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Effect of electromagnetic field on cyclic adenosine monophosphate (cAMP) in a human mu-opioid receptor cell model

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\textbf{ABSTRACT}

During the cell communication process, endogenous and exogenous signaling affect normal as well as pathological developmental conditions. Exogenous influences such as extra-low-frequency electromagnetic field (EMF) have been shown to effect pain and inflammation by modulating G-protein receptors, down-regulating cyclooxygenase-2 activity, and affecting the calcium/calmodulin/nitric oxide pathway. Investigators have reported changes in opioid receptors and second messengers, such as cyclic adenosine monophosphate (cAMP), in opiate tolerance and dependence by showing how repeated exposure to morphine decreases adenylate cyclase activity causing cAMP to return to control levels in the tolerant state, and increase above control levels during withdrawal. Resonance responses to biological systems using exogenous EMF signals suggest that frequency response characteristics of the target can determine the EMF biological response. In our past research we found significant down regulation of inflammatory markers tumor necrosis factor alpha (TNF-\textalpha) and nuclear factor kappa B (NF\textkappa B) using 5 Hz EMF frequency. In this study cAMP was stimulated in Chinese Hamster Ovary (CHO) cells transfected with human mu-opioid receptors, then exposed to 5 Hz EMF, and outcomes were compared with morphine treatment. Results showed a 23\% greater inhibition of cAMP-treating cells with EMF than with morphine. In order to test our results for frequency specific effects, we ran identical experiments using 13 Hz EMF, which produced results similar to controls. This study suggests the use of EMF as a complementary or alternative treatment to morphine that could both reduce pain and enhance patient quality of life without the side-effects of opiates.

\textbf{Introduction}

Tissue injury results in the production of inflammatory mediators, several of which sensitize primary afferent nociceptors (Davis et al., 1993; Reuff and Dray, 1993), resulting in hyperalgesic pain (Ferreira, 1981; Fierreira et al., 1978). Hyperalgesic pain is often associated with inflammatory pain (Schultz et al., 2003). The largest family of receptors for pharmaceutical agents is the G-protein-coupled receptors (GPCRs), whose signal transduction pathway is well understood. For example, G\alpha \textsubscript{s} (stimulating) subunit increases adenylyl cyclase (AC) activity, thereby stimulating the production of cyclic adenosine monophosphate (cAMP); whereas the G\alpha \textsubscript{i} (inhibitory) subunit decreases AC activity and inhibits the production of cAMP. The second messenger cAMP activates a specific number of tissue-specific cAMP-dependent protein kinases, ultimately affecting intracellular processes such as ion channel activity, release of neurotransmitters, regulation of transcription factors, and numerous other processes. GPCRs determine ligand binding and selectivity (Karlsmark et al., 2003). A number of ligands inhibit the function of specific enzymes by competitive or non-competitive inhibition (Sen et al., 2009). A ligand that binds to the same active catalytic site as the endogenous substrate is a competitive inhibitor. Ligands that bind at different sites on the enzyme, alter the shape of the molecule, and reduce its catalytic activity, are called non-competitive inhibitors. Extracellular environments, and to some extent transmembrane regions, determine ligand binding (Brenner and Steven, 2010). A substantial amount of literature suggest that hyperalgesia induced by tissue damage is initiated by the activation of AC – cAMP – protein kinase A (PKA) second messenger cascade, acti-vated at the mu-opioid receptor (MOR) site (England et al., 1996; Khasar et al., 1995; Malmberg et al., 1997; Taiwo and Levine, 1989, 1990, 1991, 1992). Agents that inhibit AC- and cAMP-dependent PKA prevent induc-tion of hyperalgesia by prostaglandin E2 (PGE2) and other inflammatory mediators.
Cyclic AMP is required for cell communication in the hypothalamus/pituitary gland axis and for the feedback control of hormones of the sympathetic nervous system (SNS) (Vargas et al., 2001). It is synthesized from adenosine triphosphate (ATP) by adenylyl cyclase (AC) located on the inside of the cell membrane, and is an important signal carrier necessary for the proper biological responses of cells to hormones and other extracellular signals that initiate inflammatory pain through the cAMP response element binding (CREB) protein cycle. Cyclic AMP is activated by a range of signaling molecules via AC stimulatory G-protein (Gs)-coupled receptors and inhibited by agonists of AC inhibitory GPCRs (Gi) (Billington and Penn, 2003; Khoury et al., 2014). GPCRs also form homo- and/or hetero-dimers, and their impact on receptor physiology and pharmacology has attracted a lot of interest (CRR, 2013). Data suggest that the MOR is a heterodimer that heterodimerizes only at the cell surface, and the oligomers of opioid receptors and heterotrimic G-proteins are the bases for MOR heterodimer phenotypes (Markova and Mostow, 2012). There is accumulating evidence that oligomerization of GPCRs can alter the selectivity and affinity of ligand binding, and this has been reported for opioid receptor heterodimers (Andreadis and Geer, 2006; Gurtner et al., 2008). Due to this phenomenon an alteration in binding properties could sensitize receptors to ligands, thereby allowing responses to lower agonist concentrations. Receptor dimerization can also result in the formation of novel binding sites. There are many documented examples of interactions between GPCRs coupling preferentially to different signaling pathways, which result in a potentiation of Ca\(^{2+}\) signaling (Fiorotto and Klish, 1991; Gourevitch et al., 2014). GPCRs are components of multi-protein signaling complexes that regulate the localization and function of receptors which form complexes with a wide variety of signaling and regulatory proteins. For these experiments we have chosen the mu-opioid receptor as our target, because it is most associated with potent analogies currently used to control pain (Tzschentke et al., 2014).

