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REVIEW

Radiofrequency Fields and Calcium Movements Into and Out of Cells

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The recent rollout of 5G telecommunications systems has spawned a renewed call to re-examine the possibility of so-called "non-thermal" harmful effects of radiofrequency (RF) radiation. The possibility of calcium being affected by low-level RF has been the subject of research for nearly 50 years and there have been recent suggestions that voltage-gated calcium channels (VGCCs) are "extraordinarily sensitive" to ambient RF fields. This article examines the feasibility of particularly modulated RF coupling to gating mechanisms in VGCCs and also reviews studies from the literature from the last 50 years for consistency of outcome. We conclude that the currents induced by fields at the ICNIRP guideline limits are many orders of magnitude below those needed to affect gating, and there would need to be a biological mechanism for detection and rectification of the extremely-low-frequency (ELF) modulations, which has not been demonstrated. Overall, experimental studies have not validated that RF affects Ca^{2+} transport into or out of cells.

INTRODUCTION

With the advent of newer telecommunications systems such as 5G and WiFi 6, there are continuing concerns regarding the adequacy of current radiofrequency (RF) guidelines (*1, 2*). Here, RF is defined as radiation in the frequency range 100 kHz–300 GHz. Although the effects of tissue heating from high-intensity RF are relatively well understood, the literature concerning possible non-thermal effects (that is, effects which cannot be attributed to tissue heating) is inconclusive, with no clear agreement on the mechanism whereby these could occur (*3*). One target for putative non-thermal effects has been cell calcium, since this ion is responsible for regulation of many cellular functions as a ''second messenger'' (*4*). This review is thus concerned with RF fields below the safety guideline levels, where a negligible temperature rise would be expected. The first article presenting evidence that weak RF fields could specifically influence the distribution of Ca^{2+} in brain cells appeared around 1975 (*5*). Discussion continues to the present day that this ion could represent the key to understanding the possibility of ''non-thermal'' interactions (*2*). This review will first briefly summarize the current state of knowledge concerning the way that Ca^{2+} enters, distributes within and leaves biological cells, emphasizing the main biophysical principles involved and therefore the mechanisms for external electric and magnetic fields by which these processes could be influenced. Experimental studies of the effects of RF on Ca^{2+} movement will then be reviewed.

All cells have mechanisms for controlling entry and distribution of small ions such as Na^+, K^+, Ca^{2+}, Cl^- and H^+; in fact for each of these there are multiple modes of ion transport. The membranes surrounding and within cells are constructed mainly of lipid, which, being oil-like, would normally be impermeable to ions and water. The route of permeation is of two broad types: via water-filled pores or channels, or via specialized protein-based transporter molecular complexes. The former type allows for up to 10^6 ions to pass per channel per s, whereas the second type is several orders of magnitude slower. The second type form the so-called ''ion pumps'' which act to restore the cell to resting levels after the ion channels have been activated to fulfill several functions, such as controlling the release of neurotransmitters or initiating muscle fiber contraction. In addition to Ca^{2+} transport, there are processes of storage within internal membrane-bound structures, such as the endoplasmic reticulum (sarcoplasmic reticulum if within muscle cells) and mitochondria. The various types of Ca^{2+} transport systems are summarized in Fig. 1, which has been adapted from a recent review (*4*). In a typical cell, the value of $[Ca^{2+}]$ in the extracellular fluid is of the order of 1 m*M*, whereas the cytoplasmic value is around 100 n*M*. Values in the endoplasmic reticulum vary, but are typically around 0.4

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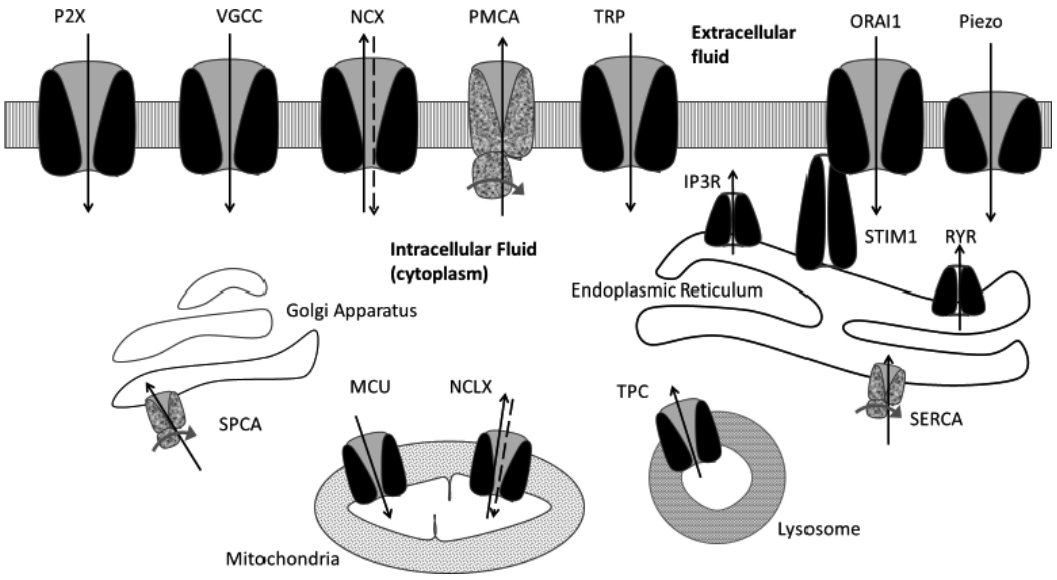

FIG. 1. Schematic diagram of variety of pathways for Ca^{2+} across the plasma membrane and internal membranes [adapted from (*4*)]. Plasma membrane-bound Ca^{2+} channels: voltage-gated calcium channels (VGCC), receptor potential (TRP) channels, ligand-gated ionotropic P2X receptors (P2X), mechanosensitive Piezo channels (Piezo). Plasma membrane-bound channels (store-operated Ca^{2+} entry pathway) mediated by stromal interaction molecule 1 (STIM1) sensor and ORAI1 channels. Internal membrane-bound channels: ryanodine receptor (RYR) and inositol 1,4,5-trisphosphate receptor (IP3R), IP3 ligand produced by the plasma membrane G protein-coupled receptor (GPCR) via Gaq and phospholipase C-β (PLCβ) proteins (not shown). Two-pore channels (TPC) regulate Ca^{2+} release from lysosomes. Mitochondrial Ca^{2+} levels are controlled by mitochondrial calcium uniporter (MCU) complex and mitochondrial Na^{+}/Ca^{2+} exchanger (NCLX). Ca^{2+} pumps (shown stippled and with a curved arrow to indicate the conversion of ATP to ADP and consequent release of energy to drive the Ca^{2+} against an electrochemical gradient): Plasma membrane Ca^{2+}-ATPase (PMCA), sarcoplasmic/endoplasmic reticulum Ca^{2+}-ATPase (SERCA), Golgi network secretory pathway Ca^{2+}/Mn^{2+}-ATPase (SPCA). Full straight arrows indicate Ca^{2+} flux; dashed arrows: Na^{+} flux.

