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Ionic factors in release of $^{45}Ca^{2+}$ from chicken cerebral tissue by electromagnetic fields

(brain/cooperative/membrane/receptor)

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ABSTRACT **Electrical stimulation with radiofrequency fields amplitude-modulated at brain wave frequencies increased $^{45}Ca^{2+}$ efflux from isolated chicken cerebral tissue. The response was not sensitive to variations of the calcium concentration (0–4.16 mM) in the bathing solution but was enhanced by addition of H^+ (0.108 mM HCl) and inhibited in the absence of normal bicarbonate levels (2.4 mM). Addition of lanthanum to the bicarbonate-free solution restored electrical responsiveness, but the stimulus decreased instead of increasing $^{45}Ca^{2+}$ efflux. It is suggested that low-frequency, weak, extracellular electric gradients may be transduced in a specific class of extracellular negative binding sites normally occupied by Ca^{2+} and susceptible to competitive H^+ binding.**

The efflux of $^{45}Ca^{2+}$ from preincubated slices of cerebral tissue is characterized by an early, fast release (first 5–10 min of exchange with nonradioactive medium), thought to represent rapidly exchangeable calcium in the extracellular space, followed by slower efflux which could result from exchanges with more sheltered calcium pools (1–3). Electrical stimulation of brain tissue with long (5 min or more) trains of square-wave pulses has been shown to increase the $^{45}Ca^{2+}$ efflux from guinea pig and rat brain slices (2, 4) and from intact cat cerebral cortex (5), and it has been suggested that the stimulus-sensitive efflux originates in the slowly exchanging pool because the tissue response could be elicited after extinction of the fast component of the $^{45}Ca^{2+}$ efflux (2). The electrical gradient induced in isolated tissue by prolonged stimulation is not known, but *in vivo* measurements in the cat brain during nonfocal repetitive stimulation (10 min, 200 Hz, 2.25 V, 1.0 msec) indicated tissue components, decreasing from 60 to 20 mV/cm, between the cortical surfaces and a depth of 20 mm (5). These electric gradients are comparable to intrinsic extracellular field potentials [the electroencephalogram (EEG)] which have gradients of 50–100 mV/cm at cellular dimensions (6), and we have sought evidence that would relate transductive coupling of such extracellular gradients to altered binding of calcium to cell surface macromolecules.

There is much evidence that behavioral, electrophysiological, pharmacodynamic, and biochemical effects observed in fish, marine vertebrates, birds, and mammals are related to interactions with weak oscillating environmental fields (7, 8, *), and these studies have focused attention on possible structural and functional bases of central and peripheral neural interactions with the fields. Because it is technically difficult to establish a tissue electric gradient large enough to simulate the EEG by imposition of low-frequency environmental electric fields, due to weak field coupling to tissue, we have applied radiofrequency fields, amplitude modulated at low frequencies (9–11).

The $^{45}Ca^{2+}$ efflux from isolated chicken cerebral hemisphere was stimulated by exposure to a weak (0.8 mW/cm^2), very high frequency (147 MHz) electromagnetic field amplitude-modulated by slow sinusoidal signals, but a significant increase occurred only for modulation frequencies between 6 and 20 Hz, with a maximal effect at 16 Hz (10). This modulation frequency specificity of the tissue response was confirmed by Blackman *et al.* (12), who also disclosed a nonlinear field-intensity response, with maximal effects around 0.75 mW/cm^2 and insignificant trends at higher and lower field powers. Studies with 450-MHz field amplitude-modulated at 16 Hz corroborated the existence of a narrow power window (0.1–1.0 mW/cm^2) within which the stimulus enhanced the $^{45}Ca^{2+}$ efflux (11). As measured with an electric field probe in cat cortex exposed to 450-MHz irradiation at 1.0 mW/cm^2, tissue gradients were of the order of 100 mV/cm (13) and thus comparable with the 50 mV/cm field induced in cat brain by more conventional electrode stimulation (5). A decrease in $^{45}Ca^{2+}$ efflux, rather than an increase, occurred after stimulation with extremely low frequency fields, with gradients of 10–60 V/m in air. The internal field strengths were several orders of magnitude smaller than the tissue gradients induced by 147- and 450-MHz fields (14), but the frequency specificity and the size of the response were the same whether the fields induced an increase or a decrease in the efflux.

