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Effects of radiofrequency fields on RAS and ERK kinases activity in live cells using the Bioluminescence Resonance Energy Transfer technique

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Running Title: Radiofrequency effect on ERK and RAS kinases

Abstract—

Purpose: The present study was conducted to re-evaluate the effect of low-level 1800 MHz RF signals (up to public exposure level for local exposure) on RAS/MAPK activation in live cells.

Material and methods: Using molecular probes based on the Bioluminescence Resonance Energy Transfer technique (BRET), we assessed the effect of Continuous wave (CW) and Global System for Mobile (GSM)-modulated 1800 MHz signals (up to 2 W/kg) on ERK and RAS kinases' activity in live HuH7 cells.

Results: We found that radiofrequency field (RF) exposure for 24h altered neither basal level of RAS and ERK activation nor the potency of phorbol-12-myristate-13-acetate (PMA) to activate RAS and ERK kinases, whatever the Specific Absorption Rate (SAR) or signal used. However, we found that exposure to GSM-modulated 1800 MHz signals at 2 W/kg decreased the PMA maximal efficacy to activate both RAS and ERK kinases' activity. Exposure with CW 1800 MHz signal at 2 W/kg only decreased maximal efficacy of PMA to activate ERK but not RAS. No effects of RF exposure at 0.5 W/kg was observed on maximal efficacy of PMA to activate either RAS or ERK whatever the signal used.

Conclusion: Our results indicate that RF exposure decreases the efficiency of the cascade of events, which, from the binding of PMA to its receptor(s), leads to the activation of RAS and ERK kinases. This effect of RF exposure is reminiscent of RF-induced adaptive response.

Keywords*— radiofrequency fields, BRET, kinases, phorbol ester, adaptive response*

1. INTRODUCTION

Over the last decades, the emergence of new technologies based on non-ionizing electromagnetic fields in the radiofrequency (RF) range has occurred in many different areas of our daily life, and RF exposure has become ubiquitous. This has triggered societal concerns regarding potential health effects. While the energy per RF photon is not strong enough to induce direct chemical changes in the biological targets, the question remains as to whether RF fields could trigger cellular stress responses (IARC 2013).

One of the major mechanisms that trigger cellular responses to extracellular stimuli is the activation of Ser/Thr mitogen-activated protein-kinase (MAPK) cascades that relays extracellular signals to intracellular processes following activation of RAS (Kim & Choi. 2015; Nussinov et al. 2018). The RAS/MAPK signalling pathway is playing a pivotal role in the regulation of various cellular processes such as gene expression, cellular growth, and survival. Within these signalling pathways, RAS activates the three MAPKs ERK, JNK, and p38. Abnormal MAPK signalling may lead to increased or uncontrolled cell proliferation and resistance to apoptosis, and has been shown to be implicated in numerous cancers (Kim & Choi. 2015).

In the last twenty years, various research groups have investigated whether RF fields activated the RAS/MAPK cell stress response using standard biochemical and proteomics assays (Caraglia et al. 2005; Lee et al. 2006; Buttiglione et al. 2007; Friedman et al. 2007; Yu et al. 2008; Huang et al. 2008; Kim et al. 2012; Sheikh et al. 2013; Zhao et al. 2014; Liu et al. 2014; Valbonesi et al. 2014; Tang et al. 2015; Wang et al. 2015; Zuo et al. 2015; Shahin et al. 2018; Szymanski et al. 2018; Tsoy et al. 2019). All articles published until 2012 were reviewed by the International Agency for Research on Cancer (IARC) working group (IARC 2013) and discussed by Kim et al. (2012) (Kim et al. 2012). Since 2012, eleven studies from eight different research groups, reported experiments in which ERK activity was found (i) to

be increased in murine microvascular endothelial cells, murine spermatocyte-derived cells (GC-2), differentiated PC-12 cells and ECV304 cell line (Sheikh et al. 2013; Liu et al. 2014; Zhao et al. 2014; Wang et al. 2015; Zuo et al. 2015), and (ii) to be decreased in rat and mice brain cells prepared from animals (Tang et al. 2015; Shahin et al. 2018; Tsoy et al. 2019) following RF exposure in the GHz range. Two studies performed in PC12-derived neuron-like cells and MCF10A human breast epithelial cells reported no effects of RF exposure on the RAS/MAPK pathway (Kim et al. 2012; Valbonesi et al. 2014) and one study reported that 900 MHz RF exposure for 4 to 24h induced either an increase, a decrease, or no effect on the level of phosphorylated ERK depending on the cell type considered (HaCAT and PCS-200-010 human keratinocyte cell lines, and human primary keratinocytes derived from atopic dermatitis patients) (Szymanski et al. 2018). The overall conclusion is that whether RF exposure is able to induce a cell stress response via the RAS/MAPK is still controversial, requiring for more sensitive quantitative methods, ideally performed on live cells.