m*M*. Although RF fields can affect ion pumps (including Ca^{2+} pumps) via a subtle low-level thermal process [see for example (*6*)], there has been much speculation on whether the fields themselves can directly interact with charged subunits associated with ion channels. This is the main thesis which has been presented elsewhere (*2*, *7, 8*) and will be a major focus of the current review. However most of the work on RF and Ca^{2+} has been less specific on the actual interaction mechanism. The channels themselves are normally ''gated'', that is, they have a method of controlling the rate of ion flow through the channel. These gates are controlled in a variety of ways: some are controlled by specific molecules such as neurotransmitters or by Ca^{2+} itself (calcium-induced calcium release), whereas others are controlled by the voltage across the membrane, normally of the order of –70 mV, (with the inside negative). The former type is known as ligand-gated and the latter voltage-gated. The normal signal for a gate to open in a voltage-gated channel is for the magnitude of the difference to fall by a certain amount (e.g., ~25 mV). There is often a separate process leading to the gate closing. More precisely, the opening and closing of single channels is a stochastic process, being either fully open or fully shut, and the characteristic rate constants being manifested in the instantaneous current through an ensemble of channels. Before focusing on voltage-gated channels, it is important to note that the permeability of certain channels is particularly sensitive to other factors, such as temperature, pressure and taste (transient receptor potential, or the TRP family of channels). These are mostly non-selective regarding which cations are allowed to permeate.

It is also worth mentioning that in addition to ion channels, some enzymes are voltage-sensitive, for example, voltage-sensitive phosphatase, where transmembrane voltage controls the rate of a reaction which cleaves phosphate ions from phosphomonoesters.

Voltage-gated calcium channels have been researched for over three decades. It is now known that voltage-gated calcium channels (VGCC) form many varieties, the major division being between high- and low-voltage activated channels. Nomenclature varies, but the former includes L-, P- and N-type (for ''long-lasting-'', ''Purkinje-'' and ''neural-'') and the latter T-type (for ''transient''). R-type (''residual'') channels are intermediate-voltage activated. In terms of greatest voltage sensitivity, the T-type of channels are of chief interest. Data reported elsewhere (*9*) show that depolarizations of around 25 mV (from a resting potential of approximately –70 mV) are required to elicit measurable currents through T-type channels, contrasting with 40 mV for R-type channels (and higher still for L-, P- and N-type). For maximal currents the depolarizations are 60 and 95 mV, respectively.

The nature and characteristics of VGCC are well known (*10*). Ca^{2+} channels are similar to Na^+ channels in that they are activated quickly, then inactivate spontaneously if they are clamped at inside-positive potentials, although the inactivation times are appreciably slower than those for Na^+ channels. Unlike the Na^+/K^+ currents associated with nerve action potentials, the influx of Ca^{2+} associated with activation of VGCCs is linked with the initiation of chemical processes, such as secretion of neurotransmitters, as well as the contraction of muscles and the opening of other gates (self-catalysis). Multiple types of VGCCs coexist in single cells and the range of other kinds of Ca^{2+}-channels are also present in the same cell, as shown in Fig. 1.

The structure of the various types of T-type VGCC has been well described, and a summary diagram is provided elsewhere (*11*). The α1 subunit (which is the main, hollow cylindrical part of the channel) exists in several varieties: Cav3.1 (CACNA1G); Cav3.2 (CACNA1H) and Cav3.3 (CACNA1I). The gene name linked to the particular variety is indicated in parentheses. T-type channel blockers are used to treat epilepsy, since lowered Ca^{2+} conductance leads to lower excitability. Other VGCC types are blocked by different specific agents [see (*12*) for further details].

This review will concentrate on two questions: 1. Is it feasible to postulate that VGCCs represent a cellular subunit with particular susceptibility to external EMFs?; 2. Does the literature on effects of EMFs on Ca^{2+} show a consistent picture in relationship to outcomes?

RESULTS AND DISCUSSION

Theoretical Considerations Affecting the Susceptibility of VGCCs to External Electric Fields

In examining whether there are grounds for suspecting that VGCCs may be susceptible to RF fields there are essentially two aspects: coupling of RF to cells and the demodulation of extremely low frequency (ELF: defined as the range 1 Hz–100 kHz) modulations from the RF carrier. With respect to the first, the cell membrane can be modeled as a parallel capacitor-resistor combination, with 10^{-2} F/m^2 and 10^{-2} $\Omega.m^2$ values, respectively (*13*). Displacement and conduction currents are approximately equal at 1.6 kHz (loss tangent $1/(\omega RC) = 1$), but since the majority of induced current is conduction current via intercellular pathways, the conduction current in bulk tissue tends to predominate up to 0.1 GHz (*13*). At 5 GHz the displacement current is several orders of magnitude greater than conduction current, but because of the dielectric properties of water, the conduction and displacement currents are about the same in the range 10–100 GHz. However, the induced current through channels is infinitesimally small and continues to fall as frequency rises. The occupational basic restriction on specific absorption rate (SAR) is 0.4 W/kg (*3*), from which the internal electric field can be deduced using the equation $E = \sqrt{(\rho.SAR/\sigma}$. Tissue conductivity ranges from 4–50 S/m for muscle and gray matter over the frequency range 5–50 GHz (http://niremf.ifac.cnr.it/tissprop/). The value corresponding to 5 GHz is thus 10 V/m. Because of the membrane capacitance short-circuiting the resistance (*13*), the field appearing across the cell membrane will be essentially the same as this average field. The actual voltage drop across an individual membrane is thus of the order of 100 nV, given that the membrane thickness is approximately 10^{-8} m. This is several orders of magnitude below the 25 mV (dc) mentioned previously for channel activation and this voltage is also alternating. With respect to alternating currents, the charge displacement required to activate the channel would only occur at frequencies up to a few kHz (*15*), which is several orders of magnitude below the frequencies considered here.

Regarding the second aspect (ELF demodulation), since most low-level RF studies claiming to show effects have used some form of modulation (see Table 1), this implies that some non-linear elements within tissue are able to detect and rectify the ELF signals. While it is true that certain ion channels show rectification properties (*10*), this is in relationship to currents in the ELF range. It is unclear whether these properties would affect RF currents in the GHz range. There have been a number of attempts at demonstrating rectification properties at frequencies relevant to telecommunications, but these have failed (*14*). The most elaborate experiment involved a very elegant exposure system (*15*) which attempted to detect signals at twice the frequency of the carrier, which would occur if there were a ''square law'' type response (*16*). These issues have been extensively reviewed (*17, 18*).