The consistency of the magnitude of the effect in all experiments (10–15% change in the efflux), together with the exquisite sensitivity of the system to narrow windows in frequency and amplitude of the field stimulus, could indicate that a specific class of calcium sites is responsive to low-frequency extracellular electric fields. It was hypothesized (14, 15) that a field-sensitive pool of calcium sites could be found in the complex binding of the ions to negative sites in the extracellular loose structure of the neuronal membrane (16, 17).

The experiments described here aimed at a better definition of the calcium sites responding to weak electrical stimulation. We first studied changes in efflux, with and without imposed electromagnetic fields, in relation to the calcium concentration in the exchanging medium, because calcium–calcium interactions have often been shown to play an important role in the regulation of calcium uptake and release (3, 18). Another approach to differentiating possible components of the $^{45}Ca^{2+}$ efflux was to attempt modification of calcium release by adding lanthanum to the bathing solution and studying effects of electrical stimulation on this new system. The trivalent lanthanum ion (La^{3+}) has been found to be extremely effective in blocking calcium flux across excitable membranes (19–22), and electron microscopy of lanthanum-treated barnacle muscle fibers (23), heart cells (24), and liver cells (25) has located the ion in close relationship to the membrane in the extracellular

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Abbreviation: EEG, electroencephalogram.

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compartment but not in the cytoplasm, an indication that La^{3+} probably does not cross the cell membrane. As a result, it was postulated (23) that La^{3+} inhibited electrical events dependent on calcium entry into the cell by displacing the ions responsible for the inward current from their specific binding sites on the membrane. The distribution of bound La^{3+} in cortical tissue is not known, but $^{45}Ca^{2+}$ release from the non-inulin-space of rat cerebral cortex slices was shown to be inhibited by the presence of 2.0 mM $LaCl_3$ in the bathing solution (26). We therefore tested a model in which La^{3+} could also displace extracellular calcium and bind to brain cell membrane in the isolated chicken hemisphere, and perhaps interfere with electrosensitive binding sites.

MATERIALS AND METHODS

Cerebral hemispheres of new born chicks (4–8 days old) were used in these experiments. Each hemisphere was incubated at 37 °C in 1 ml of physiological solution (155 mM NaCl/5.6 mM KCl/2.16 mM $CaCl_2$/2.4 mM $NaHCO_3$/10 mM D-glucose)† containing 0.2 μCi of $^{45}Ca^{2+}$ (specific activity, 0.735 Ci/mmol). After $^{45}Ca^{2+}$ loading, the hemispheres were rinsed three times in radioactivity-free solution and either transferred to 1 ml of fresh solution or immediately dissolved in a digestive medium (Soluene 350, Packard). The $^{45}Ca^{2+}$ uptake was determined by liquid scintillation counting of the dissolved brain diluted in a scintillation cocktail (Packard Dimilume), and the $^{45}Ca^{2+}$ efflux was obtained by sampling the radioactivity of the exchanging solution in 0.2-ml aliquots also diluted in Dimilume. Fig. 1 summarizes the results of preliminary experiments in which uptake and release of $^{45}Ca^{2+}$ were measured at fixed time intervals. Loading and efflux curves exhibited both the fast (from 0 to 10 min) and the slower components of the exchanges characteristic of calcium movements as studied *in vitro* with radiocalcium (1, 2, 26). In all these experiments, the hemispheres were incubated with $^{45}Ca^{2+}$ for 30 min, rinsed three times, and then bathed for 20 min in the testing solution before being dissolved for radioassay.

The physiological testing solution was normally maintained at pH 7.6 ± 0.1 at 37°C, with an osmolarity of 310–313 mosM. It was kept within these limits by replacing the solutions every 1.5–2 hr with freshly made solutions. For experiments in which the pH was kept at 6.8 or 7.2, the solutions were changed every hour and maintained within 0.1 pH unit of the original pH. The ionic composition of the testing solution was varied according to the protocol of each experimental series. Calcium concentration was manipulated in two series of experiments: in one, calcium was omitted; in the other, calcium was added to the standard solution to bring it to 4.16 mM Ca^{2+}. Lanthanum concentrations of 0.5 and 2.0 mM were used in these studies, and omission of HCO_3^- was necessary to avoid precipitation (19, 23). To minimize the possibility of lanthanum salt formation at the surface of the brain, the samples were rinsed in bicarbonate-free solution before being transferred to solutions containing $LaCl_3$. Changing the ionic composition of the testing solution did not alter the osmolarity, and removal or addition of calcium did not affect the pH of the solution. Omitting the bicarbonate fraction and adding lanthanum resulted in a decrease of almost 2 pH units. The pH of the nonbuffered solution was increased to and maintained at 7.3 by addition of 0.150 mmol of NaOH per liter; 0.676 mmol per liter was necessary to maintain this pH value when lanthanum was added to the solution. In control experiments, the pH was decreased to 6.8 by addition of 0.108 mM HCl to the usual physiological bathing medium.

