue pairs exposed to 147-MHz radiation, sinusoidally amplitude modulated at 9 Hz, as a function of calculated power density. Four tubes containing brain tissue and six tubes containing only an equivalent amount of medium were exposed at a time. The error bars represent one standard error (SE), the numbers above each bar indicate the number of samples, and * and *** indicate significant differences between the exposed and sham groups at $p < 0.05$ and $p < 0.001$, respectively.

Table 1.

A.M. FREQ. (Hz)	POWER DENSITY, (mW/cm^2)	NUMBER TUBES IN CHAMBER	Vt/Vc, SHAM			Vt/Vc, EXPOSED			P
			N	MEAN	S.E.	N	MEAN	S.E.	
16	0.11	4	32	1.081	0.033	32	1.109	0.046	0.619
16	0.55	4	52	1.061	0.040	52	1.072	0.028	0.824
16	0.83	4	63	1.000	0.023	71	1.120	0.025	< 0.001
16	1.11	4	28	1.052	0.051	28	1.100	0.052	0.515
16	1.38	4	44	1.093	0.032	32	1.135	0.051	0.466
16	0.11	10	56	1.095	0.034	55	1.125	0.030	0.504
16	0.55	10	28	0.969	0.035	36	1.087	0.034	0.021
16	0.83	10	64	1.024	0.028	64	1.168	0.032	< 0.001
16	1.11	10	78	1.038	0.025	78	1.127	0.034	0.028
16	1.38	10	64	1.066	0.027	64	1.174	0.036	0.017
16	1.66	10	64	1.080	0.025	64	1.086	0.026	0.863
9	0.11	10	32	1.076	0.047	32	1.147	0.054	0.328
9	0.55	10	63	1.055	0.029	63	1.148	0.027	0.019
9	0.83	10	28	1.003	0.026	32	1.199	0.047	< 0.001
9	1.11	10	32	1.018	0.040	32	1.154	0.035	0.013
9	1.38	10	32	1.038	0.043	32	1.086	0.041	0.427
0	0.83	10	64	1.098	0.037	64	1.084	0.030	0.765
0	0.00	10	32	1.060	0.034	32	1.066	0.033	0.888

WHERE: Vt DENOTES THE counts per minute FOR THE TREATED BRAIN HALF.
Vc DENOTES THE counts per minute FOR THE CONTROL BRAIN HALF.

Figure 1.