A Review of the Literature Concerning the Possible Influence on RF Fields on Ca^{2+} Levels

As mentioned at the outset, there has been longstanding concern over possible effects of RF on Ca^{2+} levels in cells, since there are clear links between disruption of cell Ca^{2+} signaling processes and disease, particularly cancer (*4*).

Studies were initially found by searching the EMF-Portal database (https://www.emf-portal.org/en) using the search term ''calcium channel'' and limiting studies to RF fields using EMF-portal search settings. In this search 38 articles were identified, of which 18 were studies on the effects of RF on Ca^{2+} levels. We further searched the reference lists of major reviews published by health authorities on RF and health (*19–22*) which identified an additional 11 studies. Lastly, we searched the reference list of all the included studies and identified one additional study. Only studies with full articles available in English were included. We have identified 30 papers using these criteria.

In each study the fractional change in a parameter related to cell Ca^{2+} was estimated (usually the difference between exposed (E) and sham (S), divided by sham value) (Table 1). Wherever possible, the Glass effect size (ES) as the

TABLE 1
Summary of Literature Relating to RF Ca^{2+} Effects

Literature	Cell type/animal exposed	Frequency	Exposure/ power level	Modulation	Result	Notes	Fractional change
(*25*)	Cats immobilized under local anesthesia	450 MHz	30 W/m^2	16 Hz AM	Field exposure increased $^{45}Ca^{2+}$ efflux	Plastic cylinder inserted against cortex: analysis of bathing solution for ^{45}Ca from preloaded tissue. Penetration depth estimated at 3 mm. No direct evidence of Ca channel involvement, because increased loss from tissue could be due to a variety of effects.	–0.9
(*26*)	Gallus domesticus cerebral cortex tissue slices and hemispheres	147 MHz	7.5 W/m^2	16 Hz AM	There was no significant effect on Ca^{2+} efflux from exposure	No effect despite similar conditions to Bawin *et al*. and Blackman *et al*. Used paired cerebral hemispheres.	0
(*27*)	Mice	800 MHz	Cell phone antenna	Presume GSM	Carcinogenesis may be induced earlier due to changes in calcium homeostasis	Not clear if phone was actually transmitting. Report higher levels of ^{45}Ca in organs in RF ''exposed'', which is opposite to Adey, Blackman.	+0.11
(*28*)	Isolated chicken cerebral tissue	450 MHz	7.5 W/m^2, 3.75 and 20 W/m^2	16 Hz AM	RF fields modulated at brain wave frequencies increased Ca^{2+} efflux	^{45}Ca levels in bathing solution enhanced by around 20% if added H+ ions, added HCO_3– produces inhibition (i.e., less efflux). Interpreted as effect on Ca^{2+}/H^+ binding sites rather than ion channels.	–0.2
(*5*)	Chick forebrain	I47 MHz	10 W/m^2	Various ELF: 16 Hz gave strongest effect	Reported increased calcium efflux from VHF exposed brains	^{45}Ca levels in bathing solution enhanced by around 14%: unaffected by HCN, which is a powerful metabolic inhibitor. No direct evidence for specific Ca channel involvement.	–0.14
(*29*)	Chick forebrain	147 MHz	8.3 W/m^2	16 Hz AM	147-MH exposure can affect calcium-ion efflux from brain tissue	^{45}Ca levels in bathing solution enhanced by around 18%, but this could have more to do with reduced efflux in shams (compared to controls).	–0.18
(*30*)	Chick brain tissue	50 MHz	15.6–23 W/m^2	50 Hz AM, also ELF alone	Enhanced calcium efflux at specific exposures	At RF, ^{45}Ca levels in bathing solution enhanced by around 20% (as previous). ELF effects are enhanced efflux (which differs from Adey findings).	–0.2
(*31*)	Chick brain tissue	50 MHz	7.2, 21.7 and 43.2 W/m^2	16 Hz AM	Efflux of calcium ions from *in vitro* brain tissue is affected by modulated RF radiation	^{45}Ca levels in bathing solution enhanced by around 20%, but this could have more to do with reduced efflux in shams (compared to controls). N.B. some shams show 15% enhancement relative to controls.	–0.2
(*32*)	Human leukemic T cells, Jurkat cells	915 MH	2 W/kg	GSM: 217 Hz PM	RF not associated with any changes in calcium levels or calcium signaling	No effects overall, but sign change in Ca^{++} frequency spectra. The form of Ca^{++} signaling studied relates to CICR in internal membranes.	0
(*33*)	Avian and feline brain tissues, human neuroblastoma cells	148 MHz	0.0005–0.1 W/kg	13–16 and 57.5–60 Hz	RF and ELF was reported to enhance calcium efflux	Results have high degree of scatter.	0
(*34*)	Human neuroblastoma cells	915 MHz	0.00–5 W/kg	16 Hz AM, other ELF	Human neuroblastoma cells are sensitive to certain narrow ranges of SAR with increased Ca^{2+} efflux	50% change in efflux at 16 Hz claimed.	–0.5
(*35*)	Cultured kidney cells	42.25 GHz	1 W/m^2	Not mentioned	Change in Ca^{2+} activation	Interpreted as a disruption of Ca^{++} cooperativity with decrease in affinity for Ca^{2+} sites. Hill plots. N.B. different effects depending on degree of initial affinity between Ca^{2+} and site.	Ranges from –3 to +2.5
(*36*)	Male Wistar albino	2.45 GHz	0.12 W/m^2	GSM: 217 Hz PM	Wifi exposure induced Ca^{2+} influx via the TRPV1 channel	TRPV1 channel Ca^{++} entry enhanced.	+0.4
(*37*)	Cultured rat cerebellar granule cells	380.8875 MHz	5, 10, 20, 50 or 400 mW/kg	TETRA modulation (17.6 Hz PM)	No evidence of any consistent or biologically relevant effect of TETRA fields on Ca^{2+}	Careful consideration given to possible sources of artefact.	0

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TABLE 1
Continued.