† In our previous publications, $NaHCO_3$ concentration was incorrectly stated as 24 mM. In these and all previous experiments, 2.4 mM $NaHCO_3$ was used.

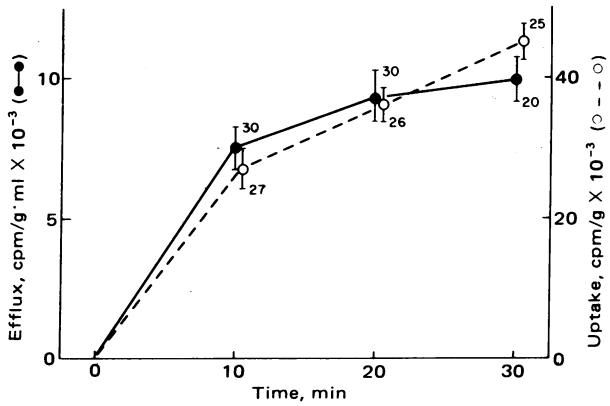

FIG. 1. Freshly isolated chick cerebral hemispheres were incubated at 37°C in a physiological solution containing radioactive calcium. The samples were rinsed in radioactivity-free solution and either immediately dissolved in a digestive medium and assayed for $^{45}Ca^{2+}$ tissue content (Uptake) or transferred to 1 ml of fresh solution sampled for radioactivity at fixed time intervals (Efflux). Vertical lines represent SD, with number of replicates shown.

All irradiations were performed for 20 min in a 450-MHz horn radiator, lined and terminated with absorbent material to form an anechoic chamber (27, 28). This chamber was seven wavelengths long, allowing exposure under semi-far field conditions in a low standing-wave field [standing-wave ratio (SWR) 1.2:1]. The radiofrequency generator was sinusoidally amplitude-modulated at 16 Hz to a depth of 90%. The chamber was maintained at 37°C and 30–40% humidity. The incident field on the tissue was 0.75 mW/cm^2; a few comparative studies were made with field powers of 0.375 and 2.0 mW/cm^2. An incident field of 0.75 mW/cm^2 has an electric gradient in air of 53 V/m. Our collaborative measurements of actual tissue gradients produced by this field, by using an implantable microdiode array (14), gave levels in the range 50–100 mV/cm, similar to that of the EEG. In previous studies at 147 MHz (11, 13) there were no effects that could be attributed to the unmodulated carrier wave. No effects are attributable to a specific very high frequency carrier frequency but, due to frequency-dependent coupling to tissues, there is a great enhancement of induced tissue gradients with radiofrequency fields over those produced by low frequency fields of the same environmental field strength. Thus, a 50 V/m 16-Hz field in air induces a tissue gradient of about 1.0 μV/cm, and tissue levels of 50–100 mV/cm with a 16-Hz field would require a gradient in air of 500 kV/m. It would thus be very difficult to achieve EEG-level gradients in tissue exposed to simple low frequency fields.

Each half brain was tested against the corresponding hemisphere, left and right sides alternately serving as control and test samples. To ensure that the changes in efflux observed after treatment were not due to abnormal $^{45}Ca^{2+}$ tissue uptake during incubation, the ratio of $^{45}Ca^{2+}$ remaining in the tissue to $^{45}Ca^{2+}$ in the exchanging solution was calculated for each sample. The effluxes were expressed in cpm/g per ml before comparison with control and statistical analysis of paired observations with a two-tailed *t* test. $^{45}Ca^{2+}$ efflux in the standard solution was the control in experiments involving calcium concentration, pH, and bicarbonate-free solutions. The efflux in the HCO_3^--free medium served as reference in experiments with La^{3+} because omission of HCO_3^- induced a considerable decrease in $^{45}Ca^{2+}$ efflux prior to addition of lanthanum.