For the last fifteen years, resonance energy-transfer (RET)-based approaches have offered new opportunities for real-time probing for the activity of an ever-growing list of proteins in live cells (Miyawaki & Niino. 2015). These techniques are based on the nonradiative transfer of energy between an energy donor and a compatible fluorescent energy acceptor. This approach allows for monitoring both constitutive and regulated inter- and intra-molecular interactions, in view of the strict dependence on molecular proximity (around 100 Å) and orientation between donor and acceptor molecules for energy transfer. Among the various RET techniques, Bioluminescence Resonance Energy Transfer (BRET) is a popular, broadly-applicable biophysical method that eliminates the need for an external light source for donor excitation. BRET thus circumvents problems inherent to Fluorescence Resonance Energy Transfer (FRET) imaging, or measurement, including (1) background fluorescence from cellular components and chemical compounds, (2) phototoxicity of excitation light, (3)

photo-bleaching of the fluorophores, (4) incompatibility with optogenetic tools, and (5) invasive procedures for in-vivo microscopy (Pfleger & Eidne. 2006). Thanks to these advantages, we recently have shown that BRET is useful for probing proteins interactions and conformational changes in live cells under chemical (Ruigrok et al. 2017) or RF exposure (Ruigrok et al. 2018).

The present study was therefore conducted to re-evaluate the effect of low-level 1800 MHz RF signals (up to public exposure level for local exposure) on RAS/MAPK activation in live cells using BRET-based molecular probes. We took advantage of already existing RAS and ERK FRET biosensors (Komatsu et al. 2011) that we modified into BRET probes. Using these new probes, we monitored both basal and phorbol-12-myristate-13-acetate (PMA)-induced ERK and RAS activities in live human hepatocarcinoma HuH7 cells exposed under isothermal conditions for 24 h to Continuous Wave (CW) and Global System for Mobile (GSM)-modulated 1800 MHz RF fields at two different Specific Absorption Rate (SAR) levels: 0.5 and 2 W/kg.

2. MATERIALS AND METHODS

Designing BRET probes-

RAS and ERK BRET sensors were developed by replacing the fluorescent energy donor (ECFP or Turquoise-GL) of the EKAREV and RaichuEV-ras FRET probes described in Komatsu et al. (2011) (Komatsu et al. 2011) with Renilla Luciferase 8 (RLuc8, (Loening et al. 2006)). Mammalian expression vector coding ERK (pEKAREV) and RAS (pRaichuEV-Ras) FRET probes were kindly provided by Dr Matsuda M (Kyoto University, Japan). RLuc8 was first amplified by PCR from the pcDNA3-YFP-EPAC-Luc vector (a kind gift of M. Bouvier, Institute for Research in Immunology and Cancer, Montreal, Canada), using the Not1-ATG-Luc-Sens primer (5'-

AAGCTGGGCGGCCGCATGACCAGCAAGGTGTACGACCCCGAGC-3') and Luc-Xba1-AS primer (5'- GCTGCAGTCTAGACTGCTCGTTCTTCAGCACTCTCTCC-3'). The Rluc8 amplicon was then cloned in place of the fluorescent energy donor, between *Not*I and *X*ba*I,* in pEKAREV and pRaichuEV-Ras expression vectors (Komatsu et al. 2011).

Reagents-

Phorbol-12-myristate-13-acetate (PMA) was acquired from Tocris (Bristol, UK), and Coelenterazine H from Nanolight Technology (Pinetop, AZ, USA).

Cell culture and transfections-

HuH7 human hepatocarcinoma cells were kindly provided by J. Rosenbaum (INSERM U889, Bordeaux University, Bordeaux, France) and were maintained in Dulbecco's modified Eagle's medium—high glucose (Cat. No. D6429; Sigma-Aldrich, St. Louis, MO) supplemented with 10% fetal bovine serum, 100 units mL^{-1} penicillin and streptomycin. Twenty hours before transfection, cells were seeded at a density of 500,000 cells/well in 6-well dishes. Transient transfections were performed using polyethylenimine (PEI, linear, Mr 25,000; Cat. No. 23966 Polysciences, Warrington, PA) with a PEI/DNA ratio of 4:1, as explained in Ruigrok et al. (2018) (Ruigrok et al. 2018). Typically, transient transfections were performed using 8 µg PEI, 1 µg of the BRET probe of interest and 1 µg empty vector. After overnight incubation, cells were then detached, and plated at a density of 10^5 cells into white-opaque 96-well plate (Greiner Bio One, les Ulis, France).