Opioid dependence has been reported to be associated with changes in the cAMP systems in experiments in vitro. For example, in neuroblastoma cells, acute treatments with morphine and other opiates inhibit adenylyl cyclase (AC) activity resulting in a decrease of cAMP levels (Sharma et al., 1975; Traber et al., 1975); however, after repeated exposure to morphine, the AC activity and cAMP levels return to control levels in the tolerant state and increased above control levels during withdrawal (Benalal and Bachrach, 1985; Sharma et al., 1975; Traber et al., 1975). These findings are the basis for cAMP as the mechanism of action for the development of morphine dependence (Mamiya et al., 2001). An increase in AC activity and cAMP levels in the brain represent biochemical associations of morphine dependence (Collier, 1980; Kuriyama et al., 1978), whereby cAMP levels are regulated by AC and phosphodiesterases (PDEs) (Thompson, 1991). It has been substantiated that 3-isobutyl-1-methylxanthine (IBMX)-modulated forskolin induces behavior that resembles morphine withdrawal syndrome in naïve rats and increases naloxone-precipitated morphine withdrawal syndrome in morphine dependent rats (Collier and Francis, 1975; Rasmussen et al., 1990).

In humans, electromagnetic field (EMF) therapy has proven to be a safe, non-invasive, easy-to-use method to treat the source of pain and inflammation (Markov, 2007; Ross and Harrison, 2013). Research has shown that therapeutic applications at extra-low frequency (ELF) EMF (1–100 Hz) levels stimulate the immune system by suppressing inflammatory responses at the cell-membrane level (O’Connor et al., 1990). Double-blind, placebo-controlled clinical trials (Stiller et al., 1992) report EMF passes through the skin into the body’s conductive tissue (Hannan et al., 1994; Gurtner et al., 2008), reducing pain and the onset of edema shortly after trauma (Chalidis et al., 2011; Rohde et al., 2009). In one such study low-frequency pulsed EMF (PEMF) therapy at 0.1 to 64 Hz was reported to improve mobility, and reduce pain and fatigue in fibromyalgia patients (Subbeyaz et al., 2009). Both human and in vitro studies report EMF to be effective in the treatment of pain and inflammation in osteoarthritis (OA) (Li et al., 2013; Sadoghi et al., 2013), without the addictive side-effects of opiates. It has been proposed that charge receptors or other kinds of sensors at the extracellular membrane could recognize EMF by their ability to resonate with varying frequencies (Funk and Monses, 2006).