RESULTS

The effluxes (mean ± SEM) referred to control in bicarbonate-containing solution are given in Table 1. This table does not include data on ratios of $^{45}Ca^{2+}$ remaining in the tissue at the end of the experiments to $^{45}Ca^{2+}$ in the testing solutions. These data are summarized here.

$^{45}Ca^{2+}$ efflux in Ca-free medium was not significantly different from control, and addition of 2.0 mM $CaCl_2$ to the testing solution induced only a small increase in the efflux. The brain-to-efflux ratio remained practically unchanged, with mean values ranging from 3.5 to 4.0, as in earlier studies (12, 27). The increase of efflux induced by field stimulation was the same in high Ca^{2+} solution as in the normal medium, and trends toward an increase were present in most observations in Ca-free solution, although the mean 10% increase failed to satisfy criteria for statistical significance. Brain-to-efflux ratios were lower in stimulated samples than in control or ionic treatment alone, reflecting increased $^{45}Ca^{2+}$ efflux.

Omission of HCO_3^- from the medium resulted in a marked decrease (10%) in the efflux, and other experiments confirmed an 8.5–11% decrease in efflux, by comparison with concurrent results for tissues in the standard solution. This decrease was not due to an early, rapid $^{45}Ca^{2+}$ efflux in the rinsing solution because levels of radioactivity measured in aliquots of the HCO_3^--free medium used to wash the $^{45}Ca^{2+}$-loaded brain samples were the same as control levels in standard solution. The brain-to-efflux ratios reflected the decrease and were slightly higher than control. Field stimulation had no effect on the decreased $^{45}Ca^{2+}$ efflux. Exploratory studies with a field intensity of 0.375 mW/cm^2 (not listed in Table 1) confirmed the lack of response of the tissue bathed in HCO_3^--free solution. Lowering the pH of the testing solution to 6.8 (addition of 0.1 mM H^+) had no sizable effect on the $^{45}Ca^{2+}$ release, but field-stimulated efflux in these conditions appeared to be larger than in standard solution. Another series of studies at 0.375 mW/cm^2 (not listed in Table 1), in which the field-induced increase in low pH medium (12.5%; $n = 30$; $P < 0.05$) was again higher than in control solution (8%; NS), corroborated these findings.

The presence of 0.5 mM $LaCl_3$ in the HCO_3^--free solution did not change the nonstimulated $^{45}Ca^{2+}$ release, but field stimulation induced a further inhibition of the efflux (7%) and a significant increase in the brain-to-efflux ratio (4.33 ± 0.17) by comparison with control in HCO_3^--free medium (3.75 ± 0.12; $P < 0.05$). The nonstimulated efflux was significantly decreased (11–14%) when the La^{3+} concentration was increased to 2.0 mV and the brain-to-efflux ratio was also significantly higher than control (4.88 ± 0.16 versus 3.85 ± 0.14 in HCO_3^--free medium, $P < 0.01$). The field-induced decrease in $^{45}Ca^{2+}$ efflux was of the same order of magnitude as in 0.5 mM La^{3+} solution and the brain-to-efflux ratio (5.48 ± 0.21) was also significantly higher than the nonstimulated control in the 2.0 mM La^{3+} medium ($P < 0.05$). By contrast, stimulation at lower and higher field intensities (0.375 and 2.0 mW/cm^2) failed to induce any change in the $^{45}Ca^{2+}$ efflux decreased by omission of HCO_3^- and addition of 2.0 mM $LaCl_3$ in the bathing solution.

DISCUSSION

The experimental results described here confirm previous findings (10–12) that amplitude-modulated (16 Hz) radiofrequency electric fields can stimulate the release of preincubated $^{45}Ca^{2+}$ from isolated brain tissue bathed in a physiological solution containing 2.16 mM Ca^{2+} and 2.4 mM HCO_3^-. Doubling the extracellular Ca^{2+} concentration increased $^{45}Ca^{2+}$ efflux by a smaller amount than noted previously in awake cat cortex (18). This difference may result from decreased metabolic activity in the isolated chick hemisphere, because energy for some membrane surface cooperative interactions, such as those with antibodies at the lymphocyte surface, are dependent on metabolic energy to establish appropriate conditions at the receptor site (29). The field-induced $^{45}Ca^{2+}$ release was not sensitive to small fluctuations of the calcium concentration (0–4.16 mM) in the bathing medium but appeared to be facilitated by addition of H^+ (0.108 mM, as HCl) even though this did not affect the $^{45}Ca^{2+}$ efflux in the absence of the field. By contrast, omission of HCO_3^- resulted in a marked decrease of the unstimulated efflux, and field stimulation had no effect on the decreased $^{45}Ca^{2+}$ release. Addition of La^{3+} to the HCO_3^--free solution restored some electrosensitivity, but the stimulus induced a decrease in efflux, unlike the increase seen in the presence of bicarbonate. Fig. 2 schematizes these results and

