

Sensitivity of calcium binding in cerebral tissue to weak environmental electric fields oscillating at low frequency

($^{45}\text{Ca}^{2+}$ efflux/cerebral organization/cooperative processes)

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ABSTRACT Weak sinusoidal electric fields modify the calcium efflux from freshly isolated chick and cat cerebral tissues bathed in Binger's solution, at 36°. Following incubation (30 min) with radioactive calcium ($^{45}\text{Ca}^{2+}$), each sample, immersed in fresh solution, was exposed for 20 min to fields at 1, 6, 16, 32, or 75 Hz, with electric gradients of 5, 10, 56, and 100 V/m in air. $^{45}\text{Ca}^{2+}$ efflux in the solution was then measured in 0.2 ml aliquots and compared with efflux from unexposed control samples. Field exposures resulted in a general trend toward a reduction in the release of the preincubated $^{45}\text{Ca}^{2+}$. Both frequency and amplitude sensitivities were observed. Maximum decreases occurred at 6 and 16 Hz (12–15%). Thresholds were around 10 and 56 V/m for chick and cat tissues, respectively. Similar but nonsignificant trends occurred during other field exposures. All results were statistically compared with matched samples of controls. Tissue gradients could not be measured, but estimates were of the order of 0.1 $\mu\text{V}/\text{cm}$. The susceptibility of the electrochemical equilibrium in the neuronal membrane to small extracellular perturbations is discussed and a possible role for weak intrinsic cerebral fields in neuronal excitability is suggested.

Calcium ions are essential in the regulation of the resting membrane potential and in the sequence of events in synaptic excitation (1–4). Anatomically, calcium appears to be differentially distributed in brain tissue. Higher concentrations occur at neuronal membranes, in synaptic regions, in neuroglial cytoplasm, and in neuronal organelles, but there is relatively little in neuronal cytoplasm (5–7). Polyanionic membrane surface glycoproteins show a strong affinity for cations, and particularly for calcium and hydrogen ions (8, 9). A calcium-sensitive fibrillar protein network lying inside the bilayer has also been described (10). In addition, there is a probable role for calcium in the transduction of far weaker events at the membrane surface, including propagation of transmembrane signals through prostaglandin molecules following binding of hormones at cell surface receptor sites (11).

Weak oscillating electric gradients occurring spontaneously in brain tissue as the electroencephalogram are of the order of 1–20 mV/cm when recorded in the extracellular medium over millimeter distances, or at cellular dimensions of 10 μm (12, 13). Environmental electric fields, both natural and artificial, produce even weaker tissue components at field frequencies below 100 Hz: a 10 V/m, 7 Hz sinusoidal field produces an average gradient of less than 0.1 $\mu\text{V}/\text{cm}$ in a phantom monkey head (14, 15).

We have previously described a sharply increased efflux of calcium from isolated chick brain tissues exposed to modulated radio frequency fields (16). These studies showed that the response depended on a narrow band of slow modulation frequencies (6–25 Hz), and not on the presence of the unmodulated carrier wave alone (147 MHz, 0.8 mW/cm²). In the present study, chick cerebral hemisphere and cat cerebral cortex were exposed, *in vitro*, to weak (5–100 V/m) extremely low frequency (ELF) fields (1–75 Hz).

MATERIALS AND METHODS

Field exposure was performed in an environmental screened chamber, between two parallel metal plates, one square meter in area, 50 cm apart. Sine wave electric fields were applied to the plates at levels of 5–100 V/m and at frequencies of 1–75 Hz. Equal voltages with respect to ground were applied to each plate.

Chick cerebral hemispheres were rapidly removed following decapitation. The hemispheres were separated and after weighing each was incubated at 36° for 30 min in 1 ml of a physiological medium [155 mM NaCl, 5.6 mM KCl, 2.16 mM CaCl₂, 24 mM NaHCO₃, and D-glucose (2 g/liter)] together with 0.2 ml of a solution containing $^{45}\text{Ca}^{2+}$ (0.2 μCi , specific activity 1.39 Ci/mmol). The incubated samples were then rinsed three times and exposed for 20 min to an environmental electric field while immersed in 1.0 ml of the physiological medium. At the conclusion of field exposure, an aliquot of 0.2 ml of the bathing solution was taken for scintillation counting. Prior to counting this aliquot was mixed with 9.0 ml of a scintillation adjutant (Packard Dimilume). The brain samples were dissolved overnight in a digestive medium (Soluene 350, Packard) and then assayed for radioactivity. For each field condition (in both frequency and amplitude) "sets" of 10 brain samples were used simultaneously in field exposure and control conditions. Control samples were tested identically to the test samples except for the field exposure. All tissues were maintained at 36° during the whole experiment (16).