In this study we hypothesize that an EMF at certain frequencies can be therapeutic, therefore we are looking for a similar down regulatory effect of EMF on cAMP as would be seen in morphine treatment. EMF has a number of well-documented therapeutic effects on cells and tissues afflicted with inflammatory pain (Ross and Harrison, 2013). Reports of resonance frequency responses of biological systems to exogenous EMF signals suggest the frequency response characteristics of the tissue can determine the EMF response (Bawin et al., 1975; Markov, 1981). EMF parameters such as frequency, field strength, and time of exposure, all account for the mechanistic pathway affecting inflammatory pain mediators. Oscillating EMF exerts forces
on free ions present on both sides of the plasma membrane which move across the cell surface through the transmembrane proteins creating a forced intracellular vibration. This force is responsible for phenomena such as the influx of extracellular calcium and the binding affinity of calmodulin (CaM) – the primary transduction pathway to second messengers such as cAMP. Because of the important ramifications for treating inflammatory pain, we investigated the effect of EMF on the cAMP second messenger pathway, which contributes to maintenance as well as the initiation of hyperalgesia.

Materials and methods

A Chinese Hamster Ovary (CHO-K1) cell line transfected with human mu(µ)-opioid receptors (catalog #6605, ChanTest Corp, Cleveland, OH) was cultured in flasks using Ham’s F-12 1X modified media with L-glutamine (Mediatech, Inc., Manassas, VA); 10% FBS, 1% non-essential amino acids (100X, #11140-050, Gibco, Grand Island, NY); 0.4 mg/ml Geneticin (G418, #10131-027, Gibco, Grand Island, NY); and 1% penicillin-streptomycin (Cellgro#30-002-CL, Fisher Scientific, Pittsburg, PA), then incubated in 5% CO₂ at 37 °C and 100% humidity until ∼80% confluent. Cells were detached from flasks using trypsin, then centrifuged at 1500 rpm for 5 min and reseeded in two separate 6-well culture plates (treatment and control) at a density of 2.5 × 10⁵ cells/mL.

Stimulation of cAMP

Cyclic AMP was stimulated by exposing µ-CHO cells to 10µM forskolin (Sigma #F6886, St. Louis, MO). Forskolin is a labdane diterpene produced by the Indian coleus plant, and is commonly used in medical research to raise cAMP levels (Gris et al., 2010). Forskolin sensitizes cell receptors by activating adenylyl cyclase (AC) to catalyze the levels of cAMP. Cells were also stimulated with 0.5 mM 3-isobutyl-1-methylxanthine (IBMX, Sigma #17018, St. Louis, MO), which is a phosphodiesterase inhibitor that will prevent the degradation of the phosphodiester bond in the second messenger molecule cAMP. All stimulation times were 15 min as is standard for this protocol (Xu et al., 2003). Duplicate experiments were performed on CHO-K1 cells used as negative control. Outcomes were measured based on EC₅₀ or half maximal effective concentration of the morphine (CatchPoint Cyclic-AMP Fluorescent Assay Kit #R8088, Molecular Devices, Sunnyvale, CA).

Experimental groups

Six individual groups were used in this experiment: (1) µ-CHO cells only (baseline) [positive control]; (2) µ-CHO cells + forskolin/IBMX stimulant [F only]; (3) µ-CHO cells + forskolin/IBMX stimulant + 0.1 mM morphine [F + M]; (4) µ-CHO cells + forskolin/IBMX stimulant + EMF treatment [F + E]; (5) µ-CHO cells + forskolin/IBMX stimulant + 0.1 mM morphine + EMF treatment [F + M + E]; and (6) CHO-K1 [negative control]. In the F + M + EMF group, morphine was added immediately before cells were exposed to EMF.

PEMF exposure

Using a commercially manufactured 11-inch diameter Helmholtz Coil (SpinCoil-11-X, Micro Magnetics, Fall River, MA), both µ-CHO cells and CHO-K1 cell groups underwent the same experimental conditions. The [F + E] and [F + M + E] groups were exposed to a 5 Hz EMF for 15 min to determine treatment effect on cAMP. The time point of 15 min was selected as it is the amount of time needed for cAMP accumulation levels to be maintained fully until the end of the incubation period. The EMF coils were driven by an alternating current power supply with adjustable frequency and amplitude. From the coil center the uniform field strength was measured to be approximately 1.5 µT (see flux density schematic in Figure 1a). Each coil carried a 50% duty sine wave in the same direction (Figure 1b). The EMF was charac-terized using a Gauss meter (Sypris Model 5180; Pacific Scientific-OECO, Milwaukee, OR). The frequency was quantified using an oscilloscope (Model TDS 20248; Tektronix Inc, Beaverton, OR). Experiments were performed under ambient conditions. After exposure to EMF, a live/dead cell assay was performed using calcein acetomethoxy (catalog # C697959; Life Technologies, Carlsbad, CA), showing no significant change in cell viability (data not shown). Measurements were taken using a Spectramax M5 plate reader (Molecular Devices LLC, Sunnyvale, CA), at 530 nm excitation level and 590 nm emission level. Control samples were kept in the same conditions without exposure to EMF. The EMF exposed cells were kept in a warm bath to ensure a consistent 37 °C temperature during these experiments. Background EMF was measured and averaged the same as Earth’s (~0.5 Gauss).