BRET measurements-

BRET measurements were done on attached cells as previously described (Ruigrok et al. 2018) except that, 6 hours before BRET measurement, the cell culture medium was

replaced by red phenol-free DMEM containing 0.2% fetal bovine serum. Briefly, following 23 hrs and 45 min of sham or RF exposures, cells were incubated for an additional 15 min with the indicated concentration of PMA under sham or RF exposure. Following PMA activation, Coelenterazine H was added to the cell culture medium at a final concentration of 5 μM and the BRET signal was immediately acquired. BRET measurements were acquired using a Tristar-2 plate reader (Berthold, Bad Wildbad, Germany).

RF field exposure system-

The RF field exposure system consisted in a tri-plate open transverse electromagnetic (TEM) cell inside which the RF signals propagate (Ruigrok et al. 2017). The HuH7 cells were exposed to RF in a 96-well plate placed on the lower ground plate of the TEM cell. RF signals were delivered to the exposure system using a vector generator (SMBV100A, Rohde & Schwarz, Munich, Germany) connected to a 10 W preamplifier and a 200 W amplifier (RF14002600-10, and RFS1800-200, RFPA, Artigues-Près-Bordeaux, France) with 39- and 9-dB gain, respectively. Investigations were carried out at two power levels and two 1800 MHz RF signals (CW and GSM). For the CW signal, the power levels delivered by the vector generator were –14.2 and –8.2 dBm corresponding to incident powers at the TEM cell input of 32.2 dBm (1.7 W) and 38.2 dBm (6.8 W). CW and GSM signals had equal mean power value while the GSM peak value was eight-fold the mean value.

Numerical and Experimental dosimetry-

The SAR was assessed through numerical and experimental dosimetry. The RF field exposure system containing the 96-well plate with HuH7 cells was numerically modelled using an in-house Finite Difference Time Domain (FDTD) software (Fig. 1A). All metallic parts were considered as perfect conductors. The 96-well plate plastic dielectric permittivity

was 2.5. The cell culture medium of 100 µl per well was modelled with a dielectric permittivity of 74.2 and an electrical conductivity of 2.64 S/m, values measured at 37° C at 1800 MHz using a dielectric probe (85070E, Agilent, USA). Simulations were performed with a spatial meshing of 200 µm for the culture medium in the 96 wells. SAR values were extracted from simulation for (i) the culture medium whole volume, ii) the bottom layer at the cells level and (iii) SAR Probe corresponding to the temperature probe measurement volume (1-mm diameter and height cylinder).

Experimental SAR values were assessed from temperature variations using the following equation (1):

$$(1) \quad SAR = 4186c \left.\frac{\Delta T}{\Delta t}\right|_{t_0}$$

where *c,* the specific heat scale factor, equals 0.95, 4186 is the sample specific heat expressed in J/(kg K), and □*T*/□*t* is the initial slope of the temperature curve.

Temperature measurements were acquired using a non-perturbing optical fibre probe (Luxtron-812, Lumasense Technologies, Santa Clara, USA) placed at the centre at the bottom of the 100-µl filled well. The temperature variation was assessed for different wells within the 96-well plate for a 39.1 dBm (8.15 W) incident power. Fig. 1B illustrates this SAR spatial distribution. The SAR distribution among the 96 wells is rather homogeneous. SAR values normalized for 1 W incident power were extracted from simulations: whole volume (0.27 ± 0.12 W/kg), and bottom layer (0.32 ± 0.05 W/kg), corresponding to the localized temperature probe (0.25 ± 0.04 W/kg). The comparison of averaged simulated and measured (0.30 ± 0.05 W/kg) localized SAR shows a good agreement. The SAR bottom layer values corresponding to the incident powers of 1.7 W and 6.8 W are 0.54±0.06 W/kg and 2.16±0.41 W/kg, respectively. No temperature difference was observed in the culture medium of cells exposed

at 0.5 W/kg while temperature increased by 0.5 °C under exposure to 2 W/Kg. This slight temperature increase was counterbalanced by decreasing the temperature of the incubator containing the RF field exposure system by 0.5 °C relative to the sham incubator that was mainained at 37 °C.