Literature	Cell type/animal exposed	Frequency	Exposure/ power level	Modulation	Result	Notes	Fractional change
(*38*)	Mice carrying human hepatocellular carcinoma xenografts	27.12 MHz	SAR 30 and 400 mW/kg	Selected freq. in range 0.5–22 kHz AM	RF antiproliferative effects mediated by calcium influx	Ca^{2+} influx through Cav3·2 T-type voltage-gated calcium channels resulting in increased Ca^{++} entry.	+0.5
(*39*)	Synaptosomes prepared from the cerebri of male SD[a] rats	450 MHz	5 W/m^2 and 43 V/m	16 or 60 Hz AM	RF-induced change in Ca^{2+} efflux probably did not involve intracellular calcium	Ca^{++} efflux from synaptosomes increased 45%, but they argue this did not arise from intracellular stores. (?) Release from membrane surface receptors.	–0.45
(*40*)	Rat dorsal root ganglion cells	900 MHz	1 W/kg	CW	Change in neuron firing rate after RF magnetic field exposure	No effects at RF, but single-channels currents measured using patch clamp, expected to yield direct evidence. However, 50 Hz magnetic fields produced significant changes.	0
(*41*)	Mice	835 MHz	1.6 W/kg and 4. W/kg	Not stated.	Changes in cellular levels of calcium flux	Calbindin and calretinin (a Ca^{2+}-binding protein) expression. Complete loss of pyramidal cells in CA1. No evidence of channel involvement.	Results variable
(*42*)	Rat brain tissue	2.45 GHz	0.12 to 2.4 W/kg	16 Hz pulse modulated: 20 ms duration	Reported no significant differences in efflux of Ca^{2+}	Brains preloaded with ^{45}Ca, carefully done.	0
(*43*)	Male Wistar albino rats	2.45GHz	1.0 mW/m^2	GSM: 217 Hz PM	Study reported that RF increased Ca^{2+} influx	TRPM2 channel involvement by patch clamp experiments. Also EEG and $[Ca]_i$ via Fura2/AM. Ca^{2+} released from stores by RF or influx increased.	+5
(*44*)	Human leukemia cells	2.45 GHz	0.1 W/kg	GSM: 217 Hz PM	RF induces proliferative effects via oxidative stress	$[Ca]_i$ via Fura2/AM: also cell numbers. Ca^{2+} released from stores by RF, although authors interpret it as increased influx.	+4
(*45*)	Human endothelial cells, PC-12 neuroblastoma and primary hippocampal neurons	900 MHz GSM	0.012–2 W/Kg	GSM: 217 Hz PM & CW	No changes in Ca^{2+} signaling	Careful measurements of $[Ca]_i$ using Fura2/AM. Also thapsigargin as positive control, etc.	0
(*46*)	Rat cortical neurons	900 MHz	2 W/kg	GSM: 217 Hz PM and CW	RF does not alter the current amplitude or the current-voltage relationship	VGCCs are target of study. Ba^{++} used to give specificity in patch-clamp experiments. Expected to yield direct evidence.	0
(*47*)	Stem cell–derived neuronal cells	0.7–1.1 GHz	0.5–5 W/kg	Not stated	Increase in Ca^{2+} spikes when exposed to RF	Used Fluo4 AM and measured rate of Ca^{++} spiking. Get doubling of rate at 800 MHz. Used nifedipine and conotoxin to block L- and N-type channels. Implies involvement of N-type channels.	+2
(*48*)	Chicks	450 MHz	10 or 50 W/m^2	16, 3 Hz AM	Possible differences in the temporal distributions of motor activity as a function of the exposure	Behavioral study, so no direct link to VGCCs. Changes not significant.	0

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TABLE 1
Continued.

Literature	Cell type/animal exposed	Frequency	Exposure/ power level	Modulation	Result	Notes	Fractional change
(*49*)	Toad hearts	240 MHz	SAR from 0.15 to 3 mW/kg	16, 0.5 Hz AM	Some alteration in efflux	Some SARs gave increased efflux from preloaded heart tissue.	–0.2
(*50*)	Human breast carcinoma cell lines and SCID mice	27.12 MHz	30 and 400 mW/kg	Selected freq. in range 0.5–22 kHz AM	Tumor inhibitory effects	Similar to Jimenez above. Both *in vivo* and *in vitro* components to study. Abstract refers to VGCCs ''acting as the cellular antenna''.	+0.5
(*51*)	Rat brain tissue	1 GHz	5–150 W/m^2	16, 32 Hz PM	No alteration in the efflux of Ca^{2+} in the rat brain	Brains preloaded with ^{45}Ca. See Merritt *et al*. above.	0
(*52*)	Isolated ventricular cardiac myocyte	900 to 1,800 MHz	1 mW/kg	16, 50 and 30 kHz, also GSM 217 Hz PM	No significant changes in cellular Ca^{2+}	Used Fura2. Only one significant change but not regarded as relevant to possible bio-effects.	0
(*53*)	Rat brain tissue	900 MHz GSM	0.023 ± 0.001 mW/kg	GSM: 217 Hz PM	No effect with RF	Epileptogenic agent did alter Ca^{2+} in hippocampus: implies that TRPV1 channels not involved.	0

[a] SD = Sprague-Dawley.

difference between the means of the exposed and sham groups divided by the standard deviation of the sham group was also computed (*23*) (Figs. 3–6).

In published studies where several frequencies or intensities were trialed, the largest value of ES was selected. In studies where values were displayed as mean ± SE, the SD was estimated from the reported number of observation for the sham value (SD = SE.$\sqrt{}$n). The sign of the ES value indicates whether the reported effect would tend to increase or decrease cell [Ca^{2+}] or alternatively increases or decreases in the amount of Ca^{2+} associated with the cell, if adsorption to glycocalyx is taken into consideration.

Table 1 summarizes the Ca^{2+}-related effects reported (0 represents not significant), along with the frequencies investigated and the modulation scheme used, if any. Wherever possible, the reported exposure, either as a power density (PD: W/m^2) or SAR (W/kg) is recorded. The ES as a function of PD or SAR is presented in Figs. 3 and 4, respectively (no study reported both PD and SAR). Following the methodology of (*24*), a quality score (QS) for each of the studies was assigned on the basis that adequate attention had been given to the following aspects: blinding, dosimetry, use of positive controls and use of shams, with 1 for yes, 0 for no and 0.5 if partial attention had been given. Figure 5 shows ES plotted against QS for 26 of the 30 studies for which estimates of ES could be made. Two studies were excluded as outliers (ES of 60 and 92, respectively). The reason for exclusion is discussed further below. Analyses were also performed on carrier frequency and type of modulation (see Table 1) versus ES (results not shown).