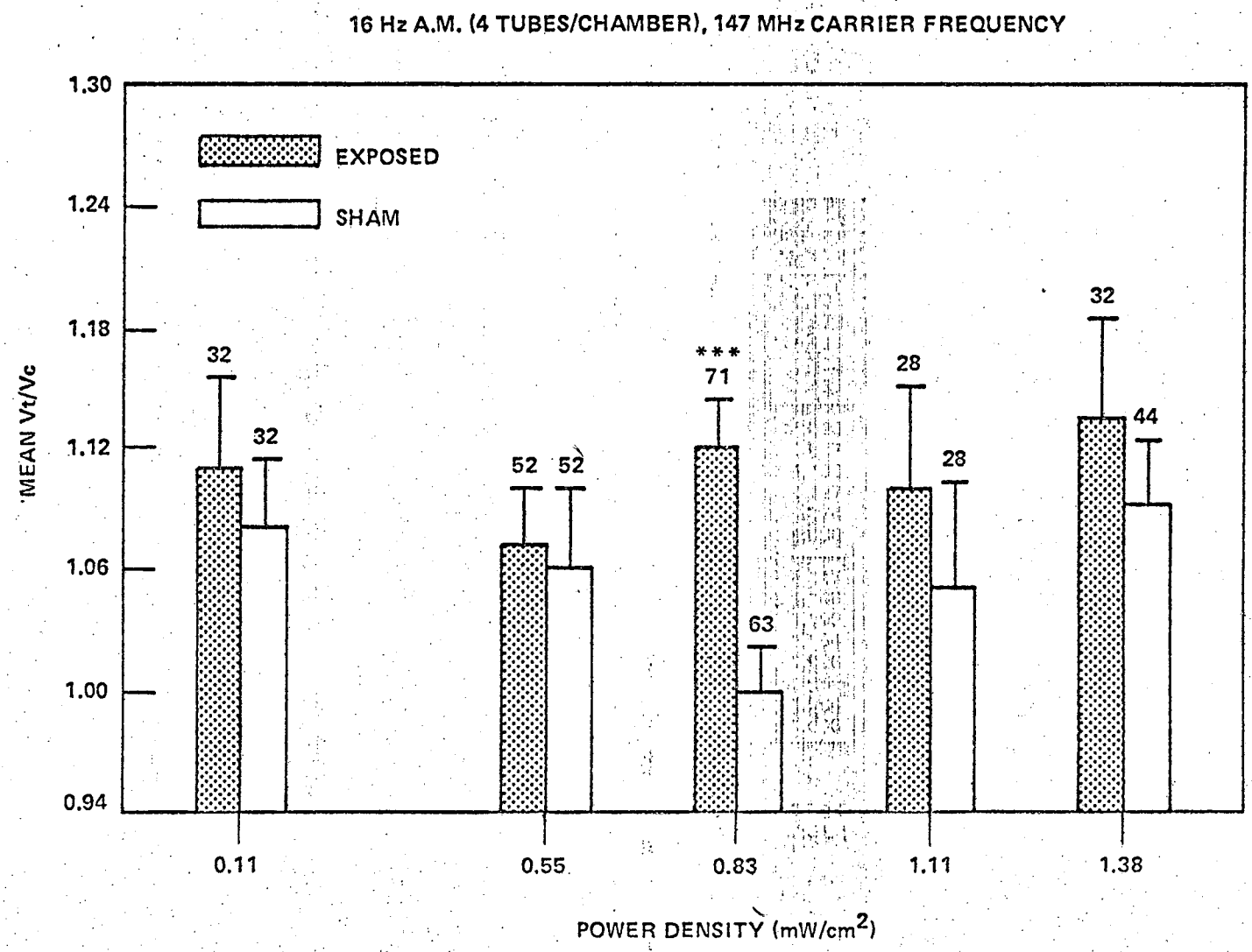

16 Hz A.M. (4 TUBES/CHAMBER), 147 MHz CARRIER FREQUENCY
EXPOSED
SHAM
MEAN Vt/Vc
1.30
1.24
1.18
1.12
1.06
1.00
0.94
32
32
52
52
***
71
63
28
28
32
44
0.11
0.55
0.83
1.11
1.38
POWER DENSITY (mW/cm2)

Figure 2.