The same experimental procedure was applied to striated muscles (lateral head of the gastrocnemius) in a series of chicks to evaluate possible effects in nonnervous tissue. Fifty muscle specimens were tested with 20 V/m, 16 Hz field and compared with nonexposed muscles (30 samples). The statistical treatment of the data was identical to that applied to brain tissues.

Samples of freshly removed cat cortex were similarly tested. Under ether anesthesia, the cerebral hemispheres were exposed. After completion of surgery, general anesthesia was discontinued and local anesthesia was instituted in all incisions and pressure points and thereafter the animal was immobilized with gallamine triethiodide (6.0 mg, intravenous). Body temperature was maintained at 37° and the tidal CO₂ levels were monitored. Cortical samples were removed 30 min after cessation of ether anesthesia. Samples were taken from visual, auditory, somato-sensory, and suprasylvian areas. Each cortical sample was bisected and the pia mater was removed before weighing. Half of the bisected samples were exposed to one of the field conditions, the other half served as control. Since the two sets of tissue had to be processed simultaneously, the controls were kept at 36° in a bath while the test samples were exposed to the fields in the environmental chamber. A series of experiments (23 paired samples) was conducted under sham conditions in the absence of irradiation to provide comparison between the

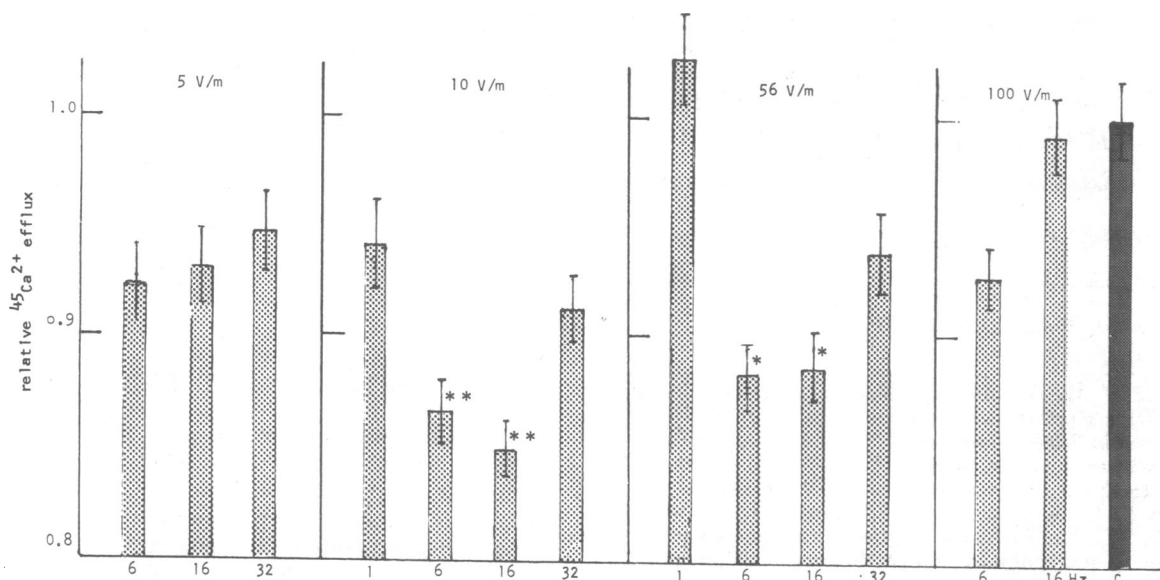


FIG. 1. Effects of extremely low frequency fields on $^{45}\text{Ca}^{2+}$ efflux from the chick forebrain. The relative $^{45}\text{Ca}^{2+}$ effluxes, given \pm SEM, are referred to the mean value of control condition (C). *, $P < 0.05$; **, $P < 0.01$ that the mean is the same as that of the control.