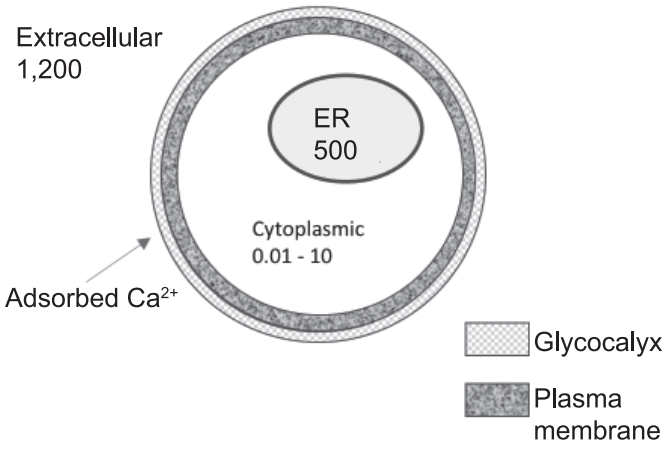

FIG. 2. Schematic diagram showing relative [Ca^{2+}] values (in μ*M*) within and surrounding typical cells, including internal stores. ER = endoplasmic reticulum.

Over the 45 years of publications of ''calcium effects'' there have been considerable changes in experimental techniques. This is reflected in Fig. 6, which is a plot of ES versus year of publication.

Are External EMFs Sufficient to Activate VGCCs?

Charge transfer for activation. Voltage gating involves the movement of protein subunits associated with the channel under the influence of imposed voltages across the ends of the channel. This movement constitutes a momentary current, which can be measured experimentally (and has been since the 1970s) (*10*). Gating phenomena for Ca^{2+} channels are essentially very similar to those of Na^+ and K^+ channels (*54*). Typically, these gating currents are of the order of 10 μA/cm^2 (0.1 A/m^2) of membrane area, with a characteristic time constant of 0.4 ms (*54*). The essential consideration here is whether these gating currents can be elicited by currents induced in tissue by external fields.

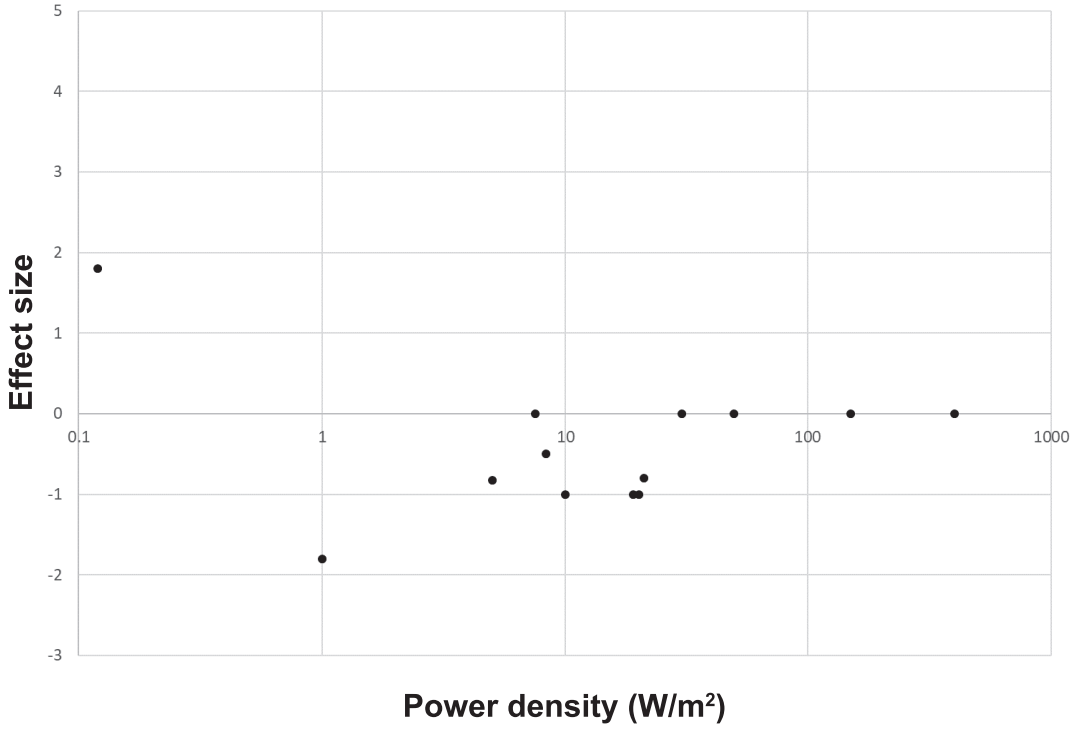

FIG. 3. Effect size (ES) expressed as the difference between the means of the exposed and sham groups divided by the standard deviation of the sham group $[(SD_E - SD_S)/SD_S]$ as a function of power density, plotted on a logarithmic axis to clearly separate low values. n = 13 studies; an outlying value of ES 60 with a PD of 0.001 W/m² was omitted.

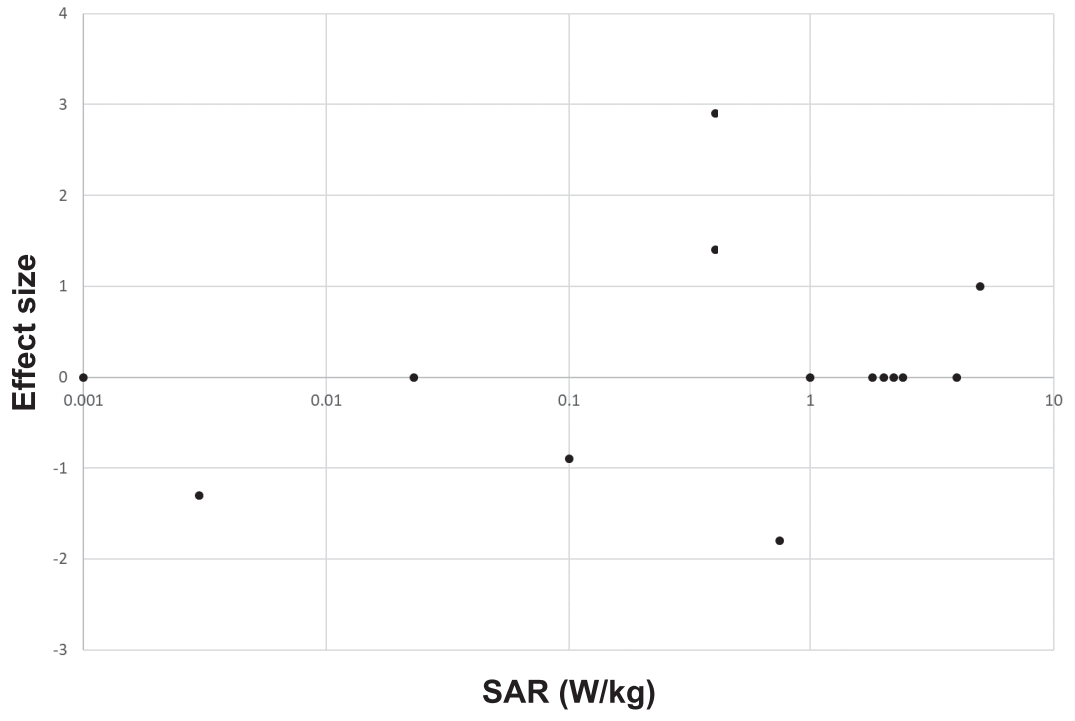

FIG. 4. Effect size (ES), as in Fig. 3, but here exposure is expressed in specific absorption rate (SAR) rather than power density. n = 14 studies; an outlying value of ES 98 at a SAR of 0.1 W/kg was omitted.