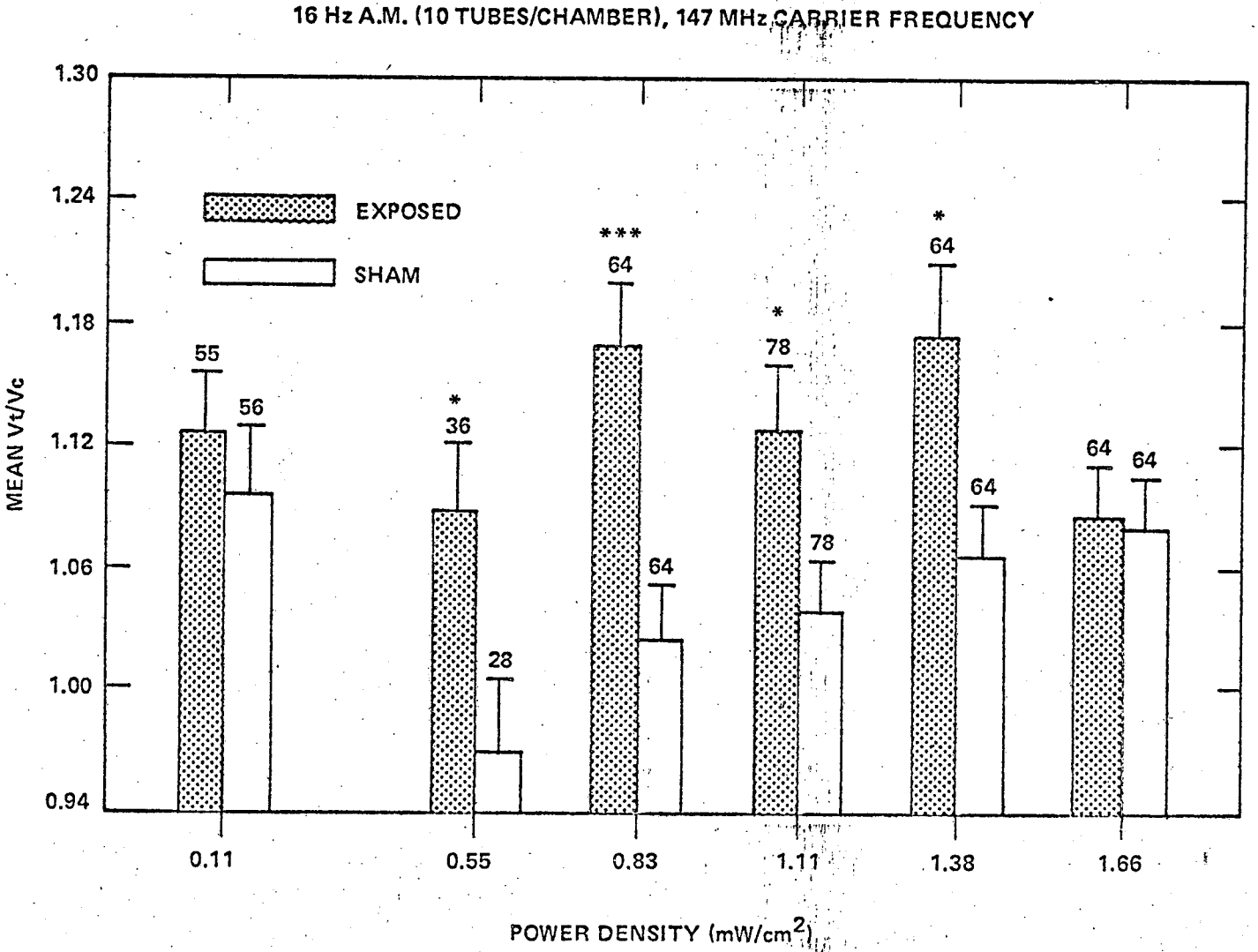
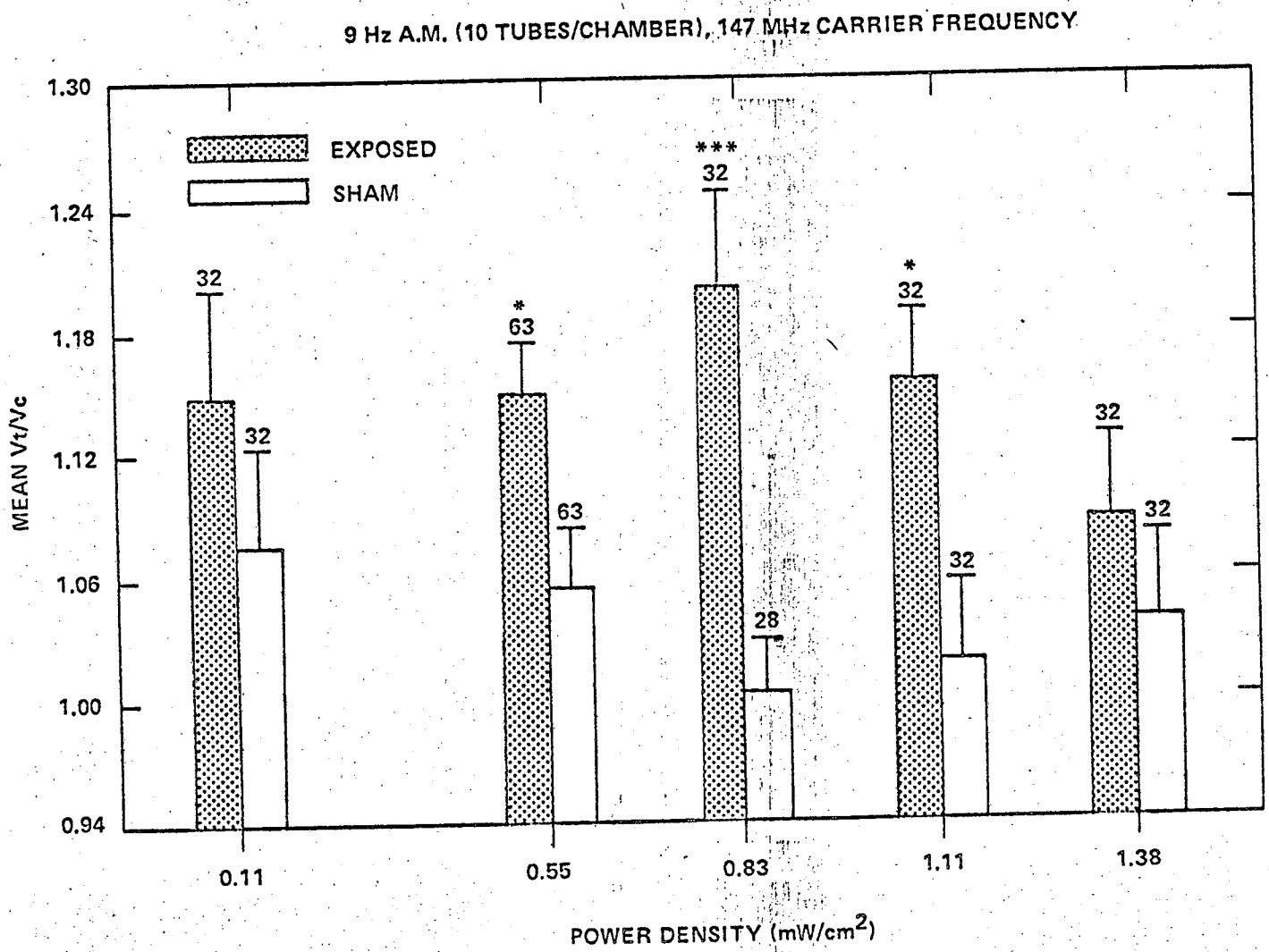

16 Hz A.M. (10 TUBES/CHAMBER), 147 MHz CARRIER FREQUENCY
1.30
1.24
1.18
1.12
1.06
1.00
0.94
MEAN Vt/Vc
EXPOSED
SHAM
55
56
*
36
28
***
64
64
*
78
78
*
64
64
64
64
0.11
0.55
0.83
1.11
1.38
1.66
POWER DENSITY (mW/cm2)

Figure 3.


9 Hz A.M. (10 TUBES/CHAMBER), 147 MHz CARRIER FREQUENCY
EXPOSED
SHAM
MEAN Vt/Vc
1.30
1.24
1.18
1.12
1.06
1.00
0.94
32
32
*
63
63
***
32
28
*
32
32
32
32
0.11
0.55
0.83
1.11
1.38
POWER DENSITY (mW/cm2)