$^{45}\text{Ca}^{2+}$ effluxes obtained during these two treatments. The sensitivity of the $^{45}\text{Ca}^{2+}$ efflux to temperature was also tested (23 paired samples), in additional experiments where half of the brain samples were processed at 30° and the other half at 36° .

Our pilot studies with radio frequency fields demonstrated that sample counts more than 40% above or below the mean of any set of 10 samples can be discarded as aberrations due to experimental errors in washing the tissue after incubation or in collecting the supernatant following the test period. In the present study, two statistical criteria applied to each set of data confirmed our previous observations. Extreme counts in any set more than 1.5 standard deviations away from the mean also satisfied the probability levels (0.1 to 0.01) in a statistical method based on the range of values [maximum ratio of extreme ranking observations (17)]. Therefore, such extreme values were eliminated from the sets (fields as well as controls) before final analysis of the data. The radioactivities (cpm/g) of all samples (supernatant and digested tissues) were referred to the mean value of the counts obtained in control effluxes. This allows direct comparison between the amounts of $^{45}\text{Ca}^{2+}$ taken up by the tissues and subsequently released during the experimental conditions. All normalized data were statistically compared (t test) with matched samples of control values.

The results are expressed in terms of the mean of all samples within a condition, plus or minus the standard error of the mean ($m \pm \text{SEM}$). 340 neonate chicks and 39 adult cats were used in this study.

RESULTS

Both frequency and amplitude sensitivities were observed in $^{45}\text{Ca}^{2+}$ efflux from the chick forebrain during field exposures (Fig. 1, Table 1). Decreased efflux occurred under most field conditions. Maximum effect occurred at frequencies of 6 and 16 Hz ($P < 0.01$) with field gradients of 10 V/m. Similar but slightly smaller effects occurred at these frequencies at 56 V/m. For fields at 5, 10, and 56 V/m, there is strong evidence of a "tuning" curve having a trough in the vicinity of 6 and 16 Hz with reduced efflux at 32 Hz. Some reduction in the efflux was noted at 1 Hz with 10 V/m fields, but this did not occur at 56 V/m. The sensitivity observed with 6 and 16 Hz fields is nota-

ble, since the reduction in efflux was between 11 and 15% at 10 and 56 V/m. The findings also suggest an amplitude window, since only nonsignificant trends occurred at 5 V/m and even these trends were essentially absent at 16 Hz with fields of 100 V/m.

Tissue counts from exposed brain tissues were not statistically different from the control values. Each field condition was tested against the corresponding no field control to insure that the decrease seen in the $^{45}\text{Ca}^{2+}$ release was not due to an accidentally low tissue uptake. The mean uptake obtained for all brain samples (across all field conditions), expressed as a ratio with respect to the mean efflux of all controls, was 3.076 with a standard error of 0.104; the control values were 3.167 and 0.090. Thus the ratio of $^{45}\text{Ca}^{2+}$ uptake in the brain tissues versus the $^{45}\text{Ca}^{2+}$ released in the bathing fluid was three to one.

Table 1. $^{45}\text{Ca}^{2+}$ Efflux from the chick forebrain

	$m \pm \text{SEM}$ (F)	$m \pm \text{SEM}$ (C)	n	t
5 V/m				
6 Hz	0.923 ± 0.036	1.000 ± 0.038	30	1.450
16 Hz	0.933 ± 0.041	1.000 ± 0.041	27	1.144
32 Hz	0.945 ± 0.038	1.000 ± 0.041	27	0.974
10 V/m				
1 Hz	0.943 ± 0.041	1.000 ± 0.038	26	1.021
6 Hz	0.866 ± 0.029	1.000 ± 0.037	26	3.069**
16 Hz	0.849 ± 0.026	1.000 ± 0.031	38	3.726**
32 Hz	0.913 ± 0.038	1.000 ± 0.037	27	1.633
56 V/m				
1 Hz	1.028 ± 0.042	1.000 ± 0.038	26	0.515
6 Hz	0.882 ± 0.032	1.000 ± 0.030	37	2.681*
16 Hz	0.889 ± 0.035	1.000 ± 0.028	36	2.489*
32 Hz	0.942 ± 0.031	1.000 ± 0.038	26	1.518
100 V/m				
6 Hz	0.928 ± 0.028	1.000 ± 0.029	36	1.735
16 Hz	0.995 ± 0.037	1.000 ± 0.037	28	0.092