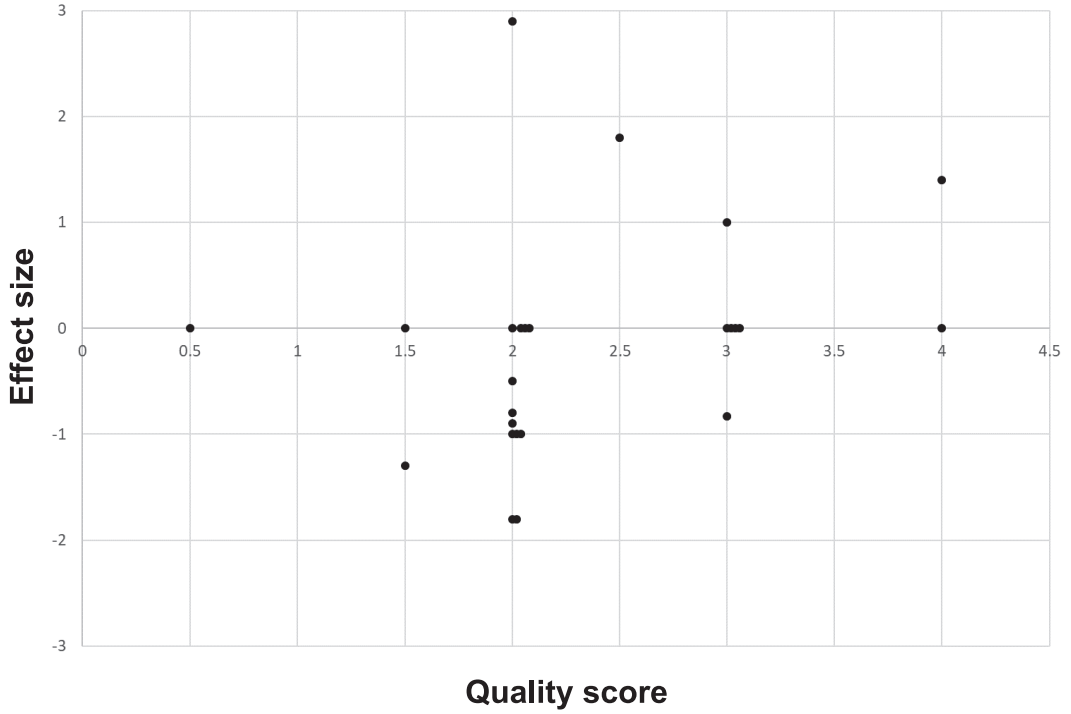

FIG. 5. Effect size (ES) (as in Figs. 3 and 4) versus ''quality'' score, as used in (*24*). Points with identical values are offset slightly to enable visibility. There is no trend to clearer outcomes as quality increases. n = 28 studies; two outliers with ES of 60 and 92 not shown in this graph.

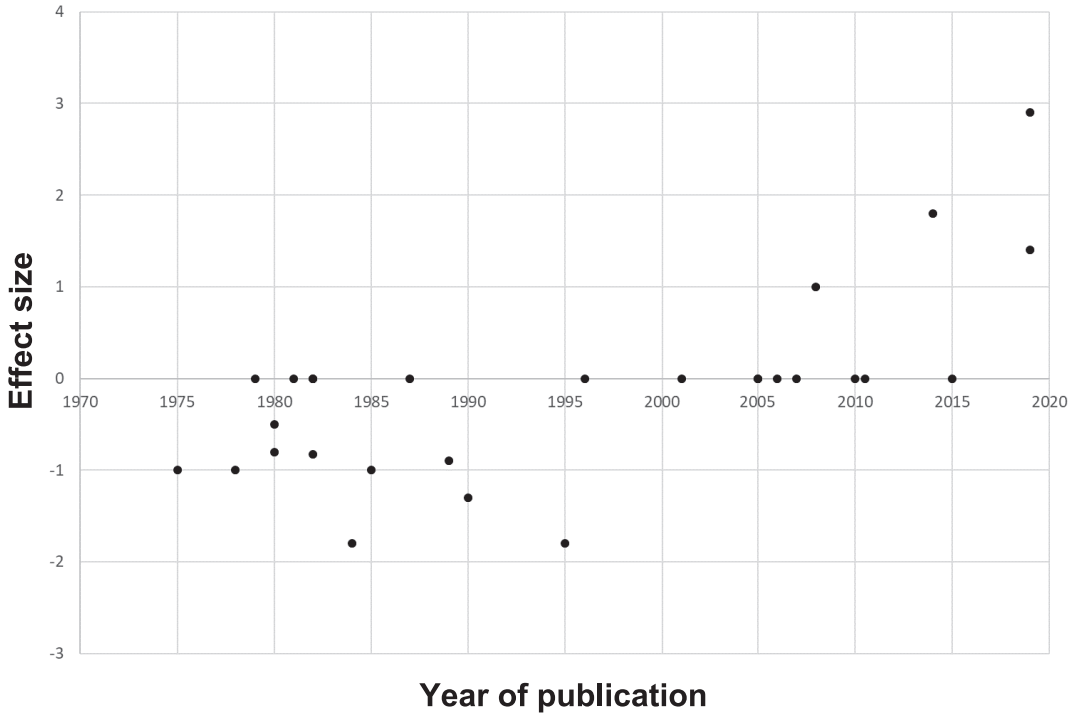

FIG. 6. Effect size (ES) (as in previous figs.) versus year of publication. More recent studies are exclusively reporting effects leading to increases in cell $[Ca^{2+}]$. n = 28 studies; two outliers with ES of 60 and 92 not shown in this graph.