Data and Statistical analysis-

GraphPad Prism v6.00 for Windows (GraphPad Software, La Jolla, CA, USA) was used for plotting dose-response curves. Statistical analyses were performed using Anastats (Rilly sur Vienne, France). The one sample Wilcoxon signed-rank test was used to assess the statistical significance against the null hypothesis of the difference calculated between Sham and RF field condition for basal BRET, PMA potency and efficacy. P-values less than 0.05 were considered as statistically significant.

3. RESULTS

The hypothesis that RF fields specifically induce RAS or ERK activity or impact chemically-induced RAS or ERK activity in live cells was tested using a BRET based assay. In that protocol, we took advantage of previously described RAS and ERK FRET probes (Komatsu et al. 2011) that we modified into BRET probes by replacing the cyan fluorescent protein energy donor with Renilla Luciferase (see material and methods). These new RAS and ERK BRET probes comprise therefore a sensor domain and a ligand domain connected by a flexible linker, a yellow Fluorescent protein (YFP) serving as energy acceptor and Renilla Luciferase serving as energy donor in place of the Cyan Fluorescent protein in the original FRET probes (see material and methods). The sensor domain changes its conformation upon action of either RAS or ERK endogenous protein. This sensitized sensor domain interacts with the ligand domain, thereby inducing a global change of the biosensor conformation and a concomitant increase of the BRET efficiency from the donor to the acceptor (Fig. 2A).

To assess the effect of RAS/MAPK activation on the BRET signal measured using both RAS and ERK BRET probes, we first transfected HuH7 cells with the cDNA coding either the EKAREV BRET probe or the Raichu-Ras BRET probe and we challenged transfected cells with different amounts of PMA for 15 min before BRET measurement. As expected, basal BRET ratio measured for RAS and ERK probes (0.623±0.012 and 0.596±0.012, respectively) dose-dependently increased following activation with PMA with potencies in the nanomolar range, demonstrating the high sensitivity of our BRET-based assay to monitor RAS and ERK activation (Fig. 2 B&C).

We then assessed the effect of increasing concentration of PMA on RAS (Fig. 3 A-D) and ERK (fig. 4 A-D) activities in HuH7 cells that were exposed for 24h to 1800 MHz CW or GSM signals at two different SARs: 0.5 W/kg and 2 W/kg, while keeping the cell culture medium at 37°C. Analysis of the resulting dose-response curve indicates that the basal BRET

ratio of RAS (Fig. 3E) and ERK (Fig. 4E) as well as the PMA potency to activate these kinases (Fig. 3F for RAS and Fig. 4F for ERK) were not modified when HuH7 cells were exposed to the RF field, whatever the SAR (0.5 and 2 W/Kg) or RF signal (CW or GSM) used. No effect of RF field exposure could also be measured on PMA maximal efficacy to activate RAS and ERK kinases, at 0.5 W/kg, whatever the signal used (CW or GSM). However, we measured a modest but statistically significant decrease (~20%) in PMA maximal efficacy to activate RAS and ERK in HuH7 cells exposed to the 1800 MHz GSM signal at 2 W/kg in comparison to the non-exposed condition (sham) (fig. 3D and G for RAS and fig.4D and G for ERK). The 1800 MHz CW signal at 2 W/kg also induced a decrease in PMA maximal efficacy to activate ERK (Fig. 4C and G), but failed to modify PMA maximal efficacy to activate RAS (fig. 3C and G).

4. DISCUSSION

In this study, we first adapted previously-described sensitive FRET probes for the detection of RAS and ERK activities in live cells using the BRET technique. Taking advantage of these new BRET probes, we then evaluated the effect of CW and GSM modulated 1800 MHz RF fields at two different SAR (0.5 and 2 W/kg) for 24 h under isothermal conditions at 37° C on live HuH7 cells. The upper SAR value corresponds to the upper limit of the standards for local exposure of the human head according to the International Commission on Non-Ionizing Radiation Protection (ICNIRP) guidelines for public exposure (ICNIRP. 1998). While we couldn't evidence a direct effect from RF radiation alone on RAS and ERK activities, we also investigated the possibility that RF fields could interact with a known inducer of RAS/ERK . In this study, we used the well-known phorbol-ester PMA as an activating chemical agent for RAS and ERK kinases. PMA is a small molecule drug that substitutes for diacylglycerol and activates the signal transduction