Effluxes obtained following field exposures (F) are compared with control results (C). n is the number of paired samples (F and C) used in the statistical analysis (t test). *, $P < 0.05$; **, $P < 0.01$.

Our previous findings indicated that striated muscle tissues were insensitive to radiofrequency fields amplitude-modulated at brain wave frequencies (6 and 16 Hz). The muscles tested in this experiment appeared again to be unaffected by a field condition (16 Hz, 20 V/m) that induced a decrease in the $^{45}\text{Ca}^{2+}$ release from brain tissues. The mean of the effluxes from exposed muscles was 0.988 with a standard error of 0.035 (44 samples), the control values were 1.000 and 0.036 (24 samples). The normalized tissue counts were respectively 1.506 ± 0.067 in the exposed muscles and 1.568 ± 0.060 in the control samples. The average uptake in muscles (25% of the initial 0.2 μCi introduced in each test tube) was approximately three times as high as in brain samples, but this could be due to differences in shape and surface area between the two tissues. The ratio of uptake versus release was 1.5 to 1, indicating a higher calcium exchange in muscles than in brain tissues.

The results obtained with isolated cat cerebral cortex also suggested a frequency sensitivity at a slightly higher threshold (Table 2). Significant reduction in $^{45}\text{Ca}^{2+}$ efflux occurred at 6 Hz ($P < 0.05$) and 16 Hz ($P < 0.01$) with 56 V/m gradients. Nonsignificant trends toward decreased efflux also occurred at 1, 32, and 75 Hz at this field strength. No significant effects were observed with 10 V/m at 6, 16, and 32 Hz, nor with 100 V/m fields at 6 and 16 Hz. Again there is evidence of a tuning curve with maximal tissue sensitivity in the vicinity of 6 and 16 Hz, and an amplitude between 56 and 100 V/m. Tissue counts of $^{45}\text{Ca}^{2+}$ uptake were tested for each condition, as in chick brain samples. The mean and standard error for all samples (across all field conditions) were respectively 2.364 and 0.084, the control values were 2.332 and 0.066.

No significant differences were noted between different cortical regions, which were arbitrarily chosen as representative of major sensory, motor, and association fields. It was noted that removal of the pia mater was essential for consistent effects in the neocortical samples. There was no difference between samples tested in the screened chamber (sham irradiation) and control tissues placed in the heated bath (23 paired samples = 1.000 ± 0.039 versus 1.000 ± 0.031 ; $t = 0.235$) or between the samples tested at two different temperatures (30° and 36°, 23 paired samples: 0.975 ± 0.034 versus 1.000 ± 0.041 , $t = 0.461$).

DISCUSSION

The present study disclosed both frequency and amplitude windows for the selective inhibition of calcium release from cerebral tissue. Direct cortical stimulation of the intact cat cortex (200 pulses/sec, 10 msec duration), with tissue electrical gradients of 20–50 mV/cm, produces a 20% increase in the efflux of preincubated $^{45}\text{Ca}^{2+}$ (18) rather than a decrease. A comparable increase occurs from isolated chick cerebral tissue exposed to radio frequency fields amplitude-modulated at frequencies between 6 and 25 Hz (16).