Table 1. $^{45}Ca^{2+}$ effluxes

Control efflux	n	Ions: Condition	Ions: Efflux	Ions + stim.
		Standard solution		
1.000 ± 0.027	40		—	1.112 ± 0.032*
1.000 ± 0.035	25	No Ca^{2+}	1.033 ± 0.046	1.098 ± 0.048
1.000 ± 0.034	20	2.0 mM Ca^{2+}	1.063 ± 0.053	1.128 ± 0.034*
1.000 ± 0.061	10	0.1 mM H^+	1.024 ± 0.039	1.215 ± 0.044*
1.000 ± 0.035	20	No HCO_3^-	0.904 ± 0.042	—
		No HCO_3^-		
0.905 ± 0.037	20		—	0.883 ± 0.024
0.917 ± 0.038	20	0.5 mM La^{3+}	0.912 ± 0.052	0.848 ± 0.030
0.890 ± 0.023	30	2.0 mM La^{3+}	0.792 ± 0.030*	0.724 ± 0.029**
—	20		0.780 ± 0.034	0.819 ± 0.044†
—	20		0.785 ± 0.039	0.798 ± 0.040‡

$^{45}Ca^{2+}$ effluxes after ionic treatment of the testing solution (Ions) and field-stimulation (Ions + stim.) were statistically compared with control efflux either in standard solution or in HCO_3^--free medium. The effluxes, all referred to control in bicarbonate-containing solution, are shown as mean ± SEM. The intensity of the 16-Hz amplitude-modulated field stimulus was 0.75 mW/cm^2 unless specified otherwise. n, Number of paired observations used in the statistical analysis. Ionic concentrations are mM.

* $P < 0.05$; ** $P < 0.01$.

† At 0.375 mW/cm^2.

‡ At 2.0 mW/cm^2.

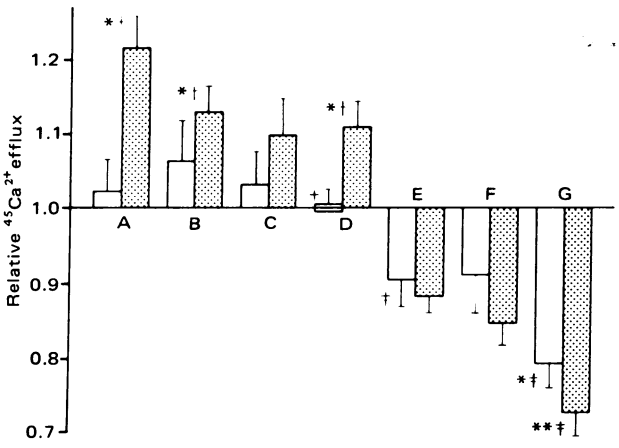

FIG. 2. Relative $^{45}Ca^{2+}$ effluxes (mean ± SEM) referred to the mean value of control efflux in HCO_3^--containing solution (control). Statistical comparisons were made either with control efflux or with efflux in HCO_3^--free solution. Open bars, stimulus off; stippled bars, stimulus on. Conditions: A, 0.1 mM H^+; B, 2.0 mM Ca^{2+} added; C, no Ca^{2+}; D, control; E, no HCO_3^-; F, 0.5 mM La^{3+}; G, 2.0 mM La^{3+}. Stimulus was 450-mHz field (16-Hz amplitude modulation) at 0.75 mW/cm^2. Statistical analysis: *, $P < 0.05$; **, $P < 0.01$; †, compared with control; ‡, compared with no HCO_3^-.

draws attention to the reversal of sign in the field effect that followed omission of HCO_3^- and addition of La^{3+}.