One of the most direct methods of investigating the properties of ion channels, including VGCCs, is the ''patch clamp'' (*10*), which is used to measure the conductance of single-ion channels (which are of the order of 10 pS) and observe the opening and shutting of the voltage-controlled gates in real time. Typically, a voltage step is applied to the patch of membrane (around 10 μm^2 in area) and then the resulting current measured. The early phase of the current represents the gate movement and the later phase the flow of particular ions through the channel (in this case Ca^{2+}). The chances of the channel being open increase as the voltage across the channel decreases, in a deterministic manner. The voltage at which T-type VGCCs are most likely to be open is around 0 mV, that is, when the resting membrane potential of –50 to –70 mV is reduced by 60 mV. (the field across the 10^{-8}-m-thick membrane reduced by 6 MV/m). Of the three studies reviewed employing patch clamping, two (*40, 46*) showed no evidence of effects of RF on Ca^{2+} fluxes and another (*43*) measured currents in TRPM2 channels, which are not voltage-gated. As just mentioned, unless electric fields can be applied directly across cell membranes (which normally involves impaling the cell with microelectrodes or the removal of membrane patches using micropipettes with suction), the fields required to cause activation are relatively large. If the field is applied in air, without direct contact with the tissue (as in the case of the sciatic nerve), the field required is far greater [see (*55*)]. Since many commentators have assumed, in emphasizing the importance of ELF modulations in the possibility of low-level RF being biologically active [see (*1*)], it is instructive to consider whether, even if demodulation were to occur, the ELF components would be sufficient to cause VGCC activation. Essentially, electric fields within tissue are attenuated by a factor of 10^5–10^6 (at ELF), thus, to stimulate nerves within tissue using external fields in the surrounding air, fields of the order of 100 MV/m are required. Whether or not the field will cause effective stimulation also depends on the time-course of the way the fields are applied, usually in the form of a square wave in the case of the sciatic nerve of amphibians, with the shape of the leading edge of the square wave a most important factor. The question of the magnitude of external fields leading to effective stimulation is discussed in many standard textbooks (*56, 57*). In terms of RF, the question of detection of ELF modulations has already been discussed, but in terms of Fourier components, the ELF amplitudes would be less than the carrier, which for the International Commission for Non-Ionizing Radiation Protection (ICNIRP) general public reference level for 6–300 GHz is 87 V/m (in air), several orders of magnitude short of the fields just mentioned.

Molecular dynamics simulations of ion channels in lipid bilayers have confirmed that there is no basis for the claim that Ca^{2+} channels are in some way extremely sensitive to external fields. Recently reported simulations of Ca^{2+} in particular (*58*) have shown that reducing the potential by 40 mV leads to the expected conformational changes allowing Ca^{2+} permeation. E-fields across the actual membrane of the order of 120–650 MV/m (as 10 ns pulses) are required (*59*) to open channels (in this case, aquaporin channels, but Ca^{2+} channels would behave similarly).

Are VGCCs unique?. The information given above is not specific to Ca^{2+} channels; many ion channels consist of α-helical subunits (usually four) with many charges within the pores formed by these subunits. Ions within the pores ''hop'' from fixed charge to fixed charge, accompanied by varying amounts of ''water of hydration''. The mechanism for K^+ translocation through specific ion channels was elucidated by the MacKinnon group (*60*), and the translocation of Ca^{2+} has been shown to be similar. As mentioned above, the structure of Ca^{2+} channels is known in surprising detail, mainly from X-ray diffraction and electron microscope studies, (*61, 62*), the latter reference giving a depth of information on the electrophysiological data on activation and inactivation.

There are two main parts to the Ca^{2+} channel: the selectivity filter and the voltage-sensitive gate. The selectivity filter confers a 400:1 selectivity to Ca^{2+} ions over Na^+, despite the latter being 70X more abundant in the extracellular environment. The protein which forms this selective filter is almost identical to that in the Na^+ channel, but with subtle changes in amino acid sequence that attracts Ca^{2+} to the outside surface of the pore. There are then three Ca^{2+} binding sites within the pore, with individual hydrated Ca^{2+} ions shuttling between occupying sites 1 and 3 or just site 2. The channel is composed of protein subunits and the charges are most likely to be within the aqueous channel itself (rather than embedded in lipid); this is certainly the location for the ''selectivity filter'' described above. The gate itself is associated with the membrane-spanning so-called S4 segment, which contains several positive charges. However, this is not unique to Ca^{2+} channels; much of the same information applies to voltage-gated potassium, proton and sodium channels. It appears that VGCCs are no more susceptible to external EMFs than other voltage-gated channels. The ''gating'' in question results from a change in shape (''conformation'') when a specific change in transmembrane potential occurs (in the case of T-type channels this is around 25 mV, as has already been mentioned). Catterall *et al.* (*61, 62*) have suggested a sliding helix mechanism, which although explaining many phenomena is still not without difficulties (*63*). This conformational change certainly takes place in a high-field environment as already mentioned, so the addition of external fields several orders of magnitude weaker are not expected to affect this process in any significant way. Hille (*10*) reviews putative mechanisms for voltage sensing, but more recent research on the structure of Ca^{2+} channels implies that the voltage sensor may consist of several interacting subunits within the channel protein (*64*). Recently published work has used computational structural dynamics (*65*) and the study of mutations (*66*) to further

elucidate the most likely mechanisms for voltage sensitivity. The movement of positively charged amino acids, such as arginine and lysine, appears to be central to voltage sensitivity. There is no suggestion of Ca^{2+} channels being especially sensitive in relationship to voltage sensors in other ion channels or in relationship to other biological functions. Indeed, certain organisms, such as fish (*67*) and monotremes (*68*), do have ''extraordinary sensitivity'' to environmental dc and ELF electric fields, which these animals use in the detection of prey. This appears to be achieved via the specific anatomical focusing, modulation and integration over many hundreds of neurons in specialized structures, with subsynaptic membranes ''well-poised'' to amplify small variations (*67*) and thus not a general property of membranes or channels. Furthermore, the work referred to above on the change in Ca^{2+} currents due to 1 and 10 Hz electric fields use 1-kV/m fields *in the media*, which would correspond to fields several orders of magnitude higher if applied to the air surrounding the biological samples. Therefore, claims that VGCCs in some way represent an amplification of forces by several orders of magnitude (*1*) appear not to be borne out by evidence.

Additional considerations. As we have already seen, there is considerable attenuation between the external fields and the fields induced in bulk tissue. In addition, the proportion of this field which adds to or subtracts from the transmembrane potential is highly dependent on cell architecture. As Reilly has pointed out, excitable cells become influenced by fields in the surrounding tissue fluid only at the ends of long cells or where they bend to be at an angle to the impressed field (*56*). This again makes the likelihood of ensembles of cells being affected by external fields at the ICNIRP limits unlikely.