enzyme protein kinase C (PKC) by directly binding to its C1 domains (Castagna et al. 1982), which can lead to PKC-dependent RAS and ERK activation in several cell lines including HuH7 (Hatzis et al. 2006). Alternatively, PMA can also stimulate the Ras/Raf/MEK/ERK pathway through direct binding and activation of RasGRPs proteins (Brose & Rosenmund. 2002). Commonly, PMA is employed as a tumour-promoting agent for skin carcinogenesis in rodents and is associated with increased cell proliferation in malignant cells from several types of tumours, such as melanoma and breast and oral cancer (Fukushima et al. 2016; Lii et al. 2016). Our results show that, under our experimental conditions, RF field exposure alone does not affect the basal activity of ERK and RAS kinases nor modifies the PMA potency to activate these kinases, whatever the signal or SAR used. However, our results indicate that GSM-modulated 1800 MHz signal at 2 W/kg can decrease the PMA maximal efficacy to activate both RAS and ERK. The maximal efficacy of PMA to activate ERK kinase, but not RAS, was also decreased following cells' exposure to unmodulated 1800 MHz RF (CW signal) emitted at 2 W/kg.

The absence of variation in the basal BRET ratio following RF field exposure (Fig. 3E and 4E) indicates that such exposure does not modify RAS and ERK expression levels nor their basal activities in absence of chemical activation. Lack of shift in the potency by which PMA, acting via various defined or undefined mechanisms of action, can lead to RAS and ERK activation (Fig. 3F and 4F) indicates that both PMA affinity for its receptors (whatever they are) and its ability to alter the functional intrinsic activity of RAS and ERK kinases are not modified by RF field exposure. Strikingly, the decrease in PMA maximal efficacy to activate RAS (Fig. 3G) and ERK (Fig. 4G) following RF field exposure of the cells at 2 W/kg is a measure of a lower capability to achieve maximal ERK and RAS activation. Our results therefore preclude any change of the PMA ligand binding probability due to low intensity RF field exposure as discussed by Chiabrera et al (2000) (Chiabrera et al, 2000). Our data instead

point to a decrease of the receptor coupling efficiency, i.e., from the cascade of events which, from the binding of PMA to its receptor(s), leads to the activation of RAS and ERK kinases.

Several studies in the last decade have shown that pre-exposure to RF fields emitted at level below the current guidelines can prime an adaptive response in the cell population, i.e. can exhibit a decrease in the genotoxic response to a subsequent high dose of ionizing radiation or chemical agent (Cao et al. 2010; Cao et al. 2011; Vijayalaxmi & Scarfi. 2014; Cao & Tong. 2014; Falone et al. 2018). Our results showing that RF fields can decrease the ERK and/or RAS kinase activity in response to PMA are in agreement with the concept of an adaptive response triggered by RF field exposure. This phenomenon is here only observed at 2 W/kg, indicating the existence of a threshold. Moreover, the differential decrease in PMA efficacy to activate ERK and/or RAS after unmodulated and GSM-modulated 1800 MHz signal exposure (2 W/kg) suggests that modulation and/or bandwidth might be an important factor in the context of RF-induced adaptive response, as indicated by a recent study (Romeo et al. 2019). To better characterize whether our results are indicative of an adaptive response, further experiments should be carried out on various parameters of RF exposure such as duration of the RF exposure, resting time between RF and chemical exposure, and characteristics of the RF signal (modulation, bandwidth, carrier frequency, SAR…).

Our observations also indicate that RF field exposure can decrease PMA-induced ERK activation independently of RAS activation. Interestingly, it was previously reported that PMA can lead to ERK activation independently of RAS activation (Ueda et al. 1996).

In summary, our results suggest that RF field exposure may affect the chemically-induced RAS/MAPK pathway at various steps and this effect seems compatible with an adaptive response. An adaptive response to low doses of radiation or chemicals is probably the result of a multifaceted cellular programme involving the concerted action of diverse stress response pathways, among which is the RAS/MAPK signalling pathway (Guéguen et

al. 2019). The description of the interplay between all these molecular mechanisms to achieve the adaptive response is still to be explored. In this context, BRET probes are powerful tools for understanding how RF fields activate an adaptive response and more generally to dissect the molecular mechanisms of an adaptive response.

5. ABBREVIATION

BRET: Bioluminescence Resonance energy Transfer

CW: Continuous wave

FDTD: Finite Difference-Time Domain method

GSM: Global System for Mobile

Luc: Luciferase

PMA: phorbol-12-myristate-13-acetate

MAPK: Ser/Thr mitogen-activated protein kinase

RF: radiofrequency.

SAR: Specific Absorption Rate

YFP: Yellow Fluorescent protein

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8. DECLARATION OF INTEREST

The authors declare no competing interests.