At low concentration (0.5 mM), La^{3+} had no effect on the $^{45}Ca^{2+}$ efflux from nonstimulated tissue bathed in HCO_3^--free solution, but at a higher concentration (2.0 mM) it inhibited the efflux, as expected from results obtained by others (23, 24, 26). Thus, La^{3+} could interfere with the release of intracellular $^{45}Ca^{2+}$ into the extracellular compartment by inhibiting calcium flux across the membrane. This would also preclude field-induced transmembrane calcium currents, and it suggests extracellular sites for field–calcium interactions. Moreover, the field-induced inhibition was of the same order of magnitude (7 and 9%) in both La^{3+} treatments. Also, this response exhibited the same specificity to field intensity as seen previously in tissues in isotonic physiological solution (13, 27), where a stimulated increase occurred only around 0.75 mW/cm^2 but vanished at higher and lower field intensities. Together, these findings support the hypothesis that a limited number of extracellular cationic binding sites are involved in the transaction of weak extracellular electrical events and suggest that the electrosensitive sites in La^{3+} treated samples are in the class of sites responsible for the field response in the control solution. However, it is not clear whether the fields enhanced the La^{3+} blockade and inhibited further the intracellular $^{45}Ca^{2+}$ release or induced sequestration of extracellular $^{45}Ca^{2+}$.

The decrease in $^{45}Ca^{2+}$ efflux that followed omission of HCO_3^- in the solution is unlikely to be due to inhibition of active transport of Ca^{2+} in mitochondria and other cell organelles (30, 31), because the HCO_3^- concentration was only 2.4 mM in the control medium (32, 33). Nor can the decreased efflux be explained by increased calcium entry into the cell, because the effect persisted in the presence of 0.5 mM La^{3+} and was enhanced at a higher concentration of it. On the other hand, inhibition of inward calcium currents in the absence of HCO_3^- could result in decreased transmembrane calcium exchange, followed by a reduction of intracellular $^{45}Ca^{2+}$ efflux (34). However, this model would not explain the absence of a response to an electric field that was effective in La^{3+}-treated tissue with the response probably mediated in the extracellular compartment. It is also possible that the decrease in efflux reflected increased binding of extracellular $^{45}Ca^{2+}$ at sites made available by removal of HCO_3^- from the cell environment.

Decreasing the pH of the bathing solution to 6.8 had no effect on the nonstimulated efflux, but the field-induced release was larger in low pH medium than in control solution, suggesting a role for H^+ binding in these field effects. A model of nerve membrane excitation has been proposed in which competitive binding of H^+ to membrane surface sites displaces Ca^{2+} to adjacent sites and causes a local alkalosis in the counterion layer (35). If the field-induced release of $^{45}Ca^{2+}$ depends on the binding of H^+ to sites previously occupied by field-displaced Ca^{2+}, the apparent unresponsiveness of the HCO_3^--free system could be explained by a decreased concentration of H^+ in the extracellular space. Moreover, the field-induced decrease in efflux in the presence of La^{3+} could result from field-displacement of La^{3+} bound to electrosensitive sites followed by binding of Ca^{2+} instead of H^+ to the vacated sites. La^{3+} has a higher affinity for phosphate and carboxyl groups than does Ca^{2+}, and the reduced size of the field-response in La^{3+}-treated tissue (7–9% of the total $^{45}Ca^{2+}$ efflux instead of 10–15% in control) could reflect the different binding strengths of the two cations. On the other hand, La^{3+} tends to form complexes, and the possibility cannot be ruled out that the field stimulation facilitated the formation of La^{3+}-Ca^{2+} complexes anchored by La^{3+} bound to the membrane (23). Lack of sensitivity of the field-induced response to increased concentration of calcium in the solution, even though added calcium (2.0 mM) triggered a small increase in nonstimulated efflux, paralleled the results seen with La^{3+} and supports the hypothesis that a specific class of negative sites, normally occupied by Ca^{2+}, participates in the transduction of low frequency, weak extracellular fields.

The amplitude range and the extremely low frequency of the modulated fields resemble those of extracellular brain waves, and thus the frequency and amplitude windows in which the isolated tissue responded to field stimulation could reflect intrinsic filtering mechanisms used by the system *in vivo*. H^+ has been shown to compete more strongly than any other biological monovalent cation for calcium binding sites on the membrane (17) and the suggestion that displacement of Ca^{2+} followed by H^+ binding at the vacated sites could transduce weak electrical fields along the membrane (36) finds support in the experimental results discussed here. Thus, it is possible that weak extracellular gradients play a role in the regulation of cell excitability by modulating calcium binding at electroresponsive sites in the membrane.

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