For both low frequency and radio frequency fields, the evidence indicates a maximal field sensitivity at "biological" frequencies, but the mode of interaction appears strongly dependent on the amplitude of the incident field. A possible basis for this amplitude selectivity may lie in the mode of calcium binding to stranded biopolymers (8) with primary bonding at sites along single strands, and secondary "ladder" formation between adjoining strands with lower energy bonds. On the other hand, no ready explanation can be offered for the "tuning" curves seen at field frequencies from approximately 6 to 20 Hz. Their congruent but mirrored relationship for low frequency and radio frequency fields suggests interaction on a common substrate. In the latter case, demodulation of the carrier may be based on asymmetry of fixed charge distribution

Table 2. $^{45}\text{Ca}^{2+}$ Efflux from the cat cortex

	$m \pm \text{SEM (F)}$	$m \pm \text{SEM (C)}$	n	t
10 V/m				
6 Hz	0.948 ± 0.023	1.000 ± 0.032	23	1.296
16 Hz	0.982 ± 0.037	1.000 ± 0.034	29	0.302
32 Hz	1.006 ± 0.054	1.000 ± 0.036	16	0.108
56 V/m				
1 Hz	0.974 ± 0.054	1.000 ± 0.036	23	0.386
6 Hz	0.855 ± 0.034	1.000 ± 0.043	21	2.600*
16 Hz	0.874 ± 0.025	1.000 ± 0.026	24	3.402**
32 Hz	0.909 ± 0.034	1.000 ± 0.040	21	1.704
75 Hz	0.932 ± 0.026	1.000 ± 0.033	22	1.600
100 V/m				
6 Hz	1.000 ± 0.025	1.000 ± 0.032	21	0.016
16 Hz	0.965 ± 0.033	1.000 ± 0.025	29	0.830

Symbols as for Table 1.

on membrane surface glycoproteins with respect to extracellular fluid and the deeper layers of the membrane (15).

The effects of these extremely weak fields on isolated brain tissues have been consistent within and between experiments. Tissue gradients were not directly measured, but in related studies, gradients of the order of 10^{-7} V/cm were induced in a phantom monkey head by fields of similar geometry (14, 15). Tissue components of the environmental fields were several orders of magnitude weaker than the intrinsic field of the electroencephalogram (EEG), which in turn is orders of magnitude smaller than transmembrane gradients of 1 kV/cm occurring in synaptic potentials. Therefore, an adequate model of field-brain interaction must account for a 15% shift in calcium efflux, on the basis of an extremely weak triggering process. Domestic and industrial environments routinely expose man to much stronger oscillating electric fields at power line frequencies without major physiological or psychological perturbations. Findings here are consistent with this absence of a proportionality between field strength and central nervous response, although our data clearly indicate both threshold and field strength/tissue response relationships within the confines of amplitude and frequency windows.

Our data suggest that these interactions could occur at different levels of cerebral organization (15). Initial interactions may occur in the long axis of the membrane, perhaps involving macromolecular conformational changes with altered calcium bonding and acting as precursors to a transmembrane response. A weak trigger at one point may initiate conformational changes over considerable distances along the membrane (19). Thereafter, transmembrane signals may trigger classic excitatory mechanisms and the release of metabolic energy.

Cooperative interactions at the membrane surface have been frequently discussed in theoretical models of neuronal excitability. In models proposed by Schwarz (20, 21), cooperativity is shown to occur in the length of a biopolymer sheet, by assuming a coherent state between neighboring segments of the polymer, based on similarity of charge states. Changeux *et al.* studied the dynamics of a two-dimensional lattice of globular lipoproteins, undergoing reversible conformational changes, and capable of specificity in the binding of biological ligands (22). The binding behavior of this model was in the class of cooperative processes and predicted "graded" and "all or none" responses, depending on the free energy of interaction between the lattice units.

Experimental evidence for chemical cooperativity involving

calcium is substantial and closely parallels observed thresholds for neurophysiological responses. The increase in efflux of both calcium and gamma aminobutyric acid from the cat cortex, *in vivo*, is a highly nonlinear response: a 1.0 mM increment of the calcium extracellular concentration is only slightly less effective than a 20 mM increment (23). Transductive coupling in retinal stimulation also appears to be a cooperative process. In the squid visual receptor, a single photon has a probability of at least 0.3 of initiating a quantized chemical response (24). Each photo-activated rhodopsin molecule releases in excess of 1000 calcium ions per disc (25, 26). Activation of a single receptor leads to responses in those surrounding it, even though the energy induced by one photon does not exceed 50 to 100 μV (27).