9. AUTHORS' CONTRIBUTIONS

YP conceived and designed the study. PL and DAC designed the device and performed the electromagnetic dosimetry. EP, LP, HJR, FPDG, AH, RR and YP collected and assembled the data. AG performed the statistical analysis of the results. YP, IL, DAC and PL wrote the manuscript. All co-authors read and approved the final manuscript.

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11. FIGURES LEGENDS

FIG. 1. RF field exposure system. A) Numerical modeling of the TEM cell containing the 96-well plate with HuH7 cells in culture medium. B) SAR spatial distribution within the bottom layer of the 96-well plate exposed to 1800 MHz, 1 W incident power.

FIG. 2. Mode of action and characterization of the intramolecular RAS and ERK BRET biosensors. A) In the presence of active endogenous kinase, which phosphorylates the substrate sequence of the biosensor, the phosphoamino acid-binding domain is prompted to bind the phosphorylated substrate peptide, thereby bringing Luc and YFP in close proximity. This intramolecular conformational change favors bioluminescent resonance energy transfer between the bioluminescent donor and the fluorescent acceptor, which results in an increase in fluorescence intensity of the acceptor, and a concomitant decrease in the bioluminescent intensity of the donor. Adapted from (González-Vera & Morris. 2015). B and C) Dose-response curves of PMA-induced change in RAS activity using the pRaichuEV-Ras BRET probe (B) and in ERK activity using the pEKAREV BRET probe (C) 36 to 48h after transfection in HuH7 cells, cells were activated for 15 min at 37 °C with increasing concentration of PMA before BRET measurement. The results represent the average ± S.E.M. of 24 (RAS) and 28 (ERK) independent experiments done in duplicate. The pEC_{50} of PMA was 8.54±0.29 for pRaichuEV-Ras BRET probe and 8.69±0.22 for pEKAREV BRET probes. PMA activated the pRaichuEV-Ras and pEKAREV BRET probes with a maximal efficacy of 0.103±0.021 and 0.121±0.020 respectively.

FIG. 3. Effect of RF field exposure on basal and PMA-induced RAS activity. A-D) HuH7 cells transfected with the pRaichuEV-Ras BRET probe were sham-exposed or exposed

to unmodulated CW 1800 MHz (A and C) or GSM-modulated 1800 MHz (B and D) signals at either 0.5 W/kg (A and B) or 2 W/kg (C and D) for 23 h and 45 min. Cells were then activated with increasing concentration of PMA under sham or RF field exposure for 15 min before BRET measurement. The results represent the average ± S.E.M. for the effect of CW RF field at 0.5 W/kg (n=5), GSM-modulated field at 0.5 W/kg (n=6), CW RF field at 2 W/kg (n=4) and GSM-modulated field at 2 W/kg (n=8). The indicated independent experiments were done in duplicate. E-G) Box and whisker plots representing the distribution of the variations of basal BRET (E), PMA potency (F) and PMA maximal efficacy (G) between the RF field exposed- (Expo) and sham- conditions derived for each experimental conditions plotted in A-D. Statistical significance of the derivation from the null hypothesis (no difference between sham and RF field exposure) was assessed using the one-Sample Wilcoxon Signed Rank Test. n.s.: not significant; *: $p<0.5$; **: $p<0.1$.

FIG. 4. Effect of RF field exposure on basal and PMA-induced ERK activity. A-D) HuH7 cells transfected with the pEKAREV BRET probe were sham-exposed or exposed to unmodulated, CW 1800 MHz (A and C) or GSM-modulated 1800 MHz (B and D) signals at either 0.5 W/kg (A and B) or 2 W/kg (C and D) for 23 h and 45 min. Cells were then activated with increasing concentration of PMA under sham or RF field exposure for 15 min before BRET measurement. The results represent the average ± S.E.M. for the effect of CW and GSM-modulated fields at 0.5 W/kg (n=6), CW RF field at 2 W/kg (n=6) and GSM-modulated field at 2 W/kg (n=8). Independent experiments were done in duplicate. E-G) Box and whisker plots representing the distribution of the variations of basal BRET (E), PMA potency (F) and PMA maximal efficacy (G) between the RF field exposed (Expo) and sham conditions derived for each experimental conditions plotted in A-D. Statistical significance of the derivation from the null hypothesis (no difference between sham and RF exposure) was

assessed using the one-Sample Wilcoxon Signed Rank Test. n.s.: not significant; *: $p<0.5$; **: $p<0.1$.