Statistical analysis

All data measured are presented as mean ± standard error of the mean (S.E.M.), for n = 4 samples. For all assays, a one-way analysis of variance (ANOVA) with
Tukey’s post hoc test was used to assess the differences between the controls and the cells exposed to the EMF, with $p < 0.05$ considered statistically significant for all tests.

**Results**

The aim of this study was to investigate the effect of a low-frequency EMF on cAMP, which is a second messenger transcription factor used for intracellular signal transduction on the AC-cAMP-PKA-dependent pathway. This pathway contributes to the initiation and maintenance of hyperalgesia. In this experiment we have chosen to study the human µ (µ) opioid receptor in particular because it belongs to the GPCR super family of receptors whose activation leads to a cascade of events that inhibit AC and decrease cAMP levels, and also has a strong binding affinity with morphine (Osugi et al., 1996). Using $n = 4$ samples for three trials, we compared the results of an EMF treatment with that of morphine. Since the opiate receptor ligand morphine has a considerably higher binding affinity for µ-opioid receptors (MORs) than for other opioid receptors, and its occupation can inhibit AC and cAMP formation, we hypothesized the EMF would affect the binding affinity of the MORs. What we found was that a statistically significant inhibition of cAMP did indeed occur in the EMF-exposed cells. EMF exposure on the MOR transfected cells reduced the amount of cAMP by 23% more as compared with the morphine-treated sample. CHO-K1 cell line produced results similar to basal (baseline) (Figure 2).

In the experimental group where MORs stimulated with forskolin increased cAMP levels, which were exposed to 5 Hz EMF [F + EMF], there was a statistically significant ($p < 0.05$ for $n = 4$ samples) decrease in cAMP levels (Figure 3). After finding an inhibitory effect of EMF on cAMP at 5 Hz, we wanted to compare it with a different frequency in order to determine if the effect was frequency dependent. Therefore, we duplicated the experiment using 13 Hz to compare with the outcome of the 5 Hz treatment. This number was chosen due to the lack of published evidence of any therapeutic value related to its frequency. As shown in Figure 2, the effect of EMF on µ-cells was more inhibitive at 5 Hz than at 13 Hz (Figure 2a and c). While we hypothesized that EMF would improve the efficacy of morphine, this did not appear to be the case.

Data represent standard error of mean (S.E.M.). A statistically significant difference ($p < 0.05$) between morphine and EMF [F + M vs. F + EMF and F + M vs. F + M + EMF] was observed in cells exposed to 5 Hz frequency, but not between EMF and morphine combined [EMF + F vs. EMF + F + M]. EMF does not appear to counteract the inhibition potential of morphine.

**Discussion**

Pain killers target membrane transport proteins, including ligand- and voltage-gated ion channels. At ligand-gated ion channels, drugs can bind at the same site as the endogenous ligand and directly compete for the receptor site. Although membrane ion channels and protein phosphorylation can be indirectly affected by GPCRs through effector proteins such as AC and second messengers such as cAMP, they can short circuit the second messenger pathway and gate the ion channels directly (Brown...
et al., 1988). This bypassing of the second messenger pathway is observed in mammalian cardiac myocytes where Ca^{2+} channels are able to survive and function in the absence of cAMP, ATP, or protein kinase C (PKC), when in the presence of the activated α-subunit of the G protein (Yatani et al., 1987). Here Gα, which is stimulatory to AC, acts on the Ca^{2+} channel directly as an effector. This short circuit is membrane-delimited, allowing direct gating of Ca^{2+} channels by G-proteins to produce effects more quickly than the cAMP cascade could (Brown et al., 1988).

Some drugs directly bind and inactivate voltage-gated ion channels; these are ion channel proteins that do not have endogenous ligands, but open or close as a function of the membrane voltage potential. This membrane voltage potential has been suggested as the mechanism of action for the biological effect of EMF through the plasma membrane (Ady, 1974; Liboff, 1985; McLeod et al., 1987) – across which the EMF signal induces a voltage change (Figure 4).