Effects Reported: Cell Calcium Levels

Figure 2 summarizes the relative concentrations of Ca^{2+} in the various parts of the cell and surrounding media. The state of calcium is of two types: ionized and sequestered. For example, in the extracellular fluid approximately 50% is ionized. The cell glycocalyx has numerous positive charges with adsorbed Ca^{2+}, which is in addition to the approximately 1 m*M* in the extracellular fluid. Thus, any changes in the dynamics of the ratio of ionized to sequestered could lead to an apparent loss of Ca^{2+} from the cell. Normally, the only route for Ca^{2+} loss would be via stimulation of Ca^{2+} pumps or gross damage to the cell membrane, as occurs in cell death. An early report suggests that pH is a major determinant on the amount of Ca^{2+} adsorbed onto phospholipid bilayers (*69*). However, there is no clear link between RF exposure and pH changes.

Taking the sign of the ES into consideration, the number of reports of increases in cell Ca^{2+} is approximately the same as the number of decreases (and the number of studies reporting no effect). The average ES value (leaving out the two outliers, which will be discussed below) is -0.15 ± 0.17 (SE). In terms of fractional change, the average value is $+0.22 \pm 0.25$.

As shown in the final two right-side columns in Table 1, the outcomes are mixed. In fact, very few of the studies were of Ca^{2+} channels *per se*; some used channel blockers to implicate specific types of VGCC, but the evidence was not direct. Those studies in which patch clamp techniques were used to directly gauge effects on VGCCs did not show significant effects due to RF, as has been discussed above. The list was compiled using certain keywords. While there may be other relevant studies, especially multiple studies from the same laboratory, which have been omitted, these omissions are unlikely to change the overall conclusions.

The estimation of ''exposure'' is to be used with caution: often it is not clear from descriptions on how PD has been estimated. Indeed, some estimates may be from quoted output power divided by estimates of aperture, without adjustment for reflections, non-uniformity or absorption by overlying media. The variable nature of the way the biological system was placed in media also makes PD a poor indicator of the actual PD at the cell membrane level. Nevertheless, an increase of ES with PD would be expected and is not observed. In Fig. 4, there are several large ES values, both for Ca^{2+} increases and decreases, but no obvious trend. Again, the SAR values used are those presented in the studies, with the possibility that erroneous assumptions were made. In some cases, SAR estimates were the result of detailed modeling, but in other instances the detailed derivation of SAR was not presented. In both Figs 3 and 4 the highest exposure estimates tend to be associated with lack of effects, with one or two exceptions. There is no consistent evidence of PD or SAR ''windows''. The two outliers referred to in the caption, with ES values of 60 and 92, report SD values (for Ca^{2+} release) of approximately 1% of the control. These are an order of magnitude lower than those in comparable studies, and thus the possibility of computational error exists.

Figure 5 indicates that there are studies with good QS values with non-zero ES. However, the bulk of studies with QS ≥ 3 are for no effect. Similarly, the scoring of ''quality'' is not without considerable error. Only 4/32 (13%) of studies were performed blind and 11/32 (34%) used positive controls. The assessment of ''exposure'' was adjusted up and down by 0.5 points for greater or lesser attempts at assessing actual SAR within the target tissue. The more recent studies have tended to pay more attention to detailed exposure assessment, including, in some cases, monitoring of temperature changes.

Analyses of ES versus carrier frequency and type of modulation (or lack of modulation) did not reveal any significant relationship. This further weakens arguments that the membrane could act as a detector and rectifier of low frequency modulations.

From Fig. 6, it is apparent that the more recent studies, using techniques concentrating on measuring cell [Ca^{2+}] (via fluorescent dyes) or channel currents directly (using

patch clamping) report either increases or no effects, whereas the earlier studies, using radioactive tracers, report mostly decreases. Possible sources of artefact in the latter method were discussed near the time the earlier studies were published, including the possible non-viability of the brain tissue and the variability in the sham measurements (*70*). However, it should be acknowledged that there are potential artefacts with the other methods as well. For example, the fluorescent dye method is susceptible to bleaching effects if a non-ratiometric dye is used. The more recent methods are likely to give a much clearer indication of changes in cell Ca^{2+}, but potential artefacts of the three methods are unlikely to explain the apparent change in direction of Ca^{2+} movement reported. In fact, earlier studies concluding increased efflux are perhaps using a misleading nomenclature, since, as mentioned, the percentage of Ca^{2+} associated with surface proteins or interstitial fluid movements could equally well account for the changes reported. Nevertheless, the more recent methods are more specific in measuring Ca^{2+} cellular movement and should be afforded greater weight. Small metabolic changes due to weak local ''hot spots'' in RF absorption could also stimulate Ca^{2+} transport, which is outward-directed (Fig. 1). On the other hand, VGCCs allow Ca^{2+} into cells if activated. However, since all of the studies reporting SAR values are below 5 W/kg, with many well below this value, thermal effects are expected to be minimal, which would make local RF ''hot spots'' unlikely as an explanation for significant effects.

CONCLUSIONS

Despite nearly 50 years of research into possible effects of RF on cellular calcium levels, results continue to be mixed and a mechanism for action, if the effect is real, elusive. Those experiments reporting changes in cell Ca^{2+} are roughly equally divided between those that can be interpreted as a loss to cytoplasmic Ca^{2+} and those as an increase. The greatest proportion (40%) report no changes at all. Furthermore, the majority of the studies with higher quality score did not report an effect. Those experiments targeting VGCC by direct measurement of cell Ca^{2+} currents do not show significant RF effects. Since GHz RF fields alternate too quickly for there to be alterations in ion flow, it is unclear how dc gating currents could be affected, with no evidence for demodulation occurring in biological membranes. The change in field across the membrane required to transition a gate from the ''closed'' to ''open'' condition is of the order of several MV/m, which is considerably higher than the modest fields induced across membranes by external EMF at the respective ELF or RF reference levels [20 kV/m and 87 V/m, respectively (*3, 71*)]. There appears to be no basis for the claim that VGCCs are extraordinarily sensitive to environmental RF-EMF. In addition, there appears to be no consistency in the relationship between ES and either PD or SAR. What is apparent is that as methods for estimating Ca^{2+} have become sophisticated, the direction of reported significant changes has moved from cytoplasmic (or membrane) loss to cytoplasmic gain. As methods for estimating subcellular $[Ca^{2+}]$, pH and temperature improve, it may be prudent to revisit the ''calcium effect'', particularly patch-clamping experiments targeting VGCCs, to endeavor to uncover reasons for disparity in outcomes. Future experiments should give even greater attention to aspects of improving ''quality'', specifically, the use of blinding, positive controls and dosimetry estimations, including modeling. It is extremely important to eliminate artefact, in particular, the possibility of localized heating.

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