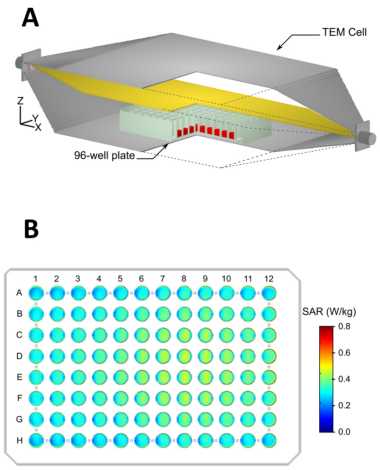
A
TEM Cell
Z
Y
X
96-well plate
B
1 2 3 4 5 6 7 8 9 10 11 12
A
B
C
D
E
F
G
H
SAR (W/kg)
0.8
0.6
0.4
0.2
0.0

Figure 1:

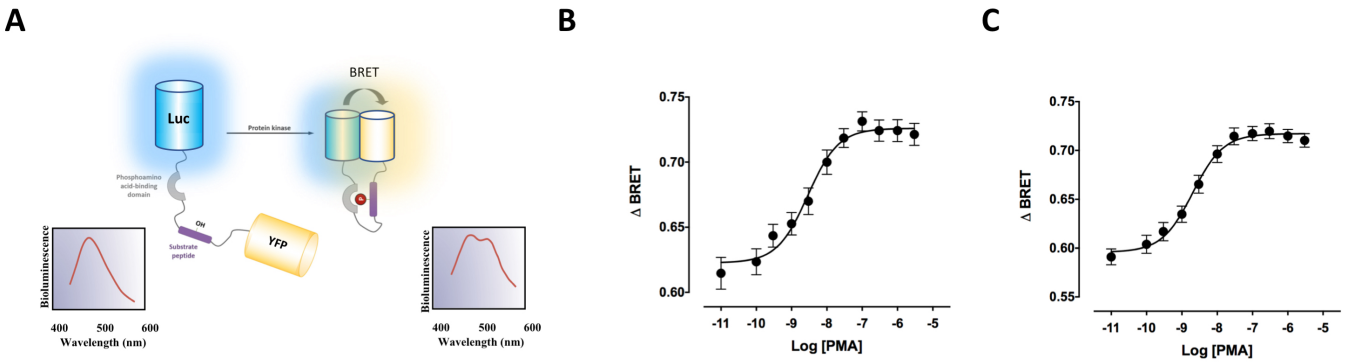
A
B
C
BRET
Luc
Protein kinase
Phosphoamino acid-binding domain
Substrate peptide
YFP
Bioluminescence
400
500
600
Wavelength (nm)
Δ BRET
0.75
0.70
0.65
0.60
0.55
-11
-10
-9
-8
-7
-6
-5
Log [PMA]

Figure 2:

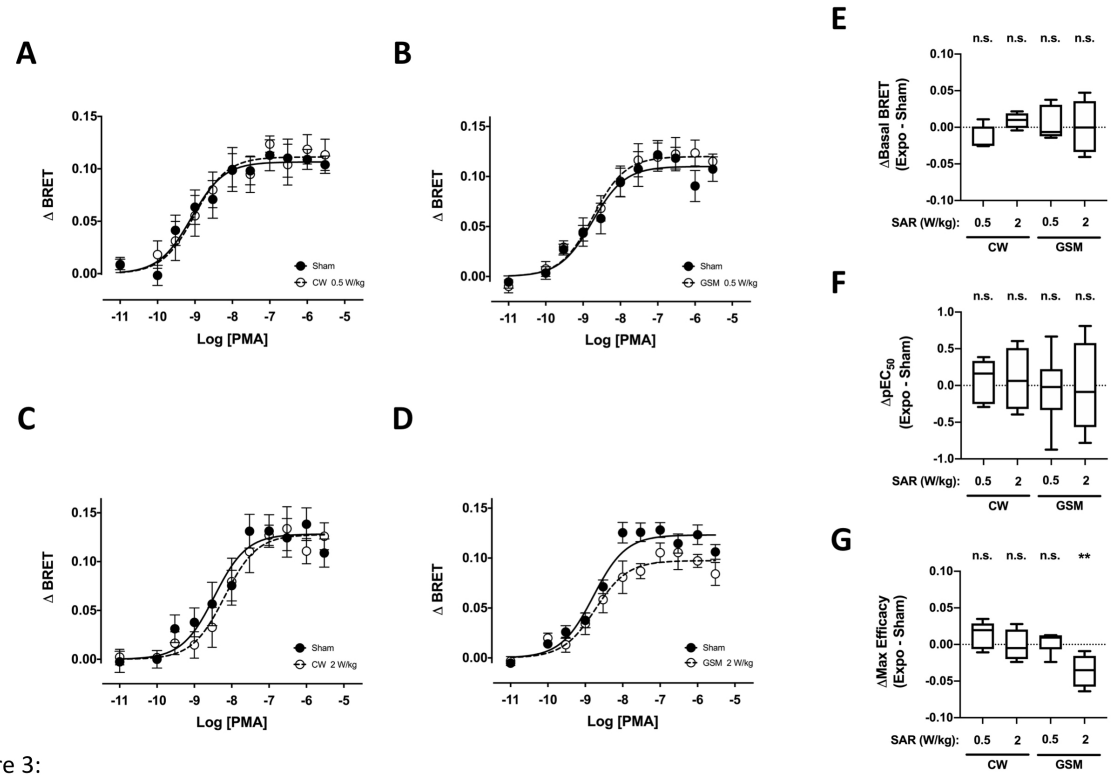
A
B
C
D
E
F
G
Δ BRET
Log [PMA]
Sham
CW 0.5 W/kg
GSM 0.5 W/kg
CW 2 W/kg
GSM 2 W/kg
ΔBasal BRET (Expo - Sham)
ΔpEC50 (Expo - Sham)
ΔMax Efficacy (Expo - Sham)
n.s.
**
SAR (W/kg):
CW
GSM