Oscillating EMF exerts forces on free ions present on both sides of the plasma membrane that move across the cell surface through transmembrane proteins, creating a forced intracellular vibration. This vibration is responsible for phenomena such as the influx of extracellular Ca^{2+}, as well as efflux of intracellular Ca^{2+}. A rise in cytosolic Ca^{2+} concentration is used as a
Figure 4. (a) Voltage-gated ion channels control intra- and extra- cellular ion flux due to positive surface charge. (b) EF can attenuate the opening and closing of these ion channels to trigger intracellular events due to negative charge (’Q) depolarizing the plasma membrane.

signaling messenger in nearly all eukaryotic cells (Berridge et al., 2000; Clapham, 2008). Oscillations in cytosolic Ca\(^{2+}\) concentration are observed in all cell types, suggesting they represent a universal signaling mode (Thomas et al., 1996). Changes in the plasma membrane potential can alter the activity of voltage-gated Ca\(^{2+}\) channels leading to bursts of Ca\(^{2+}\) entry (Parekh, 2011), which can result directly in Ca\(^{2+}\) oscillations which can trigger second messengers through the activation of cell-surface receptors that activate enzymes such as AC, modulator of cAMP. Regardless of the mechanism, oscillations in cytosolic Ca\(^{2+}\) concentration can be supported for a few minutes in the absence of external Ca\(^{2+}\), showing that Ca\(^{2+}\) recycling across the intracellular stores is the primary mechanism for driving them (Parekh, 2011). Oscillation triggered in Ca\(^{2+}\)-free solution does decrease with time, because a fraction of the Ca\(^{2+}\) released during each cycle is transported out of the cell, causing less Ca\(^{2+}\) to be available to support the next oscillation. To sustain oscillation requires Ca\(^{2+}\) entry, and this is accomplished through Ca\(^{2+}\) channels in the plasma membrane (Parekh, 2010; Parekh and Putney, 2005).

EMF has also been reported to be instrumental in the binding affinity of calmodulin (CaM) – the primary transduction pathway to second messengers cAMP, and cyclic guanosine monophosphate (cGMP), found to influence inflammatory pain (Pilla et al., 2011). Since EMF decreased the amount of cAMP by 23% compared with morphine, it is either causing a change in the conformation of the GPCRs, or it is affecting the second messenger cAMP via the voltage-gated calcium channels (VGCCs). This action could be achieved through several high thresholds, slowly activating Ca\(^{2+}\) channels in neurons which are regulated by G-proteins (Morris and Malbon, 1999). The activation of α-subunits of G-proteins has been shown to cause rapid closing of voltage-dependent Ca\(^{2+}\) channels, which causes difficulties in the firing of action potentials (Stryer et al., 2007). This inhibition of voltage-gated Ca\(^{2+}\) channels by GPCRs has been demonstrated in the dorsal root ganglion of a chick and other cells lines (Morris and Malbon, 1999). Other studies have indicated roles for G\(^{\alpha}\) subunits in the inhibition of Ca\(^{2+}\) channels (Jeon et al., 2013; Zhang et al., 2008). Since receptors normally pick up signals in the cells’ environment, and G-proteins couple these signals to the effectors (sending the signal to the cytoplasm), it is feasible that the EMF was able to short circuit the second messenger pathway and gate the ion channels directly. This would account for a moderately stronger down-regulation of cAMP with EMF treatment than with morphine.

Conclusion

The therapeutic effects of low-frequency EMF have been reported for years; however, a mechanism of action has yet to be elucidated. Here we compared the effects of EMF on cAMP at both 5 Hz and 13 Hz. The 5 Hz frequency showed a stronger inhibitory effect in cAMP expression on [F + EMF] than the 13 Hz frequency. EMF appears to competitively inhibit cAMP as morphine does; however, EMF exposure does not have the addictive side-effects of morphine. If EMF changes the conformation of the MOR receptor, along with stabilizing Ca\(^{2+}\) flux, then it would explain the outcomes we observed. If EMF is able to induce
homeostasis via the stabilization of Ca$^{2+}$, then it appears to be frequency specific as our study suggests.

**Declaration of interest**

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**References**