Nevertheless, caution is necessary in attempting to explain the observed field-brain interactions in terms of long-range, cooperative phenomena. Evidence so far available does not support molecular sensitivities in such a narrow low frequency range. Fröhlich (28, 29) has described a model of long-range coherence and energy storage in biological membrane based on dipole interactions and the recurrence of certain bonds (such as hydrogen bonds) in macromolecules, but with much higher frequencies of oscillation in the range 10^{11} to 10^{12} Hz. Another prime problem lies in the extremely weak tissue electric gradients that are effective stimuli.

It may appear that thermal noise at normal tissue temperature is substantially larger than the tissue components of the imposed electric fields. For typical conductors in the biological temperature range, the Boltzmann (kT , where k is the Boltzmann constant and T , the absolute temperature) noise is of the order of 0.02 electron volts. However, this expression gives little concept of the extent to which electric gradients in tissue may be established by thermal atomic or molecular perturbations, nor of the way in which components of this noise may be transferred to distant sites within tissue. In metallic conductors, the transfer function for this noise energy has an essentially infinite bandwidth, a condition that does not pertain in tissue.

The transfer function for thermoelectric noise in tissue has yet to be studied. The following model is therefore offered tentatively, but does provide interesting points of resemblance to observed neurochemical and behavioral thresholds. This model relates realistically to the observed frequency dependence and limited frequency bandwidth of ionic conductances in oscillating fields in the counterion layer along the membrane surface. Dielectric constants in excess of 10^6 at frequencies below 1.0 kHz occur at porous surfaces of micrometer-sized resin particles (30), and relate to electric fields emerging from fixed charge sites within the porous surface. The Boltzmann equation may be written in terms that model the tissue as a low pass filter: $e^2 = 4kTBR$ where the transfer function for the root mean square noise voltage (e) is a function of the frequency bandwidth (B) and the specific resistance of the noise pathway (R). With a specific resistance for brain tissue of the order of $300 \Omega \text{ cm}$ and an effective frequency bandwidth from 0 to 100 Hz, the equivalent noise voltage gradient would be of the order of 10^{-8} V/cm (typical tissue components of the environmental fields imposed in the present study were estimated to be 10^{-7} V/cm). This model must be considered tentative, but it offers a stimulus to further study, in view of observed sensitivities to fields of 10^{-8} V/cm in marine vertebrates (31, 32), and the behavioral effects seen in birds (33), man, and other primates attributable to oscillating tissue gradients less than an order of magnitude larger (34-38).

Grodsky (39, 40) has modeled the plasma membrane as a sheet of dipoles under electrical strain attributable both to the

dipoles' mutual interactions and to the local electric field generated by the presence of cations in the polyanionic structure of the outer membrane. This model is mathematically similar to an antiferromagnetic crystal in an external magnetic field. For any fixed temperature below a critical point (Néel temperature), the modeled membrane can undergo long-range order changes (phase transition) in response to sudden fluctuations of a surrounding electric field. The lowest frequencies of the natural modes of the system approach zero with increasing wave amplitude near a phase transition, so that most of the total energy is then contained in a narrow frequency band. External oscillating fields matching these frequencies would be resonantly absorbed by the system. Grodsky postulated that the low frequencies of the permissible modes of this system could be the basis of spontaneous oscillations of intrinsic electric fields in cerebral tissues and the phase transitions experienced by the model would be analogous to action potentials.

To the extent that extremely weak electrical fields have been shown to modify brain states, they raise questions concerning the role of the intrinsic cerebral low frequency oscillating fields. It would be expected that a stimulus capable of modifying the efflux of an ion essential in excitation and regulatory functions by 15% would be associated with dramatic changes in perception and behavior. The patent lack of such effects may serve to emphasize the endless complexity of the intrinsic field, even at cellular dimensions, and the improbability that imposition of an essentially uniform gradient over large tissue masses would replicate significant elements of the intrinsic fields. It remains to be shown that the intrinsic field does indeed provide a mechanism by which cerebral neurons can "whisper together" (41), but this possibility is made more likely by demonstration of such an extremely high chemical sensitivity to the global effects of environmental electric fields. Specificity of these effects in cerebral tissue is suggested by their apparent absence in striated muscle.

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