Figure 3:

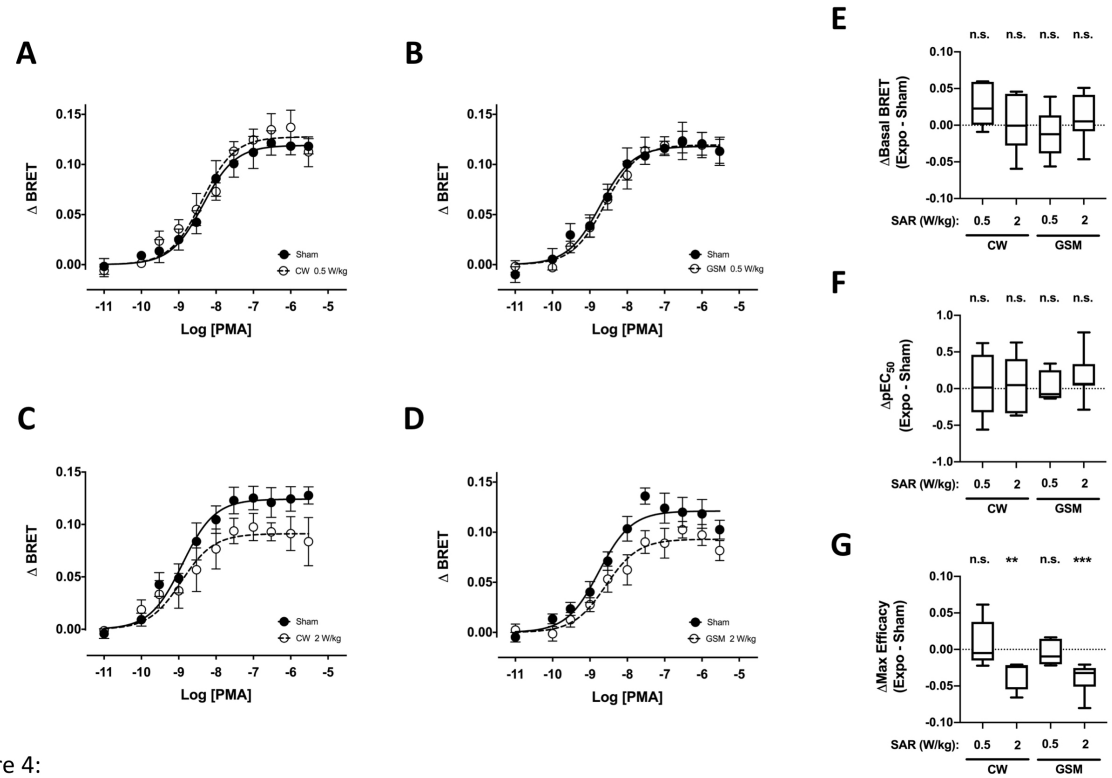
A
B
C
D
E
F
G
Δ BRET
Log [PMA]
Sham
CW 0.5 W/kg
GSM 0.5 W/kg
CW 2 W/kg
GSM 2 W/kg
ΔBasal BRET (Expo - Sham)
ΔpEC50 (Expo - Sham)
ΔMax Efficacy (Expo - Sham)
n.s.
**
***
SAR (W/kg):
CW
GSM

Figure 4: